From the DEPARTMENT OF LABORATORY MEDICINE
DIVISION OF PATHOLOGY
Karolinska Institutet, Stockholm, Sweden

TARTRATE-RESISTANT ACID PHOSPHATASE/ACP5 AS A DRIVER OF CANCER: DISSECTION OF ITS ONCOGENIC MECHANISMS AND IDENTIFICATION OF SMALL MOLECULE INHIBITORS

Anja Reithmeier, MSc

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Tartrate-resistant acid phosphatase/ACP5 as a driver of cancer: Dissection of its oncogenic mechanisms and identification of small molecule inhibitors

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Anja Reithmeier, MSc

Principal Supervisor:
Prof. Göran Andersson
Karolinska Institutet
Department of Laboratory medicine
Division of Pathology

Co-supervisor:
Barbro Ek-Rylander, PhD
Karolinska Institutet
Department of Laboratory medicine
Division of Pathology

Opponent:
Prof. Maréne Landström
Umeå Universitet
Department of Medical Biosciences
Division of Pathology

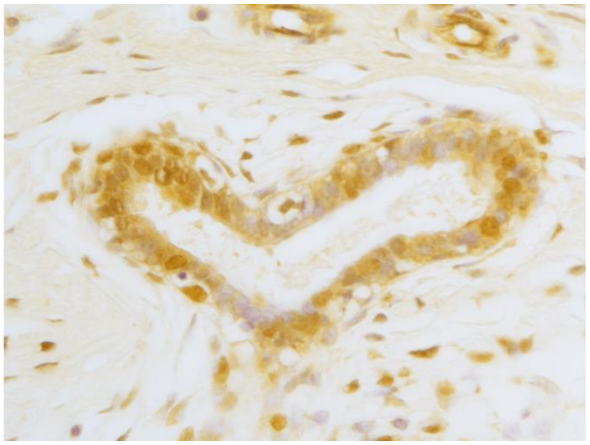
Examination Board:
Associate Prof. Jonas Fuxe
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology (MTC)

Associate Prof. Katalin Dobra
Karolinska Institutet
Department of Laboratory medicine
Division of Pathology

Associate Prof. Lars-Göran Mårtensson
Linköping Universitet
Department of Physics, Chemistry and Biology (IFM)

The public defense of this thesis will take place on
Friday, the 15th of December, 9:30 a.m.
Karolinska University Hospital, Huddinge, SE-141 86 Stockholm
Visiting address: Hälsovägen, Flemingsberg
C1:87, C1 building 8th floor

To the commitment, support and unconditional love
of my family and friends.
To every person behind the disease.



Breast cancer tissue stained for TRAP

POPULAR SCIENCE ABSTRACT

HOW A BONE ENZYME HELPS CANCER CELLS TO SPREAD IN THE BODY

A bone enzyme that could reveal the risk for the spreading of cancer. Research has identified that the enzyme TRAP changes the program of cancer cells. This causes them to be more aggressive and potentially spread to other sites of the body (´metastasis´). Using this knowledge, we could reduce the risk of death by inhibiting the cancer´s invasion of our body.

Cancer is an abnormal growth of cells - cells that have changed their genetic information and their way of communicating. Thereby, they gradually become aggressive by overcoming all safety checks in our body. When cancer cells start to spread in the body (´metastasize´), a patient has a high risk of dying. According to statistics, only one out of ten patients wins the fight against the uncontrolled disease when diagnosed with metastases. Even though many cancers can be treated nowadays, one of the remaining big challenges of research is to understand why a cancer starts spreading.

TRAP makes cancer cells more aggressive

Research has recently made an important finding that could help us come one molecule closer to understanding why and how cancer cells can spread. The enzyme TRAP that is usually involved in rebuilding bone is also highly active in cancer cells that have metastasized - a fact that supports the assumption that the enzyme is most likely a helper for cancer cell spreading. Concomitantly, the main question for this doctoral thesis was: *´What is TRAP doing in cancer cells and how can we inhibit it from doing so?´* - To address these questions, the scientists imitated the process seen in patients. They genetically modified cancer cells to produce high amounts of TRAP. These cells were then used in experimental studies to look at how they behave and how TRAP communicates within the cell.

The outcome was both interesting but also highly concerning. Cancer cells that have a specific form of TRAP that has high enzyme activity can both grow and move quicker but also pass barriers more easily. These phenomena are described as features of cancer cells, which are more aggressive, and increase their potential for spreading. *´But how did the cells do that?´* - Therefore, the researchers looked at ´proteomics data´, a method to catalog all proteins, protein changes and connections within the cells. These data showed that TRAP redirects the communication with other molecules in the cell in a manner, different from how cells without TRAP do. Interestingly, when this communication was blocked, certain features of the cancer cells normalized, *i.e.* the cells moved more slowly again.

Small molecule inhibitors to reprogram cancer cells

To be able to understand further, how the enzyme TRAP makes cancer cells more aggressive and moving, the scientists used small molecule inhibitors. These are drug-like compounds that can specifically shut down the activity of TRAP and thereby could reprogram the cells to become less aggressive again. Additionally to a previously reported inhibitor, the researchers

looked into a big library of chemical compounds and found several other candidates that could be used to inhibit TRAP. When cells with high TRAP activity were treated with these compounds, they also moved more slowly again.

Finding compounds that are specific for TRAP can be used to resolve the role of TRAP also in other diseases, such as diseases of the bone. Finally, this discovery can assist us in predicting, which cancers are at risk to spread and threaten a patient´s life. Using small molecule inhibitors for TRAP has a potential use in the development of drugs that could eventually be applied in the treatment of patients with diseases involving TRAP.

ABSTRACT

Cancer patients diagnosed with metastasis have an increased risk of dying. To be able to predict and target tumors with an increased risk for spreading, underlying molecular events need to be better dissected and understood.

TRAP is a metalloenzyme existing in two isoforms - a precursor form (TRAP 5a) and a highly enzymatically active form (TRAP 5b). TRAP expression was detected in cancer cells of several primary and metastatic tumors and expression levels were raised with increasing malignancy. TRAP expression was further correlated to clinical parameters of aggressiveness such as reduced tumor- and metastasis-free survival. Underlying molecular processes remain unclear and only a limited amount of studies is addressing the respective role of the TRAP isoforms in cancer.

In this thesis, *two major milestones* were addressed tackling the role of TRAP (isoforms) in cancer cell metastasis. **(I)** Identification and characterization of previously reported and novel small molecule inhibitors of TRAP **(II)** Characterization of functional alterations and cellular mechanisms induced by TRAP perturbation.

The TRAP inhibitor 5-phenylnicotinic acid (5-PNA/CD13) was previously identified by fragment-based screening. In ***Paper I***, 5-PNA was further characterized for its ability to inhibit the mammalian TRAP, its selectivity for TRAP and its cytotoxicity in a cellular model. TRAP-dependent migration was inhibited by 5-PNA, shown to be selective for the TRAP 5b isoform.

By small molecule screening of a library containing drug-like compounds in ***Paper II***, several inhibitors for TRAP activity were found and selected based on a strict filtration strategy. Orthogonal validation, full-concentration responses and isoform selectivity were assessed for a selection of hit compounds. Six potential lead structures were characterized for molecular docking modes. The compound CBK289001 rendered valid as inhibiting TRAP-dependent migration in a cell system and initial structure-activity relationships were derived.

The aggressiveness of cancer cells with perturbations of TRAP expression was assessed by functional studies in ***Paper III***. TRAP had a promotive effect on cancer cell elongation, proliferation, migration and invasion. Proteomics and Phospho-proteomics outlined changes in the cellular network associated with extracellular matrix modulation and cell adhesion, and a regulation of TGFβ and CD44 signaling. A list of potential TRAP substrates was generated.

The role of TRAP 5b isoform in cancer cell aggressiveness and Cathepsin K (CtsK) in its generation and processing was investigated in ***Paper IV***. TRAP 5b was significantly increased compared to TRAP 5a in cells overexpressing TRAP. Inhibition of CtsK, an enzyme shown to be able to cleave TRAP, resulted in an intermediate processed TRAP 5b form with similar activity and promotive effect on migration. CtsK colocalized highly with TRAP 5b and cleaving changed the subcellular localization of TRAP 5b.

In summary, the work presented in this thesis is contributing to the knowledge about the role of TRAP in cancer metastasis. Specifically, we were able to show a connection of TRAP 5b to metastasis-related cell functions and the involvement of TGFβ/CD44 signaling. Possible starting points for the development of potent and TRAP-specific inhibitors are provided.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following peer-reviewed publications and manuscripts, referred to in the text by their roman numerals.

I. Michael Krumpel[#], **Anja Reithmeier**[#], Teresa Senge, Toni Andreas Bäumler, Martin Frank, Per-Georg Nyholm, Barbro Ek-Rylander, Göran Andersson

The small chemical enzyme inhibitor 5-phenylnicotinic acid/CD13 inhibits cell migration and invasion of Tartrate-resistant acid phosphatase/*ACP5*-overexpressing MDA-MB-231 breast cancer cells

Experimental Cell Research (2015) 339:154–162

II. **Anja Reithmeier**, Thomas Lundbäck, Martin Haraldsson, Martin Frank, Barbro Ek-Rylander, Per-Georg Nyholm, Anna-Lena Gustavsson, Göran Andersson

Identification of novel inhibitors of Tartrate-resistant acid phosphatase (TRAP/*ACP5*) activity by small molecule screening

Manuscript

III. **Anja Reithmeier**[#], Elena Panizza[#], Michael Krumpel, Lucas Orre, Rui M. Branca, Janne Lehtiö, Barbro Ek-Rylander, Göran Andersson

Tartrate-resistant acid phosphatase (TRAP/*ACP5*) promotes metastasis-related cell properties via the TGFβ-pathway and CD44 in human breast cancer cells

BMC Cancer (2017) 17:650

IV. **Anja Reithmeier**, Tuomas Näreoja, Maria Norgård, Barbro Ek-Rylander, Göran Andersson

Tartrate-resistant acid phosphatase (TRAP/*ACP5*) activity promotes migration of MDA-MB-231 breast cancer cells independent of Cathepsin K processing

Manuscript

[#] Equal contribution

Related articles published by the defendant, not included in the thesis.

Carian E. Boorsma, T. Anienke van der Veen, Kurnia S. S. Putri, Andreia de Almeida, Christina Draijer, Thais Mauad, Gyorgy Fejer, Corry-Anke Brandsma, Maarten van den Berge, Yohan Bossé, Don Sin, Ke Hao, **Anja Reithmeier**, Göran Andersson, Peter Olinga, Wim Timens, Angela Casini, Barbro N. Melgert

A potent Tartrate resistant acid phosphatase inhibitor to study the function of TRAP in alveolar macrophages

Scientific Reports (2017) 7:12570

CONTENTS

LIST OF ABBREVIATIONS

1KBP	Crystal structure of the red kidney bean TRAP
1QFC	Crystal structure of the rat proteolytically cleaved TRAP
1QHW	Crystal structure of the rat uncleaved TRAP
1UTE	Crystal structure of the phosphate-uteroferrin/pig TRAP complex
1WAR	Crystal structure of the human TRAP
5-PNA	5-phenylnicotinic acid /CD13
ACP5	Tartrate-resistant acid phosphatase/ Acid phosphatase 5 gene
ALK	Activing-receptor-like kinase
Asn	Asparagine
BSP	Bone sialoprotein
CD44	Cluster of differentiation-44
CID	Compound identification number
COPD	Chronic obstructive pulmonary disease
CtsK	Cathepsin K
CtsL	Cathepsin L
Cys	Cysteine
DMP1	Dentin matrix protein 1
DMSO	Dimethylsulfoxide
DSPP	Dentin sialophosphoprotein
ECM	Extracellular matrix
eiF3	Eukaryotic translation initiator factor 3
EMT	Epithelial-to-mesenchymal transition
ERM	Ezrin, Radixin, Moesin
ETA1	Early T-lymphocyte activation 1
FAK	Focal adhesion kinase
GAG	Glycosaminoglycan
HA	Hyaluronan/hyaluronic acid
HAS	Hyaluronan synthase
HiREF	High Resolution Isoelectric focusing
His	Histidine
HRE	Hemin response elements
IgG	Immunoglobulin G
LAP	latency-associated protein
LCI	Live cell imaging
LTBP	latent TGFβ-binding proteins
MAPK	Mitogen-activating protein kinase
MEPE	Matrix extracellular phosphoglycoprotein
MET	Mesenchymal-to-epithelial transition
MG	Malachite green
MITF	Microphthalmia-associated transcription factor
MMP	Matrix metalloproteinase
OPN	Osteopontin
PAINS	PAN assay-interfering compounds
PAP	Purple acid phosphatase
PI3K	Phosphatidylinositol-3 kinase
pNP	Para-nitrophenol
pNPP	Para-nitrophenylphosphate
PPA1	Serine/ threonine protein phosphatase 1
PPA2	Serine/ threonine protein phosphatase 2
PU.1	PU-box binding protein
PVDF	Polyvinylidene difluoride
RANKL	Receptor-activator of NFκB Ligand
REOS	Rapid elimination of Swill
RGD	Arginine-glycine-aspartic acid-recognition sequence
sCD44	Standard isoform of CD44
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
Ser	Serine
Sf9	Spodoptera frugiperda insect cells
SIBLING	Small integrin-binding ligand N-linked glycoprotein
SILAC	Stable isotope labeling by/with amino acids in cell culture
SMAD	Mothers against decapentaplegic
SPENCD	Spondyloenchondrodysplasia
SPP1	Secreted phosphoprotein 1
TGFβ	Transforming growth factor β
TMT	Tandem Mass Tag
TRAP	Tartrate-resistant acid phosphatase
TRIP-1	Transforming growth factor β receptor-interacting protein-1
Tyr	Tyrosine
TβR	Transforming growth factor β receptor
uPA	Urokinase-type plasminogen activator
vCD44	Variant isoform of CD44

I PREFACE

People all around us are being diagnosed with diseases, treated, cured - sometimes they die, sometimes they suffer. Every person around us is in either way affected - everybody knows somebody that knows somebody. One out of three individuals will eventually be diagnosed with cancer.

Cancer is an intimidating disease that intersects everybody´s life at some point and it is often stigmatized with anticipations. People have lost family members to it; cancer leaves patients and their relatives generally with a feeling of lost influence and helplessness. It is not surprising that the first reactions after the diagnosis of cancer usually involve questions, such as ´*What does that mean?´* or ´*Can I be treated? ´*

For a variety of diseases science has made such a great progress that going to the pharmacy, being treated in a medical facility, taking medication once a week, being able to continue a normal life, has become an expectation that cannot be taken for granted. Not always is there a golden standard of treatment. Not always is there a good prognosis to be cured or even to survive.

Only due to laborious effort, persistent, creative and devoted scientists and medical doctors, we have nowadays reached those crucial breakthroughs and acquired that necessary information allowing for a better understanding of the disease, for the identification of new treatment strategies and for providing the optimal care of every specific patient.

Nevertheless, we are still missing pivotal puzzle pieces and need further research to create majorly missing knowledge about mechanisms that often lead to a poor prognosis.

´Every field in medicine has had a moment in history that has been transforming. The moment, where the knowledge that was required to change the field became available.´

Dr. José Baselga, Memorial Sloan Kettering Cancer center
In Ken Burns CANCER: THE EMPEROR OF ALL MALADIES, a film by Barak Goodman.

This is where I see the framework of my thesis to set in - a seemingly little puzzle piece in the big picture - a puzzle piece that might for every now and then be underrated in its significance. In the end, this work is built upon the strong belief that creating basic knowledge about molecular mechanisms and treatment strategies eventually will result in the success of beating cancer - by providing just that one little puzzle piece that fits together in the big picture.

Inspired by Siddhartha Mukherjee's 2010 Pulitzer Prize-winning book, ´The Emperor of All Maladies: A Biography of Cancer.´

II BACKGROUND

1 Cancer

1.1 Definition and Epidemiology

Cancer is a generic term for a group of complex diseases, commonly also termed as malignant tumor or neoplasm. The disease is based on the abnormal development and growth of cells, leading to outgrowth and spreading into distant sites. Failed treatment or late detection of cancer will finally lead to general organ failure, ultimately resulting in the death of the patient.

Cancer is attributable for being one of the leading causes of deaths in the world with approximately 14.1 million new cases and 8.2 million deaths in 2012 [1]. The most common types are represented by cancers of the lung (1.82 million), breast (1.67 million), and colon (1.36 million), whereas the most common causes of cancer death are lung cancer (1.6 million deaths), liver cancer (745,000 deaths) and stomach cancer (723,000 deaths) [1]. Accounting for more than 3.7 million new cases and 1.9 million deaths each year, cancer represents the second most important cause of death and morbidity in Europe [2]. Importantly, 30-50% of cancer can be prevented through prevention programs and by early diagnosis [3].

1.2 History

Already 3000 years before Christ, cancer was for the first time described in an ancient Egyptian text book, elaborating on eight ulcers of the breast being removed by surgical procedures, however incurable [4]. Only from much later, though, we have proof that the disease was given a name – around 460–370 before Christ, Hippocrates, the "father of medicine", described and created drawings of the disease he termed "*carcinos/carcinoma*". Hippocrates applied the latter naming, as the tumor reminded him of a crab, with veins stretched on all sides of the body of the tumor [4,5]. Only with the description of the cellular theory and the discovery of cellular pathology by Robert Virchow in the 19th century ("*omnis cellula e cellula*") [6], diseases were no longer diagnosed merely based on symptoms but could be defined by anatomical changes. Finally, by the finding of the chemical structure of DNA in the 20th century, gene mutations and repair mechanisms of the cell, cancer scientists have understood that cancer is a disease that originates from modulations of our gene material [4]. These DNA changes can be caused by cancerogenic agents, such as physical (e.g. radiation), chemical (e.g. asbestos, components of tobacco smoke or dietary habits) and biological carcinogens (e.g. viruses and bacteria) [3]. Next to inherited and environmental factors as the reason for cancer-driving mutations, a recent study claimed that one third of cancer driver mutations would be due to unavoidable DNA replicative errors [7]. In light of this, early detection would claim an even bigger role in patient care.

Early treatment attempts mainly relied on the removal of the cancer, a strategy that has remained applicable until the current days of clinical practice. With the detection of X-rays and radioactive elements by Wilhelm Conrad Roentgen and Marie and Pierre Curie in the 19th century, radiation therapy was put in the spotlight of cancer treatment. Chemotherapy was invented in the course of World War II experiments, as several alkylating agents, such as

nitrogen mustard [8] proofed to kill rapidly replicating cells via DNA damage allowing for the targeting of small cancer masses, unable to be surgically removed. For instance, aminopterin, a precursor of the nowadays commonly applied chemotherapeutic methotrexate, showed to induce remission in children with acute leukemia [9]. These findings provided the basis for novel and more directed chemo-guided strategies that are nowadays applied for a more patient-specific treatment. For example, several efforts have been funneled into the development of combination chemotherapy, drug delivery techniques, multi-resistance treatments and chemoprotective treatments. Given the increasingly better understanding of cancer biology, the latest strategies of cancer treatment include immunotherapeutics or targeted therapies, such as T-cell therapy, anti-cancer vaccines or antibody-mediated therapy [4].

1.3 Cancer biology

1.3.1 Tumor-Suppressor genes and Proto-Oncogenes

Cell growth is under physiological conditions highly regulated at the molecular level, and several regulatory machineries are involved in repairing dysregulated events. During cancer development, these machineries can be either rescinded or get dysfunctional, or even overridden by promotional forces. Accompanying the findings of genes, scientists introduced the terms of tumor-suppressor genes and proto-oncogenes in the 1970s. Tumor-suppressor genes and proto-oncogenes are genes encoding for proteins that are involved in the regulation of cell division that either lose their suppressor activity or promote the progression of cancer, when mutated [5]. They are defined as commonly belonging to either class of growth factors (I), growth factor receptors (II), signal transduction proteins (III), transcription factors (IV), pro- or anti-apoptotic proteins (V), cell cycle control proteins (VI) or DNA-repair proteins (VII). Whereas mutations of one allele of a gene were reported to be sufficient to change a proto-oncogene into an oncogene, mutations in both alleles were thought to be necessary to drive cancer in the case of a tumor-suppressor gene (*two-hit hypothesis*) [10]. Nevertheless, the current theory of a tumor evolving is manifested on a *sequential accumulation of mutations* in several genes, as suggested in the early 1990s for the development of colorectal cancer by Bert Vogelstein [11,12].

1.3.2 Cancer hallmarks: Invasion and Metastasis

In 2006, Hanahan and Weinberg have suggested a framework for the classification of capabilities of cancer during the transformation from a normal cell to a malignant one [13] – the "*hallmarks of cancer*". These alterations of normal cell physiology represented by most types of cancers have been termed as (I) self-sufficiency in growth signals, (II) insensitivity to growth-inhibitory (antigrowth) signals, (III) evasion of programmed cell death (apoptosis), (IV) limitless replicative potential, (V) sustained angiogenesis, and (VI) tissue invasion and metastasis. Later, in 2011, these hallmarks have been updated and completed with a second generation of enabling and emerging hallmarks [14]. The additional hallmarks, (VII) genome instability and mutation, (VIII) tumor-promoting inflammation, (IX) avoiding immune destruction and (X) deregulation of cellular energetics were then added to the conceptual framework [14].

When cancer cells acquire respective traits allowing for *tissue invasion and metastasis*, most cancers ultimately render incurable and lead to the death of the patient. Specifically, most of the currently reported deaths of cancer are associated with the development of metastases, as current strategies of treatment are not able to completely abrogate or inhibit this process [3].

Similar principles with regard to the hallmarks of cancer have been earlier conceptualized with the description of the *stages of metastatic progression*. Here, Gupta and Massagué [15] described the different traits a cancer cell has to accommodate on its way to the metastatic site. A simplistic two-step description of these would be the (I) physical dissemination of the primary cancer cells to distant sites, and the (II) adaptation of these cells to the environment at the metastatic site. Several concepts important for cancer invasion and metastasis to overcome the obstacles barring metastasis have been addressed and are organized by a complex network of extracellular and intracellular signals and mechanisms. These include e.g. the concept of stem cells and dormancy, cellular heterogeneity of a tumor mass, the evolvement of mechanisms to overcome intrinsic and extrinsic barriers (hypoxia, nutritional limitations, physical forces, anoikis and senescence programs), the recruitment of tumor-promoting mesenchyme and inflammatory cells, stromal crosstalk, the ´angiogenic switch´ and homing of disseminated cancer cells and hematopoietic progenitor cells.

As relevant in relation to the research covered in this thesis, specific concepts are pointed out based on the summaries and interpretations of Hanahan and Gupta [14,15].

1.3.2.1 Epithelial-mesenchymal plasticity

Both during the initial step of dissemination, intravasation, and when reaching a potential metastatic site, cells undergo a transition between an epithelial and mesenchymal state. The initial epithelial-to-mesenchymal transition (EMT) is a program that resembles processes associated with embryonic morphogenesis and wound healing [16]. A set of transcription factors and effector proteins has been postulated as markers of EMT that are induced by cells exposed to either the tumoral or the stromal microenvironment. Amongst them, proteins associated to increased mobility, cell adhesion molecules and matrix-degrading enzymes are part of the signature in cells undergoing EMT [14]. Nevertheless, it has been postulated that the same cells need to either be in a temporal state of mesenchymal plasticity or undergo partial EMT (hybrid) to be able to colonize metastatic sites (**Figure 1**) [17].

1.3.2.2 Extracellular matrix modulation

Primary cells need to be able to get invasive to overcome barriers, such as the basement membrane, a dense network of glycoproteins and proteoglycans and endothelial cell structures or when they proceed through the adjacent matrix in the primary and metastatic site [14]. The matrix provides the tumor with a variety of growth factors and cytokines that, when extracted, can promote tumoral progression. Matrix proteases, such as urokinase-type plasminogen activator (uPA) and matrix metalloproteases (MMPs) can be either expressed by cancer cells or promoting stromal cells to assist in the degradation and modulation of the surrounding matrix [13] during *in situ* invasion or homing at the metastatic site. As several tumor types showed a tendency to metastasize to specific secondary sites (Paget´s "*seed-and-soil hypothesis*", [18]), it was postulated that the cellular program of the metastasizing cells is either already adapted to the metastatic site or that soluble factors promote the homing/tropism and precondition of the metastatic niche. Here, e.g. premetastatic induction

of MMPs in distal sites was postulated to prepare a niche, more compliant with the needs of the metastatic cancer cells. Additionally, osteolytic lesions in breast metastasis to bone often involve a crosstalk between cancer cells and cells involved in bone remodeling, such as osteoclasts.

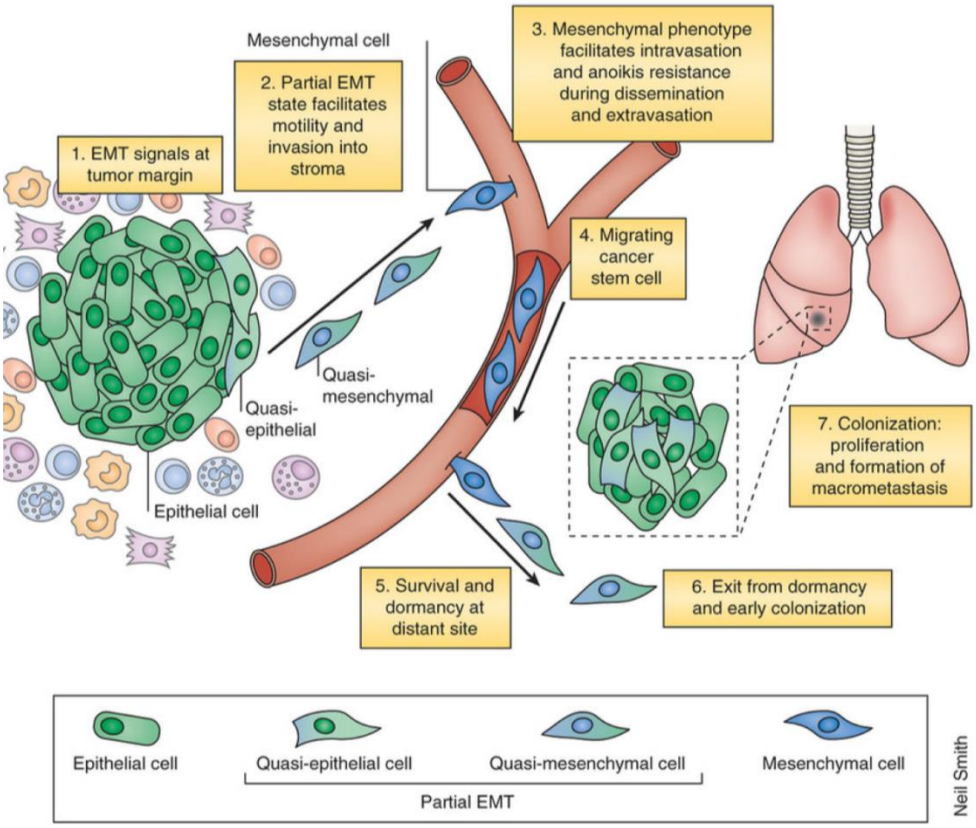


Figure 1 *In response to EMT-promoting signals, a subpopulation of epithelial cells at the invasive edge of the tumor may lose epithelial traits. As these cells detach from the bulk of the tumor, they become less exposed to epithelial signals and acquire mesenchymal properties in the presence of EMT signals supplied by stromal cells. A fully mesenchymal phenotype facilitates invasion into surrounding tissues and intravasation into blood capillaries or draining lymphatic vessels. After arrival at a distant organ, the mesenchymal phenotype facilitates extravasation and invasion into the foreign tissue. Here, disseminated cells are exposed to signals different from those of the primary tumor, and the mesenchymal state may confer survival advantages to single cancer cells or alternatively may support long-term dormancy. When the appropriate contextual signals become available, disseminated cells may undergo MET and gradually reacquire epithelial properties. Epithelial signals are reinforced through autocrine and paracrine signals. This facilitates the outgrowth of macrometastases that are composed predominantly of epithelial cells.*
Reprinted with permission from Tam WL, Weinberg RA. The epigenetics of epithelial-mesenchymal plasticity in cancer. Nat. Med. Nature Publishing Group; 2013;19:1438–49, Copyright © 2013

1.3.2.3 Remodeling of Cell adhesion complexes

Additionally to matrix modulation, tumor cells rebuild their set of adhesion molecules to adapt to the specific microenvironment and to overcome barriers. Commonly observed is the downregulation of the epithelial adhesion marker E-Cadherin that is also part of the EMT program. Other specific examples of commonly dysregulated cell adhesion molecules are integrins [13,19]. Integrins were observed to form uncommon α- and β-complexes and enable interaction of the tumoral cell with ligands specifically expressed at distinct target sites. Both during dissemination of the primary tumor and the colonization of micro-metastases, the cells need to adapt to the local environment or to a local supportive stroma. Additionally, the rearrangement of adhesive complexes assists in the rearrangement of the cellular cytoskeleton, important for example for cell motility.

2 Tartrate-resistant acid phosphatase/TRAP/*ACP5*

2.1 Classification

Tartrate-resistant acid phosphatase (TRAP) is an enzyme belonging to the subclass of acid phosphatases (**Figure 2**), hydrolyzing phosphate ester bonds and anhydrides in the presence of water and acidic conditions into alcohols and phosphates [20]. Emerging as the 5th band separated by electrophoresis on an acidic native acrylamide gel and as the only acid phosphatase in serum insensitive to tartrate, it subsequently was designated TRAP/*ACP5* [21].

Enzymes of this subclass are characterized by a binuclear iron center, rendering a purple color and thus the alternative name purple acid phosphatases (PAPs) [20]. Among the phosphatases, also other enzymes with two metal ions in their active site exist, e.g. the serine/threonine protein phosphatase 1 (PP1) and 2A (PP2A), combining them under the term binuclear (metallo-) phosphatases. Next to the resistance to tartrate and the purple color, PAPs can also be distinguished from other acid phosphatases by their molecular weights and a high isoelectric point (pI> 9) [20,22].

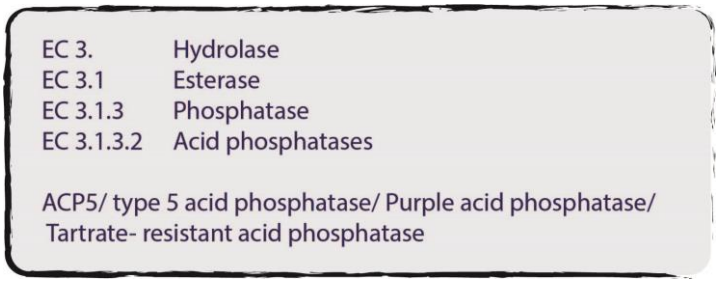


Figure 2 *TRAP enzyme classification*

PAPs were isolated from microbes [23–25], plants [26,27] and animals [28–30]. PAPs found in plants are predominantly displaying molecular weights of 110 kDa with two homodimeric 55 kDa subunits, whereas enzymes of the mammalian species are monomeric low molecular weights forms of around 35 kDa.

Mammalian PAPs (bovine spleen and pig uterine fluid/´uteroferrin´) and TRAPs have been independently characterized for several decades, and therefore are referred to by multiple names. Only in the 1990s cDNA cloning, protein sequencing and immunological cross-reactivity provided evidence that TRAP and PAP is actually the same enzyme [31–34].

2.2 TRAP gene

2.2.1 Chromosomal location and gene structure

The human TRAP gene has been mapped on a region on Chromosome 19p (19p13.2-13.3) [32,35], whereas the murine TRAP gene is located on chromosome 9 [36], rat TRAP gene on chromosome 8 [37] and pig TRAP gene (uteroferrin) on chromosome 2 [38], encoding one single gene.

Mammalian TRAP genes have been cloned from mouse [36,39], human [32,39,40], rat [31] and pig [34]. The gene structure is overall highly conserved between species [34,41–43]; built up by 5 exons and the ATG-start-codon/translation initiation site at the beginning of exon 2, the promotor region is located on exon 1. Transcription gives rise to a ~1.5 kb mRNA with open reading frames of 975 bp in human macrophages [32] and 981 bp in the rat cDNA [31].

2.2.2 Promotor regions

Studies of the 5´-flanking region in the murine gene suggest the presence of tissue-specific promotors resulting in three different mRNA transcripts, differing only in the non-coding exon 1 sequence [43,44]. The human gene displays a similar genomic structure, with four transcripts, differing in the exon 1 sequence [43]. Little sequence conservation was however noted upstream of the proximal promotor in the mouse and human gene [44].

2.2.3 Transcriptional regulation

Within the human 5´-flanking region, several potential binding sites for transcription factors (e.g. PU.1, MITF) [20,45,46] have been identified, as well as two HRE (hemin response elements) motifs [47,48].

2.3 TRAP protein

2.3.1 Amino acid sequences

TRAP protein varies between 325, 327 and 340 amino acids when isolated and cloned from human macrophages, rat bone and pig uterus, respectively (**Figure 3 A**) [31,32,49]. In the rat protein a 21 amino acid signal sequence was identified resulting in a mature protein of 306 amino acids [50]. A signal sequence of 19 amino acids was predicted in human macrophages by von Heijne´s rule [32]. Comparison of the amino acid sequences of isolations from different mammalian sources revealed a 81-94% of homology between species (**Figure 3 B**).

It needs to be noted that amino acid numbering is derived from the mature sequences (21 amino acid signal peptide subtracted) and deviates between the TRAPs crystallized from different species due to differences in the in- or exclusion of two additional N-terminal residues of the rat/pig [51,52] and the human [53] crystal structures.

2.3.1 Crystal structures

Earlier, crystal structures have been derived for the 55 kDa dimeric plant enzyme from red kidney beans in a resolution of 2.9 Å (Protein data bank code KBP) [54,55], followed by crystallization of mammalian TRAP in a phosphate-enzyme complex (1.55 Å; 1UTE) [51]. Furthermore crystal structures were generated for the proteolytically cleaved (2.7 Å; 1QFC) and uncleaved (2.2 Å; 1QHW) forms of TRAP from rat bone [52,56]. Finally, crystal structures for the human TRAP with and without the repression loop (2.2 Å; 1WAR; **Figure 4 A**) have been retained [53].

Comparing all structures, conservation of a bisymmetrical organization with a βαβαβ-motif in each domain has been resolved, connected via a disulphide bond at residues Cys 142 and Cys 200 [52,56]. In this motif, the catalytic domains with the dimetal centers are located at the C-terminal ends and the metals ions are positioned in an octahedral geometry [53]. Binuclear iron centers in mammalian TRAPs are comprised by a chromophoric (Fe^{3+}) and a divalent metal ion (Fe^{2+}), which are redox active and display a pink color with absorption at λ_{max} ~515 nm. Upon oxidation, the color shifts to purple (λ_{max} ~550 nm) and an inactive differric (Fe^{3+}- Fe^{3+}) enzyme [57,58].

A flexible repression loop domain of 20 amino acids (1UTE, 1QHW, amino acid sequence 145-164) (1WAR amino acid sequence 143-163) is located close to the active site in an exposed region [51,56].

A **CLUSTAL O(1.2.4) multiple sequence alignment**

```
NP_999374.1 PIG       MDTWTVLLILQASLVLPGAVGTRTNTRTAPTPILRFVAVGDWGGVPNAPFHTAREMANAK 60
sp|P13686|PPA5_HUMAN  MDMWTALLILQALLLPSL------ADGA--TPALRFVAVGDWGGVPNAPFHTAREMANAK 52
sp|Q05117|PPA5_MOUSE  MDSWVVLLGLQIIWLPLL------THGTAPTPTLRFVAVGDWGGVPNAPFHTAREMANAK 54
1QHW_A RAT            MDTWMVLLGLQILLLPLL------AHCTAPASTLRFVAVGDWGGVPNAPFHTAREMANAK 54
                      ** * .** **   :           :  :  **************************

NP_999374.1 PIG       AIATTVKTLGADFILSLGDNFYFTGVHDAKDKRFQETFEDVFSDPSLRNVPWHVLAGNHD 120
sp|P13686|PPA5_HUMAN  EIARTVQILGADFILSLGDNFYFTGVQDINDKRFQETFEDVFSDRSLRKVPWYVLAGNHD 112
sp|Q05117|PPA5_MOUSE  EIARTVQTMGADFIMSLGDNFYFTGVHDASDKRFQETFEDVFSDRALRNIPWYVLAGNHD 114
1QHW_A RAT            EIARTVQIMGADFIMSLGDNFYFTGVHDANDKRFQETFEDVFSDRALRNIPWYVLAGNHD 114
                      ** **: :*****:***********:* .************** :**::**:******* 

NP_999374.1 PIG       HLGNVSAQIAYSKISKRWNFPSPYYRLRFKIPRSNVSVAIFMLDTVTLCGNSDDFVSQQP 180
sp|P13686|PPA5_HUMAN  HLGNVSAQIAYSKISKRWNFPSPFYRLHFKIPQTNVSVAIFMLDTVTLCGNSDDFLSQQP 172
sp|Q05117|PPA5_MOUSE  HLGNVSAQIAYSKISKRWNFPSPYYRLRFKIPRTNITVAIFMLDTVMLCGNSDDFASQQP 174
1QHW_A RAT            HLGNVSAQIAYSKISKRWNFPSPYYRLRFKVPRSNITVAIFMLDTVMLCGNSDDFVSQQP 174
                      ***********************:***:**:*::*::********* ******** **** 

NP_999374.1 PIG       ERPRNLALARTQLAWIKKQLAAAKEDYVLVAGHYPVWSIAEHGPTHCLVKQLLPLLTTHK 240
sp|P13686|PPA5_HUMAN  ERPRDVKLARTQLSWLKKQLAAARERDYVLVAGHYPVWSIAEHGPTHCLVKQLRPLLATYG 232
sp|Q05117|PPA5_MOUSE  KMPRDLGVARTQLSWLKKQLAAAKEDYVLVAGHYPIWSIAEHGPTRCLVKNLRPLLATYG 234
1QHW_A RAT            EMPRDLGVARTQLSWLKKQLAAAKEDYVLVAGHYPIWSIAEHGPTRCLVKNLRPLLAAYG 234
                      : **:: :*****:*:*******:**********:*********:****:* ***::: 

NP_999374.1 PIG       VTAYLCGHDHNLQYLQDENGLGFVLSGAGNFMDPSKKHLRKVPNGYLRFHFGAENSLGGF 300
sp|P13686|PPA5_HUMAN  VTAYLCGHDHNLQYLQDENGVGYVLSGAGNFMDPSKRHQRKVPNGYLRFHYGTEDSLGGF 292
sp|Q05117|PPA5_MOUSE  VTAYLCGHDHNLQYLQDENGVGYVLSGAGNFMDPSVRHQRKVPNGYLRFHYGSEDSLGGF 294
1QHW_A RAT            VTAYLCGHDHNLQYLQDENGVGYVLSGAGNFMDPSVRHQRKVPNGYLRFHYGSEDSLGGF 294
                      ********************:*:************* :* ***********:*:*:***** 

NP_999374.1 PIG       AYVEITPKEMSVTYIEASGKSLFKTKLPRRARSEHQHRRA      340
sp|P13686|PPA5_HUMAN  AYVEISSKEMTVTYIEASGKSLFKTRLPRRARP-------      325
sp|Q05117|PPA5_MOUSE  THVEISPKEMTIIYVEASGKSLFKTSLPRRPRP-------      327
1QHW_A RAT            TYVEIGSKEMSITYVEASGKSLFKTSLPRRPRP-------      327
                      ::***  ***:: *:********** **** *
```

B **Percent Identity Matrix - created by Clustal2.1**

%	NP_999374.1 PIG	sp|P13686|PPA5 HUMAN	sp|Q05117|PPA5 MOUSE	1QHW_A RAT
NP_999374.1 PIG		84.92	81.35	81.96
sp|P13686|PPA5 HUMAN	84.92		85.54	85.85
sp|Q05117|PPA5 MOUSE	81.35	85.54		93.88
1QHW_A RAT	81.96	85.85	93.88	

Figure 3 (A) *Protein amino acid sequence alignment of pig TRAP, human TRAP, murine TRAP and rat TRAP 5a, respectively * = amino acid identity; : = amino acid change in one sequence; . = amino acid change in more than one sequence. Loop area is marked in bold. Sequences were derived from NCBI or Uniprot and numbering includes a predicted signaling sequence of 21 amino acids marked with a vertical line, respectively.* ***(B)*** *Percent of identity of different mammalian TRAP structures.*

2.3.2 Post-translational modifications

2.3.2.1 Glycosylation

Mammalian TRAPs are glycoproteins containing up to two conserved surface exposed N-glycosylation motifs at Asn 97 and Asn 128 located in the N-terminal fragment of the two subunit form (represents Asn 95 and Asn 126 in human TRAP, **Figure 4 A**) [31]. Interestingly, these glycosylation sites were found to influence latency and catalytic properties of the recombinant monomeric TRAP, as mutations with a single oligosaccharide chain exhibited lower substrate affinity and catalytic activity [59]. Differences in molecular weights of the enzymes can be partially explained by differences in their glycosylation patterns.

2.3.2.2 TRAP isoforms

TRAP exists as two isoforms, which was identified first due to a differential electrophoresis profile of serum samples [60]. TRAP is transcribed as a monomeric proenzyme and activated by posttranslational cleavage [30,61].

The proteolytically cleaved two-subunit form TRAP 5b was isolated from rat and human bone [31,62], osteoclastomas [63] and human and bovine spleen [30,64]. After purification from bovine spleen homogenates, the single polypeptide as well as the heterogeneous subunits of the cleaved TRAP 5b form were compared [30]. Despite high homology in the protein, the monomeric proenzyme, also called TRAP 5a contains a flexible loop region, which is cleaved and released in the dimeric TRAP 5b isoform (**Figure 4 A**) [61]. Moreover, the TRAP 5a isoform displays several different biochemical characteristics compared to the TRAP 5b isoform. TRAP 5a contains sialic acid residues, which are not present in TRAP 5b [65,66]. The pH optimum for TRAP 5a is lower (pH ~5.2) than for TRAP 5b (pH ~5.8) as well as the specific activity of TRAP 5a, which is ~10X lower than that of TRAP 5b [60,65,67]. Substrate affinity is moreover lower in TRAP 5a, as K_m values are increased, when compared to TRAP 5b [30,68].

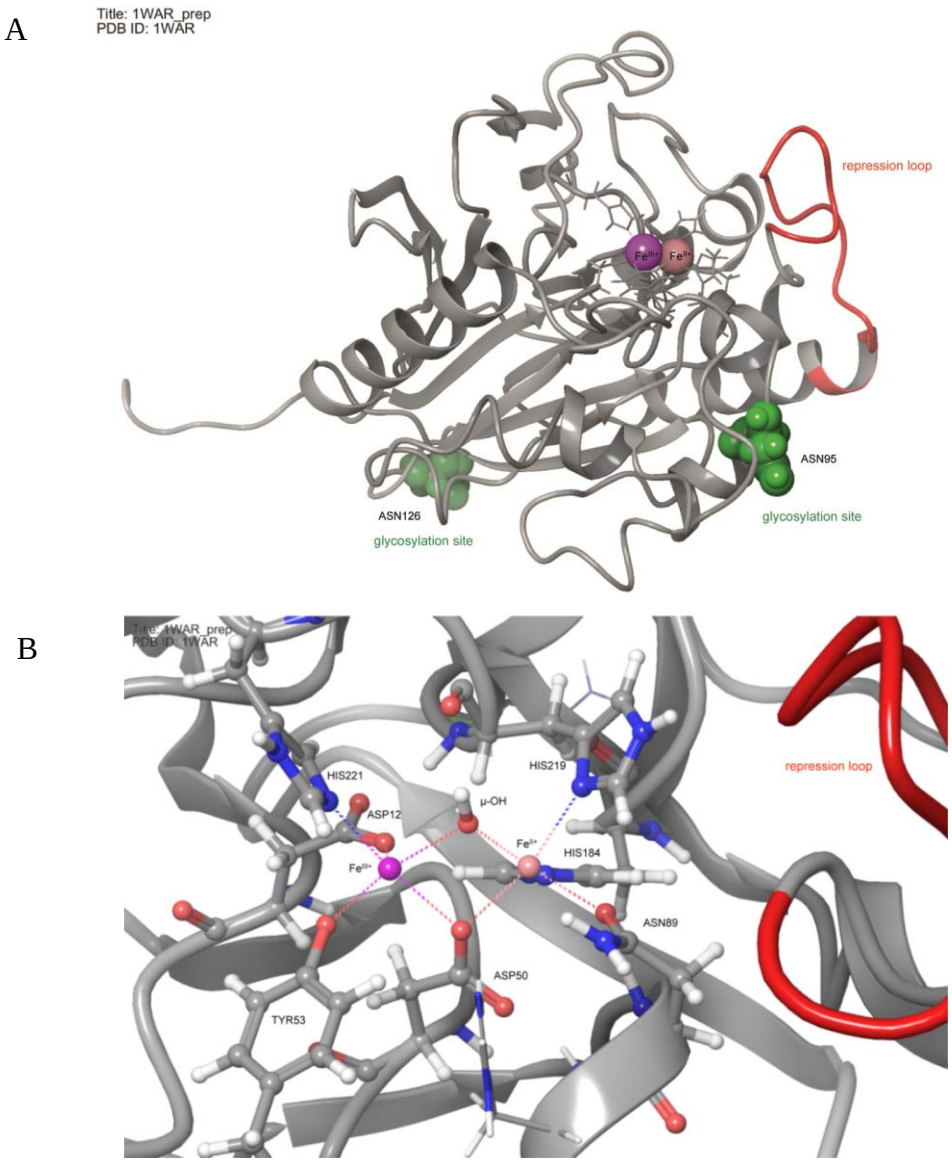


Figure 4 *Recombinant human TRAP,* ***(A)*** *protein structure, resolution 2.2 Å, PDB ID 1WAR,* ***(B)*** *Active site, Graphics by Biognos AB, Martin Frank, 2015*

2.3.2.3 Proteolytic cleavage

Orlando *et al.* were first in stating that cleavage of a five amino acid sequence in the loop region amino acid sequence 155-160 was suggested to be a process resulting in the two-subunit structures, subsequently performed and proven by digestion with the serine proteinases trypsin and chymotrypsin [30]. Nevertheless, trypsin as a physiological enzyme processing TRAP in tissues and body fluids was regarded unlikely [69]. Later, several other cleavage enzymes, amongst them cysteine proteases and MMPs were tested in their capacity to cleave the proenzyme, as physiologically expressed in the osteoclast microenvironment [61,68]. Enzyme activity, sequence analysis, measurements of substrate specificity and site-directed mutagenesis showed that cleavage with Cathepsin K and L, enzymes of the class of cysteine proteinases resulted in activation, comparable to the native bone enzyme. The amino acids Ser 145 and Asp 146 were pointed out to be important in the repressive function of the loop upon electrostatic interaction with amino acids Asn 91 and His 92 of the active site [69–71].

Altogether, proteolytical cleavage was proposed to function as a regulatory mechanism for the activation and degradation of the TRAP enzyme [61].

2.3.3 Enzymatic activity

2.3.3.1 Active site structure and Catalytic reaction

In reviews from 2012 and 2013 about the mechanisms employed by TRAPs, the configuration of the active site and an step-wise model of the catalytic mechanism of TRAP were suggested [72,73].

In the active site, next to the conditional presence of water ligands, the ferric iron (Fe^{3+}) is coordinated by a tyrosine (Tyr 55/Tyr 53), histidine (His 223/His 221) and an aspartate residue (Asp 14/Asp 12). The divalent metal iron (Fe^{2+}) is coordinated by two histidine residues (His 221/His 219, His 186/His 184) and an asparagine (Asn 91/Asn 89). Aspartate (Asp 52/Asp 50) is bridging the iron ions [51–53,55] (**Figure 4 B**).

(As previously noted, amino acid numbering in the respective studies deviates between the TRAPs crystallized from different species due to differences in the in- or exclusion of two additional N-terminal residues of the rat/pig [51,52] and the human [53] crystal structures.)

Catalyzation of the hydrolysis of a monophosphate ester bonds was described as follows [72,73]:

I Substrate binding to the enzyme in a second coordination sphere, formation of a pre-catalytic complex

II Substrate rearrangement, coordination to the divalent metal ion for the facilitation of a nucleophilic attack by μ-OH on the phosphorus atom of the substrate

III Esterolysis of the substrate, free alcohol, phosphate remains bound

IV Release of phosphate, regeneration of the resting state of the enzyme, reformation of the μ-OH bridge

2.3.3.2 Redox activation

TRAP is activated generally by mild reducing agents such as β-mercaptoethanol, ascorbate, glutathione, dithiothreitol and Fe^{2+} converting the redox active Fe^{3+} ion to a Fe^{2+} concomitant with a color change from purple to pink [28,62,74,75]. A low concentration of ascorbate in combination with Fe^{2+} reduces the TRAP enzyme optimally and increases the catalytic rate [62]. Strong reducing agents bleach the TRAP enzyme upon removal of the ferric ions irreversibly [63]. Oxidizing agents only inactivate the enzyme via conversion of the Fe^{2+} ion to a Fe^{3+}, for instance by treatment with hydrogen peroxide [76].

2.3.3.3 Substrates

In its reduced state, TRAP catalyzes the hydrolysis of various phosphate monoesters and anhydrides under acidic conditions [20]. In a study using recombinant and non-recombinant mammalian enzyme sources, TRAP exhibited high catalytic efficiency towards pyrophosphate and phosphotyrosine-containing peptides [77]. Phosphoserine and phosphothreonine-containing proteins, such as osteopontin and bone sialoprotein can moreover be dephosphorylated efficiently [78]. TRAP was suggested as an osteopontin phosphatase, given that it is colocalized with TRAP and that dephosphorylation of OPN has shown implications in adhesion and migration of osteoclasts and human choriocarcinoma cell lines [79–81]. Finally, dephosphorylation of focal adhesion kinase (FAK) autophosphorylation and global tyrosine dephosphorylation of FAK and Paxillin was shown in TRAP-overexpressing melanoma cells [82].

As TRAP has proven to exhibit relatively low substrate specificity, localization, activation by cleavage and a reducing and acidic environment of the enzyme can be suggested to stipulate the mechanism controlling its functional reach.

3 Cathepsin K/CtsK

3.1 Classification and Enzymatic activation

Cathepsin K (CtsK) is a lysosomal enzyme with high homology in its primary sequence to members of the papain cysteine protease superfamily including cathepsins S, L, and B [83]. The CtsK gene is localized on chromosome 1q21 in humans [84] and on chromosome 3 in mouse [85]. CtsK protein is produced as a catalytically inactive pre-pro-enzyme, containing a signal sequence of 15 amino acids followed by a pro-leader sequence of 99 amino acids and a 214 amino acid long catalytic domain [83]. The pro-enzyme displays a molecular weight of 37 kDa and enzymatic activity is increased upon conversion to a 27 kDa mature enzyme. The pro-peptide in the pro-enzymatic form is required for correct folding of the catalytic domain, redirection to the lysosome and for maintaining the enzyme inactive [86,87]. CtsK is activated by either an autocatalytic mechanism [88] or in an acidic environment [83]. Nevertheless, activation of its collagenolytic and elastinolytic activity was also inducible at a physiological pH through allosteric modification by sulfated glycoaminoglycans [89].

3.2 Pathological implications and Pharmacological targeting

CtsK degrades predominantly collagen type I, the major extracellular matrix protein of the bone, but was also shown to degrade other matrix proteins, such as osteonectin [83,90]. Initially, CtsK was assumed to have an exclusive role in bone resorption, as its abundant expression in comparison to other cysteine proteinases was specifically detected for osteoclasts (**Figure 5**) [91]. CtsK furthermore regulated the activation and intracellular localization of TRAP in osteoclasts [92] and colocalized with TRAP in resorptive compartments [68].

CtsK has been studied regarding its role in a variety of *pathological conditions* related to bone, where CtsK levels were perturbed. In particular, lack of CstK function was associated to the bone related condition osteopetrosis [93,94] and the autosomal recessive gene disorder pycnodysostosis [95]. Contrary to that, increased CtsK activity, mRNA or protein levels were later also reported in a variety of primary tumors and CtsK found to be overexpressed in invading cancer cells at metastatic sites, such as the bone [96–99].

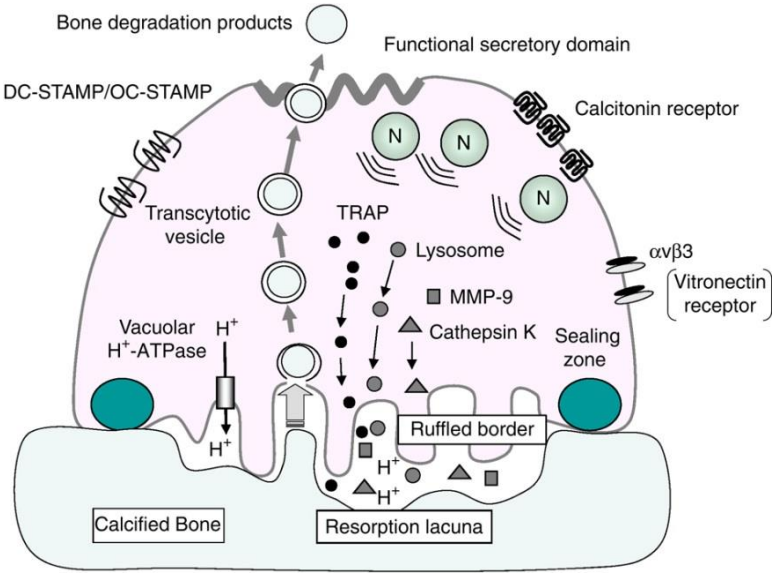


Figure 5 *Bone-resorbing osteoclasts have multiple nuclei and a large number of vacuoles and lysosomes and form sealing zones. Vacuolar H^+-ATPase localized in the ruffled border is involved in the transport of protons into the resorption lacunae, making it acidic. Enzymes such as cathepsin K, MMP9 and TRAP are secreted into the resorption lacuna to degrade bone matrix proteins. Matrix degradation products are endocytosed, packaged into transcytotic vesicles and secreted from the functional secretory domain. Osteoclasts express large numbers of receptors, which are e.g. involved in the cell–cell fusion of osteoclasts. Reprinted with permission from Takahashi N, Udagawa N, Suda T. Vitamin D endocrine system and osteoclasts. Bonekey Rep., Springer Nature; 2014;3., Copyright © 2013*

With regard to its involvement in matrix degradation in bone, CtsK has served as a target for *pharmacological inhibition* in postmenopausal osteoporosis therapy, a condition of pathologically increased bone resorption [100,101]. Several attempts have been made to develop clinically tested anti-resorptive drugs by targeting CtsK, to retain osteoclast differentiation and bone formation [101–104]. Given that CtsK was also overexpressed in invasive cancer cells, the assumption has been made that CtsK implements a similar matrix degradation function also in the development of metastatic lesions. Hence, CtsK inhibitors were applied in mouse studies, where they effected a reduction in tumor burden and local metastasis, osteolytic lesions, as well as extracortial tumor growth [105,106]. Metastatic bone

disease was furthermore diminished in female patients with breast cancer, when the CtsK inhibitor Odanacatib was tested in a double-blind, randomized, controlled trial [107].

3.3 TRAP and CtsK in bone remodeling.

Both TRAP and CtsK have been intensively studied regarding their involvement and processing in activated osteoclasts, resorbing bone. TRAP is, together with Cathepsin K, secreted during the bone-resorbing process into the sealed ruffled border area of the osteoclast and activated by conversion to TRAP 5b [68,92,108]. After degradation of the organic bone matrix by proteinases, TRAP 5b is, together with particles of the matrix, endocytosed by the osteoclast and transported in vesicles via the transcytotic route to the functional secretory domain for secretion into the extracellular space (**Figure 5**) [109].

4 Osteopontin/OPN

4.1 Structure and Regulation

Osteopontin is a secreted and intracellular protein of the class of small integrin-binding ligand N-linked glycoprotein (SIBLING) proteins. This family comprises five glycophosphoproteins, further including bone sialoprotein (BSP), dentin matrix protein 1 (DMP1), dentin sialophosphoprotein (DSPP) and matrix extracellular phosphoglycoprotein (MEPE). OPN is further denoted as secreted phosphoprotein 1 (SPP1), early T-lymphocyte activation 1 (ETA1) protein or bone sialoprotein 1 (BSP1) [110].

The *structure* of the OPN protein is defined by several highly conserved domains and functional motifs (**Figure 6**), such as the conserved arginine-glycine-aspartic acid-recognition (RGD) sequence, ELVTDFP and SVVYGLR sequences, a thrombin cleavage site and cleavage sites for MMPs, an aspartic acid rich sequence at its N-terminal, calcium binding sites and heparin binding domains at its C-terminal [111,112]. RGD, ELVTDFP and SVVYGLR motifs (available after cleavage with thrombin) enable binding and signaling via integrins [113]. An RGD-independent binding was shown for interaction with the CD44 receptor [114] (chapter II.5).

Regulation of OPN is highly complex, as the protein is subjected to extensive modification on the translational level and posttranslationally [115]. So far, three isoforms or splice variants have been identified, consisting of the full-length OPN-a, OPN-b lacking exon 5 and OPN-c lacking exon 4. Additionally, two further isoforms have been annotated. Isoform 4/d is lacking two alternate in-frame exons (missing protein sequence at location 95-116) and isoform 5 (missing protein sequence at location 59-72). Other splice variants have been mentioned, however, not yet been validated to a similar extent [116]. Additionally to the secreted version, alternative translation results in the generation of an intracellular form of OPN, a truncated version of the full-length protein lacking the signal sequence [117]. Further posttranslational modifications include e.g. cleavage, crosslinking mediated by transglutaminase, sialylation, phosphorylation, N- and O-glycosylation and sulfation, resulting in different functional forms (**Figure 6**) [118].

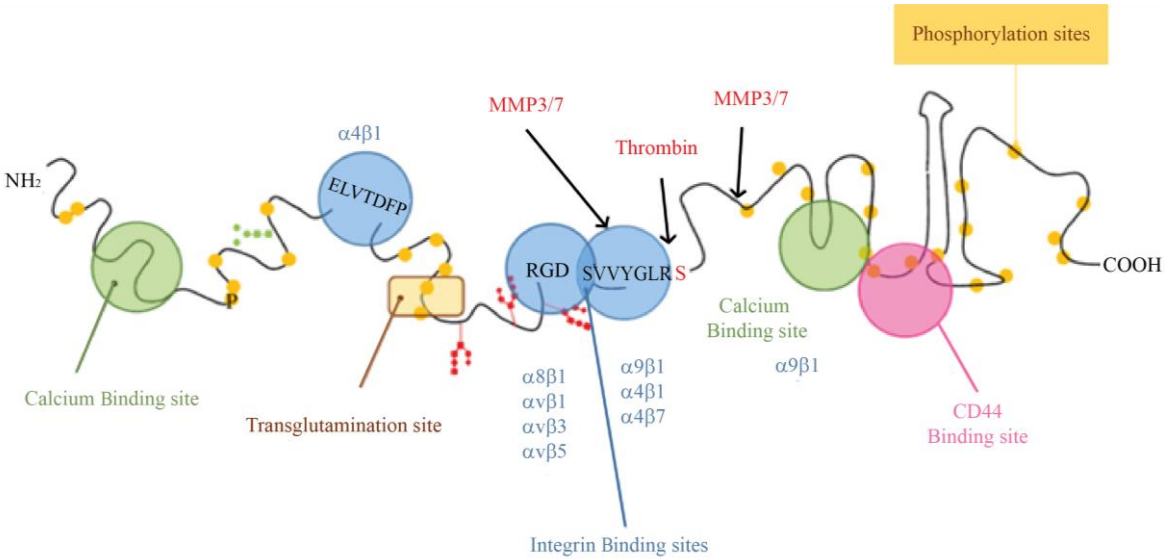


Figure 6 *Main domains of OPN. The cartoon depicts the functional parts of OPN. OPN binds two different classes of receptors, integrins (in blue) and CD44 (in pink). It can also interact with calcium (green). The SVVYGLR sequence is usually masked in the full-length molecule, but it becomes available upon thrombin cleavage of OPN. OPN undergoes several posttranslational modifications including glycosylation (red and green sugars), phosphorylation (yellow dots), crosslinking mediated by transglutaminase and protease cleavage (thrombin and MMPs). Each of these modifications can alter OPN functions.*
Modified with permission from Castello LM, Raineri D, Salmi L, Clemente N, Vaschetto R, Quaglia M, et al. Osteopontin at the Crossroads of Inflammation and Tumor Progression, Mediators Inflamm. Hindawi; Jul 9, 2017 p. 1–22, Copyright © 2017

4.2 Functional implications of OPN

Osteopontin is expressed in a variety of cell types, such as bone and immune cells, epithelial, stromal and endothelial cells and was found in various tissues and secreted fluids [112].

It has been associated to a variety of functions and conditions related to *bone remodeling* and *inflammatory responses*. For a brief introduction, several review articles were selected, presenting OPN´s role in e.g. rheumatoid arthritis, systemic lupus erythematosus, osteoarthritis, inflammation in cancer and adipose tissue and bone homeostasis [112,119–124]. During inflammation, OPN is triggering several immune cells to maintain the immunological response by inducing cytokine secretion [112]. On a cellular level, OPN was reported to be involved in osteoclast, macrophage and endothelial cell migration [80,125–127], acting as coupling factor between cells and matrix. Based on the presence of crosslinking sites, it is also thought to be a stabilizing component of the bone matrix, produced by bone-building osteoblasts, as being able to crosslink to other components of the matrix, such as collagen type I [128].

Osteopontin has moreover attracted interest as a marker of *cancer* development due to strong correlation with advanced stage in many cancer types. Several tumors types showed overexpression of OPN [110,129,130], whereas most cancers expressed hypophosphorylated OPN [131]. Numerous reviews furthermore elaborated in detail about the differential involvement of OPN as a tumoral and stromal protein in cancer [110–112,118,128,132,133]. The clinical relevance of OPN has been addressed e.g. suggesting it as a serum marker monitoring malignant pleural mesothelioma [134] and attributing it prognostic significance in

limited-stage small cell lung cancer [135]. OPN has furthermore been reported to upregulate the expression of genes associated to EMT in aggressive breast cancer [136] and induced migration, invasion and EMT in endometrial carcinoma cells [137]. Splice variant OPN-c, the only variant that cannot form polymeric complexes, induced anchorage-independent growth in human breast cancer cells [138] and was repeatedly reported to be associated with cancer progression [116,139,140]. Further, OPN expression rescued MDA-MB-231 breast cancer cells from sensitivity to chemotherapeutic treatment and increased their proliferative potential [141]. OPN was also suggested to induce stem-cell like behavior in cancer cells [142,143]. Finally, cancer cells were shown to produce or stimulate stromal cells to produce osteoclast-stimulating factors, such as OPN in the metastatic environment of the bone to enable niching and bone destruction [144].

4.3 TRAP as an OPN phosphatase

TRAP was shown to be colocalized with the bone matrix protein osteopontin [145], and migration as well as adhesion of osteoclasts impaired upon dephosphorylation by TRAP [78,80]. Colocalization was further reported in macrophages and dendritic cells [146]. TRAP had the highest activity, when compared to bone alkaline phosphatase, as it was able to remove all phosphates of OPN [147]. This dephosphorylation further inhibited bone mineralization by OPN [147]. Low abundance of TRAP protein and activity caused by mutations in the *ACP5* gene in patients with SPENCD increased ratios of phosphorylated OPN, resulting in an autoimmune phenotype [148]. Similarly, increased levels of phosphorylated OPN were detected in rheumatoid arthritis synovia, when TRAP activity ratios were decreased [149]. OPN was altogether suggested to be a major target of TRAP, affecting its functionality by dephosphorylation [81].

5 Cluster of differentiation-44/CD44

5.1 Structure and Splicing

CD44 is a transmembrane glycoprotein, encoded by a single highly conserved gene. Nevertheless, CD44 proteins are a group of extremely heterogenic molecules, derived from extensive alternative splicing and proteolytical processing [150]. Furthermore, CD44 is extensively modified by phosphorylations, glycosylation, palmitoylations, sulfation and attachment of glycosaminoglycans (GAGs).

The CD44 gene contains 20 exons, whereas translation of exons 1-5 and 16-18 are constant in all isoforms, representing the standard isoform sCD44 [151]. Variant forms of CD44 (vCD44) include exons that respectively elongate the extracellular variable isoform insertion site (**Figure 7 A**) [152,153]. Variable splicing of the v exons is observed in epithelial cells, endothelial cells, and inflammatory monocytes and commonly associated to aggressiveness and stem cell properties in tumors [153,154]. The simplistic structure of the transmembrane molecule is constituted by an (I) amino-terminal ectodomain, (II) a variable stem structure, (III) a transmembrane domain and (IV) an intracellular cytoplasmic tail (**Figure 7 B**) [151].

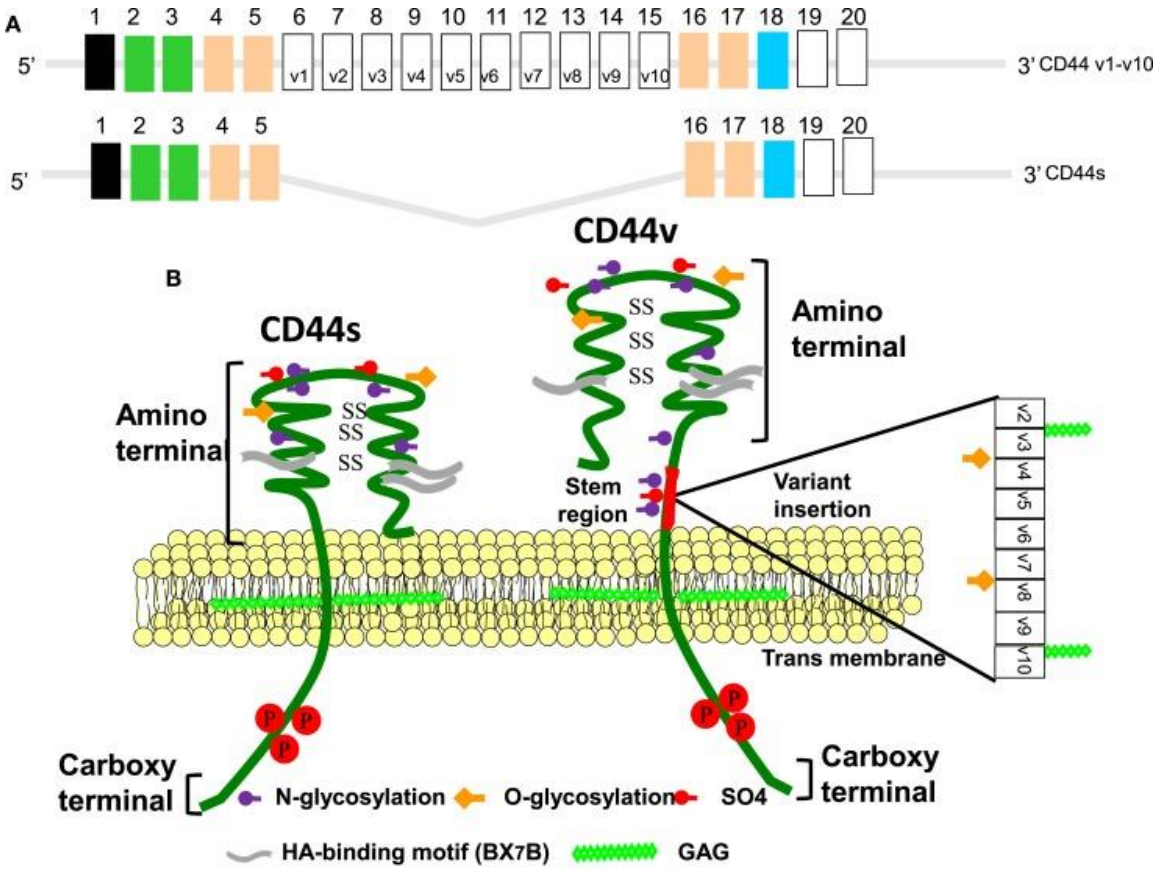


Figure 7 *(**A**) Model structure of alternative splicing in CD44. CD44 pre-mRNA is encoded by 20 exons. CD44s form has a serine motif encoded in exon 5 that can initiate synthesis of a chondroitin sulfate or dermatan sulfate chain. Alternative splicing of CD44 predominantly involves variable insertion of 10 extra exons with combinations of exons 6–15 and spliced in v1–v10 into the stem region, of which v3 encodes a substitution site for a heparan sulfate chain. (**B**) Model structure of alternatively spliced CD44 proteins. CD44 is composed of an extracellular N-terminal domain, a stem region in the extracellular domain close to the transmembrane region, where the variant exon products (red/violet circles) are inserted, the transmembrane region, and the carboxyl terminal cytoplasmic tail. There are multiple sites for N-glycosylation (purple circles) and O-glycosylation (orange circles), and a sulfation domain. The N-terminal portion contains highly conserved disulfide bonds as well as 2 BX7B motifs, both of which are essential for HA binding. The C-terminal cytoplasmic tail contains several phosphorylation sites that regulate the interaction of CD44 with the cytoskeletal linker proteins, as well as with SRC kinases*
Reprinted with permission from Misra S, Hascall VC, Markwald RR, Ghatak S. Interactions between hyaluronan and its receptors (CD44, RHAMM) regulate the activities of inflammation and cancer. Front. Immunol. Frontiers; 2015, 17 Oct 2, p.201, Copyright: © 2015

Whereas the amino-terminal domain serves as a docking site of extracellular matrix (ECM) components, such as hyaluronan (HA), osteopontin, collagen, laminin and fibronectin, the variable stem structure contains putative cleavage sites for MMPs and other sheddases, and is smallest in sCD44. The transmembrane domain is assumed to assist in the association with adaptor proteins and cofactors, whereas the cytoplasmic tail contains motifs for the anchoring of cellular skeleton proteins such as ERM (Ezrin, Radixin, Moesin) proteins and Merlin [150]. Upon proteolytical cleavage in both extracellular and intracellular domain the extracellular stem region of CD44 can be released as a soluble molecule whereas the intracellular domain can act as a nuclear signaling mediator [150,155].

5.2 Receptor-ligand interactions and Signaling

CD44 can induce a variety of signaling cascades that can be clustered into several routes. CD44 can induce signaling (I) directly, (II) by acting as a coreceptor or (III) through accumulation and apposition of other proteins [152].

Several ligands have been proposed to bind to the ectodomain of CD44, inducing signaling. Hyaluronan (HA) is a cell surface-associated glycosaminoglycan (GAG) and is the major and best-characterized ligand to CD44 [155]. It is synthesized and secreted by HA synthetases (HAS) and subjected to cleavage by hyaluronidases. It is also a major component of the ECM. Hyaluronan can bind both sCD44 and vCD44 isoforms and thereby activate the cytoskeleton or MMPs [155]. Binding to CD44 can results in uptake of hyaluronan to regulate its bioavailability [150] and high expression levels of both ligand and receptor were suggested as prognostic markers [156].

Osteopontin is bound by both integrins and CD44 [114,127] (compare II.4). It was suggested that OPN/CD44 signaling is significantly involved in inflammatory processes and cell migration.

Localization of proteolytically active membrane-degrading proteases, such as MMPs to the cell surface is important during invasion and metastasis and can be mediated by CD44. Matrix-bound MMPs are protected from inhibitors and can additionally act on CD44 by proteolytical release of domains to induce signaling [155]. For instance, TGFβ induced upregulation of membrane type 1-MMP and thereby released sCD44 via cleavage [157]

Furthermore, colocalization of CD44 with the TGFβ receptor was shown to induce signaling and increase stability [158,159]. Moreover, complex formation with receptors of the Src-family, Rho-family and the ErbB and c-Met receptor has been reported [151].

Intracellularly CD44 can interact with the cytoskeleton via e.g. ERM proteins [150]. CD44 does not have an intrinsic kinase activity but induces signaling via accumulation of kinases and adaptor proteins.

5.3 Functional implications in cancer

CD44 has been suggested to be a cancer stem cell marker as it serves to integrate a wide variety of extracellular signals and signaling modes. It was shown to be functionally engaged in hematopoietic cell homing, matrix assembly, EMT and apoptosis and drug resistance in cancer cells [160]. It is also associated to cell migration and invasion, angiogenesis and suggested to function as a transcription factor [155]. Nevertheless, CD44 is implicated in functions inhibiting cancer progression [161]. This discrepancy is in part explained by the wide variability of CD44 proteins, the interaction factors and the stage of tumor progression, contribution to the complexity of CD44 involvement in cancer progression.

Specifically, in hepatocellular carcinoma, sCD44 is upregulated, correlated to poor prognosis and reduced disease-free and overall survival. Similarly, it rendered valid as a upstream regulator of TGFβ-mediated EMT, as loss of sCD44 abrogated the TGFβ-mediated expression of vimentin, elongated morphology and tumor invasiveness [162].

6 Transforming growth factor β/TGFβ

6.1 Activation

The TGFβ family is a group of proteins that are part of family of structurally related secreted cytokines [163–165]. TGFβ ligands exist in three isoforms, TGFβ1, TGFβ2 and TGFβ3 that are highly homologous. The most abundant form, TGFβ1, is a protein of 390 amino acids, while TGFβ2 and TGFβ3 contain 412 amino acids [166]. Commonly secreted is an form of TGFβ that is bound to the latency-associated protein (LAP) in an inactive complex. Association of latent TGFβ-binding proteins (LTBP) with the LAP/TGFβ complex assists in proper folding and secretion of the TGFβ precursor protein and regulates its bioavailability through binding to the extracellular matrix (ECM) [167]. The latent complexes are activated by proteolytical cleavage and traction forces through e.g. integrins, proteases, such as MMPs and thrombospondin-1 [168,169] (**Figure 8**).

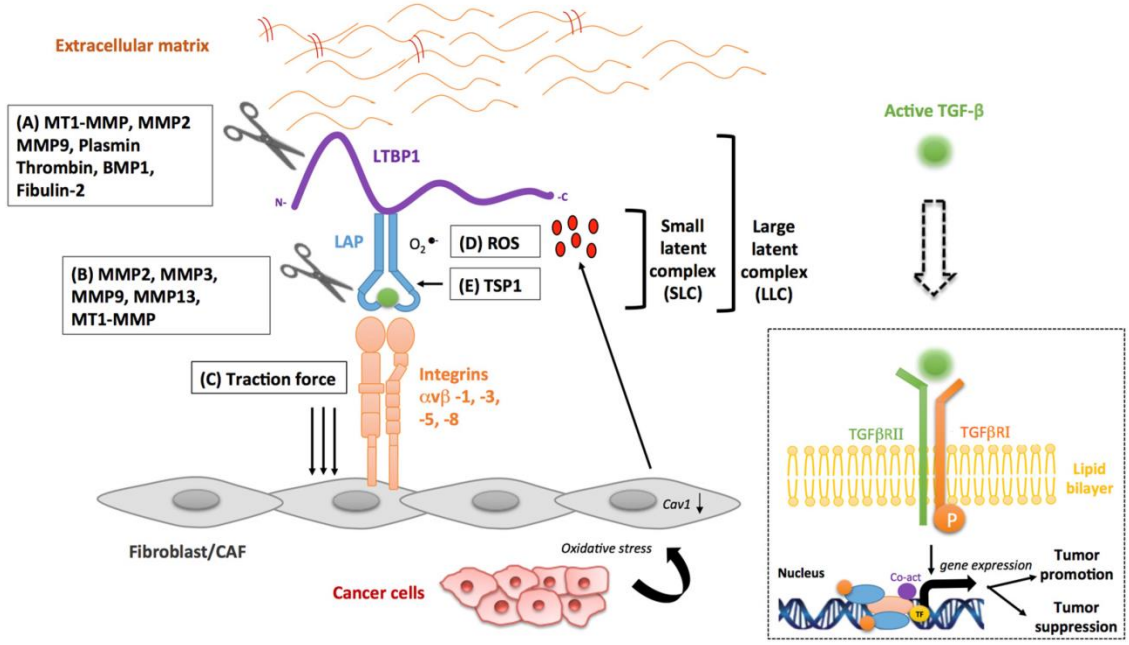


Figure 8 *Stromal activators of TGF-β in the tumor microenvironment* ***(A)*** *MT1-MMP, MMP2 and MMP9, Plasmin, thrombin, BMP1 and fibulin-2* ***(B)*** *MMP2, MMP3, MMP9 and MMP13* ***(C)*** *Integrins αvβ* ***(D)*** *Reactive oxygen species/ROS and* ***(E)*** *Thrombospondin-1 (TSP-1). The mature (active) form of TGF-β can then bind to its cognate receptor and exert its tumor promoting and tumor suppressive properties. Dashed arrow indicates recruitment of the mature TGF-β protein to its cognate receptor.*
Reprinted with permission from Costanza B, Umelo I, Bellier J, Castronovo V, Turtoi A. Stromal Modulators of TGF-β in Cancer. J. Clin. Med.. Multidisciplinary Digital Publishing Institute; 2017;6:7.

6.2 Signaling

TGFβ proteins bind to transmembrane serine/threonine kinases, the TGFβ receptors (TβR). They are constituted of a class of seven type I (activing-receptor-like kinases/ ALKs) and five type II receptors. TβRII is a constitutively active receptor that can undergo autophosphorylation at Ser 213 and Ser 409, while Ser 416 phosphorylation has an inhibitory effect [170]. Ligand binding to TβRII allows for the formation of a hetero-oligomeric complex with a TβRI [168]. TβRII immediately transphosphorylates TβRI at its GS domain

(TTSGSGSG). Mutations in this motif can either lead to an impairment of the kinase activity or results in a partial activation [170]. Phosphorylation of TβRI then leads to the recruitment and phosphorylation of intracellular effector proteins, ultimately leading to the transcription of various target genes.

Intracellular signaling mediated through TβRI/II is regulated either via the canonical mothers against decapentaplegic (SMAD)- (**Figure 9 A**) or non-SMAD-mediated pathways (**Figure 9 B**) [165,170–173]. SMAD proteins are categorized in three classes: receptor-regulated SMADs (SMAD-1, -2, -3, -5, -8), a common mediator SMAD-4 and inhibitory SMAD-6 and SMAD-7 [166]. Upon phosphorylation by TβRI, SMAD-2- and SMAD-3 associate with SMAD-4 and mediate nuclear translocation. Non-SMAD-mediated pathways include the activation of mitogen-activating protein kinase (MAPK), TRAF6, TAK1, phosphatidylinositol-3 kinase (PI3K) and small GTPases such as Rho [166,174–179].

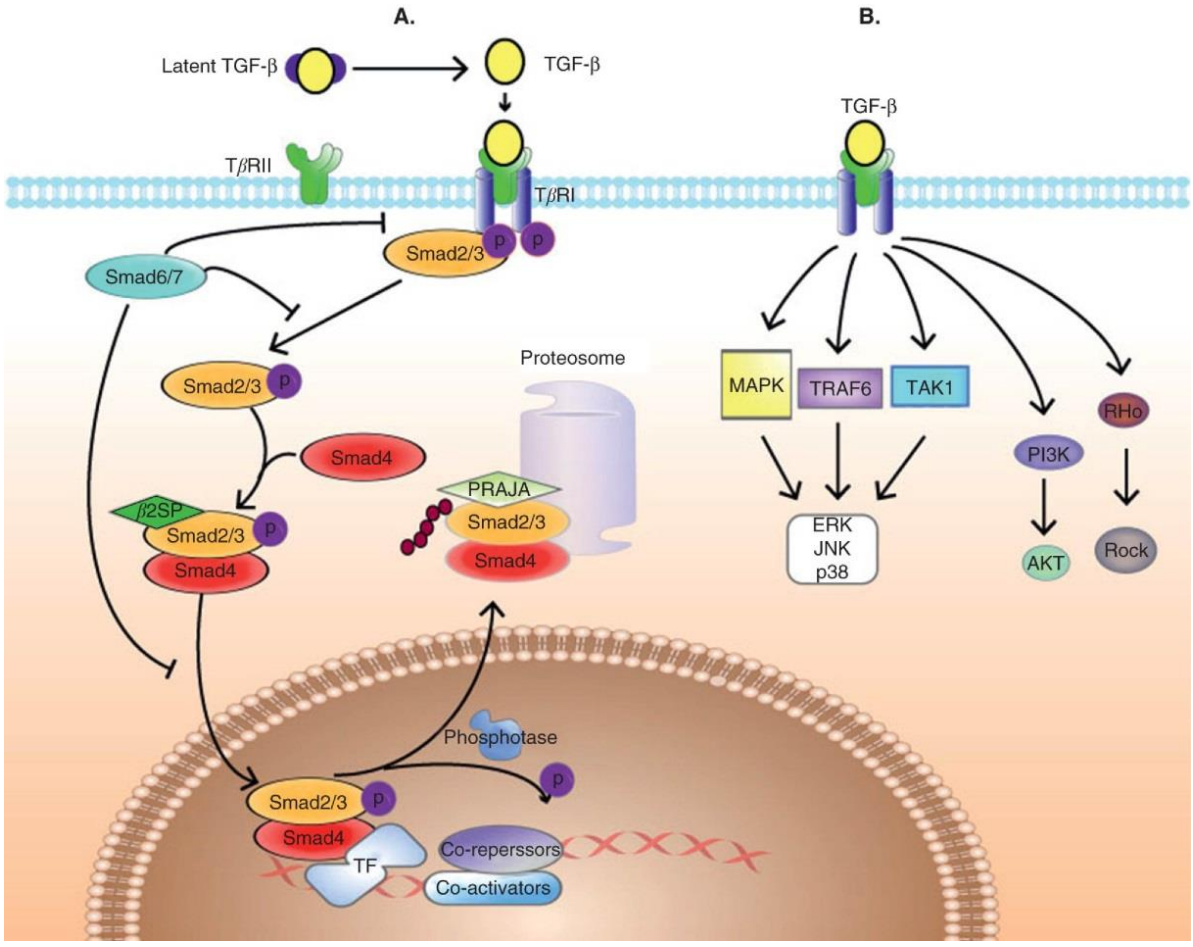


Figure 9 *TGF-β signaling pathway* ***(A)*** *TGF-β ligands signal through distinct receptors and SMADs that are modulated by adaptor proteins, such as β2-spectrin (β2SP), and ubiquitinators. At all levels, SMAD modulation occurs through adaptor proteins as well as E3 ligases such as PRAJA and Smurfs, generating diverse and complex signals.* ***(B)*** *SMAD-independent signaling, including mitogen activated protein kinases (MAPKs), phosphoinositide 3' kinase (PI3K), TRAF6-TAK1-p38/JNK, Rho-Rock, among others. Such alternative signal transducers often regulate the SMAD pathway. TF: transcription factor*
Reprinted with permission from Katz LH, Li Y, Chen J-S, Muñoz NM, Majumdar A, Chen J, et al. Targeting TGF-β signaling in cancer. Expert Opin. Ther. Targets. Taylor & Francis; 2013;17:743–60. Copyright ©2013

Interestingly, TGFβ signaling was coupled to transactivation and interaction with other signaling receptors and pathways, such as CD44 or integrins.

For instance, TβRI was shown to contain a single cytoplasmic binding site for CD44 and binding of hyaluronan to CD44 stimulated the kinase activity of TβRI, which in turn increased SMAD-2/3 phosphorylation in metastatic breast cancer cells [158]. TGFβ1 moreover upregulated hyaluronan synthases (HAS) and promoted the expression of CD44 during EMT induction through HA/CD44 [180]. On the contrary, microarray analysis identified TGFβ2 as a downstream transcriptional target of CD44 signaling in the presence of hyaluronan in breast cancer cells [181] Furthermore, TNFα stimulated the formation of the HA/CD44/moesin complex, which lead to an interaction and activation of SMAD-dependent TβR signaling, resulting in ECM production and cell motility in a fibrosis model [182]. Contradictory to that, CD44 stabilized and thereby inhibited TβRI signaling in normal dermal fibroblasts [159]. Finally, integrin signaling was shown to potentiate TGFβ-dependent effects on EMT [183].

6.3 TGFβ in tumor suppression and progression

TGFβ proteins and signaling is an immensely studied field. Searching for publications in 2017 in the field of cancer involving either transforming growth factor or TGF in the title delivers hundreds of reports.

Generally, TGFβ acts as a pleiotropic cytokine, though, has a bidirectional role during cancer progression. In early stages and normal tissues TGFβ represses tumorigenesis, whereas it acts as an oncogene in late stages [164]. A plethora of effects and observations has been made and attributed to the TGFβ proteins and signaling in the regulation of cancer development (**Figure 10**).

- Increased TGFβ expression in tumor cells correlating with disease progression, tumor stage or survival [184–187]
- Development of treatment resistance [188,189]
- Angiogenesis [190–193]
- Immunosuppression [194–197]
- Master regulator of EMT [198–201]
- Autocrine regulation of its own signaling [202]

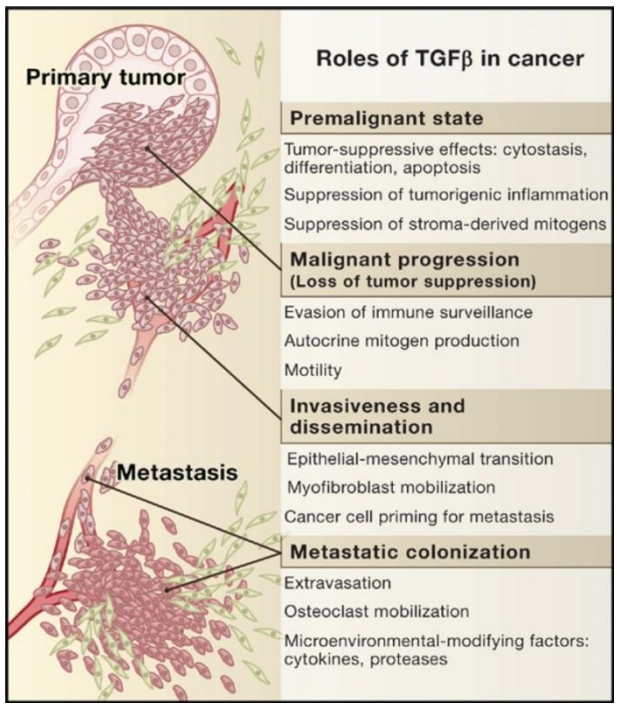


Figure 10 *In normal and premalignant cells, TGFβ enforces homeostasis and suppresses tumor progression directly through cell-autonomous tumor-suppressive effects (cytostasis, differentiation, apoptosis) or indirectly through effects on the stroma (suppression of inflammation and stroma-derived mitogens). However, when cancer cells lose TGFβ tumor-suppressive responses, they can use TGFβ to their advantage to initiate immune evasion, growth factor production, differentiation into an invasive phenotype, and metastatic dissemination or to establish and expand metastatic colonies.*
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6.4 TRIP-1, a bridge between TGFβ and TRAP

TβR-interacting protein-1 (TRIP-1) is an intracellular protein identified as a phosphorylation target of the TβRII kinase and as a functional component of eukaryotic translation initiator factor 3 (eiF3) multiprotein complex [203]. TRIP-1 was shown to negatively modulate TGFβ-dependent functions, such as the induction of EMT and plasminogen activator inhibitor-1 expression [203,204]. Secreted into the ECM via exosomes, it was shown to increase matrix mineralization in bone upon binding to collagen type I [205].

TRAP was shown to interact intracellularly with TRIP-1 thereby activating TβRII and osteoblast differentiation through the SMAD-2/3 pathway at sites of prior bone resorption [24]. Furthermore, TRIP-1 knockdown abrogated osteoblast differentiation and proliferation [25]. Finally, TRAP 5a interaction with TRIP-1 has been demonstrated in mouse pre-adipocytes [26].

7 Physiological and pathological implications of TRAP

7.1 Cell and tissue expression

Upon search in the human protein atlas database, expression of RNA and protein is mainly found in bone marrow and the immune system, the lung and the kidney and urinary bladder (*https://www.proteinatlas.org/ENSG00000102575-ACP5/tissue*) [206]. TRAP mRNA expression has been found in e.g. the thymus, liver, small intestine, lymph nodes and the bone marrow [207]. Human epithelial cell expression has been detected in a variety of tissues, such as e.g. spleen, skin, lung, colon and ileum [207]. Apart from that expression was found in tissue-specific macrophages such as in adipose tissue [208], in the liver [209] and in the lung [210]. TRAP was also expressed during maturation of dendritic cells [207] and in bone-resorbing osteoclasts [211]. Moreover, TRAP expression was found in the nervous system of rats [212]. A detailed look into isoform selectivity in rat tissues revealed that monocytes in the spleen, thymus, liver and colon were majorly positive for TRAP 5b, whereas epithelial cells in colon, lung and kidney were positive for the monomeric TRAP 5a variant [213].

7.2 TRAP isoform processing, Intracellular localization and Secretion

In a mammalian cell line stably overexpressing TRAP, monomeric TRAP 5a is a precursor, distinctly separated early in the endoplasmatic reticulum of cells to either provide a pool for secretion or to be subjected to proteolytic processing for generation of the intracellular isoform TRAP 5b [214]. Concomitant with that, in macrophages and dendritic cells TRAP 5a gets secreted, whereas TRAP 5b retains intracellularly [66,215]. In osteoclasts, monomeric TRAP 5a is secreted via the secretory pathway and the ruffled border area into the resorption lacunas and gets cleaved extracellularly by cysteine proteases [68] (**Figure 11**). As TRAP is a lysosomal enzyme, supplied with a mannose-6-phosphate lysosomal targeting sequence, it was assumed that this sequence must presumably be cleaved or modified for TRAP to be secreted [20].

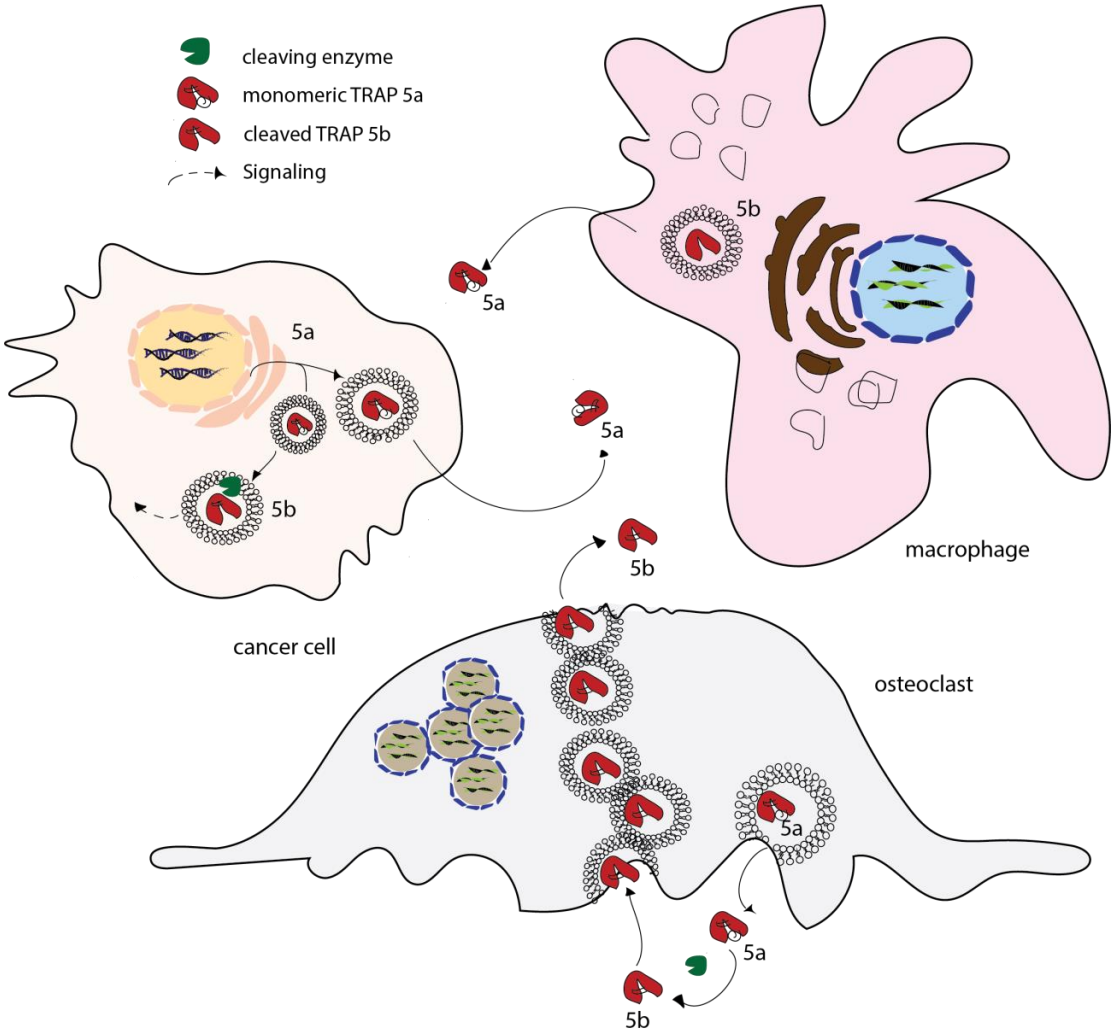


Figure 11 *TRAP isoform location and processing in TRAP-overexpressing cancer cells, macrophages and osteoclast*

7.3 Proposed functions

A particular function for TRAP has so far not been identified, nevertheless, TRAP was associated to a series of processes, both involved in physiological and pathological conditions:

Phosphorylation-independent:

- Generation of reactive oxygen species [216–219]
- Iron transport [220–222]
- Growth and differentiation factor [223–226]
- Regulation of immune responses [146,148,217,227]

Phosphorylation-dependent:

- Marker of osteoclast activity and bone resorption [211,228,229]
- Regulation of osteopontin phosphorylation and bioactivity [81,146]
- Osteoclast and epithelial cell migration [80,230]
- Provinvasion driver in cancer cells [82]
- Transport of lysosomal proteins [231,232]

7.4 TRAP pathology

7.4.1 Conditions associated to bone metabolism

TRAP 5b is highly expressed in osteoclasts and a marker of osteoclast activity [229]. Elevated levels of TRAP have been reported in Paget´s disease, a chronic disorder associated with disorganized bone remodeling [233]. A uncoupling of bone formation and resorption is present in osteoporosis and elevated serum levels of TRAP were found in postmenopausal women with osteoporosis [234]. In osteolytic lesions of multiple myeloma, levels of TRAP 5b were furthermore found to be increased [235].

Additionally, mice lacking TRAP expression displayed disrupted endochondral ossification and mild osteopetrosis [228], whereas TRAP overexpression led to an increased bone turnover [236].

7.4.2 Macrophages and Inflammatory diseases

Macrophages extensively expressed TRAP compared to their monocytic precursors [32] and in an activated or inflammatory state [237]. TRAP expression in macrophages was associated with an enhanced capacity of bacterial killing [216,238]. Restricted expression of TRAP 5a in inflammatory macrophages lead to the suggestion of TRAP as a marker of chronic inflammation [237]. TRAP protein levels were increased, however, specific TRAP activity was decreased in rheumatoid arthritis, suggesting the involvement of TRAP 5a derived from inflammatory macrophages [239]. Next to rheumatoid arthritis, TRAP was also detected in other autoimmune diseases like e.g. the lysosomal storage disease Gaucher´s disease [21,60,240]. Recently, in mouse models and patients diseased with chronic obstructive pulmonary disease (COPD) and asthma, higher expression and activity of TRAP was found, associated to expression in alveolar macrophages [241].

TRAP 5a overexpression was furthermore associated with hyperplastic obesity in mice and induced proliferation and differentiation of adipocyte precursor cells [242]. This could be possibly explained by findings on a molecular level that showed TRAP 5a binding to the membrane of pre-adipocyte mediating a colocalization with glycosaminoglycans (GAGs) and subsequent calveolae-mediated endocytosis [224]. TRAP secreted by macrophages in the subcutaneous adipose tissue suggested involvement of TRAP in lipid accumulation and adipose tissue inflammation [208].

7.4.3 Spondyloenchondrodysplasia (SPENCD)

SPENCD is regarded primarily as a skeletal disorder, recently associated with biallelic mutations in the human TRAP gene leading to a loss of protein or enzyme activity [148,243]. Additionally to the characteristic metaphyseal and vertebral bone lesions, patients displayed a variety of autoimmune phenotypes linked to the accumulation of phosphorylated osteopontin (OPN) in serum, urine and cells. Deregulation of OPN bioactivities as a result of impaired dephosphorylation by TRAP was connected to the activation of dendritic cells and an enhanced inflammatory cytokine signature [148]. Disturbed processing concomitant with accumulation of phosphorylated OPN was moreover detected in TRAP-deficient mice [244].

7.4.4 Cancer and Metastasis

7.4.4.1 Histochemical marker

Initially, among blood-derived cells, only reticuloendothelial cells of leukemic reticuloendotheliosis (hairy cell leukemia) showed exclusive expression of acid phosphatase band 5, *i.e.* TRAP [240]. Soon after, restrictive expression of TRAP in hairy cell leukemia cells and absence in other hematopoietic cells was proven [245], suggesting the usefulness of TRAP as a marker enzyme for the detection of hairy cell leukemia. Later, TRAP could be isolated from spleen [64] and was identified by means of immunohistochemistry in lymphoid cells in the bone marrow [246] in cases of hairy cell leukemia. Nowadays, histological TRAP staining has been routinely used in diagnostics for hairy cell leukemia for more than four decades.

7.4.4.2 Serological marker in bone metastases

Bone metastases typically derive from several types of cancer increasing morbidity and prognosis, breast and prostate cancer representing about 80% thereof [247]. Clinical applicability of serum TRAP isoform 5b was widely suggested for the diagnosis of metabolic and pathological bone diseases, reflecting the extent of lytic bone metastasis and tumor burden. The development of specific TRAP 5b immunoassays highly promoted diagnosis of bone metastasis [248]. Specifically, TRAP 5b has been proposed as a serum marker in several studies addressing bone metastases in breast cancer [248–252], prostate cancer [253–255], multiple myeloma [235] and non-small cell lung cancer [256].

7.4.4.3 Cancer-associated inflammation and Cachexia

Macrophage expression of TRAP detected by immunohistochemistry in colon cancer was associated with improved outcome, increasing the 5-year survival rate and a risk-reduction in disease specific death [257]. Only specific macrophages were positive, when anti-CD68 staining was applied, however, expression could not be restricted to the M1/M2-classification.

Very recently, TRAP 5a was rendered a role as a chronic inflammatory marker and a possible prognostic marker for cancer cachexia in different types of cancer [258]. TRAP 5a activity to protein levels were elevated in cancer patients suffering from cachexia with its serum activity significantly correlated to a decreased patient survival.

7.4.4.4 Cancer cell expression and Clinical relevance

Several studies of the last decade found TRAP expression in cancer cells, redirecting the general assumption that serum levels of TRAP in cancer patients with bone metastases would primarily derive from an increase in osteoclastic resorption or macrophage expression.

In a study from 2006 assessing serum levels of cancer patients, Honig *et al.* [259] detected TRAP expression in breast patients regardless of the presence of bone metastases. To pursue in this direction, several cancer cell lines, primary cancer cells as well as cancer tissues were analyzed for TRAP mRNA and protein levels. Expression was found in breast, ovarian, cervical cancer and malignant melanoma, whereas benign sites were either low in or absent of TRAP protein [259]. Moreover, expression of TRAP was detected in cells cultured from pleural effusions and malignant ascites [259]. In parallel, TRAP activity and protein was identified in several commercially available breast cancer cell lines. In another study, protein

expression, evaluated by immunohistochemistry, was increased in human breast tumors with advancing malignancy [260].

Apart from expression, TRAP was also associated to clinically relevant parameters of cancer progression in patients and was assessed for its oncogenic potential in mouse models.

TRAP was initially identified as one of six proinvasion oncogenes during a genetic screen for invasion and metastasis drivers in *melanoma* [82]. As a functional and clinical validation to this finding, Scott *et al.* showed that TRAP (I) induced tumorigenicity and metastasis development in various mouse models (II) expression was increased when comparing benign to malignant and primary to metastatic tumors (III) reduced tumor- and metastasis-free survival in melanoma significantly. In *hepatocellular carcinoma*, increased expression of TRAP was found in tumor tissues when compared to adjacent tissue [261]. TRAP furthermore correlated to microvascular invasion, poor differentiation and higher tumor–node–metastasis (TNM) stage. It decreased overall survival and increased the incidence of metastasis in both patients and mouse models and served as a risk factor for disease recurrence [261]. Later on, in *gastric cancer*, TRAP was attributed a clinically relevant function as an independent risk factor for peritoneal dissemination and was associated with lymph node metastasis and shorter survival [262]. Expression was again shown to be significantly higher in cancer tissues than adjacent normal mucosa. TRAP was moreover upregulated in *lung adenocarcinoma* and high expression significantly related to lymph node status, TNM stage, and differentiation [263]. High TRAP expression served as an independent prognostic factor for overall survival.

Altogether, these studies support the clinical relevance of TRAP during cancer development and progression. They furthermore prove that tumor-derived TRAP is a relevant source contributing to elevated enzyme activity in serum.

7.4.4.5 TRAP isoforms in cancer

In cancer specimens, metastatic samples from patients with different kind of primary tumors, e.g. prostate, breast, lung and kidney displayed expression of both TRAP 5a and TRAP 5b isoforms. Interestingly, a novel TRAP 5a 42 kDa isoform restricted to metastatic cancer tissue was indicated. Monomeric isoform 5a was predominantly expressed by metastatic cancer cells, whereas the cleaved isoform TRAP 5b was expressed in tumor-associated macrophages and multinucleated giant cells in the tumor stroma [264].

8 TRAP inhibitors

Similarly to other phosphatases, TRAP can be inhibited by *fluoride and tetrahedral oxyanions* such as molybdate, tungstate, arsenate, vanadate and phosphate by binding into the active site [55,58,62,265–267]. However, such compounds are ineffective as selective inhibitors, often weak, toxic and lacking drug-like characteristics. In addition, they commonly affect other phosphatases as well.

Several attempts have been made to identify more selective and potent substances to inhibit the TRAP enzyme:

8.1 Phytochemicals

In an attempt to find anti-osteoporosis phytochemicals, terpenes and sterols from the fruits of *Prunus mume* were isolated and their effects on TRAP activity and osteoclast differentiation was investigated [268]. Nevertheless, none of the substances was able to minimize TRAP activity more than by 50%. Furthermore, flavonoids, such as phytoestrogens have been shown to reduce osteoclast precursor differentiation via acting on Receptor Activator of NFκB Ligand (RANKL), a stimulator of TRAP expression [269]. Following, these results were continued showing a reduction of TRAP activity in osteoclast-conditioned medium obtained by treatment with selected flavonoids, concordantly with a reduction in pit formation [270].

8.2 Substrate mimics

Several efforts have been funneled also into the design and optimization of substrate mimics of TRAP. TRAP was shown to dephosphorylate phosphotyrosine and related peptides [77,271]. Substrate derivates were built based on modifications and structural optimizations of the latter peptides and replacement with more stable phosphotyrosyl analogues. The selected peptides displayed inhibitory constants in the µM range and docked into the active site [272]. Additionally, bone pit formation activity of osteoclasts was inhibited when treated with inorganic polyphosphates, associated with a potent inhibition of TRAP activity [273]. Here, the longer phosphate side chains affected the inhibition potency positively, without introducing any changes in cell number or differentiation.

8.3 Gold coordination compounds

Gold compounds have been applied in the past to treat medical conditions with inflammatory reactions, such as rheumatoid arthritis. Given that the reactive mechanism has never been understood and several side effects were observed, its application was discontinued in the clinics. Nevertheless, gold compounds rendered valuable as inhibitors of TRAP. Gold-chloride was reported as a potent non-competitive inhibitor by inhibiting the phosphorylation of OPN [274]. In contrary, the gold compounds aurothiogluccose and aurothiomalate proved ineffective on TRAP activity [274]. Additionally to this study, we recently showed applicability of the gold coordination compound AubipyOMe on TRAP activity, as it was able to inhibit TRAP activity in macrophage lysates and lung tissue extracts. Furthermore the gold compound inhibited migration in macrophages stimulated to express TRAP by RANKL, without affecting unstimulated macrophages [241].

8.4 Rational drug design: phosphonic acids

Based on the scaffold of 1-naphtylmethyl phosphonic acids, synthesized as inhibitors of TRAP [275], more compounds were designed by computational modelling and rational drug design in follow-up studies [276,277]. This approach of finding inhibitory compounds is based on the strategy to predict appropriate derivatives of lead compounds followed by empirical testing and evaluation for bioactivity. Several derivates were chemically produced with respect to their anticipated binding pattern, leading into a family of α-alkoxy-naphtylmethyl phosphonic acids tested to be active in the pig and plant TRAP enzyme [276]. A second generation of this family, *i.a.* acyl derivates, furthermore proved valid as active inhibitors of TRAP, dependent on the acyl chain length, as predicted by high docking scores [277]. Here, α-alkoxy-naphthylmethyl phosphonic acids, predicted to display the highest binging affinities also rendered most potent, when tested on the recombinant enzymes.

8.5 Fragment-based screening: lead structures

Fragment-based screening was further applied in a study from Feder *et al.* [278], with the aim of finding lead structures from a library with drug-like characteristics that bind into the active site of TRAP. Screening approaches are a second approach commonly applied to identify potential small molecule inhibitors that enable structural optimization by modulations based on the leads structures. Three fragments were found, amongst them 5-phenylnicotinic acid (5-PNA), inhibiting the TRAP enzyme in the µM range and displaying favorable parameters and high ligand binding efficiencies applying crystallography and assessing docking modes. Despite similarities of the applied plant enzyme to the human enzyme, none of the lead structures were assessed for their functionality on the human TRAP or in their applicability in a cell based system.

III PRESENT INVESTIGATION

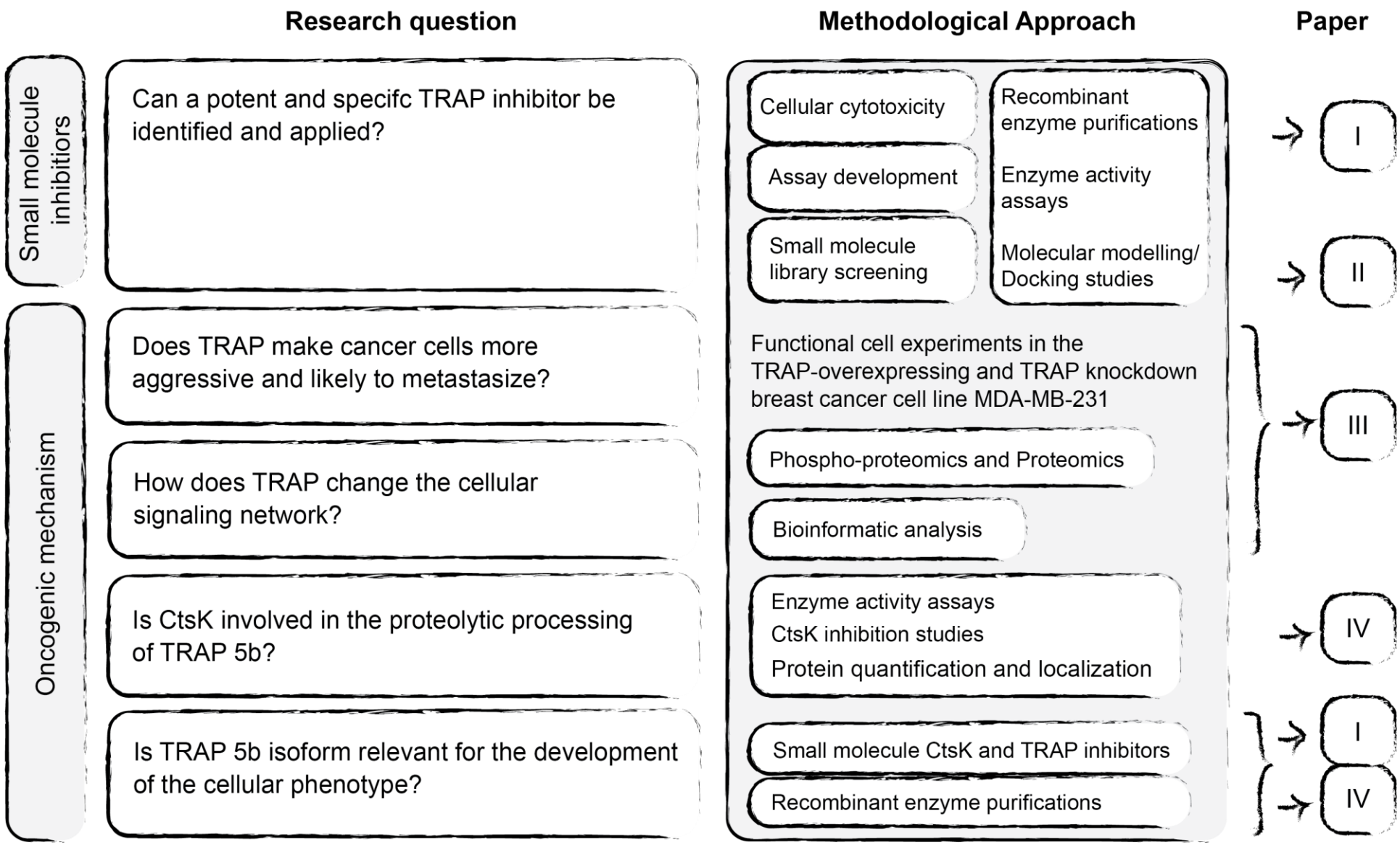
1 Aims of the thesis

Overarching aim:

These studies addressing the role of TRAP enzyme in the development of cancer were founded on two major milestones. We aimed (I) to identify and/or characterize novel and potent small molecule compounds inhibiting the activity of TRAP and (II) to dissect molecular mechanisms of TRAP action in cancer cells overexpressing TRAP.

Specific aims:

- To further characterize the inhibition potency and applicability of the small molecule inhibitor 5-PNA on the two TRAP isoforms and to test its functionality on the mammalian enzyme and in a cancer cell system
- To screen for potent small molecule inhibitors applicable to either or both TRAP isoform
- To specify functional effects of TRAP overexpression at a cellular level
- To outline signaling and network perturbations upon modulation of TRAP expression
- To identify possible TRAP substrates
- To evaluate the involvement of Cathepsin K in the generation of TRAP isoform 5b
- To dissect the role of TRAP isoforms 5a and 5b during cellular transformation


Research question
Methodological Approach
Paper
Small molecule inhibitiors
Can a potent and specifc TRAP inhibitor be identified and applied?
Cellular cytotoxicity
Assay development
Small molecule library screening
Recombinant enzyme purifications
Enzyme activity assays
Molecular modelling/ Docking studies
I
II
Oncogenic mechanism
Does TRAP make cancer cells more aggressive and likely to metastasize?
How does TRAP change the cellular signaling network?
Functional cell experiments in the TRAP-overexpressing and TRAP knockdown breast cancer cell line MDA-MB-231
Phospho-proteomics and Proteomics
Bioinformatic analysis
III
Is CtsK involved in the proteolytic processing of TRAP 5b?
Enzyme activity assays
CtsK inhibition studies
Protein quantification and localization
IV
Is TRAP 5b isoform relevant for the development of the cellular phenotype?
Small molecule CtsK and TRAP inhibitors
Recombinant enzyme purifications
I
IV

2 Considerations on the Methodology

2.1 Ethical considerations

In all studies included in this thesis, recombinant proteins or commercially available cell lines were applied. No animal studies or patient material was used. Therefore, ethical approval was not necessary and general guidelines for research ethics consulted.

2.2 Small molecules and Blocking antibodies

In the course of inhibition experiments and for delineation of the relative involvement of candidate molecules, small molecule inhibitors and blocking antibodies were applied. In control conditions, respective concentrations of DMSO solvent or IgG antibodies were applied.

2.2.1 Small molecule libraries

To screen for TRAP inhibitors we made use of a highly specialized and characterized library of small molecule compounds supplied by the Chemical Biology Consortium Sweden (CBCS, *www.cbcs.se*). This library is originally derived from the pharmaceutical industry (Biovitrum AB) and is consistently updated, expanded and filtered for small molecules free from unwanted characteristics such as promiscuity, compound interferences, aggregators, PAINS and REOS. All compounds display drug-like characteristics with respect to molecular weight, hydrogen bond accepting and donating groups, lipophilicity and polar surface area. For ***Paper II***, we applied a primary screening set, consisting of both proprietary and commercially available compounds with chemical diversity. Original compound batches were stored in concentrations of 10 mM in DMSO and consistently tested for identity and purity. Compounds were further handled by subsequent dispensing.

2.2.1 TRAP-inhibitor 5-PNA

The small molecule inhibitor 5-phenylnicotinic acid (5-PNA/CD13) is a chemical compound of 200 Da and was initially found to be a TRAP inhibitor for red kidney bean TRAP with drug-like properties within an approach using fragment-based screening [278]. This inhibitor was shown to block TRAP activity of the plant and pig enzyme at K_{ic} values around ~40 µM. 5-PNA co-crystallized into the active site of the plant TRAP enzyme upon interaction with the iron residues assessed by X-ray structures and molecular modelling. In ***Paper I***, we further characterized the applicability of 5-PNA and then used it as a specific inhibitor to target TRAP function in ***Paper III***. Considerations regarding the use of 5-PNA have been addressed in the results and discussion of ***Paper I*** (chapter III. 3.1.1 and 3.1.2).

2.2.2 Inhibitors and Blocking antibodies for TβR, TGFβ2 and CD44

To target the involvement of selected candidates in ***Paper III***, small molecules and blocking antibodies to inhibit respective effects of TβR, TGFβ2 and CD44 were applied. To target TβR, LY2109761 (Santa Cruz, CAS 700874-71-1, PUBCHEM CID 11655119) a selective dual inhibitor of TGF-β receptor type I/II (TβR I/II) activity was administered, proven to negatively affect the phosphorylation of SMAD-2. To normalize levels of TGFβ2, the blocking antibody AB-12-NA (R&D systems) was applied. Similarly, a CD44-blocking

antibody (Hermes-1, NBP2-22530, Novus Biologicals) was used to assess the relative effect of CD44. Antibodies detecting the phosphorylation sites, Ser 179, Ser 180 and Ser 183 in the intracellular portion of the CD44 receptor were not available.

2.2.3 Cathepsin K inhibitors

The highly potent and irreversible cysteine proteinase inhibitor E-64 has been used in earlier studies to dissect CtsK involvement, e.g. in the activation of TRAP in osteoclasts and cancer cells [92,214]. E-64 is a pan-cysteine proteinase inhibitor, as targeting also e.g. Papain, Cathepsin L and Cathepsin B [279]. Despite not being an optimal candidate for the use as a drug, E-64 is widely applied as a research tool for the characterization of cathepsins and to clock proteinase activity.

In ***Paper IV***, we applied the small molecule Cathepsin K inhibitor Odanacatib (MK0822, Selleckem). Odanacatib was developed based on a structural modification of a previously tested compound and has rendered to be similarly effective but more selective against other Cathepsins [100,280,281]. Odanacatib is a reversible active site inhibitor [280] and discontinuation of treatment restored osteoclastic bone resorption and CtsK secretion [282]. Several attempts have made to assess the relevance of blocking Cathepsin K in diseases with high Cathepsin K levels and activity such as postmenopausal osteoporosis [100] and breast cancer with bone metastases [105–107]. Despite reduction of CtsK-associated bone fractures, the clinical phase III study with Odanacatib [283] was discontinued, as recurrent side effects, *i.e.* increased risk for stroke were observed.

2.3 Enzyme preparations

TRAP enzyme was purified from various sources, cleaved and applied in ***Paper I***, ***II*** and ***IV***.

2.3.1 Monomeric TRAP 5a purification

Recombinant human or rat TRAP was derived from conditioned medium from Baculovirus-infected *Spodoptera frugiperda* (Sf9) insect cell culture (obtained from GenScript USA. Inc.), concentrated, complemented with protease inhibitors and removed from any cell debris. Similarly, TRAP was extracted from conditioned medium of TRAP-overexpressing cancer cells. TRAP was purified by column separation within an ÄKTA purifier^{TM} 10 FPLC system according to a protocol derived from several publications [214,266,284], described in detail in the Material and methods section of ***Paper I***. Briefly, supernatants were loaded onto Sulphopropyl-Sepharose HiPrep SP FF16/10 columns and eluted on a linear gradient from 0.1 M to 0.5 M NaOAc, pH 6.5. Protein containing fractions were further applied onto a HiPrep Phenyl FF 16/10 column and TRAP eluted on a linear gradient from 50 mM NaOAc and 1.1 M ammonium sulphate towards 50 mM NaOAc, pH 5.0. The pooled fractions were then dialyzed against a buffer containing 0.1 M NaCL, 0.005% Triton X-100, 20 mM Tris-HCl pH 7.2 and loaded on a HiTRAP Heparin HP column followed by an additional elution on a linear gradient from 0.1 M NaCl towards 1 M NaCL. Fractions were pooled and frozen at -80 °C and used within a limited number or freeze-thaw cycles to inhibit spontaneous cleavage.

2.3.2 Generation of TRAP 5b by proteolytic cleavage

To obtain recombinant TRAP 5b, TRAP 5a purifications were incubated with human liver Cathepsin L for 2-3 h a 37 °C. The reactions were stopped on ice and by addition of protease inhibitors, such as cysteine proteinase inhibitor E-64. Complete cleavage was confirmed by Western Blotting and activity measurements. Aliquots were frozen and used within a limited number of freeze-thaw cycles to inhibit degradation and activity loss.

2.3.3 Purification of native cleaved TRAP 5b

Native TRAP 5b derived from bone tissue were used from previous isolations [266,285]. A comparison of the TRAP derived from bone with its recombinant counterpart, extracted from Sf9 cells was documented in a study from Ek-Rylander *et al.* [266].

2.4 Cell lines

Adams *et al.* [260] previously reported expression and activity of TRAP in human cancer cell lines and tissues. In their study three breast cancer cell lines (MCF-7, T47-D, MDA-MB-435) and mammary luminal epithelial cells Hb4a displayed TRAP enzyme activity and MDA-MB-435, the most tumorigenic of these line, has two-fold higher TRAP activity than the other cell lines. In a study from Honig *et al.* [259] MDA-MB-468, MCF-7, BT20 and HBL-100 breast cancer cell lines expressed TRAP. Increased TRAP expression was additionally assessed in 1205Lu and M619 melanoma cell lines [82] and SMMC7721 hepatocellular cell lines [261]. The invasive breast cancer cell line MDA-MB-231 was used in ***Paper I-IV*** as a model system to characterize the impact of levels of TRAP expression on several parameters in breast cancer cells. As this cell line expresses low levels of endogenous TRAP, it was surmised that cellular processing of TRAP was highly similar to the one in cells with high expression. Low expression levels assured that TRAP expression would only derive from the overexpression vector. This cell line has not been applied to studies assessing the effect of TRAP on cancer progression, and its use further provided a model to assess the effect of TRAP in late tumor progression.

2.4.1 TRAP-overexpressing MDA-MB-231 breast cancer cells

The TRAP-overexpressing cell line was previously generated by stable transfection with a plasmid containing the full rat sequence [214]. TRAP derived from different mammalian sources reveals high homology in amino acid sequences (**Figure 3B**) and identical biochemical properties [31,32,49,286]. Effects induced by overexpression of the rat sequence are thus comparable to a human TRAP overexpression. Zenger *et al.* [214] previously characterized the cell population overexpressing TRAP and described the biogenesis, processing and channeling of the TRAP isoforms within these cells. Within this study, however not published, clonal cell populations were generated, whereof several clones were picked and further characterized within ***Paper III***. Four clones with either high or low TRAP enzyme activity or protein expression in cell lysate or in the medium were used. The highly TRAP-overexpressing clonal cell population TRAP3^{high} was mostly applied in ***Paper I-IV*** as a representative for TRAP overexpression studies.

2.4.2 TRAP knockdown in MDA-MB-231 breast cancer cells

The TRAP3^{high} cell population was furthermore used to create cell populations with a knockdown of TRAP, to directly study if effects are reversible. For this, MISSION® shRNA pLKO.1-puro Plasmid DNAs targeting rat TRAP were amplified in One Shot® TOP10 Chemically Competent *E. coli* after heat shock transformation, purified, controlled for sequence identity by restriction enzyme digestions. Several plasmids were then transfected into TRAP3^{high} cells together with scrambled shRNA and selected by culture in puromycine-containing medium. Knockdown efficiencies were assessed on RNA and protein level by qPCR and Western blotting, respectively.

2.5 Protein quantification and Localization studies

2.5.1 Antibodies

2.5.1.1 TRAP

As TRAP 5a and TRAP 5b molecules are exclusively distinguished by the presence or absence of a loop domain, present in TRAP 5a, respectively, it is not feasible to generate antibodies selectively detecting the TRAP 5b isoform, lacking the loop. Nevertheless, isoforms can be distinguished by combined application of an antibody detecting both TRAP isoforms and a loop-recognizing antibody selectively identifying monomeric TRAP 5a. Rabbit antibody sera against total TRAP or monomeric TRAP 5a were raised by immunization of New Zealand rabbits with either recombinant TRAP [266] or with the synthetic loop-peptide corresponding to amino acids 167–183 (DDFASQQPKMPRDLGVA) in the mouse TRAP sequence [213,266], respectively. High sequence homology allows for detection of TRAP derived from human, mouse and rat sources.

2.5.1.2 Others

Antibodies for β-Actin (mouse; Cat# 8224, Abcam), TGFβ2 (rabbit; Cat# 113670, Abcam) Integrin α5 (rabbit, Cat# ab150361, Abcam), Integrin β4 (mouse, Cat# ab29042, Abcam) and Cathepsin K (goat, Cat# ab77396) were purchased. A polyclonal antibody serum derived from New Zealand rabbits immunized with a peptide for Cathepsin K (C-KTHRKQYNNKVDE) as previously described [287].

2.5.2 Immunocytochemistry (ICC)

For protein quantification and (co-)localization studies, as well as to determine cell proliferation (chapter III.2.7.2) in ***Paper III and IV***, ICC was performed. Cells were grown in glass 8-well chambers (Labtek II, 154534). Medium was respectively enriched with blocking antibodies or inhibiting compounds or deprived from serum. The cells were fixed and stained with fluorescently labeled antibodies or based on click-chemistry. Confocal images were acquired in the Nikon A1+ confocal laser microscope system and image batch analysis performed in the ImageJ or Nis Elements Advance research imaging software 4.1.0 (Nikon). Confocal microscopy allowed for the generation of optical sectioning and increased resolution. Dependent on the measurements, different settings were applied during image acquisition and image analysis, regarding laser intensities, thresholding or signal intensities.

2.5.3 Western blotting

In ***Paper I-IV***, immunoblotting was applied to detect and quantify isoforms or precursor and mature forms of proteins. Briefly, lysates were prepared from cells that had conditionally undergone treatment. Cell pellets were lysed in RIPA buffer enriched with complete protease inhibitor cocktail and homogenized. Protein debris was removed by centrifugation and total protein content was determined. Respective conditioned media from cells prepared for lysis were separated from cell debris by centrifugation and immediately processed at 4 ˚C. Cell lysates and normalized volumes of corresponding medium were subjected to SDS-PAGE and transferred to PVDF membranes. Unspecific binding was blocked by 3% bovine serum albumin in PBS. Protein bands were detected by incubation with primary antibodies and infrared-labelled secondary antibodies, visualized in the Licor Odyssey Fc Imager system and quantified by densitometry with the Licor Image Studio software 3.1.4 (Licor Biosciences, Lincoln, NE, U.S.) upon normalization to β-Actin expression.

2.6 Enzyme activity measurements

2.6.1 pNPP assay

Lysates prepared in a mild extraction buffer (0.15 M KCl, 0.1% Triton X-100) and fractions derived from Fast protein liquid chromatography (FPLC) were analyzed for TRAP-specific activity. Simultaneously, recombinant enzyme batches were assayed. Here, measurement of the released pNP product, emitting at a wavelength of 405 nm was used as an indirect correlation to released phosphate/TRAP activity. Addition of tartrate and molybdate allowed for discrimination from related TRAPs. The assay was conducted at a pH optimum for TRAP 5b (pH 5.8) and conditionally enriched with sodium ascorbate and iron to reduce the irons in the active site of the enzyme (Fe^{2+}/Fe^{3+}) and thereby activate it. In ***Paper II***, the assay was optimized to be adaptable to high-throughput format.

2.6.2 Malachite green

As an orthogonal assay, Malachite green was applied to complex with free phosphate derived from dephosphorylation of e.g. the experimental substrate ATP or the natural substrate osteopontin by TRAP in ***Paper I and II***. As the malachite green assay is conducted in an acidic range, spontaneous phosphate release of pNPP contributed to high background levels and therefore excluded the use of pNPP as a substrate. This assay was besides using different substrates also measured at a different wavelength (630 nm), which allows for direct evaluation of assay interference, when compared to the pNPP assay, as performed in ***Paper II***.

2.7 Functional cell experiments

Essential alterations in cell physiology that characterize cancer cells as malignant have been proposed as the hallmarks of cancer in 2000 and 2011 by Hanahan and Weinberg [13,14].

Common strategies to assess the ´behavior´ and capabilities of malignant cell lines related to these hallmarks are e.g. to assess morphological changes, replication potential and the ability to migrate and invade. To identify functional capabilities of cells with TRAP overexpression, several methods were set up, optimized or incorporated as part of ***Paper I-IV***.

2.7.1 Morphology

Morphological changes of cells are typical during dedifferentiation and in the course of progressing to a malignant state. Cancer cells often undergo (partial) epithelial-to-mesenchymal transition (EMT) [201], accompanied by a cellular elongation, comparable to the morphology of fibroblastic cells. MDA-MB-231 cells are of elongated morphology and *per se* already fibroblast-like, when kept in cell culture. By measuring length to width ratios by image analysis in randomly selected cells, changes in elongation of cells with overexpression or knockdown of TRAP were assessed.

2.7.2 Cell growth

To assess the role of TRAP overexpression on the replicative potential of cancer cells we applied several different assays. By measuring cell growth with trypan blue, staining for acidic components such as nucleic acid and polysaccharides [288], we assessed both the potential of increased cell division and DNA production. Toluidine blue quantities were measured by absorbance at 630 nm of the dissolved color. A colony formation assay was used to evaluate the potential of cells growing under anchorage-independent conditions, increased hypoxia and limited access to nutrition, such as during the colonization of a metastatic site. For visualization, the DNA-intercalating dye Crystal violet was applied and image analysis consulted for the assessment of colony sizes and numbers. A proliferation assay, measuring the number of cells, going into S-phase by incorporation of the nucleic acid analog EdU, allowed us to test for multiplication of DNA-material.

2.7.3 Migration and Invasion

During several steps of the metastatic cascade, cancer cells become motile and invade into surrounding tissue. These capabilities were addressed applying both wound migration and transwell migration and invasion assays. Wound migration assays were conducted under live cell imaging in cell culture conditions, where cellular movement was recorded as an images series for 30 h under conditional treatment. Cells were first allowed to form confluent layers and migration induced by removal of cells by scratching. Migration in serum-free medium reduced the contribution of proliferation. For Transwell migration assays, the underside of the inserts was coated with basement membrane and extracellular matrix proteins. A serum gradient was adjusted by seeding cells in serum-deficient medium and adding serum-containing medium in the lower well. Additional to the serum-gradient, it is likely that the coating attracts the cells both based on a chemo-gradient and allows direct contact through the membrane pores. For different proteins, specific times, when the assay was stopped were assessed and the amount of cells on the lower side of the insert quantified by Crystal violet stain dissolved and measured by absorbance at 600 nm.

To test invasion, basement membrane-precoated transwell inserts were applied and cells allowed to invade for a specific amount of time through the layer towards the lower site of the insert. The invaded cells were then detached and quantified by luminescence.

2.8 Phospho-proteomics and Proteomics analysis

Mass spectrometry-based proteomics and phospho-proteomics analyses were applied as part of a collaborative effort to investigate global changes of the protein and phosphoprotein network introduced by TRAP perturbation. A detailed elaboration on the method with respect to analytical depth and current protocol standards as well as interpretation of the results are discussed in ***Paper III*** and the doctoral thesis of Elena Panizza [289].

Briefly, proteins were extracted in a detergent solution aimed to extract a variety of proteins, including cellular and membrane proteins and the proteins were digested by trypsination. For multiplex analysis either SILAC or TMT labeling was consulted. In case of SILAC labeling, the cells were first cultured in medium containing stable heavy isotope or light isotope-labeled amino acids. For TMT labeling, peptide samples were incubated after digestion with TMT10plex reagents allowing for similar analysis of up to 10 samples. Phosphoproteins and proteins were pre-fractioned by High Resolution Isoelectric focusing (HiREF) to increase the analytical depth. To enrich for phosphorylation sites conditionally an ultra-acidic strip was applied. Peptides were pre-fractioned and analyzed in a LTQ Orbitrap Velos mass spectrometer. Computational methods, such as hierarchical clustering based on Euclidian distance, Gene ontology analysis (Gorilla) and network analysis (Cytoscape, STRING) were conducted to analyze and interpret the results for the formulation of biological hypotheses.

2.9 Molecular docking studies

In ***Paper I and II***, molecular modelling of selected compounds was performed within collaboration with the company Biognos AB, Gothenburg. Receptor structures were prepared in a protein preparation wizard (Maestro, LeadIT), based on the mammalian crystal structures of human (1WAR) and rat TRAP (1QFC) [52,53]. The inhibitors were docked to assess putative binding modes into the active site of the TRAP molecule with the program Glide or FlexX. Poses were scored using HYDE.

Based on missing knowledge about co-crystallization of the molecules with the enzyme and limitedly defined crystal structures (2 Å and 2.7 Å, respectively), all binding modes described are *de novo* and putative. Nevertheless, binding into the active site suggests potential interaction points and modes of inhibitory action.

3 Results and Discussion

3.1 Can potent and specific TRAP inhibitors be identified and applied?

Protein phosphorylation plays a key role in a variety of physiological processes and deregulation can be coupled to pathological conditions. Orchestrating a variety of cellular functions via their activity to phosphorylate and dephosphorylate signaling mediators, both kinases and phosphatases represent interesting candidates for drug targeting.

Protein kinases have been the more popular drug targets, whereas phosphatases are underrepresented in the development of inhibitors and clinical trials. Phosphatases have been earlier categorized as undruggable targets as not fitting into the conventional "lock and key" model [290]. Nonetheless, advances in the development of phosphatase inhibitors have been made with the progression of knowledge about structural features, computational modelling programs or enhanced libraries [290].

Tartrate-resistant acid phosphatase (TRAP) is a ubiquitously expressed enzyme and cellular location is attributed to vesicular like structures, such as lysosomes. Furthermore, TRAP is an enzyme containing a redox-sensitive bimetal iron center in the active site and irons susceptible to reduction or oxidation affect TRAP´s activity [20]. In the conventional view, these characteristics make TRAP a challenging drug target, nevertheless, an attractive drug target, due to its involvement in the pathogenesis of diseases, such as osteoporosis and cancer. Finally yet importantly, limitations that would render inhibitors inapplicable in a clinical setting do not necessarily exclude the use of the compound to target functions in a cellular system.

3.1.1 Characterization and identification of TRAP inhibitors

Feder *et al*. identified 5-PNA next to two other compounds as a TRAP inhibitor by fragment-based lead discovery [278]. When these compounds were retested, 5-PNA was the only compound inhibiting recombinant human and rat TRAP in ***Paper I***. K_{ic} values for the inhibition of TRAP could be reproduced from various sources and with the application of several possible substrates (~100 µM). Importantly, 5-PNA inhibited TRAP 5b and TRAP 5b activity in lysate, selectively.

In TRAP-overexpressing MDA-MB-231 breast cancer cells, TRAP is early in the secretory pathway diverged to provide a pool of monomeric TRAP 5a destined for cleavage [214]. It could be excluded that the reduction of TRAP activity by 5-PNA derives from an inhibition of cleavage or modulated secretory pathway. Either of these possibilities would result in higher levels of the low active TRAP 5a at the expense of TRAP 5b. 5-PNA did not exert any of these effects, further assessing the specificity of 5-PNA on inhibition of TRAP activity (**Figure 12**).

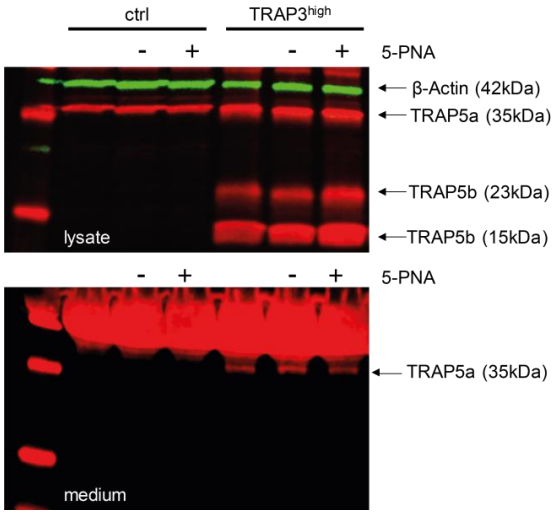


Figure 12 *TRAP isoform expression in control and TRAP-overexpressing MDA-MB-231 cells under treatment with 200 µM 5-PNA for 24 h.*

Next to the characterization of 5-PNA, small molecule library screening was applied to identify additional compounds with a potential for TRAP enzyme inhibition (***Paper II***). In this approach, among several thousands of compounds, meticulously assessed for drug-like characteristics and promiscuous hitters, 22 compounds and thereof six cluster representatives were filtered for inhibition of TRAP 5a and 5b activity in a range of ~1-100 µM.

As 5-PNA rendered only potent for TRAP 5b and previous studies did not differentiate between the TRAP isoforms, our interest focused also on the identification of isoform-selective/unselective inhibitors. Additionally, the compounds were assessed for inhibition of the recombinant enzymes in either mixed-valent or oxidized state of the iron atoms in the active site. No previous study has addressed possible differences in inhibition dependent on the redox state of the active site. The majority of activity assays was performed under non-reducing conditions [63,241,270,273,278], which increases the likeliness that the enzyme was in the oxidized state. Additionally, in ***Paper II***, all compounds had a higher potency of inhibiting TRAP 5b and the enzyme when the iron atoms were oxidized**.** In contrast, 5-PNA did show minimal preferences for inhibiting the TRAP 5b enzyme in the mixed-valent state (unpublished results).

Different modes of inhibition in different redox states could suggest a modulation of the active site dependent on redox state of the irons. Compounds can commonly render as unspecific inhibitors by oxidizing enzymes with redox-active sites. As all six cluster representatives displayed more potent inhibition on the oxidized enzyme, it is unlikely that they act via changing the redox activity of TRAP.

5-PNA (***Paper I*****)**, and the six novel compounds identified in ***Paper II***, were subjected to molecular docking studies to predict a possible binding mode to the active site of TRAP. 5-PNA, which was already proven to bind into the active site by X-ray crystallography, interacted with the iron atoms in a fashion similar to substrates (***Paper I***). In contrast to that, all six compounds in ***Paper II*** bound into the active site without any close interaction with the iron atoms.

3.1.2 Application of the TRAP inhibitors in a cellular system

The applicability of 5-PNA and the novel six TRAP inhibitors in a cell system was investigated by assessing their potential to block TRAP-dependent breast cancer cells characteristics. In ***Paper I,*** 5-PNA inhibited TRAP-dependent migration and invasion at 200 µM without inducing cytotoxicity up to mM concentrations. In ***Paper III***, TRAP-dependent proliferation and increased expression of TGFβ promoted by TRAP was further abrogated by 5-PNA.

Furthermore, RAW264.7 macrophages treated with receptor-activator of NFκB ligand (RANKL), upregulate TRAP expression. When TRAP was inhibited with 200 µM 5-PNA in these cells, RANKL-stimulated migration was abrogated [241].

Amongst the six novel TRAP-inhibitors of ***Paper II***, only the compound CBK289001 was able to inhibit TRAP-dependent migration of MDA-MB-231 cells, similarly to 5-PNA. The control cells were not affected and the cells did not show any obvious signs of cytotoxicity. Why only one compound inhibited migration in the cell system, whereas all six compounds inhibited TRAP *in silico* can currently not be explained by molecular docking studies but could eventually be resolved by molecular dynamic simulations.

3.1.3 Advantages and limitations of the TRAP inhibitors

Earlier, flavonoids reduced secreted TRAP activity and osteoclastic pit formation with IC_{50} <10 µM, while maintaining cell viability [270]. Polyphosphates with long chain lengths inhibited TRAP phosphatase activity at IC_{50} < 5 µM. Nevertheless, concentrations of 1 mM were applied for the reduction of osteoclastic pit formation [273]. Finally, the gold coordination compound AubipyOMe inhibited both isoforms of TRAP at IC_{50} < 2 µM and TRAP at IC_{50} < 4 µM, when applied to cells. When treating RANKL-stimulated RAW264.7 macrophages with 80 nM of the gold compound, migration was inhibited without exerting any cytotoxic effects up to mM concentrations [241].

Despite previously suggested inhibitors displaying higher potencies in K_{ic} and IC_{50} values, 5-PNA and CBK289001 are one of the first inhibitors effective in the inhibition of TRAP-dependent cancer cell functions. Furthermore, 5-PNA is a TRAP 5b selective inhibitor, whereas CBK289001 was able to inhibit both isoforms.

In spite of testing for and applying a library with drug-like characteristics in ***Paper I and II,*** the current results bear limitations.

5-PNA was tested for its activity on closely related phosphatases, such as PP2A and PP1 and cytotoxic effects up to high concentrations in the MDA-MB-231 cell line. It was further assessed for drug-like characteristics based on prediction analysis and binding shown by X-ray crystallography. On the other hand, CBK289001 was shown to be non-promiscuous and did not belong to classes of PAINS, REOS or aggregators, but has not yet been experimentally tested for promiscuity, cytotoxicity or binding. Thus, the TRAP-inhibitors need to be further assessed for unwanted interactions with other molecules and their cytostatic effects in a variety of cancer cell lines. Moreover, comparably high concentrations of the inhibitors were necessary to result in inhibition of the recombinant enzyme and in the abrogation of TRAP-dependent functions. The latter could be explained due to a ´non-optimized´ structure, as e.g. 5-PNA is only a compound fragment. Limited cell permeability and delivery to the enzyme site or a less potent binding with active site residues could further be optimized by structural modifications. Further tests and chemical optimizations are therefore necessary to allow for the multifunctional application of 5-PNA as a potent and specific TRAP inhibitor. Last but not least, studies on structural analogs of CBK289001 and 5-PNA can help in the development and chemical optimization for more potent substances.

3.2 Does TRAP make cancer cells more aggressive and likely to metastasize?

A growing body of evidence has been accumulated in the recent years supporting the idea that TRAP 5b serum levels from bone lesions do not only derive from resorbing osteoclasts. Even that the knowledge about TRAP expressing tumors, *i.e.* leukemic tumors was not new [291], detection of TRAP expression in several cancer cell lines and primary cells underscored the involvement of TRAP in cancer cell function [259,260].

TRAP was associated to disease progression, as levels were high in metastatic lesions of the breast, ovary, skin, prostate, lung and kidneys [259,264]. Furthermore, expression of TRAP showed to be increased with progressive disease status [82,259–261]. Conclusively, several studies attributed TRAP expression a significant role in the development of the disease, as correlated to a variety of clinically relevant parameters and addressed in mouse models.

Specifically, TRAP was associated to a shortened patient survival, lymph node metastasis, peritoneal dissemination, TNM stage, probability of recurrence, tumor size and encapsulation, microvascular invasion and tumor differentiation in melanoma, hepatocellular carcinoma and gastric and lung cancer [82,261–263].

In vitro studies assessed the effects of TRAP expression on the cellular level. For instance, TRAP overexpression increased invasion of melanoma cells and downregulation reduced the potential to form colonies in soft agar [82]. In hepatocellular carcinoma cells, TRAP stimulated cell migration and invasion [261]. Similarly, overexpression of TRAP in metastatic breast cancer cells promoted proliferation, anchorage-independent growth, migration and invasion (***Paper III***). Cellular morphology was elongated, whereas TRAP overexpression resulted in cell rounding and spreading in previous studies [82,214]. Interestingly, morphology and migration were regulated level-dependently when comparing TRAP-overexpressing clones with different expression and activity levels, whereas proliferation and invasion were upregulated independent of the levels of TRAP protein and activity (***Paper III***). Migration was moreover selectively increased on extracellular matrix proteins osteopontin, fibronectin, collagen IV and laminin. Proliferation was furthermore increased compared to control cells after serum starvation. Unpublished results support a possible upregulation of the EMT-markers Snail and Vimentin. Abrogation of TRAP by small chemical inhibition or knockdown reversed the effects in ***Paper I, II and III***.

In summary, cellular characteristics associated to metastasis were positively regulated in several cancer cells and can directly be associated to the data acquired from clinical studies. TRAP upregulation promotes a metastatic phenotype of cancer cells that could ultimately be coupled to increased spreading and infiltration.

3.3 How does TRAP change the cellular signaling network?

3.3.1 Signaling pathways affected by TRAP

Melanoma cells overexpressing TRAP were shown to express reduced levels of focal adhesion kinase (FAK) autophosphorylation at Tyr 397 and tyrosine phosphorylation of Paxillin at Tyr 118. These results could generally suggest an involvement of integrin signaling. Also in ***Paper III,*** downregulation of integrin β4, integrin α6 and upregulation of integrin α5 upon TRAP upregulation could be observed by proteomics analysis and verified by immunoblotting (**Figure 13**, unpublished data).

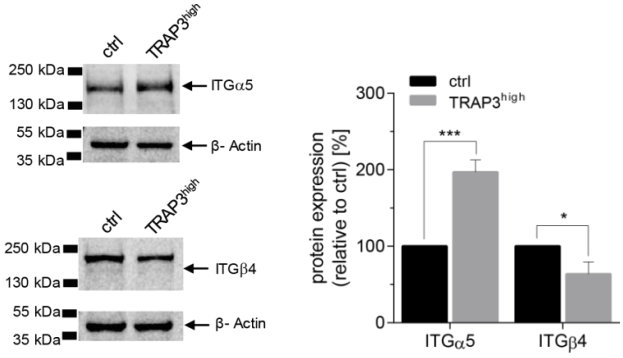


Figure 13 *Integrin α5 (ITGα5) and β4 (ITGβ4) expression in control and TRAP-overexpressing MDA-MB-231 breast cancer cells.*

TRAP signaling independent of cancer cells was earlier associated with TβR-interacting protein-1 (TRIP-1). TRIP-1 is an intracellular protein that is phosphorylated by TβRII kinase [203]. Intracellular interaction of TRIP-1 with TRAP was activating TβRII and osteoblast differentiation through the SMAD-2/3 pathway at sites of prior bone resorption [24]. Independent of TRIP-1, ***Paper III*** could point out that TRAP overexpression is modulating TGFβ signaling by upregulating TGFβ2 and TβRI, shown by (phospho-)proteomics analysis. CD44 phosphorylation in the intracellular domain was furthermore highly upregulated. CD44 has been related to TRAP via its substrate OPN, which is a ligand for CD44 [81,114]. Moreover, CD44 can with its intracellular domain interact with TβRI [158], thereby stimulating multiple signaling pathways mediating, e.g. migration. Indeed, blocking of TGFβ2 and TβRI/II activity was able to abrogate the promotive effect on migration in ***Paper III***, whereas exclusive blocking of CD44 was not sufficient (**Figure 14**).

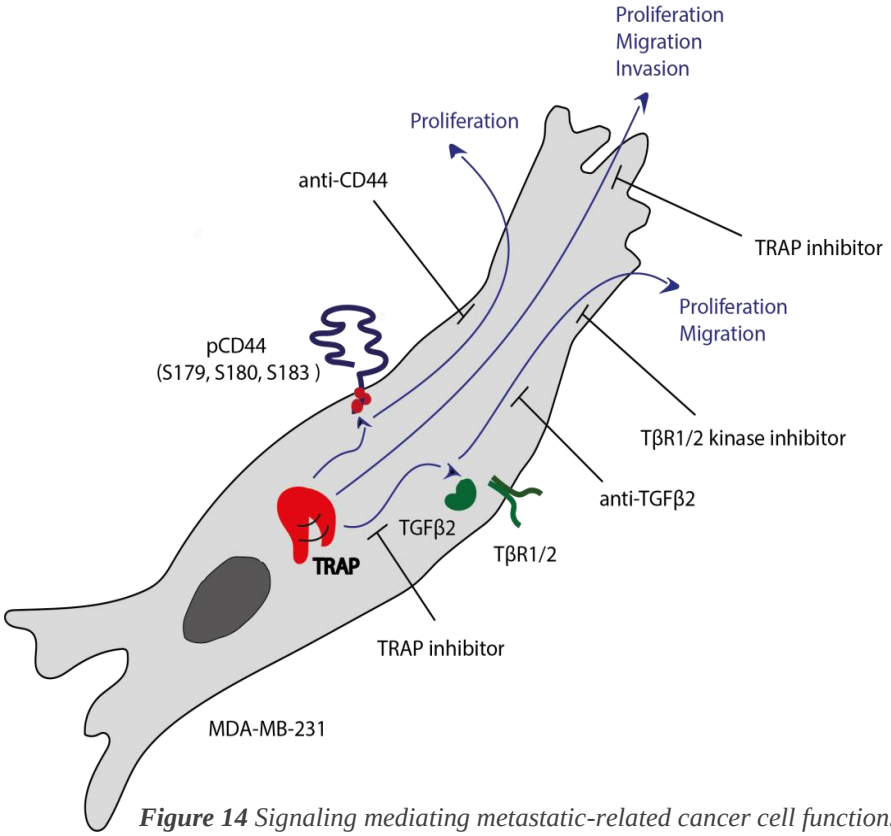


Figure 14 *Signaling mediating metastatic-related cancer cell functions*

Additionally, several proteins associated with extracellular matrix modulation and cell adhesion were regulated by TRAP overexpression/downregulation. These included proteins, such as β-Catenin-1, MMPs, collagens, laminin, lumican, vitronectin, thrombospondin 5, moesin (ERM family) and ROCK-1. Importantly, TRAP is already known to be involved in extracellular matrix regulation during bone remodeling.

Despite the fact that ***Paper III*** verified only a very limited amount of signaling mediators and connections, it provides a huge set of data, including relevant candidates that have either previously been connected to TRAP or with functions associated to TRAP. The reported network of proteins regulated upon TRAP perturbation, provides a starting point to further dissect molecular signaling sequences induced upon TRAP overexpression.

3.3.2 Possible substrates of TRAP in cancer cells?

Phospho-proteomics was used in ***Paper III*** to depict phosphorylation events modulated by TRAP perturbation. It needs to be noted that the applied method of phosphosite enrichment was based on high isoelectric focusing (HiREF) on an ultra-acidic strip. This results in enrichment, mainly detecting multiple phosphorylated peptides or peptides with several acidic residues. Furthermore, dephosphorylated peptides are generally low in abundance, limiting the detection level and spectrum of phosphopeptides (compare thesis of Elena Panizza, [289]), thereby possibly excluding significant and direct substrates of TRAP.

Nevertheless, the phosphorylation sites significantly regulated upon TRAP overexpression were associated with GO molecular functions, such as cell adhesion molecule binding, and cadherin binding. They were further allocated to the GO cell components nuclear part, cell junction, anchoring junction, adherens junction and cell-substrate junction. Despite including upregulated phosphoevents, these data corroborate a role of TRAP in cell adhesion and cytoskeletal modulation upon dephosphorylation of un-annotated substrates.

Interestingly, two serine phosphorylation sites of integrin β4 (1448, 1451), three phosphorylation sites of plectin (S4253, 4476, 4254), a protein interlinking different elements of the cytoskeleton and two phosphorylation sites of ROCK1 (S1105, S1102) are candidates, associated with cell migration and cytoskeletal changes.

Similarly to tyrosine peptides being able to be dephosphorylated by TRAP, melanoma cells overexpressing TRAP displayed modulations in tyrosine phosphorylations [82].

Interestingly, despite osteopontin (OPN) being proposed as a main substrate for TRAP, it was not identified as a significantly downregulated phosphoevent. OPN is an acidic protein with an abundance of negatively charged amino acids [292]. Thus, it is unlikely that the previously mentioned detection limits of the phosphosite enrichment are solely responsible for OPN not being detected. Nevertheless, OPN was earlier shown to be a substrate present in the extracellular matrix for TRAP [78–81]. This could possibly explain why cellular levels were not changed, possibly due to differences in localization.

Localization differences have to be further considered with regard to phosphosubstrates, as TRAP is stored in vesicular compartments, e.g. making a direct interaction with substrates, such as PAX and FAK [82] unlikely. Earlier, TRAP was reported to be an enzyme with low substrate specificity and it was suggested that localization and posttranslational control would define its functional reach. This notion could be further supported by immunohistochemistry staining of monomeric and total TRAP from ***Paper IV***, where a disturbance of TRAP cleavage resulted in relocalization within the cellular compartments. Also Zenger *et al.* showed earlier, that MDA-MB-231 cells overexpressing TRAP diverted TRAP to be cleaved early in the secretory pathway [214].

3.4 Is Cathepsin K involved in the proteolytic processing of TRAP 5b?

Previous studies applying *in silico* experiments, osteoclasts and Cathepsin knockout mice have led to the hypothesis of Cathepsin K (CtsK) being involved in processing of TRAP. Whereas cleavage by other unidentified enzymes was suggested to be necessary for further processing by Cathepsins, CtsK was hypothesized to mediate the final step of TRAP 5b processing.

Initially, several enzymes were tested for their ability to generate and cleave TRAP 5a to TRAP 5b *in silico* (chapter II.2.3.2.3). Amongst them, serine/threonine proteinases, MMPs and Cathepsins were tested [30,61,68]. Amino acid sequencing resolved that processing of both bone and CtsK-cleaved TRAP 5b was identical in N- and C-terminal sequences, whereas Cathepsin L (CtsL) cleavage removed fewer amino acids in the N-terminal site [68].

3.4.1 Codependent regulation of CtsK and TRAP

A variety of results stimulated the idea of a concerted regulation of CtsK and TRAP. Both molecules are co-expressed in the osteoclast microenvironment and several compartments of the osteoclast [68]. In the latter, CtsK was predominantly expressed amongst the cathepsins, whereas CtsL was expressed at much lower levels [68,91,109]. Furthermore, both CtsK and TRAP enzyme are involved in the bone degradation process by either degrading collagen type I or dephosphorylating non-collagenous bone matrix proteins, such as the phosphoproteins osteopontin or osteonectin, respectively. Absence of CtsK in knockout mice was further compensated by upregulation of TRAP activity and mRNA, suggesting a possible feedback loop [92]. In contrast, other reports supported a regulation by different mechanisms, as CtsK and TRAP were recruited to the resorption lacuna of activated osteoclasts in a sequential manner [108].

A variety of cancers display upregulation of CtsK [96–99], and CtsK levels were significantly increased when compared to CtsL [97]. Nevertheless, studies assessing a possible correlation of TRAP and CtsK levels in patients are lacking to date. In ***Paper IV***, we could show that pre- and mature CstK was expressed in MDA-MB-231 cells. Unpublished results demonstrate further expression of both CtsK forms in other cancer cell lines (**Figure 15**).

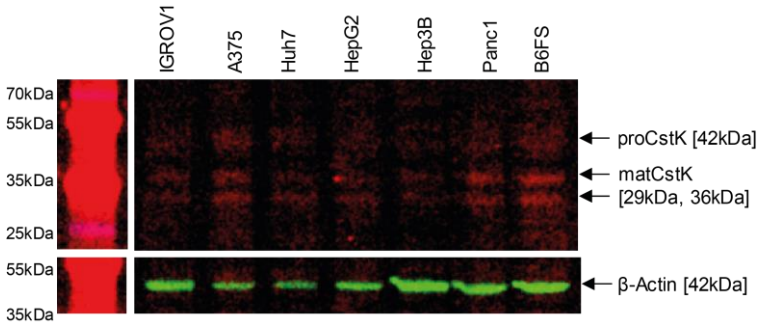


Figure 15 *CtsK expression in cancer cell lines*

A TRAP-dependent regulation of CtsK, reverse to what was denoted in osteoclasts could not be argued for, given that TRAP upregulation or inhibition by 5-PNA did not elicit any obvious changes in CtsK expression (unpublished data). Treatment with the CtsK inhibitor Odanacatib did neither increase the levels of total TRAP in the TRAP-overexpressing cells.

3.4.2 Mechanism of CtsK-dependent cleavage of TRAP

FPLC can be used to separate monomeric and differentially processed TRAP forms [68]. Commonly, three peaks can be observed, where the first peaks at lowest salt concentrations represents the monomeric TRAP 5a isoform (L1a, L1b) and two following peaks at higher salt concentrations represent differently cleaved versions of TRAP 5b. For instance, when cell lysates were applied, e.g. from osteoclasts or TRAP-overexpressing breast cancer cells,

several forms of cleaved TRAP were identified, such as and intermediate product (L2) and a fully cleaved (L3) TRAP 5b form (**Figure 16 A**, lysate –E-64) [92,214].

Importantly, this intermediate form (L2) was increased when cysteine proteinases were inhibited by E-64 in RAW264.7 cells [92] or TRAP-overexpressing breast cancer cells (**Figure 16 A**, lysate +E-64) [214]. Increased levels of the intermediate TRAP 5b form were further detected in bone lysates of CtsK knockout mice [92].

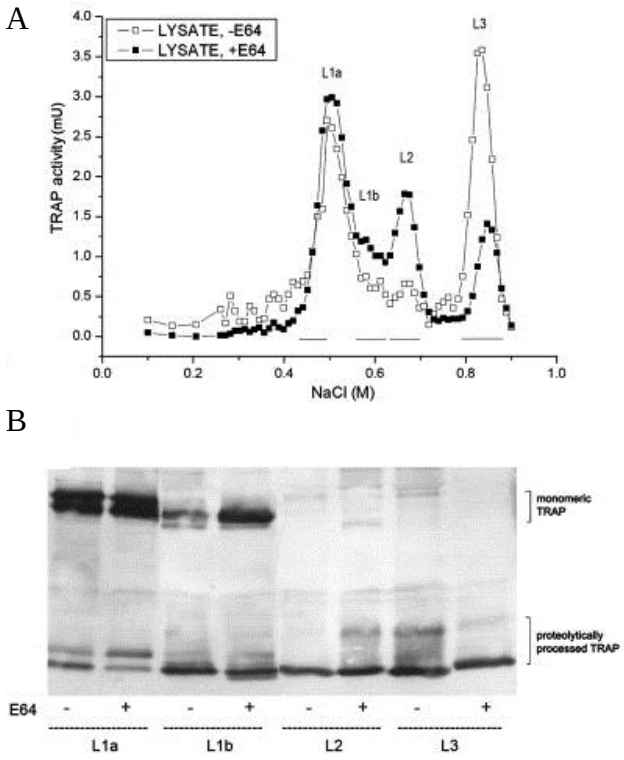


Figure 16 *Effect of the cysteine proteinase inhibitors E-64 on proteolytic processing of TRAP expressed by MDA-C11 cells.* ***(A)*** *Elution pattern from FPLC Heparin chromatography of cellular TRAP expressed by MDA-C11 cells following treatment with E-64. Lysates containing equal amounts of protein were loaded onto the column for each group. Horizontal bars under the peaks highlight the fractions pooled for further analysis of the peaks.* ***(B)*** *Western blot analysis of eluted intracellular TRAP peaks of MDA-C11 before and after treatment with E-64. Reprinted with permission from Zenger S, Ek-Rylander B, Andersson G. Biogenesis of tartrate-resistant acid phosphatase isoforms 5a and 5b in stably transfected MDA-MB-231 breast cancer epithelial cells. Biochim. Biophys. Acta - Mol. Cell Res. Elsevier B.V.; 2010;1803:598–607. Copyright: ©2010*

In ***Paper IV*** the involvement of CtsK could be pinpointed, by treating TRAP-overexpressing MDA-MB-231 cells with a CtsK-specific inhibitor. Even that it remains to be shown that Odanacatib treatment elicits a similar intermediate peak in the FPLC elution profile, it increased the 23 kDa specific band reminiscent to the blotting profile of the FPLC-purified intermediate TRAP 5b form in E-64 treated cells (**Figure 16 B**, L2 +E-64).

3.4.3 TRAP trafficking affected by CtsK cleavage

Interestingly, next to changes in TRAP processing intracellularly, a reduction in TRAP 5a protein secretion could be seen upon CtsK-specific inhibition in ***Paper IV***. Similarly, CtsK deficient knockout mice were unable to secrete monomeric TRAP 5a [92].

The change in secretion in TRAP-overexpressing breast cancer cells was coherent with intracellular differences in TRAP localization. Changes in localization of TRAP have previously also been detected in osteoclasts of CtsK knockout mice [92]. Whereas hardly any colocalization was seen of total and monomeric TRAP 5a in wildtype mice, total and monomeric TRAP 5a was evenly distributed in CtsK knockout mice. This is similar to the TRAP-overexpressing cells that were treated with the CtsK-specific inhibitor Odanacatib (***Paper IV)***. Monomeric TRAP 5a was generally retained in vesicular-like structures, whereas total TRAP (representing supposedly TRAP 5b) showed diffuse distribution. Here CtsK

inhibition only affected total TRAP staining, by reducing the diffuse distribution and inducing retention in vesicular-like structures, *i.e.* similar to the TRAP 5a –like pattern. This altogether suggests that CtsK enzymatic activity influence the localization of cleaved TRAP 5b in cells.

3.4.4 Other enzymes involved in TRAP cleavage

The presence of intermediate cleaved forms of TRAP 5b in a variety of conditions is suggesting a common mechanism of TRAP processing. Nevertheless, CtsK could not be exclusively claimed responsible for the complete cleavage of TRAP. This was for instance supported by the observation that cleaved TRAP 5b was still present in CtsK-deficient mice [92]. Also both Cathepsin K and L enzymes were earlier shown to similarly cleave TRAP in a two-step process *in vitro* [68], suggesting the possible involvement of several enzymes in the cleaving sequence. CtsL, present at low quantity in acidic vesicles, was then suggested to be responsible for the initial processing of TRAP. TRAP would then be further processed upon fusion with vesicles containing CtsK. Nevertheless, neither complete blocking of Cathepsins by E-64, nor selective blocking of CtsL was sufficient to inhibit full cleavage of TRAP in several cell types [214,293], ruling out that sequential processing by Cathepsins would be sufficient for full cleavage of TRAP. Other enzymes are potentially involved in complete cleavage of TRAP and remain to be identified.

3.4.5 Parameters affecting CtsK-dependent cleavage of TRAP

As isoform processing and secretion displays to be different in various cell types, further suggestions have been made regarding factors determining TRAP cleavage.

For instance, increasing levels of Cathepsins added to recombinant TRAP resulted in increasing levels of fully cleaved TRAP 5b concomitant with a reduction in intermediate TRAP 5b [68]. This led to the assumption that relative levels of CtsK and CtsK activity in the compartments could determine the efficiency of TRAP processing. High levels of CtsK are reported in osteoclasts, which associate with increased TRAP 5b levels in the resorption lacuna. Furthermore, we could show high percentage of colocalization and similarly high levels of TRAP 5b in TRAP-overexpressing cells. Coherently, after a recovery period of 24 h after treatment, the reduction in Odanacatib concentration (reversible CtsK inhibitor) resulted in a reversible effect on TRAP processing and secretion in ***Paper IV***.

Finally, also other parameters could steer activation of TRAP by cleavage. As increased TRAP 5b ratios were predominantly seen in ´TRAP-overexpressors´, such as osteoclasts, it is speculated that TRAP 5a cleavage mainly occurs in cells with high TRAP expression. As we could not detect any difference in mature CtsK ratios in the TRAP-overexpressing cells, the determining factor could lie in the initial processing of TRAP that might be regulated by TRAP levels.

Finally, the activation of TRAP is dependent on localization, as TRAP is mainly stored in vesicular structures. A site-dependent role for CtsK in TRAP processing was for instance also observed in bone tissue of mice [294].

3.5 Is TRAP 5b isoform relevant for the development of the cellular phenotype observed in TRAP-overexpressing cells?

The different TRAP isoforms have been proposed for their relevance in different diseases and functions (chapter II.7.3 and II.7.4). Posttranslational modifications, such as proteolytical cleavage, are highly regulated mechanisms to control the exertion of protein functions. When these processes are dysregulated, pathological conditions commonly appear.

With reference to the similarities in its function in osteoclasts, TRAP 5b was regarded as the isoform being more likely involved in conferring cancer cell features. Similar to the matrix degradation necessary for cellular invasion, enzymes are secreted by the osteoclasts to degrade the surrounding bone matrix. Here, TRAP was secreted and rapidly cleaved by extracellular enzymes to dephosphorylate non-mineralized phosphorylated bone matrix proteins, such as osteopontin or osteonectin [295]. Additionally, TRAP 5b was involved in the attachment and migration of osteoclasts on the phosphorylated bone matrix [78,80].

With the detection of TRAP expression in cancer cells [82,259–261], TRAP 5b levels suggested as serum markers of cancer patients with bone lesions, were no longer only attributable to increased activity of osteoclasts, but also likely to be derived from cancer cell populations.

3.5.1 Isoform expression in cancer cells

Most cancer-related studies commonly did not specify the respective expression and contribution of either isoform and only Zenger *et al.* reported about isoform differences in breast cancer [264]. When analyzing the distribution of TRAP isoforms, it was found that cancer cells mainly expressed TRAP 5a, whereas 5b was present in stromal cells. Also in a majority of cancer cell lines, mainly TRAP 5a expression was detected intracellularly (**Figure 17**).

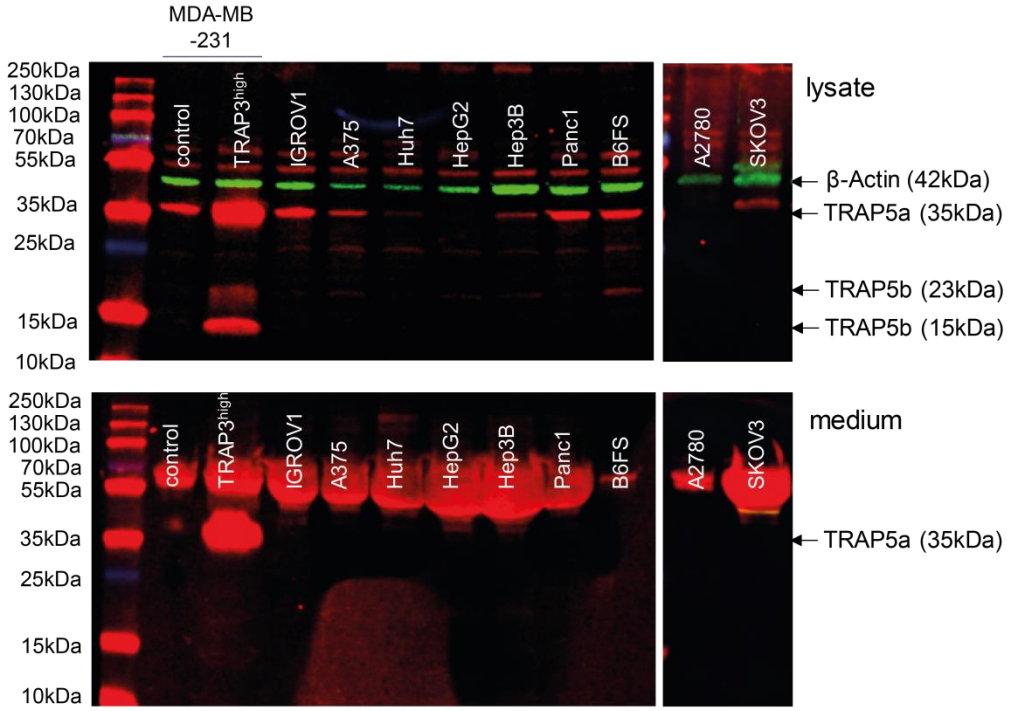


Figure 17 *TRAP expression in lysate and medium of various cancer cell lines. Cell pellets and conditioned media were kindly provided by the groups of Prof. Mikael Björnstedt (IGROV1, A375, Huh7, HepG2, Hep3B, Panc1), Assoc.Prof. Katalin Dobra (B6FS) and Assoc.Prof. Maria Shoshan (A2780, SKOV3).*

Contrary to these observations, the levels of TRAP 5b/5a were significantly increased in the TRAP-overexpressing cells used as a model system for this thesis (***Paper IV***). Importantly, the levels of TRAP 5a were not significantly changed within the parental and TRAP-overexpressing cells. Hence, TRAP 5b activity in TRAP-overexpressing cancer cells was hypothesized to be responsible for the promotion of aggressiveness. TRAP-overexpressing cells displayed cell characteristics commonly associated to metastasis that were less pronounced in the control cells (***Paper III***).

Given that most cell lines with TRAP expression do not process TRAP 5a in a similar manner as TRAP-overexpressing MDA-MB-231 cells, intracellular TRAP is thus likely to be cleaved under unknown but specific circumstances, as previously noted in chapter III.3.4.5.

3.5.2 Inhibition of TRAP 5b-activity

A limited amount of studies investigating the role of TRAP in cancer cells modulated the expression and activity of TRAP. Hereby strategies such as blocking antibodies, siRNA, shRNA-mediated knockdown or upregulation models were applied [82,230,261].

Importantly, we could expand these data in ***Paper I and III*** by inhibiting the activity of the TRAP 5b isoform with 5-PNA next to up- and downregulating the TRAP (and considerably the TRAP 5b) protein. Metastasis-related functions, such as proliferation, migration, invasion and overexpression of TGFβ2 could be normalized upon 5-PNA treatment. This normalization was associated to a reduced enzymatic activity intracellularly, where mostly TRAP 5b contributes to cellular activity.

Even that CBK289001, the compound identified as a TRAP-inhibitor in ***Paper II***, inhibited both isoform activities under non-reducing conditions, TRAP 5b was favored when activated by reduction. Additionally, TRAP 5b was more potently inhibited than TRAP 5a under both conditions. CBK289001 abrogated TRAP-dependent migration, similarly to 5-PNA, level-dependently. These data altogether underscore that inhibition of TRAP 5b activity by two independent compounds is able to normalize the TRAP-dependent phenotype in cancer cells.

Initially, it was hypothesized that inhibition of TRAP processing by Odanacatib would increase TRAP 5a levels concordantly with reduced TRAP 5b levels. Nevertheless, CtsK inhibition was not sufficient to inhibit TRAP cleavage fully or reducing TRAP 5b/5a levels in ***Paper IV***. In contrast, treatment of TRAP-overexpressing cells with Odanacatib increased the 23 kDa TRAP 5b, possibly attributable to an intermediate cleaved from of TRAP 5b. Importantly, the intermediate form of TRAP 5b did not elicit a reduced activity, as total TRAP activity in lysate was unchanged (***Paper IV***). Also in a previous publication, total TRAP activity, calculated by the sum of FPLC peak activity derived from intermediate and fully cleave TRAP 5b after E-64 treatment, was similar to the untreated condition [214]. Addition of CtsK to the intermediate cleaved TRAP 5b form was not able to further increase activity [214]. Related to indifferent changes in phosphatase activity, the difference in the intracellular TRAP 5b profile did not result in functional changes, such as modulation of migration (***Paper IV***).

Nevertheless, it should be noted that treatment with the CtsK inhibitor Odanacatib resulted in a differential localization of the intermediate TRAP 5b within the cell. This would consequently have a pivotal effect on subcellular substrates and signaling mediators available to TRAP activity. Finally yet importantly, migration was level-dependently regulated by

TRAP (***Paper III***). It can therefore not be excluded that despite similar activity and differential location of the intermediate TRAP 5b form, levels of the fully cleaved TRAP 5b remaining after CtsK inhibition are sufficient to promote migration. Neither can one exclude that other functions, such as growth and invasion would similarly be unaffected by changes in the cleavage pattern of TRAP.

3.5.3 Contribution of extracellular TRAP

TRAP is processed and secreted by a variety of cells (chapter II.7.1 and II.7.2). Importantly most cell types, such as TRAP-overexpressing MDA-MB-231 breast cancer cells (***Paper I, III and IV***) and macrophages secrete monomeric TRAP 5a, whereas the monomeric TRAP 5a secreted into the resorption lacuna by osteoclasts is rapidly processed to the cleaved TRAP 5b by osteoclast-derived CtsK (**Figure 5 and 11**). Most other cancer cells with TRAP expression were not reported or shown to secrete TRAP (**Figure 17**) [82,259–261]. As in the parental MDA-MB-231 breast cancer cells TRAP 5a was secreted, this alternative supply of TRAP can similarly be attributed to eliciting characteristics specific for TRAP-overexpressing cells.

TRAP 5a has so far mainly been reported to function as a growth and differentiation factor [242], which could disclose, why growth and proliferation was also promoted in TRAP overexpressing cells in ***Paper III***. As binding to the surface of adipocytes and endocytosis was shown [224], it cannot be excluded that secreted TRAP 5a could act as an autocrine factor that stimulates proliferation/migration/invasion via a receptor-mediated signaling process. Currently, limited knowledge is present regarding possible receptors that TRAP can bind to, but attempts have been made to identify them [223]. Also, unpublished studies, performed by Christina Patlaka (personal communication), showed that TRAP 5a can bind to the surface of MDA-MB-231 cells.

Treatment with 5-PNA reduced proliferation, migration and invasion in TRAP-overexpressing cells (***Paper I and III***). This inhibitor is inefficient in inhibiting TRAP 5a activity, but can bind to both isoforms, shown by docking studies and crystallography [278]. Assuming that TRAP 5a exerts its role via binding, we cannot exclude that, despite inefficiency in inhibiting the activity of TRAP 5a, 5-PNA is also able to block surface-receptor binding and thereby inhibiting the possible TRAP 5a role.

Contradictory to a role of extracellular TRAP 5a, however, addition of TRAP 5a to the parental cells left migration unchanged (***Paper IV***). Concentrations of TRAP 5a were adapted to levels similar to the secreted levels in TRAP-overexpressing MDA-MB-231 cells. Additionally, the reduction in secreted TRAP upon treatment with Odanacatib failed to change, *i.a.* reduce the migration of TRAP-overexpressing cells.

In summary, it can be excluded that secreted TRAP 5a has an effect on TRAP-dependent migration. A possible autocrine signaling of TRAP 5a and its contribution to growth and proliferation or invasion in cancer cells remains to be investigated.

4 Conclusions

Can potential and specific TRAP inhibitors be identified and applied?

- 5-PNA is a small molecule inhibitor despite low sensitivity specific for TRAP 5b activity and applicable in a cellular system
- By small molecule screening, six potential candidates for TRAP 5a and TRAP 5b inhibition, and especially CBK289001, were outlined that can be further used to develop more potent inhibitors of TRAP

Does TRAP make cancer cells more aggressive and likely to metastasize?

- Overexpression of TRAP promotes metastasis-related cell properties in MDA-MB-231 breast cancer cells that can be reverted by TRAP inhibition or knockdown.

How does TRAP change the cellular signaling network?

- Overexpression of TRAP significantly increases levels of TGFβ2, TβRI and intracellular phosphorylation of CD44, attributable for increased migration and proliferation in MDA-MB-231 breast cancer cells.
- Overexpression of TRAP modulates Gene ontology terms "Cellular adhesion", "Extracellular matrix modulation" and "Mitochondrial termination"
- Overexpression of TRAP downregulates a variety of phosphosites, representing possible candidates as TRAP substrates

Is Cathepsin K involved in the proteolytic processing of TRAP 5b?

- Cathepsin K is involved in proteolytical processing of TRAP 5b from an intermediate form to the fully cleaved from.
- The intermediate TRAP 5b form has most likely a different cellular localization of TRAP, similar activity and a promotive effect on cell migration.

Is TRAP 5b isoform relevant for the development of the cellular phenotype?

- TRAP 5b intracellular, but not TRAP 5a extracellular is responsible for increased migration in TRAP-overexpressing MDA-MB-231 breast cancer cells.

5 Future perspective

In the curse of targeted cancer therapy, well-established prognosis and prediction markers are pivotal to enable patient-focused therapy.

In this thesis, a substantial contribution has been made to the knowledge of the role and mechanism of TRAP during cancer development. Further, promising candidates for the inhibition of TRAP activity have been identified and characterized.

Two compounds and structures with TRAP-inhibiting properties were provided. Both compounds inhibited TRAP activity and TRAP-dependent functions in the low micromolar range. By binding into the active site in different manners and displaying highly structural differences, they provide two independent fragments for structural optimizations as previously performed for the phosphonic acids [275–277]. Further experimental approaches are necessary to assess the selectivity and potency of the TRAP inhibitors, and to be able to extent its application to several (cancer) cell systems and *in vivo* models. In a next step, both analogs and the identified TRAP inhibitors can for example be tested in *ex vivo* cultured patient samples to assess their effect in a 3D-system.

Studies on the molecular mechanisms of TRAP are detrimentally necessary to further understand and assess the involvement of TRAP in the disease.

That TRAP might also have effects on other cancer cell hallmarks has been early noticed, when analyzing omics data and the phenotype of TRAP-overexpressing cancer cells. Specifically, TRAP seems to affect cancer cell metabolism and might play a role in cancer-associated inflammation. Specific interest lies in the identification of the detailed role of TRAP isoforms in the interplay of the cancer microenvironment. Furthermore, the data on TRAP role on invasion and metastasis can be supplemented by *in vivo* mouse models.

Molecular players identified by proteomic analysis revealed a strong connection and a possible context is reasonable to assess in a next step. The suggested involvement of TRIP-1 in the recruitment and activation of TGFβ and CD44 signaling by TRAP could for instance be investigated in mechanistic studies.

Furthermore, TRAP expression in cancer patient material can be completed by assessing the distribution of the TRAP isoforms, with a specific focus on TRAP 5b in cancer cells. The identification of unknown enzymes in the activation process of TRAP could further provide a possible targeting strategy for the tackling of TRAP-expressing cancers.

Finally, TRAP has rendered an important and crucial target in the understanding of molecular changes introduced during cancer progression. Supported by a strong connection to earlier reported cancer-markers, it displays an interesting and potent candidate for research behind the goal of understanding cancer cell processes and patient-directed treatment.

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´Who knows where all the time goes, years just slip away.
There's so many things I haven't done today.
But I'll always recall everyone that made my world better
And though some may be gone
It´s so hard to be sad, I´m so happy to be where you are.´

´Right from the start´, Rip It Up, Thunder, 2017
Text: Luke Morley

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´Hold out your hand 'cos friends will be friends.

Right till the end.´

´ Friends Will Be Friends´, A Kind of Magic, Queen, 1986
Text: Freddie Mercury and John Deacon

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Wir können alles sein.

Woher wir kommen, ist nicht wichtig,
wichtig ist, wohin wir gehen.
Wir müssen nichts verstecken,
wenn wir zu uns selbst stehen.
Und wir können alles sein.

Unsere eigenen Helden, unsere eigenen Freunde,
unser eigener Investor in unsere eigenen Träume.
Unsere eigenen Beschützer, unsere Schüler, unsere Lehrer,
unsere eigenen Vorbilder, unser Halt, unsere Verehrer.
Und wir können alles sein.

Querdenker, Quereinsteiger, Grenzgänger, Wegweiser,
Party People, große Kinder, Erfinder und Familiengründer,
Alles-Erdachte-möglich-Macher, Lebenslauf-Collagenbastler,
oder Master of Disaster an der Uni Kopenhagen.

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Grenzen sind Phantome.

Gebaut von Angst. Bloß in Gedanken.
Wenn du einmal um dich siehst, stehen da nirgends Schranken.
Dein Weg ist frei, ganz bis zum Horizont, und bietet klare Sicht,
nur gehen musst du alleine. Das übernimmt keiner für dich.
Die beste Zeit ist immer jetzt.

Julia Engelmann, Poetry-Slam

CURRICULUM VITAE

Anja Reithmeier was born 1987 in Augsburg, Germany as the second child of three siblings to Sofie and Josef Reithmeier. She grew up in Feldheim, Bayern, Germany and finalized her A-grades at the Gymnasium Donauwörth in 2006. After graduation, she conducted a Voluntary Social Service year at the Bayerisches Rotes Kreuz, where she assisted with principle medical and nursing competencies in the Kreiskrankenhaus Erding, Bayern, Germany to gain practical insight in the field of medicine.

In 2008, the Technische Universitet München accepted Anja for conducting her bachelor studies in biology. There, she acquired next to the basic knowledge in the biological and chemical field, practical experience in the labs and participated in mathematics, economics and business administration courses. After finishing the Bachelor of Science degree with a thesis focusing on electrophysiology to detect neuronal systems in the auditory midbrain of chicken in 2010, she was accepted to pursue her master studies at the Technische Universitet München in biology, with focus on biomedical sciences. Having acquired necessary background and gained practical experiences in the field of pathology and medicine, she was funded by an Erasmus exchange program in 2011/12 to perform a practical laboratory project in the group of Prof. Göran Andersson, Division of Pathology, Department of Laboratory medicine, Karolinska Institutet, Sweden. After returning from Sweden, Anja finished here master of science degree *magna cum laude* with a thesis project on collagen IV in the development of pancreatic ductal adenocarcinoma in the group of Prof. Dr. Irene Esposito, Institut für Allgemeine Pathologie und Pathologische Anatomie, Technische Universitet München in the end of 2012.

Anja returned to the Karolinska Institutet, Sweden in 2013 to perform her postgraduate studies in the group of Prof. Göran Andersson, where she was registered for PhD studies in August 2013. In her projects, she focused on the role of the metalloenzyme Tartrate-resistant acid phosphatase in the progression of cancer and the identification and characterization of small molecule inhibitors, e.g. by small molecule screening. During her time at the division of pathology, she acquired besides participation in relevant courses, deep experience in research and laboratory methodology, knowledge in the organization of teaching courses, skills in grant writing and competences in presentation strategies. She participated in several conferences and international meetings by presentation of posters and talks about her research and was able to acquire travel funding. Anja is defending her thesis with the title "*Tartrate-resistant acid phosphatase/ACP5 as a driver of cancer: Dissection of its oncogenic mechanisms and identification of small molecule inhibitors*" on December 15th in 2017 at the Karolinska University hospital, Huddinge.