From Department of Laboratory Medicine
Division of Pathology
Karolinska Institutet, Stockholm, Sweden

Biogenesis of tartrate-resistant acid phosphatase isoforms 5a and 5b in cell and animal models

Serhan Zenger

Karolinska Institutet

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ABSTRACT

Tartrate-resistant acid phosphatase (TRAP) is an iron-containing binuclear acid metallohydrolase that belongs to the purple acid phosphatase (PAP) family. It is a glycoprotein translated as a monomeric protein but is commonly isolated from tissues as a proteolytically processed dimeric form with increased enzyme activity. In human serum, the enzyme exists as two isoforms, monomeric *TRAP 5a* and proteolytically-processed *TRAP 5b*. Traditionally, the enzyme has been used as a marker for osteoclasts and participates in bone resorption. Activated macrophages, dendritic cells, certain neurons and epithelial cells as well as several cancer cell lines and tissues have also been shown to express TRAP.

TRAP 5a and 5b isoforms are compartmentalized differently by different cell types and their serum levels correlate independently with different human diseases, consistent with the notion that the TRAP isoforms exert different functions. The aims of the present investigation were to characterize biogeneration of these two isoforms through proteolytic cleavage and differential intracellular sorting, and their differential expression in physiological and pathological settings.

Cathepsin K (Ctsk) is an essential cysteine proteinase in bone matrix degradation by osteoclasts. The enzyme can efficiently activate TRAP through proteolytic processing in vitro and these two proteins co-localize in osteoclasts. Analysis of Ctsk-deficient mice revealed that absence of Ctsk was associated with increased abundance of monomeric TRAP in osteoclasts of long bones as well as calvaria. In long bones, this increase was more pronounced in osteoclasts of the distal compared to the proximal part of the metaphyseal trabeculae, suggesting a site-dependent role for Ctsk in TRAP processing in vivo and highlighting functional differences between bone-resorbing osteoclasts within the trabecular metaphyseal bone. In addition, proteolytic processing of TRAP was more extensive in long bones than calvaria which correlated with higher cysteine proteinase activity and protein expression of Ctsk. Absence of Ctsk was also associated with altered intracellular distribution and secretion of TRAP, suggesting a novel role for Ctsk in the regulation of intracellular trafficking in osteoclasts.

Biogenetic relationship between TRAP 5a and 5b was further studied in a stably transfected TRAP-overexpressing breast cancer epithelial cell line. The study demonstrated that distinct monomeric TRAP populations are diverted early in the secretory pathway either giving rise to a secreted, monomeric TRAP 5a or to an intracellular, proteolytically processed TRAP 5b. In support of different functional roles for TRAP 5a and 5b, the majority of the intracellular monomeric TRAP was destined for secretion as TRAP 5a, while a minor portion provided the putative precursor for intracellular TRAP 5b.

Examination of breast cancer bone metastases revealed that TRAP 5a was the predominant form expressed by metastatic cancer cells, whereas tumor-associated macrophages expressed the proteolytically processed TRAP 5b. Moreover, a previously unstudied TRAP 5a variant was identified in cancer cells, which may have functional and diagnostic implications.

LIST OF PUBLICATIONS

I. **S. Zenger** [1], K. Hollberg [1], J. Ljusberg, M. Norgard, B. Ek-Rylander, R. Kiviranta, G. Andersson
Proteolytic processing and polarized secretion of tartrate-resistant acid phosphatase is altered in a subpopulation of metaphyseal osteoclasts in cathepsin K-deficient mice.
Bone, 2007, 41:820-832

II. **S. Zenger,** B. Ek-Rylander, G. Andersson
Long bone osteoclasts display an augmented osteoclast phenotype compared to calvarial osteoclasts.
Biochem Biophys Res Commun, 2010, 394:743-749

III. **S. Zenger,** B. Ek-Rylander, G. Andersson
Biogenesis of tartrate-resistant acid phosphatase isoforms 5a and 5b in stably transfected MDA-MB-231 breast cancer epithelial cells.
Biochim Biophys Acta, 2010, 1803:598-607

IV. W. He [1], **S. Zenger** [1], B. Ek-Rylander, D. Vassiliou, R. Wedin, H. Bauer, G. Andersson
Differential expression of tartrate-resistant acid phosphatase isoforms 5a and 5b by tumor and stromal cells in human metastatic bone disease.
Submitted manuscript

[1] Shared first authorship

ABBREVIATIONS

3D	-	Three dimensional
BfA	-	Brefeldin A
BSP	-	Bone sialoprotein
CGN	-	Cis Golgi Network
CP	-	Cysteine proteinase
CTR	-	Calcitonin receptor
Ctsk	-	Cathepsin K
Ctsl	-	Cathepsin L
ER	-	Endoplasmic Reticulum
FACIT	-	Fibril-associated collagens with interrupted triple helices
FSD	-	Functional secretory domain
FPLC	-	Fast performance liquid chromatography
IGF	-	Insulin-like growth factor
kD / kDa	-	kilo Dalton
M6P	-	Mannose-6-phosphate
M-CSF	-	Macrophage colony stimulating factor
MITF	-	Microphthalmia transcription factor
MMP	-	Matrix metalloproteinases
Mw	-	Molecular weight
OPG	-	Osteoprotegerin
OPN	-	Osteopontin
PAP	-	Purple acid phosphatase
PTH	-	Parathyroid hormone
RANKL	-	Receptor activator of nuclear factor kappa B ligand
RA	-	Rheumatoid arthritis
RGD	-	Arginine-glycine-aspartic acid
ROS	-	Reactive Oxygen Species
SDS-PAGE	-	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TEM	-	Transmission electron microscopy
TGF	-	Transforming growth factor
TGN	-	Trans Golgi Network
TRAP	-	Tartrate-resistant acid phosphatase
Uf	-	Uteroferrin

1. INTRODUCTION

1.1 Bone

1.1.1 Anatomy and structure

Bone is a dynamic connective tissue that together with cartilage forms the skeletal system occupying 15-20% of the body weight in humans. Besides providing structural support and protection of inner organs, bones are essential for maintenance of mineral homeostasis, i.e. homeostatic regulation of serum calcium and phosphate concentrations, as well as in acid-base balance [2]. Moreover, bones harbor bone marrow where hematopoiesis takes place postnatally. Recently, bone-derived proteins have been shown to act as hormones regulating energy metabolism, establishing bone as an endocrine organ as well [3].

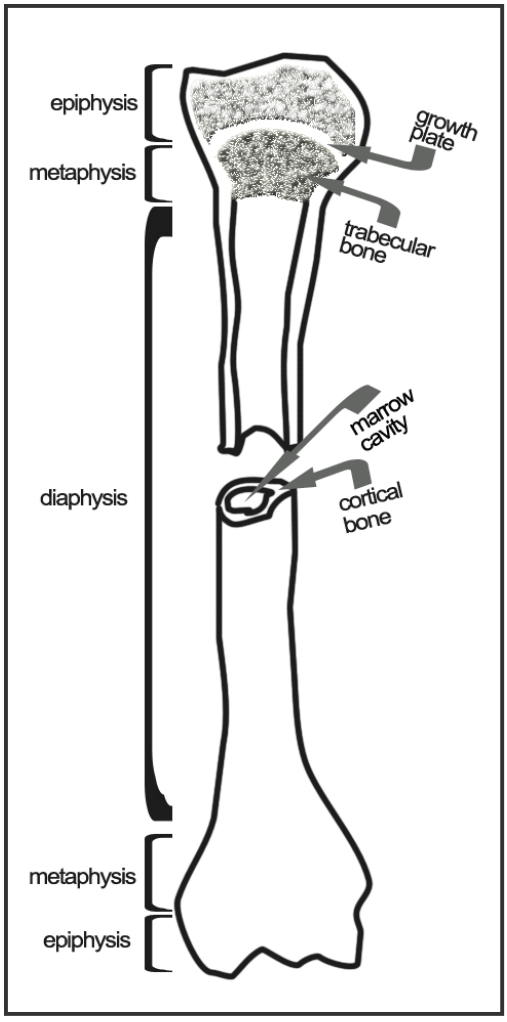

Figure 1. Structure of a long bone

The adult human skeleton contains over 200 bones shaped by a process called modeling. Moreover, they are constantly renewed throughout lifetime by a process termed remodeling, which helps to strengthen the skeletal system by repairing the fatigued bone. Proper bone remodeling requires the tight regulation of bone formation and bone resorption processes and imbalance in this regulation results in various metabolic bone diseases, such as osteoporosis.

Anatomists distinguish between two major types of bones: long bones such as the femur and tibia, and flat bones such as the skull and scapula. Morphologically, bones can be divided into two forms: cortical and trabecular (***Fig. 1***). Cortical bone forms the hard exterior while internal spaces are

filled by the trabecular bone, which makes the overall organ lighter while allowing room for blood vessels and marrow. Trabecular bone accounts for overall 20% of total bone mass, but has nearly ten times the surface area of cortical bone and is the most metabolically active part in a mature long bone. The shaft of the long bones is called diaphysis. Metaphysis is the wider portion of a long bone and separates diaphysis from epiphysis, i.e. rounded ends of a long bone. The metaphysis and epiphysis of a long bone is separated by a zone of cartilage called the growth plate where longitudinal growth takes place in young bones (***Fig. 1***).

1.1.2 Bone formation

The mammalian skeleton is formed by two distinct types of developmental processes, i.e. intramembranous and endochondral ossification. In endochondral ossification, migration and condensation of mesenchymal cells during embryonic development gives rise to the cartilage models of the future bones that are eventually replaced by bone. In intramembranous ossification, on the other hand, bone is deposited directly without an intermediate cartilage phase. While long bones are formed by a combination of these two processes, most flat bones such as calvaria are solely formed by intramembranous ossification.

1.1.3 Bone matrix

Bone matrix is composed of an organic and an inorganic mineral phase. The major constituent of the organic matrix is collagen I, which is the most abundant protein of the bone matrix accounting for 90% of the total protein mass. However, trace amounts of type III, V and FACIT collagens may also be present in the matrix during certains stages of bone formation and are thought to influence the regulation of collagen fibril diameter [4]. The collagen fibers constitute the major protein framework upon which inorganic hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] crystals are deposited which in turn enhance the strength and stiffness of the bone. The rest of the organic phase contains several other non-collagenous proteins and proteoglycans. RGD-containing proteins constitute an important subgroup of noncollagenous bone matrix proteins and includes bone sialoprotein (BSP), osteopontin (OPN), fibronectin and vitronectin. These proteins mediate cell-matrix attachment through binding of integrins to RGD motif. Other functions performed by non-collagenous proteins of the bone matrix include the regulation of mineralization and bone remodelling. There are also several types of

polypeptide growth factors, e.g. TGF-β and IGF-II, embedded in the mineralized bone matrix, which influence bone cell activity.

The relative amounts of collagen and non-collagenous proteins as well as the level of collagen cross-linking has been shown to differ between bone types formed by different modes of ossification, e.g. calvaria and long bones [5], reflecting the different mechanical demands on different bone types.

1.1.4 Bone cells

1.1.4.1 Osteoblast lineage cells

Osteoblasts are specialized connective tissue cells responsible for bone matrix formation. They originate from mesenchyme-derived osteoprogenitor stem cells and their differentiation is regulated by a plethora of hormones and cytokines including parathyroid hormone (PTH), estrogen, PTH-related peptide, insulin-like growth factors, glucocorticoids and vitamin D. Their differentiation can also be influenced by local factors such as TGF-β, bone morphogenic proteins and fibroblast growth factors. Mature osteoblasts express and secrete high amounts of alkaline phosphatase, one of the earliest markers of the osteoblast phenotype, and type I collagen. Bone matrix laid down by osteoblasts is eventually mineralized which leads to up-regulated expression of several other non-collagenous proteins such as osteocalcin, BSP and OPN, which are regarded as mature osteoblast markers. Of these, osteocalcin is used as biomarker for the bone formation process. Yet another crucial function of osteoblasts in bone modeling/remodeling is the regulation of osteoclastogenesis and osteoclast maturation through the RANKL/RANK/OPG system (see 1.1.4.2.1).

Subsequent to completing bone formation, three possible fates await osteoblasts. Some undergo apoptosis whereas others adopt a quiescent phenotype. These quiescent cells with flat and elongated morphology are called bone-lining cells and are characterized by moderate rough ER and Golgi apparatus and express intercellular adhesion molecule-1 (ICAM-1) [6]. Bone-lining cells have been proposed proposed to have an essential role in coordination of bone resorption and formation processes [6]. Another possible fate for an osteoblast is to be trapped in bone matrix subsequent to bone deposition. These trapped osteoblasts are called osteocytes. Osteocytes occupy a small cavity in the matrix and communicates with surrounding cells through tiny channels called canaliculi, radiating from each cell as a cellular extension. Although the exact role of these cells in bone metabolism is not clear, they have been advocated to be

mechanosensors of stress and strain on bone. Other functions proposed for osteocytes include the regulation of bone remodeling and mineral homeostasis. [7]

1.1.4.2 The Osteoclast

The word "osteoclast" is derived from the Greek words "oston" meaning bone, and "klastos" meaning "to break", literally meaning bone-breaker. Osteoclast is a large multinucleated phagocyte derived by fusion of mononuclear precursors of the monocyte/macrophage lineage and is the exclusive bone resorptive cell so far identified. Mature osteoclasts are large cells with two to ten nuclei. Defective osteoclast formation and/or function results in a condition called osteopetrosis, i.e. increased bone mass, and inspections of naturally-occuring and bioengineered mutations resulting in this condition have provided important insights into the factors regulating osteoclast formation and function.

1.1.4.2.1 Osteoclastogenesis

Development of mononuclear precursors into fully functional osteoclasts is dependent on the presence of two essential cytokines: macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor kappa B ligand (RANKL), both of which are either presented to osteoclasts by bone marrow stromal cells and osteoblasts through intercellular contact or as soluble factors [8] (***Fig. 2***). M-CSF is crucial for the survival and proliferation of osteoclast precursors and increases the expression of receptor activator of nuclear factor kappa B (RANK), thereby rendering these cells more responsive to RANKL. RANKL, a member of the TNF superfamily, is essential for the later stages of osteoclastogenesis and osteoclast maturation. The discovery of RANKL was preceded by identification of its inhibitor OPG, an osteoblast-secreted decoy receptor competing with RANK for RANKL. The RANKL/OPG ratio seems to be critical for osteoclastogenesis and is influenced by a variety of hormones and cytokines such as parathyroid hormone (PTH), 1.25-dihydroxyvitamin D, estrogen, thyroid hormones, TNF-α, IL-1 and PGE-2. By suppressing OPG expression while simultaneously enhancing RANKL expression, pro-inflammatory cytokines such as TNF- α and IL-1 increase osteoclastogenesis, but whether these cytokines, alone, prompts osteoclastogenesis is controversial. In addition to these factors, calcitonin, a polypeptide hormone produced by parafollicular cells of the thyroid gland in response to hypercalcemia, regulates

osteoclast activity by directly binding to its receptor, i.e. calcitonin receptor (CTR), expressed by mature osteoclasts, and terminates osteoclast activity.

Under the action of RANKL and M-CSF, precursors go through the transcriptional programming leading to the expression of functionally important osteoclast proteins (***Fig. 2***). PU.1 and MITF, essential transcription factors for initial macrophage differentiation, have been shown to be involved in the commitment of hematopoietic cells into myeloid lineage and thus mediate the earliest events in osteoclastogenesis [9]. Other transcription factors demonstrated to be involved in osteoclastogenesis includes NFκB[10], c-Fos[11] and NFATc1. Of these, NFATc1 is the transcription factor most potently induced by RANKL [12] and regarded as the master transcription factor in osteoclastogenesis. NFATc1 is a major substrate for the enzyme calcineurin, a calmodulin-dependent phosphatase activated by RANKL-induced release of Ca^{2+}. Its subsequent nuclear translocation and activation leads to the direct regulation of several osteoclast-specific genes such as TRAP [12], CTR [12], β3 integrin [13] and cathepsin k (Ctsk) [14].

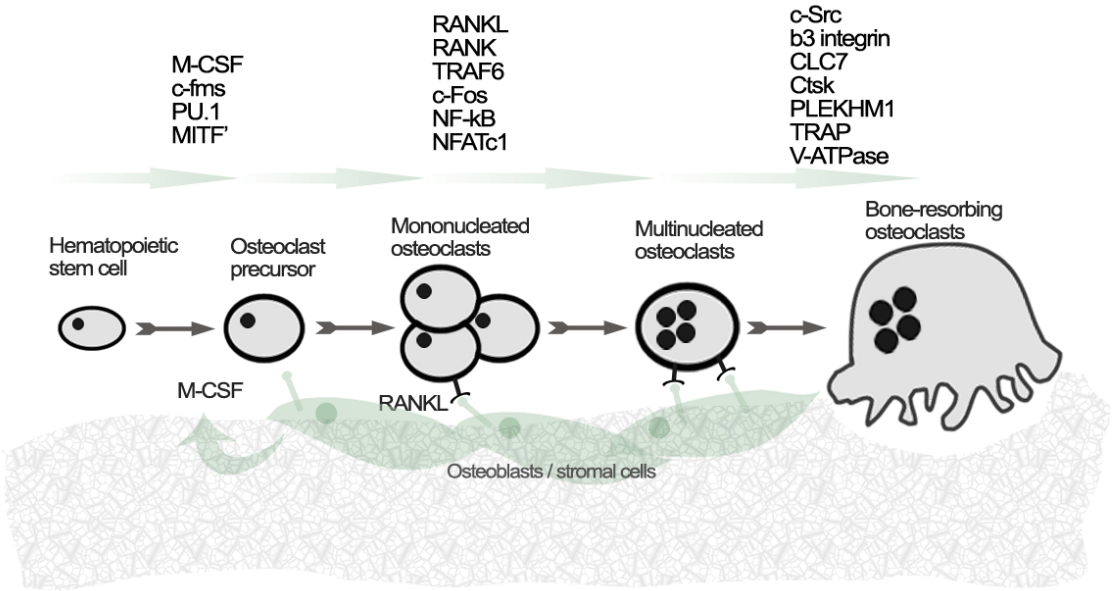

Figure 2. Important steps and factors in osteoclast differentiation and function. Figure reproduced from Negishi-Koga et al. [1].

1.1.4.2.2 Osteoclastic bone resorption

1.1.4.2.2.1 Polarization & Vesicular transport

Osteoclastic bone resorption involves demineralization of bone followed by degradation of the organic matrix by collagenolytic proteases. Bone resorption by osteoclasts is initiated by attachment of the cells to the sites to resorb on the bone matrix via integrin $\alpha_v\beta_3$ [15, 16] that recognizes the amino acid tripeptide -RGD- motif of bone phosphoproteins such as OPN and BSP. Transmission of outside-in signals through integrins leads to cell polarization and subsequent formation of four specialized membrane domains: sealing zone, ruffled border, basolateral domain and the functional

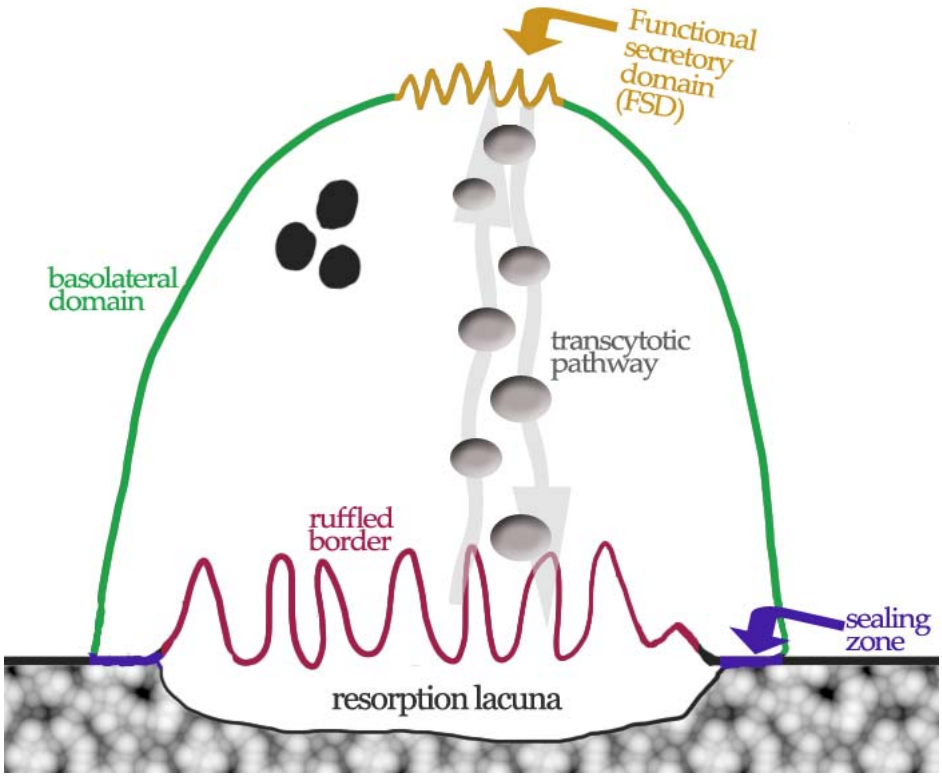

Figure 3. Functionally distinct membrane domains of a polarized osteoclast and the transcytotic pathway

secretory domain (FSD) [17-20] (***Fig. 3***). Establishment of a specific microenvironment between the underlying bone matrix and itself is the key to the resorptive activity of osteoclasts. This is achieved through formation of a sealing zone as a result of extensive re-organization of the cytoskeleton and formation of actin rings upon integrin binding

[19]. Once sealed, acidification of this isolated microenvironment called resorption lacuna via vectorial transport of hydrochloric acid by the V-ATPase and chloride transporter CIC-7 leads to dissolution of the inorganic matrix. This acidified mileu also provides the optimal environment for degradation of the organic matrix by proteases such as Ctsk and MMP9 delivered to the resorption lacuna by osteoclasts. The highly invaginated membrane facing the bone matrix of a resorbing osteoclast is called ruffled border, which is the product of the extensive fusion of acidified vesicles thought to be transported along microtubules [20]. The FSD is the central portion of the basolateral membrane and is thought to correspond to the apical membranes of epithelial cells. Degraded bone matrix products are endocytosed and transported through the transcytotic pathway and finally targeted to FSD and released to the bloodstream [21, 22]. Finally, elevated extracellular calcium levels in the resorption lacuna leads to the inhibition of bone resorption through osteoclast dysfunction and apoptosis [23, 24].

1.1.4.2.2.2 Cysteine proteinases and cathepsin K in bone resorption

Cysteine proteinases, which represent an essential group of the lysosomal protease repertoire, were implicated in bone resorption for the first time in 1980 when Delaisse et al. demonstrated that cysteine proteinase inhibitors can inhibit bone resorption in tissue culture and later in isolated osteoclasts [25-27]. Several members of this family have been shown to be expressed by osteoclasts [28]. However, cathepsin B and L were thought to be the main actors as both were shown to cleave in the telopeptide region of triple helical collagens [29], and due to their ubiquitous expression, attracted little attention as pharmacological targets. Later on, another cathepsin, named cathepsin K (Ctsk), was discovered and shown to be predominantly expressed by osteoclasts [30, 31].

We now know that Ctsk is a critical bone resorbing protease with unique ability of cutting within the triple helix of collagen type I as well as in the N- and C-terminal telopeptides at an acidic pH [31, 32], which in turn renders the collagen vulnerable to further degradation by other proteases. It is abundantly expressed by osteoclasts near the ruffled border membrane as well as in intracellular vesicles of the transcytotic pathway [33]. The inactive pro-form of the enzyme is autocatalytically activated before secretion into the resorption lacuna [34, 35]. Mutations in the Ctsk gene has been reported to be the causative factor behind a rare autosomal recessive osteosclerosing skeletal disorder called pycnodysostosis [36]. In patients with the disorder, large areas of demineralized nondigested bone matrix was observed adjacent to actively resorbing osteoclasts [37]. Later, generation of knockout [38-41] and transgenic mice [42, 43] further

confirmed the essential role of this enzyme in bone metabolism (see 2.2.1.1.). These findings rendered the enzyme as an attractive target for osteoporosis therapy. Several cathepsin K inhibitors have now passed preclinical studies and are currently in clinical trials at different stages of advancement.

Although the main substrates for Ctsk during bone resorption are the fibrillar types I and II collagen [31, 44], the non-collagenous matrix protein osteonectin[45] has been shown to be cleaved by Ctsk in vitro. Moreover, the osteoclast enzyme TRAP has also been shown to be a substrate for Ctsk (see 1.4.3.2.1).

1.1.4.2.3 Phenotypic heterogeneity of osteoclasts

A growing body of data in the literature suggests that osteoclasts of different bone types exhibit phenotypic differences [46]. For instance, studies on isolated osteoclasts seeded on bone in the presence or absence of inhibitors for cysteine proteinases (CPs) or matrix metalloproteinases (MMPs) demonstrated that calvarial osteoclasts employ both CPs and MMPs in bone resorption, whereas bone resorption in long bones depends primarily on CPs [47]. In line with these findings, structural assessment of long bones from MMP1 resistant collagen type I mouse revealed no major differences, whereas calvarial bones were much thicker in the mutant mouse [47]. Relevantly, significantly higher levels of cathepsin B, K and L activities were found in long bones compared to calvaria [47, 48].

The concept of osteoclast heterogeneity is also supported by circumstantial evidence from cases where osteopetrosis is bone-site specific. Saftig et al. reported that resorption defects observed in long bones of $Ctsk^{-/-}$ mice was not observed in calvarial bones [40]. Recently, it was demonstrated that $Ae2_{a,b}$-deficient mice (deficient in three of five isoforms of anion exchanger-2) exhibit osteopetrosis of long bones but not of calvaria, which was shown to express an additional sodium transporter (SLC4a4) [49], possibly compensating for the lack of Ae2.

Osteoclast heterogeneity appears to occur even within the same bone. Comparison of ultrastructural features of osteoclasts involved in cartilage breakdown at the epiphyseal/mataphyseal border with those of bone/cartilage-resorbing osteoclasts revealed that the former cells do not form ruffled borders and clear zones to the same extent as the latter [50]. These results suggest that those two populations of osteoclasts differ, not only with regard to site of resorption but also by mode of action. Recently, two different populations of osteclasts were identified in rat long bones differing in their size,

number of nuclei and adhesive ligand specificity [51]. Small osteoclasts with 3-4 nuclei showed weak resorption and adhered to both prothrombin and OPN, whereas large osteoclasts with 6-7 nuclei resorbed bone much more efficiently and adhered only to OPN. Based on the findings, the authors concluded that the small osteoclast may represent either an immature precursor to the large osteoclast or alternatively constitute a distinct population with a specific role in bone.

It is still not known if different osteoclast populations arise from different precursors or whether the matrix they resorb dictates the phenotypic behavior of the osteoclast. However, the observation that osteoclasts isolated from different sites retained their characteristics upon seeding on different types of bones, e.g. bovine skull or cortical bone, led to the hypothesis that functionally different subpopulations of osteoclasts originate from different sets of progenitors [47].

1.2 Cancer and Bone Metastasis

Cancer development is a multistep process of genetic alterations that drive the progressive transformation of normal cells into malignancy. The fundamental abnormality leading to cancer is dysregulated cell proliferation, differentiation and apoptosis. There is a general agreement that most cancers acquire the same set of functional capabilities during their development: self-sufficiency in growth signals, insensitivity to anti-growth signals, evading apoptosis, sustained angiogenesis, limitless replicative potential and tissue invasion & metastasis [52]. During the development of most cancers, certain cells of the primary tumor mass acquire the ability of invading adjacent tissues and subsequently leave the primary site to establish new colonies in a distant secondary organ. Metastasis of primary tumor cells to distant organs is the primary cause of cancer-related mortality in most cancer patients.

Bone is the third most common site of metastases, preceeded by only the liver and the lung. Advanced breast and prostate cancers have a high prevalence of bone metastases in the range of 60-70% [53]. Bone metastases are mainly classified as osteolytic (excessive bone resorption) and osteoblastic (excessive bone formation), based on their radiographic appearance [54]. In osteolytic lesions, PTH-rP produced by cancer cells indirectly induces osteoclastic bone resorption by increasing RANKL expression and inhibiting OPG secretion from osteoblasts. In turn, growth factors embedded in bone matrix, e.g. TGFβ are released and further stimulates bone resorption, creating a vicious cycle. Bone metastases from prostate cancer are predominantly osteoblastic,

whereas those of breast cancer can be either type, or a mixture of the two. Purely lytic bone lesions develop only in bone metastases of multiple myeloma. Regardless of the lesion type, the end result is a change in bone architecture predisposing the patient to a variety of skeletal complications such as fractures, life-threatening hypercalcemia and various nerve compression syndromes. Radiological methods are commonly used for diagnosis, treatment response and prognosis of the disease; however biochemical markers are also being evaluated as an alternative for these purposes. Current treatments of bone metastasis include chemotherapy, hormonal therapies, radiation, surgical interventions and biphosphonates with the aim of slowing the progression of this incurable disease.

1.3 Protein Sorting and Transport – The ER, Golgi, and Lysosomes

All eukaryotic cells possess a constitutive biosynthetic-secretory pathway which is responsible for the continuous delivery of plasma membrane proteins to the cell surface, excretion of certain soluble proteins to the extracellular space, as well as sorting of certain proteins to the vesicular compartments. Major components of this pathway include endoplasmic reticulum (ER), Golgi, endosomal/lysosomal compartments and secretory vesicles. Transport between different compartments is carried out by transport vesicles budding from one and fusing to another. Newly synthesized proteins enter the biosynthetic-secretory pathway via translocation to the ER from the cytosol. The pathway leads outward from the ER toward the Golgi apparatus and cell surface, with a side route leading to endosomal/lysosomal compartments. Thus, proteins translocated into the ER is either retained within the ER or transported to Golgi apparatus from where they are transported to lysosomes, the cell membrane, or the extracellular space via secretory vesicles.

The ER is composed of a network of membrane-enclosed tubules and sacs extending from the nuclear membrane throughout the cytoplasm. In the ER, oligosaccharide chains are attached to certain asparagine residues in the consensus sequence Asn-X-Ser/Thr (N-linked glycosylation) of the translocated proteins, and a quality-check process ensures that only properly folded and assembled proteins are allowed to the next destination, i.e. the Golgi apparatus. The Golgi consists of ordered stacks of membrane-enclosed cisternae. ER-derived molecules enter the cis-Golgi network (CGN) and exit from the trans-Golgi network (TGN). During their passage

through Golgi, proteins may undergo an ordered series of covalent modifications including further processing of N-linked oligosaccharides attached to the proteins in ER. Two major classes of N-linked glycans are found attached to most glycoproteins, i.e. complex-type oligosaccharides and high-mannose type oligosachharides. N-linked carbohydrate chains attached to proteins leaving ER for Golgi are of the high-mannose type. Complex-type chains are generated by a combination of trimming of these original high-mannose type oligosaccharides and the addition of further sugars such as galactose and sialic acid. This conversion only happens if the chains are accessible to the processing enzymes of the Golgi and might be influenced by the position of the glycan chain on the protein. Moreover, different glycoproteins can be modified to different extents in Golgi, depending on both the abundance of processing enzymes and the structure of the protein. Complex-type chains are resistant to attack by a highly specific exogenous endoglycosidase called EndoH and treatment with the enzyme is commonly used by cell biologists to distinguish between high-mannose and complex-type oligosaccharides. Proteins can also be modified by the addition of N-acetyl-galactosamine to certain serine and threonine residues followed by other carbohydrates such as galactose and sialic acid (O-linked glycosylation).

Golgi also functions as a sorting and dispatching station for the ER-derived molecules. All proteins passing through the Golgi, with the exception of resident ER and Golgi proteins, are sorted in the TGN to their final destination. Lysosomal hydrolases and lysosomal membrane proteins are tagged with a unique identifier molecule called mannose-6-phosphate (M6P), which is added in CGN. Recognition of these moieties in TGN by M6P receptors (M6PRs) results in the sorting of lysosomal proteins into late endosomal compartments that later mature into lysosomes. There are two types of M6PRs, the cation-dependent M6PR (CD-M6PR) and the cation-independent M6PR (CI-M6PR). Both types share the ability of delivering newly synthesized lysosomal enzymes with the M6P-tag to lysosomes. In addition to this shared function, the CI-M6PRs have been implicated in several other physiological processes. For instance, by taking a detour to the plasma membrane, they can capture certain growth factors such as IGF-II from the extracellular fluid and internalize for degradation in lysosomes. There is also evidence that some lysosomal enzymes are sorted to the lysosomes in a mannose 6-phosphate-independent manner. The proteins with no sorting signals are either secreted to extracellular space or incorporated into the cell membrane [55, 56].

1.4 Tartrate-Resistant Acid Phosphatase

1.4.1 Introduction

Purple acid phosphatases (PAPs) constitute a subclass of acid phosphatases (EC 3.1.3.2) and have been isolated from plants, animals, fungi and bacteria [57, 58]. The members of this enzyme family are characterized by a binuclear iron center and a light absorption maximum at 500-560 nm associated with a pink/purple color, hence the name `purple´acid phosphatase. The PAP family comprises both high and low Mw-forms. Homodimeric proteins of 110 kD are predominantly found in plants, wheras mammalian species express a monomeric PAP form of ~35 kD. Regarding their secondary structure and folding, the C-terminal domain of the plant PAPs exhibits similarities with mammalian PAPs, indicating a common genetic origin. A low Mw-type PAP with similar secondary structure to mammalian PAPs was also found in plants [59]. Moreover, a human gene product encoding a tartrate-resistant acid phosphatase distantly related to the plant PAPs was recently identified and characterized [60].

Mammalian PAP is referred to by multiple names, i.e. tartrate-resistant acid phosphatase (TRAP), type 5 acid phosphatase (Acp 5, EC 3.1.3.2) and uteroferrin. TRAPs are characterized by and distinguished from other mammalian acid phosphatases by their resistance to inhibition by tartrate, high isoelectric points and molecular weights [61, 62].

1.4.2 Gene

TRAP protein is encoded by a single gene (Acp5) in mammals [63]. Inter-species comparisons revealed an intron-exon structure consisting of five exons with the translation initiation site located at the beginning of exon 2. The gene is highly conserved through mammalian evolution [64-66]. In the mouse, three alternative non-coding first exons, denoted as 1A, 1B and 1C, have been identified and seems to be involved in tissue- and cell-restricted regulation of TRAP gene expression [65]. Human TRAP gene also exhibits a similar genomic structure as the mouse counterpart [65].

In osteoclasts, the gene promoter is synergistically regulated by several transcription factors including macrophage/osteoclast transcription factor PU.1[67], osteoclast commitment factor MITF [68, 69] and the master transcription factor of osteoclast gene regulation NFATc1[12]. Among the negative transcriptional regulators of

TRAP are the Myc oncoprotein [70] and Eos [71]. Other factors shown to be involved in Acp5 gene regulation are Yin Yang-1 (YY1) [72], as well as USF-1 and -2 [73] .

The gene promoter of pregnancy-associated uteroferrin has been shown to contain steroid hormone response elements [74]. Moreover, similar elements were found in mouse and human Acp5 genes [66]. TRAP expression was found to be induced by iron at the transcriptional level through iron regulatory elements in the TRAP promoter [64, 75-78].

Interestingly, DNA oligonucleotides and RNA molecules have recently been shown to increase TRAP expression through TLR-independent, yet unidentified mechanisms in cells of monocyte-macrophage lineage [79], suggesting a role for TRAP in innate immunity (see 1.4.7.2).

1.4.3 Protein

1.4.3.1 Amino acid sequence & 3D crystal structure

The mRNA transcript of rat TRAP predicts a protein of 327 amino acids, with an unglycosylated relative Mw of 34.35 kD. Of these, a signal peptide of 21 amino acids is removed, giving rise to a mature protein of 306 amino acids [80] ***(Fig. 4)***. At the protein level, comparison of the amino acid sequences from various mammalian sources indicated an overall identity of 89-94% [63, 80, 81].

TRAP enzyme is translated as a monomeric protein [82, 83] with a disulfide bond between Cys142 and Cys200 [84, 85]. The crystal structure of TRAP has been resolved by three independent groups [84-86] and revealed that the molecule exhibits a twofold symmetry with each half consisting of a βαβαβ motif. Two metal-binding domains in each site contain critical amino acid residues coordinating the diferric center [86, 87]. The enzyme contains a repressive loop domain of 20 amino acids (at amino acids 145-164 in uteroferrin and rat TRAP, and 143-162 in human TRAP) located in an exposed region in close proximity to the active site [85, 88] ***(Fig. 4)***.

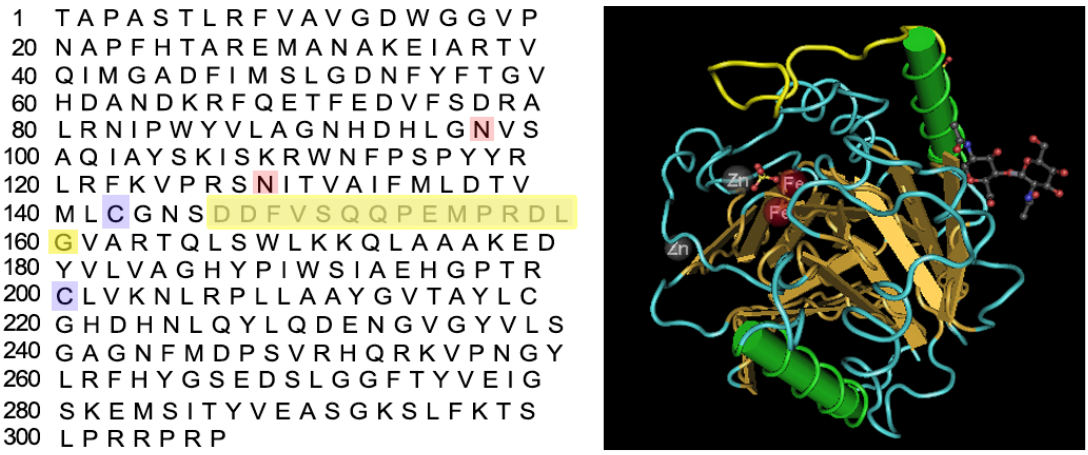

Figure 4. Amino acid sequence and 3D structure of rat TRAP. The loop sequence is marked as yellow both in the sequence and 3D structure. N-glycosylation sites and the cysteines forming the disulfide bond is marked as red and blue, respectively, in the sequence. 3D structure was obtained from PDB and modified using Cn3D, an open-source Java viewer.

1.4.3.2 Post-translational modifications

1.4.3.2.1 Proteolytic Processing

Although TRAP is translated as a monomeric protein with low activity [82, 83], it is isolated from various sources such as giant cell tumors[89], rat[80] and human[90] bones and bovine spleen[91, 92] as a proteolytically cleaved two-subunit form consisting of a 20-23 kDa N-terminal domain disulfide linked to a 16-17 kDa C-terminal domain. Moreover, absence of the loop sequence between Ser145 and Val161 observed in a crystallized rat TRAP preparation, suggested proteolytic removal of the loop region [84].

It is now clear that the two-subunit form is generated by proteolytic cleavage in the loop domain, that represses the active site by abolishing the interaction between the loop residue Asp146 and a ligand to the redox-sensitive iron of the active site, i.e. Asn91 or His92 [93-95]. Several proteinases of the serine proteinase family, e.g. trypsin [92, 94, 96] and chymotrypsin [92], have been shown to cleave at a single peptide bond in the repressive loop domain and moderately increase the enzymatic activity of TRAP in vitro. However, only cysteine proteinase family members, e.g. papain, cathepsin B, cathepsin L

and cathepsin K can make the enzyme reach similar specific activity as the TRAP purified from bone [83, 93, 97, 98], presumably due to complete excision of the loop domain.

Proteolytic processing of the protein is associated with an up to 10-fold increase in enzymatic activity and a shift to more basic pH optimum [83, 97]. Interestingly, proteolytic processing appears to increase the enzymatic activity through rendering the enzyme more susceptible to activation by reducdants [97, 98], suggesting that redox state and ambient pH of the enzyme´s microenvironment could be critical determinants of enzyme action in vivo.

1.4.3.2.2 Glycosylation

The mammalian TRAP sequence contains two putative N-glycosylation sites at Asn_{97} and Asn_{128}, both located in the N-terminal fragment of the two-subunit form [80]. Studies on crystal structures demonstrated substitution with oligosaccharides only at Asn_{97} [84, 85, 88]. However, uteroferrin secreted by endometrial explants [99] and TRAP expressed by Sf9 cells [100] were shown to contain two N-linked oligosaccharides. TRAP purified from bovine spleen was also shown to carry two non-sialylated oligosaccharide chains per molecule [92], suggesting an oligosaccharide at Asn_{128} may also be present but not detected in the crystallized proteins. Regarding the nature of the attached glycans, both high-mannose type and multi-antennary complex type oligosaccharide chains have been reported to substitute these sites [99, 101-103].

Interestingly, catalytic properties of the enzyme were affected by the extent of glycosylation as mutants lacking either glycosylation site exhibit a lower substrate affinity and catalytic activity [100], indicating that N-glycans could influence the interaction of the repressive loop domain with active site. Other suggested functions for N-linked glycans include promoting uptake of uteroferrin by reticuloendothelial cells of the fetal liver [102] and a role in the overall stability of the molecule [100]. Intriguingly, recombinant TRAP expressed in E. Coli, devoid of N-linked glycans, display altered conformation of the loop domain [86].

As TRAP localization has been reported in a plethora of subcellular compartments (see 1.4.6), N-glycans are likely to be involved in the proper targeting of the enzyme to various subcellular locations as well.

1.4.3.3 Enzyme Activity

1.4.3.3.1 Phosphatase activity

TRAP has been shown to catalyze the hydrolysis of a variety of natural and synthetic phosphate esters with a preference for aromatic phosphomonoesters and pyrophosphate under acidic conditions [89, 91, 104, 105]. TRAP can also hydrolyze phosphotyrosine and phosphotyrosine-containing peptides in vitro [83, 106-108].

Despite being unable to use phosphoserine or phosphoserine-containing peptides as substrates [107], purified TRAP can dephosphorylate acidic phosphoserine-containing bone phosphoproteins such as OPN, BSP and casein at high efficiency [83, 109]. The ability of TRAP to dephosphorylate phosphoserines only in the context of a protein such as OPN provides a compelling circumstantial support for an in vivo relation between these proteins.

TRAP has also been shown to be able to dephosphorylate the recognition marker of lysosomal enzymes, i.e. mannose-6-phosphate, and experimental evidence supports an in vivo role for this function (see 1.4.7.3.3). Among the other potential natural substrates for TRAP are nucleotide di- and tri-phosphates [110, 111].

Mild reducing agents such as β-mercaptoethanol, dithiotreitol, ascorbate, and iron in combination with ascorbate can enhance[89-91, 112-114] , whereas inorganic phosphates and tetrahedral oxyanions such as molybdate, vanadate, tungstate and arsenate inhibits [89, 114-116] phosphatase activity of TRAP.

1.4.3.3.2 Reactive Oxygen Species (ROS) generation

In addition to its phosphatase activity, the diferric active site of TRAP is also capable of generating reactive oxygen species (ROS) through the Fenton reaction [117, 118], where the Fe^{2+} ion reacts with hydrogen peroxide to generate highly reactive hydroxyl radicals in the continuos presence of hydrogen peroxide.

Unlike phosphatase activity, ROS-generating activity of TRAP is optimal at neutral pH. Moreover, these two activities appear to be functionally independent as site-directed mutagenesis of His_{92} and His_{195} of rat recombinant TRAP severely inhibited phosphatase activity while ROS-generating activity remained unaffected [119], suggesting that TRAP can act as a Fenton catalyst.

1.4.4 Tissue and Cell Expression

1.4.4.1 Physiological conditions

Abundant TRAP expression, at both the mRNA and protein levels have been reported in bone, liver, spleen, the linings of gastrointestinal tract, lung, thymus and skin [65, 77, 98, 120-122], whereas trace amounts were detected in testis, muscle and heart [120].

In bone, it is widely accepted that TRAP expression is restricted to osteoclasts [123], although a few studies reported TRAP expression in osteoblasts and osteocytes [124, 125]. In lung and liver, tissue-specific macrophages, i.e. alveolar macrophages [126] and Kupffer cells [127], have been shown to be the primary cell types expressing TRAP. In most other tissues, TRAP expression is primarily due to widely distributed dendritic cells [120]. Apart from the cells of the myeloid cell lineage, certain epithelial cells have been shown to express TRAP as well. A well-known example is uteroferrin which is expressed and secreted by porcine endometrium [128, 129]. Intriguingly, TRAP expression has been reported in epithelioid cells of human placenta as well [130]. In lung, significant expression of monomeric form was noted in certain bronchiolar epithelial cells [98]. TRAP protein expression was also detected in intestinal epithelia, respiratory epithelial cells resembling Clara cells in the bronchiolar epithelium of lungs and distal tubular epithelium in kidney [98]. Superficial epithelial cells of human urothelium also exhibit strong expression of TRAP [131]. Other epithelial cell types shown to express TRAP are hepatocytes and keratinocytes [120].

Interestingly, TRAP expression declines with age in bone and liver, whereas in lymphoid organs such as spleen and thymus, the expression is constant or even increased [98]. Declined TRAP expression in bone has been attributed to diminishing numbers of osteoclasts as bone growth/modeling is reduced with age.

Another cell type shown to express TRAP is neurons. TRAP protein was detected in the cytoplasm of neuronal cell bodies in certain areas of both the central and the peripheral nervous system in rats [132].

1.4.4.2 Pathological conditions

TRAP has been used as a diagnostic marker for hairy cell leukemia for over four decades. Moreover, elevated TRAP expressions have been employed in histochemical characterizations of other pathological conditions such as Gaucher´s disease [133] and osteoclastoma [89]. Moreover, elevated serum concentrations of TRAP were reported for

Paget´s disease [134], hyperparathyroidism [135] rheumatoid arthritis [136] and chronic kidney disease .

Patients with bone metastases of breast cancer and multiple myeloma exhibit elevated levels of TRAP activity in their serum [137-139], mostly attributed to increased osteoclast activity. Intriguingly, elevated levels of serum TRAP were reported in patients with breast and ovarian cancer, even in the absence of metastatic bone disease[140], suggesting TRAP is expressed and secreted by tumor cells as well. Notably, several human breast cancer cell lines and tissues express TRAP and the intensity of histochemical activity has been reported to correlate with degree of tumorigenicity [140, 141].

1.4.5 Isoforms 5a and 5b

TRAP exists as two isoforms in human serum, denoted as TRAP 5a and 5b. These isoforms were first identified by their differential electrophoretic migration on polyacrylamide gels [142], which was due to the presence of sialic acid residues on TRAP 5a [143]. Further biochemical characterization of the isoforms revealed that TRAP 5a and 5b had different pH optima, i.e. 5.2 and 5.8, respectively [142]. Moreover, they exhibit differential substrate preferences for synthetic substrates [144, 145] and are differentially inhibited by heparin [146]. However, the major structural difference between TRAP 5a and 5b appears to be the proteolytic processing of the repressive loop domain [147]. TRAP 5a is an uncleaved monomer whereas TRAP 5b is a proteolytically processed two-subunit form, explaining the different kinetic properties of the two isoforms (see 1.4.3.2.1). A comparison of the major structural and biochemical properties of TRAP 5a and 5b can be found in ***Table 1***.

High abundance of uteroferrin, a momomeric TRAP enzyme with low phosphatase activity and low pH optimum [148], is present in allantoic fluid of pregnant pigs and in culture supernatant of porcine endometrial explants. Moreover, the majority of circulating TRAP in human serum is monomeric isoform 5a with low pH optimum and phosphatase activity (***Table 1***) and TRAP 5a and 5b has been shown to contain distinct sugar moieties [101]. These findings overall suggest that monomeric 5a may be a mature protein with extracellular functions distinct from those of cleaved 5b.

Table 1. Comparison of TRAP 5a and 5b. Reproduced from [149].

	TRAP 5a	TRAP 5b
Protein structure	Monomeric	Proteolytically processed di-sulfide linked two-subunit
Specific activity	<200 U/mg	>1000 U/mg
pH optimum	5.0 – 5.2	5.8 – 6.0
Sialic acid	+	-
Abundance in human serum [150]	• 80-90% of total TRAP protein • ~50% of total TRAP activity	• 10-20% of total TRAP protein • ~50% of total TRAP activity
Expression	• Secreted to resorption lacuna by osteoclasts. • Secreted by macrophages and dendritic cells in vitro.	• Secreted to blood circulation through FSD by osteoclasts • Retained intracellularly by macrophages and dendritic cells.

1.4.6 Intracellular localization / Secretion

Immunofluoresence costaining studies on isolated osteoclasts cultured on bone demonstrated that TRAP colocalizes with endocytosed bone matrix products and cathepsin K in transcytotic vesicles [33, 118]. Moreover, TRAP has been shown to colocalize with Rab9, a GTPase known to be involved in the vesicular trafficking between late endosomes and trans-Golgi network [33]. In these in vitro studies, TRAP was not detected in resorption lacuna or at the ruffled border area. Ultrastructural immunohistochemistry on bone sections, on the other hand, detected TRAP in intracellular cytoplasmic vesicles, vacuoles and small dense granules in the mitochondria-rich part of the cytoplasm as well as ruffled border and resorption lacuna [97, 151-153]. The reason for this discrepancy over TRAP localization in resorption area is still not clear but could be due to differences in the microenvironment between in vivo and in vitro culture settings [154]. However, several lines of evidence support the secretion of TRAP to the resorption lacuna. Firstly, ultrastructural immunohistochemistry of TRAP on bone sections from TRAP$^{-/-}$ mice was negative in contrast to wildtype mice, confirming the specificity of TRAP staining in resorption area [154]. Secondly, in animal models with impaired secretory pathway in osteoclasts, e.g. ia/ia rat mutants, TRAP accumulates in intracellular vesicles whereas enzyme was not detected in extracellular matrix [155, 156]. A loss-of-function mutation in

Plekhm1 resulting in a truncated protein was recently shown to underlie the phenotype of ia/ia rats [157]. Plekhm1 encodes a non-secretory adaptor protein that localizes to endosomal vesicles and appears to have a role in vesicular acidification in osteoclasts. Mutations in the human homologue of the gene, i.e. PLEKHM1, was also associated with an osteopetrotic phenotype with altered TRAP secretion, indicating a role for the protein in TRAP secretion in osteoclasts [158].

Later ultrastructural immunohistochemistry studies employing monomeric TRAP (TRAP 5a)-specific antibodies revealed that monomeric TRAP was transported in the secretory pathway and secreted in the ruffled border area whereas the proteolytically processed TRAP (TRAP 5b) was the predominant isoform in phagocytic vacuoles and bone matrix [97]. This finding, combined with the detection of co-localization of TRAP with Ctsk in resorption area, led to the hypothesis that Ctsk cleaves and activates monomeric 5a in the resorption lacuna. The active 5b form is then partly internalized during matrix degradation and released from FSD to the circulation [97]. These findings are in accordance with the study of Janckila et al. [159] where the authors demonstrated that cultured osteoclasts secrete both TRAP 5a and 5b to the media, with the predominance of the latter. Both isoforms were also present intracellularly.

In macrophages, TRAP is partially colocalized with late endosomal/early lysosomal Rab7-positive vesicles, lysosomal marker Lamp1, antigenic-peptide binding MHC II molecules and phagocytosed S. Aureus [120, 160]. In immature dendritic cells, TRAP colocalized with lysosomal membrane glycoprotein CD63, whereas in mature cells TRAP remained intracellular while CD63 was translocated to the cell surface. Macrophages and dendritic cells have been shown to preferentially secrete TRAP 5a while retaining TRAP 5b intracellularly [159, 161].

As touched upon in section 1.4.4.1., the monomeric TRAP enzyme, uteroferrin is produced and secreted in large amounts by porcine endometrium during pregnancy. Glycosylation analysis revealed that the enzyme carries the lysosomal recognition marker mannose-6-phosphate [103, 162]. However, mannose-6-phosphate is covered by N-acetylglucosamine, thereby not recognized by mannose-6-phosphate receptor, resulting in the secretion of the enzyme to the extracellular fluid [103].

The mechanisms of differential compartmentalization of TRAP isoforms in macrophages, dendritic cells and osteoclasts is still largely unknown.

1.4.7 Proposed Functions

1.4.7.1 Involvement in bone metabolism

Osteoclasts are characterized by high expression of TRAP and the enzyme has long been used as a histochemical marker for osteoclasts [123, 163]. Although, to date no skeletal disorder has been linked to mutations in TRAP in humans, several lines of evidence support a role for TRAP in osteoclastic bone resorption. TRAP activity accumulates in the culture medium upon culturing osteoclasts on bone slices and neutralizing antibodies towards TRAP have been shown to reduce bone resorption in osteoclast cultures [164]. Moreover, TRAP-deficient mice exhibit mild osteopetrosis and increased mineral density of the axial and appendicular skeleton as well as disrupted endochondral ossification [165]. Transgenic mice overexpressing TRAP, on the other hand, were found to be mildly osteoporotic associated with a decreased trabecular density and increased bone turnover in long bones [166]. These data collectively indicate a role for TRAP in bone resorption and turnover, however the exact mechanisms of action is still not delineated.

1.4.7.1.1 Regulation of bone phosphoprotein bioactivity

The TRAP substrates bone phosphoproteins OPN and BSP contain an RGD motif and are involved in attachment of the osteoclast to the bone surface via its $\alpha_v\beta 3$ integrins [167, 168]. TRAP has been shown to co-localize with both proteins in the resorption lacuna [169]. However, only OPN has been shown to be concentrated in bone opposite the clear zone of osteoclasts, marking sites for osteoclast attachment, and in vivo observations did not support a major role for BSP in osteoclast binding [169]. Ek-Rylander et al. [109] demonstrated that removal of phosphate groups from OPN by TRAP was associated with decreased osteoclast binding to the protein. A recent study demonstrated that the presence of neutralizing antibodies to TRAP inhibited osteoclast migration on phosphorylated OPN, indicating that the process is regulated by endogenous TRAP [170]. Moreover, TRAP-overexpressing mouse osteoclasts migrated faster on bovine milk OPN than their wildtype counterparts (***Fig. 5***). Furthermore, both phosphorylated OPN[171] and TRAP [165] increased bone resorption in pit resorption assays, which is in support of the proposed interaction between the two proteins in the promotion of bone resorption. These findings overall led to the hypothesis that the main biological role of the

phosphatase activity of TRAP is to regulate bioactivity of OPN via dephosphorylation [154].

TRAP has also been proposed to contribute to the degradation of OPN during bone matrix digestion, as accumulation of OPN adjacent to actively resorbing osteoclasts was observed in TRAP-deficient mice [48, 172]. It was also proposed that degradation of phosphoproteins of bone matrix by dephosphorylation may represent a preliminary stage in bone matrix degradation which in turn allows proteinases to access and act upon these proteins more easily [173].

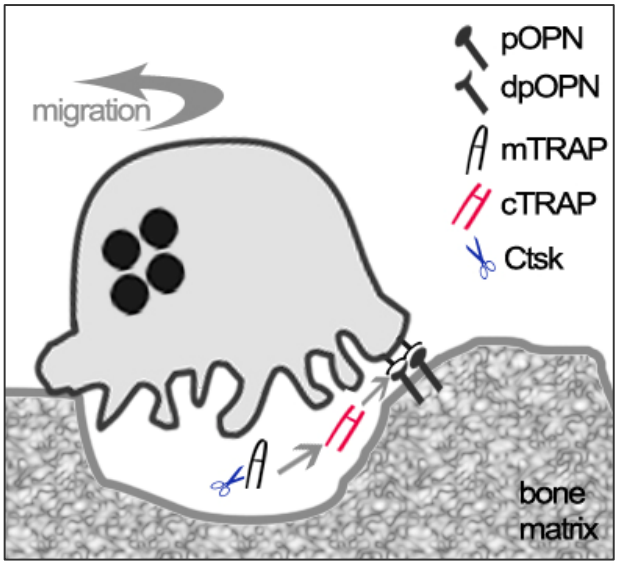

Figure 5. Role of TRAP in osteoclast migration through dephosphorylation of OPN. Monomeric TRAP (mTRAP) is secreted to the resorption lacuna, processed by cathepsin K (Ctsk) to cleaved TRAP (cTRAP) and activated. cTRAP dephosphorylates OPN (pOPN). Decreased affinity of dephosphorylated OPN (dpOPN) for $\alpha_v\beta3$ integrins leads to the release of the osteoclast from the bone surface and migration of the osteoclasts is promoted.

1.4.7.1.2 Intracellular bone matrix degradation

Observation of intracellular accumulation of vesicles containing filamentous material in TRAP-deficient osteoclasts [172] led to the hypothesis that these osteoclasts are unable to digest the internalized bone degradation products due to lack of TRAP-generated ROS. In support of this hypothesis was the finding that ROS generated by TRAP are capable of degrading type I collagen in vitro [118]. Moreover, Ctsk digestion increased the ROS generating activity of TRAP [33] and TRAP co-localized with Ctsk and bone degradation products in the transcytotic vesicles where further degradation of endocytosed material was proposed to take place [33]. The hypothesis is further supported by the observation that some transcytotic vesicles exhibit neutral pH, which favors ROS-generating activity of TRAP over its phosphatase activity [33].

1.4.7.1.3 Bone collagen metabolism

Investigation of structural and functional properties of bone collagen in TRAP-deficient mice demonstrated an abnormal type I collagen and mineral metabolism in

bone in the absence of TRAP [174]. These defects were associated with altered resistance to fracture due to mechanical stress, implicating a role for TRAP in correct formation and functioning of intact collagen in the skeletal system.

1.4.7.1.4 Participation in intracellular vesicular transport

The dimension of the ruffled border is thought to be determined by regulated fusion and retrieval of vesicular membranes [175]. Ultrastructural examination revealed that the relative area of ruffled borders is increased in osteoclasts from TRAP-deficient mice [176]. This, along with the observation of intracellular accumulation of vesicles in mutant osteoclasts [172, 176] may point to a novel function for TRAP in modulating intracellular vesicular transport in osteoclasts.

1.4.7.1.5 Coupling factor between osteoclasts and osteoblasts

TRAP has been advocated as a growth and differentiation factor for osteoblasts. Sheu et al. [177] demonstrated that TRAP activation of TGF-β receptor interacting protein (TRIP-1) evokes a TGF-β-like differentiation process in osteoblasts, which in turn increases the expression and activity of several osteoblast markers. In the same study, TRAP-containing osteoclast lacuna was shown to drive osteoblast cultured on it towards differentiation into a mature bone-forming phenotype. Thus, it was hypothesized that TRAP could be a coupling factor between osteoclasts and osteoblasts.

1.4.7.2 Involvement in immune responses

Given the widespread expression of TRAP in cells of monocyte/macrophage family, it is not surprising that several roles have been proposed for TRAP in the immune system.

1.4.7.2.1 Dendritic cell maturation

Human dendritic cells upregulate their TRAP expression upon induction of maturation by LPS, suggesting a role for TRAP in the immune response [122]. In support of this finding, dendritic cells from TRAP-deficient mice display impaired maturation and defective Th1 responses [178]. LPS exhibited reduced ability in upregulating the expression of MHCII and CD80 in TRAP-deficient dendritic cells. Moreover, the cells

showed an altered cytokine secretion profile, indicating an important role for TRAP in the process of dendritic cell maturation.

1.4.7.2.2 Pathogen clearance

TRAP-deficient mice exhibit signs of impaired macrophage function including altered cytokine secretion profile and reduced clearance of the microbial pathogen *S. aureus* after intraperitoneal inoculation [179]. Relevantly, recombinant TRAP in the presence of hydrogen peroxide was shown to be more efficient in killing bacteria than hydrogen peroxide alone [180]. Moreover, in TRAP-overexpressing mice, the macrophages displayed increased production of free radicals, associated with an increased capacity for bacterial killing [180]. However, TRAP-deficient peritoneal macrophages and neutrophils are able to normally phagocytose and kill the bacteria in vitro, suggesting that TRAP acts by influencing recruitment of macrophages to the site of microbial invasion rather than participating directly in degradation of bacteria.

1.4.7.2.3 Antigen processing and presentation

In alveolar macrophages, TRAP colocalize with antigenic-peptide binding MHC II molecules and phagocytosed S. Aureus [160]. TRAP, by virtue of its ROS-generating activity, has been proposed to be involved in antigen processing in the antigen presentation route of activated macrophages [118]. Relevantly, antigen presentation pathway of activated macrophages is analogous to the transcytotic pathway in osteoclasts (see 1.4.6).

It has also been proposed that TRAP may be an important regulator of OPN bioactivity in the immune system [179].

1.4.7.3 Miscellaneous

1.4.7.3.1 Growth Factor

TRAP has been advocated as a growth and differentiation factor in adipocytes as well as in osteoblasts (see 1.4.7.1.5), both of which are of mesenchymal origin. A substrain of TRAP-overexpressing mice was shown to display early onset obesity [181]. The obesity was found to be due to adipose tissue hyperplasia accompanied by a low-grade inflammatory reaction in the adipose tissue. Monomeric TRAP, but not the cleaved isoform, secreted from adipose tissue macrophages was suggested to induce the hyperplastic obesity with normal adipocyte lipid metabolism and insulin sensitivity.

1.4.7.3.2 Iron transport

Uteroferrin in pig uterus was suggested to serve as an iron transporter between the mother and the developing fetus [129, 182].

1.4.7.3.3 Lysosomal enzyme targeting

A role for TRAP in dephosphorylation of the M6P lysosomal targeting marker was first proposed by Bresciani et al.[183] through in vitro studies of uteroferrin. However, fibroblasts from mice doubly deficient in TRAP and LAP (lysosomal acid phosphatase) were shown to retain their ability of dephosphorylating endocytosed arylsulfatase A [172], suggesting that neither enzyme plays a role in dephosphorylation of lysosomal proteins. This view is challenged by a recent cell-based study disclosing that TRAP is essential for the removal M6P marker [184]. This apparent discrepancy might simply be reflecting selectivity of TRAP for certain lysosomal proteins in removal of the M6P marker. Whether TRAP could have a similar role in osteoclasts remains an intriguing question.

1.4.7.3.4 Participation in epithelial cell migration

A siRNA screening approach performed as a systematic analysis of genes regulating epithelial cell migration identified Acp5 as one of the genes whose knockdown impaired the migration of epithelial cells [185]. During a wound-healing assay, TRAP-deficient epithelial cells exhibited abnormally stretched morphology and nuclear rotation, suggesting a role for TRAP in polarization of the microtubule organizing center. The exact mechanism behind this role still remains to be elucidated.

1.4.8 Clinical significance

1.4.8.1 TRAP as a histochemical marker

In an attempt to develop clinically useful cytochemical markers for blood cells, non-denaturing acidic polyacrylamide gel analysis seperated the leukocytic acid phosphatases into seven different types, one of which was type 5 TRAP [61, 186]. Among blood-derived cells, only leukaemic reticuloendotheliotic cells, also known as leukemic hairy cells (HCs) showed almost exclusive expression of TRAP [61]. This finding led to the development of a cytochemical test based on the detection of TRAP activity [187] for the disease which is, after 40 years, still used in the diagnosis of the disease.

1.4.8.2 TRAP as a serological marker

Development of an antiserum towards TRAP purified from a human spleen affected by HC led to the finding that TRAP expressed by HCs was also highly expressed in osteoclasts [188] and TRAP activity in human serum can be used as a serological marker of osteoclast function, i.e. bone resorption [123]. Soon, it was shown that TRAP activity in serum was elevated in normal growing children compared to those of adults [142, 189] as well as in patients with a plethora of metastatic [106, 190] and metabolic [106, 135, 191-193] bone diseases. Further detailed characterization of the enzyme revealed that it exists as two different isoforms in human serum, the bone-derived 5b and the non-bone-derived 5a (see 1.4.5.). As mentioned in section 1.4.6, the two isoforms appears to be differentially expressed/secreted/compartmentalized by different cell types of the monocyte/macrophage family. Thus, the distinction of TRAP 5a and 5b is becoming increasingly important since growing lines of evidence indicate these two isoforms possess unique properties and are of different clinical significance.

1.4.8.2.1 Development of TRAP immunoassays

Early biochemical assays measuring serum TRAP activity were not sufficiently specific to use as a marker of bone metabolism due to the presence of non-bone-derived TRAP activity in serum. The production of monoclonal antibodies for TRAP made it possible to develop more sensitive assays based on two-site sandwich enzyme immunoassay principle [194-198]. Proving the clinical sensitivity of the assays, TRAP activity in serum was shown to be significantly elevated in postmenopausal women, patients with Gaucher´s disease, and patients with secondary hyperparathyroidisim, all known to be associated with increased bone resorption.

The sensitivity of the assays for TRAP 5bwas further improved by development of assay conditions where interference by TRAP 5a was significantly reduced through use of:

1) Substrates with greater specificity for TRAP 5b [199]
2) Heparin as a specific 5a inhibitor [146]
3) pH conditions favoring 5b activity over that of 5a [150, 200, 201].

Two different immunoassays for TRAP 5b activity based on the above-mentioned principles have been commercialized with the brand names BoneTRAP® and Metra TRAP5b®. A recent comparison of these two commercial assays revealed no difference in clinical sensitivity between the two [202].

In addition to immunoassays for TRAP 5b, immunoassays for TRAP 5a have also been developed through the use of 5a-specific monoclonal antibodies [136], although not made commercially available yet.

1.4.8.2.2 TRAP 5b as a marker of osteoclast number and osteoclastic activity

As mentioned previously in section 1.4.6., TRAP 5b is the predominant isoform secreted by osteoclasts [161]. Elevated serum TRAP 5b activity has been observed in various physiological and pathological conditions associated with increased bone resorption including metastatic bone diseases, hyperparathyroidism, osteoporosis, Paget´s and Gaucher´s diseases [194, 203]. Evaluation of TRAP 5b assays in bone diseases revealed that serum TRAP 5b activity strongly correlates with collagen markers of bone resorption [204]. Moreover, TRAP 5b levels showed a specific and significant response to anti-resorptive treatments [200], proving the usefullness of TRAP 5b in the assessment of metabolic status of bone in a variety of pathological conditions. There is also evidence that serum TRAP 5b can be used in the assessment of fracture risk, as increased levels of baseline serum TRAP 5b was shown to be associated with increased risk of vertebral fracture in elderly women [205].

Not surprisingly, observations of elevated serum TRAP activity in conditions where bone resorption is augmented led to the assumption that TRAP activity is positively correlated with bone resorption activity. However, serum TRAP 5b activity has also been shown to be elevated in malignant infantile osteopetrosis[206] and type II autosomal dominant osteopetrosis [207], conditions where osteoclast function is compromised. Alatalo et al. [208] reported a direct correlation between TRAP 5b activity released by non-resorbing osteoclasts cultured on plastic and the number of osteoclasts formed. These findings formed the basis for the notion that TRAP 5b activity is a marker of systemic osteoclast number rather than bone resorption. CTX is a marker of systemic bone resorption and Rissanen et al. [209] recently proposed the use of CTX/TRAP 5b ratio as an estimate of the bone resorption activity per osteoclast.

1.4.8.2.3 TRAP 5a as a marker of chronic inflammation

Janckila et al.[150] found that TRAP activity and TRAP protein did not correlate in serum of rheumatoid arthritis (RA) patients. These patients exhibited elevated serum TRAP protein but normal TRAP activity. Further analysis of the serum samples revealed that elevated TRAP protein in serum from RA patients was due to isoform 5a, explaining

the unchanging TRAP activity as 5a isoforms has a much lower specific activity compared to 5b [210]. Moreover, it was recently shown that TRAP 5a protein is elevated significantly in patients on maintenance hemodialysis, which is known to be associated with chronic inflammation [211]. In light of the previous observations that TRAP 5a is abundantly secreted by macrophages in vitro, these findings led to the hypothesis that serum TRAP 5a is derived from inflammatory macrophages and reflect the systemic burden of inflammatory macrophages and chronic inflammation.

2. PRESENT INVESTIGATION

2.1 Aims of the Investigation

TRAP 5a and 5b isoforms differ in post-translational modifications including proteolytic processing of the repressive loop domain and carbohydrate content. Phosphatase and ROS-generating activities of TRAP are differentially affected by proteolytic processing suggesting that regulation of proteolytic processing can be employed for differential use of these two activites. Moreover, these two isoforms are compartmentalized differently by different cell types and correlate independently with different human diseases. There is also evidence suggesting that the repressive loop domain not only affects the kinetic properties of the enzyme but could also take a non-enzymatic role as an essential structural domain of monomeric TRAP acting as a growth/differentiation factor (see 1.4.7.3.1).

The major aim of this thesis was to characterize biogeneration of these two isoforms through proteolytic cleavage and differential intracellular sorting, and their differential expression in physiological and pathological settings. This aim has been divided into sub-aims:

- In vivo assessment of the involvement of cathepsin K in the generation of TRAP 5b in osteoclasts.
- To investigate the mechanisms of biogeneration and intracellular routing of TRAP 5a and 5b in a TRAP-overexpressing human epithelial cell line.
- To investigate the relative expression of TRAP 5a and 5b in osteoclast populations of different bone types, in relation to cathepsin K expression.
- To investigate the differential expression of TRAP 5a and 5b by constituent cell types in breast cancer bone metastasis tissue.

2.2 Comments on methodology

2.2.1 Animal & Cell models used in the investigation

2.2.1.1 Cathepsin K knock-out mice

Ctsk-deficient mice were generated as previously reported[39]. Mutations in the Ctsk gene has been reported to be the causative factor behind a rare autosomal recessive osteosclerosing skeletal disorder called pycnodysostosis. Ctsk-deficient mice, generated by several groups independently [38-41], is a phenocopy of this disorder in several aspects. They display osteopetrosis of the long bones and vertebrae and abnormal joint morphology [38, 40]. They also exhibit splenomegaly and reduced bone marrow cellularity [38]. Although osteoclasts appear to be fully differentiated, they demonstrate an altered ultrastructural appearance with incomplete ruffled border morphology accompanied by an impaired resorptive activity in vitro [40]. Increase in the RANKL/OPG ratio has been reported to compensate for the impaired bone resorption in these mice through enhancing osteoclastogenesis and expression of other proteases [39].

2.2.1.2 RAW264.7 cell line

Identification of the essential roles of RANKL and M-CSF in osteoclast differentiation [212, 213] has revolutionized the field of osteoclast biology by allowing to generate osteoclasts by culturing bone marrow-derived precursor cells in the presence of recombinant M-CSF and RANKL. RAW264.7 is a well-established mouse macrophage cell line transformed by Abelson leukemia virus. These cells express M-CSF continuously, and thus can be easily differentiated into osteoclast-like cells in the presence of soluble RANKL alone [214]. Phenotypically, RANKL-differentiated multinucleated osteoclast-like RAW264.7 cells exhibit high expression of typical osteoclast markers such as TRAP, cathepsin K and CTR and readily form resorption lacuna on bone slices, an essential test in the evaluation of osteoclastic phenotype of multinuclated TRAP-positive cells [214]. Advantages such as relatively easy transfectability and being able to generate multinucleated osteoclast-like cells in a relatively short time have led to widespread use of this cell line in studies of osteoclastogenesis as well as osteoclast activation and function.

2.2.1.3 MDA-c11 : TRAP-overexpressing MDA-MB-231 cell line

In the current investigation, the rationale behind opting for MDA-MB-231, a human breast cancer epithelial cell line, to study the biogenesis of TRAP isoforms monomeric 5a and cleaved 5b was severalfold:

- Glandular epithelium of porcine endometrium was reported to exhibit exclusive secretion of monomeric 5a form of TRAP, i.e. uteroferrin [129]. Also evidence was presented showing that epithelial cells of breast carcinoma retain cleaved TRAP 5b intracellularly [141]. Thus, we reasoned that an epithelial cell model could provide a simple, yet physiologically relevant model for the investigation of TRAP biogenesis, as two isoforms appeared to be distinctly routed and compartmentalized in epithelial cells. This is in contrast to osteoclasts and macrophages where both isoforms were present both intracellularly and in conditioned media (see 1.4.6).
- As a cell line derived from human, where TRAP 5a and 5b denotation was established, physiological relevance of MDA-MB-231 to the purpose of the study was better fulfilled.
- As overexpression of TRAP was detected in the cells of various different tumors of epithelial origin and advocated to be a marker of progression of malignancy [140, 141], characterization of TRAP expression in this cell line might have implications for studies of TRAP in cancer.
- Unlike other breast cancer cell lines which were shown to express low levels of TRAP activity [141], pilot studies confirmed that MDA-MB-231 cells do not express significant levels of endogenous TRAP, ensuring that TRAP protein in the cells are derived exclusively from the transfected gene.

2.2.2 Chromatographic separation of TRAP isoforms with Heparin ion-exchange FPLC

Igarashi et al. [215] was the first to demonstrate that heparin column chromatography can be used to separate TRAP 5a and 5b from human serum. The authors attributed the differential heparin binding properties of 5a and 5b to the presence of sialic acid on TRAP 5a. Later, the technique was shown to efficiently separate the monomeric and cleaved isoforms of recombinant rat TRAP expressed by Sf9 cells [100]. The absence of sialic acid residues on both forms, demonstrated by a lack of reaction with sialic acid-recognizing lectins [100], suggested that properties other than the

presence of sialic acid could be the determinant in differential separation of cleaved and monomeric TRAP isoforms by Heparin chromatography.

Running bone-derived TRAP as well as Ctsk and Ctsl-cleaved recombinant *rat* TRAP on Heparin FPLC generates three different TRAP activity peaks [97]; one for monomeric TRAP and two for differentially-cleaved TRAP forms. The presence of differentially-cleaved forms was due to sequential proteolytic processing of TRAP by cathepsin K and L. Proteolytic processing of TRAP by cathepsin K and L takes place in a stepwise manner and proceeds through an intermediate fragment that could be separated from the final cleavage product by using heparin FPLC. For cathepsin K, these two forms differed by only two amino acids in the N-terminus of the small fragment, which was sufficient to substantially alter the affinity of TRAP to heparin. From these studies, altered surface charge properties upon proteolytic cleavage appears to be the main determinant of differential separation of TRAP isoforms by Heparin FPLC, and Heparin chromatography can be employed to differentially separate TRAP cleavage isoforms.

In ***paper I*** *of* the present investigation where *mouse* was used as a model organism, Heparin FPLC was also used to separate TRAP fragments. Prior to starting experiments, it was confirmed that mouse recombinant TRAP digested with Ctsk *in vitro*

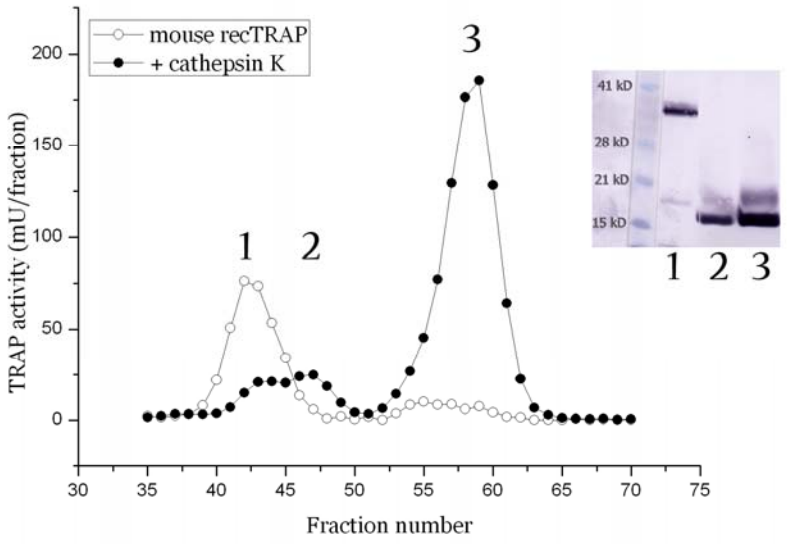

Figure 6.
Heparin FPLC elution profile of *mouse* recombinant TRAP after digestion with cathepsin K.

elutes in three separable peaks, similar to rat TRAP (***Fig. 6***).

2.2.3 Antibodies recognizing TRAP

Two polyclonal antibodies recognizing monomeric and total, i.e. both monomeric and two-subunit form, were used in the present investigation.

The antibody recognizing *total TRAP* was made in rabbits against the recombinant monomeric rat TRAP as antigen, i.e. the antigen contained the loop region as well as N- and C-terminal parts of TRAP, therefore detects both monomeric and proteolytically processed TRAP forms. The antibody used to detect *monomeric* TRAP, on the other hand, was generated in rabbits by using a peptide (^{146}DDFASQQPKMPRDLGVA^{162}) corresponding to the loop domain according to mouse TRAP sequence as the immunogen. This sequence contains the major part of an exposed loop domain in TRAP, which is only present in the monomeric protein.

Monomeric TRAP can be detected by both monomeric and total TRAP antibodies, whereas proteolytically processed TRAP lacking the loop domain can only be detected by the total TRAP antibody.

2.3 Results and Discussion

2.3.1 Proteolytic processing of TRAP by Cathepsin K in vivo (Paper I, II ,III)

Of cysteine proteinases (CPs) known to proteolytically process and activate TRAP, cathepsin K (Ctsk) is an essential protease in bone matrix degradation and has been shown to colocalize with TRAP in resorptive compartments of osteoclasts [97] as well as in intracellular vesicles of the transcytotic route [33]. Moreover, protein sequencing analysis revealed that the C-terminal sequence of TRAP isolated from rat bone was consistent with Ctsk-mediated processing in vivo [97]. Overall in vitro data strongly suggests a potential physiological role for Ctsk in proteolytic processing of TRAP in osteoclasts.

In the present investigation, analysis of $Ctsk^{-/-}$ mice revealed that the absence of Ctsk in mice was associated with significantly higher TRAP activity as well as mRNA levels in long bones, indicating that TRAP expression was up-regulated at the transcriptional level ***(paper I)***. Kiviranta et al.[39] reported that impaired bone resorption in $Ctsk^{-/-}$ mice is partially compensated for by enhanced osteoclastogenesis and increased expression of other proteases via increased RANKL/OPG ratio (see 1.1.4.2.1.). RANKL has been shown to up-regulate the expression of several osteoclastic enzymes including TRAP [216]. Therefore, increased osteoclast numbers and TRAP mRNA expression due to increased RANKL/OPG ratio potentially explains the increased TRAP activity in long bones from Ctsk-deficient mice.

Not only the total TRAP activity, but also the abundance of monomeric TRAP (mTRAP) in relation to the cleaved isoform was increased in long bones from $Ctsk^{-/-}$ mice ***(paper I)***. Heparin FPLC analysis of bone-purified TRAP revealed that proteolytic processing of TRAP was prematurely arrested at an intermediate stage in $Ctsk^{-/-}$ mice, as evident by an increase in the height of the FPLC peak for the intermediate TRAP fragment (imTRAP) at the expense of that of the final cleavage product (fcTRAP) (*see 2.2.2. for the discussion on the use of Heparin FPLC for separating differentially cleaved TRAP forms*). The same pattern was also observed in calvaria from Ctsk-deficient mice, indicating a role for Ctsk in proteolytic processing of TRAP in long bones ***(paper I)*** as well as calvaria ***(paper II)***.

Absence of Ctsk was also associated with disturbed intracellular trafficking of TRAP in mice ***(paper I)***. Double immunofluorescence staining of osteoclasts with

monomeric and total TRAP antibodies suggested that mTRAP accumulates in the cytoplasm of Ctsk-deficient osteoclasts. This was possibly due to disturbed vesicular trafficking as examination of bone sections by TEM demonstrated that Ctsk-deficient osteoclasts exhibited disturbed ruffled border formation as well as accumulation of cytoplasmic vacuoles. Monomeric TRAP has been shown to be secreted through the ruffled border and the (presumably Ctsk-generated) cleaved TRAP to accumulate in the matrix [97]. Therefore, it was of interest to assess whether accumulation of mTRAP in Ctsk-deficient osteoclasts was associated with disturbances in the secretion of the enzyme to the resorption lacuna. Ultrastructural immunohistochemistry using the protein A-colloidal gold labeling revealed that Ctsk-deficient osteoclasts are unable to secrete mTRAP through the normal secretory pathway, but are able to deliver, possibly as a compensatory mechanism, the enzymatically active, cleaved TRAP to the resorption area by alternate transport pathways. Hollberg et al. [153] reported that secretion of TRAP was virtually absent in the polarized osteoclasts of fulminant rickets. Moreover, a similar absence of TRAP secretion into the resorption lacuna was described in resorbing osteoclasts cultured on bone slices [33], indicating the essential role of the composition of the bone surface to initiate the secretion of mTRAP. In light of these findings, it could be argued that disturbed bone degradation and consequent alterations in the composition of the bone surface in Ctsk-deficient osteoclasts prevents the secretion of the monomeric TRAP to the resorption lacuna through the normal secretory pathway.

Table 2. Phenotypic comparison of wild-type and Ctsk-deficient mice with regard to TRAP expression and intracellular trafficking

	Wild-type mice	Ctsk-deficient mice
TRAP mRNA	-	↑
TRAP activity	-	↑
mTRAP/cTRAP	-	↑
fcTRAP/imTRAP (intracellular)	-	↓
TRAP in RB	mTRAP	cTRAP
Predominant TRAP form in serum	fcTRAP	imTRAP

RB, Ruffled border; mTRAP, monomeric TRAP; cTRAP, cleaved TRAP; fcTRAP, final cleavage form of TRAP; imTRAP, intermediate TRAP form.

As mentioned earlier (see 1.4.6), osteoclasts release proteolytically processed TRAP into the circulation through the transcytotic pathway [33]. Heparin FPLC analysis of cleavage profile of TRAP

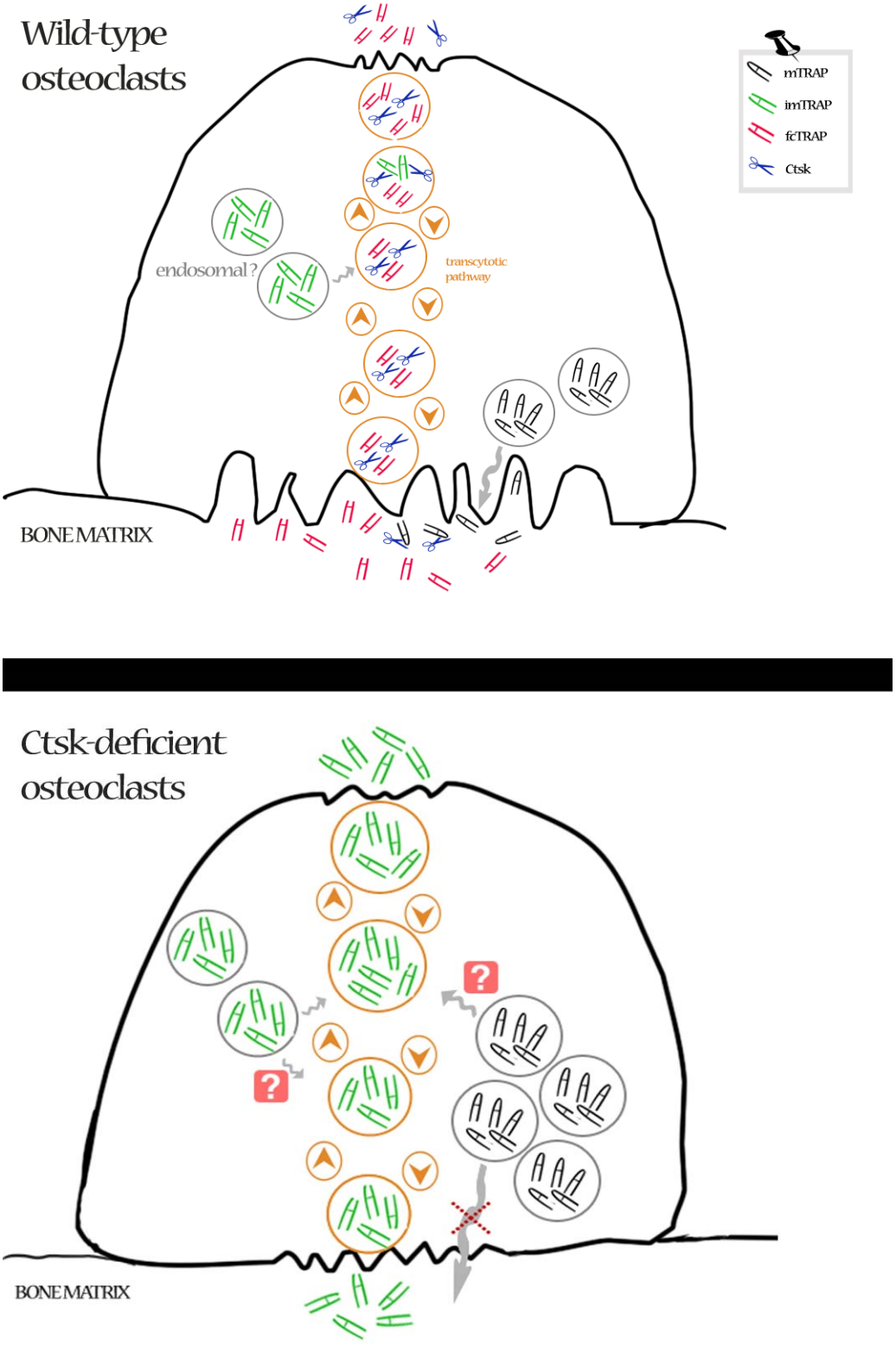

Figure 7. Hypothetical model of TRAP trafficking and proteolytic processing in osteoclasts

in the serum revealed that fcTRAP dominates over imTRAP in wild-type mice, whereas the profile was reversed in Ctsk-deficient mice. These data identify Ctsk as the main protease generating the final cleavage product, i.e. fcTRAP in serum.

Based on the data presented in ***paper I*** *(as summarized in* ***Table 2****)* and previous studies by others [33, 97, 118, 151-153], a hypothetical model was constructed detailing proteolytic processing in relation to the intracellular trafficking of TRAP in osteoclasts **(*Fig. 7*)**. This model predicts the presence of distinct pools of TRAP-containing vesicles in osteoclasts. The vesicles carrying mTRAP fuse with the ruffled border membrane during bone resorption and release their cargo into the resorption lacuna where TRAP is proteolytically processed by Ctsk as proposed by Ljusberg et al.[97]. Ctsk-generated fcTRAPs are then endocytosed, along with bone matrix remnants and Ctsk, and transported through the transcytotic pathway to be released from FSD to the circulation. It has been shown that most intracellular TRAP staining in osteoclasts is not in transcytotic vesicles, but rather in acidic vesicles which was proposed to fuse with the transcytotic vesicles [33]. Moreover, co-localization of TRAP with Rab9 [33], a GTPase involved in vesicular trafficking between TGN and late endosomes [217], supports the notion of the presence of a distinct pool of acidic TRAP-carrying vesicles with endosomal characteristics. In these vesicles, TRAP is partially digested into imTRAP by proteases other than Ctsk (see also below). Eventual fusion of these vesicles with the transcytotic vesicles results in the completion of the proteolytic processing of imTRAP fragments by Ctsk in the transcytotic pathway and release of fcTRAP into the circulation. Secretion of cleaved TRAP into resorptive compartments in Ctsk-deficient osteoclasts can potentially be explained by the presence of TRAP-containing endosomal vesicles, as ruffled border of osteoclasts is known to receive material from cytoplasmic endosomes in order to compensate for the membrane loss caused by the transcytotic secretory pathway [175]. Another possibility is that some of the accumulating mTRAP-containing vesicles of Ctsk-deficient osteoclasts fuse with the transcytotic vesicles, proteolytically processed there and secreted to the resorption lacuna as well as to the circulation through FSD.

Intriguingly, involvement of other proteases in TRAP proteolytic processing was implicated by the presence of cleaved TRAP in Ctsk-deficient mice ***(paper I)***. Moreover, the fact that cleaved TRAP subunits were not detectable when probed with loop-specific antibody indicates the excision of the loop domain through a Ctsk-like cleavage. It could be argued that this protease could be Ctsl. There are several lines of evidence indicating that Ctsl is expressed in osteoclasts [28, 97, 218], albeit at much lower levels than Ctsk

(***paper I***, [97, 219]). In support of the involvement of Ctsl in proteolytic processing of TRAP, the increased TRAP activity in long bones lacking Ctsk was not observed in long bones lacking both Ctsk and Ctsl [48]. Ljusberg et al.[97] elegantly demonstrated that TRAP can be cleaved and fully activated in vitro by both Ctsk and Ctsl at similar efficiencies. Both these cathepsins process the loop domain of TRAP in a sequential manner, proceeding through an intermediate cleavage form, i.e. imTRAP, whose further processing leads to the full excision of the loop domain, generating the final cleavage product, i.e. fcTRAP. Importantly, but not surprisingly, relative levels of imTRAP and fcTRAP upon digestion by these cathepsins are dependent on protease concentration, so that with increasing protease to TRAP ratio, a transition from imTRAP to fcTRAP occurs. According to the model in ***Fig. 7***, TRAP is partially digested by Ctsk-like CPs, e.g. Ctsl, in TRAP-containing acidic vesicles, where CPs exhibit optimal enyzmatic activity. This digestion taking place in the presence of relatively low levels of a Ctsk-like protease results in a cleavage profile with the predominance of imTRAP over fcTRAP. In normal osteoclasts, fusion of these vesicles with Ctsk-containing transcytotic vesicles results in the conversion of imTRAPs into fcTRAPs, that predominate in serum. However, in the absence of cathepsin K, this final processing cannot occur, explaining the predominance of imTRAP over fcTRAP in the serum from Ctsk-deficient mice.

Importantly, inhibition of the total CP activity with E64 in RANKL-differentiated, osteoclast-like RAW264.7 cells resulted in an even more pronounced depression of fcTRAP accompanied by increased levels of both imTRAP and mTRAP, in comparison to the absence of Ctsk in mice ***(paper I)***. This finding supports the involvement of other CPs such as Ctsl in proteolytic processing of TRAP in osteoclasts. Moreover, contribution by proteases other than CPs to TRAP processing was also implicated by these results as TRAP processing was not abolished even when CP activity was completely inhibited. This is in accordance with Wang et al.´s study [220] where authors demonstrated that proteolytic processing of TRAP over-expressed in the fibroblast-like CHO-K1 cell line was shown to be independent of CP activity. However, it cannot be ruled out that residual CP activity in E64-treated RAW264.7 cells could be sufficient for generation of imTRAP.

The effects of Ctsk on proteolytic processing and intracellular trafficking of TRAP were more pronounced in the osteoclasts of the distal compared to the subepiphyseal proximal metaphysis **(*paper I*;** see also 2.3.2**)**. Stereological analysis of osteoclasts using TRAP antibodies revealed that mTRAP was detectable in the entire population of

osteoclasts in distal metaphysis of Ctsk$^{-/-}$ mice, whereas mTRAP was hardly detected in the same region of wild-type mice. On the other hand, quantitative distribution of monomeric form in proximal metaphysis did not significantly differ between wild-type and knockout mice. Immunohistochemical co-staining of the distal metaphysis with total and monomeric TRAP antibodies revealed that Ctsk$^{-/-}$ osteoclasts of this region, but not of proximal metaphysis, exhibit intracellular accumulation of mTRAP. In previous ultrastructural studies, one notable difference between the osteoclasts of the proximal subepiphyseal region and those of the distal metaphysis was a less complete development of ruffled borders and lower levels of secretion of TRAP to the ruffled border in the osteoclasts of proximal region [50]. It could be argued that the secretion of mTRAP into the ruffled border does not occur as extensively in the osteoclasts of the proximal metaphysis as in those of the distal metaphysis, explaining the intracellular accumulation of mTRAP in the Ctsk-deficient osteoclasts of the distal metaphysis, but not of the proximal part. Despite the presence of Ctsk in both populations, the role of Ctsk in subepiphyseal clasts may be limited to the final processing of TRAP (from imTRAP to fcTRAP in transcytotic vesicles), whereas the protease could have a more essential or complete role in fully activating the enzyme secreted into the ruffled border by proteolytic processing in osteoclasts of the distal metaphysis. These findings support the notion that TRAP enzyme exerts its function(s) at several subcellular sites in resorbing osteoclasts and suggest that different osteoclast populations can differentially employ these functions through differential regulation of TRAP trafficking.

Both Western Blot analysis and immunohistochemistry showed that Ctsk protein expression was higher in osteoclasts from long bones versus calvaria ***(paper II)***. Moreover, TRAP proteolytic processing was more extensive in long bones in comparison to calvarial bones. These observations, along with the finding that Ctsk is involved in proteolytic processing of TRAP in both bone types ***(paper I, II)*** suggest that higher expression of Ctsk in long bones could explain the more extensive processing of TRAP in this bone type.

Interestingly, the effect of cysteine proteinase inhibitor E64 on proteolytic profile of TRAP in TRAP-overexpressing breast cancer epithelial cells judged by Heparin FPLC ***(paper III)*** was reminiscent of RANKL-differentiated osteoclast-like RAW264.7 cells ***(paper I)***, indicating a role for cysteine proteinases in proteolytic activation of TRAP in breast cancer epithelial cells as well. Cathepsin L and cathepsin B are two cysteine

proteinases capable of proteolytically activating TRAP and known to be abundantly expressed in breast carcinoma cells.

However, treatment of TRAP-overexpressing breast carcinoma cells with cathepsin B/L inhibitor CA-074-Me had only a slight effect on proteolytic processing of TRAP, suggesting that another cysteine proteinase/s must be involved in the process ***(paper III)***. Ctsk is yet another candidate for the role as MDA-MB-231 cells express mature cathepsin K protein ***(Fig. 8)***. Moreover, cleaved isoforms of TRAP expressed by these cells were not further activated upon digestion with Ctsk *in vitro* ***(paper III)***. This suggests that Ctsk, or related proteases could be involved in TRAP proteolytic processing not only in osteoclasts but also other cell types, e.g. epithelial cells, as well.

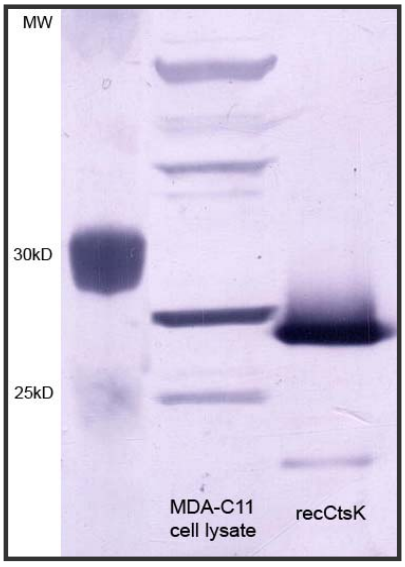

Figure 8. Demonstration of Ctsk protein expression in MDA-C11 cells by Western blotting.

2.3.2 Osteoclast heterogeneity with regard to TRAP expression (Paper I, II)

A growing body of evidence suggests that osteoclasts of different bone types exhibit phenotypic differences [46], among which is differential use of cysteine proteinases in degradation of bone matrix. As shown in ***paper II***, protein expression and proteolytic processing profile of TRAP, which is a long-known histo- and biochemical marker of osteoclasts, also differs between osteoclasts of different bone sites, i.e. calvaria and long bones. TRAP enzyme activity was 4- to 7-fold higher in long bones than calvaria in both young (3-week old) and adult (12-week old) rats, as well as in mice ***(Table 3)***.

Immunohistochemical analysis disclosed that long bone osteoclasts contain higher amounts of TRAP protein than those of calvaria, further substantiating biochemical data at a cellular level. Moreover, densitometric analysis of Western blot analysis of bone extracts as well as Heparin FPLC analysis revealed that abundance of monomeric TRAP in relation to cleaved isoforms was higher in calvaria than long bones, indicating that proteolytic processing of TRAP is more pronounced in long bone osteoclasts. Since Ctsk is involved in TRAP proteolytic processing in both long

Table 3. Comparative TRAP activity in long bone vs. calvarial homogenates

	Calvaria	Femur	Mean fold change
3-week old rats	242 (mU/mg protein) Paper II	1179 (mU/mg protein) Paper II	*4.9*
	3.96 (mU/mg bone weight) Paper II	26.85 (mU/mg bone weight) Paper II	*6.8*
12-week old rats	151 (mU/mg protein) Paper II	863 (mU/mg protein) Paper II	*5.7*
4-week old mice	6.5 (mU/mg bone weight) Paper II	29 (mU/mg bone weight) Paper I	*4.5*

bones ***(paper I)*** and calvaria ***(paper II)***, higher abundance of monomeric TRAP in relation to cleaved isoforms in calvaria can potentially be explained by lower protein expression of Ctsk, and possibly of other cysteine proteinases in osteoclasts of this bone type.

Interestingly, two differently sized isoforms were detected for both cathepsin K and monomeric TRAP in bone extracts and different bone types exhibited differential expression of these isoforms ***(paper II)***. Physiological determinant(s) of this heterogeneity is not clear, however it could be reflecting the differential processing and/or compartmentalization of TRAP and Ctsk by different osteoclast populations.

There is evidence that not only osteoclasts of different bone types but also populations of those of the same bone type may exhibit heterogeneity. For instance, long bone osteoclasts constitute a heterogeneous population, e.g. with respect to their size or adhesive ligand specificity [51]. In ***paper I***, the effect of the absence of Ctsk on TRAP proteolytic processing and intracellular distribution was more pronounced in the distal compared to proximal metaphysis (see also 2.3.1). In contrast to distal metaphysis where osteoclast numbers were increased, decreased osteoclast numbers were observed in the proximal part, indicating differences in osteoclast recruitment, regulation and/or function between the different sites.

2.3.3 Biogenetic relationship between 5a and 5b (paper I, III)

Post-translational proteolytic processing of TRAP appears to be an important mechanism for regulating the catalytic activity of the enzyme towards different substrates [83] as well as for enhancing ROS generation [33]. However, physiological importance of proteolytic removal of the repressive loop domain appears to extend beyond just enzyme activation or regulation of the catalytic rate. For instance, only the monomeric 5a isoform has been shown to act as a growth factor for osteoblastic [177] cells and adipocytes [181], but not the cleaved 5b isoform. Besides exerting distinct functions, serum levels of these two isoforms, i.e. 5a and 5b, correlate independently with different human diseases. Differential compartmentalization of these two isoforms have been observed in a variety of cell types. For example, macrophages and dendritic cells secrete monomeric 5a isoform while retaining TRAP 5b intracellularly [159, 161, 221]. Osteoclasts secrete monomeric TRAP to the ruffled border, wheras a cleaved isoform is detected in the resorption lacuna [97], endosomal vesicles as well as in the transcytotic pathway [33]. TRAP exhibits characteristic features of a lysosomal enzyme, e.g. carrying a Man-6-P[103] and exhibiting optimal enzymatic activity at acidic pH. On the other hand, TRAP is commonly secreted by a variety of cell types. However, still not much is known about the posttranslational mechanisms regulating the intracellular trafficking and biogenesis of TRAP 5a and 5b.

To address the issue, a TRAP-overexpressing sub-line of MDA-MB-231 breast cancer epithelial cell line was generated ***(paper III)***. This cell line, denoted as MDA-C11, secreted only monomeric 5a-like TRAP isoform to the media while retaining 5a- and 5b-like isoforms intracellularly. Heparin FPLC purification of homogenates separated intracellular TRAP expressed by MDA-C11 into four fractions denoted as L1a, L1b, L2 and L3. Single TRAP activity peak generated by conditioned media was denoted as M1.

Biogenetic relationship between these different forms was assessed by utilizing differences in their molecular weight and N-glycan moieties. The analysis revealed that secreted TRAP (M1) is derived from L1a fraction, whereas the intracellularly located cleaved forms L2 and L3 are generated by proteolytic processing of the monomeric L1b form ***(Fig. 9)***. Treatment with BfA, an inhibitor of ER-Golgi transport, reduced the secretion of TRAP up to 80% and attenuated the L1a fraction but not L1b, L2 and L3. This indicates that L1a isoforms are transported through the ER-Golgi-plasma membrane axis. L1a and secreted M1 fractions consist of both high molecular weight (HMW) as well as low molecular weight (LMW) TRAP isoforms. BfA treatment results in

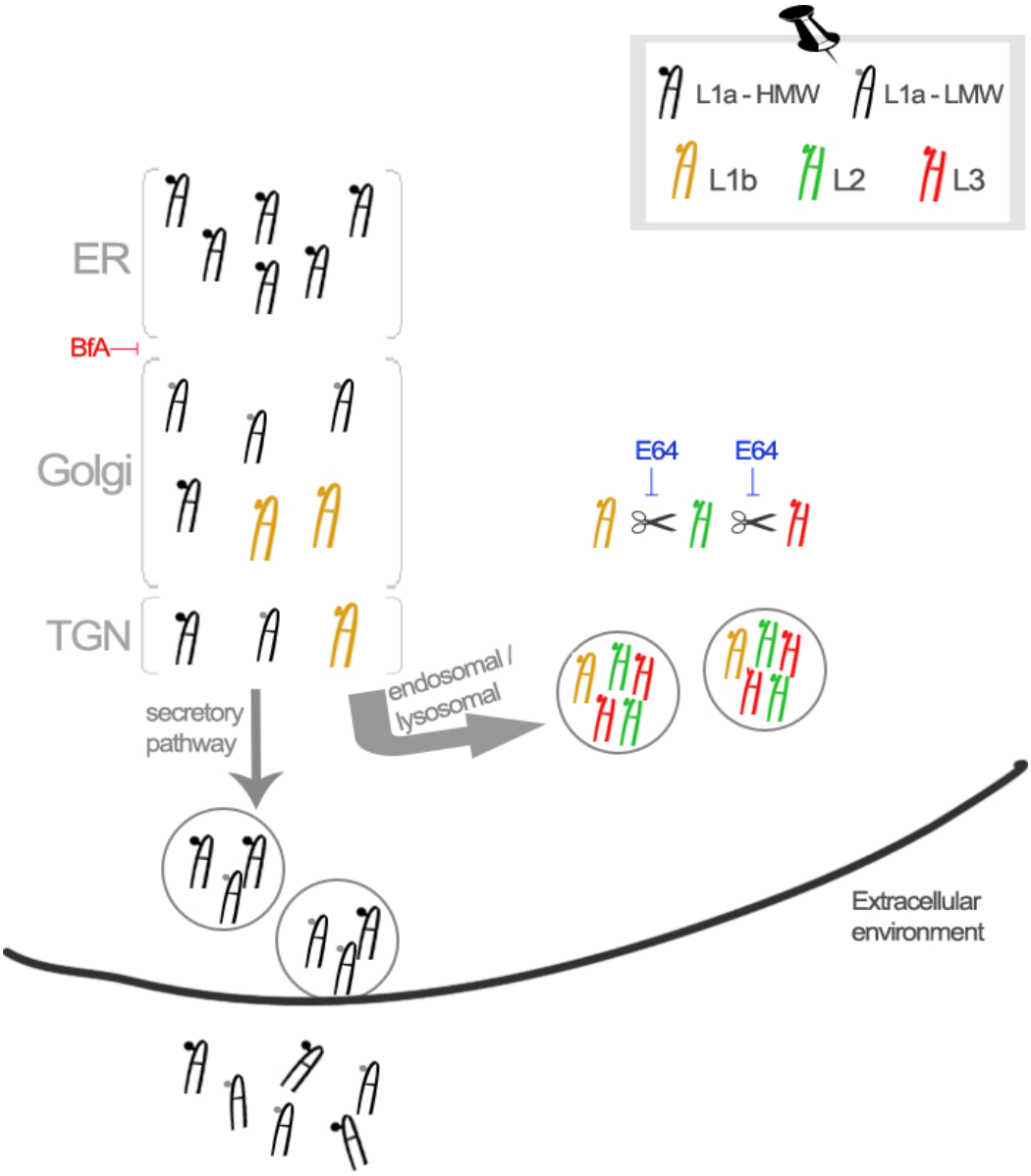

Figure 9. Hypothetical model of intracellular trafficking of TRAP in TRAP-overexpressing epithelial cells

an intracellular accumulation of HMW-isoforms wheras LMW-isoforms are secreted into the media. As BfA acts by blocking the ER to Golgi transport, it can be argued that HMW isoforms of L1a represent the TRAP population in the ER, whereas LMW isoforms are presumably generated in the Golgi compartments by processing of high-mannose type oligosaccharide chains added in the ER.

The absence of an attenuating effect of BfA on L1b, L2 and L3 isoforms, on the other hand, suggests that these forms are transported through and/or localized in a different intracellular pathway. It stands to reason that these isoforms are located in endosomal/lysosomal compartments since TRAP possesses the mannose-6-phosphate lysosomal targeting sequence and has been shown to localize to lysosomes [120, 151, 222]. Moreover, levels of only L1b, L2 and L3 isoforms are affected upon treatment of MDA-C11 cells with cysteine proteinase inhibitor E64 ***(paper III)***. This provides circumstantial evidence to the assumption since cysteine proteinases require acidic pH of lysosomes for optimal enzymatic activity. In support, TRAP activity was only detected in vacuolar compartments in these cells and no obvious co-localization was observed between TRAP activity and the ER-marker calnexin ***(paper III)***. From these data, we hypothesize that distinct monomeric TRAP populations are diverted early in the secretory pathway either giving rise to a secreted, monomeric 5a-like TRAP isoform or to an intracellular, proteolytically processed 5b-like TRAP isoform ***(Fig. 9)***.

TRAPs, like most lysosomal enzymes, possess the M6P lysosomal targeting sequence. However, in order to permit the secretion of the enzyme, M6P must either be cleaved/modified or not incorporated into the molecule at all. Secreted monomeric TRAP enzyme uteroferrin synthesized by explanted endometrium was shown to contain M6P-tagged N-linked oligosaccharides [103, 162]. However, the M6P-tag was covered by N-acetylglucosamine that blocks the interaction of the M6P tag with its receptor that in turn leads to the secretion of the enzyme [103]. Moreover, Ling and colleagues [223] demonstrated that overexpression of TRAP results in its high rate of secretion from transfected fibroblasts and proposed that high production of a lysosomal enzyme such as TRAP may overwhelm the intracellular enzymatic and receptor systems that are normally employed to target acid hydrolases to lysosomes, resulting in secretion. Although not assessed in the present investigation, it could be argued that similar mechanisms could also operate in MDA-MB-231 cells leading to the formation and diversion of distinct monomeric TRAP populations in the Golgi.

Western blot analysis showed that monomeric precursor to cleaved TRAP isoforms, i.e. L1b, has a lower molecular weight than secreted monomeric isoforms expressed by MDA-C11 cells ***(paper III)***. Similar to the monomeric L1b isoform of MDA-C11 cells, the monomeric TRAP isoform accumulating in the absence of Ctsk in long bone homogenates of $Ctsk^{-/-}$ mice ***(paper I)***, has a lower molecular weight than the monomeric TRAP isoform predominating in wild-type mice. Interestingly, abundance of

this high-molecular-weight monomeric isoform was not dependent on whether cathepsin K was present or not. This suggests that our "TRAP biogenesis" model could apply not only to epithelial cells but also to other TRAP-"overexpressers" such as osteoclasts. On that note, similarities between models of TRAP trafficking in osteoclasts (***Fig. 8***) and in epithelial cells (***Fig. 9***) should also be noted ***(****see* ***Fig. 10*** for a side-by-side comparison***).***

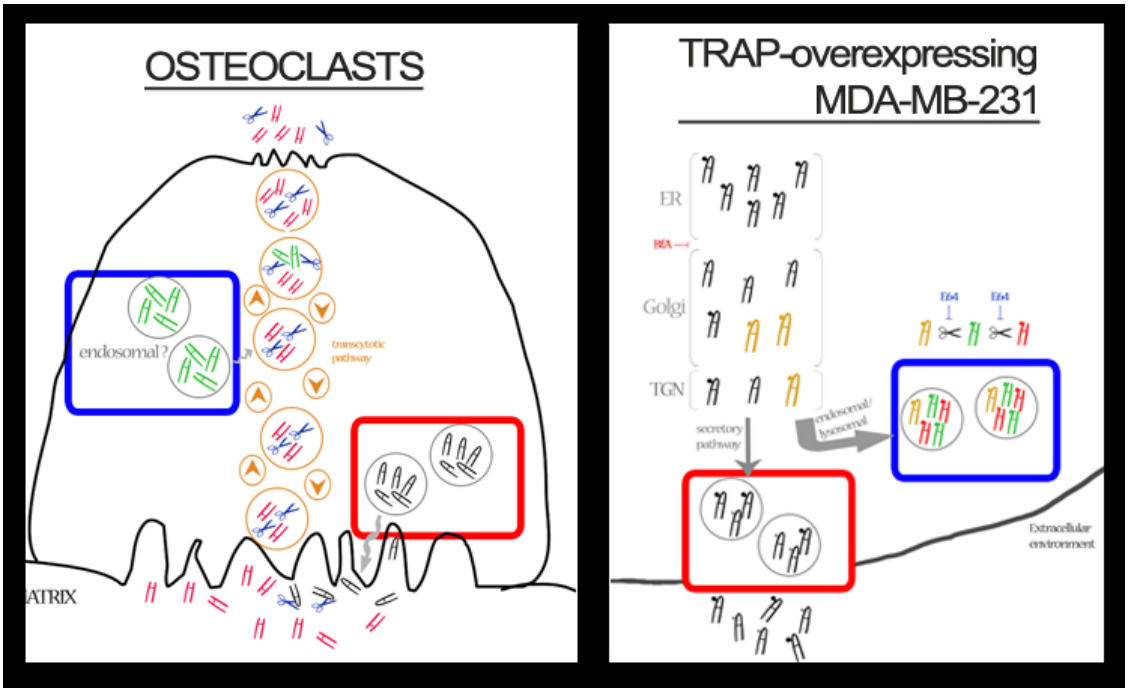

Figure 10. Similarities between epithelial cells and osteoclasts with regard to the intracellular trafficking of TRAP. Analogous TRAP-containing compartments were highlighted with blue and red boxes based on the hypothetical models in Fig.8 and Fig.9.

2.3.4 *Expression of TRAP isoforms in cancer. Pathophysiological and clinical implications (paper lll, lV)*

TRAP 5b has been studied extensively as a serum marker of bone resorption in cancer patients [137, 224-226]. What is relatively new is that TRAP is expressed by cancer tissue as well. Several cancer cell lines and tissues have been shown to express TRAP in a manner that their degree of tumorigenicity correlates with the intensity of histochemical activity [140, 141]. Intriguingly, elevated levels of serum TRAP were reported in patients with breast and ovarian cancer, even in the absence of metastatic bone disease [140],

suggesting that TRAP secreted by tumor cells can substantially contribute to serum TRAP levels. These studies indicate that TRAP can be utilized as a marker for progression of metastatic bone disease and suggest that the enzyme could also be a potential target for future cancer therapies. However, the expression and clinical utility of different TRAP isoforms in the pathogenesis of metastatic bone disease has not previously been reported.

In ***paper IV***, the previous observations of TRAP expression in cancer tissue were extended by showing that cancer cells of bone metastases originating from prostate, lung, kidney and breast express TRAP protein. Immunohistochemical analysis employing total and monomeric TRAP antibodies revealed that tumor cells and tumor-infiltrating macrophages exhibit predominant expression of monomeric 5a and cleaved 5b isoforms, respectively, in breast cancer bone metastasis tissue. This is in line with ***paper III*** where monomeric TRAP was shown to be the major isoform expressed intracellularly by TRAP-overexpressing breast cancer epithelial cells and previous studies reporting TRAP 5b to be retained intracellularly by macrophages[159, 161, 221]. It remains to be determined which isoform is secreted by constituent cell types of a TRAP-positive tumor. Based on the information that both macrophages[159] and TRAP-overexpressing breast carcinoma cells ***(paper III)*** secrete monomeric TRAP 5a to the medium, it stands to reason that TRAP 5a could be the predominant isoform secreted by tumor cells and infiltrating macrophages. Intriguingly, a novel previously unreported monomeric 5a TRAP variant was identified in ***paper IV***. This variant has a higher molecular weight (42 kDa) than the monomeric TRAP from human bone (37 kDa) and was shown to be present in tumor homogenates from prostate, kidney and lung cancer bone metastases. It remains to be determined whether cancer cells secrete this tumor-specific novel 42 kDa variant of TRAP 5a, possibly differing from 37 kDa isoform in post-translational modifications such as N-glycan maturation and/or proteolytic processing. If this is the case, the use of this novel 5a isoform as a marker in disease progression in cancer could be evaluated.

2.4 Conclusions

- TRAP is proteolytically processed by cathepsin K in long bone and calvarial osteoclasts *in vivo*.
- Cathepsin K is involved in the regulation of intracellular trafficking of TRAP in osteoclasts.
- Osteoclasts of different anatomical sites in trabecular metaphyseal bone exhibit heterogeneity with regard to proteolytic processing of TRAP and the role of cathepsin K in the process.
- Proteolytic processing of TRAP is more extensive in long bones than calvaria, which correlates with higher cysteine proteinase activity and protein expression of cathepsin K.
- In TRAP-overexpressing epithelial cells, distinct monomeric TRAP populations are diverted early in the secretory pathway either giving rise to a secreted, monomeric TRAP 5a or to an intracellular, cleaved 5b.
- Metastatic breast cancer tissue exhibits differential expression of TRAP 5a and 5b. TRAP 5a is the predominant form expressed in metastatic cancer cells, whereas tumor-associated macrophages express TRAP 5b.
- A previously unstudied TRAP 5a variant is expressed by metastatic cancer cells of different histogenetic origins.

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