JACOBS UNIVERSITY

Roles of cysteine cathepsins in intestinal homeostasis

by

Maria Arampatzidou

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Approved Dissertation Committee

Prof. Dr. Klaudia Brix

Prof. Dr. Georgi Muskhelishvili

Prof. Dr. Matthias Ullrich

Prof. Dr. Margarete Heck

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School of Engineering and Science

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“Man is a goal-seeking animal. His life only has meaning if he is reaching out and striving for his goals.”

Aristotle (Greek philoshopher, 384-322 B.C.)

Publications, Oral presentations, Conferences

Peer-reviewed research publications

1. **Arampatzidou M**, Mayer K, Iolyeva ME, Asrat SG, Ravichandran M, Günther T, Schüle R, Reinheckel T, Brix K (2011). Studies of intestinal morphology and cathepsin B expression in a transgenic mouse aiming at intestine-specific expression of Cath B-EGFP. Biological Chemistry. 392(11):983-93
2. **Arampatzidou M**, Rehders M, Dauth S, Yu DMT, Tedelind S, Brix K (2011). Imaging of protease functions-current guide to spotting cysteine cathepsins in classical and novel scenes of action in mammalian epithelial cells and tissues. Italian Journal of Anatomy and Embryology 116:1-19
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Oral presentations

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5. **Arampatzidou M** (Mar 2010). Investigating the role of cathepsins in the intestine under physiological and pathological conditions. MoLife Seminar, Jacobs University Bremen, Germany.
6. **Arampatzidou M**, Pampalakis G, Zoumpourlis V, Sotiropoulou G, Brix K (Apr 2008). The role of human kallikrein-related peptidase 6 in breast cancer. Invited ICTS Seminar, Jacobs University Bremen, Germany.

Poster presentations (presenting author underlined)

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2. Tamhane T, Mayer K, Iolyeva ME, Vreemann A, **Arampatzidou M**, and Brix K (Feb 2012). Cysteine cathepsins trafficking in intestine epithelial cells. 29th Winter School on Proteinases and their Inhibitors. Tiers, Italy.
3. Brix K, Rehders M, Dauth S, **Arampatzidou M**, Bonetti MI, Yu DMT, Tedelind S (Oct 2011). Compartmentalized actions of endo-lysosomal cysteine peptidases in physiology and pathology. 7th General Meeting of the International Proteolysis Society-IPS, San Diego, CA USA.
4. **Arampatzidou M**, Tacke M, Vreemann A, Reinheckel T, Brix K (Oct 2009). Intestinal cathepsins and their role in ECM turnover. 6th General Meeting of the International Proteolysis Society-IPS, Gold Coast, Australia.

Involvement in international Conferences

1. To come: Discussion Leader at the Gordon Research Seminar on Proteolytic Enzymes & Their Inhibitors 2012. June 15-16, 2012, Lucca (Barga), Italy.
2. Co-chair of the session ‘Therapeutic Strategies’ at the 7th General Meeting of the International Proteolysis Society-IPS, October 16-20 2011, San Diego, CA USA.
3. Tutor at the Workshop ‘Imaging of Proteases in Living Cells’. 7th General Meeting of the International Proteolysis Society-IPS, October 15-16 2011, San Diego, CA USA.

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Abbreviations

ABP	activity based probe
APN	aminopeptidase N
bp	base pair
BSA	bovine serum albumin
C	colon
CA-074	cathepsin B-specific inhibitor
CathB	cathepsin B
cDNA	complementary DNA
Cdx	caudal-related homeobox
CHO	chinese hamster ovary
CMF	calcium and magnesium free
CNS	central nervous system
Ctsb$^{-/-}$	cathepsin B-deficient
Ctsd$^{-/-}$	cathepsin D-deficient
Ctsk$^{-/-}$	cathepsin K-deficient
Ctsl$^{-/-}$	cathepsin L-deficient
Ctsz$^{-/-}$	cathepsin X/Z/P-deficient
D	duodenum
dd	double distilled
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DsRed	red fluorescent protein from Discosoma sp.
DTT	dithiotreitol
E64	cysteine proteases inhibitor
ECM	extracellular matrix
EGFP	enhanced green fluorescent protein
ER	endoplasmic reticulum
FCS	fetal calf serum
g	acceleration of gravity (9,81 m/sec)
g	gram
GFP	green fluorescent protein
GI	gastrointestinal
h	hours
HEPES	4-(2-hydoxyethyl)-1-piperazineethanesulfonic acid
HRP	horseradish peroxidise
I	ileum

Abbreviations

IBD	inflammatory bowel disease
IEC	intestinal epithelial cell
Ig	immunoglobulin
J	jejunum
kDa	kilo Dalton
l	liter
L	liver
LSM	laser scanning microscope
m	meter
M	molarity
mg	milligram
MHC	major histocompatibility complex
min	minutes
mRNA	messenger RNA
Muc2	mucin 2
ng	nanogram
NS-173	cathepsin B-specific activity based probe
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PFA	paraformaldehyde
RNA	ribonucleic acid
rpm	rounds per minutes
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulphate
SE	standard error
sec	seconds
Tg	transgenic
TGN	trans-Golgi network
TNFa	tumor necrosis factor alpha
TX	Triton X
U	unit
WT	wild type
µg	microgram

Table of contents

1 Abstract

Cysteine cathepsins are endo-lysosomal proteases expressed in a variety of cells and tissues, including the gastrointestinal tract. A variety of experimental approaches have been developed to enable visualization and trafficking studies of proteases *in vitro* and *in vivo*. Such approaches include fluorescent protein tagging experiments and use of activity based probes for monitoring of proteolytically active enzymes. Based on these strategies we generated a transgenic mouse model aiming at intestine-specific expression of cathepsin B-EGFP in order to elucidate the functions and transport pathways of cathepsin B *in situ*. Trafficking and localization studies have changed the classical view of cysteine cathepsins as enzymes acting only intracellularly and now it is well established that these proteases can also be secreted and exert extracellular functions. For example, extracellular cathepsin K has been recently indentified as an intestinal antibacterial factor with anti-inflammatory potential. Our studies on cathepsin K-deficient mice demonstrated that absence of cathepsin K is accompanied by elevated levels of other cysteine cathepsins, namely cathepsins B, L, and X. In addition, cathepsin K-deficient mice exhibited higher protein levels of collagen IV, an observation also shown for cathepsin B- and cathepsin L-deficient mice. These findings made us propose a role of cysteine cathepsins in ECM remodeling through both direct and indirect effects. Moreover, this study revealed that cathepsin K is important for the intestinal barrier function, since absence of this protease resulted in impaired distribution and expression of intercellular junction proteins. An important and very interesting observation was that cathepsin-deficiency affects each part of the gastrointestinal tract differently. For the first time each part of the small and large intestine was analyzed separately and our observations revealed that the proteolytic profile is unique and characteristic for each intestinal part. Interestingly, elevated levels of cathepsin X were found only in the duodenum and colon of cathepsin B-deficient mice when compared to wild type controls, while no alterations were observed in the case of jejunum and ileum. Hence, the conditions required for compensatory tasks of cathepsin X might be present only in specific parts of the gastrointestinal tract. Overall, this study demonstrates the key role of cysteine cathepsins in intestinal homeostasis and highlights the necessity to investigate each part of the gastrointestinal tract independently and not as an entity.

2 Introduction

2.1 The intestine

The gastrointestinal (GI) tract serves as a barrier between the body and the external environment. This organ is responsible for digestion and absorption of nutrients and fluids. The GI tract starts from the oral cavity and includes the oesophagus, stomach, small and large intestine [Bhagavan, 1992; Schneeman, 2002]. The small intestine is anatomically divided into three segments, namely the duodenum, jejunum, and ileum that differ in morphology and function. Duodenum is the first part of the small intestine that receives the partially digested food from the stomach and also secretions from the pancreas and liver. In the jejunum the breakdown of the food is completed and absorption of nutrients takes place. The ileum is the last part before the large intestine and here the remaining nutrients are absorbed [Feldman and Schiller, 1997]. In the large intestine and more precisely in colon, absorption of water and minerals in addition to storage of feces takes place. The colon, in contrast to the small intestine, is characterized by a denser and more diverse microbiota that includes more than 10^{12} different microorganisms. Some of those microorganisms are harmful and have been associated with pathological conditions of the intestine whereas others are beneficial for the human organism [Schneeman, 2002; Turner and Turner, 2010].
The intestinal wall consists of four layers i.e. mucosa, submucosa, muscularis externa, and serosa.

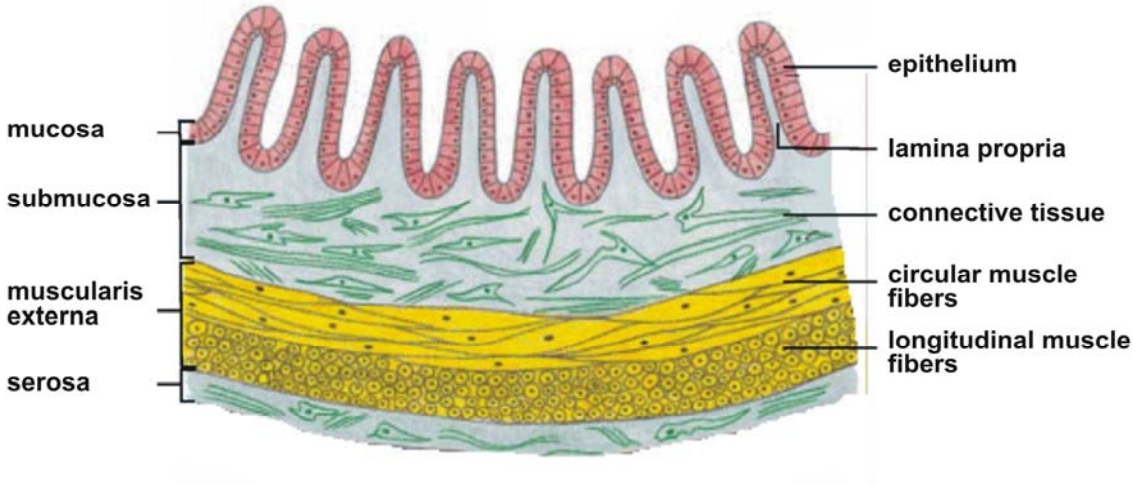

Figure 1: Composition of the intestinal wall modified after [Alberts, 2002]. The intestinal wall is composed of mucosa, submucosa, muscularis externa, and serosa. The mucosal epithelium, the lamina propria, the connective tissue, and the circular and longitudinal muscle layers are also depicted.

The mucosa is in direct contact with the intestinal lumen and it is involved in absorption of nutrients and in mucus secretion. The absorptive area of the mucosa is increased by finger-like projections, called villi. The lamina propria is a thin layer of loose connective tissue that forms the inner support of the villi. Furthermore, foldings of the epithelium are found at the

basis of the villi and are known as intestinal crypts [Alberts, 2002]. The crypts are responsible for cell renewal by hosting intestinal stem cells. These stem cells will then differentiate into Paneth cells and enteroendocrine cells that are found in the crypts base. Intestine epithelial and goblet cells are also derived from the stem cells but migrate upwards to the villus tip from where eventually cell shedding into the lumen occurs [Sato et al., 2009]. A major difference between the small and large intestine is that the colonic mucosa has no villi and it is only organized into crypts.

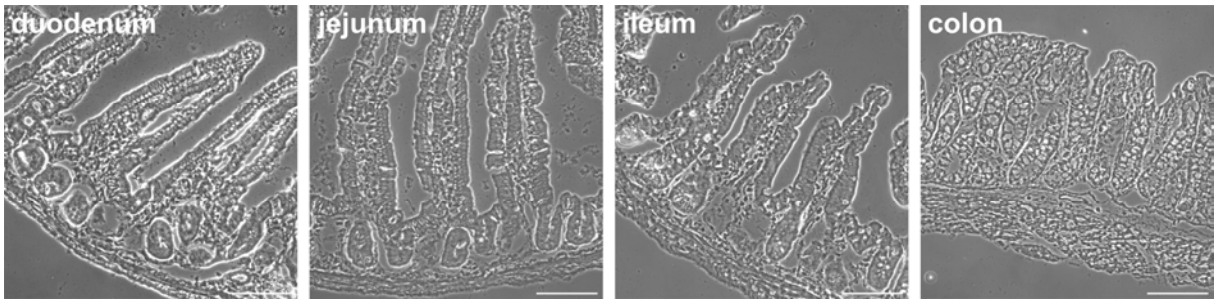

Figure 2: Morphology of the small and large intestine. Phase contrast micrographs of cryosections prepared from mouse duodenum, jejunum, ileum, and colon. The duodenum and jejunum are characterized by long villi. The villi become shorter in the ileum and they finally disappear in the colon where only colonic crypts are observed. Bars: 100 µm.

The intestinal wall is further composed of the submucosa, a dense layer of connective tissue attached to the mucosa. The following layer is the muscularis externa, which consists of two layers of smooth muscles, a circular and a longitudinal. These muscle layers are responsible for contraction and peristalsis of the intestinal tract. Lastly, there is another layer of connective tissue that is called serosa, enwrapping the intestinal tube.

A major function of the intestine is the barrier function that is vital for the human organism since it orchestrates the symbiosis of commensal bacteria with the host. An important determinant of intestinal barrier function is the mucus layer which serves as a physical barrier between the luminal bacteria and the underlying intestinal epithelium. The main component of intestinal mucus is the only gel-forming mucin, Muc2, that is produced and secreted by goblet cells [Johansson et al., 2011b]. The colonic mucus consists of two well-defined layers. In mouse, the inner layer is approximately 50 µm thick, stratified, and firmly attached to the colonic epithelium. Under physiological conditions, this layer is devoid of bacteria. On the other hand, the outer mucus layer is 3 to 4 times thicker, loose, and in direct contact with the luminal content. It has been shown that the expansion and conversion of the inner to the outer layer is mediated by proteolytic events [Johansson et al., 2011a]. Interestingly, the mucus in the small intestine is discontinuous and consists only of a single layer that has similar properties with the loose layer of the colon [Johansson et al., 2008]. The results of this difference in mucus features might be represented by bacteria residing much longer in

the colon in contrast to their fast passage through the small intestine. Therefore, the protective mechanisms have to be strongest in the colon since in this part potential pathogens have time to increase in number and jeopardise intestinal homeostasis.

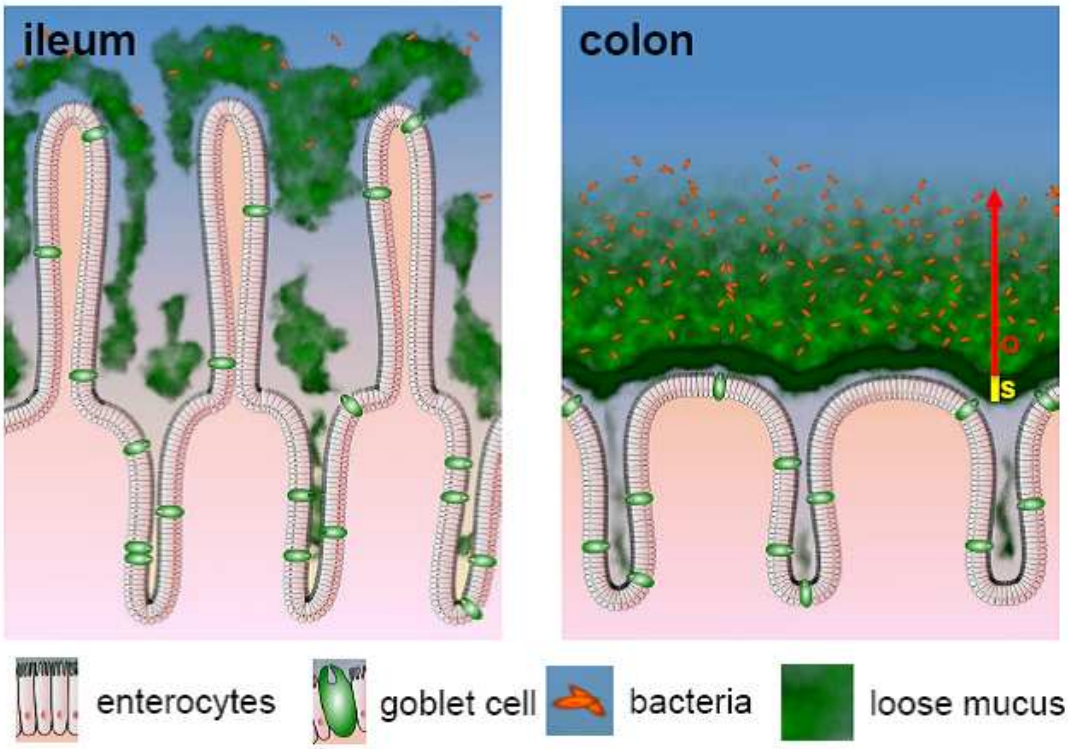

Figure 3: Mucus organization in the mouse small and large intestine [Arampatzidou et al., submitted]. The mucus layer in the small intestine is constituted by a single and less organized layer, whereas the colon mucus consists of two well-defined layers, i.e. the outer loose mucus layer (O) and the inner stratified mucus layer (S).

However, in some cases the enteric pathogens manage to overcome the obstacle of the mucus barrier and invade the colonic epithelium. This invasion can lead to pathological conditions of the intestine, such as inflammation. The intestinal microorganisms are using various mechanisms in order to accomplish this task, including proteolytic degradation of the mucin polymeric network. For instance, it has been shown that the parasite *Entamoeba histolytica* is able to disrupt the mucus layer by secreting cysteine proteases [Lidell et al., 2006].

2.2 Endo-lysosomal cathepsins

Proteases are multifunctional enzymes that play fundamental roles in diverse biological processes, such as protein turnover, proliferation, migration, adhesion, and differentiation. However, they have also been associated with pathological conditions, including cancer, inflammatory bowel disease, neurodegenerative disorders, and osteoporosis [Turk et al., 2001; Mohamed and Sloane, 2006; Buth et al., 2007; Lopez-Otin and Matrisian, 2007; Brix et

al., 2008; Arampatzidou et al., 2011a]. In humans, around 600 different proteases are known that are distributed into five catalytic classes, i.e. aspartic, cysteine, metallo, serine, and threonine. An interesting observation is that in rodents there are more proteases in comparison to humans, a finding that highlights the diversity of proteolytic systems found in different species [Puente et al., 2003; Puente and Lopez-Otin, 2004; Rawlings et al., 2006].
This study focuses on cysteine cathepsins that belong to the C1A subfamily of papain-like enzymes in clan CA [Rawlings and Barrett, 1994; Barrett and Rawlings, 2001]. There are 11 cysteine cathepsins identified in humans, namely cathepsins B, C, F, H, K, L, O, S, V, W, and X/Z/P. Rodents have a total of 18 cysteine cathepsins since they also express placenta-specific cathepsins that are not found in other species [Deussing et al., 2002; Sol-Church et al., 2002; Mason, 2008; Brix et al., 2008].

Table 1: Overview of human and mouse cysteine cathepsins

Human cathepsins	Chromosome	Mouse cathepsins	Chromosome
cathepsin B	8	cathepsin B	14
cathepsin C	11	cathepsin C	7
cathepsin F	11	cathepsin F	19
cathepsin H	15	cathepsin H	9
cathepsin L	9	cathepsin L	13
cathepsin K	1	cathepsin K	3
cathepsin O	4	cathepsin O	4
cathepsin S	1	cathepsin S	1
cathepsin V	9	not expressed	-
cathepsin W	11	cathepsin W	19
cathepsin X/Z/P	20	cathepsin X/Z/P	2
not expressed	-	cathepsin M	13
not expressed	-	cathepsin P	13
not expressed	-	cathepsin Q	13
not expressed	-	cathepsin R	13
not expressed	-	cathepsin 1	13
not expressed	-	cathepsin 2	13
not expressed	-	cathepsin 3	13
not expressed	-	cathepsin 6	13

Some of these enzymes, like cathepsins B and L, are ubiquitously expressed in contrast to others that are distributed in a cell- or tissue-specific manner. Initially, cysteine cathepsins were considered to act exclusively within the compartments along the endocytic pathway, since the proteolytic activity of most of the enzymes is optimal at slightly acidic pH and reducing conditions. However, it is now well established that these proteases and variants thereof exhibit functions in versatile locations, including even the nucleus, mitochondria but also the peri- and extracellular space [Turk et al., 2000; Brix et al., 2008; Tedelind et al., 2011]. The scenes of action for a specific protease can differ between physiological and pathological conditions. Safe-guarding mechanisms are responsible for regulating the proteolytic activity of enzymes found in unwanted locations or at abnormally elevated levels. Inhibitors, the natural counter-players of proteases, represent such a regulatory mechanism that contributes in tissue homeostasis. Endogenous inhibitors of cysteine cathepsins play pivotal roles in the regulation of their activity and include cytosolic stefins as well as cystatins and kininogens in extracellular fluids [Turk et al., 1997; Abrahamson et al., 2003; Rawlings, 2010].

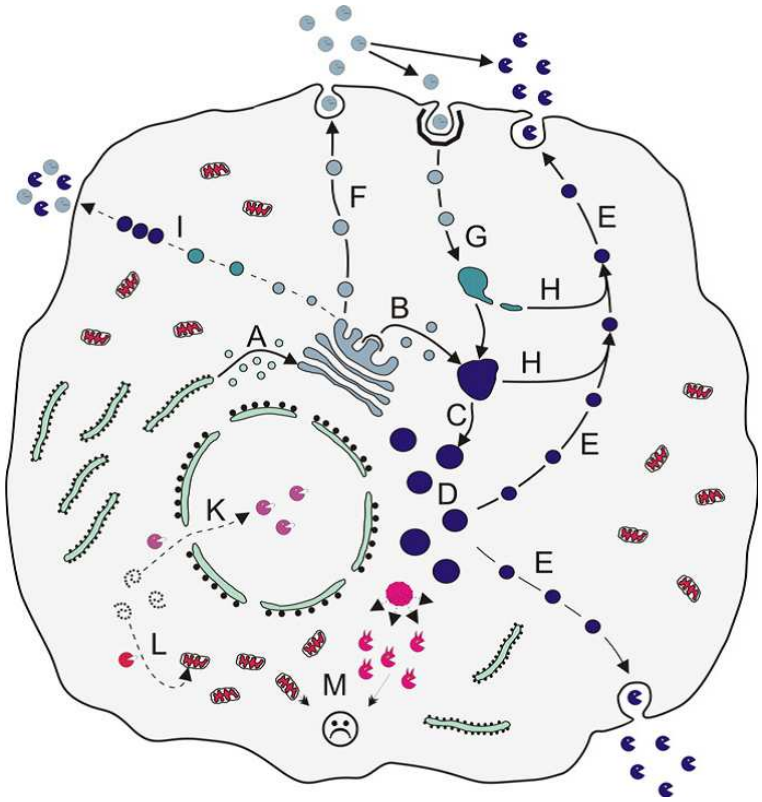

Figure 4: Transport pathways of cysteine cathepsins in health and disease [Brix et al., 2008]. Schematic depiction of biosynthetic pathways and trafficking of cysteine cathepsins in a non-polarized eukaryotic cell. Cysteine cathepsins are synthesized as preproenzymes at the rough endoplasmic reticulum (ER). By leader sequences they are targeted for entry into the ER lumen, and they are subsequently processed to proenzymes. The proforms of cysteine cathepsins are then transported to the Golgi apparatus for further post-translational modifications and sorting into transport vesicles in the trans-Golgi network (TGN) (A). From the TGN, procathepsins are then transferred via clathrin-coated vesicles to late endo-lysosomes where proteolytic processing of the proforms occurs, resulting in generation of mature and enzymatically active cathepsins (B). The final destination of the mature enzymes is the lysosome (C and D). However, in specific types of cells active cathepsins can be recruited out of the late endo-lysosomes and subsequently transported to the plasma membrane for secretion (E). It is also possible to have transport of procathepsins along the secretory pathway that results in secretion of enzymatically inactive proforms into the extracellular space (F). These secreted procathepsins can be re-internalized and transported along the endocytic pathway (G). Recycling pathways are also observed (H). In neurosecretory cells, procathepsins can be directed into secretory granules for maturation and subsequent secretion (I). N-terminally truncated cathepsins (found for example in cancer cells) can end up in unusual locations, such as the nucleus (K) or the mitochondria (L). Under pathological conditions lysosomes can become leaky, resulting in release of cathepsins into the cytosol (M) where they are considered to contribute to the induction of cell death pathways.

Cysteine cathepsins are known to exert versatile tasks in both health and disease states. Cathepsin B for example has been shown to contribute to prohormone processing in the thyroid gland [Friedrichs et al., 2003] while it is also able to degrade ECM constituents and promote tumor growth and metastasis [Buck et al., 1992; Yano et al., 2001; Vasiljeva et al., 2006; Chan et al., 2010]. Another cysteine cathepsin known to be involved in cancer, is the recently identified cathepsin X, also known as cathepsin Z or cathepsin P [Nagler et al., 2004; Buhling et al., 2004]. Cathepsin X has been suggested to play a role in cell adhesion and migration processes [Lechner et al., 2006] as well as in maturation of dendritic cells [Obermajer et al., 2008]. In contrast to cathepsin B, that exhibits both endo- and carboxypeptidase activity [Musil et al., 1991; Nagler et al., 1997], cathepsin X can only act as a carboxypeptidase [Nagler et al., 1999; Puzer et al., 2005].

Although cathepsins B and X display distinct activity profiles against substrates and inhibitors [Menard et al., 2001], these two proteases are known to exhibit compensatory effects in breast carcinoma and gastric epithelial cells [Vasiljeva et al., 2006; Bernhardt et al., 2010]. Compensatory phenomena have been observed for more cathepsins, e.g. for cathepsins K and L in the thyroid gland [Friedrichs et al., 2003]. Cathepsin K has recently attracted special attention since the cathepsin K inhibitors are currently in phase III clinical trials for osteoporosis treatment [Podgorski, 2009; Rachner et al., 2011; Dauth et al., 2011a]. This protease was first recognized for its high expression in osteoclasts [Bromme and Okamoto, 1995] and due to its collagenolytic activity, cathepsin K has a prominent role in bone remodeling [Gelb et al., 1996; Saftig et al., 1998]. Several osteoclast-specific therapeutic strategies have focused on cathepsin K [Deaton and Tavares, 2005; Yasuda et al., 2005; Desmarais et al., 2009; Podgorski, 2009]. Apart from osteoclasts, however, cathepsin K is also present in other types of cells and tissues, including thyroid gland [Tepel et al., 2000; Jordans et al., 2009], brain [Dauth et al., 2011b], GI tract [Haeckel et al., 1999; Mayer et al., 2006], and macrophages [Punturieri et al., 2000].

2.3 Cathepsin-deficient animals

Elucidating the roles of cathepsins *in vivo* is of great significance since these proteases are associated with diverse disorders and therefore have a great potential as therapeutic targets [Mohamed and Sloane, 2006; Vasiljeva et al., 2007; Reiser et al., 2010]. The cathepsin-deficient mice represent an excellent tool for such studies and they have been widely used by the scientific community. In several cases, cathepsin-deficiency does not alter the overall phenotype of the animals, while in other cases absence of a specific cathepsin, like for instance of cathepsin D, can be lethal [Reinheckel et al., 2001; Saftig et al., 1995]. When studying cathepsin-deficient mice, one should always keep in mind the major differences between the human and mouse proteolytic systems. For example mice have 8 additional cathepsins that are not found in humans and they lack human cathepsin V. Therefore, direct

conclusions from studies on mouse models should not be drawn without taking into account the unique proteolytic identity of each species.

Table 2: Overview of phenotypes and characteristics of the various cathepsin-deficient mice

Genotype	Phenotype / Characteristics	References
***Ctsb*$^{-/-}$**	No obvious phenotype Challenged mice: less severe experimental acute pancreatitis less susceptible to TNFa-induced hepatocyte apoptosis Thyroid alterations	[Halangk et al., 2000; Guicciardi et al., 2000; Reinheckel et al., 2001; Friedrichs et al., 2003]
***Ctsd*$^{-/-}$**	Lethal Die approximately at postnatal day 26 due to atrophy of intestinal mucosa and destruction of lymphoid cells	[Saftig et al., 1995]
***Ctsk*$^{-/-}$**	Osteopetrosis Resistant to experimental autoimmune encephalomyelitis Defective toll-like receptor 9 signaling Thyroid alterations CNS alterations Learning and memory impairments Reduced anxiety	[Saftig et al., 1998; Friedrichs et al., 2003; Asagiri et al., 2008; Dauth et al., 2011a]
***Ctsl*$^{-/-}$**	Altered hair follicle morphogenesis and cycling (periodic hair loss) Dilated cardiomyopathy Impaired MHC class II antigen presentation Thyroid alterations	[Roth et al., 2000; Reinheckel et al., 2001; Stypmann et al., 2002; Friedrichs et al., 2003]
***Ctsz*$^{-/-}$**	No phenotype reported	
***Ctsb*$^{-/-}$*/Ctsl*$^{-/-}$**	Lethal Die within 2-4 weeks after birth due to neuronal loss and brain atrophy	[Felbor et al., 2002]
***Ctsb*$^{-/-}$*/Ctsz*$^{-/-}$**	No phenotype reported	
***Ctsk*$^{-/-}$*/Ctsl*$^{-/-}$**	Hypothyroidism	[Friedrichs et al., 2003]

2.4 Cathepsins in the intestine

Cysteine cathepsins exert diverse tasks in the GI tract and they also exhibit variable localization patterns. In human intestine cathepsin B was localized within endo-lysosomes, in contrast to cathepsins K and L that were predominantly detected in association with the apical plasma membrane. Moreover, cathepsins K and L were found to be secreted from goblet cells, along with the intestinal mucus [Mayer et al., 2006; Brix et al., 2008]. This different localization of cysteine cathepsins in the intestine suggests also different functions for these proteases not only intracellularly but also in the extracellular space.

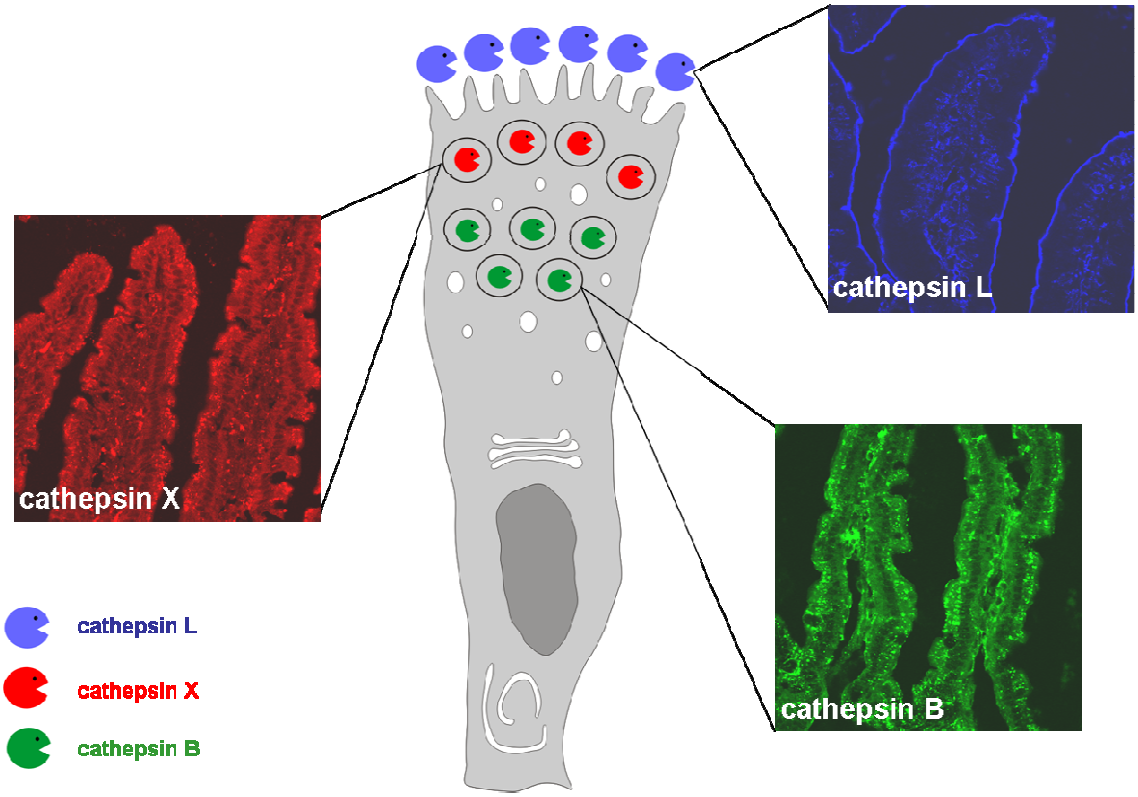

Figure 5: Localization of cysteine cathepsins in mouse intestine. Cryosections of mouse intestine immunostained for cathepsin B (green), cathepsin X (red), and cathepsin L (blue). Cathepsins B and X are localized within endo-lysosomes and cathepsin X-containing vesicles appear closer to the apical plasma membrane in comparison to vesicles containing cathepsin B. Cathepsin L is mainly detected in association with the apical plasma membrane. The schematic drawing depicts localization of these enzymes in a mouse intestinal epithelial cell.

Our studies have demonstrated that absence of cathepsins B, K, or L is accompanied by elevated levels of collagen IV in the mouse intestine [Vreemann et al., 2009; Dauth et al., 2011a]. Therefore, a role in ECM remodeling has been suggested for these cysteine cathepsins and has been further investigated, thereby taking all segments along the lengths of the GI tract into account. Cathepsins are also considered to be involved in MHC class II-mediated antigen presentation in intestinal epithelial cells and in M-cells [Hershberg et al.,

1997; Beers et al., 2005]. In addition, cathepsin K has recently been identified as an intestinal antibacterial factor with anti-inflammatory potential [Sina et al., 2012].

However, cathepsins have often been associated with pathological conditions of the intestine and it is well established that alterations in the proteolytic network can lead to such conditions [Medina and Radomski, 2006]. In particular, in a mouse model of colitis it has been shown that inhibition of cathepsins B and L can reduce the mucosal damage [Menzel et al., 2006]. Furthermore, *in vitro* and *in vivo* studies demonstrated a non-physiological release of active cathepsin B after intestinal trauma [Mayer et al., 2009; Vreemann et al., 2009]. In addition, several studies have highlighted the involvement of cathepsin B in colorectal carcinoma invasion and metastasis. Cathepsin B is overexpressed in colon cancer and its expression is associated with poor prognosis and increased mortality [Campo et al., 1994; Jessup, 1994; Guzinska-Ustymowicz et al., 2004; Chan et al., 2010].

2.5 Intercellular junction proteins and brush border enzymes as markers of intestinal polarity and differentiation

The intestine is a highly polarized epithelium that undergoes rapid and constant self-renewal. Therefore, maintenance and repair of the intestinal barrier is required and impairment of this barrier function is often associated with pathological conditions. The intercellular junctions have profound roles in maintenance of intestinal polarity and tightness. It has been shown that alterations in the expression and localization patterns of junctional complex constituents occur concomitant with intestinal inflammation [Gibson et al., 1995; Hanby et al., 1996; Karayiannakis et al., 1998; Schmitz et al., 1999; Gassler et al., 2001]. There are different types of intercellular junctions, such as tight and adherens junctions.

Tight junctions are the most apically localized junctional proteins that regulate paracellular permeability across the intestinal epithelium [Anderson and Van Itallie, 1995]. The major components of tight junctions are occludin and claudin-1 that are associated with integral plasma membrane proteins known as *Zonula occludens* (ZO) proteins [Hartsock and Nelson, 2008]. Localization shifts of occludin and claudin-1 away from the tight junctions have been reported in several studies and have been linked with inflammatory bowel disease [Boudreau et al., 2007; Poritz et al., 2011; Noth et al., 2011].

Adherens junctions have a more basal distribution and they are detected at the lateral plasma membrane sub-adjacent to tight junctions. E-cadherin is their major constituent and it is known to bind to cytosolic catenins [Halbleib and Nelson, 2006]. E-cadherin is considered to have pivotal roles in intestinal homeostasis since it is involved in diverse processes, including adhesion, differentiation, maturation, and positioning of intestinal cells [Schneider et al., 2010]. Interestingly, cathepsins have been found to influence the expression and distribution of intercellular junctions. For instance, it has been shown that inhibition of cathepsin L promotes redistribution of claudin-1 in the nucleus of intestinal epithelial cells

[Boudreau et al., 2007]. Moreover, E-cadherin has been identified as a substrate of several cathepsins [Gocheva et al., 2006].

Another important characteristic of intestinal epithelial cells is the brush border that is located at their apical pole. The brush border is composed of numerous microvilli and its main function is terminal digestion and absorption of nutrients [Holmes and Lobley, 1989]. Several enzymes are embedded in this brush border and they are therefore referred to as brush border enzymes. APN is such a brush border enzyme that is implicated in a variety of functions, like proliferation, adhesion, apoptosis, antigen presentation, and enzymatic regulation of peptides [Mina-Osorio, 2008]. APN is also involved in differentiation, therefore in several studies it serves as differentiation marker of polarized epithelia. The distribution of APN differs between the small and large intestine. More precisely, in the small intestine APN is found at the apical plasma membrane whereas in the colon its polarity is reversed [Arampatzidou et al., 2011b].

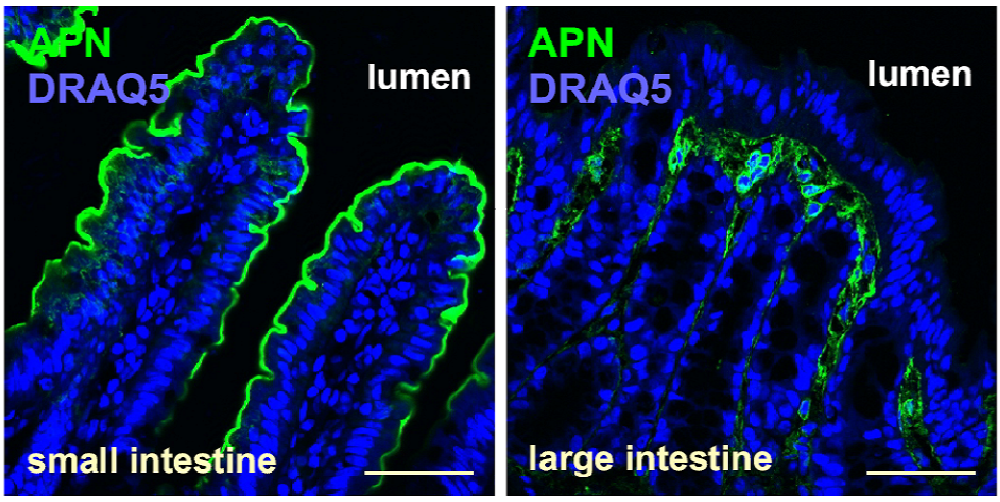

Figure 6: Distribution of APN in the small and large intestine modified after [Arampatzidou et al., 2011b]. Cryosections of mouse intestine after staining with APN-specific antibodies. In the small intestine (left) APN is localized at the apical plasma membrane. This distribution is replaced by basolateral plasma membrane localization in the colon (right). Cell nuclei are stained with DRAQ5 (blue). Bars: 50 µm.

2.6 Extracellular matrix and basal lamina constituents

Apart from intercellular junctions, a profound role in intestinal homeostasis is exerted also through interactions between intestinal cells and ECM macromolecules. The ECM provides structural support and it is essential for processes like cell growth, cell differentiation, and cell migration [Beaulieu, 1997; Montgomery et al., 1999]. Integrins are cell surface receptors that are specific for ligation of ECM constituents such as collagens, laminins, and fibronectins [Beaulieu, 1999]. The expression patterns of integrins and their ligands during renewal of the

intestinal epithelium is crucial and alterations in these patterns have been associated with pathological conditions of the intestine [Teller and Beaulieu, 2001]. The intestinal epithelium lies on a thin and flexible network of glycoproteins, proteoglycans, and glycosaminoglycans named basal lamina. The basal lamina defines the microenvironment required for cellular functions including proliferation, apoptosis, and migration. It regulates cell behaviour and mediates tissue compartmentalization, while it also serves as scaffold during tissue regeneration [Timpl and Brown, 1996]. Major components of the basal lamina are collagen IV, laminin, perlecan, and entactin. Perlecan and entactin are proteins responsible for stabilizing the network build up by collagen IV and laminin [Paulsson, 1992; Timpl and Brown, 1996].

The collagen family consists of 28 members of fibrous and non-fibrous proteins that contain at least one triple-helical domain. Collagens are the most abundant proteins in mammals known to play important roles in many tissues since they define their structural and mechanical properties. Moreover, collagens are the main components found in connective tissue [Ricard-Blum, 2011]. Collagen IV, the major constituent of basal lamina, belongs to the non-fibrous collagens and it is a heterotrimeric molecule. Besides collagens, laminins are also essential for the assembly of basal lamina. They are trimeric glycoproteins that form independent networks which are then associated with collagen IV networks through small connecting proteins. It has been shown that the expression patterns of laminins are altered in pathological conditions of the intestine such as IBD and cancer [Teller and Beaulieu, 2001].

Interestingly, cysteine cathepsins are identified as important players in ECM remodeling since they are able to degrade ECM and basal lamina constituents, such as collagen IV, laminin, and fibronectin [Buck et al., 1992; Koblinski et al., 2000; Lombardi et al., 2005; Buth et al., 2007]. In the intestine, cathepsins B, K, and L are considered to be involved in ECM turnover since the respective cathepsin-deficient mice exhibit significantly elevated levels of collagen IV in their intestinal tissue [Vreemann et al., 2009].

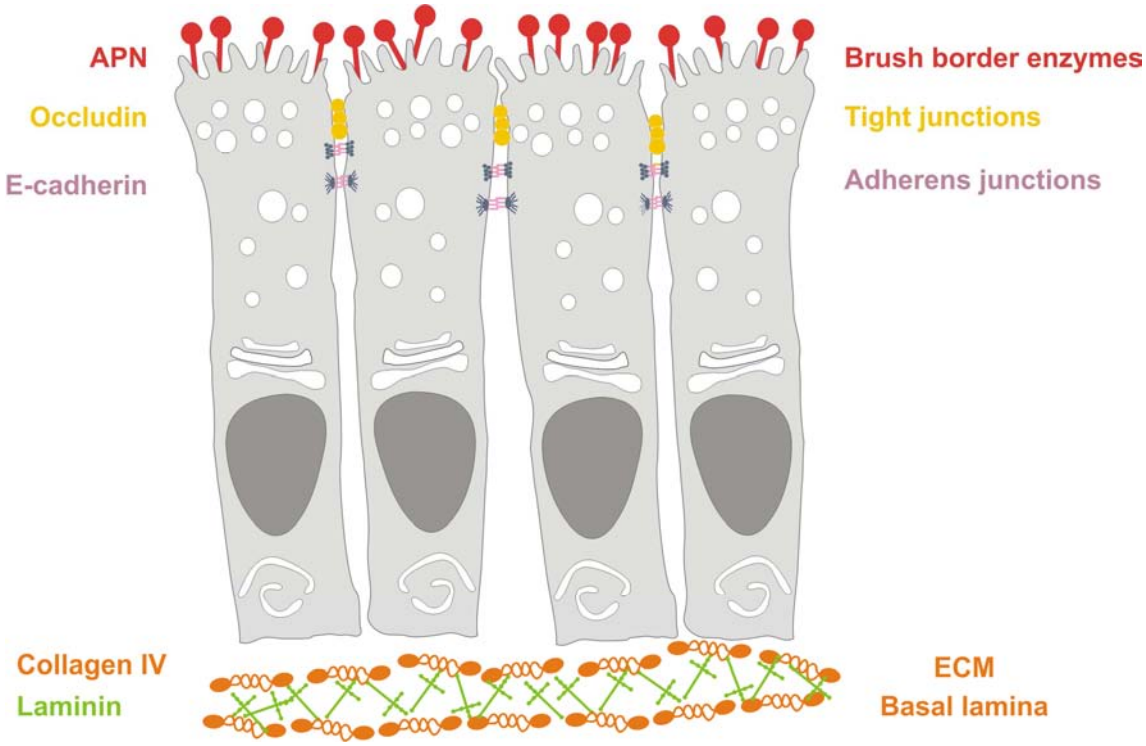

Figure 7: Overview of brush border enzymes, intercellular junctions, and ECM constituents in the intestinal epithelium modified after [Vreemann, 2009]. Schematic drawing illustrating the localization of the brush border enzyme APN (red), the tight junction protein occludin (yellow), the adherens junction protein E-cadherin (purple) and the ECM constituents collagen IV (orange) and laminin (green).

2.7 Monitoring protease activity with activity based probes

Proteases represent promising targets for drug development because they are involved in a variety of pathologies and disorders. However, successful design of protease inhibitors as efficient drugs for the clinics remains still a challenge and the major restraints are low specificity and unwanted off-target effects [Vasiljeva et al., 2007; Reiser et al., 2010]. In order to overcome these obstacles we need to be able to monitor the activity of proteases and spot their scenes of actions in living cells and organisms. The recent development of activity based probes (ABPs) has significantly contributed towards this direction over the last years [Greenbaum et al., 2002; Kato et al., 2005; Blum et al., 2009; Arampatzidou et al., 2011a; Deu et al., 2012].

ABPs are small reporter molecules able to penetrate biological membranes thereby reaching proteolytically active proteases within cells and tissues. They consist of three parts, the warhead that reacts with the active site of the target protease, the spacer (recognition element) that directs the probe to the target enzyme, and the tag that is usually a fluorescent dye or an affinity handle [Deu et al., 2012].

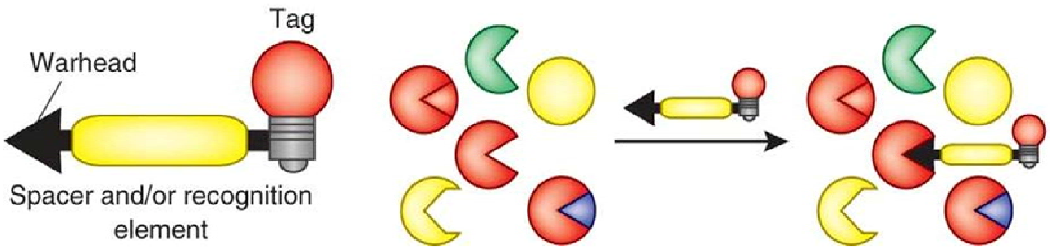

Figure 8: Structural elements of ABPs modified after [Deu et al., 2012]. ABPs are chemical probes consisting of three elements: warhead, spacer, and tag. ABPs can bind to specific proteases and tag them only when these enzymes are active.

ABPs are substrate analogs. Upon binding they are cleaved and remain covalently attached to the target protease. Some ABPs target whole families of proteases, like DCG-04 that binds to active cysteine proteases [Greenbaum et al., 2000], while others are designed to target a specific protease, like NS-173 that is a cathepsin B-specific ABP [Schaschke et al., 2000]. The advantage of quenched in comparison to non-quenched ABPs is that the first become fluorescent only upon cleavage, thus offering better signal-to-noise ratios [Blum et al., 2005; Brix and Jordans, 2005; Blum et al., 2007]. An impressive and highly promising application of ABPs is whole-animal non-invasive imaging that has successfully been tested in mice and could in the future contribute in human diagnostics [Blum et al., 2007; Blum et al., 2009; Deu et al., 2012].

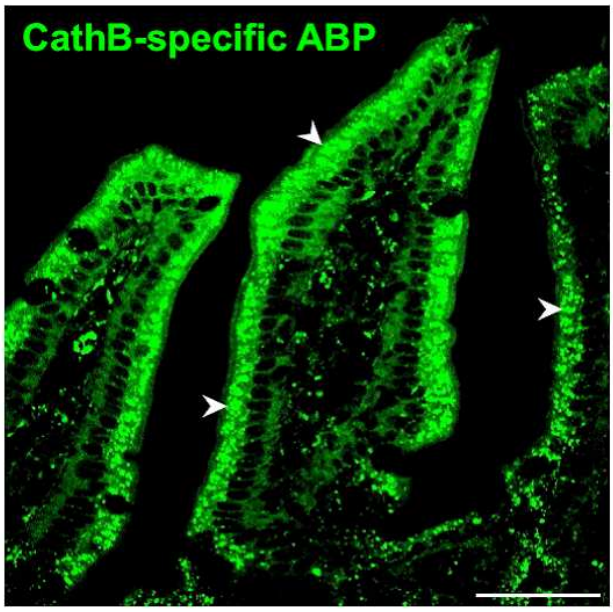

Figure 9: *In situ* detection of active cathepsin B by using ABPs. The non-quenched cathepsin B-specific ABP, NS-173, was applied on cryosections prepared from mouse intestine. Active cathepsin B (green) is visualized within endo-lysosomes (arrowheads). Bars: 50 µm.

3 Aim of the study

The main goal of this study was to investigate the contribution of cysteine cathepsins in intestinal homeostasis, with a primary interest for cathepsins B, K, and X. Therefore, *Ctsb*$^{-/-}$, *Ctsk*$^{-/-}$, *Ctsz*$^{-/-}$, and *Ctsb*$^{-/-}$/*Ctsz*$^{-/-}$ mice were used in order to identify potential alterations and differences between these animals and WT controls. The double deficient mice for cathepsins B and X represent an ideal model system since these two proteases are known to exhibit compensatory functions. Thus, we wanted to analyze *Ctsb*$^{-/-}$/*Ctsz*$^{-/-}$ mice in order to detect possible alterations that are latent in single deficient mice due to compensation. Indeed, we found that cathepsin X protein levels are elevated in some parts of the small and large intestine of *Ctsb*$^{-/-}$ mice, which are rich in cathepsin B in WT mice. This finding demonstrates for the first time that compensatory phenomena of cathepsins B and X occur also in intestinal tissue. For these studies we followed an approach in which every part of the gastrointestinal tract, e.g. duodenum, jejunum, ileum, and colon was analyzed separately although most studies tend to use the whole mouse intestine for analysis. Interestingly, the results of this study highlighted the exclusive properties of each intestinal part and emphasized the importance of taking into consideration the major functional and structural differences observed along the lengths of the gastrointestinal tract. Moreover, our biochemical and morphological analyses focused on the expression and localization pattern of ECM constituents, intercellular junction proteins, and brush border enzymes in an effort to elucidate the effects of cathepsin-deficiency in intestinal function and architecture. This study confirmed our initial hypothesis that cathepsins are involved in ECM remodeling since *Ctsb*$^{-/-}$ and *Ctsk*$^{-/-}$ mice displayed alterations in the protein levels of ECM constituents, such as collagen IV and laminin. We concluded that the expression pattern of collagen IV as well as its response to cathepsin deficiency varies along the length of the gastrointestinal tract and appears to be characteristic for the respective intestinal part. Furthermore, absence of cathepsin K led to altered protein levels and distribution patterns of adherens and tight junction proteins that are important players in the maintenance of intestinal tightness and integrity. These findings made us address the question whether there is a general impairment of intestinal barrier function in the intestine of *Ctsk*$^{-/-}$ mice. For this purpose, we used an *ex vivo* approach developed for the analysis of intestinal mucus organization and growth and our results revealed similar properties of mucus layers between *Ctsk*$^{-/-}$ and WT mice. However, since the protein levels of other cysteine cathepsins were found to be significantly elevated in the intestine of *Ctsk*$^{-/-}$ mice, implying compensatory mechanisms, we can not exclude the possibility that cathepsin K is involved in mucus processing. It is crucial to further investigate the tasks that cathepsin K exerts in the gastrointestinal tract since cathepsin K inhibitors are currently in clinical trials and the question whether it is safe to treat patients with such inhibitors remains still open.

4 Results

The results of this study are included in three research articles (one published, one submitted, and one in preparation) and two review articles that are published in peer-reviewed journals. A brief description of each article is given below.

1. **Arampatzidou M**, Rehders M, Dauth S, Yu DMT, Tedelind S, Brix K (2011). Imaging of protease functions-current guide to spotting cysteine cathepsins in classical and novel scenes of action in mammalian epithelial cells and tissues. Italian Journal of Anatomy and Embryology 116:1-19 (published; invited and peer-reviewed review article)

This review article describes in detail the refined methodology needed for visualization and localization of proteases in cells and tissues. Apart from localization we are also able to detect and image the proteolytic activity of these enzymes. The activity based probes represent a great tool for visualizing protease activity both *in situ* and *in vivo*.
Moreover, the use of fluorescent protein tagging enables us to elucidate the transport pathways and the trafficking of proteases within cells. Such studies are of great importance since in many pathological conditions, like in cancer, the trafficking of specific proteases is altered. In addition, this article discusses and compares different fixation procedures and protocols for immunolabeling, highlighting the importance of choosing the best and most suitable method for each model system.
This review focuses on cysteine cathepsins that have a great potential as drug targets because of their involvement in a variety of disorders and pathological processes. Therefore, it is of great importance to spot these enzymes in all possible locations within and outside cells in an optimal and precise way.

Contributions: MA carried out the experiments described in Figure 1 (C and C′) and Figure 4 (C and C′), was involved in discussion and interpretation of data, and participated in manuscript writing.

2. Dauth S, **Arampatzidou M**, Rehders M, Yu DMT, Führer D, Brix K (2011). Thyroid cathepsin K - roles in physiology and thyroid disease. Clinical Reviews in Bone and Mineral Metabolism 9:94-106 (published; peer-reviewed review article)

Cathepsin K is a cysteine protease mainly known for its bone remodeling tasks. The main focus of this review however was on the functions that cathepsin K exhibits in the physiology and pathology of the thyroid gland. Initially, the role of cysteine cathepsins in thyroglobulin processing is discussed and cathepsin K substrates in bone and thyroid are compared.

Results

Immunolabeling experiments demonstrating cathepsin K localization in human and mouse thyroid are also included in this article. Furthermore, possible consequences of cathepsin K inhibition or deletion for thyroid and thyroid target tissues, such as bone, CNS, skin, and intestine are described and discussed. Since cathepsin K inhibitors are in phase III clinical trials, the potential off-target effects of their use are speculated and the importance of elucidating the roles of cathepsin K in cells and tissues in general is highlighted.

Contributions: MA carried out the experiments described in Figure 4, was involved in discussion and interpretation of data, and participated in manuscript writing.

3. **Arampatzidou M**, Mayer K, Iolyeva ME, Asrat SG, Ravichandran M, Günther T, Schüle R, Reinheckel T, Brix K (2011). Studies of intestinal morphology and cathepsin B expression in a transgenic mouse aiming at intestine-specific expression of Cath B-EGFP. Biological Chemistry. 392(11):983-93 (published; peer-reviewed; selected for cover illustration of the November 2011 issue)

Cathepsin B is a ubiquitously expressed cysteine protease, known to be involved in a variety of physiological and pathological conditions. Among the various tissues of action, cathepsin B has been suggested to play an important role in the gastrointestinal tract which is the tissue of interest in this article.
In an effort to visualize the function and trafficking of cathepsin B *in situ*, we generated a transgenic mouse model that was expected to express cathepsin B-EGFP in an intestine-specific setting. For this purpose, we used the A33-antigen promoter as a tool for Cdx1-dependent cathepsin B-EGFP expression, an approach that was successfully used in previous *in vitro* studies. However, *in vivo*, no expression of chimeric cathepsin B could be detected in the intestine of transgenic mice. The investigations revealed that although the transgene was maintained over several generations, it was not expressed, suggesting that possibly a problem at the transcriptional level had occurred.
Nevertheless, the overall intestinal phenotype as well as expression and localization patterns of various proteins, such as endogenous cathepsin B, APN, and A33 antigen, were not altered due to A33-CathB-EGFP transgene integration.

Contributions: MA contributed to the design of the study, carried out the experiments described in Figures 1B, 2, 4, 5, 6, 7, and 8, was involved in discussion and interpretation of data, and participated in manuscript writing.

4. **Arampatzidou M**, Schütte A, Hansson GC, Saftig P, Brix K. Effects of cathepsin K deficiency on intercellular junction proteins, luminal mucus layers, and ECM constituents in mouse colon. (Submitted to Biological Chemistry, May 2012)

In this article we investigated the role of cathepsin K in mouse colon by using *Ctsk*$^{-/-}$ mice. Extracellular cathepsin K has recently been identified as an intestinal antibacterial factor with anti-inflammatory potential. In order to further investigate the tasks that cathepsin K exerts in mouse intestine, we performed a series of biochemical and morphological studies.
The results of this article showed that absence of cathepsin K leads to alterations in the proteolytic network of mouse colon, characterized by elevated levels of cathepsins B, L, and X. Moreover, cathepsin K deficiency had an impact on ECM constituents, like collagen IV and laminin, as well as on the expression and distribution of intercellular junction proteins. For example, mislocalization of occludin was observed in the colon of *Ctsk*$^{-/-}$ mice when compared to WT controls. In addition, this article includes results of an *ex vivo* method used for studying the patterns of mucus organization and growth.
In conclusion, cathepsin K seems to play an important role in intestinal function and homeostasis and the findings highlight the need for cautious assessments regarding the use of orally administered cathepsin K inhibitors.

Contributions: MA contributed to the design of the study, carried out the experiments described in Figures 1, 2, 3, 4, 5, and 6, was involved in discussion and interpretation of data, and participated in manuscript writing.

5. **Arampatzidou M**, Gerganova V, Schaschke N, Peters C, Reinheckel T, Brix K. The activity and localization patterns of cathepsins B and X in cells of the mouse gastrointestinal tract differ along its length. (Manuscript in preparation)

The localization and distribution of cysteine cathepsins in the intestine differs, suggesting also different functions for these proteases. The scenes of their actions are found not only within endo-lysosomes but also in the extracellular space. In this article we focused on cathepsins B and X and for the first time we studied each intestinal part, namely duodenum, jejunum, ileum, and colon separately. Compensatory effects have been previously shown for cathepsins B and X in various tissues, thus in addition to *Ctsb*$^{-/-}$ and *Ctsz*$^{-/-}$ mice, we also studied the double *Ctsb*$^{-/-}$/*Ctsz*$^{-/-}$ animals. The results of this article demonstrated that the activity and expression pattern of cathepsins B and X are different along the length of the gastrointestinal tract and that compensatory functions seem to be segment-specific.

Furthermore, we showed that cathepsin-deficiency influences the expression pattern of collagen IV and APN in an intestinal segment-specific way.
Overall, this article highlights the unique features of each intestinal part in terms of function and morphology, and highlights that the proteolytic network as well as the expression pattern of various proteins differ along the length of the gastrointestinal tract.

Contributions: MA contributed to the design of the study, carried out the experiments described in Figures 1, 2, 3, 4, and 5, was involved in discussion and interpretation of data, and participated in manuscript writing.

4.1 Visualization of protease functions and activity

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Invited Review in Histology and Cell Biology*

Imaging of protease functions – current guide to spotting cysteine cathepsins in classical and novel scenes of action in mammalian epithelial cells and tissues

Maria Arampatzidou, Maren Rehders, Stephanie Dauth, Denise M. T. Yu, Sofia Tedelind and Klaudia Brix**

School of Engineering and Science, Jacobs University Bremen, 28759 Bremen, Germany

Submitted December 28, 2010; accepted February 20, 2011

Summary

The human genome encodes some hundreds of proteases. Many of these are well studied and understood with respect to their biochemistry, molecular mechanisms of proteolytic cleavage, expression patterns, molecular structure, substrate preferences and regulatory mechanisms, including their endogenous inhibitors. Moreover, precise determination of protease localisation within subcellular compartments, peri- and extracellular spaces has been extremely useful in elucidating biological functions of peptidases. This can be achieved by refined methodology as will be demonstrated herein for the cysteine cathepsins. Besides localisation, it is now feasible to study *in situ* enzymatic activity at the various levels of subcellular compartments, cells, tissues, and even whole organisms including mouse.

Key words

Activity based probes, enzyme cytochemistry, green fluorescent protein, immunofluorescence, protein trafficking, endo-lysosomal proteases.

Introduction

Proteases belong to various families, i.e. the aspartic, cysteine, glutamic, metallo-, serine or threonine peptidases (Puente et al., 2003). However, some proteolytic enzymes remain unassigned and unclassified as yet. The most comprehensive overview of our present knowledge about proteases and their inhibitors can be found in the Merops database (Rawlings et al., 2010) at merops.sanger.ac.uk. This website is a valuable tool in approaching investigations on the biochemistry and molecular biology of proteases throughout all kingdoms of living species such as *Archaea*, *Eubacteria* and *Eukaryota*. In addition, viral proteases are included because they have gained increased attention in recent years due to their importance in a variety of infectious diseases. Inhibitors of proteases, the natural counter-parts of proteases, are important in safe-guarding mammalian cells, by regulating proteolytic enzymes in excess or in unwanted locations in diseased cells or tissues.

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**Corresponding author. Phone: +49 421 2003246, Fax: +49 421 2003249, E-mail: k.brix@jacobs-university.de.

http://www.fupress.com/ijae

This review article will focus on cysteine peptidases. In particular, we will discuss cysteine cathepsins that belong to the C1A subfamily of papain-like enzymes in clan CA (Rawlings et al., 1994; Chapman et al., 1997; Barrett et al., 2001; Reinheckel et al., 2001; Turk et al., 2001; Nagler et al., 2003; Choe et al., 2006; Mohamed et al., 2006; Vasiljeva et al., 2007; Brix et al., 2008). Endogenous inhibitors of mammalian cysteine cathepsins comprise the family of cystatins (Barrett et al., 1981; Barrett et al., 1986; Bode et al., 1988; Abrahamson et al., 1994) and the propeptide-mimicking cytotoxic T-lymphocyte antigens (CTLA) -2a and -2b (Denizot et al., 1989). Cystatins function as intra- and extracellular proteins that integrate into the active site cleft of cysteine cathepsins in a wedge-shaped edge-like fashion (Abrahamson et al., 1987; Stubbs et al., 1990; Jenko et al., 2003). In contrast, the CTLAs bind to the active site of family C1 cysteine peptidases in reverse orientation, similar to their propeptides. This binding involves a chaperoning mechanism, also used during biosynthesis and trafficking of cysteine cathepsins, in order to retain the immature proenzyme in zymogen form until it reaches its target compartment (Guay et al., 2000; Wiederanders et al., 2000). The precise knowledge of the molecular structures of proteolytic enzymes and their endogenous inhibitors, along with the notion of their interactions in forming protease-inhibitor complexes in a reversible fashion, has stimulated increasing interest in using natural (e.g. E64) or synthetic inhibitors and other small molecule derivatives in order to target proteolytic activity (Barrett et al., 1982; Baruch et al., 2004).

Cysteine cathepsins have been considered as useful targets to treat a large diversity of disorders: atherosclerosis, cancer, rheumatoid and osteoarthritis, osteoporosis, neurodegenerative disorders such as Alzheimer's disease and, more recently, inflammation and obesity (Turk et al., 2006; Vasiljeva et al., 2007; Bromme et al., 2009). This impressive list of cysteine cathepsin involvement in pathological processes highlights their functional versatility, and the strategic move towards better tackling of specific protease inhibition has further entailed the development of invaluable tools to visualize and image proteolytic functions of cysteine cathepsins, through the use of activity based probes (ABPs) (Jeffery et al., 2003; Baruch et al., 2004; Berger et al., 2004; Brix et al., 2005; Fonovic et al., 2007; Blum et al., 2008; Paulick et al., 2008).

Visualization of proteolysis – a challenging task

Proteolysis is as complex as biology in general. Hence, elucidating expression patterns of proteases and their inhibitors is a basic priority when approaching an understanding of protease functions. A variety of experimental set-ups have been well established in order to investigate the genome, transcriptome and proteome systems, and to compile a comprehensive list of proteases and inhibitors that enable cellular and tissue functions.

Genome sequencing projects have been instrumental in learning more about the so-called degradome, the entire set of proteases expressed in a given organism (Lopez-Otin et al., 2002; Puente et al., 2003; Quesada et al., 2009). Because proteases are regulated by their endogenous inhibitors, these are also covered in The Mammalian Degradome Database, provided at degradome.uniovi.es. Proteolytic enzymes are encoded by 2% of the human genome, a huge proportion of all genes. Thus, it is hardly surprising that almost 90 different hereditary diseases known to date are derived from mutations

in protease genes (Lopez-Otin et al., 2008). Degradomics enable a systems biology analysis of all proteases, protease homologues and protease inhibitors through the use of DNA microarrays (Overall et al., 2006). This approach has been further taken to establish metadegradomics, which includes mass spectrometry for quantitative *in vivo* degradomics and the introduction of post-translational modifications (Doucet et al., 2008).

The next important step in understanding protease functions is to localize proteolytic enzymes in an *in situ* context with their natural substrates and their endogenous inhibitors within cells and tissues. While this sounds like a classical domain of cell biologists, it became clear that a wider spectrum of disciplines is needed to provide the tools necessary for grasping the mammalian cell's repertoire of proteases. With respect to the broader picture, it will be important to keep in mind that proteolysis is the most important post-translational protein modification process, because proteolytic cleavage is irreversible, thus determining the fate of cells in their most critical turning point of deciding between life and death.

The study of protease functions must encompass determination of protease activities, which has often been achieved through the basic use of synthetic substrates in biochemical approaches. However, we are now entering an era where the more complex tasks of monitoring substrate cleavage and tracking protease activities in intact cells (Tab. 1) are not only possible but reveal an overwhelming wealth of data.

Spotting proteolytic enzymes at high resolution by immunolabeling for light and electron microscopy

Immunolocalization is the best known and most straightforward experimental approach to detect proteases in subcellular compartments and to localize secreted enzymes in tissues. Most protocols include fixation procedures that are based on formaldehyde fixation for light microscopical detection, or fixation with glutaraldehyde for electron microscopy. While both fixation techniques bear the clear advantage of being very well established and easy-to-use routines, it might be worthwhile considering an alternative fixation strategy, namely alcohol (ethanol or methanol) -based dehydration procedures. For example, it is well known that keratinocytes and oligodendrocytes exhibit a strikingly different membrane lipid composition in comparison to cells such as fibroblasts. Accordingly, our own experience, as summarized in Tab. 2, has convinced us to compare methanol/acetone-based dehydration techniques carried out at low temperatures with paraformaldehyde-based protocols conducted at room temperature or at 37 °C, when visualizing cysteine cathepsins that are thought to reside within endo-lysosomal compartments of mammalian cells (Brix et al., 1996; Tepel et al., 2000; Linke et al., 2002b; Friedrichs et al., 2003; Buth et al., 2007; Jordans et al., 2009; Mayer et al., 2009; Vreemann et al., 2009; Tedelind et al., 2010). For the majority of cell types such as fibroblasts, thyroid epithelial cells, enterocytes or macrophages, our preferred method is chemical cross-linking with mono- or bifunctional aldehydes, whereas keratinocytes are best treated with methanol. Other important measures that improve structural preservation are washing procedures before and after fixation, as well as paying attention to the composition of buffers used in combination with certain fixatives. In our hands, calcium and magnesium-free phosphate buffered saline (CMF-PBS) is best combined with methanol/acetone solutions, where-

Table 1 – Sample preparation for various methods of cysteine cathepsin detection.

1. **Transfection** with vectors encoding fluorescent protein tagged protease
2. **Fluid phase** uptake to label endocytic compartments:
   wash in PBS, incubate with 1 µM LysoTracker Red DND-99 in culture medium for 5 min (endosomes) or 30 min (lysosomes) at 37°C, chase in complete culture medium for 120 min – 24 h (lysosomes, only) at 37°C, wash in PBS, fix as desired, e.g. 3% PFA in PBS or HEPES for 30 min at 37°C
3. **Activity Based Probes**
   - label pericellular proteases by incubation at 37°C with non-permeable ABP or protease substrate analogs, use inhibitors like E64d or CA-074d as control;
   - alternatively, incubate with cell-permeable ABP or protease substrate analogs, use inhibitors as control, incubate at 4°C to block endocytosis, then, wash and perform first minutes of fixation also at 4°C
   - peri- and intracellular proteases can be labeled by incubation at 37°C with cell-permeable ABP or protease substrate analogs, incubate with inhibitors as control; ABPs come as non-quenched or quenched probes; principle of labeling: ABPs are cleaved by active protease, releasing quenching group and resulting in covalently labeled protease
     – non-quenched ABPs: fluorescence where probe binds, i.e. background could be a problem
     – quenched ABPs: fluorescence only where the probe binds specifically to active proteases, i.e. better signal-to-noise ratio is achievable

Notes:
Protease-specific inhibitors:
E64 inhibits all cysteine peptidases, use at 1-5 µM; **CA-074** inhibits cathepsin B specifically, use at 1-5 µM; **Z-FY(t-Bu)-DMK** (Z-Phe-Tyr(t-Bu)-diazomethylketone) inhibits cathepsin L specifically, use at 1-5 µM
Activity Based Probes (ABPs):
NS-173 or **NS-196** = rhodamine-conjugated or biotinylated cathepsin B-specific probe (Schaschke et al., 2000), use at 500 nM in culture medium
DCG-04, e.g. Green-DCG-04, fluorophore-conjugated probes (Greenbaum et al., 2000), use at 500 nM in culture medium
GB-111 and **GB-117**, non-quenched and quenched probes (Blum et al., 2005), a variety of next generation ABPs have been synthesized recently (Blum et al., 2009), use at 500 nM in culture medium

as HEPES or PIPES are our preferred biogenic amine-based buffers used in combination with freshly prepared paraformaldehyde for fixation.

In this context, it is interesting to note that many researchers appear to be more concerned about the properties of specific antibodies rather than structural preservation of the primary antigen when choosing fixation protocols. Certainly, antibodies that are commercially available often prefer immunoidentification of their antigens in a specific context, such as in formaldehyde-fixed cell or tissue preparations without or after paraffin embedding, or as well exposed antigens and structurally well preserved molecules in frozen tissues. However, we further suggest taking the distinct lipid membrane composition of the cell type of interest into account. For illustration of the results achieved with identical antibodies in different cell types, examples are provided of cysteine cathepsin B localization in keratinocytes after methanol-acetone fixation (Fig. 1A), in thyroid carcinoma cells after fixation with paraformaldehyde (Fig. 1B), and in cryo-sections of mouse intestine tissue (Fig. 1C'). In all cases, the

Table 2 – Procedures for indirect immunofluorescence after different fixation procedures.

	Methanol/Acetone	**Paraformaldehyde (PFA)**
cell culture	e.g. on cover glasses	e.g. on cover glasses
PBS	X	X
fixation	Methanol, 8 min, -20°C Acetone, 8 min, -20°C	4 - 8% PFA in 200 mM HEPES, pH 7.4, 30 min, RT
HEPES	---	X
CMF-PBS	X	X
0.2 - 1% Triton X-100 in CMF-PBS, 5 min, RT	---	optional depending on whether interested in pericellular proteases = w/o TX100, or intracellular proteases = w/ TX100
CMF-PBS	X	X
block non-specific binding sites	3 mg/ml BSA in CMF-PBS, 30 min, RT	3 mg/ml BSA in CMF-PBS, 30 min, RT
0.1 mg/ml BSA in CMF-PBS	X	X
1° ab in 0.1 mg/ml BSA in CMF-PBS	90 min / over night, 37°C / 4°C	90 min / over night, 37°C / 4°C
0.1 mg/ml BSA in CMF-PBS	X	X
2° ab coupled to FITC / DTAF / Alexa 488 / Alexa 543 / Alexa 633 / Cy2 / Cy3 in 0.1 mg/ml BSA in CMF-PBS	60 min, 37°C	60 min, 37°C
CMF-PBS	X	X
ddH_2O	X	X
mounting in 33% glycerol, 14% mowiol in 200 mM Tris (pH 8.5) + 5% 1,4-diazabicyclo(2,2,2) octan	X	X
view @ excitation as required by fluorophore	X	X

X, step required; ---, step not required; RT, room temperature; ddH_2O, double distilled water; PBS, phosphate-buffered saline; CMF, calcium- and magnesium-free

Notes: DRAQ5™ for counter-staining of nucleic acids can be applied with 2° ab at final concentration of 5 µM, view at 633 nm excitation. **Propidium iodide** (2 µg/µl in CMF-PBS) is applied to cell cultures before fixation to stain dead cells with ruptured membranes, when applied after fixation and TX100 permeabilisation, it stains all cellular DNA, view at 543 nm excitation.

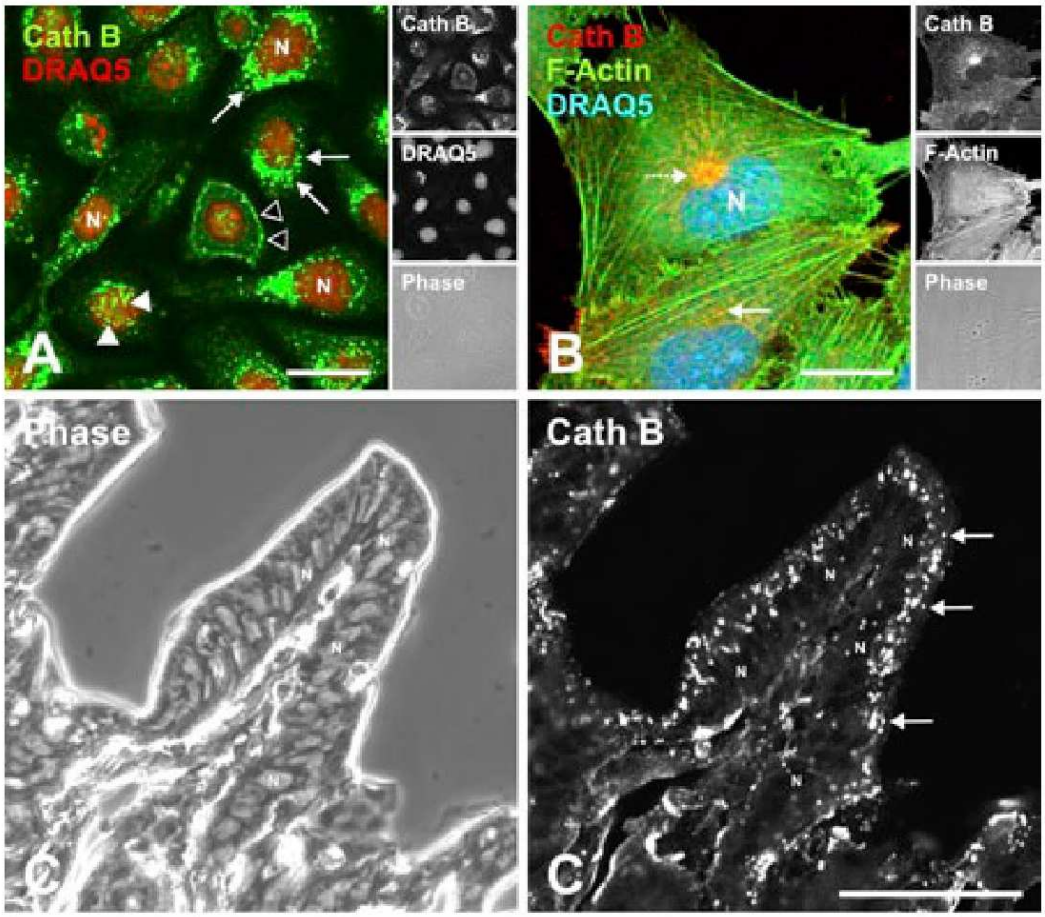

Figure 1 – Visualization of cathepsin B in cells and tissue using different fixation methods and indirect immunofluorescence.

(A) Human HaCaT keratinocytes were fixed with methanol and acetone for 8 min each, at -20°C before immunolabeling with primary anti-cathepsin B and secondary, fluorophore-conjugated antibodies (green). Nuclei were counter-stained with the nucleic acid-specific dye DRAQ5™ (red). Endo-lysosomal (arrows), nuclear (arrowheads) and plasma membrane (open arrowheads) localizations of cathepsin B were readily observed. For further details on subnuclear labeling patterns, see (Tedelind et al., 2010).

(B) Human thyroid carcinoma KTC-1 cells were fixed with paraformaldehyde before immunolabeling with primary anti-cathepsin B and secondary fluorophore-conjugated antibodies (red). Nuclei were counter-stained with DRAQ5™ (blue) and cytoskeletal F-actin was visualized by FITC-phalloidin staining (green). Cathepsin B accumulated in the perinuclear Golgi region of thyroid carcinoma cells (broken arrow) and the protease was additionally sorted into vesicular structures spreading from the cell center in a star-like fashion (arrow), characteristic for vesicles transported along microtubules during protease trafficking with destination to endo-lysosomes.

(C and C') Mouse intestine tissue was paraformaldehyde-fixed before cryo-sectioning and immunolabeling with primary anti-cathepsin B and secondary fluorophore-conjugated antibodies (C'). Cathepsin B was detectable within endo-lysosomes of intestine epithelial cells (arrows) and the protease was abundantly immunolabeled within endocytic vesicles of cells in the *lamina propria*.

In all three examples the same primary anti-cathepsin B-specific antibody was used to detect its differently fixed antigen. Single channel and merged fluorescence micrographs in A and B are shown in false-colors; right hand panels in A and B and pictures in C and C' depict single channel micrographs and phase contrast images as indicated. N, nucleus; scale bars represent 20 µm in A and B, and 50 µm in C'.

cysteine cathepsin-containing structures could be easily visualized by fluorescence microscopy and are compared to well-preserved cellular structures as viewed in the corresponding phase contrast images (Fig. 1).

Since cysteine cathepsins are redundantly expressed in both a ubiquitous and cell type-specific manner (Chapman et al., 1997; Nagler et al., 2003; Brix et al., 2008), it may also become important to simultaneously visualize other closely related enzymes. In such cases, we prefer to immunolabel in a consecutive fashion, meaning first to label the proteases present in small amounts with green fluorophores and then to visualize the more abundant proteins with employment of red fluorophores (Tepel et al., 2000; Jordans et al., 2009). This strategy ensures that the brightness of each fluorophore as measured by its characteristic extinction coefficient and fluorescent quantum yield is applied for the best detection of rare proteins as bright spots by the emitted green fluorescence, on a usually more dimmed background fluorescence derived from labeling abundantly present proteolytic enzymes with red fluorophores.

The results of such co-localization studies demonstrate that it is now possible to visualize even small amounts of rare nuclear cysteine cathepsin variants, which maximally constitute 5-10% of all cysteine peptidases in e.g. thyroid carcinoma cells (Tedelind et al., 2010), and which have escaped immunodetection for many decades. Nonetheless, with improved detection methods it is now possible to also study such rare proteases that are likely to be crucial for cell differentiation and cell fate determinations.

Likewise, co-localization studies have allowed the investigation of protein composition of specific vesicles of endocytic compartments and have revealed the existence of different vesicle populations, such as in thyroid epithelial cells that are loaded with distinct sets of proteases (Tepel et al., 2000; Jordans et al., 2009; Tedelind et al., 2011). Such results are not at all trivial since they raise important questions; for example, how loading of vesicles with soluble enzyme cargo is achieved in mammalian cells. Clearly, thyroid epithelial cells are able to selectively recruit specific proteases that are present in different vesicle populations in particular biological contexts, for example in resting or thyroid stimulating hormone (TSH) -stimulated conditions (Brix et al., 1996; Brix et al., 2001; Linke et al., 2002a; Linke et al., 2002b). This enables cells to fine-tune their protease cocktails in a subcellular compartment or even subcompartment, so that they can cope with the necessity of degrading a huge variety of internalized molecules that are likely to differ in resting or stimulated conditions.

In order to detect cysteine cathepsins with high spatial resolution in various cellular compartments, immunoelectron microscopy is a useful tool. Visualization of cysteine cathepsins with specific antibodies has been performed by classical approaches that involve post-Epon-embedding-labeling techniques or cryo-immunoelectron microscopy procedures as well as by rather unusual approaches which include immunolabeling of whole-mount preparations (Brix et al., 1996).

Following protease tracks using fluorescent protein tagging experiments

Cysteine cathepsins are not exclusively present within endo-lysosomes; they are detectable in all compartments of the secretory route and in vesicles targeted to reach the compartments of the endocytic pathway. In addition, cysteine cathepsins are often actively secreted from cells in processes such as cell migration (Buth et al., 2004), tis-

sue repair (Buth et al., 2007) or invasion of carcinoma cells (Mohamed and Sloane, 2006), where the secreted enzymes are thought to be involved in ECM remodeling processes. Secreted cysteine cathepsins can further accumulate in structures such as caveolae (Cavallo-Medved et al., 2009) and they may also re-associate with the cell surface as shown in Fig. 1A. In peri-cellular locations such as in the vicinity of the apical plasma membrane of epithelial cells, cysteine cathepsins have been shown to contribute to limited proteolysis of molecules en-route to endosomes and lysosomes (Brix et al., 1996; Brix et al., 2001; Friedrichs et al., 2003; Buth et al., 2004; Mayer et al., 2009; Vreemann et al., 2009; Dauth et al., 2011) and hence, act in the direct pericellular environment. In addition, rare variants of cysteine cathepsins have been detected which appear to serve key functions in unusual locations such as the mitochondrial matrix (Muntener et al., 2004) or the nucleus (Goulet et al., 2004; Ong et al., 2007; Duncan et al., 2008; Maubach et al., 2008; Tedelind et al., 2010).

These observations illustrate that trafficking studies are important in elucidating the transport pathways of proteases within cells, which in turn provide clues to their function. Such studies are best performed by fluorescent protein tagging, which allows tracking of protease transport, from the rough endoplasmic reticulum (rER) where *de novo*-biosynthesis of the full-length forms occurs, to their main cellular destination within endo-lysosomes (Mach et al., 1994; Mort et al., 1997; Linke et al., 2002a; Brix et al., 2008). One must bear in mind to tag the proteases in such a way that intrinsic protein sequences, essential for protein targeting and correct transport, will not become affected by introduction of the fluorescent protein-encoding sequences. Cysteine cathepsins can be tagged easily and without obvious mistargeting or loss-of-function effects when the green fluorescent protein (GFP) or other fluorescent proteins are attached to the C-terminus via a spacer peptide (Linke et al., 2002a; Katayama et al., 2008; Mayer et al., 2008). Cathepsin B has for example been localized to endo-lysosomes through visualization of the eGFP-tagged chimeric protein (Linke et al., 2002a) in thyroid carcinoma KTC-1 cells (Tedelind et al., 2011), in the absence or presence of LysoTracker® that accumulates in endo-lysosomal compartments (Fig. 2). A variety of fluorescent proteins are available, some of which might also be useful in studying the precise timing of transport reactions, while others might be instrumental in determining interactions of proteases with substrates or other partners, such as split GFP (Waldron et al., 2008) or fluorescence resonance energy transfer (FRET) approaches, which are achievable and in use (Chudakov et al., 2010).

However, fluorescent protein tagging can be used as well to study secretion pathways of the zymogen forms along the secretory route in particular cells, e.g. cancer cells (Moin et al., 2000; Sameni et al., 2001; Mohamed and Sloane, 2006), or to analyze the strategies that epithelial cells use to recruit mature cysteine cathepsins out of late endosomes/lysosomes for retrograde trafficking and secretion into the extracellular space (Linke et al., 2002a; Linke et al., 2002b; Brix et al., 2008; Dauth et al., 2011). Interestingly, many cell types have been recognized as being competent in secretion of endo-lysosomal enzymes (Brix et al., 1994; Andrews et al., 2000; Brix et al., 2008). However, the precise transport pathways are not always studied in sufficient detail. Macrophages and dendritic cells are especially exceptional in the transport of proteases because they need to adapt quickly to situations such as infection and inflammation, where they serve as first-line defence and antigen-presenting cells respectively (Bogyo and Ploegh, 1998; Honey and Rudensky, 2003; Beers et al., 2005; Hsing et al., 2005).

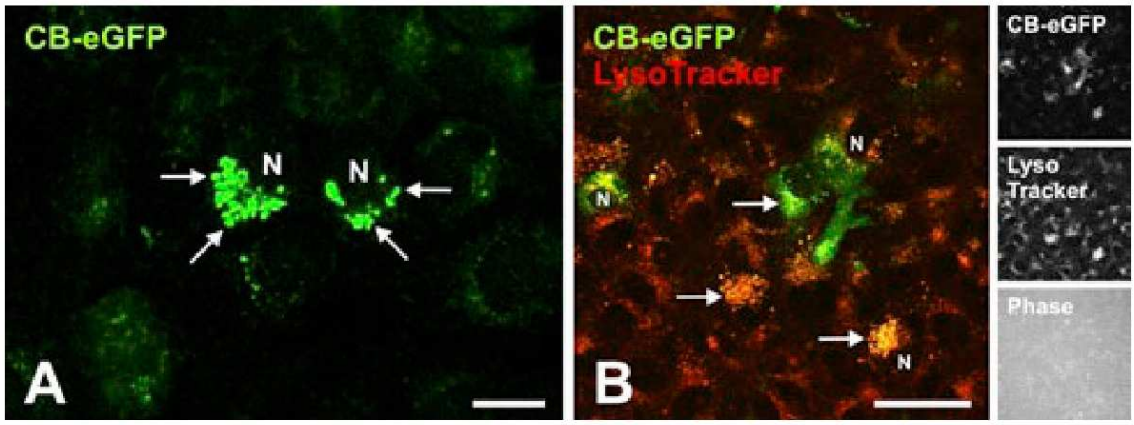

Figure 2 – Localizing cathepsins in living cells using enhanced green fluorescent protein tags.
Human thyroid carcinoma cells were transfected with a vector coding for cathepsin B-eGFP chimeras (A, green). One day post-transfection, the vital stain LysoTracker was applied at 37°C in complete culture medium for fluid phase uptake and accumulation within endo-lysosomes in the perinuclear region (B, red). Cells were viewed through live cell imaging with a confocal laser scanning microscope. Thyroid carcinoma cells transiently expressing cathepsin B-eGFP displayed a vesicular staining pattern of the GFP-tagged cathepsin B peptidase (green) in a pattern characteristic for endo-lysosomal compartments in the peri-nuclear region (A and B, arrows). The vesicles containing eGFP-tagged cathepsin B were partially co-localized with those that acquired the fluid phase marker (B, yellow as a result of overlapping green and red signals), indicating that GFP-tagging did not interfere with protease transport to endo-lysosomes.
Single channel and merged fluorescence micrographs in A and B are shown in false-colors; right hand panel in B depicts corresponding single channel micrographs and phase contrast as indicated. N, nucleus; scale bars represent 20 µm in A and 50 µm in B.

Moreover, fluorescent protein-tagged cysteine cathepsins might become useful in determining the exact timing and to quantify the extent of lysosomal membrane rupture. That is, for instance, essential for release of mature cathepsin B into the cytosol which eventually leads to induction of apoptotic cell death by an alternative intrinsic induction pathway due to Bid-cleavage (Houseweart et al., 2003; Turk et al., 2009) and may be involved in killing of tumor cells by irradiation to induce lysosomal burst (Kroemer and Jaattela, 2005).

Caught in the act – monitoring protease activities and substrate cleavage in cells and tissues

From the above, it becomes clear that proteases are a group of extremely versatile enzymes that function in all possible locations within and outside cells. The classical view of cysteine cathepsins purely as lysosomal enzymes is now clearly outdated. Therefore, strategies are needed to visualize and image proteases in sites of action (Baruch et al., 2004; Blum et al., 2005; Brix and Jordans, 2005; Brix et al., 2008; Blum et al., 2009).

A variety of such strategies can include enzyme cyto- and histochemistry encompassing various photo conversion methods (Spiess et al., 1994). However, the principal possibility of combining immunolabeling with enzyme cytochemistry has

rarely been realized (Brix et al., 1996). The procedures that may be used to precisely determine the locations of cysteine cathepsin actions are summarized in Fig. 3. The protocols for detection of cysteine cathepsins are detailed in Tabs 1 to 3, and can be varied such that specific substrates in combination with enzyme-specific inhibitors may be used for ultrastructural detection, which are then verified by post-embedding immunolabeling procedures (Brix et al., 1996; Brix et al., 2008). Furthermore, a variety of antibodies can be tested by immunogold labeling techniques. However, those protocols bear the obvious disadvantage of inducing alterations of protease activities that may be caused by harsh fixation methods and other drastic chemical treatments of cells.

Hence, protease activities are best investigated by fluorescence microscopy methods that are optimal for exclusive use in living cells. In former times, fluorescently labelled extracellular matrices have been used to track substrate cleavage during processes such as cell migration (Friedl et al., 2000; Jedeszko et al., 2008; Cavallo-Medved

Table 3 – Comparison of live and fixed cell procedures for enzyme cytochemistry procedures.

	live cells (pericellular activity)	**fixed cells**
cell culture, e.g. on cover glasses	X	X
PBS	X	X
fixation: 1% PFA in PBS, 20 min, RT	---	X
PBS	---	X
reactivation: reaction buffer (RB = 0.2 M ammonium acetate, 0.125 mM ß-mercaptoethanol, 0.1 mM EDTA-Na_2, pH 6.2 or pH 7.2 with 0.4 M Na_2HPO_4), 5 min, 40°C without O_2	X	X
reaction at 40°C without O_2 for 15 - 60 min: 1 mM protease-specific substrate coupled to 4-methoxy-ß-naphthylamine in RB + 0.5 – 1 mM 2-hydroxy-5-nitrosalicylaldehyde (NSA); controls: 1 mM E64 in RB, or omit substrate from RB + NSA	X	X
PBS or CMF-PBS	X	X
fixation: 1% PFA in PBS, 20 min, RT	X	---
PBS	X	---
ddH_2O	X	X
mounting in 33% glycerol, 14% mowiol in 200 mM Tris (pH 8.5)	X	X
view at excitation 488 nm	X	X

X, step required; ---, step not required; RT, room temperature; O_2, oxygen; ddH_2O, double distilled water

Protease-specific substrates

cathepsin B: 0.5 – 1 µM Z-Arg-Arg-4MßNA ± CA-074 (pH 6.0 – optimum); controls with E64

cathepsin K: 0.5 – 1 µM Z-Gly-Pro-Arg-4MßNA ± 10 µM CA-074; controls with E64

cathepsin L: 0.5 – 1 µM Z-Phe-Arg-4MßNA ± 1.5 µM CA-074 (pH 5.5 – optimum); controls with CA-074 + Z-FY(t-Bu)-DMK

et al., 2009). A number of small molecules are now available that have accelerated the field of cysteine cathepsin biology significantly in recent years (Greenbaum et al., 2000; Greenbaum et al., 2002; Blum, 2008; Blum et al., 2009). The basic idea of activity based probes (ABPs) is the synthesis of small molecules that can easily penetrate biological membranes (see, Tab. 1), therefore reaching all potential locations of proteolytic enzymes within cells and tissues.

In general, ABPs bear a so-called warhead, i.e. they are functionalized to bind to only one group of proteases, because the mechanism of action is specific targeting of ABPs to one family of enzymes (Liu et al., 1999; Kidd et al., 2001; Baruch et al., 2004; Kato et al., 2005; Sieber et al., 2006; Sexton et al., 2007; Wright et al., 2009; Yang et al., 2009). Upon binding, ABPs are cleaved since most of them represent substrate analogs, thereby attaching themselves covalently to the targeted proteases. ABPs are additionally functionalized with fluorophores, iodinatable groups, biotin moieties

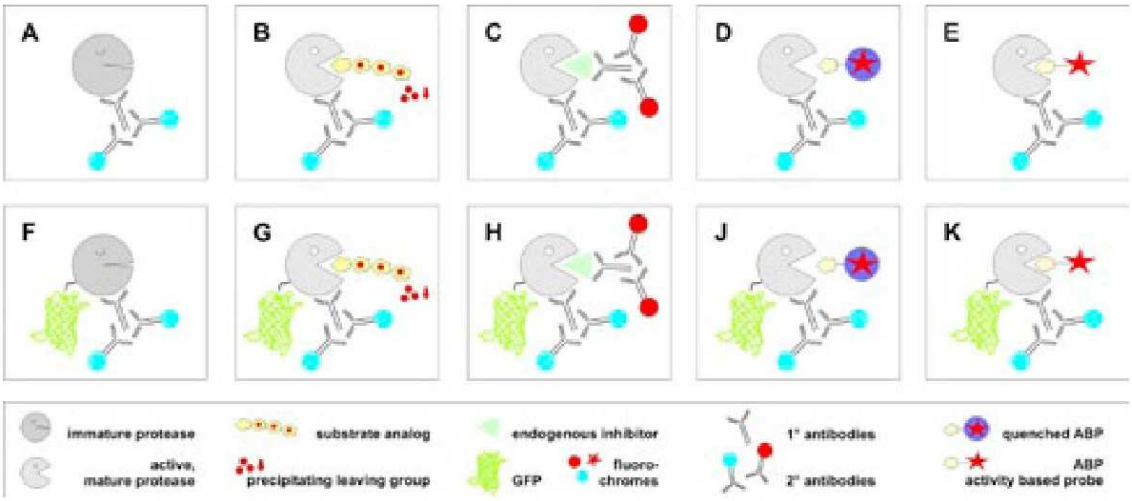

Figure 3 – Schematic representation of experimental strategies for imaging of cysteine cathepsin functions. An appropriate choice from the selection of detection methods available for cysteine peptidase localization is dependent on factors such as the nature of the experimental question, type of specimen and specimen preparation. Most established protocols of indirect immunofluorescence employ the use of antibodies which can effectively detect both zymogens (A and F) and active cathepsins (B – E and G – K). Cathepsin activity can be visualized through the use of substrate analogs that are cleaved upon encountering active protease with the leaving groups precipitating at the spot of enzymatic activity (B and G). Leaving groups may be visualized through signal enhancement by incubation with nitrosalicyladehyde in fluorescence microscopy, or pararosanilin and OsO_4 in electron microscopy (Brix et al., 1996). Active cysteine cathepsins in complex with their endogenous inhibitors such as the cystatins, can be detected using cystatin-specific antibodies (C and H). A very valuable tool for detecting active cathepsins at their sites of action is the use of quenched (D and J) or non-quenched activity-based probes (ABP; E and K). Quenched ABPs are designed to mimic the natural substrate and covalently interact with the protease in its active site, whereby the fluorescent signal is generated upon proteolytic cleavage. Non-quenched ABPs are fluorescent before interacting covalently with the active protease in a 1-to-1 ratio, thereby fluorescently highlighting enzymatic activities on the spot (E and K; Brix and Jordans, 2005). Another useful approach of studying cathepsins in living cells is the use of GFP-tagged chimeras, which constitutively track the position of the proteins regardless of whether immature (F) or enzymatically active (G – K) proteases are tagged with GFP (Brix et al., 2008). GFP fusion at the C-terminus of cysteine cathepsin B has been shown not to interfere with protease trafficking. GFP tagging of proteases can be combined with immunofluorescence or enzyme cytochemical or ABP labelings of all kinds (F – K).

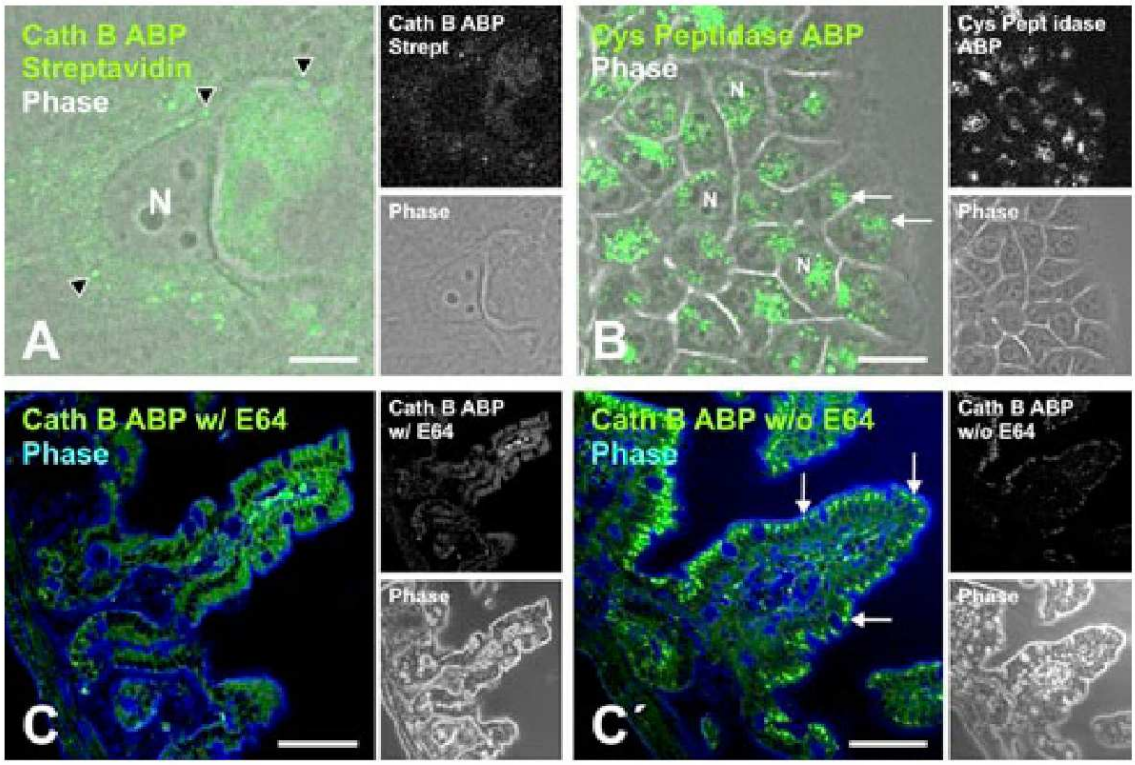

Figure 4 – Employment of activity-based probes for spotting enzymatic activity of cathepsins at their sites of action *in vivo* and *in situ*.
(A) Live cell imaging through confocal fluorescence and phase contrast microscopy of human HaCaT keratinocytes after treatment with a membrane impermeable, biotinylated cathepsin B-specific activity based probes (ABP; Schaschke et al., 2000) and incubation with fluorophore-conjugated streptavidin (green), visualizing cathepsin B activity at the cell surfaces and at junctions between neighboring cells (open arrowheads).
(B) Live cell imaging through confocal fluorescence and phase contrast microscopy of human HaCaT keratinocytes after treatment with a membrane permeable, fluorophore-conjugated cysteine peptidase-specific ABP (Greenbaum et al., 2000), visualizing cathepsin B activity within endo-lysosomes (green; arrows) accumulating in the perinuclear region of HaCaT cells.
(C and C') Confocal fluorescence images (green) merged with corresponding phase contrast micrographs (dark blue) of cryosections prepared from paraformaldehyde-fixed mouse intestine tissue and after incubation with a cathepsin B-specific ABP (green; Schaschke et al., 2000). The cryosections were pre-incubated with freshly prepared L-cysteine for re-activation of cysteine peptidase activity before ABP treatment; E64 incubation served as a control for ABP-specificity as the fluorescence signal within endo-lysosomes (arrows in C') was clearly diminished in inhibitor-competed ABP stainings of cryosections (C). A number of fluorescent dots in cells present in the lamina propria are most likely due to lipofuscins accumulating within cells, e.g. macrophages or neutrophils.
Merged pictures of single fluorescence channels with corresponding phase contrast micrographs are shown in false-colors; right hand panels depict corresponding single channel micrographs and phase contrast images. N, nucleus; scale bars represent 10 µm in A, 20 µm in B, and 50 µm in C and C'.

etc., which are attached to the opposite end of the small molecule backbone. Hence, ABPs target protease families rather than individual enzymes as shown for the membrane permeable and fluorophore-conjugated DCG-04 that visualizes active cysteine peptidases in human keratinocytes (Fig. 4B). An alternative approach combines

propeptide-like with substrate-like binding properties in a synthetic small molecule (Schaschke et al., 2000). These probes are suitable in targeting individual enzymes in larger families of related proteases. For instance, a cathepsin B-specific probe has been designed which is able to clearly distinguish between the most abundantly expressed cathepsins B and L by an impressive factor of 2,000 (Schaschke et al., 2000; Schaschke et al., 2002). The fluorophore-conjugated ABP specific for cathepsin B, NS173, enabled the visualization of active, plasma membrane associated cathepsin B in human keratinocytes (Fig. 4A), and it can be used additionally for visualization of active cathepsin B *in situ* in mouse intestine (Fig. 4C´) while pre-incubation with the cysteine cathepsin inhibitor E64 serves as a control for probe selectivity (Fig. 4C).

Quenched ABPs provide better signal-to-noise ratios than non-quenched probes because they become fluorescent only upon cleavage and covalent attachment to the target protease (Blum et al., 2005; Brix and Jordans, 2005; Blum et al., 2007; Blum et al., 2009). This elegant approach has now reached as far as the design of ABPs for diagnostic use in mouse (Blum et al., 2007; Blum et al., 2009). The long-term goal is to utilize them in translational approaches of rational drug design as therapeutics.

These most promising features of ABPs point to their future applications as useful tools to visualize excess protease activities and to even treat diseases like cancer at the molecular level. However, the molecular features of ABPs and their mechanism of action also bear a serious disadvantage to this approach. Namely, ABPs act as inhibitors of the targeted proteases. ABPs bind and covalently attach to their target proteases in a one-to-one fashion. Hence, for therapy they must be used in extremely high concentrations, thereby increasing the risk of toxicity and induction of off-target effects. On one hand, since they are protease inhibitors, treatment of a cell with ABPs may elegantly target protease activities; on the other hand, a pharmacological knockdown can be easily produced.

In future, ABPs and related molecules could become as useful as gene knockdown or knock-out strategies in altering the composition of proteolytic networks on a long-term basis. It would then also be possible to challenge protease activities and actions by using animal models of various diseases. The goal is to target, inhibit or initiate protease activities, in order to learn more about their true biological significance as enzymes mediating quick cuts that irreversibly change the fate of proteins.

Conclusions and Perspectives

Tracking and localizing proteases in living cells and co-localizing proteases with their natural substrates in an entire organism is our ultimate objective in specifying the significance of proteolysis *in vivo* (see, Fig. 3). This aim is not far away. Numerous experimental achievements have demonstrated that pharmacological inhibition, gene targeting through knock-outs, knock-ins, transgenic over-expression or knock-downs, and combinations with imaging studies of protease functions in living cells have been conducive in answering several important questions. These include, but are not limited to, issues on the *in vivo* importance of a certain protease in any given biological context. To this end, we will continue in our aim of clarifying how post-translational proteolytic modifications are involved in biological processes during development, adulthood and in ageing. Proteases encoding two percent of the entire human genome must be expressed for fun-

damentally crucial reasons - to enable the molecular basis of decision-making processes of cells in tissues and in all conditions of life, disease, repair and death.

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4.2 Cathepsin K in thyroid physiology and disease

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ORIGINAL PAPER

Thyroid Cathepsin K: Roles in Physiology and Thyroid Disease

Stephanie Dauth · Maria Arampatzidou · Maren Rehders · Denise Ming Tse Yu · Dagmar Führer · Klaudia Brix

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Abstract The human genome encodes 11 cysteine cathepsins belonging to the papain-like family of cysteine peptidases that are known predominantly as endo-lysosomal enzymes. However, it is now understood that the functions and activities of cysteine cathepsins are not limited to endo-lysosomal compartments, as they are also active in the peri- and extracellular space. The thyroid gland is an endocrine organ where such intra- and extracellular proteolytic activities are required to solubilize the prohormone thyroglobulin from its luminal, covalently cross-linked storage forms for subsequent processing into smaller protein fragments and thyroid hormone liberation. Cathepsin K has been identified as one of the cysteine cathepsins with a crucial role in thyroglobulin processing. However, cathepsin K has mainly been a key focus of attention in the last few years because of its high expression in osteoclasts and due to its essential role as collagenase and elastase important for bone remodelling. Besides its remarkable function as an endopeptidase acting on high-molecular mass, covalently cross-linked extracellular substrates such as type I collagen, elastin or thyroglobulin, cathepsin K is also one of the very few proteolytic enzymes that is able to directly liberate thyroxine from thyroglobulin fragments by exopeptidase action. Thus, thyroid cathepsin K is now accepted as a cysteine peptidase with a vital role in liberation of thyroid hormones, which in turn are essential for homoeostasis by triggering a number of important biological processes, ranging from growth and brain development in young vertebrates to tissue remodelling events during morphogenesis or wound healing, as well as control of metabolic pathways and thermoregulation in adults. This review focuses on thyroid cathepsin K and will discuss how localization and trafficking within thyroid epithelial cells explain its thyroid-specific functions. The effects of targeted cathepsin K gene ablation will be summarized from the perspective of the thyroid gland, and we will propose potential consequences of short- and long-term inhibition of thyroid cathepsin K activity for the main thyroid hormone target tissues, namely bone, cardiovascular and immune systems, intestine, and the central nervous system, in addition to the thyroid gland itself.

Keywords Cysteine cathepsins · Extracellular proteolysis · Thyroid hormones · Thyroglobulin

S. Dauth · M. Arampatzidou · M. Rehders · D. M. T. Yu · K. Brix (✉)
School of Engineering and Science, Research Center MOLIFE, Molecular Life Science, Jacobs University Bremen, Campus Ring 6, Research II, 28759 Bremen, Germany
e-mail: k.brix@jacobs-university.de

S. Dauth
e-mail: s.dauth@jacobs-university.de

M. Arampatzidou
e-mail: m.arampatzidou@jacobs-university.de

M. Rehders
e-mail: m.rehders@jacobs-university.de

D. M. T. Yu
e-mail: d.yu@jacobs-university.de

D. Führer
Universitätsklinikum Leipzig Medizinische Klinik III, 04103 Leipzig, Germany
e-mail: dagmar.fuehrer@medizin.uni-leipzig.de; dagmar.fuehrer@uk-essen.de

D. Führer
Klinik fuer Endokrinologie, Zentrum fuer Innere Medizin, 45147 Essen, Germany

Thyroid-Specific Functions of Cysteine Cathepsins in Thyroglobulin Processing: Comparison to Substrates of Bone Tissue

Cathepsin K belongs to the cysteine cathepsins that bear important functions in maintaining physiological processes directed by thyroid hormones (TH), because the prohormone thyroglobulin (Tg) has been recognized as one of the natural substrates of cathepsin K [1–3]. Peptidases other than cathepsin K which are known or have been suggested as Tg-processing enzymes comprise the cysteine cathepsins B, C (DPP-I), H, L, and S, the aspartic cathepsin D, and the metalloprotease aminopeptidase N and serine proteases dipeptidylpeptidases II and IV [4–15]. The precise cleavage sites in the sequence of Tg are known for some of these proteolytic enzymes, namely for most of the cathepsins [3, 4, 11, 15], with the important remark that processing of the prohormone Tg depends on where it happens within thyroid follicles. Hence, thyroid functions are strongly linked to the trafficking pathways of the proteolytic enzymes for interaction with their substrate Tg and its cleavage by sequential endo- and exopeptidatic modes.

Tg is a difficult substrate for proteolytic processing because it is a huge molecule of 2,755 amino acids in humans. Tg is N- and O-glycosylated to an extent of up to 30%, and it exists in situ in dimeric to oligomeric forms. The largest Tg-aggregates found in the thyroid gland are represented by the so-called thyroid globules, which consist of up to 800 mg Tg per ml in supra-molecular arrangements that are made of covalently cross-linked Tg molecules. Intra- and intermolecular cross-linking of Tg depends on species-specific molecular mechanisms including disulfide bond formation, dityrosine bonding and transglutaminase-mediated isopeptide cross-links [11, 16–20]. Thus, the task of proteolytic processing of Tg can be compared with that of some other well-known and physiologically important cathepsin K substrates, namely collagen I and elastin of the extracellular matrix (see Fig. 1). We propose that cathepsin K is special among the cysteine cathepsins in that it seems to belong to a group of proteolytic enzymes that can cope with highly aggregated substrates and which exhibit their proteolytic activity under conditions where other proteases fail, namely in the extracellular space and in the presence of high concentrations of glycosaminoglycans, proteoglycans and glycoproteins (featuring complex and high-mannose type carbohydrate modifications) [21–23]. Cathepsin K can thus overcome the protective role of glycosylation that is believed to serve as a posttranslational modification preventing premature degradation of extracellular matrix constituents or blood serum proteins.

Requirements of Localized Proteolytic Activities in the Thyroid

Maintenance of constant thyroid hormone levels in the circulation is the predominant task of the thyroid gland. Thyroid follicles are the functional units of this butterfly-shaped endocrine gland found in the neck region of vertebrates. Evolutionary earlier species than vertebrates synthesize Tg-like prohormones and thereby thyroid hormones as well, but a proper thyroid gland is not always established [24, 25]. Each lobe of the thyroid gland consists of millions of spherical structures, the thyroid follicles, that are composed of a monolayer of thyroid epithelial cells, thyrocytes, surrounding an extracellular follicle lumen that

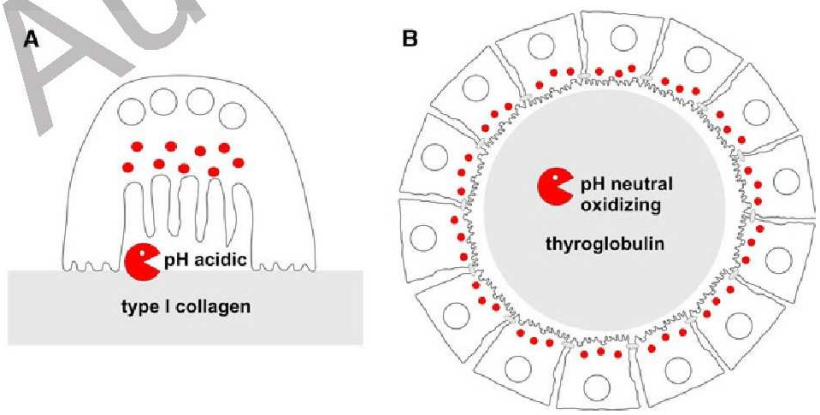

Fig. 1 Comparison of cathepsin K action in bone and thyroid. Cathepsin K is an unusually versatile cysteine cathepsin under various conditions, making it an important enzyme for degradation of natural substrates. For instance, osteoclast cathepsin K is secreted into the acidified resorption lacuna and degrades extracellular bone matrix components such as type I collagen (**a**). In thyroid (**b**), cathepsin K is also secreted into the extracellular space, the thyroid follicle lumen, to degrade Tg resulting in the liberation of T4. These actions, however, take place in pH neutral, oxidizing conditions

serves as the main Tg-storage compartment [11, 18, 26]. Tg, as the highly iodinated precursor of thyroid hormones, is extracellularly stored in a condensed, covalently cross-linked and thereby osmotically inert form in the extracellular lumen of thyroid follicles [4, 17]. Covalently cross-linked Tg forms large globules of 20–120 μm in diameter [18] that are impossible to be taken up by thyroid epithelial cells as an entity. Therefore, extracellular mechanisms of Tg solubilization preceding endocytosis of Tg by thyroid epithelial cells and its delivery to endo-lysosomes are required [3, 10, 11]. Hence, extracellular proteolysis was postulated as the mechanism of Tg solubilization for subsequent utilization of the prohormone by liberation of thyroid hormones [6, 7, 10, 11]. This led to the discovery that extracellular proteolysis of Tg is mediated by secreted endo-lysosomal cysteine proteinases, a regulated process that results in the rapid liberation of thyroxine (T4), involving extra-, peri- and intracellular stations that Tg passes through when being transported from the very interior of thyroid follicles to endo-lysosomes [1–3, 10]. Following the limited extracellular proteolytic Tg solubilization steps, fragments of the precursor molecule Tg are observed to become internalized by thyroid epithelial cells for complete degradation and liberation of both T4 and triiodothyronine (T3) within endo-lysosomes [10]. From these observations, we concluded that endo-lysosomal cysteine proteinases are involved in extracellular proteolysis of Tg under physiological conditions before continuing their actions on the pre-processed substrate within endo-lysosomes.

In more general terms, the cysteine cathepsins B and K, in particular, have qualified during the past years as potential competitors of matrix metalloproteinases, which were usually considered first in regard to the cleavage of substrates in an extracellular environment. Cathepsin B seems to be of utmost importance for proteolytic activation of pericellular protease cascades and for degradation of extracellular matrix constituents in cancer conditions [27–29]. Cathepsin K, on the other hand, was one of the first cysteine cathepsins for which an important and extracellular function in the maintenance of human physiology was proved, as it is responsible for type I collagen degradation during bone matrix removal [30, 31] and because it mediates Tg processing in the thyroid.

It is of note that the task of degrading Tg in the secluded extracellular lumen of thyroid follicles differs from the task of degrading the proteinaceous bone matrix type I collagen in the resorption lacuna formed transiently by osteoclasts tightly adhering to calcified bone (see Fig. 1). While the resorption lacuna built by activated osteoclasts provides an acidified and reducing environment for proteolytic cleavage, the thyroid follicle lumen is more demanding in that it exhibits neutral and oxidizing conditions. Besides such distinct functions mediated by versatile enzymes like cathepsin K of osteoclasts or thyrocytes, similarities must exist in the trafficking pathways that explain delivery of endo-lysosomal cysteine cathepsins towards extracellular locations in both bone and thyroid (see below).

Cathepsin K in Thyroid Follicles

The cysteine proteases cathepsins B, C, H, K, L, S and V have been detected to be present in the thyroid [4, 6, 11, 32, 33]. Cathepsin K sequences, like those of the other cysteine cathepsins, are reported to be highly homologous across mammalian species [21, 22]. Based on this assumption, we had used RT-PCR approaches to clone a cDNA coding for the cathepsin K protein using mRNA derived from primary cultures of porcine thyrocytes [2]. The porcine cathepsin K cDNA was then compared with that of various species, revealing highest homologies of porcine cathepsin K to the human, monkey and rabbit enzymes with overall sequence identities of 93–95%. Homologies with cDNAs encoding rat and mouse cathepsin K were 87–88%, and porcine cathepsin K cDNA was only 56% identical to chicken cathepsin K [2]. The cDNA of porcine cathepsin K coded for a 37-kDa proform which was predicted to yield the 35-kDa mature enzyme upon proteolytic processing of the precursor within late endosomes. As expected, both the proform and mature cathepsin K were detected by immunoblot in homogenates of porcine thyrocytes. At the subcellular level, the mature form of cathepsin K was found to be enriched in lysosomal fractions prepared by differential centrifugation, demonstrating the presence of mature cathepsin K in lysosomes.

This description of thyroid cathepsin K belonged to the first of many to follow that verified cathepsin K's expression in epithelial cells, i.e., in cell types other than osteoclasts, at a time shortly after the crucial importance of cathepsin K for bone remodelling in mouse and man was discovered [21, 22, 30, 31, 34]. Meanwhile, cathepsin K has also been detected in various other tissues including colon, heart, lung, placenta, skeletal muscle, small intestine and most recently in brain [2, 21, 22, 35–38]. Therefore, it is not surprising that cathepsin K has now been demonstrated to not only participate in bone turnover and thyroid functions, but this protease is also believed to be involved in a variety of physiological processes and pathological conditions such as rheumatoid arthritis, osteoarthritis, atherosclerosis, reproduction, development of skeletal muscles and heart tissue, lung inflammation, obesity, cancer and immune system, to name only some of the best studied examples [22, 32, 34, 36, 39].

Immunolabeling of cultured porcine thyrocytes with cathepsin K-specific antibodies revealed the abundance of this protease in vesicular structures [2]. Such cathepsin K-positive vesicles were detected in the perinuclear region of thyroid epithelial cells, and they were identified as

endo-lysosomes by colocalization with cathepsin D, an excellent marker of endo-lysosomal compartments in the thyroid [1, 2, 10]. Of particular interest was the observation of cathepsin K's secretion into the extracellular space of cultured porcine thyroid epithelial cells and the detection of cathepsin K-like activity at the surface of thyroid epithelial cells by means of enzyme cytochemistry [2]. Hence, cathepsin K was secreted from thyrocytes in proteolytically active form, and it re-associates with plasma membrane constituents for its concentration in the direct peri-cellular space at the apical pole of thyrocytes, where it has been shown to be active in Tg processing at slightly acidic and even at neutral pH conditions [1–3]. Likewise, cathepsins B and L often colocalize with cathepsin K and exhibit similar Tg-processing skills in the sense that cleavage patterns are comparable, whereas cathepsin S as another closely related enzyme was the most active of the four in cleaving Tg in oxidizing environments [3]. Both cathepsins K and S are able to cleave Tg as exopeptidases for direct thyroid hormone liberation from Tg fragments derived from preceding processing steps as endopeptidases [2, 3].

Moreover, secretion of active forms of cathepsin K from a variety of cell types other than osteoclasts or thyrocytes has been described in numerous reports from our own and other laboratories [21, 22, 32, 36], making it likely that many more extracellular substrates of cathepsin K await their future discovery.

Species-Specific Differences in Thyroid Cysteine Cathepsin Distribution Patterns

Although there seem to be differences in the localization patterns of cathepsin K across various tissues and cell types, thyroid cathepsin K can be considered to act within endo-lysosomes as well as in the extracellular follicle lumen and in the pericellular space at the apical plasma membrane domain of thyroid epithelial cells of cattle, mouse, pig, rat [2, 3, 32, 40] and man (Fig. 2).

In thyrocytes, localization of cathepsin K and most other thyroid cysteine cathepsins has been observed mainly in vesicles of various sizes that are typically located close to the apical cell pole. Cathepsin S in the human thyroid, however, is an exception since it seems to often localize to distinct vesicle populations, whereas cathepsins B, K and L typically reside in identical vesicles [3].

When comparing the localization of cathepsin K in human and porcine thyroids [2], cathepsin K was detected

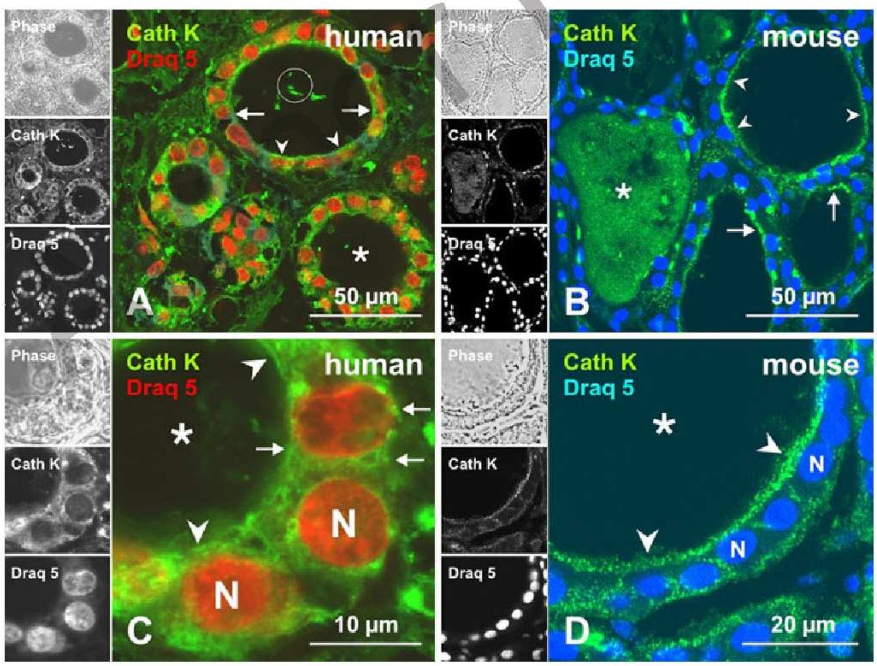

Fig. 2 Cathepsin K localization in human and mouse thyroid. The most intense staining for cathepsin K was found in thyrocytes, particularly within endo-lysosomes (*arrows*) and at the apical cell surface (*arrowheads*). Cathepsin K is also localized within the follicle lumen (**a**, *circle* and **b**, *asterisk*) where Tg is stored. Draq5 nuclear staining is depicted for human (**a**, **b**) and mouse thyroid (**c**, **d**). Merged fluorescence micrographs are shown in *false* colours, while *left*-hand panels depict phase contrast and single channel images of cathepsin K and Draq5 staining as indicated

more often within the extracellular follicle lumen of human thyroid (Fig. 2, asterisks and circle in a, c) where it was also associated in high amounts with the apical plasma membrane (arrowheads). Double-labelling of cathepsin K and Tg often revealed a colocalization of both proteins [2], once again supporting the notion that Tg is a natural substrate for cathepsin K. To investigate this in further depths, purified Tg was incubated in vitro with increasing amounts of cathepsin K, and results demonstrated that cathepsin K indeed mediated limited proteolysis of Tg under neutral pH conditions in oxidizing environments, thereby even resulting in the liberation of T4 [2, 3].

In mouse thyroid epithelial cells, cathepsin K was similarly localized within vesicles as well as in the follicle lumen (Fig. 2, b, d). Taken together with the observation that cathepsin K-deficient mice exhibit a mild hypothyroid phenotype [1], mouse thyroid cathepsin K must be considered an effective Tg-degrading protease similar to the human and porcine enzyme. Overall, vesicular localization of cathepsin K within thyrocytes (Fig. 2, arrows) was found to be similar in human, mouse and pig, whereas colocalization of cathepsin K and Tg in extracellular locations was more frequently observed in the human and mouse thyroid glands.

Finally, it should be noted that Tg proteolysis in the thyroid has been proposed to take place in different steps and by a combinatory action of cathepsin K and related cysteine cathepsins. Solubilization of extracellularly stored Tg from covalently cross-linked globules is believed to be mainly mediated by cathepsins B and L [1]. Soluble Tg is then thought to be subjected to limited proteolysis mediated by cathepsins K and L for Tg utilization, i.e., extracellular T4 liberation. Afterwards, partially degraded Tg re-enters thyroid epithelial cells by endocytosis and reaches endo-lysosomes for its complete degradation by the action of several lysosomal enzymes, i.e., cathepsins B, D, H, K, L, S, etc., which eventually also leads to T3 liberation [1].

Protease Trafficking Explains How Thyroid Cysteine Cathepsins can Conduct their Physiological Tasks Efficiently and in a Regulated Fashion

Targeting of proteolytic enzymes to endo-lysosomes is believed to be a directive, instructing a cell to retain proteases in their current positions. However, many endo-lysosomal proteases are secreted by a variety of cell types, which include individually acting cells like macrophages and collectively functioning cells such as epithelial cells [32, 40–42]. Secretion of endo-lysosomal proteases is not confined to pathological conditions such as cancer, but, as a regulated process, it can well be part of defence responses performed by macrophages to maintain healthy physiology of mammals. In addition, secretion of endo-lysosomal proteases also occurs to fulfil physiological tasks such as processing of prohormones or growth factors [11, 43].

In keeping with this notion, the onset of T4 liberation in the thyroid gland is achieved by rapid and regulated secretion of endo-lysosomal enzymes into the extracellular follicle lumen (Fig. 3) [12, 44]. T4 liberation by cysteine cathepsin-mediated proteolysis was observed to be transient and lasted for 4 h with a peak at 2 h in a culture model comprising primary porcine thyrocytes [44]. Similar observations clearly indicated that T4 liberation in situ is a fast process and belongs to the so-called short-term responses to TSH, the thyroid stimulating hormone (TSH) that regulates many aspects of thyroid epithelial cell biology. Accordingly, the proteases needed for rapid T4 liberation must be available before TSH hits the cells (see Fig. 3). During the initial TSH response (Fig. 3, steps 2–6), these proteases need to be transported in a specific manner to reach their substrate for extracellular cleavage [12, 44]. Indeed, cathepsin B, which was studied as a model cysteine cathepsin, is first transported to late endosomes and lysosomes for maturation before being sorted into retrograde trafficking vesicles directed towards the apical cell surface (Fig. 3, steps 4–6) [12, 44, 45]. Hence, this transport step is induced by TSH in a physiologically meaningful manner in that TSH triggers an increase of cytosolic calcium (Fig. 3, steps 4 and 5), which results in scattering and fusion of peripherally located vesicles with the apical plasma membrane within a couple of minutes upon stimulation. Prohormone processing and thyroid hormone liberation then results from Tg solubilization from its covalently cross-linked storage forms and subsequent limited proteolytic processing of Tg by cysteine cathepsins such as cathepsin K (Fig. 3, steps 7–9). The latter fact is also deduced from the observation that Tg processing by extracellular proteolysis can be efficiently inhibited by E64 [10], a broad-spectrum cysteine peptidase inhibitor. Thereafter, however, it is conceivable that there is a replenishment of the intracellular protease pool by either reinternalization from within the extracellular follicle lumen and/or by up-regulation of their de novo biosynthesis (Fig. 3, steps 10 and 11). Hence, the Tg-processing proteases might as well be regulated by TSH stimulation [11, 44, 46–48], as is the biosynthesis and cleavage of the prohormone itself. Long-term TSH-regulated stimulation of de novo protein biosynthesis of Tg is known to start after several hours (Fig. 3, steps 12–14).

Hence, regulation of extracellular proteolysis can occur at several levels. Cysteine cathepsin secretion can be brought about solely by their signal-triggered sorting into retrograde transport vesicles, which enable trafficking of the enzymes from late endosomes/lysosomes to the cell surface for subsequent secretory delivery to the extracellular space [10, 12, 32, 40, 44]. It is unclear, however, whether such vesicles are selectively loaded with specific proteases by putative, intrinsic sorting signals, or whether

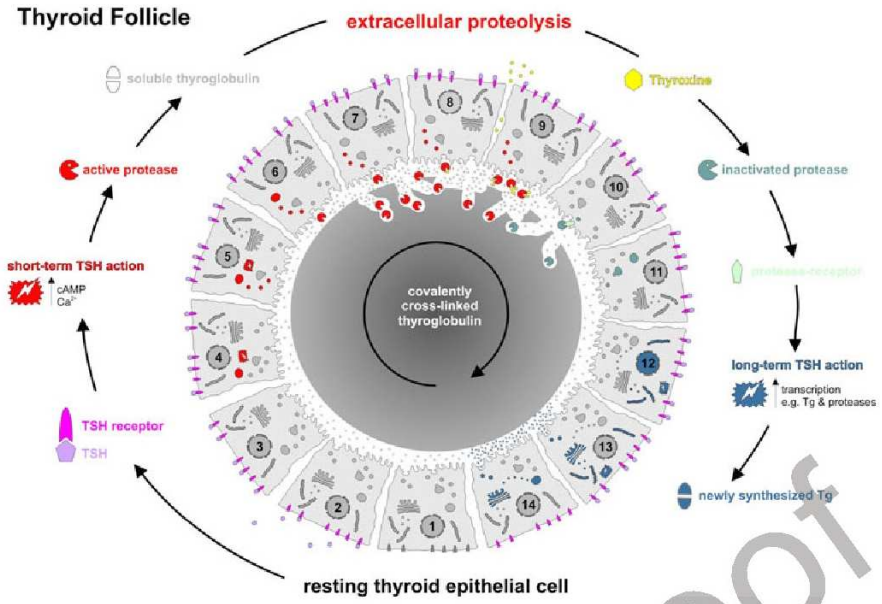

Fig. 3 Schematic diagram of Tg processing and thyroid hormone liberation by the action of proteases. The release of thyroid hormones from stored covalently cross-linked Tg in the extracellular lumen of thyroid follicles is a tightly regulated process, involving the activation of thyrocytes from their resting state (*step 1*) by TSH signalling via basolateral TSH receptors (*steps 2* and *3*). Active cathepsin K and other Tg-degrading endo-lysosomal proteases are subsequently transported to the apical plasma membrane by retrograde trafficking before being secreted into the extracellular space (*steps 4–6*). The proteases like the cysteine cathepsins B, K and L then degrade stored Tg for T4 liberation (*steps 7–9*). The binding of inactivated enzyme to apically located thyrocyte receptors might then lead to re-internalization of these proteases and termination of Tg proteolysis (*steps 10* and *11*). Tg is newly synthesized by long-term TSH-regulated stimulation (*steps 12–14*)

the vesicles contain an arbitrary mixture of proteases due to non-selective loading. In the thyroid, evidence for the latter proposal is accumulating because not all cysteine cathepsins behave the same, i.e., individual proteases may follow different transport logistics and kinetics.

In conclusion, inhibition of cathepsin K activity in the thyroid gland is hypothesized to affect Tg proteolysis at different locations and with variable efficacies. Because extracellular steps of cathepsin K-driven Tg proteolysis do not seem to be as effective as intracellular cleavages [3], inhibitors of cathepsin K would first and foremost inhibit endo-lysosomal Tg proteolysis by this protease. However, it has to be taken into consideration that thyroid epithelial cells belong to a category of one of the most transport-active cells known [11, 49], since they perform vivid transport of cargo along the secretory and endocytic routes while also being well known as transcytotically active cells. Hence, cysteine cathepsin inhibitors would have numerous possibilities not only to interfere with thyroid functions of the targeted proteases, but they may also find escape pathways bypassing delivery to endo-lysosomes when entering transcytotic vesicles, which would re-deliver the drugs into the blood circulation or shuttle them to the lymphatic system. Both blood and lymph vessels are extremely abundant in the thyroid gland [26].

Developmental Versus Short-Term Effects of Impaired Cathepsin K Activities: Thyroid-Specific Compensatory Mechanisms

Inspection of the thyroid phenotype of cathepsin K-deficient ($Ctsk^{-/-}$) mice revealed a number of alterations in both cysteine cathepsin expression levels and transport pathways [1].

In $Ctsk^{-/-}$ mice, the distribution of cathepsin B, typically present in vesicles of mouse thyroid epithelial cells, over the follicle lumen and associated with the apical plasma membrane, became altered such that its cell surface association was no longer observed in the thyroid gland of $Ctsk^{-/-}$ mice although the protein levels remained unchanged. This observation was also true for cathepsin L-deficient ($Ctsl^{-/-}$) as well as for $Ctsk^{-/-}/Ctsl^{-/-}$ double-deficient mice, indicating that deficiencies in cathepsins K and/or L resulted in the redistribution of binding partners of cathepsin B from the apical plasma membrane to other

cellular locations [1]. In wild-type (WT) mice, cathepsin L was usually detected in vesicles and in association with the apical plasma membrane of thyrocytes, but not prominently as a soluble enzyme distributed over the lumen of mouse thyroid follicles. This distribution changed in a similar direction as that of cathepsin B upon targeted gene ablation of *Ctsb*, *Ctsk* or both *Ctsb* and *Ctsk*. Again, the cell surface binding of cathepsin L was no longer observed, but the protease was now distributed over the follicle lumen of thyroid glands prepared from $Ctsb^{-/-}$, $Ctsk^{-/-}$ and $Ctsb^{-/-}/Ctsk^{-/-}$ mice. Cathepsin K itself was found to be localized within vesicles of mouse thyroid epithelial cells as well as in the follicle lumen. In cathepsin B-deficient animals, however, cathepsin K was no longer detectable in the lumen, but at the apical plasma membrane of thyrocytes. Furthermore, cathepsin K-positive vesicles appeared smaller in cathepsin B-deficient mice compared to those of WT. In cathepsin L-deficient mice, cathepsin K was again detected in the follicle lumen, but cathepsin K-containing vesicles appeared larger than in WT. Overall, these results suggested that deficiencies in cysteine proteases in mouse thyroid epithelial cells led to an extensive rearrangement of the endocytic system and to a redistribution of related enzymes. In particular, the changes of the proteolytic potential in the apical pericellular space of thyroid epithelial cells are proposed to bear important consequences on Tg proteolysis (see Fig. 3).

In line with this proposal, cathepsin K- and/or L-deficient mice suffered from a goiter-like enlargement of the thyroid gland, whereas significantly decreased blood serum levels of T4 were detected in $Ctsl^{-/-}$ and $Ctsk^{-/-}/Ctsl^{-/-}$ double-deficient mice only [1]. Thus, $Ctsl^{-/-}$ and $Ctsk^{-/-}/Ctsl^{-/-}$ animals are considered mildly hypothyroid. While these observations point to the prominent roles of all three cysteine cathepsins, i.e., cathepsins B, K and L, in Tg proteolysis, the detection of enhanced cathepsin L levels in thyroid tissue from $Ctsk^{-/-}$ mice and, vice versa, those of cathepsin K in thyroid glands of $Ctsl^{-/-}$animals proved that both enzymes were able to compensate for one another with respect to their functions in a tissue-specific manner. Keratinocytes and fibroblasts of the identical mouse strains exhibited alterations in cysteine cathepsin protein levels; however, those did not reproduce the changes observed in the thyroid gland [43, 50].

Moreover, thyroid epithelial cells of cathepsin B-, K-, L-, B/K- and K/L-deficient mice compared to WT displayed shape changes from prismatic to cubic and flattened appearances, which are considered morphological indications of thyrocyte activity. In euthyroid vertebrates, a need for thyroid hormones is signalled by a rise in TSH levels in the blood as a result of negative feedback-regulation [51, 52]. Under conditions of acute TSH stimulation, appearance of thyroid epithelial cells changes from cubic to prismatic, which is reminiscent of enhanced thyrocyte activity and therefore enhanced Tg turnover [11]. Hence, flattening of epithelial cells in conditions of cysteine cathepsin deficiencies indicated a reduction in thyroid functional activity [1, 10, 11], a conclusion that was shared with observations from mouse models in which the animals suffered from impaired thyroid hormone transport mechanisms and thereby severe impairments of CNS development [53].

Hence, while further investigations are required in order to fully explain the thyroid phenotype of cathepsin K-deficient mice, subtle changes in thyroid parameters and morphological signs of hypothyroidism may skip attention when only mild effects are expected, such as those that might arise when cathepsin K inhibitors are given as anti-osteoporotic treatment. It is hard to predict what the precise outcomes of transient or even long-term cathepsin K activity impairment by inhibitors could be, because even Pycnodysostosis patients are not reported (to our knowledge) to exhibit severe thyroid phenotypes. Considering the wide range of thyroid hormone actions influencing almost all tissues and major metabolic processes, it seems, however, wise to have a closer look on potential side-effects which may be induced by cathepsin K-specific inhibition, and which could include thyroid-mediated effects as well.

Perspectives: Cathepsin K Inhibitors and Possible Consequences for Thyroid Target Tissues

Cathepsin K has not only been reported to be expressed in a variety of tissues other than bone and thyroid, but importantly, it is also involved in crucial physiological and pathological conditions. Furthermore, it has to be taken into consideration that thyroid hormones affect several target tissues, such as bone, brain, cardiovascular and immune systems, skin and the gastro-intestinal tract. Hence, although cathepsin K is a promising therapeutic target for osteoporosis treatment, it is important to consider possible consequences of cathepsin K inhibition on various organ systems, not only due to direct tasks of this protease in specific tissues but also indirectly, via disturbances of its thyroid function. Thus, further investigations on the functions of cathepsin K in general and in tissues affected by thyroid hormones will allow a better understanding of safe medical applications of cathepsin inhibitors.

Odanacatib is to date the most promising cathepsin K inhibitor and is currently in phase III clinical trials [54, 55]. Previous work on various cathepsin K inhibitors such as L-006235 and Balicatib revealed off-target effects, such as increased activities of cathepsin K related enzymes like cathepsins B and L in liver, kidney and spleen but also in the central nervous system (CNS) [56]. In contrast,

Odanacatib exhibits high selectivity for cathepsin K and only minimal off-target effects [57]. Clinical studies with odanacatib in comparison to placebo demonstrated reduced serum concentrations of the bone resorption marker C-terminal telopeptide of type I collagen (CTX) and an increase of bone mineral density (BMD) in the lumbar spine and hips to extents depending on dosage and administration of the drug, indicating that osteoporosis treatment with this cathepsin K inhibitor is based on its osteoclast-selective, anti-resorptive effects [58, 59]. Adverse reactions to Odanacatib were rare and similar to those observed in patients treated with placebo. Importantly, scleroderma-like cutaneous lesions, which were reported in response to first-generation cathepsin K inhibitors, were not detectable upon Odanacatib treatment [59]. Phase III clinical studies are currently conducted in which postmenopausal women are treated with Odanacatib for assessment of anti-fracture efficacy of the drug [59].

A major advantage of cathepsin K inhibitor treatment over commonly used bisphosphonates in osteoporosis therapy is considered in the drug-mediated suppression of osteoclast function while preserving viability of the same cells [59]. In turn, this might also affect signalling between osteoclasts and osteoblasts for maintenance of normal bone formation during suppressed bone resorption, whereas biphosphonates are known to enhance osteoclast apoptosis [59–62]. It has been pointed out, however, that long-term safety and implications of inhibition of cathepsin K activity are unknown at this point [59, 63]. As mentioned already above, it is crucially important to keep in mind that cathepsin K is not only expressed and active in the bone, but also in other tissues, including the thyroid. Thus, inhibition of cathepsin K might lead to various consequences, beneficial, neutral or negative, not only for the thyroid but also for thyroid hormone target tissues such as those discussed below, i.e., bone, CNS, skin and intestine.

Bone as a TH Target Tissue

Altered thyroid status has well-known and profound consequences for skeletal development and growth as well as for adult bone maintenance [64, 65]. Hypothyroidism is known to result in delayed bone age, growth arrest and short stature when occurring in childhood [66, 67]. Reduced bone turnover and increased fracture risk are the features of hypothyroidism during adulthood [68, 69]. Hyperthyroidism during childhood, on the other hand, causes advanced bone age, accelerated growth, premature fusion of growth plates, short stature and craniosynostosis [64, 70]. Consequences of hyperthyroidism during adulthood are high bone turnover, osteoporosis and increased fracture risk [65, 71–73].

Cathepsin K and related cysteine cathepsins play a role not only in bone remodelling but also in ECM remodelling in general. Such tasks may be brought about directly by processing of ECM constituents and indirectly by initiating proteolytic cascades in cancer invasion and metastasis [28, 29, 32, 39, 54, 74]. Undoubtedly, cathepsin K is one of the cysteine peptidases with such functions in cancer [54, 74–77]. However, ECM degradation besides type I collagen processing in bone turnover has also been shown to occur in physiology for tissue remodelling during wound healing of the skin or the gastro-intestinal tract and to be mediated by a number of different cysteine cathepsins, namely cathepsins B, D and L [22, 29, 32, 78].

Moreover, tissue remodelling processes depend to quite some extent on healthy thyroid status [26, 63, 64, 66, 78, 79]. Vice versa, hyperthyroid conditions may be considered a negative factor in bone disease as a result of tumour cells metastasizing to bone because administration of high doses of thyroid hormones has been reported to be associated with low bone density [80, 81]. In line with this proposal, a group of male patients with differentiated thyroid cancer (DTC) that were on suppressive levothyroxine therapy were studied in comparison to a group of healthy euthyroid men with respect to serum levels of cathepsin K and a number of other markers of bone metabolism. The results indicated that cathepsin K was increased in the patients on suppressive levothyroxine therapy. Although cathepsin K levels were decreased in patients with older age, the generally high levels of cathepsin K were considered likely to contribute to accelerated bone degradation in these patients suffering from DTC on suppressive levothyroxine therapy [80]. In turn, it could even be hypothesized that cathepsin K inhibitor treatment may have beneficial effects on both symptoms, osteoporosis and bone disease as well as on hyperthyroidism caused by different triggers including autoimmune diseases or thyroid cancer, because the cathepsin K inhibitors would affect the enzyme's activities in all tissues involved, bone, thyroid, immune system and circulating cathepsin K in the blood, thereby possibly resulting in the patient's relief from more than one complication of the respective disease.

CNS as a TH Target Tissue

Numerous studies demonstrated the importance of thyroid hormones for proper brain development and function [53, 82, 83]. In rodents, deprivation of thyroid hormones during development affects various processes such as glial cell proliferation, neuronal differentiation, outgrowth and migration, myelination and synaptogenesis [82, 83]. In humans, untreated congenital hypothyroidism is often associated with intellectual deficits, abnormal balance and fine motor skills as well as spasticity and deafness [84–86].

It is clear at this point that for thyroid hormone-mediated effects on brain development and cognitive functions all phases of life are important, and particularly early childhood is of main significance. Although most reports nowadays focus on severe developmental consequences of thyroid dysfunction, it has become increasingly clear that even during adulthood mood disturbances, cognitive impairments as well as other psychiatric symptoms can emerge due to non-optimal thyroid function [85]. It will therefore be important to closely monitor thyroid function parameters under cathepsin K inhibitor treatment, in order not to overlook mild and not immediately overt side-effects that are possible due to inhibition of thyroid cathepsin K functions at all stages of embryonic development, childhood and in adults.

Skin as a TH Target Tissue

Skin and hair are both well-known thyroid hormone target [87–90]. The hair cycle is under direct control of the endocrine system including thyroid hormones [87, 91]. In hyperthyroidism, skin changes including erythema, palmoplantar hyperhidrosis, acropathy and infiltrative dermopathy can occur. Furthermore, Graves' disease may be associated with generalized pruritus, chronic urticaria, *alopecia areata*, diffuse skin pigmentation and vitiligo. In contrast, hypothyroidism results in skin of dry and pasty appearance that is hypothermic, while the epidermis is hyperkeratotic and thin [91–93]. Moreover, hypothyroid states result in impaired wound healing since T3 is also important for wound healing [94]. Likewise, cysteine cathepsin B at least is involved in mediation of keratinocyte migration during wound healing [32, 78, 95]. Whether similar functions in type IV collagen processing during keratinocyte migration would be carried out by cathepsin K is unclear at present, but can be considered likely as also deducible from the side-effects that were triggered by a first-generation cathepsin K inhibitor. Balicatib has been demonstrated to cause incidences of skin rashes and scleroderma, a form of fibrosis, and it was therefore withdrawn from clinical trials of potential osteoporosis treatment [56].

Gastro-Intestinal Tract as a TH Target Tissue

Another tissue that is significantly influenced by thyroid hormones is the gastro-intestinal tract. It has been shown that T3 has a role in maturation and homeostasis of the intestinal epithelium [96, 97] and that it regulates proliferation of intestinal epithelial cells through the Wnt/β-catenin signalling pathway [98, 99]. Thyroid hormones seem to be important not only during intestinal development but also in the adult gastro-intestinal tract of mammals [100, 101]. Thyroid disturbances have various manifestations on the gastro-intestinal tract [102]. Hyperthyroidism may result in digestive symptoms, but persistent abdominal pain, intractable vomiting, weight loss and altered bowel habits are also designated as signs of apathetic hyperthyroidism [102]. Consequences of hypothyroidism on the gastro-intestinal tract seem to be multifactorial, with possible alterations in hormone receptors, neuromuscular disorders and even myopathy [102]. However, reduction of peristalsis is considered a main pathophysiological effect of hypothyroidism [103]. Furthermore, thyroid hormone deficiency may influence transepithelial flux transport by inhibiting chloride-carbonate-anion exchange with subsequent negative effects on intestinal motility [104].

Cathepsin K has been shown to be expressed in the gastro-intestinal mucosa of human embryos as well as in chronically inflamed gastric mucosa [21, 22]. The specific expression of cathepsin K in parietal cells of the intestine suggests its function in the proteolytic processing of factors and thereby in processes which are mediated by these cells, such as release of pepsinogen or proton pump activation [105]. Furthermore, in previous studies, we observed that cathepsin K was localized at the apical plasma membrane of human enterocytes, and it was also detected in the mucus of goblet cells [106]. This localization suggested secretion and extracellular functions of cathepsin K in intact intestine of man. Additional experiments, performed in mouse, suggested a role of cathepsin K in ECM remodelling since we observed higher protein levels of the ECM-constituent type IV collagen in the intestine of $Ctsk^{-/-}$ mice in comparison to the WT animals (Fig. 4). Higher levels of type IV collagen were found for both the small intestine (duodenum, jejunum, ileum) and large intestine (colon) of $Ctsk^{-/-}$ mice. Moreover, we have observed that cathepsin K-deficient mice have longer small intestines when compared to WT animals, which was another indication that cathepsin K might exhibit a supportive role on gastro-intestinal structure due to its ECM remodelling tasks.

Another important scenario becomes conceivable from the notion of secreted cathepsin K detectable in the intestinal mucosa of patients [32, 106], in that secreted cathepsin K—if secreted in the proteolytically active form and stable in the lumen of the gastro-intestinal tract—could interact with cathepsin K inhibitors before they are absorbed through the intestinal wall to reach the blood circulation. Thus, cathepsin K in the lumen of the gastro-intestinal tract may counterbalance the beneficial effects of orally administered cathepsin K inhibitors and may even lead to the requirements of higher dosages, which then bears higher risks of complications caused by off-target effects. The cathepsin K-deficient mouse model [1, 31] provides a useful tool in our opinion to study these possible aspects in further detail on the cellular and molecular levels.

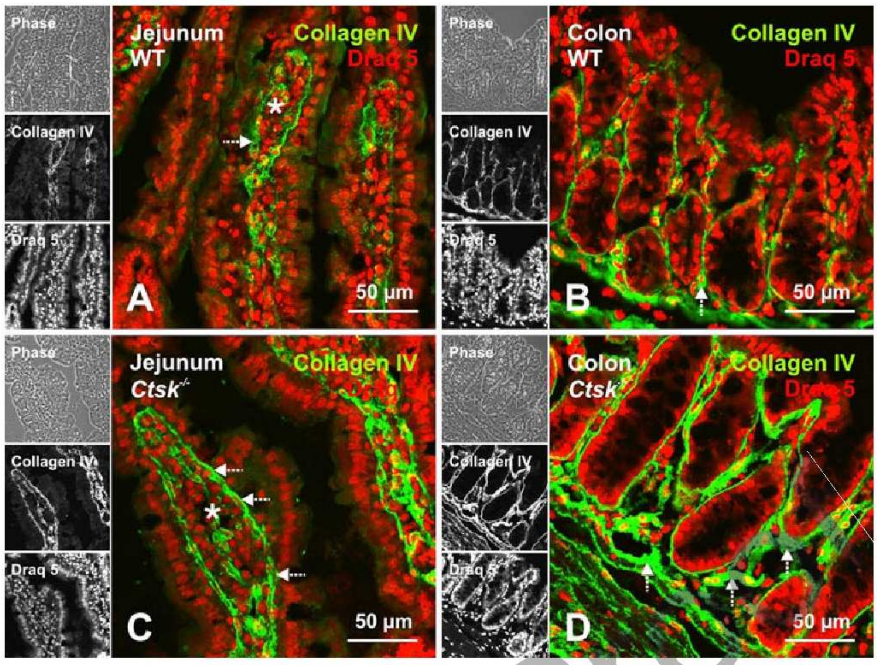

Fig. 4 Cathepsin K-deficient mice exhibit higher *collagen IV* levels in the intestine in comparison to wild type animals. The gastro-intestinal tract is a major thyroid hormone target tissue. Confocal laser scanning micrographs of immunofluorescence labelling of collagen IV on cryosections prepared from the intestine of wild type (**a**, **b**) and cathepsin K-deficient mice (**c**, **d**). Note the presence of collagen IV in the small (**a** and **c**) and large intestine (**b** and **d**). Cathepsin K-deficient mice demonstrated more intense collagen IV staining compared to wild type mice, indicating higher expression of collagen IV deposited in the lamina propria of villi (*asterisks*) and in the basal lamina (*broken arrows*) in the absence of cathepsin K. Draq5 nuclear staining is shown. Merged fluorescence micrographs are shown in *false* colours, while *left*-hand panels depict phase contrast and single channel images of collagen IV and Draq5 staining as indicated

Conclusions

In summary, further investigations are clearly required to gain more detailed information about the multiple functions that cathepsin K exhibits in a variety of organ systems. Special attention must be given to the cross-talk of the tissues that might be affected by cathepsin K inhibitor treatment of osteoporosis or osteoarthritis. While beneficial effects are obvious, milder side-effects may be less overt but may carry important consequences in the long run. For instance, long-term inhibition could be required to treat bone phenotypes caused by increased cathepsin K activities. Moreover, such prolonged exposure of other cathepsin K-depending organs to inhibitory drugs may lead to impairments that become noticeable only after lag-periods of several months. Especially when the endocrine system involvement is discussed, the many targets of thyroid hormones must be inspected for potential off-target effects. Therefore, seen from the focus of this review and in general terms, it may be proposed that cathepsin K inhibitors may reduce the risks of osteoporosis and bone disease both directly and indirectly via suppressing thyroid functions; a hypothesis that may account mainly in hyperthyroidism but may not be so prevalent in the more frequent conditions of hypothyroidism. Whether cathepsin K-inhibitor treatment is safe in the long run or whether the drugs would influence endocrine disorders remains elusive up to now, but will be an engaging and worthwhile future field of investigations.

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Conflicts of interest The authors have declared that no conflict of interest exists.

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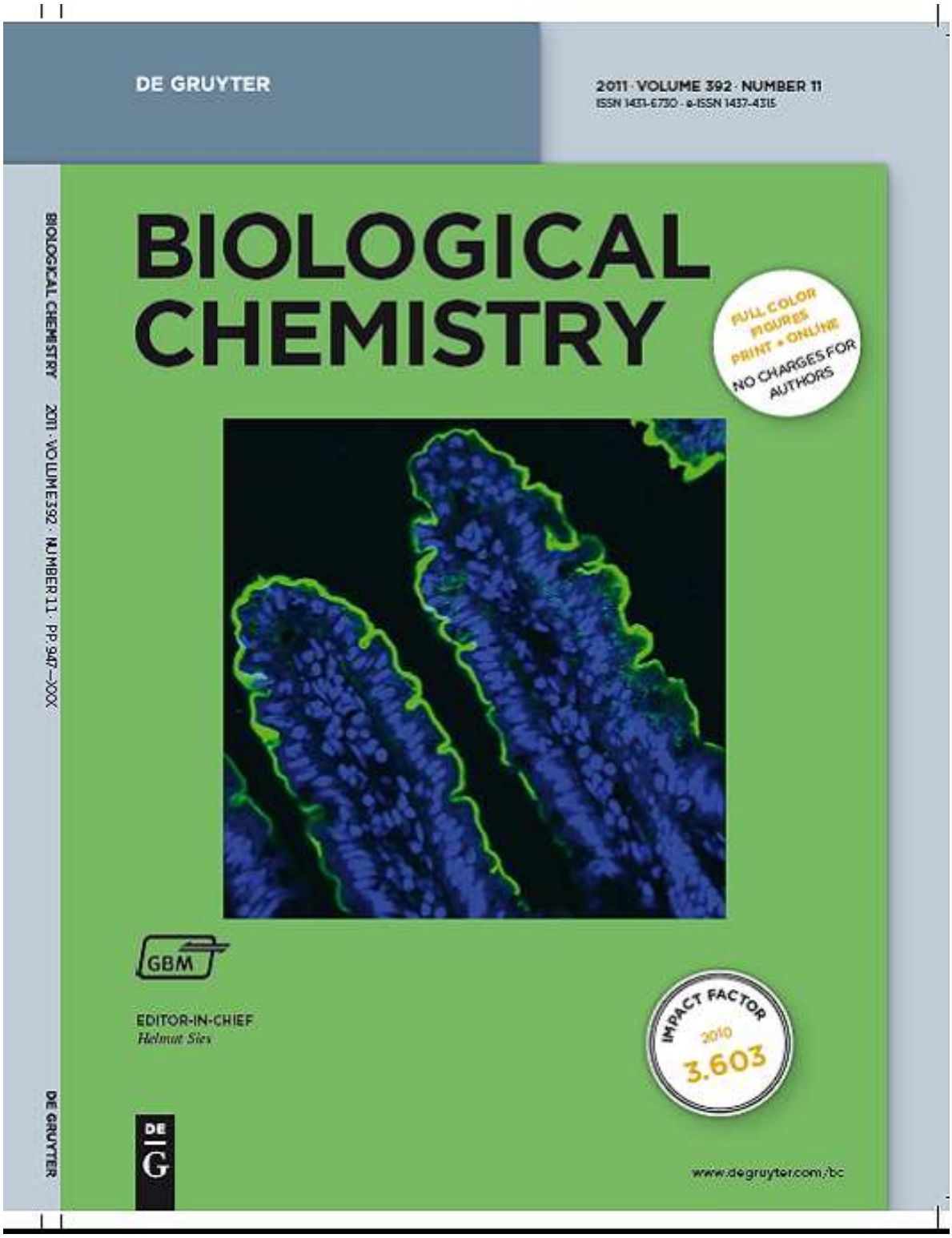
4.3 Intestine-specific expression of cathepsin B-EGFP



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Studies of intestinal morphology and cathepsin B expression in a transgenic mouse aiming at intestine-specific expression of Cath B-EGFP

Maria Arampatzidou[1], Kristina Mayer[1], Maria E. Iolyeva[1], Seblewongel Gebre Asrat[1], Mirunalini Ravichandran[1], Thomas Günther[2], Roland Schüle[2], Thomas Reinheckel[3] and Klaudia Brix[1,*]

[1] School of Engineering and Science, Research Center MOLIFE – Molecular Life Science, Jacobs University Bremen, Campus Ring 6, D-28759 Bremen, Germany
[2] Urologische Klinik/Frauenklinik und Zentrale Klinische Forschung, Klinikum der Universität Freiburg, Breisacherstrasse 66, D-79106 Freiburg, Germany
[3] Institut für Molekulare Medizin und Zellforschung, Albert-Ludwigs-Universität Freiburg, Stefan-Meier-Strasse 17, D-79104 Freiburg, Germany

* Corresponding author
e-mail: k.brix@jacobs-university.de

Abstract

Cathepsin B has been shown to not only reside within endo-lysosomes of intestinal epithelial cells, but it was also secreted into the extracellular space of intestinal mucosa in physiological and pathological conditions. In an effort to further investigate the function of this protease in the intestine, we generated a transgenic mouse model that would enable us to visualize the localization of cathepsin B *in vivo*. Previously we showed that the A33-antigen promoter could be successfully used *in vitro* in order to express cathepsin B-green fluorescent protein chimeras in cells that co-expressed the intestine-specific transcription factor Cdx1. In this study an analog approach was used to express chimeric cathepsin B specifically in the intestine of transgenic animals. No overt phenotype was observed for the transgenic mice that reproduced normally. Biochemical and morphological studies confirmed that the overall intestinal phenotype including the structure and polarity of this tissue as well as cell numbers and differentiation states were not altered in the A33-CathB-EGFP mice when compared to wild type animals. However, transgenic expression of chimeric cathepsin B could not be visualized because it was not translated *in situ* although the transgene was maintained over several generations.

Keywords: A33-antigen promoter; cysteine cathepsins; enhanced green fluorescent protein; intestine.

Introduction

In the gastrointestinal tract, like in any other tissue, a complex proteolytic network is responsible for controlling and regulating tissue homeostasis. Cathepsins are part of this network and are classified as aspartic, cysteine, and serine proteases. Elucidating the roles of these enzymes is of crucial importance since cathepsins are known to exhibit diverse functions in human physiology but also under pathological conditions, such as cancer (Riese and Chapman, 2000; Turk et al., 2001; Friedrichs et al., 2003; Buth et al., 2007; Vasiljeva et al., 2007; Brix et al., 2008). There are 11 cysteine cathepsins known to be encoded by the human genome whereas rodents express a larger group of these enzymes due to additional cysteine cathepsin family members that are placenta-specific and not found in other species (Deussing et al., 2002; Sol-Church et al., 2002; Mason, 2008). Some of these proteolytic enzymes, such as cathepsin B, are ubiquitously expressed, whereas others are considered to be cell- or tissue-specific in their tasks (Brix et al., 2008; Reiser et al., 2010; Dauth et al., 2011). Because of their functional diversity, cysteine cathepsins have been viewed as promising targets for the treatment of various disorders, including neurodegenerative diseases, osteoporosis and a variety of malignancies (Mohamed and Sloane, 2006; Turk, 2006; Bromme and Lecaille, 2009; Arampatzidou et al., 2011). Among them, cathepsin B gained significant attention since it is not only an endopeptidase, like most of the cysteine cathepsins, but it can also act as an exopeptidase (Musil et al., 1991; Mort and Buttle, 1997; Nagler et al., 1997; Kos et al., 2005; Brix et al., 2008). In the intestine, cathepsin B has been proposed to have a direct role in collagen degradation for extracellular matrix (ECM) remodeling (Vreemann et al., 2009), while it has also been identified as an important player during inflammatory bowel disease (Menzel et al., 2006). In intestinal epithelial cells, cathepsin B has been predominantly localized within endo-lysosomes (Mayer et al., 2006; Arampatzidou et al., 2011), whereas other cysteine proteases such as cathepsins K and L were found in association with the apical plasma membrane (Mayer et al., 2006) and affected the structural integrity of the intestine by interfering with the constitution of the basal lamina (Dauth et al., 2011). These observations suggested different trafficking routes for the various cathepsins within the cells of the intestinal mucosa that could also imply different functions for each of these enzymes.

Therefore, we wanted to study the transport pathway of cathepsin B by using enhanced green fluorescent protein (EGFP) tagging in an intestine-specific setting. For this purpose, we chose the A33-antigen promoter since the expression of genes under control of this promoter depends on the presence of the intestine-specific transcription factor Cdx1 (Johnstone et al., 2002). The A33 antigen is a transmembrane

protein that is expressed only in the intestine and is specifically localized at the basolateral membrane of intestinal epithelial cells of all lineages (Johnstone et al., 2000). The A33-antigen promoter contains positive *cis*-regulatory elements, including caudal-related homeobox (Cdx1) binding sites. Cdx1 is an intestine-specific transcription factor which is crucial for A33-antigen promoter activation and could thereby induce expression of the chimeric protein cathepsin B-EGFP (Johnstone et al., 2000). By proof-of-principle studies we confirmed that the A33-antigen promoter can serve as a tool for induction of Cdx1-dependent CathB-EGFP expression *in vitro* (Mayer et al., 2008). In the present study, we used a similar approach for expressing CathB-EGFP in the intestine of a transgenic mouse model that was expected to enable us to elucidate and directly visualize the function and trafficking of cathepsin B *in vivo*.

Results

Generation of transgenic mice expressing cathepsin B-EGFP under the control of the A33-antigen promoter

A transgenic mouse model was established in order to be able to study the trafficking and function of cathepsin B *in situ*. These transgenic animals were expected to express the chimeric protein cathepsin B-EGFP under the control of the A33-antigen promoter, using the principle of intestine-specific expression of the A33 antigen (Mayer et al., 2008). C57BL/6NCrl mice were used for this study and the linearized A33-CathB-EGFP construct was microinjected into the male pronucleus of fertilized oocytes. After integration of transgene was proven to be successful, one transgenic founder was used for generation of transgenic offspring which were comparable to wild type littermate controls with respect to weight, size and breeding behavior. In addition, A33-CathB-EGFP transgenic mice appeared at the expected Mendelian frequency. Tail biopsies of the transgenic animals were analyzed in order to examine the incorporation of the transgene by using semi-quantitative polymerase chain reaction (PCR) analysis. Different amounts of transgene were detected in the A33-CathB-EGFP mice of different generations (Figure 1A) but the transgene was kept over the three generations analyzed (Figure 1B).

Expression of A33-CathB-EGFP transgene

The next step was to check the expression of the A33-CathB-EGFP transgene for presence in the intestine of transgenic mice. For this purpose, intestinal tissue was obtained from both transgenic and wild type mice and was divided into duodenum, jejunum, ileum, and colon. Liver was also obtained since it served as negative control due to the absence of Cdx1 as an A33-inducing factor. Total RNA was isolated from all different segments and liver and was used for RT-PCR. It was found that the A33-CathB-EGFP mice did not express the transgene in any of the intestinal parts (Figure 2, upper panel) while CHO-K1 cells co-transfected with pCdx1-DsRed-Express and pA33-CathB-EGFP gave the expected band at 1606 bp. These data suggested that there was possibly a problem at the transcriptional level that resulted in a lack of expression of the transgene in the A33-CathB-EGFP mice. We then checked the expression of the endogenous mouse cathepsin B and no difference was observed between wild type and transgenic mice (Figure 2, middle panel), showing that integration of the A33-CathB-EGFP construct did not influence expression of the endogenous protease.

A33-CathB-EGFP mice do not translate the chimeric protein cathepsin B-EGFP in their intestine

Immunoblotting experiments with a GFP-specific antibody were performed in order to confirm the lack of cathepsin

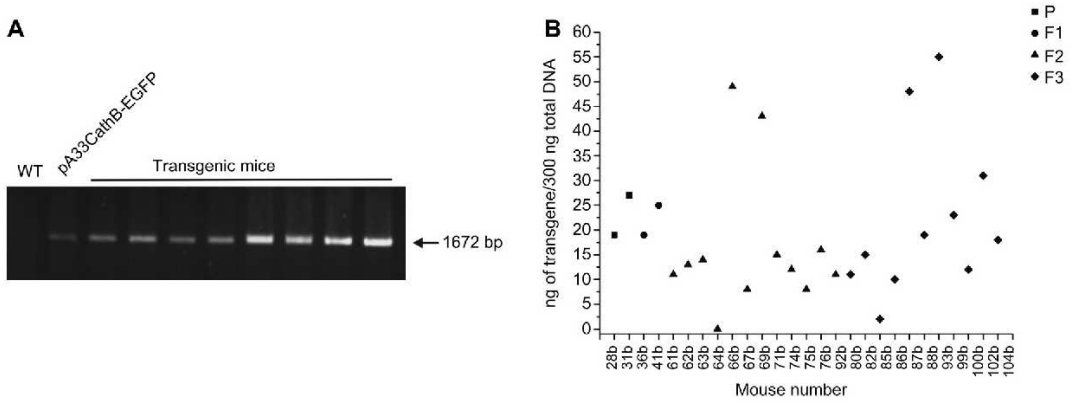

Figure 1 Genotyping of the A33-CathB-EGFP transgenic mice.
(A) Semi-quantitative PCR analysis using a transgene-specific primer pair shows different amounts of transgene in the various A33-CathB-EGFP transgenic mice. No PCR product was detected for wild type mouse (WT) while the plasmid pA33-CathB-EGFP served as positive control. (B) Scattergram of the analyzed A33-CathB-EGFP mice indicating the amount of transgene through three generations. Note that the transgene was kept and present in comparable copy numbers over the different generations.

Figure 2 Expression of A33-CathB-EGFP transgene and endogenous cathepsin B.
Total RNA isolated from intestinal segments (D=duodenum, J=jejunum, I=ileum, C=colon) and liver (L) of wild type and transgenic mice was used for RT-PCR with two pairs of primers that can distinguish between chimeric rat cathepsin B-EGFP and endogenous mouse cathepsin B. CHO-K1 cells co-transfected with pCdx1-DsRed-Express and pA33-CathB-EGFP were used as positive control. By using the rat cathepsin B-specific primers, an RT-PCR product of 1606 bp was only detected for co-transfected CHO-K1 cells but not for the wild type or any of the transgenic mice. Mouse cathepsin B-specific primers gave the expected product of 67 bp in both wild type and transgenic mice but not in co-transfected CHO-K1 cells. The β-actin gene product was used as a control for RNA integrity.

B-EGFP also at the protein level. Intestine tissue extracts were prepared from wild type and transgenic mice, as well as cell lysates from non-transfected, single (pCdx1-DsRed-Express) or co-transfected (pCdx1-DsRed-Express and pA33-CathB-EGFP) CHO-K1 cells. The latter cell lysates served as positive controls of cathepsin B-EGFP expression under the control of the A33-antigen promoter. The band at approximately 60 kDa corresponding to the molecular mass of the chimeric protein was detected only in lysates of co-transfected CHO-K1 cells, thereby reproducing the results of our previous proof-of-principle experiments (Mayer et al., 2008). However, no signal corresponding to cathepsin B-EGFP was detected in the intestine tissue extracts prepared from both wild type and transgenic mice (Figure 3). These results confirmed that the chimeric protein was not translated in the intestine of A33-CathB-EGFP mice even though its coding sequence was integrated into the mouse genome and kept over three generations at well detectable levels.

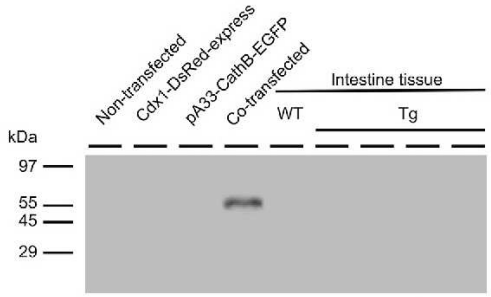

Figure 3 Transgenic mice do not express cathepsin B-EGFP chimeric protein.
Immunoblot analysis was performed using GFP-specific antibody on cell lysates and tissue extracts. Cell lysates were prepared from non-transfected, single or co-transfected CHO-K1 cells while intestine tissue from wild type and transgenic mice was used for preparation of tissue extracts. The band at approximately 60 kDa corresponding to the molecular mass of cathepsin B-EGFP chimeras was only detected in lysates of CHO-K1 cells co-transfected with pCdx1-DsRed-Express and pA33-CathB-EGFP plasmids, but not in non-transfected or singly transfected cells or wild type or transgenic mice. Molecular mass markers are given in the left margin.

Cryosections of small and large intestine and also liver were prepared from wild type and transgenic mice and were checked for green fluorescence by microscopic analysis. Green fluorescence signal was detected in a few cells (Figure 4, arrows) but the intensity was equal in the tissue of both wild type (Figure 4, A–E) and transgenic mice (Figure 4, F–L), suggesting that such signals were due to tissue auto-fluorescence and not to the presence of the chimeric protein cathepsin B-EGFP. In an effort to enhance a potentially weak signal of cathepsin B-EGFP, an antibody specific for GFP was used for immunolabeling of intestine tissue cryosections of transgenic and wild type control animals. In this case we used a secondary antibody coupled with red-emitting fluorophore in order to avoid cross-talk with the signal from the chimeric protein. However, no fluorescence signal was observed in the intestinal tissue of transgenic mice stained with anti-GFP antibodies (Figure 5, F–L). The same result was also observed in the wild type animals (Figure 5, A–E). These studies therefore confirmed the lack of translation of cathepsin B-EGFP chimeric protein in our transgenic mouse model.

A33-CathB-EGFP and wild type mice revealed comparable intestinal morphology

Morphological studies were performed in an effort to investigate whether the A33-CathB-EGFP mice had a generally altered intestinal phenotype due to integration of the transgene. We first stained the nuclei with DRAQ5™ (Biostatus Limited, Shepshed, Leicestershire, UK) in order to be able to compare the cell numbers of the different intestinal parts and also of the liver between transgenic and wild type mice. By this experiment we expected to also observe potential alterations in the intestinal structure. We observed that the

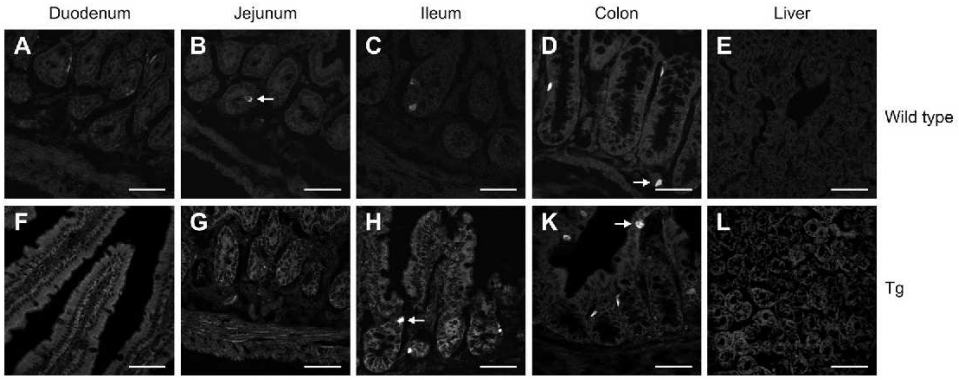

Figure 4 Lack of green fluorescence in the intestine of transgenic mice.
Cryosections of intestine segments and liver isolated from wild type (A–E) and transgenic mouse (F–L). Tissue cryosections were inspected by fluorescence microscopy under blue light excitation required to directly detect the introduced EGFP tag without any prior immunolabeling. Liver served as negative control. Green fluorescence signals were detected in a few cells (arrows) but these were of equal intensities for both wild type and transgenic mice, indicating that such fluorescence signals were due to tissue auto-fluorescence. Bars, 50 μm.

cell numbers found in the intestine and liver of transgenic mice were comparable to those found in the control animals (Figure 6). Moreover, the structure of the intestine did not show any alterations in the A33-CathB-EGFP mice, implying that integration of the transgene did not result in any developmental changes during tissue morphogenesis (Figure 6, phase contrast pictures). In addition, an antibody that can detect both the endogenous (mouse) and the chimeric (rat) cathepsin B was used in order to check whether the localization and expression pattern of the endogenous protein was altered in the transgenic mice. However, the amounts and localization of endogenous cathepsin B were comparable between wild type (Figure 7, A–E) and transgenic (Figure 7, F–L) animals.

The expression pattern and localization of the brush border enzyme aminopeptidase N (APN) was also analyzed. APN is an integral plasma membrane protease with a broad substrate specificity that serves as an apical plasma membrane marker in the small intestine (Mina-Osorio, 2008). The reason we wanted to study such markers was to check whether the polarity of the intestinal epithelium was affected in the transgenic mouse model. Cryosections of intestine and liver tissue were used for immunofluorescence analysis and confocal laser scanning micrographs prepared from transgenic and wild type mice revealed a steady expression of APN (green color) without changes in the distribution of this protein in both transgenic (Figure 8, F–L) and wild type (Figure 8, A–E) mice. Note that localization of APN at the apical plasma membrane in the small intestine (Figure 8, A–C and F–H) is replaced by basolateral plasma membrane localization in the large intestine (Figure 8, D and K). The results from both the small and large intestine suggested that the overall structure and polarity of this tissue was not affected in A33-CathB-EGFP mice.

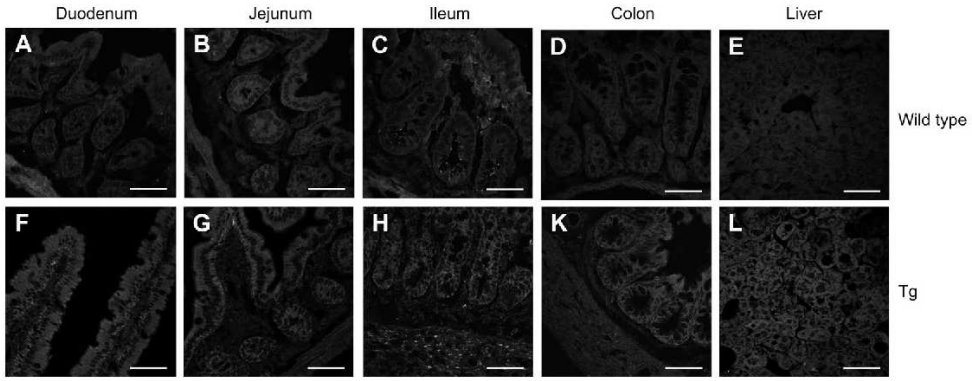

Figure 5 Enhancement of GFP signal by immunolabeling.
In order to enhance a potentially weak green fluorescence emitted by CathB-EGFP chimeras, tissue cryosections from wild type (A–E) and transgenic mice (F–L) were immunolabeled with GFP-specific and red fluorophore-conjugated secondary antibodies. No specific red fluorescence was detected, again confirming lack of cathepsin B-EGFP protein. Bars, 50 μm.

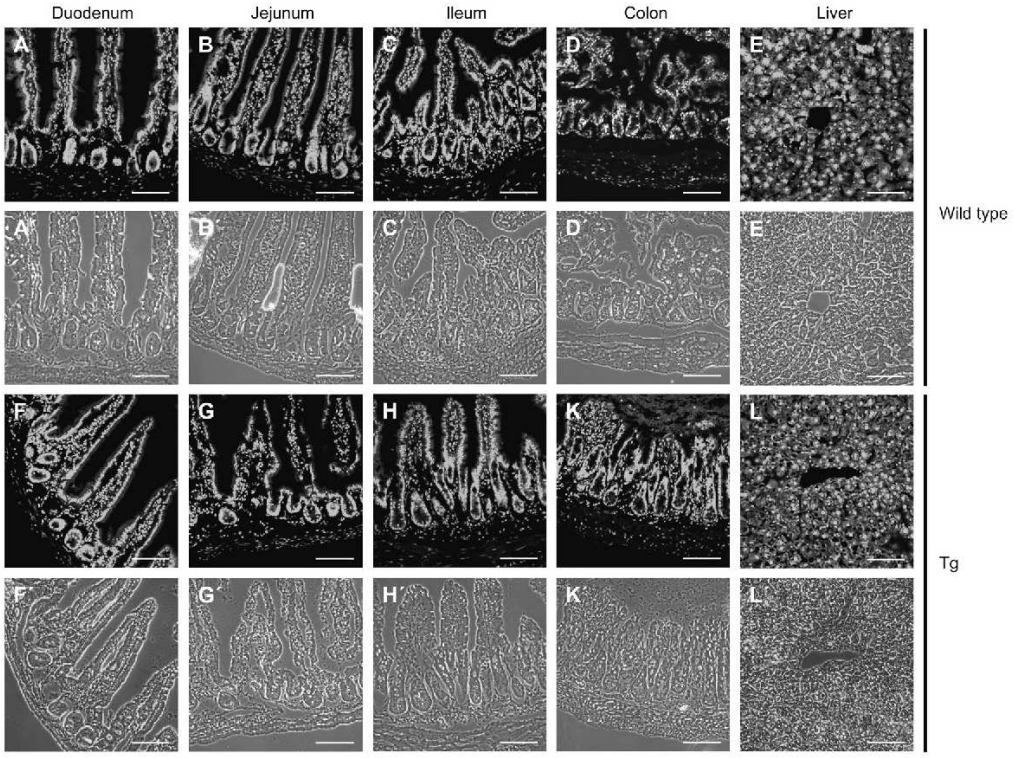

Figure 6 Comparison of intestinal morphology by nuclei staining.
The cell numbers of the different intestinal segments and the liver were analyzed by fluorescence microscopy after staining with the nuclear marker DRAQ5™. Corresponding phase contrast micrographs are also shown. No morphological alterations of intestine or liver tissue were observed in the transgenic animals (F–L) when compared to the wild type controls (A–E). Bars, 50 μm.

Expression and localization of Cdx1 and A33 antigen in the intestine of A33-CathB-EGFP mice

In an attempt to identify reasons for the non-expression of cathepsin B-EGFP chimeric protein in the intestine of A33-CathB-EGFP mice, we next wanted to check whether a general problem with the regulation of the A33-antigen promoter occurred upon transgene incorporation. For this purpose, we focused our studies on the intestine-specific transcription factor Cdx1 that is crucial for A33-antigen pro-

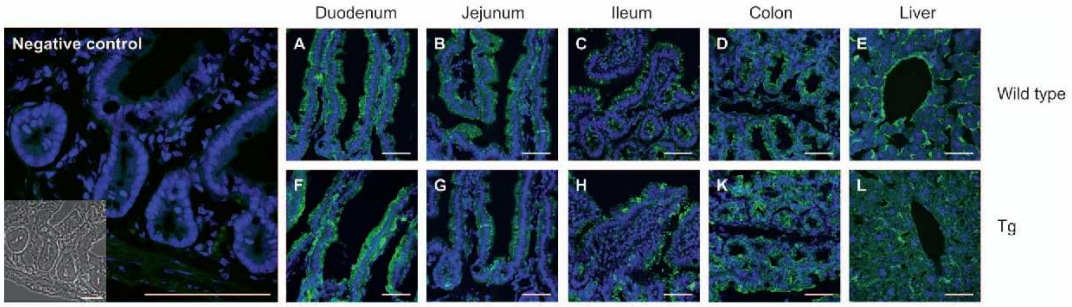

Figure 7 Cathepsin B follows the same expression and localization pattern in wild type and transgenic mice.
Immunofluorescence analysis was performed using a polyclonal cathepsin B-specific antibody that recognizes both the endogenous (mouse) and the chimeric (rat) protein. The expression levels as well as the localization of endogenous cathepsin B (green fluorescence) remained unaltered in the transgenic mice in comparison to wild type animals, confirming normal intestinal morphology of those animals. DRAQ5™ was used as nuclear counter-stain (blue fluorescence). Negative controls are shown on the left. Bars, 50 μm.

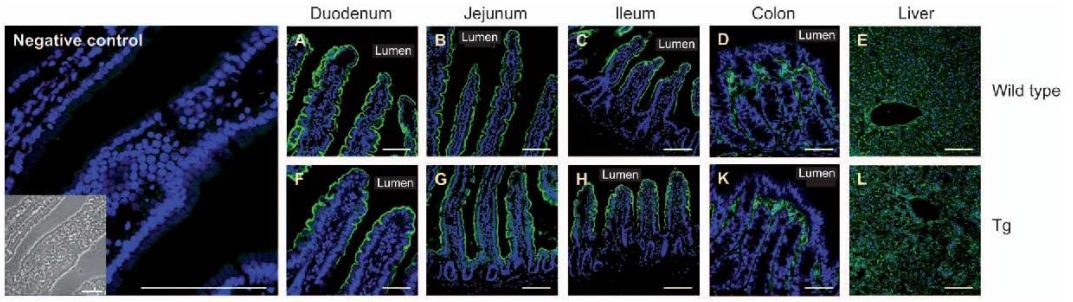

Figure 8 Expression and localization of the brush border enzyme aminopeptidase N.
Confocal laser scanning micrographs of cryosections prepared from wild type (A–E) and transgenic (F–L) mice after staining with APN-specific antibody. A steady expression of APN (green fluorescence) without changes in the localization of this enzyme was observed in both wild type (A–E) and transgenic (F–L) mice. Note the localization of APN at the apical plasma membrane in the small intestine (A–C and F–H) that is replaced by basolateral plasma membrane localization in the colon (D and K). DRAQ5™ was used as nuclear counterstain (blue fluorescence). Negative controls are shown on the left. Bars, 50 μm.

moter activation, and we also investigated distribution of the intestinal protein A33 antigen which is under the control of this same promoter. Cryosections of intestine tissue isolated from transgenic mice were used for these studies in which the presence of Cdx1 (Figure 9A and B) was confirmed in the intestine, but not in tissues such as heart and liver which are known to be Cdx1-negative in mice (Figure 9C and D). Cdx1 protein was present in differentiated cells of the intestinal epithelium only, but absent from the stem cells at the crypt base (Figure 9B), as expected. Furthermore, the Cdx1-response gene A33 antigen was expressed as obvious from the presence of A33 antigen on the protein level in the intestine of the transgenic mice (Figure 9E). Both Cdx1 and A33 antigen were localized as expected in the nuclei of enterocytes and at the basolateral plasma membrane of intestinal epithelial cells, respectively. These observations ruled out the possibility that lack of transgenic cathepsin B-EGFP expression in the intestine of A33-CathB-EGFP mice was due to

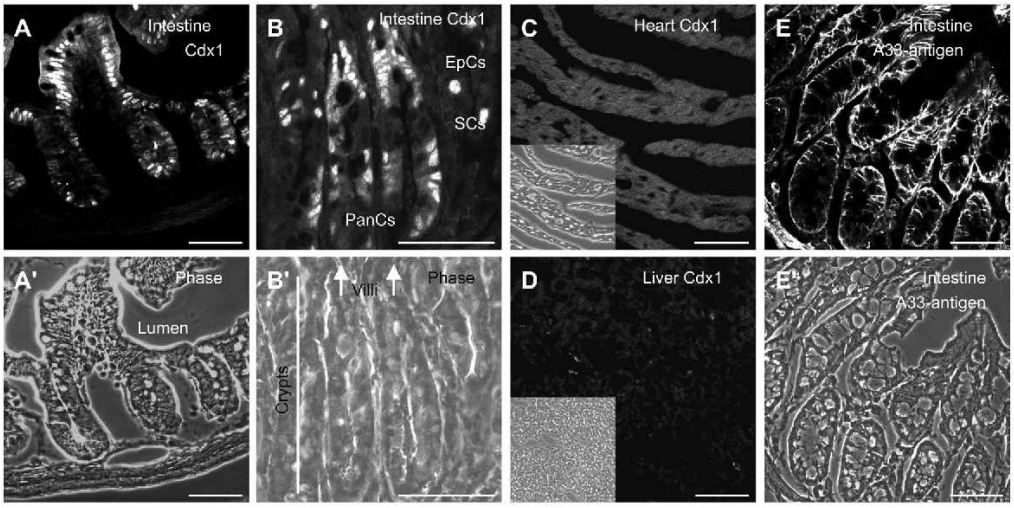

Figure 9 Presence and localization of Cdx1 and A33 antigen in the intestine of A33-CathB-EGFP mice.
Confocal laser scanning micrographs of cryosections prepared from intestine (A, B, E), heart (C) and liver (D) tissue of A33-CathB-EGFP mice after staining with Cdx1 and A33 antigen-specific antibodies as indicated. Cdx1 was observed within the nuclei of enterocytes (A and B) whereas the A33 antigen was found in the basolateral plasma membrane domain of intestinal epithelial cells (E). Corresponding phase contrast micrographs are also shown (A′–E′). Higher magnification of the crypt regions (B) revealed absence of Cdx1 in stem cells at position +4 but its presence in differentiated cells of the mouse intestinal mucosa as expected. Cdx1 was absent from heart and liver tissue (C–D). The presence of Cdx1 and A33 antigen in the transgenic mouse intestine and their localization patterns following the expected distribution reject the possibility of altered and non-functional regulation in general of the intestine-specific Cdx1-driven expression of genes under the control of the A33-antigen promoter. EpCs, epithelial cells; SCs, stem cells and PanCs, Paneth cells. Bars, 50 μm.

altered regulation of the A33-antigen promoter, or was caused by alterations in the differential expression pattern of Cdx1, its inducing and intestine-specific transcription factor.

Discussion

Cysteine cathepsin-deficient and transgenic mouse models for analysis of proteolytic functions of this diverse family of proteolytic enzymes

Cathepsin-deficient mice have been important tools in elucidating the roles and functions of these proteases *in vivo* (Reiser et al., 2010). Among them, cathepsin B-deficient animals exhibit a normal phenotype and cannot be distinguished from wild type littermates unless they are challenged (Guicciardi et al., 2000; Halangk et al., 2000; Reinheckel et al., 2001; Friedrichs et al., 2003; Brix et al., 2008). In this study we wanted to create a transgenic mouse model that would enable us to investigate the physiological role and trafficking of cathepsin B *in vivo*. The A33-CathB-EGFP mice were generated based on a principle previously proved functional in an *in vitro* cell culture model (Mayer et al., 2008) for expression of the chimeric protein cathepsin B-EGFP under the A33-antigen promoter that was induced by co-expression of the intestine-specific transcription factor Cdx1. Furthermore, it was initially envisaged that, apart from being able to monitor and visualize cathepsin B *in situ*, the transgenic mouse model would also serve as a system to analyze transgenic over-expression of cathepsin B. As such the A33-CathB-EGFP mice would be ideal for comparison with cathepsin B-deficient mice, since they would be two opposite cases, one of cathepsin B over-expression and one of cathepsin B absence.

Moreover, the reason we were interested in investigating the function of cathepsin B specifically in the intestine was previous studies of our group suggesting a role of cathepsin B in the ECM remodeling of mouse intestine (Vreemann et al., 2009). More specifically, it was suggested that cathepsin B might have a role in collagen IV degradation. Collagenase activity has also been shown for other cathepsins, for example cathepsin K, which is known to be involved in bone remodeling (Saftig et al., 1998; Li et al., 2002). Recently, we have shown that cathepsin K deficiency affects the gastrointestinal tract structurally (Dauth et al., 2011). Therefore a comparison of the intestinal tasks of cathepsin B that resides within endo-lysosomes of human enterocytes versus cathepsin K that is secreted from intestinal cells (Mayer et al., 2006) would have been interesting and within reach. Another aspect of interest would be to elucidate potential alterations in the overall intestinal tissue architecture and function due to over-expression of cathepsin B. It is well established that misbalance of expression or localization of proteases and their inhibitors is often associated with pathological conditions of the gastrointestinal tract, such as inflammation of the intestine (Medina and Radomski, 2006). In a mouse model of colitis, for example, it was shown that inhibition of cathepsins B and L or of cathepsin D can result in reduced damage of mucosal tissue (Menzel et al., 2006). Hence, cysteine cathepsins may not simply be considered endo-lysosomal proteolytic enzymes important for general protein turnover, but they may entail more specific functions in gastrointestinal tissues.

Transgenic mice develop normally

The first step in characterizing the generated A33-CathB-EGFP mice was to confirm incorporation of the transgene. We found that the transgene was successfully integrated and was kept over three generations while the A33-CathB-EGFP mice had no obvious phenotype and showed normal breeding behavior. For our further studies, we focused on each intestinal segment (duodenum, jejunum, ileum, and colon) separately since these parts are known to have unique functional and morphological features. Moreover, the expression pattern of various intestinal proteins is graded along the anterior-posterior axis, as in the case of the intestine-specific transcription factor Cdx1, for which the highest expression levels are observed in the distal colon (Duluc et al., 1997). Thus, we investigated the different parts of the intestine for expression of the A33-CathB-EGFP transgene, because the highest protein levels could have been predicted to be expressed in the colon due to the high amounts of Cdx1. However, no expression of the chimeric protein was observed in any of the intestinal segments of the A33-CathB-EGFP transgenic mice. On the other hand, expression of the endogenous mouse cathepsin B was detectable in all intestinal parts, and reassuringly without any differences observed between wild type and A33-CathB-EGFP mice.

Based on these results we continued our studies at the protein level. Immunoblotting experiments showed lack of the chimeric protein cathepsin B-EGFP in the intestine of A33-CathB-EGFP mice, a finding that was also confirmed by morphological studies in which no green fluorescence was observed.

The above results then led us to a more general question of whether the A33-CathB-EGFP mice had an overall altered intestinal phenotype due to transgene integration. Could it be that the A33-CathB-EGFP transgene was incorporated in a site leading to expression differences of other proteins associated with tissue structure, polarity and function? To answer this question we performed morphological studies in which the cell numbers of the different intestinal parts were compared between wild type and A33-CathB-EGFP mice as well as their general structure. Since no alterations were observed, we suggest that integration of the transgene had no effect during development and tissue morphogenesis. Furthermore, immunostainings for cathepsin B confirmed our *in vitro* studies (Mayer et al., 2008) where we had shown that the localization and trafficking of the endogenous cathepsin B is not altered due to the presence of the chimeric CathB-EGFP.

An important function of the gastrointestinal tract is to serve as a barrier, protecting the organism from the various pathogens found in the intestinal lumen (Schneeman, 2002; Turner and Turner, 2010). This barrier function strongly depends on the polarity of the intestinal epithelium, which is why we wanted to check for potential changes in such polarity-associated markers. One of those markers is the

APN also known as CD13. APN is a brush border enzyme that serves as an apical plasma membrane marker in the small intestine but in the large intestine it is found in the basolateral plasma membrane domain. APN, also called moonlighting enzyme, is of major interest since multiple functions, including differentiation, proliferation, apoptosis and signal transduction, have been assigned to this protein (Mina-Osorio, 2008). The localization and expression pattern of APN was not changed in the A33-CathB-EGFP mice, another finding suggesting that these mice were comparable to wild type littermates and that transgene integration was not compromising homeostasis of the intestine.

Conclusions

Since our analyses showed that the transgenic mouse model generated in this study had no dramatic alterations in the overall intestinal phenotype and that the animals were similar to wild type mice, the key question remaining is why we were not able to detect cathepsin B-EGFP?

In order to approach this question in more detail we wanted to check whether a potentially altered regulation of the A33-antigen promoter had led to a lack of cathepsin B-EGFP translation in the intestine of transgenic mice. For instance, a potential absence of Cdx1 could have resulted in non-expression of the chimeric protein because this intestine-specific transcription factor is known to be crucial for the activation of the A33-antigen promoter (Johnstone et al., 2002). Moreover, the levels of Cdx1 would also affect the extent of expression of one of its target genes, the A33 antigen in the mouse intestine. However, by immunofluorescence analysis we confirmed the presence of both Cdx1 and A33 antigen, as well as their expected localization patterns in the intestine of A33-CathB-EGFP animals. These results strongly suggested that Cdx1-driven gene expression and translation of proteins under the control of the A33-antigen promoter were still functional in the transgenic animals that were generated in this study.

We concluded that problems in the regulation and activation of the A33-antigen promoter were not the reason for the observed lack of cathepsin B-EGFP. On the other hand, a possible reason for this would be that the transgene was integrated in a site where it was silenced, resulting in no transcription. Another possibility could be the production of unstable mRNA, which we were not able to detect due to rapid degradation. One crucial factor for RNA degradation is the truncation of the polyA-tails of transcription products (Couttet et al., 1997; Brown and Johnson, 2001). The pA33-CathB-EGFP plasmid was linearized prior to its microinjection into the pronuclei of developing zygotes, meaning that, indeed, the polyA-tail might have been shortened or eliminated during the process. Another likely explanation for the lack of chimeric protein translation is the absence of an internal sequence that would promote splicing during transcript generation, and hence premature disintegration of transcripts was possibly involved (Choi et al., 1991; Palmiter et al., 1991; Auerbach, 2004). In another recent study in which the A33-antigen promoter was used in order to express human *TGF-βRII* specifically in the intestine of transgenic mice [Flentjar et al., 2007], such splicing was planned for and transgenic expression in an intestine-specific manner was achieved. We therefore conclude that expression of soluble cysteine cathepsins under the control of a promoter that drives intestine-specific expression of a typical enterocyte transmembrane protein was not productive.

Further experiments are planned to better understand the regulation of cysteine cathepsin expression in intestinal tissues. So far, it is known that immunosuppressive transforming growth factor-β1 (TGF-β1) results in down-regulation of cathepsin B and L expression, whereas the proinflammatory cytokine IL-6 leads to dose-dependent up-regulation of cysteine cathepsin expression (Gerber et al., 2001; Reisenauer et al., 2007). Similarly, IL-13 is known to stimulate the expression of matrix metalloproteinases but also of cathepsins in the lungs of mice suffering from emphysema (Zheng et al., 2000). Furthermore, activation of specific, pro-inflammatory caspases was proposed to be modulated by cathepsin-mediated proteolysis (Schotte et al., 1998). It is therefore a possible hypothesis (but beyond the scope of this report) that in addition to Cdx1, further factors, normally accounting for an efficient immune response during acute or chronic inflammation, would have been required to regulate the expression levels of cysteine peptidases such as cathepsin B in the transgenic mouse model realized in this study. Our own results with a trauma model of surgical manipulation of the mouse intestine argue in favor of this latter proposal, because only transient and localized inflammatory responses were observed after surgery while cathepsin B mRNA levels remained constant throughout the regeneration phase of several days (Vreemann et al., 2009). The localization pattern of cathepsin B was, however, dramatically changed within a few hours, both in our *in vitro* (Mayer et al., 2009) and in the *in vivo* model of intestinal manipulation (Vreemann et al., 2009). The findings of these and of the present study may thus indicate that a redundant system of cysteine peptidases in the gastrointestinal tract ensures rapid reactions to challenging conditions by protease re-location rather than by alteration in the protease expression levels.

Materials and methods

Generation of A33-CathB-EGFP transgenic mice

In order to establish a transgenic mouse model, pA33-CathB-EGFP (Mayer et al., 2008) was linearized by digestion of 100 μg plasmid DNA with ApaLI in Y^+ Tango buffer (both from MBI Fermentas, St. Leon-Rot, Germany) for 1 h at 37°C. Inactivation of the restriction enzyme was performed for 20 min at 65°C, the linearized plasmid DNA was purified using a PCR Purification Kit (Qiagen, Hilden, Germany) and used for microinjection into the pronuclei of developing zygotes. Pronuclear injection into fertilized C57BL/6NCrl oocytes was performed by T.G. and R.S. at the Transgenic Core Facility of the Freiburg University Medical Center (Freiburg, Germany), yielding the founder transgenic mice, A33-CathB-EGFP, which were transferred to the Jacobs University Bremen. Housing and breeding of wild type and transgenic animals were conducted in accordance with institutional guidelines and took place in the S1-

laboratories of Jacobs University Bremen, Germany, registered as SfAFGJS Az. 513-30-00/2-15-32 and 522-27-11/3-1, 05-A20 and A21. Mice were housed under standard conditions, with a 12 h/12 h light/dark cycle with lights on from 07:00 to 19:00, and *ad libitum* water and food.

Genotyping of the A33-CathB-EGFP transgenic mice

Tail biopsies of the transgenic animals were analyzed in order to examine the incorporation of the transgene by using semi-quantitative PCR. Total DNA was isolated with DNeasy Blood and Tissue Kit (Qiagen) and 300 ng were used as a template for PCR reactions. The primers used for genotyping were designed based on the published sequence of pEGFP-N1 (Clontech Laboratories, Heidelberg, Germany). The forward primer (5′-GTT ATC CCC TGA TTC TGT GG-3′) is located upstream of the AscI restriction site and the reverse primer (5′-GTG GCG ACC GGT GGA TC-3′) binds downstream of the BamHI restriction site that was used for generation of pCathB-EGFP (Linke et al., 2002; Mayer et al., 2008). PCR products were separated on 1% agarose gels and visualized by inclusion of 0.3% ethidium bromide.

Tissue preparation

A33-CathB-EGFP and wild type mice were anesthetized, the abdominal and thoracic cavities were opened and the abdominal aorta was cut. For perfusion via the heart, 0.9% NaCl supplemented with 200 IU heparin (Braun Melsungen AG, Melsungen, Germany) was used. Subsequently, the small and large intestines were isolated and kept on ice. The mesenteries were removed and the intestines were washed with ice-cold 0.9% NaCl solution. The small intestine was further divided into its three segments, duodenum, jejunum, and ileum. Apart from the small and large intestine (colon), the heart and liver were also obtained serving as negative controls. Each intestinal segment was divided into two parts. The anterior part was fixed using 4% paraformaldehyde (PFA) in 200 mM 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES), pH 7.4, and used for morphological studies while the posterior part was snap-frozen in liquid nitrogen and used for biochemical analysis.

Analysis of transgene expression

Total RNA was isolated from the different intestinal segments and the liver using the RNeasy Mini Extraction Kit and subjected to DNase treatment according to the manufacturer's instructions (Qiagen). Reverse transcription was performed with the Omniscript Reverse Transcription Kit (Qiagen) at 37°C for 1 h. Each reaction contained 2 μg RNA, 0.5 mM dNTPs, 1 μM random oligonucleotide primers, and 4 U Omniscript Reverse Transcriptase. The cDNAs were amplified with the following primers: 5′-CCT TGA TCC CTC TCT CTT GCC TGC-3′ (forward) and 5′-TGG TTG TCG GGC AGC AGC AC-3′ (reverse) to include the coding regions of the entire chimera consisting of cathepsin B and EGFP. With this primer combination the endogenous mouse cathepsin B is not amplified. PCR reactions were performed in a total volume of 20 μl in the presence of 2 μl RT reaction buffer, 100 pmol of each specific primer, 2 U *Taq* DNA polymerase, 1.25 mM $MgCl_2$ and 0.2 mM dNTPs (MBI Fermentas). In addition, primers amplifying the endogenous mouse cathepsin B were used (forward, 5′-TGC GTT CGG TGA GGA CAT AG-3′ and reverse, 5′-CGG GCA GTT GGA CCA TTG-3′), as well as primers specific for β-actin (forward, 5′-GCT CGT CGT CGA CAA CGG CTC-3′ and reverse, 5′-CAA ACA TGA TCT GGG TCA TCT TCT C-3′).

Immunoblot analysis

Total tissue extracts were isolated with lysis buffer PBS containing 0.5% Triton X-100, and homogenization of the samples was done using a Potter S homogenizer (Sartorius, Göttingen, Germany) at 1000 rpm for 5 min on ice. Homogenates were kept in a rotary mixer for 45 min while all steps were performed at 4°C. After centrifugation for 10 min at 10 000 *g*, supernatants were stored at -20°C. Protein concentration was determined using BSA as a protein standard (Neuhoff et al., 1979). For each sample, 10 μg of tissue extract was boiled in sample buffer consisting of 10 mM Tris-HCl, pH 7.6, 0.5% (w/v) SDS, 25 mM DTT, 10% (w/v) glycerol, and 25 μg/ml bromophenol blue. Prestained protein standards were used as molecular mass markers (MBI Fermentas). Samples were analyzed on 8–12.5% gradient acrylamide gels and then blotted onto nitrocellulose membranes using a semi-dry blotting procedure. After blocking overnight at 4°C with 5% milk powder in PBS containing 0.3% Tween-20, primary antibodies were applied. The antibodies used were goat anti-mouse cathepsin B (Neuromics, through Acris Antibodies, Herford, Germany), mouse anti-GFP (Roche Diagnostics, Mannheim, Germany), and rabbit anti-mouse A33 antigen (Abcam, Cambridge, UK). After several washing steps, membranes were incubated for 1 h at room temperature with HRP-conjugated rabbit anti-goat, goat anti-mouse, and goat anti-rabbit secondary antibodies (all from Southern Biotech, Birmingham, Alabama, USA). Visualization of immunoreactions was achieved by using enhanced chemiluminescence substrate on CL-XPosure film (both from Pierce through Perbio Science Europe, Bonn, Germany).

Morphological analysis by immunolabeling

The three small intestine segments, the colon, and the liver were fixed in 4% PFA in 200 mM HEPES buffer, pH 7.4 and left overnight at 4°C. PFA was washed out and samples were incubated overnight in 200 mM HEPES buffer (pH 7.4). Intestine samples were then cut into pieces of approximately 1 cm length and segments representing duodenum, jejunum, and ileum were taken from the beginning, middle, and end parts, respectively. The above pieces were incubated overnight in Tissue Freezing Medium (Jung, through Leica Microsystems, Nussloch, Germany), and were subsequently frozen on dry ice.

Cryosections of 5 μm were prepared from each sample by using a Leica CM1900 cryostat (Leica Microsystems) and mounted on microscope slides. Prior to staining, slides were incubated with PBS overnight at 4°C in order to remove the remaining embedding material. Non-specific binding sites on sections were blocked with 3% bovine serum albumin (BSA) which was followed by incubation with primary antibodies diluted in 0.1% BSA in calcium- and magnesium-free (CMF) PBS.

Specific primary antibodies were rabbit anti-GFP (Abcam), goat anti-mouse cathepsin B (Neuromics), rabbit anti-human Cdx1 (Abcam), rabbit anti-mouse A33 antigen (Abcam), and goat anti-mouse aminopeptidase N (R&D Systems, Wiesbaden, Germany). After washing, sections were incubated for 1 h at 37°C with secondary Alexa-543-coupled goat anti-rabbit and rabbit anti-goat IgG (Invitrogen through Molecular Probes, Karlsruhe, Germany). DRAQ5™ (Biostatus Limited, Shepshed, Leicestershire, UK) in a final concentration of 5 mM served as a nuclear counter-stain. Negative controls were prepared in which the specific primary antibodies were omitted and sections were incubated only with secondary antibodies and DRAQ5™. Immunolabeled samples were viewed with a Zeiss LSM 510 META laser scanning microscope equipped with Argon and Helium-Neon lasers (Carl Zeiss GmbH, Jena, Germany). Optical sections were obtained with a pinhole

setting of 1 Airy unit and at a resolution of 1024×1024 pixels and were further analyzed by using LSM 510 software, Release 3.2 (Carl Zeiss).

Cell lines and transfections

CHO-K1 cells were used for transfection with pCdx1-DsRed-Express and pA33-CathB-EGFP vectors in order to serve as positive control for RT-PCR reactions and for immunoblotting. Cell culture and transfection procedures were performed as previously described (Mayer et al., 2008).

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4.4 Cathepsin K in mouse colon

Effects of cathepsin K deficiency on intercellular junction proteins, luminal mucus layers, and ECM constituents in mouse colon

Maria Arampatzidou[1], André Schütte[2], Gunnar C. Hansson[2], Paul Saftig[3], Klaudia Brix[1]

[1] School of Engineering and Science, Research Center MOLIFE – Molecular Life Science, Jacobs University Bremen, Campus Ring 6, D-28759 Bremen, Germany
[2] Department of Medical Biochemistry, University of Gothenburg, Medicinaregatan 9A, 41390, Gothenburg, Sweden
[3] Institute of Biochemistry, Christian-Albrechts Universität zu Kiel, Olshausenstraße 40, 24118 Kiel, Germany

Email addresses of authors
MA: m.arampatzidou@jacobs-university.de
AS: andre.schuette@medkem.gu.se
GH: gunnar.hansson@medkem.gu.se
PS: psaftig@biochem.uni-kiel.de
KBr: k.brix@jacobs-university.de

*Corresponding Author
Dr. Klaudia Brix, Professor of Cell Biology
School of Engineering and Science
Research Center MOLIFE – Molecular Life Science
Jacobs University Bremen
Campus Ring 6, D-28759 Bremen, Germany
Phone: +49 (0)421 200 3246
Fax: +49 (0)421 200 3249
E-mail: k.brix@jacobs-university.de

Abstract

Cathepsin K is a cysteine protease expressed in a variety of tissues, including the gastrointestinal tract. Recently, it has been shown that cathepsin K can exhibit antimicrobial and anti-inflammatory activity in the mouse intestine. In our effort to further elucidate the roles of this enzyme in the large intestine, we used *Ctsk*$^{-/-}$ mice for biochemical and morphological studies. We demonstrated that absence of cathepsin K was accompanied by elevated protein levels of other cysteine cathepsins, namely cathepsins B, L, and X. However, no obvious changes were observed in the sub-cellular localization of these proteases indicating that trafficking was unaltered. We observed that cathepsin K deficiency had an impact on extracellular matrix constituents, such as collagen IV and laminin. In addition, the expression and localization pattern of intercellular junction proteins, like E-cadherin and occludin were altered in the colon of *Ctsk*$^{-/-}$ mice, suggesting potential impairment of the barrier function. In order to further investigate the properties of the intestinal barrier, we used an *ex vivo* method for measuring the growth of the two colon mucus layers. Our findings suggested that absence of cathepsin K had no influence on mucus organization or on its growth.

Keywords: cysteine cathepsins, colon, intestinal barrier, intercellular junctions, mucus

Introduction

Cathepsin K is a cysteine protease first recognized for its high expression in ovary and osteoclasts [Bromme and Okamoto, 1995]. Cathepsin K of osteoclasts is essential for bone remodeling, because it exhibits collagenolytic activity and it has been shown to degrade type I collagen during bone matrix removal [Gelb et al., 1996; Saftig et al., 1998]. This cathepsin has been in the focus of osteoclast-specific therapeutic strategies, aiming at an effective treatment of pathological conditions, such as osteoporosis [Deaton and Tavares, 2005; Yasuda et al., 2005; Desmarais et al., 2009; Podgorski, 2009]. Apart from osteoclasts, cathepsin K has been found to be present also in other cell types and tissues, such as macrophages [Punturieri et al., 2000], thyroid epithelial cells [Tepel et al., 2000; Jordans et al., 2009; Dauth et al., 2011a], brain parenchyma with neuronal and non-neuronal cells [Dauth et al., 2011b], and cells of the gastrointestinal tract [Haeckel et al., 1999; Mayer et al., 2006]. In human intestine, we observed brush border localization of cathepsin K, while it was also detected in the mucus of goblet cells [Mayer et al., 2006]. This localization suggested secretion of cathepsin K and its re-association with the apical plasma membrane of enterocytes with potential extracellular functions in the pericellular environment and the intestinal lumen.

Extracellular functions of cathepsin K are conceivable because the proteolytic activity is optimal at acidic pH but still detectable even at neutral to alkaline conditions [Tepel et al., 2000; Jordans et al., 2009]. Extracellular cathepsin K has recently been shown to act as an intestinal antibacterial factor with anti-inflammatory potential [Sina et al., 2012].

In an effort to investigate further the role of this cathepsin in intestinal physiology and homeostasis, we focused our studies on cathepsin K-deficient mice. In the present study we observed alterations in the intestinal proteolytic network, characterized by up-regulation of other cysteine cathepsins, such as cathepsins B, L, and X. Moreover, we demonstrated that cathepsin K deficiency has an impact on extracellular matrix constituents in the colon, thereby supporting our previous observations of increased collagen IV in the small and large intestine of *Ctsk*$^{-/-}$ mice [Dauth et al., 2011a]. These findings suggested a role of cathepsin K in ECM remodeling in the mouse intestine, which has also been shown for other cathepsins, namely cathepsins B and L [Vreemann et al., 2009].

Since cathepsin K inhibitors are already in phase III clinical trials [Lewiecki, 2009; Podgorski, 2009], it is very important to elucidate the roles and functions of this protease in the intestine, which represents one of the major off-target tissues when oral administration routes are aimed at. Treatment of mice with inhibitors of cathepsin D and/or cathepsins B and L resulted in reduced damage of mucosal tissue in a mouse model of colitis [Menzel et al., 2006].

In most cases of intestinal inflammation, impairment of the barrier function is observed. Important players in the maintenance of intestinal tightness and epithelial integrity are the intercellular junctions [Gibson et al., 1995; Hanby et al., 1996; Karayiannakis et al., 1998; Schmitz et al., 1999; Gassler et al., 2001]. Therefore, our studies focused on tight and adherens junction proteins in the colonic epithelium, in order to investigate whether cathepsin K deficiency has an impact on the expression and localization patterns of these proteins.
Another important role of the intestinal barrier in orchestrating the symbiosis of commensal bacteria with the host, meaning the human body, is the intestinal mucus [Backhed et al., 2005; Johansson et al., 2011]. The major component of mucus is the gel-forming protein mucin 2 (Muc2), which is produced and secreted by goblet cells. In the mouse colon, this molecule forms a 50 µm thick, firmly attached network on top of the epithelial cells which is converted to a 3 to 4 times thicker looser outer layer towards the intestinal lumen due to proteolytic events [Johansson et al., 2008]. The inner mucus layer of the colon is well organized and free of bacteria. In contrast, the mucus in the small intestine only consists of a single layer which has similar properties as the outer layer in colon [Johansson et al., 2008; Johansson et al., 2011b]. For the structural conversion from the inner to an outer loose layer and the expansion of the Muc2 protein to a wide and loose network, proteolytic events performed by endogenous enzymes are suggested from studies on germ free mice [Johansson et al., 2008]. One example of a pathogen-mediated proteolysis of the Muc2 protein is a cysteine protease secreted by *Entamoeba histolytica* which leads to the disruption of the mucus network and thus to the dissolution of the mucus layer [Lidell et al., 2006]. Since cathepsin K has recently been found in the goblet cells and the mucus layers [Sina et al., 2012] and thus must be colocalized with the Muc2 mucin, we compared the colon mucus layers properties of WT and *Ctsk*$^{-/-}$ mice in this study and observed similar patterns of mucus organization and growth in both genotypes.

Results

Proteolytic network of cysteine cathepsins in mouse intestine

Misbalance of proteases and their inhibitors can influence intestinal homeostasis and lead to pathological conditions such as inflammation [Medina and Radomski, 2006]. In the colon of *Ctsk*$^{-/-}$ mice, we observed alterations in the proteolytic network due to the absence of cathepsin K protein. By immunoblotting analysis, we found that the protein levels of cathepsins L, B, and X (Figure 1A, C, and D, respectively) were significantly elevated in the colon of *Ctsk*$^{-/-}$ mice when compared to WT controls. In addition, cryosections prepared from WT and *Ctsk*$^{-/-}$ mice were stained with cathepsin L-specific antibodies and this protease

(green signals) was localized at the apical plasma membrane of intestinal cells in both WT (Figure 1B, arrows) and *Ctsk*$^{-/-}$ mice (Figure 1B, arrowheads), without obvious differences in its localization. Thus translational regulation or altered turnover rates but not sorting or trafficking were affected by the lack of cathepsin K activity in the mouse colon.

Absence of cathepsin K has an impact on the extracellular matrix of mouse colon

Previous studies of our group have suggested a role of cathepsins B and L in ECM remodeling [Vreemann et al., 2009]. Since the protein levels of cathepsins B and L were found to be altered in the colon of *Ctsk*$^{-/-}$ mice (see Figure 1), we wanted to investigate whether this alteration in the proteolytic network would influence ECM constituents like collagen IV and laminin. Indeed, we found that the protein levels of collagen IV (Figure 2A) were significantly elevated in the colon of *Ctsk*$^{-/-}$ mice (Figure 2B, grey bars) when compared to WT controls (white bars). These results were also in line with our previous immunofluorescence analysis in both the small and large intestine [Dauth et al., 2011a]. Here we conducted immunofluorescence analysis (Figure 2C) for laminin (green signals, arrowheads) and we found higher levels of this ECM protein in the colonic muscular part of *Ctsk*$^{-/-}$ mice. These observations suggested a role of various cathepsins, including cathepsin K, in the remodeling of ECM and in the integrity of the basal lamina in the mouse intestine.

Comparison of intestinal architecture between WT and *Ctsk*$^{-/-}$ mice

Because the ECM serves as an important structural support, our next step was to study whether the observed alterations in the proteolytic network and the basal lamina of the colon of *Ctsk*$^{-/-}$ mice had influenced the overall tissue morphology and architecture. We compared cryosections prepared from the colon of WT and *Ctsk*$^{-/-}$ mice and no morphological changes were observed regarding the thickness of mucosa, submucosa, and muscle layer (Figure 3A and B). After staining with the nuclear marker DRAQ5™ (Figure 3A′ and B′) we were able to analyze the total cell numbers and we obtained comparable results for both WT and *Ctsk*$^{-/-}$ mice.

Cathepsin K-deficient mice display alterations in the expression and localization patterns of intercellular junctions in their colonic epithelium

Intercellular junctions, such as tight and adherens junctions play a crucial role in the maintenance of intestinal integrity and stability. We initially checked for the expression and localization pattern of the adherens junction protein E-cadherin. By immunoblotting analysis (Figure 4A), we found significantly elevated levels of E-cadherin in the colon of *Ctsk*$^{-/-}$ mice (Figure 4B, grey bars) when compared to WT controls (white bars). By immunofluorescence microscopy (Figure 4C) we then analyzed the distribution of E-cadherin (green signals,

arrowheads) and we observed localization to cell junctions in WT and *Ctsk*$^{-/-}$ mice. In addition, the stronger immunolabeling of E-cadherin observed in *Ctsk*$^{-/-}$ mice, confirmed higher levels of this protein in their colonic epithelial cells and more abundant adherens junctions.

We then focused on the multispan tight junction protein occludin. Interestingly, when we stained colon cryosections from WT and *Ctsk*$^{-/-}$ mice we observed an altered distribution of occludin (Figure 5A, green signals). More precisely, in WT mice occludin was detected as expected at the apical poles of the colonic epithelial cells (Figure 5A, arrowheads), whereas in the *Ctsk*$^{-/-}$ mice it was found throughout the cell (Figure 5A, arrows). Several studies have demonstrated localization shifts of tight junction proteins, such as occludin and claudin-1, in chronically inflamed intestine. Thus, in most cases these alterations have been associated with pathological conditions of the intestine, including IBD [Boudreau et al., 2007; Poritz et al., 2011; Noth et al., 2011].

The integrity of intercellular junctions is not only a sign for differentiation of intestinal mucosa but, more so, junctional complexes enable and maintain the functional states of intestine cells. In order to identify whether the observed changes in the gastrointestinal tract of *Ctsk*$^{-/-}$ mice had an effect on the differentiation process, polarity, and function of intestinal epithelial cells, we studied the localization pattern of the differentiation marker and brush border enzyme aminopeptidase N (APN). In the small intestine it is found at the apical plasma membrane whereas in the colon the cellular polarity is reversed [Arampatzidou et al., 2011b]. By immunofluorescence microscopy, we found that APN (Figure 5B, green signals) marked the basolateral plasma membrane in both WT and *Ctsk*$^{-/-}$ mice, and without altered protein levels. These results demonstrated that the functional polarity and differentiated states of intestinal epithelial cells were not affected by the lack of cathepsin K protein.

Cathepsin K deficiency had no impact on the patterns of mucus organization and growth

To investigate a possible role of cathepsin K on formation or secretion of the mucus layers, we performed immunofluorescence microscopy on cryosections of different parts of the small and large intestine as well as *ex vivo* measurements of the mucus in the colon of *Ctsk*$^{-/-}$ mice. With the latter technique we determined the increase of the mucus thickness growth in the two mucus layers over the time span of 45 minutes. The secretion rate and the regeneration of the mucus layers in the colon of *Ctsk*$^{-/-}$ mice were comparable to WT animals (Figure 6A). Accordingly, no significant deviation was detectable for each time point.

Immunostaining analysis of the Muc2 mucin, which is the main glycoprotein and structural compound of the mucus layers, showed a normal morphology in *Ctsk*$^{-/-}$ colon like in WT mice (Figure 6B). We also observed a well-structured mucus layer in ileum as seen in WT mice

(data not shown). Figure 7 shows a schematic representation of the mucosal organization in the ileum and colon of WT mice. The ileum contains a loose non-adherent mucus layer, which was also observed in the *Ctsk*$^{-/-}$ mice. The colonic mucus comprises two separate layers, an inner and an outer loose adherent layer. Even in the *Ctsk*$^{-/-}$ mice these two layers were readily distinguishable and organized in the same way as in WT animals.
In summary, we could not observe any specific phenotype in composition and assembly of the mucus layers in the ileum and colon of *Ctsk*$^{-/-}$ mice compared to WT mice.

Discussion

Cysteine cathepsins are known to exhibit a variety of functions in diverse tissues, including the small and large intestine. The localization pattern of these proteases differs in the intestine, suggesting both intra- and extracellular scenes of action [Brix et al., 2008; Arampatzidou et al., 2011a]. In human enterocytes, cathepsin B was found to reside within endo-lysosomes, whereas procathepsin L and cathepsin K were detected in association with the apical plasma membrane, and cathepsin K was also abundant in and secreted by goblet cells [Mayer et al., 2006].
One of the roles that have been suggested for cathepins in the mouse intestine is their involvement in the remodeling of extracellular matrix and more precisely in the turnover of collagen IV [Vreemann et al., 2009]. Collagenase activity of cathepsin K has also been shown to be vitally important in bone and in lung tissue [Gelb et al., 1996; Saftig et al., 1998; Srivastava et al., 2008].
On the other hand, cysteine cathepsins beyond and including cathepsin K have been associated with pathological conditions of the intestine, like for example chronic inflammatory disease [Medina and Radomski, 2006; Menzel et al., 2006]. Hence, balance and proper regulation of the proteolytic network is crucial for maintaining intestinal homeostasis and integrity.
Recently, cathepsin K has attracted special attention since the cathepsin K inhibitor Odanacatib, designed for osteoporosis treatment, is in phase III clinical trials [Podgorski, 2009; Rachner et al., 2011; Dauth et al., 2011a]. However, previous studies on other cathepsin K inhibitors revealed off-target effects in various tissues, including the central nervous system, kidney, and liver [Desmarais et al., 2008]. Although Odanacatib exhibits minimal off-target effects due to its high selectivity for cathepsin K [Gauthier et al., 2008], it is still unknown what the long-term effects of cathepsin K inhibition will be in non-targeted tissues, such as the intestine. For instance, it has been shown that deficiency or pharmacological inhibition of cathepsin K leads to reduced body weight gain and decreased

insulin and glucose circulating levels in mice [Yang et al., 2008]. Cathepsin K is also known to play a vital role in the liberation of thyroid hormones which are involved in a variety of physiological processes, including regulation of metabolic pathways and body weight [Tepel et al., 2000; Dauth et al., 2011a]. Moreover, it has been demonstrated that the intestinal microbiota is altered in cathepsin K-deficient mice and an antimicrobial role has been suggested for cathepsin K [Sina et al., 2012]. Thus, it is important to consider the potential consequences that may result from oral drug administration and off-target inhibition of cathepsin K that might rely on both, direct and indirect effects of this protease. Consequently, it is of great interest to elucidate the roles that cathepsin K fulfils in the intestine and to determine potential alterations induced by lack of this cysteine protease.

Cathepsin K-deficient mice represent a suitable model for this type of studies and the colon of these mice was used for both biochemical and morphological analysis. We demonstrated that absence of cathepsin K from mouse colon leads to alterations in the proteolytic network, with protein levels of specific cathepsins (B, L, and X) being significantly elevated. Our results suggest a compensatory effect among cysteine cathepsins in colon, which is already known for other tissues. For example, cathepsins K and L are already known to compensate the function of each other in the thyroid [Friedrichs et al., 2003]. In addition, it was shown that in the thyroid of *Ctsk*$^{-/-}$ mice the distribution and localization of cathepsin L was altered due to absence of cathepsin K. Moreover, previous studies of our group have suggested a role for cathepsins B and L in ECM remodeling. More precisely, we found that non-directed, traumatic release of cysteine cathepsins resulted in massive ECM damage while cathepsin B- and cathepsin L-deficient mice have significantly increased protein levels of collagen IV in their intestine compared to WT animals [Mayer et al., 2009; Vreemann et al., 2009].

In the present study, we demonstrated that absence of cathepsin K has an impact on ECM constituents, such as collagen IV and laminin. In the colon of *Ctsk*$^{-/-}$ mice the levels of these two basal lamina components were significantly higher compared to WT animals, suggesting a role for cathepsin K in the remodeling of ECM and basement membrane. The basement membrane plays an important role in maintaining intestinal structure and compartmentalization, while it is also involved in various cellular processes, including adhesion, proliferation, and differentiation [Timpl, 1996; Mahoney et al., 2008]. Hence, alterations in the basement membrane components could influence the overall tissue architecture and function. Morphological studies enabled us to verify whether this scenario was true for the *Ctsk*$^{-/-}$ mice. Our results revealed no differences between WT and *Ctsk*$^{-/-}$ mice, regarding the colonic epithelial cells, goblet cells, submucosa, and muscularis layer, as well as the total cell numbers, in accordance with recent findings [Sina et al., 2012].

A major function of the intestine is the barrier function. Several studies have shown that intercellular junctions contribute in maintaining this intestinal barrier while impairment of their

structure and function has been associated with inflammatory responses [Gibson et al., 1995; Hanby et al., 1996; Karayiannakis et al., 1998; Schmitz et al., 1999; Gassler et al., 2001]. Therefore, we analyzed the expression and localization pattern of E-cadherin and occludin. The adherens junction protein E-cadherin has a pivotal role in cell survival, proliferation, and apico-basal polarity [Lien et al., 2006]. Absence of cathepsin K resulted in elevated E-cadherin protein levels in the colon of *Ctsk*$^{-/-}$ mice. Various cathepsins, namely cathepsins B, L, and S have been shown to act on E-cadherin as a substrate when the proteases are secreted into the extracellular space in tumor conditions [Gocheva et al., 2006]. Therefore, the elevated levels of E-cadherin in the colon of *Ctsk*$^{-/-}$ mice could possibly be explained by the notion that this protein can be cleaved also by cathepsin K. However, the localization of E-cadherin was comparable in WT and *Ctsk*$^{-/-}$ mice and followed the expected basolateral pattern.

In contrast, the localization pattern of the tight junction protein occludin, serving as molecular fence between the apical and the basolateral plasma membrane domains, was altered in the colon of *Ctsk*$^{-/-}$ mice. Cathepsin K deficiency resulted in redistribution of occludin away from the tight junctions and towards the cytoplasm. Several studies have demonstrated such localization shifts of tight junction proteins, like occludin and claudin-1, and have associated them with pathological conditions of the intestine [Boudreau et al., 2007; Poritz et al., 2011; Noth et al., 2011]. Interestingly, it was shown that inhibition of cathepsin L promotes nuclear localization of claudin-1 in intestinal epithelial cells [Boudreau et al., 2007]. Moreover, posttranslational phosphorylation of occludin has also been suggested to mediate the function and localization pattern of this tight junction protein [Paris et al., 2008].

In addition, the integrity of intercellular junctions is also important for proper differentiation and maintenance of the functional state of the intestinal epithelium. In an effort to investigate whether the observed changes regarding tight junctions had influenced intestinal cell differentiation, we studied the localization pattern of APN [Howell et al., 1993; Mina-Osorio, 2008] in both WT and *Ctsk*$^{-/-}$ mice. We observed that cathepsin K deficiency induced no alterations in the distribution of this differentiation marker.

Another important determinant of intestinal barrier function is the mucus layer that is mainly organized by a single protein, the Muc2 mucin. It has been shown that a cysteine protease from *Entamoeba histolytica* is able to cleave this mucin and disrupt the mucus layers [Lidell et al., 2006]. However, the addition of a protease inhibitor cocktail *in vivo* can inhibit the secretion and thereby the increase of mucus thickness in rat colon [Johansson et al., 2008]. Furthermore, it has been recently described that cathepsin K can be found inside goblet cells and the mucus layers of both man and mouse [Sina et al., 2012]. Thus, it has to be colocalized with the Muc2 mucin and could - as an endogenous cysteine protease - likely be involved in mucus layer formation or organization. To address possible functions of cathepsin

K inside the mucus layers we used a newly established *ex vivo* method to measure the mucus growth in the colon of *Ctsk*$^{-/-}$ mice [Gustafsson et al., 2011].

But the absence of cathepsin K does not seem to have an effect on the growth and remodeling of colon mucus layers in the measured time interval of 45 minutes. In addition, immunofluorescence analysis of Muc2 in colon cryosections of WT and *Ctsk*$^{-/-}$ mice confirmed the observations from the mucus measurements since no differences were demonstrated between the two genotypes. Nevertheless, since other cysteine cathepsins were shown to be up-regulated in the colon of *Ctsk*$^{-/-}$ mice, thus pinpointing to compensatory processes, we can not completely exclude that cathepsin K is involved in mucus processing. Further research is required in order to investigate the functions of cathepsin K, as well as other proteases in the colonic mucus layers.

In conclusion, our results indicated that the tasks of cathepsin K in the turnover of ECM and junctional complex proteins are more important than its extracellular and luminal contributions to intestinal homeostasis. Taking this further, one still has to consider potential scavenging of orally administered cathepsin K inhibitors by the high amounts of this protease on the luminal side of the epithelium, indicating that high inhibitor dosage might be required for achieving on-target effects. The question whether it is safe to treat human patients with cathepsin K inhibitors remains open, since the complexity of proteolytic networks makes it even more difficult to predict what the overall outcome will be after such treatment.

Materials and methods

Animals

Cathepsin K-deficient mice (*Ctsk*$^{-/-}$) were generated at the University of Göttingen, Germany, and genotyping was performed as described elsewhere [Saftig et al., 1998]. All *Ctsk*$^{-/-}$ and wild type (WT) C57Bl/6J mice were 6 months-old and males. Housing and breeding of animals were conducted in accordance with institutional guidelines and took place in the S1-laboratories of Jacobs University Bremen, Germany, registered as SfAFGJS Az. 513-30-00/2-15-32 and 522-27-11/3-1, 05-A20 and A21. Mice were housed under standard conditions, with a 12 h/12 h light/dark cycle with lights on from 07:00 to 19:00 h, and *ad libitum* water and food.

Tissue sampling and preparation of tissue extracts

Ctsk$^{-/-}$ and WT mice were anesthetized, the abdominal and thoracic cavities were opened and the abdominal aorta was cut. For perfusion via the heart, 0.9% NaCl supplemented with 200 IU heparin (Braun Melsungen AG, Melsungen, Germany) was used. Subsequently, the colon

was isolated and washed with ice-cold 0.9% NaCl solution. Each colon was divided into two parts. The anterior part was fixed using 4% paraformaldehyde (PFA) in 200 mM 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES), pH 7.4, and used for morphological studies while the posterior part was snap-frozen in liquid nitrogen and used for biochemical analysis. Total tissue extracts were isolated with lysis buffer PBS containing 0.5% Triton X-100, and homogenization of the samples was done using a Potter S homogenizer (Sartorius, Göttingen, Germany) at 1000 rpm for 5 min on ice. Homogenates were kept in a rotary mixer for 45 min while all steps were performed at 4˚C. After centrifugation for 10 min at 10 000 *g*, supernatants were stored at -20˚C. Protein concentration was determined using BSA as a protein standard [Neuhoff et al., 1979].

SDS-PAGE and immunoblotting

Total tissue extracts were normalized to equal amounts of protein (16 µg each), loaded onto 8% or 12.5% SDS-polyacrylamide gels and the separated proteins were then semi-dry blotted onto nitrocellulose membranes. For detection of collagen IV, gel electrophoresis and immunoblotting were performed under native conditions [Vreemann et al., 2009], since the collagen IV-specific antibody recognizes only its non-denatured antigen. Blocking was performed overnight at 4˚C using 5% milk powder in PBS containing 0.3% Tween-20. After blocking, the following primary antibodies were applied: rabbit anti-mouse collagen IV (Rockland, Philadelphia, USA), goat anti-mouse cathepsin B (Neuromics, through Acris Antibodies, Herford, Germany), goat anti-mouse cathepsin X (R&D Systems, Wiesbaden, Germany), goat anti-mouse cathepsin L (Neuromics), and rabbit anti-human E-cadherin (Santa Cruz Biotechnology Inc., Santa Cruz, CA USA). Membranes were subsequently washed and incubated for 1 h at room temperature with HRP-conjugated antibodies. The secondary antibodies used were goat anti-rabbit and rabbit anti-goat (both from Southern Biotech, Birmingham, Alabama, USA). Immunoreactions were visualized by using enhanced chemiluminescence substrate on CL-XPosure film (both from Pierce through Perbio Science Europe, Bonn, Germany).

Densitometry and statistical analysis

Densitometry analysis of immunoblots was performed using TINA software version 2.09d (Raytest Isotopen-Messgeräte GmbH, Straubenhardt, Germany). Background intensity was subtracted and all measured optical density/mm^2 values were given as mean of intensities per area. The results were expressed as percentage of protein expression in WT controls using Coomassie-stained protein per lane for normalization to account for differences between independent experiments. Two-tailed Student's t-test of Origin® 7.0 SR0 (OriginLab Corp., Northampton, MA, USA) was used in order to assess differences between *Ctsk*$^{-/-}$ and

WT mice and determine levels of significance. All data are shown as mean ± standard error of the mean.

Cryosectioning and immunolabeling

After fixation with 4% PFA in 200 mM HEPES, colon tissue was washed and samples were incubated overnight in 200 mM HEPES buffer (pH 7.4). From each colon sample, pieces of approximately 1 cm length were cut and incubated in Tissue Freezing Medium (Jung, through Leica Microsystems, Nussloch, Germany). After overnight incubation, samples were frozen on dry ice and cryosections of 5 µm were prepared by using a Leica CM1900 cryostat (Leica Microsystems). Cryosections were mounted on microscope slides, blocked with 3% bovine serum albumin (BSA) in calcium-magnesium-free (CMF) PBS, and immunolabeled with primary antibodies. Specific primary antibodies were rabbit anti-human cathepsin L (RD Laboratories, Diessen, Germany), goat anti-human laminin (Santa Cruz), rabbit anti-human E-cadherin (Santa Cruz), goat anti-human occludin (Santa Cruz), goat anti-mouse aminopeptidase N (R&D Systems). After several washing steps with 0.1% BSA in CMF-PBS, sections were incubated with secondary antibodies for 1 h at 37˚C. Alexa 488 or Alexa 546-coupled goat anti-rabbit IgG, Alexa 546 donkey anti-goat IgG and Alexa 488 rabbit anti-goat IgG secondary antibodies were used (all from Invitrogen through Molecular Probes, Karlsruhe, Germany). DRAQ5™ (Biostatus Limited, Shepshed Leicestershire, UK) was applied together with the secondary antibodies and served as nuclear counter-stain. Negative controls were prepared in which the specific primary antibodies were omitted and sections were incubated only with secondary antibodies and DRAQ5™.

Microscopy

Immunolabeled colon sections were viewed with a Zeiss LSM 510 META laser scanning microscope equipped with Argon and Helium-Neon lasers (Carl Zeiss GmbH, Jena, Germany). Optical sections were obtained with a pinhole setting of 1 Airy unit and at a resolution of 1,024×1,024 pixels and were further analyzed by using LSM 510 software, Release 3.2 (Carl Zeiss).

Mucus mesearuments

Mucus measurements in *Ctsk*$^{-/-}$ and WT mice have been performed as previously described [Gustafsson et al., 2011]. The colon was dissected from the mice, opened along the mesenteric border and mounted between two acrylic plates in an Ussing type chamber after the removal of the longitudinal muscle layer. Inside the basal chamber, oxygenized KREBS' solution (136 mM NaCl, 4. mM KCl, 1.4 mM $MgSO_4$, 1.6 mM KH2PO_4, 1.5 mM $CaCl_2$, 27 mM $NaHCO_3$, 10 mM D-Glucose, pH 7.4) was applied with a constant perfusion of 5 ml per min

(Pump33, Harvard apparatus, USA). The luminal side of the colon sample was directed towards the apical, i.e. upper chamber with a fixed surface diameter of 2.5 mm and covered with 150 µl of KREBS' solution, but completed with 10 mM D-mannitol instead of 10 mM D-glucose. The whole chamber system was heated to 37˚C (dual channel heater TC-344B, Warner Instrument Corporation, USA).

By the addition of charcoal particles to the KREBS' mannitol-solution in the upper chamber the usually transparent mucus was visualized under a stereo microscope (Leica MZ75, Leica, Wetzlar, Germany). The actual measuring process was performed with a glass micropipette (diameter 5-10 µm), fixed in an angle of 45˚. Attached to a micromanipulator with a connected digimatic indicator (Mitutoyo, Japan), the micropipette was used to measure the distance between the epithelial surface and the charcoal particles on top of the mucus layer. To calculate an average mucus thickness, each measurement comprised five data points on both the mucus layer and the epithelial surface. From this data, the actual vertical mucus thickness was calculated by multiplying the measured distance with the *cosinus* of 45.

After the initial measurement, the mucus was removed with a pipet adjusted to a fixed volume of 150 µl. To obtain the mucus layer regeneration over 60 min, the measurements were repeated every 15 min. After 45 min, the mucus layer was removed again and the thickness of the remaining mucus was determined.

Figure legends

Figure 1 Alterations in the proteolytic network of *Ctsk*$^{-/-}$ mouse colon

(A, C, D) Densitometry analysis and immunoblots of lysates prepared from the colon of WT (white bars) and *Ctsk*$^{-/-}$ (grey bars) mice. Protein expression is given as percentage of WT controls after normalization to total Coomassie-stainable protein as means ± standard error. Note that the protein levels of cathepsin L (A), cathepsin B (C), and cathepsin X (D) were significantly elevated in the colon of *Ctsk*$^{-/-}$ mice when compared to WT controls. Molecular mass markers are given in the left margin. Levels of significance are indicated as * for $p<0.05$, and ** for $p<0.01$.

(B) Confocal laser scanning micrographs of cryosections prepared from WT and *Ctsk*$^{-/-}$ mice after staining with cathepsin L-specific antibodies. Cathepsin L (green signals, lower panels, middle) was localized at the apical plasma membrane of intestinal cells in both WT (arrows) and *Ctsk*$^{-/-}$ mice (arrowheads) but *Ctsk*$^{-/-}$ mice showed stronger immunolabeling, confirming higher cathepsin L protein levels. Corresponding phase contrast micrographs are shown in lower panels, left. DRAQ5™ was used as nuclear counter-stain (blue signals, lower panels, right). Bars, 50 µm.

Figure 2 Differences in the extracellular matrix of WT and *Ctsk*$^{-/-}$ mice

(A) Immunoblot of lysates prepared from the colon of WT (white bars) and *Ctsk*$^{-/-}$ (grey bars) mice, and (B) densitometry analysis. Protein expression is given as percentage of WT controls after normalization to total Coomassie-stainable protein as means ± standard error. Note that the protein levels of the ECM constitute collagen IV were significantly elevated in the colon of *Ctsk*$^{-/-}$ mice when compared to WT controls. Levels of significance are indicated as ** for $p<0.01$. (C) Confocal laser scanning micrographs of cryosections prepared from WT and *Ctsk*$^{-/-}$ mice after staining with laminin-specific antibodies. Laminin (green signals, lower panels, middle) was present in the colonic ECM (arrowheads) in both WT and *Ctsk*$^{-/-}$ mice. Stronger immunolabeling was observed in the colon of *Ctsk*$^{-/-}$ mice, indicating higher levels of laminin compared to WT controls. Corresponding phase contrast micrographs are shown in lower panels, left. DRAQ5™ was used as nuclear counter-stain (red signals, lower panels, right). Bars, 50 µm.

Figure 3 Comparison of intestinal morphology in the colon of WT and *Ctsk*$^{-/-}$ mice

Phase contrast micrographs of cryosections prepared from WT and *Ctsk*$^{-/-}$ mice (A and B). Confocal laser scanning micrographs of cryosections after staining with the nuclear marker DRAQ5™ (A′ and B′). No morphological alterations, such as total cell numbers, thickness of

mucosa, submucosa, and muscularis layers (ML) were observed in the colon of *Ctsk*$^{-/-}$ mice when compared to WT controls. Bars, 100 µm.

Figure 4 Expression and localization of E-cadherin in mouse colon

(A) Immunoblot of lysates prepared from the colon of WT (white bars) and *Ctsk*$^{-/-}$ (grey bars) mice, and (B) densitometry analysis. Protein expression is given as percentage of WT controls after normalization to total Coomassie-stainable protein as means ± standard error. Protein levels of E-cadherin were significantly elevated in the colon of *Ctsk*$^{-/-}$ mice when compared to WT controls. Molecular mass markers are given in the left margin. Levels of significance are indicated as *** for $p<0.001$. (C) Confocal laser scanning micrographs of cryosections prepared from WT and *Ctsk*$^{-/-}$ mice after staining with E-cadherin-specific antibodies. E-cadherin (green signals, lower panels, middle) exhibited a basolateral localization (arrowheads) in both WT and *Ctsk*$^{-/-}$ mice. Stronger immunolabeling was observed in the colon of *Ctsk*$^{-/-}$ mice, confirming higher E-cadherin protein levels. Corresponding phase contrast micrographs are shown in lower panels, left. DRAQ5™ was used as nuclear counter-stain (blue signals, lower panels, right). N, nucleus; bars, 20 µm.

Figure 5 Localization patterns of occludin and aminopeptidase N in the colon of WT and *Ctsk*$^{-/-}$ mice

Confocal laser scanning micrographs of cryosections prepared from WT and *Ctsk*$^{-/-}$ mice after staining with occludin and aminopeptidase N-specific antibodies as indicated. (A) Occludin (green signals, side panels, middle) showed a labeling typical for tight junctions at the apical plasma membrane in the colonic epithelium of WT mice (arrowheads). This localization pattern was disrupted in *Ctsk*$^{-/-}$ mice (arrows), suggesting alterations in the colonic tight junctions due to cathepsin K-deficiency. Corresponding phase contrast micrographs are shown in side panels, top. DRAQ5™ was used as nuclear counter-stain (red signals, side panels, bottom). N, nucleus; bars, 20 µm. (B) Aminopetidase N (green signals, side panels, middle) was localized at the plasma membrane in the colon of both WT and *Ctsk*$^{-/-}$ mice and no differences were observed in the signal intensity. Corresponding phase contrast micrographs are shown in side panels, top. DRAQ5™ was used as nuclear counter-stain (blue signals, side panels, bottom). Bars, 50 µm.

Figure 6 No changes were induced in the colon mucus layers due to absence of cathepsin K

(A) Mucus growth in the colon of WT and *Ctsk*$^{-/-}$ mice within 45 minutes. White bars represent the regeneration of the mucus thickness in WT mice and grey bars in *Ctsk*$^{-/-}$ mice. t=0 determines the measurement after initial removal of the outer mucus layer by aspiration.

Likewise, t=45' defines the second removal of the outer layer after the measurements. The dotted line corresponds to the inner mucus layer which is not removable by aspiration. (B) Confocal laser scanning micrographs of cryosections prepared from *Ctsk*$^{-/-}$ mice after staining with Muc2-specific antibodies. Muc2 (green signals, lower panels, middle) showed comparable localization and signal intensity in the colonic epithelium of WT and *Ctsk*$^{-/-}$ mice. Corresponding phase contrast micrographs are shown in lower panels, left. DRAQ5™ was used as nuclear counter-stain (red signals, lower panels, right). Bars, 50 µm.

Figure 7 Mucus organization in the small and large intestine

Schematic view of the organization of the mucus layers in the murine ileum and colon. The ileum contains a loosely, non-adherent mucus, whereas the colonic mucus comprises two layers: one inner stratified layer (S) and an outer loose layer (O), which is the bacterial habitat simultaneously. Mucus is mainly build of the Muc2 mucin, which is solely produced in goblet cells.

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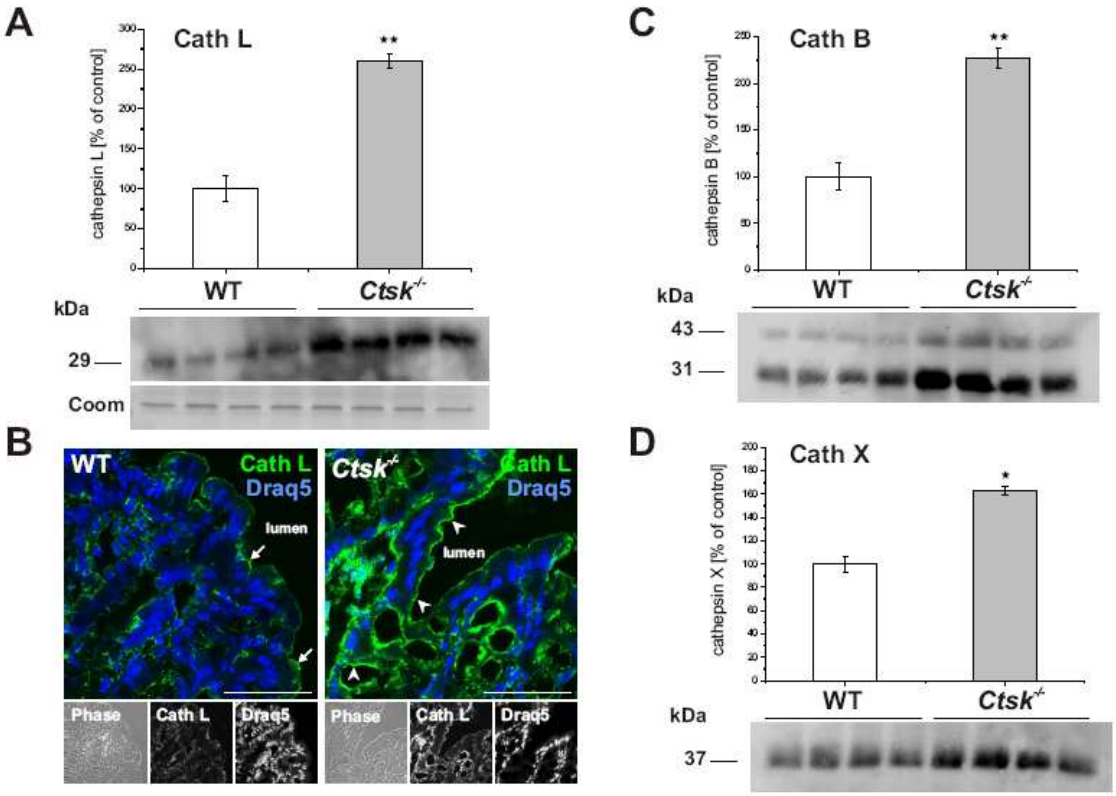
A
Cath L
cathepsin L [% of control]
**
WT
Ctsk-/-
kDa
29
Coom
B
WT
Cath L
Draq5
lumen
Ctsk-/-
Phase
Cath L
Draq5
C
Cath B
cathepsin B [% of control]
**
WT
Ctsk-/-
kDa
43
31
D
Cath X
cathepsin X [% of control]
*
WT
Ctsk-/-
kDa
37

Figure 1

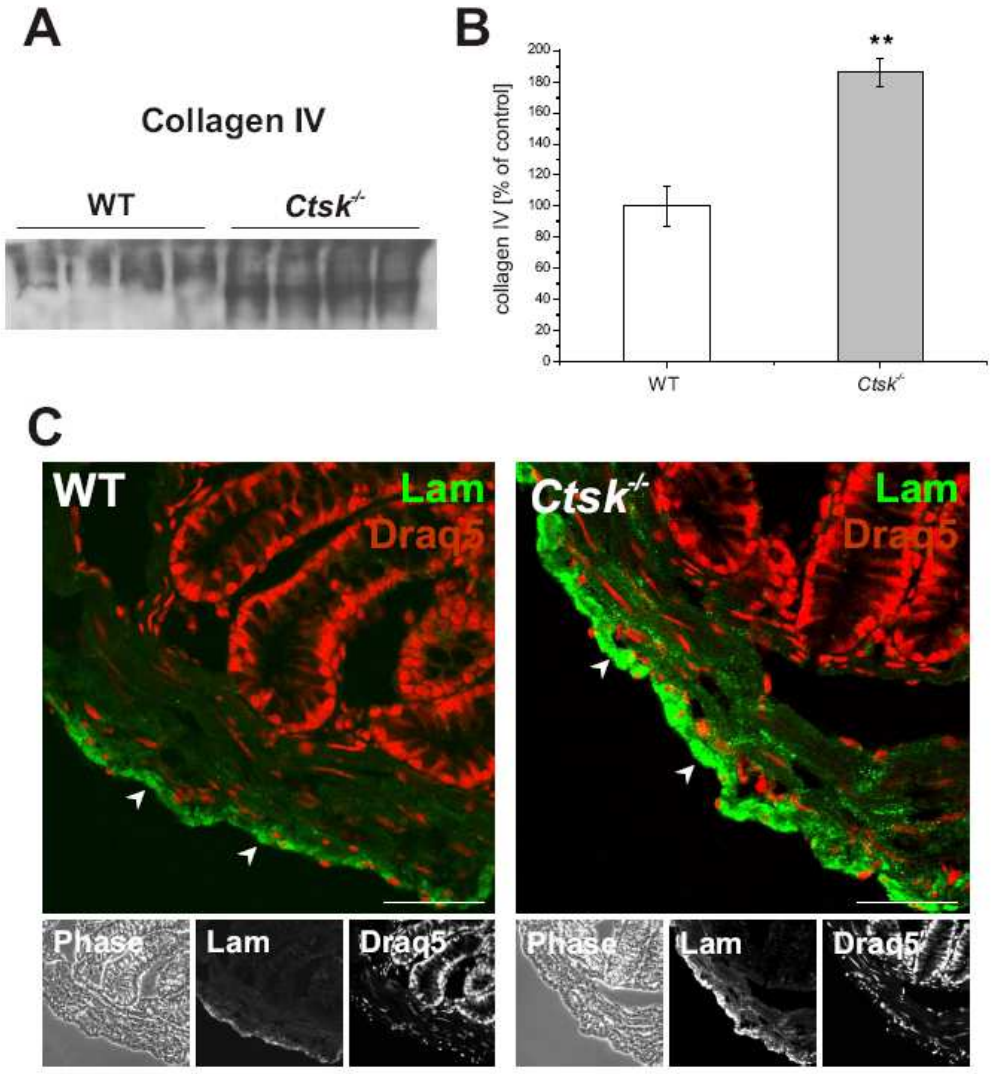
A
Collagen IV
WT
Ctsk-/-
B
collagen IV [% of control]
200
180
160
140
120
100
80
60
40
20
0
**
WT
Ctsk-/-
C
WT
Lam
Draq5
Ctsk-/-
Lam
Draq5
Phase
Lam
Draq5
Phase
Lam
Draq5

Figure 2

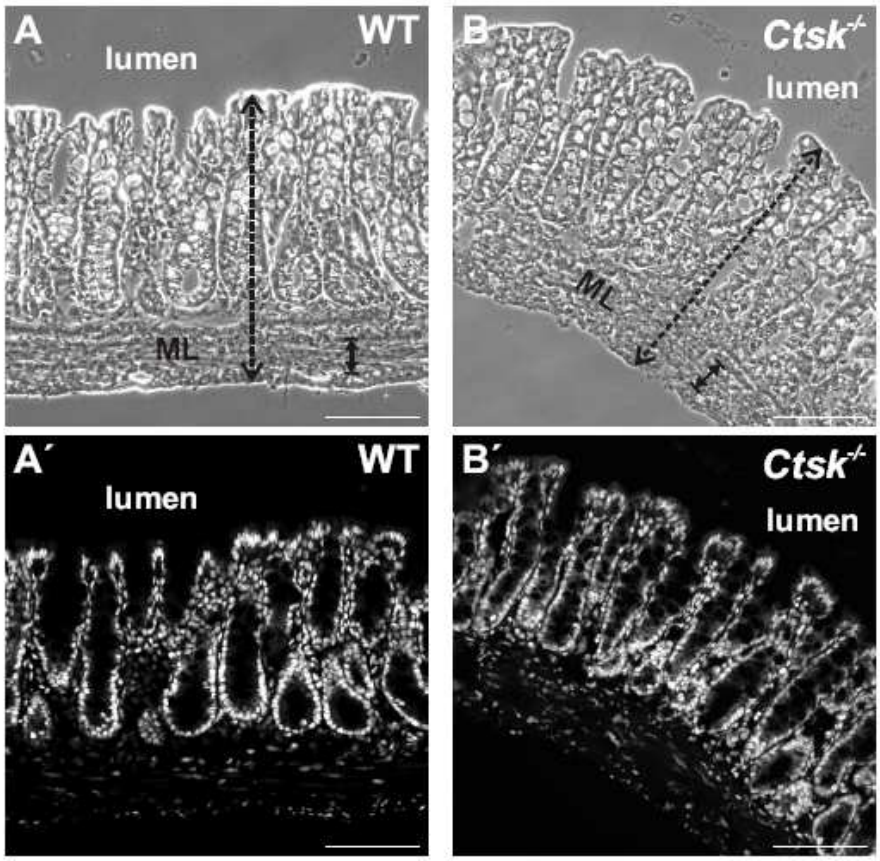

Figure 3

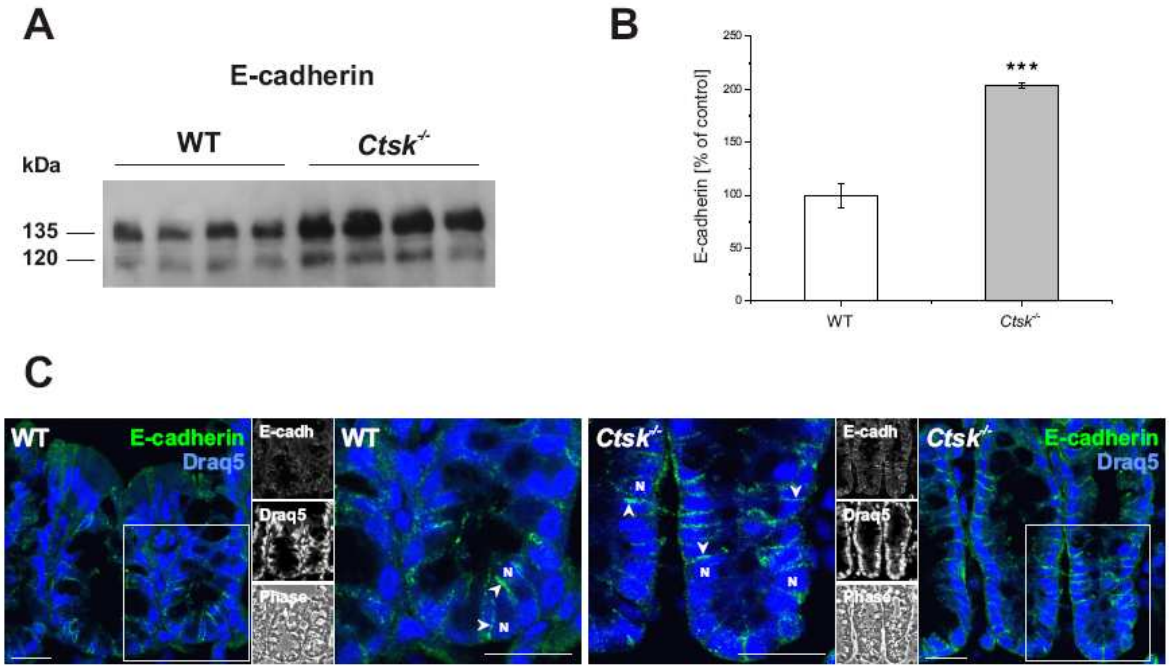
A
E-cadherin
WT
Ctsk-/-
kDa
135
120
B
E-cadherin [% of control]
250
200
150
100
50
0
***
WT
Ctsk-/-
C
WT
E-cadherin
Draq5
E-cadh
Draq5
Phase
WT
N
N
Ctsk-/-
N
N
N
E-cadh
Draq5
Phase
Ctsk-/-
E-cadherin
Draq5

Figure 4

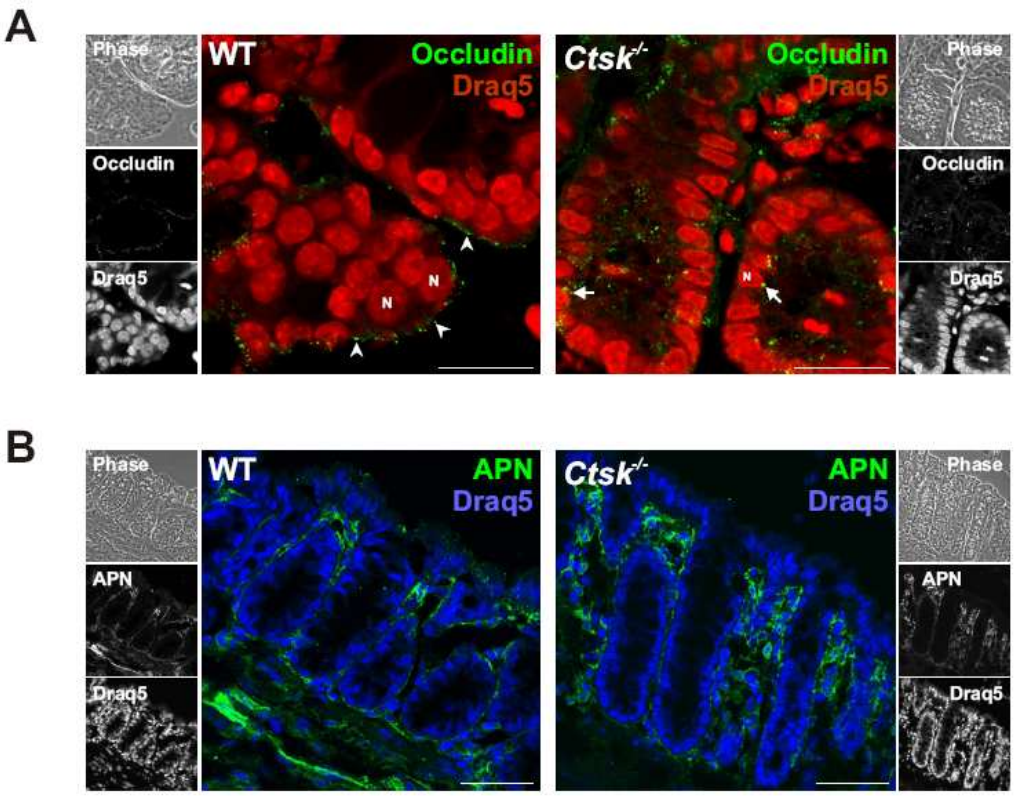
A
Phase
Occludin
Draq5
WT
Occludin
Draq5
N
N
Ctsk-/-
Occludin
Draq5
N
Phase
Occludin
Draq5
B
Phase
APN
Draq5
WT
APN
Draq5
Ctsk-/-
APN
Draq5
Phase
APN
Draq5

Figure 5

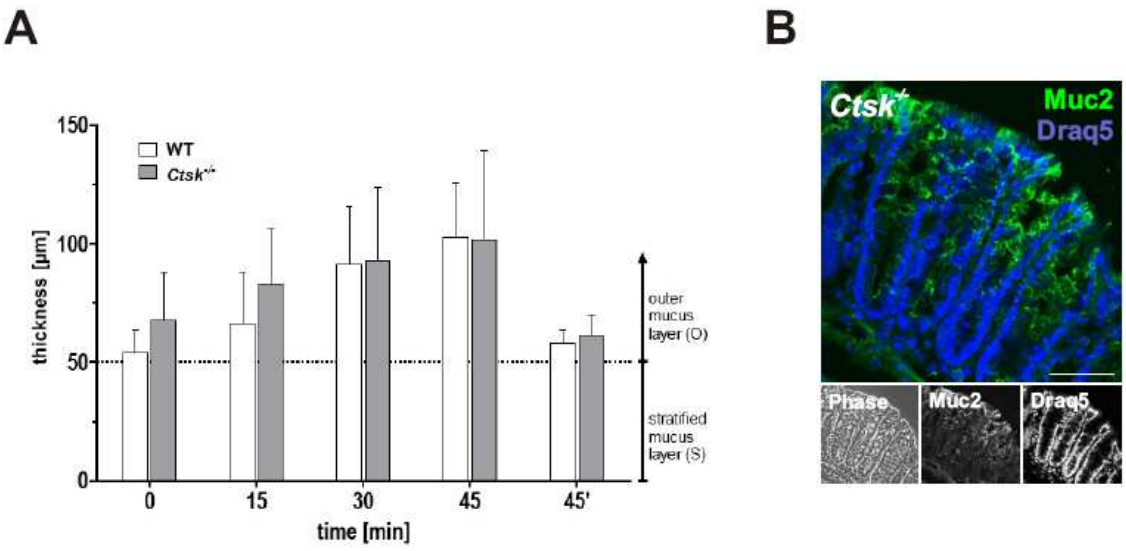
A
B
150
100
50
0
thickness [µm]
WT
Ctsk-/-
outer mucus layer (O)
stratified mucus layer (S)
0
15
30
45
45'
time [min]
Ctsk-/-
Muc2
Draq5
Phase
Muc2
Draq5

Figure 6

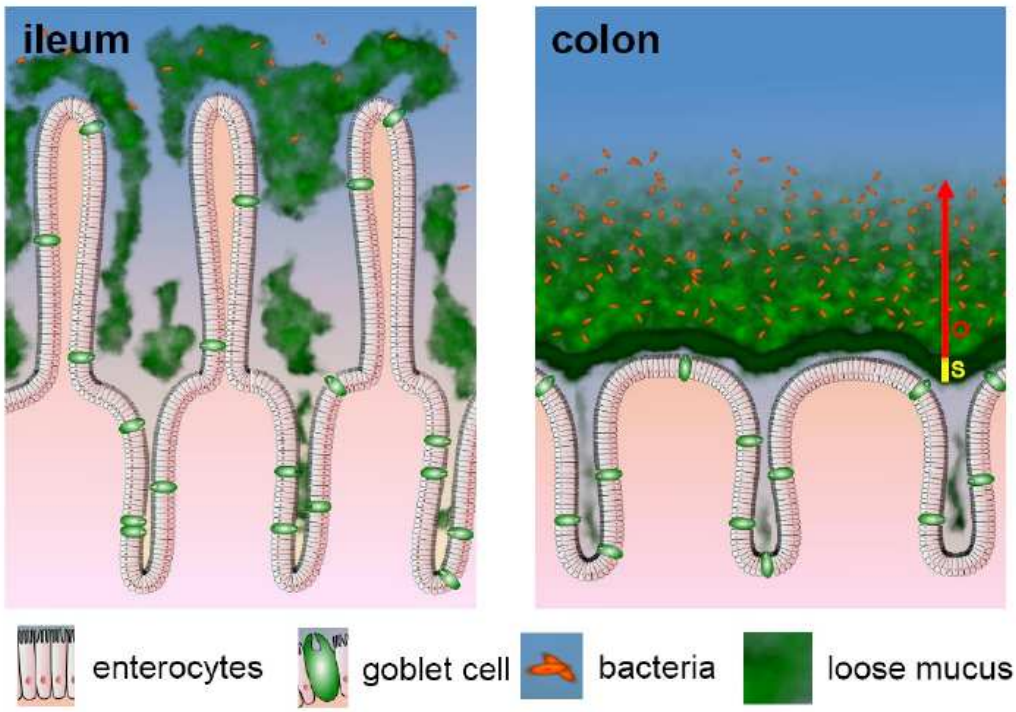
ileum
colon
O
S
enterocytes
goblet cell
bacteria
loose mucus

Figure 7

4.5 Cathepsins B and X along the gastrointestinal tract

The activity and localization patterns of cathepsins B and X in cells of the mouse gastrointestinal tract differ along its length

Maria Arampatzidou[1], Veneta Gerganova[1], Norbert Schaschke[2], Christoph Peters[3], Thomas Reinheckel[3], and Klaudia Brix[1]

[1] School of Engineering and Science, Research Center MOLIFE – Molecular Life Science, Jacobs University Bremen, Campus Ring 6, D-28759 Bremen, Germany
[2] Fakultät für Chemie, Universität Bielefeld, Universitätsstrasse 25, D-33615 Bielefeld, Germany
[3] Institut für Molekulare Medizin und Zellforschung, Albert-Ludwigs-Universität, Stefan-Meier-Str. 17, D-79104 Freiburg, Germany

Email addresses of authors
MA: m.arampatzidou@jacobs-university.de
VG: v.gerganova@jacobs-university.de
NS: norbert.schaschke@uni-bielefeld.de
CP: christoph.peters@mol-med.uni-freiburg.de
TR: thomas.reinheckel@uniklinik-freiburg.de
KBr: k.brix@jacobs-university.de

*Corresponding Author
Dr. Klaudia Brix, Professor of Cell Biology
School of Engineering and Science
Research Center MOLIFE – Molecular Life Science
Jacobs University Bremen
Campus Ring 6, D-28759 Bremen, Germany
Phone: +49 (0)421 200 3246
Fax: +49 (0)421 200 3249
E-mail: k.brix@jacobs-university.de

Keywords: cysteine cathepsins, small intestine, colon, activity-based probe

Abstract

Cysteine cathepsins are expressed in a variety of tissues, including the gastrointestinal tract. Previous studies of our group revealed an involvement of cathepsin B in turnover of extracellular matrix proteins during regeneration of the mouse intestine after traumatic injury. In this study, we investigated the expression and localization pattern of cathepin B and its closest relative, cathepsin X along the length of the intact, non-traumatized gastrointestinal tract, and determined the effects of their absence on this tissue. Initially, we observed that cathepsin B protein levels were highest in duodenum and lowest in jejunum of the intact gastrointestinal tract of wild type mice. Thus, we then inspected cathepsin X expression levels because this enzyme can compensate for the function of cathepsin B, and indeed cathepsin X was most abundant in jejunum and detected in decreasing levels in ileum, colon, and duodenum. However, our findings further showed elevated levels of cathepsin X in the duodenum and colon of *Ctsb*$^{-/-}$ mice but not in the other intestinal parts, namely jejunum and ileum. Therefore, potential compensatory effects of cathepsin X for lacking cathepsin B might take place in a segment-specific manner. Intestinal segment-specific expression patterns were also observed for collagen IV in the WT mice. We further showed that cathepsin-deficiency affected the protein levels of collagen IV differently among the various intestinal parts. This was also true for the brush border enzyme, aminopeptidase N which exhibited enhanced protein levels only in the duodenum of *Ctsz*$^{-/-}$ and *Ctsb*$^{-/-}$/*Ctsz*$^{-/-}$ mice but not of *Ctsb*$^{-/-}$ animals. In conclusion, we observed different activity levels of cysteine peptidases along the length of the small and large intestines in a segment-specific manner. Thus, localization and distribution of specific proteases varies significantly in the intestine mucosa, suggesting different *in situ* functions of these enzymes in specific parts of the gastrointestinal tract. These findings highlight the notion and importance of a delicate balance of proteolytic activities in intact gastrointestinal tissue.

Introduction

It has been shown that the expression and proteolytic activity of cysteine cathepsins are crucial for a variety of physiological processes and pathological conditions [Riese and Chapman, 2000; Turk et al., 2001; Friedrichs et al., 2003; Vasiljeva et al., 2007; Brix et al., 2008]. The majority of these proteases are ubiquitously expressed, like for example cathepsins B and L, whereas some of them are expressed only by specific cell types [Deussing et al., 2002; Brix et al., 2008]. Uncovering the roles of cysteine cathepsins in different tissues and cell types is of importance since they bear a significant potential as drug

targets for novel therapeutic approaches in bone and cartilage disorders, cancer, inflammation, and neurodegenerative diseases [Deaton and Tavares, 2005; Mohamed and Sloane, 2006; Vasiljeva et al., 2007; Reiser et al., 2010]. For example, cathepsin K inhibitors for osteoporosis treatment are currently in phase III clinical trials [Podgorski, 2009; Rachner et al., 2011], while cathepsin B inhibitors are assessed for arthritis, osteoarthritis, and Alzheimer's disease treatment [Reiser et al., 2010].

Cathepsin B is special among the cysteine cathepsins because it exhibits both endopeptidase and carboxypeptidase activity [Musil et al., 1991; Nagler et al., 1997; Kos et al., 2005]. Cathepsin B has been mainly associated with tumor progression and metastasis [Buck et al., 1992; Yano et al., 2001; Mohamed and Sloane, 2006; Vasiljeva et al., 2006; Gocheva et al., 2006; Sevenich et al., 2010]. The more recently identified cathepsin X, also known as cathepsin Z [Santamaria et al., 1998; Nagler et al., 1999], is a protease very similar to cathepsin B, which is also involved in cancer. More precisely, elevated levels of cathepsin X were found in prostate and gastric cancers [Nagler et al., 2004; Buhling et al., 2004]. In contrast to cathepsin B, cathepsin X exhibits only carboxypeptidase activity and it does not act as an endopeptidase [Nagler et al., 1999; Puzer et al., 2005]. Furthermore, the procathepsin X is the only cysteine cathepsin that contains an RGD (Arg-Gly-Asp) motif in an exposed region of the propeptide. This integrin-binding motif suggests a role of procathepsin X in regulating cell adhesion during migration processes [Lechner et al., 2006].

Previous studies of our group have focused on the roles of cysteine cathepsins in mouse intestine [Mayer et al., 2009; Vreemann et al., 2009; Arampatzidou et al., 2011b; Dauth et al., 2011a]. We have shown that *Ctsb*$^{-/-}$, *Ctsk*$^{-/-}$, and *Ctsl*$^{-/-}$ mice exhibit higher levels of collagen IV in their intestine when compared to WT controls, therefore suggesting involvement of these proteases in extracellular matrix (ECM) remodeling. In addition, we found that absence of cathepsin K results in significantly elevated levels of laminin, another basement membrane protein, in the mouse colon. Furthermore, we observed that cathepsin K-deficieny has an impact on the expression and localization pattern of protein constituents of intercellular junctions, such as E-cadherin and occludin [Arampatzidou et al., submitted]. Moreover, cathepsin K has been recently shown to exhibit antimicrobial and anti-inflammatory actions in the mouse intestine [Sina et al., 2012].

In the present study, we focused on the functions of cathepsins B and X along the length of the gastrointestinal tract. Apart from *Ctsb*$^{-/-}$ and *Ctsz*$^{-/-}$ mice, we also studied the double-deficient *Ctsb*$^{-/-}$/*Ctsz*$^{-/-}$ mouse model. Our interest for the *Ctsb*$^{-/-}$/*Ctsz*$^{-/-}$ mice relies on previous studies that have demonstrated compensatory effects of cathepsins B and X in breast carcinoma and gastric epithelial cells [Vasiljeva et al., 2006; Bernhardt et al., 2010]. Intestine tissue isolated from the various cathepsin-deficient mice was divided into segments (duodenum, jejunum, ileum, and colon) and used for both biochemical and morphological

analysis. We determined the activity and expression patterns of cathepsins B and X in cells of the mouse intestine and we found that these patterns are segment-specific. Segment-specific expression pattern was also shown for collagen IV and aminopeptidase N. Our findings highlight the unique features and functions of each intestinal part, in terms of proteolytic events and physiological processes like ECM remodeling.

Results

The activity pattern of cathepsin B differs along the length of the mouse gastrointestinal tract

By using a cathepsin B-specific activity based probe (ABP) [Schaschke et al., 2000] we wanted to visualize the activity levels of cathepsin B in the various intestinal segments, namely duodenum, jejunum, ileum, and colon. We found that the activity of cathepsin B (Figure 1, green signals) varied among the different parts of the gastrointestinal tract. More precisely, we observed more intense staining representative of active cathepsin B in the jejunum of WT mice (Figure 1A, arrowheads) when compared to the duodenum (Figure 1B), ileum (Figure 1C), and colon (Figure 1D). When intestinal tissue of *Ctsb*$^{-/-}$ or *Ctsb*$^{-/-}$/*Ctsz*$^{-/-}$ mice was reacted with the ABP no signal was detectable confirming its specificity (data not shown). The cathepsin B-specificity of this ABP was further confirmed by incubation with the cathepsin B selective inhibitor CA-074 before ABP treatment, because no signal was observed in this control (Figure 1E).

Cathepsin X protein levels are elevated in the intestine of *Ctsb*$^{-/-}$ mice

By immunoblot analysis we initially studied the expression pattern of cathepsin B in all intestinal segments, i.e. duodenum, jejunum, ileum, and colon of cathepsin-deficient mice. No alterations in the protein levels of cathepsin B were observed between WT and *Ctsz*$^{-/-}$ mice (Figure 2A). Absence of cathepsin B was confirmed in *Ctsb*$^{-/-}$ and *Ctsb*$^{-/-}$/*Ctsz*$^{-/-}$ mice (Figure 2A). In contrast, elevated levels of cathepsin X were found in the duodenum and colon of *Ctsb*$^{-/-}$ mice when compared to WT controls (Figure 2B). However, no alterations were observed in the case of jejunum and ileum, suggesting that the compensatory effects of cathepsin X are intestinal segment-specific. The absence of cathepsin X was confirmed in *Ctsz*$^{-/-}$ and *Ctsb*$^{-/-}$/*Ctsz*$^{-/-}$ mice as expected (Figure 2B).

The expression pattern of collagen IV varies among the intestinal parts and between different genotypes of cathepsin-deficient mice

By immunoblot analysis we studied the protein levels of the basal lamina constituent collagen IV in duodenum, jejunum, ileum, and colon of WT, *Ctsb*$^{-/-}$, *Ctsz*$^{-/-}$, and *Ctsb*$^{-/-}$/*Ctsz*$^{-/-}$ mice (Figure 3A). The protein levels of collagen IV differed among the various intestinal parts, with highest protein levels found in the duodenum of WT mice. Interestingly, the levels of collagen IV were decreased in the duodenum and elevated in the jejunum of *Ctsb*$^{-/-}$ mice when compared to WT controls (Figure 3A), thereby contrasting the levels of cathepsin X in these segments of *Ctsb*$^{-/-}$ intestines (see Figure 2). However, no alterations were observed in the ileum and colon of *Ctsb*$^{-/-}$, *Ctsz*$^{-/-}$, and *Ctsb*$^{-/-}$/*Ctsz*$^{-/-}$ mice when compared to WT. In order to study the tissue distribution of collagen IV, immunofluorescence analysis was conducted and collagen IV was detected in the basal lamina (Figure 3B, green signals, arrowheads), as expected. Regarding the signal intensity among the various intestinal parts and for different cathepsin-deficient mice, our findings were in accordance with the immunoblot results. Overall, the findings suggested that the expression pattern of collagen IV depends not only on the genotype of the animals inspected, but it is also strictly dependent on the specific intestinal segment.

Absence of cathepsins B and X had no impact on the protein levels of E-cadherin

Cathepsin B has been shown *in vitro* to cleave E-cadherin [Gocheva et al., 2006]. Therefore, we wanted to investigate whether absence of cathepsins B and/or X would influence the protein levels of this adherens junction protein in the mouse intestine. By immunoblot analysis we found that the protein levels of E-cadherin remained unaltered in *Ctsb*$^{-/-}$, *Ctsz*$^{-/-}$, and *Ctsb*$^{-/-}$/*Ctsz*$^{-/-}$ mice when compared to WT controls, irrespectively of the intestinal segments (Figure 4). There were, however, differences in the banding pattern, such that E-cadherin appeared differently processed or posttranslationally modified in duodenum and jejunum versus ileum and colon (Figure 4). The presence of different molecular forms of E-cadherin was found to be independent of the analyzed genotypes.

Aminopeptidase N exhibits higher protein levels in the duodenum of *Ctsz*$^{-/-}$ and *Ctsb*$^{-/-}$/*Ctsz*$^{-/-}$ mice when compared to WT controls

The next protein that we studied was the differentiation marker and brush border enzyme aminopeptidase N. By immunofluorescence microscopy (Figure 5), it was found that aminopeptidase N-specific antibodies (green signals) resulted in stronger staining of the duodenum of *Ctsz*$^{-/-}$ and *Ctsb*$^{-/-}$/*Ctsz*$^{-/-}$ mice when compared to WT controls. No such enhancements were observed in signal intensity in the other intestinal segments, namely jejunum, ileum, and colon. However, aminopeptidase N was less abundant in the ileum of

Ctsb$^{-/-}$ and *Ctsb*$^{-/-}$/*Ctsz*$^{-/-}$ mice. In the small intestine, i.e. duodenum, jejunum, and ileum, aminopeptidase N was found at the apical plasma membrane of mucosal cells, whereas in the large intestine, i.e. colon, this polarity was reversed.

Discussion

Intestinal cathepsins

The contribution of cysteine cathepsins to intestinal function and homeostasis has been highlighted in various studies [Mayer, 2006; Menzel et al., 2006; Mayer et al., 2009; Vreemann et al., 2009; Arampatzidou et al., 2011b; Sina et al., 2012; Arampatzidou et al., submitted]. Moreover, it has been shown that alterations in the proteolytic network can lead to pathological conditions of the intestine such as inflammation [Medina and Radomski, 2006]. For example, inhibition of cathepsins B and L was found to reduce the mucosal damage in a mouse model of colitis [Menzel et al., 2006]. On the other hand, cathepsin K has recently been shown to play a protective role against chronic DSS-induced colitis. The same study identified extracellular cathepsin K in the lumen of the gastrointestinal tract as an antibacterial factor since the microbiota found in the intestine of *Ctsk*$^{-/-}$ mice was altered when compared to WT controls, and because ectopic application of cathepsin K on the colon protected from colitis-induced tissue damage [Sina et al., 2012]. Moreover, our previous studies on the colon of *Ctsk*$^{-/-}$ mice showed that absence of cathepsin K has an important impact on the distribution and protein levels of ECM constituents and proteins of intercellular junctions [Arampatzidou et al., submitted]. Apart from cathepsin K, we have also demonstrated the involvement of cathepsin B in ECM remodeling during regeneration of the gastrointestinal tract from traumatic injury simulating the life-threatening condition of post-operative ileus [Vreemann et al., 2009].

In the current study, we focused on *Ctsb*$^{-/-}$, *Ctsz*$^{-/-}$, and *Ctsb*$^{-/-}$/*Ctsz*$^{-/-}$ mice in an effort to elucidate the roles of cathepsins B and X in the gastrointestinal tract, and to determine potential compensatory functions of these two proteases. Such compensatory mechanism has been already reported for cathepsins B and X in other tissues [Vasiljeva et al., 2006; Bernhardt et al., 2010], and our question in this study was whether this is also true for the intestine. Furthermore, this is the first report examining the effects of cathepsin-deficiency along the length of mouse intestine in a segment-specific manner. In contrast to other studies that focused only on a specific intestinal part or on the whole intestine as an entity, we chose to analyze each segment separately. More precisely, our biochemical and morphological analysis included duodenum, jejunum, and ileum as representing the small intestine, and the large intestine, i.e. colon.

Segmental differences of proteolytic enzymes in the mouse intestine

It is already known that morphological and functional differences occur between different cell types and among the various intestinal parts. However, our findings now add another, important level to the unique features of each part of the gastrointestinal tract. By using a cathepsin B-selective ABP, we demonstrated that the activity of cathepsin B is not the same among the different intestinal parts. These findings suggest that the proteolytic network found in each intestinal segment is unique and characteristic for the specific region. Interestingly, we also found that cathepsin X protein levels were elevated only in the duodenum and colon of *Ctsb*$^{-/-}$ mice, another observation confirming differences in the proteolytic status of each intestinal part. If indeed cathepsin X can compensate the function of the lost cathepsin B in duodenum and colon, why is it not able to exert the same action in jejunum and ileum as well? The answer to this question might reside within the complexity of the proteolytic systems that are characterized by both, direct and indirect effects. In other words, it is possible that the proteolytic environment as represented by specific proteases and their inhibitors allows cathepsin X to exhibit compensatory tasks in mouse duodenum and colon, whereas in the jejunum and ileum the conditions are not permissive. We conclude that it is important to determine the ratios of specific proteolytic enzymes not only on the cellular or tissue levels but also for distinct tissue segments in order to deduce *in situ* functions of the proteases.

Contributions of cathepsins B and X to establishing polarity of intestinal mucosa cells

We studied the expression and distribution of collagen IV in mouse intestine and found a different expression pattern among the various segments. Moreover, we demonstrated that the protein levels of collagen IV were decreased in the duodenum and elevated in the jejunum of *Ctsb*$^{-/-}$ mice when compared to WT controls. Combining with the above results we concluded that the lower levels of collagen IV in the duodenum of *Ctsb*$^{-/-}$ mice are found in parallel with a dramatic increase in the protein levels of cathepsin X. However, in the case of jejunum absence of cathepsin B and unaltered protein levels of cathepsin X resulted in elevated levels of collagen IV. Since cathepsin X is lacking endopeptidase activity it is unlikely that this protease can play a direct role in ECM degradation [Nagler et al., 1999; Lechner et al., 2006]. Therefore, the observation of elevated levels of cathepsin X in the duodenum of *Ctsb*$^{-/-}$ mice as accompanied by decreased levels of collagen IV could only be assigned to indirect effects of this cysteine cathepsin, and not to direct degradation processes. Maybe cathepsin X is involved in the regulation of other proteases that can exhibit collagenase activity in the mouse intestine. Alternatively, the described and quite unique feature of secreted procathepsin X to interfere with cell adhesion through regulating

interaction of integrins with basal lamina components via RGD motives in focal contacts, make it tempting to speculate that cathepsin X together with the ECM-remodeling cathepsin B are important for the structural integrity of the intestinal mucosa. Another interesting observation in this respect was that the protein levels of aminopeptidase N were found to be elevated only in the duodenum of *Ctsz*$^{-/-}$ and *Ctsb*$^{-/-}$/*Ctsz*$^{-/-}$ mice when compared to WT controls. Both aminopeptidase N and cathepsin X are proteins involved in cell adhesion [Lechner et al., 2006; Mina-Osorio, 2008], thus it would be interesting to investigate whether there is a correlation between these two peptidases in terms of regulation and function.

Conclusions

Overall, our findings highlight for the first time the major differences of protease distribution among various parts of the mouse intestine. We showed that the activity, expression, and distribution patterns of cysteine cathepsins B and X, ECM constituents, and brush border enzymes differ along the length of the gastrointestinal tract, while E-cadherin remained unaffected. Furthermore, the outcomes of cathepsin B and cathepsin X-deficiency depend on the specific regions of the small and large intestine, pointing to the importance of further studies aiming at more detailed and improved assessments of drug safety when cathepsin inhibitors are thought to be orally administered.

Materials and methods

Generation of cathepsin-deficient mice

All cathepsin-deficient mice (*Ctsb*$^{-/-}$, *Ctsz*$^{-/-}$, and *Ctsb*$^{-/-}$/*Ctsz*$^{-/-}$) were generated and genotyped at Albert-Ludwigs-Universität Freiburg, Germany as described elsewhere [Halangk et al., 2000; Sevenich et al., 2010]. All wild type (WT) and cathepsin-deficient mice were 4 months-old and males. The maintenance and breeding of the animals used in this study was performed in accordance with the institutional guidelines in the laboratories of TR and CP approved by local authorities. Tissue handling in Bremen followed institution guidelines and was conducted in S1-certified laboratories.

Tissue samples

WT, *Ctsb*$^{-/-}$, *Ctsz*$^{-/-}$, and *Ctsb*$^{-/-}$/*Ctsz*$^{-/-}$ mice were anesthetized, the abdominal and thoracic cavities were opened and the abdominal aorta was cut. For perfusion via the heart, 0.9% NaCl supplemented with 200 IU heparin (Braun Melsungen AG, Melsungen, Germany) was used. Subsequently, the small and large intestines were dissected and kept on ice. The mesenteries were removed and the intestines were washed with ice-cold 0.9% NaCl solution.

The small intestine was then divided into its three segments, duodenum, jejunum, and ileum. Each intestinal segment was divided into two parts. The anterior part was fixed using 4% paraformaldehyde (PFA) in 200 mM 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES), pH 7.4, and used for morphological studies while the posterior part was snap-frozen in liquid nitrogen and used for biochemical analysis.

Total tissue extracts were isolated with lysis buffer, PBS containing 0.5% Triton X-100, and homogenization of the samples was done using a Potter S homogenizer (Sartorius, Göttingen, Germany) at 1000 rpm for 5 min on ice. Homogenates were kept in a rotary mixer for 45 min while all steps were performed at 4˚C. After centrifugation for 10 min at 10 000 *g*, supernatants were stored at -20˚C. Protein concentration was determined using BSA as a protein standard [Neuhoff et al., 1979].

SDS-PAGE and immunoblotting

Total tissue extracts were normalized to equal amounts of protein, i.e. 16 µg per lane, and loaded onto 8% or 12.5% SDS-polyacrylamide gels. The separated proteins were then semi-dry blotted onto nitrocellulose membranes. In order to detect collagen IV, gel electrophoresis was performed under native conditions [Vreemann et al., 2009], since the collagen IV-specific antibodies recognize only non-denatured antigen. Blocking was performed overnight at 4˚C using 5% milk powder in PBS containing 0.3% Tween-20. After blocking, the following primary antibodies were applied: rabbit anti-mouse collagen IV (Rockland, Philadelphia, USA), goat anti-mouse cathepsin B (Neuromics, through Acris Antibodies, Herford, Germany), goat anti-mouse cathepsin X (R&D Systems, Wiesbaden, Germany), and rabbit anti-human E-cadherin (Santa Cruz Biotechnology Inc., Santa Cruz, CA USA). Membranes were subsequently washed and incubated for 1 h at room temperature with HRP-conjugated antibodies. The secondary antibodies used were goat anti-rabbit IgG and rabbit anti-goat IgG (both from Southern Biotech, Birmingham, Alabama, USA). Immunoreactions were visualized by using enhanced chemiluminescence substrate on CL-XPosure film (both from Pierce through Perbio Science Europe, Bonn, Germany).

Morphological analysis-immunofluorescence

After fixation with 4% PFA in 200 mM HEPES, all intestinal parts (duodenum, jejunum, ileum, and colon) from WT, *Ctsb*$^{-/-}$, *Ctsz*$^{-/-}$, and *Ctsb*$^{-/-}$/*Ctsz*$^{-/-}$ mice were washed and samples were incubated overnight in 200 mM HEPES buffer (pH 7.4). From each tissue sample, pieces of approximately 1 cm length were cut and incubated in Tissue Freezing Medium (Jung, through Leica Microsystems, Nussloch, Germany). After overnight incubation, samples were frozen on dry ice and cryosections of 5 µm each were prepared by using a Leica CM1900 cryostat (Leica Microsystems). Cryosections were mounted on adhesive microscope slides,

blocked with 3% bovine serum albumin (BSA) in calcium-magnesium-free (CMF) PBS, and immunolabeled with primary antibodies. Specific primary antibodies used in this study were goat anti-mouse cathepsin B (Neuromics, through Acris Antibodies, Herford, Germany), goat anti-mouse cathepsin X (R&D Systems, Wiesbaden, Germany), rabbit anti-mouse collagen IV (Novotec, Saint Martin La Garenne, France), and goat anti-mouse aminopeptidase N (R&D Systems). After several washing steps with 0.1% BSA in CMF-PBS, sections were incubated with secondary antibodies for 1 h at 37˚C. As secondary antibodies Alexa 488-coupled goat anti-rabbit IgG and rabbit anti-goat IgG were used (both from Invitrogen through Molecular Probes, Karlsruhe, Germany). As nuclear counter-stain DRAQ5™ (Biostatus Limited, Shepshed Leicestershire, UK) was applied together with the secondary antibodies. In negative controls, specific primary antibodies were omitted and sections were incubated only with secondary antibodies and DRAQ5™.

Direct labeling of active cathepsin B in tissue cryosections

A cathepsin B-specific activity based probe, i.e. compound NS-173; R = NH-$(CH_2)_6$-NH-rhodamine B was used for detection of active cathepsin B [Schaschke et al., 2000]. Cryosections of duodenum, jejunum, ileum, and colon were prepared as described elsewhere [Buth et al., 2004; Arampatzidou et al., 2011a]. In brief, the sections were initially incubated with freshly prepared L-cysteine for 10 min at 37˚C to reactivate cysteine peptidase activities and then the ABP was applied for 30 min at 37˚C. After incubation with the ABP several washing steps in CMF-PBS were performed. In controls, cryosections were pre-incubated with the cathepsin B-specific inhibitor CA-074 (Merck, Darmstadt, Germany) before ABP treatment. Both the ABP and the inhibitor were applied at a concentration of 500 nM.

Microscopy

Immunolabeled and ABP-treated cryosections were viewed with a Zeiss LSM 510 META laser scanning microscope equipped with Argon and Helium-Neon lasers (Carl Zeiss GmbH, Jena, Germany). Optical sections were obtained with a pinhole setting of 1 Airy unit and at a resolution of 1,024×1,024 pixels and were further analyzed by using LSM 510 software, Release 3.2 (Carl Zeiss).

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Figure legends

Figure 1 Visualization of cathepsin B activity in the intestine of WT mice

Confocal laser scanning micrographs of cryosections prepared from jejunum (A), duodenum (B), ileum (C), and colon (D) of WT mice after incubation with a cathepsin B-specific ABP (green signals). Note that the activity of cathepsin B, shown in a vesicular pattern (arrowheads), was higher in the jejunum in comparison to the other segments. In the control (E), the cryo-sections were pre-incubated with the inhibitor CA-074 before ABP treatment. No activity was detected in this control. Corresponding phase contrast micrographs are shown (A′-E′). Asterisks indicate the position of lamina propria of the villi. Bars, 50 µm.

Figure 2 Expression pattern of cathepsins B and X in mouse intestine

Immunoblot analysis of lysates prepared from the intestine, i.e. D=duodenum, J=jejunum, I=ileum, and C=colon of WT, *Ctsb*$^{-/-}$, *Ctsz*$^{-/-}$, and *Ctsb*$^{-/-}$/*Ctsz*$^{-/-}$ mice as indicated. No alterations were observed in cathepsin B protein levels between WT and *Ctsz*$^{-/-}$ mice (A). Note that cathepsin X protein levels were elevated in the duodenum and colon of *Ctsz*$^{-/-}$ mice but not in the jejunum and ileum (B). Molecular mass markers are given in the left margins.

Figure 3 Localization and expression pattern of collagen IV along the length of mouse intestine

(A) Immunoblot analysis of lysates prepared from the intestine, i.e. D=duodenum, J=jejunum, I=ileum, and C=colon of WT, *Ctsb*$^{-/-}$, *Ctsz*$^{-/-}$, and *Ctsb*$^{-/-}$/*Ctsz*$^{-/-}$ mice as indicated. Note that the protein levels of collagen IV were decreased in the duodenum and elevated in the jejunum of *Ctsb*$^{-/-}$ mice when compared to WT controls. No alterations were observed in the ileum and colon of *Ctsb*$^{-/-}$, *Ctsz*$^{-/-}$, and *Ctsb*$^{-/-}$/*Ctsz*$^{-/-}$ mice when compared to WT. (B) Confocal laser scanning micrographs of cryosections prepared from duodenum, jejunum, ileum, and colon of WT and *Ctsb*$^{-/-}$ mice after staining with collagen IV-specific antibodies. Collagen IV (green signals, side panels, middle) was present in the basal lamina (arrowheads), and less intense labeling was observed in the duodenum of *Ctsb*$^{-/-}$ mice when compared to WT controls. In the jejunum of *Ctsb*$^{-/-}$ mice, collagen IV exhibited stronger staining when compared to WT. No differences in signal intensity were observed between WT and *Ctsb*$^{-/-}$ mice in the case of ileum and colon. Corresponding phase contrast micrographs are shown in side panels, top, or as insets. DRAQ5™ was used as nuclear counter-stain (blue signals, side panels, bottom). Bars, 50 µm.

Figure 4 E-cadherin protein levels in the intestine of WT and cathepsin-deficient mice

(A) Immunoblot analysis of lysates prepared from the intestine, i.e. D=duodenum, J=jejunum, I=ileum, and C=colon of WT, *Ctsb*$^{-/-}$, *Ctsz*$^{-/-}$, and *Ctsb*$^{-/-}$/*Ctsz*$^{-/-}$ mice as indicated. No alterations were observed in the protein levels of E-cadherin in all intestinal segments of cathepsin-deficient mice when compared to WT controls. Molecular mass markers are given in the left margin.

Figure 5 Distribution and localization of aminopeptidase N in the mouse intestine

Confocal laser scanning micrographs of cryosections prepared from the intestine, i.e. D=duodenum, J=jejunum, I=ileum, and C=colon of WT, *Ctsb*$^{-/-}$, *Ctsz*$^{-/-}$, and *Ctsb*$^{-/-}$/*Ctsz*$^{-/-}$ mice as indicated, after staining with aminopeptidase N-specific antibodies. Note the localization of aminopeptidase N (green signals) at the apical plasma membrane in duodenum (A1-A4), jejunum (B1-B4), and ileum (C1-C4) that is replaced in the colon (D1-D4) by the reverse orientation. Stronger staining of aminopeptidase N was observed in the duodenum of *Ctsz*$^{-/-}$ (A3) and *Ctsb*$^{-/-}$/*Ctsz*$^{-/-}$ (A4) mice when compared to WT (A1), while weaker staining was detectable in the ileum of *Ctsb*$^{-/-}$ (C2) and *Ctsb*$^{-/-}$/*Ctsz*$^{-/-}$ (C4) mice in comparison to WT controls. In the other intestinal segments no alterations were observed between WT and cathepsin-deficient mice. Bars, 50 µm.

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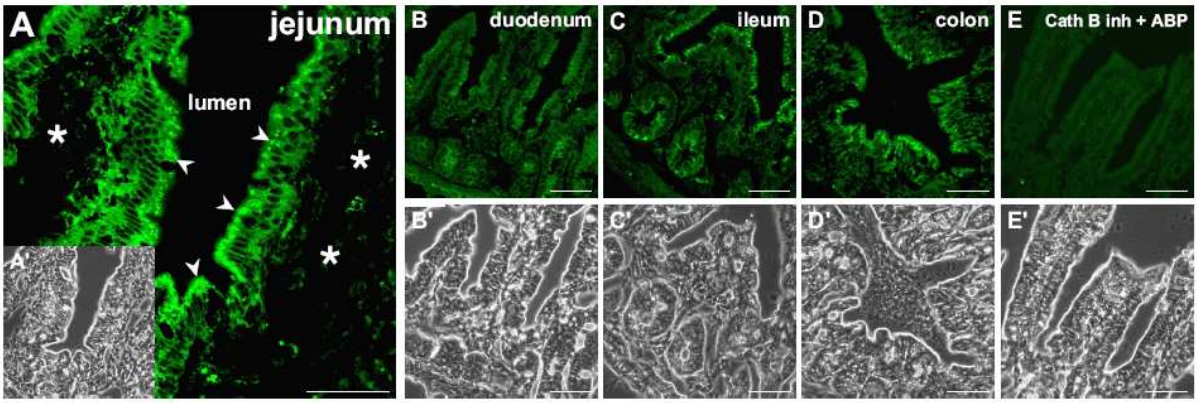
A
jejunum
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A'
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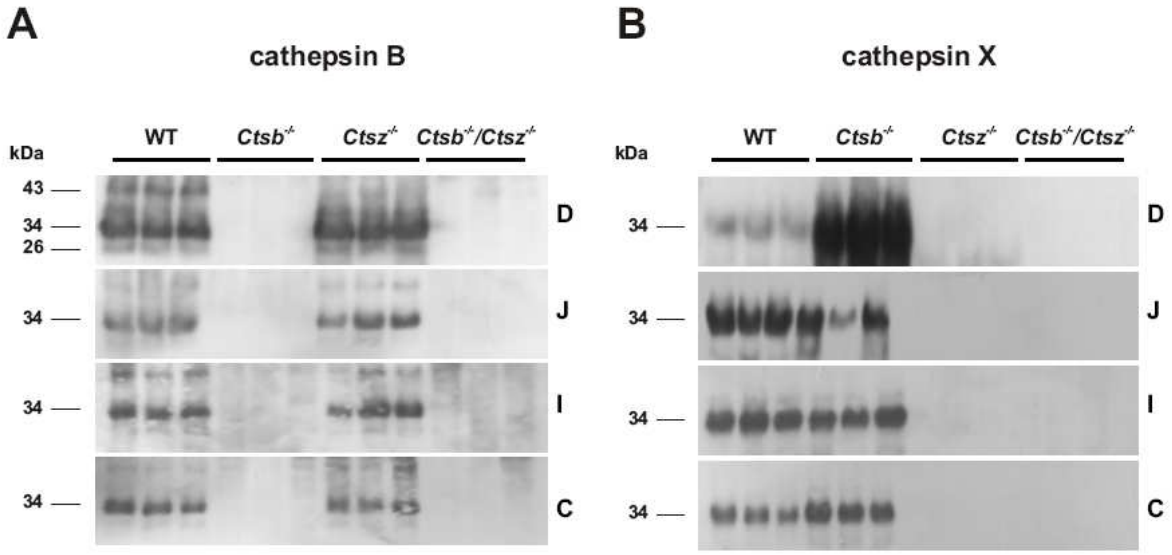
Figure 1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cathepsin X
WT
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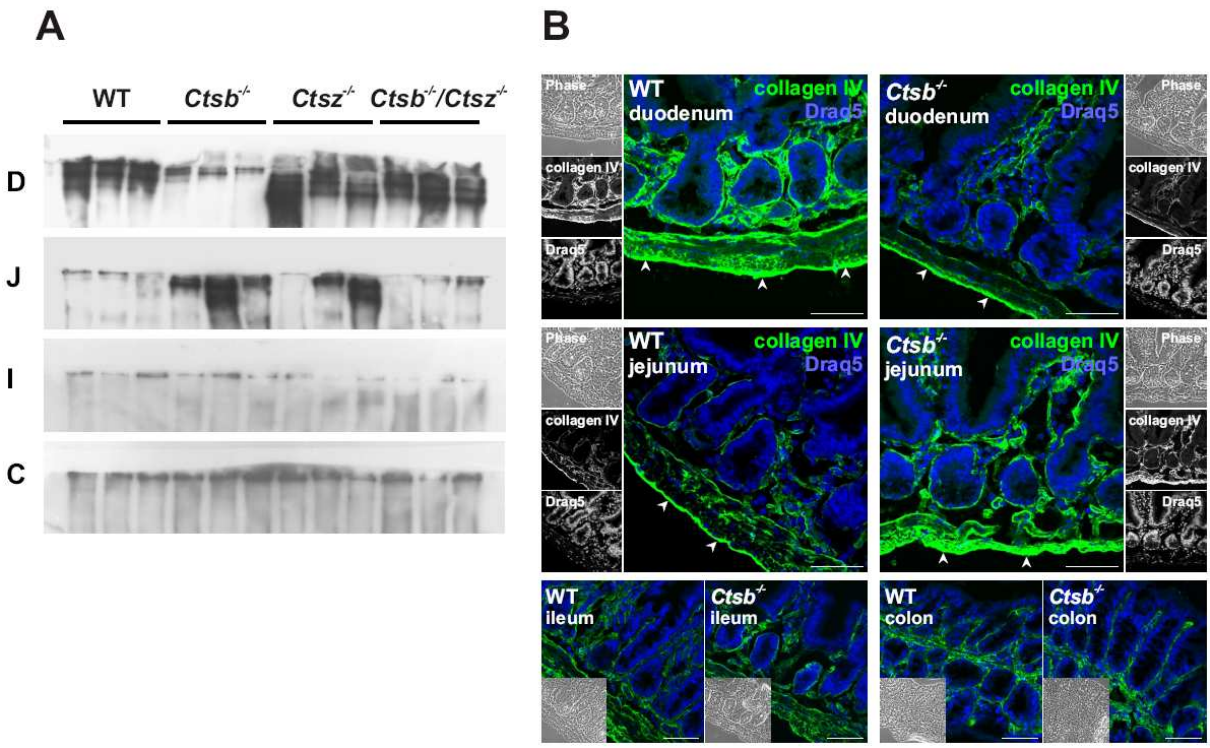
Figure 2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duodenum
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Figure 3

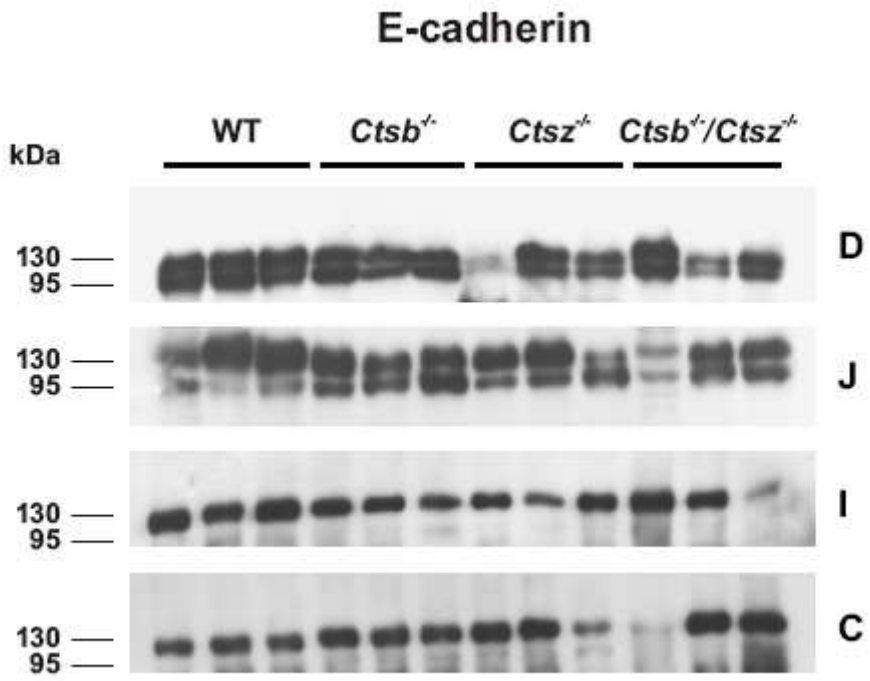
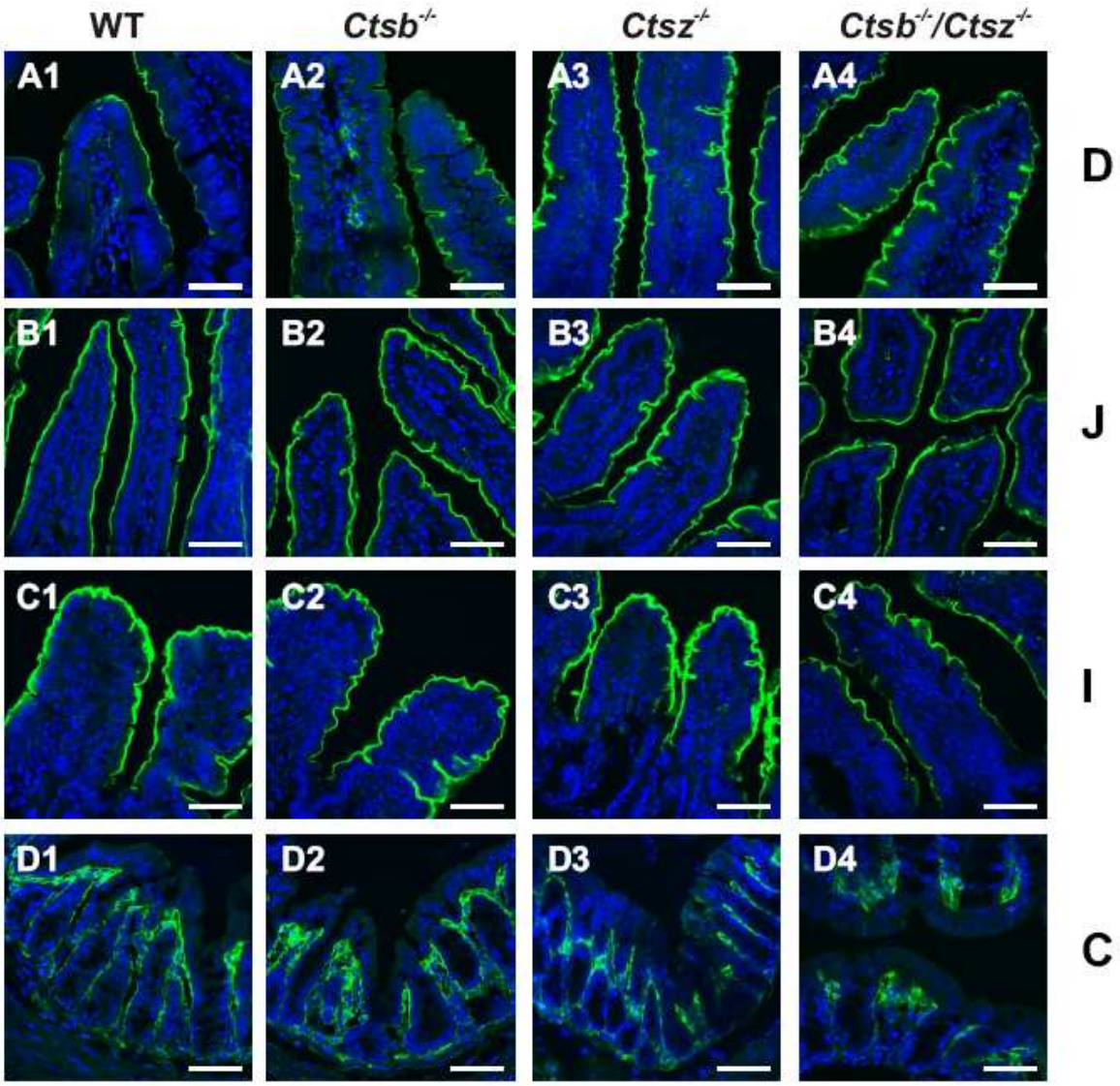


Figure 4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C1
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C3
C4
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D1
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Figure 5

5 Discussion

The results of this study revealed an important contribution of cysteine cathepsins to intestinal homeostasis through their involvement in diverse processes. Such physiological processes include remodeling of the ECM and turnover of proteins constituting junctional complexes that are crucial for intestinal function and integrity of the tissue structure. Further studies are required in order to elucidate the molecular mechanisms underlying the actions of cysteine cathepsins in the intestine and to determine whether they rely on direct or indirect effects. Furthermore, we highlighted major differences along the length of the gastrointestinal tract and emphasized the unique morphological, functional, and proteolytic properties of each intestinal part.

5.1 Distribution of cysteine cathepsins in the intestine

Cysteine cathepsins exhibit different localization and distribution patterns in the intestine revealing a variety in scenes of actions for these proteolytic enzymes. In particular, cathepsins B and X were localized within endo-lysosomes, but the distribution of cathepsin B- and cathepsin X-containing vesicles was quite different. Vesicle populations loaded with distinct sets of proteases have been previously described in other cell types such as thyroid epithelial cells [Tepel et al., 2000; Jordans et al., 2009; Tedelind et al., 2011]. On the other hand, cathepsins K and L were mainly found in association with the apical plasma membrane of intestinal epithelial cells and were also detected in the secretions of goblet cells [Mayer et al., 2006; Brix et al., 2008]. Therefore, secretion of distinct cysteine cathepsins towards the intestinal mucus layer and the luminal space could be foreseen.

Several studies have contributed in changing our view of cysteine cathepsins as proteases exclusively found within the endo-lysosomes. It is now well established that these enzymes and their molecular variants exert their tasks in diverse locations, including mitochondria, nucleus, and the extracellular space [Muntener et al., 2004; Goulet et al., 2004; Brix et al., 2008; Tedelind et al., 2011; Sina et al., 2012]. The trafficking and localization patterns of cathepsins depend also on the status of the respective cell or tissue since it has been shown that in disease states, like for example in malignancies, these patterns can be altered [Mohamed and Sloane, 2006; Tedelind et al., 2011]. As far as pathological conditions of the intestine are concerned, previous studies demonstrated a non-regulated release of cathepsin B after intestinal traumatic injury [Mayer et al., 2009; Vreemann et al., 2009].

5.2 Cathepsin-deficient mice as valuable model system

In order to elucidate the roles of cathepsins in a given tissue or cell type it is crucial to perform studies with suitable *in vitro* and *in vivo* models. A relevant model system to address what are the functions of specific cathepsins *in vivo* is the cathepsin-deficient mouse. In our

studies we used *Ctsb*$^{-/-}$ and *Ctsz*$^{-/-}$ mice (chapter 4.5) that do not have any overt phenotype, as well as *Ctsk*$^{-/-}$ mice (chapter 4.4). The *Ctsk*$^{-/-}$ mice are characterized by phenotypic alterations and they develop disorders such as osteopetrosis [Saftig et al., 1998]. Although cathepsin K is known to be expressed in the intestinal tract, the effects of cathepsin K-deficiency on this tissue have not been previously studied in detail. Therefore, we compared intestinal tissue from WT and *Ctsk*$^{-/-}$ mice and we observed differences that are associated with properties of the ECM and characteristics of intercellular junctions (see chapter 4.4). We also studied mice doubly-deficient for cathepsins B and X since compensatory phenomena have been shown for these two proteases. Like *Ctsb*$^{-/-}$ and *Ctsz*$^{-/-}$ mice, the *Ctsb*$^{-/-}$/*Ctsz*$^{-/-}$ animals have a phenotype comparable to WT without any obvious differences. In general, the use of double cathepsin-deficient mice could provide a better understanding of complex proteolytic networks in which redundancy and compensatory mechanisms exist. However, in some cases single or double cathepsin-deficiency can be lethal, like for example the *Ctsd*$^{-/-}$ and the *Ctsb*$^{-/-}$/*Ctsl*$^{-/-}$ animals that die within the first weeks after birth [Saftig et al., 1995; Felbor et al., 2002].

Since cathepsins have been associated with a variety of pathological conditions, cathepsin-deficient mice are often used in combination with diseased mouse models to address whether absence of a specific cathepsin could ameliorate the severity of the investigated disease. The cathepsin-deficient mice have significantly contributed in gaining substantial knowledge regarding the involvement of cathepsins in health and disease. Nevertheless, there are some drawbacks regarding this model system that should be taken into consideration. The major problem is that the proteolytic networks of human and mice are not identical because each species has a unique set of cathepsins and proteases in general. Therefore, it is difficult to predict what the influence of cathepsin-deficiency would be in a completely or even in a slightly different proteolytic environment. It is important to keep in mind that studies on mouse models, including cathepsin-deficient mice, can give us research directions and indications rather than conclusions directly applicable in investigations on human tissue from patients.

5.3 Visualization and trafficking studies enable imaging of protease functions *in vitro* and *in vivo*

A variety of experimental strategies have been developed to enable spotting of proteases in classical and novel scenes of action (see [Arampatzidou et al., 2011a], chapter 4.1). Such approaches allow visualization and monitoring of proteolytic activity both *in vitro* and *in vivo*. For instance, fluorescent protein tagging experiments are often used to elucidate trafficking and transport pathways of proteases with the aim to reveal unknown biological functions of these enzymes. In general, cysteine cathepsins can be tagged by attaching fluorescent proteins to the C-terminus via a spacer peptide, in such a way that intrinsic protein

sequences essential for proper protein targeting and transport are not affected [Linke et al., 2002; Katayama et al., 2008; Mayer et al., 2008]. In chapter 4.3 [Arampatzidou et al., 2011b] we described the generation of a transgenic mouse model that was expected to express cathepsin B-EGFP in an intestine-specific manner. This mouse model would enable us to investigate the trafficking and distribution of cathepsin B *in situ* in order to better understand the functional roles of this protease in intestinal homeostasis.

Apart from studying the localization of proteases, it is also important to monitor their proteolytic activity. Recently, small reporter molecules, known as activity based probes (ABPs), were developed which are used for studying protease activities and substrate cleavage in cells and tissues [Greenbaum et al., 2002; Kato et al., 2005; Blum et al., 2009; Deu et al., 2012]. The advantage of ABPs is that these molecules can penetrate biological membranes thereby reaching proteolytically active enzymes within living cells and in whole organisms [Blum et al., 2007; Deu et al., 2012].

5.4 Proteolytic network and compensatory effects of cysteine cathepsins in mouse intestine

Cysteine cathepsins are part of a highly complex and dynamic proteolytic network that participates in variable processes both in health and disease states. Apart from proteases, this proteolytic system includes also their natural counter-parts, namely endogenous inhibitors that regulate their enzymatic activity. Proteolytic misbalance has been associated with many pathological conditions, including cancer, neurodegenerative disorders, osteoporosis, and intestinal inflammation [Medina and Radomski, 2006; Menzel et al., 2006; Mohamed and Sloane, 2006; Reiser et al., 2010]. Moreover, the set of proteases and inhibitors found in each tissue or cell type is unique because some proteases exhibit tissue or cell-specific expression while other proteolytic enzymes are ubiquitously distributed. Thus, observations regarding proteolytic events and functions made in a particular tissue should not be considered identical in different experimental settings in which other tissues or cell types are studied. Additionally, an important characteristic of proteolytic networks is the compensatory mechanisms developed to protect overall cellular and tissue homeostasis. Compensation, also known as redundancy, has been demonstrated for several cysteine cathepsins in a variety of cells and tissues. For example, cathepsins K and L were shown to exhibit reciprocal compensation in the thyroid gland [Friedrichs et al., 2003]. Moreover, redundancy was shown for cathepsins B and X in breast carcinoma and gastric epithelial cells [Vasiljeva et al., 2006; Bernhardt et al., 2010].

The results presented in chapters 4.4 and 4.5 revealed that cathepsin-deficiency leads to altered proteolytic networks in mouse intestine. More precisely, we found significantly elevated levels of cathepsins B, L, and X in the colon of *Ctsk*$^{-/-}$ mice when compared to WT. Another interesting finding was that cathepsin X protein levels were elevated in the

duodenum and colon of *Ctsb*$^{-/-}$ mice but not in the other intestinal segments, namely jejunum and ileum. In addition, we observed differential expression and activity patterns of cathepsins B and X along the length of the gastrointestinal tract (see chapter 4.5). Taken together, these observations made us speculate that the proteolytic environment of mouse duodenum and colon allows cathepsin X to exert its compensatory tasks in contrast to non-permissive conditions found in jejunum and ileum. It is important to note, however, that cathepsin X could only partially compensate the function of cathepsin B since it can only act as a carboxypeptidase and does not have endopeptidase activity like cathepsin B [Nagler et al., 1999; Puzer et al., 2005].

5.5 Involvement of cysteine cathepsins in ECM remodeling

Various studies have demonstrated the ability of cysteine cathepsins to degrade ECM components and promote cell migration. For instance, cathepsin B contributes in remodeling of ECM during keratinocyte migration [Buth et al., 2004; Buth et al., 2007]. It has been shown that cathepsin B can degrade ECM constituents such as collagen IV, laminin, and fibronectin at both acidic and neutral pH [Buck et al., 1992; Kos et al., 2005]. However, the ability of cathepsin B to degrade ECM is associated not only with physiological processes but also with pathological conditions including tumor invasion and metastasis [Hazen et al., 2000; Mohamed and Sloane, 2006; Vasiljeva et al., 2006; Chan et al., 2010]. Moreover, cathepsin B has been suggested to play a role in turnover of ECM proteins in traumatized intestinal tissue [Vreemann et al., 2009]. In this study we focused on the intact, non-traumatized gastrointestinal tract and we showed that cathepsin B is also involved in remodeling of intestinal ECM under physiological conditions (see chapter 4.5). In addition, our studies on *Ctsb*$^{-/-}$ mice revealed that absence of cathepsin B has different influence along the gastrointestinal tract that is characterized by elevated or decreased protein levels of collagen IV.

Interestingly, the decreased levels of collagen IV in the duodenum of *Ctsb*$^{-/-}$ mice were accompanied by dramatically increased protein levels of cathepsin X. In contrast, the levels of cathepsin X in the jejunum of *Ctsb*$^{-/-}$ mice were not altered while collagen IV protein levels were increased in this intestinal part (see chapter 4.5). It is important to note that cathepsin X acts as a carboxypeptidase and has no endopeptidase activity hence a direct contribution of this cysteine cathepsin in ECM degradation is unlikely [Nagler et al., 1999; Puzer et al., 2005; Lechner et al., 2006]. A possible explanation for the simultaneous elevated and decreased protein levels of cathepsin X and collagen IV, respectively, could be an indirect involvement of cathepsin X through proteolytic regulation of other proteases that are able to degrade ECM constituents like the collagenases. Moreover, since procathepsin X is known to be important modulator of cell adhesion and migration processes by regulating interaction of integrins with basal lamina constituents [Lechner et al., 2006], it is tempting to speculate that

together with cathepsin B, these two proteases are crucial for intestinal structure and integrity. Further studies are required to investigate in depth such protein functions of cathepsin X that are independent of proteolytic activity but still important for tissue homeostasis.

Another cysteine cathepsin with a pivotal role in ECM degradation is cathepsin K, known to participate in bone remodeling [Bromme and Okamoto, 1995; Saftig et al., 1998]. The results of chapter 4.4 suggested that cathepsin K is involved in turnover of intestinal ECM since we found elevated levels of collagen IV and laminin in the colon of *Ctsk*$^{-/-}$ mice when compared to WT controls. Besides colon, *Ctsk*$^{-/-}$ mice exhibited higher levels of collagen IV also in their small intestine (see chapter 4.2) [Dauth et al., 2011a]. Uncovering the roles of cathepsin K in intestinal homeostasis is of special importance because inhibitors of this protease are currently in clinical trials for treatment of bone disorders [Lewiecki, 2009; Podgorski, 2009; Rachner et al., 2011]. Therefore, it is crucial to elucidate the effects of cathepsin K inhibition in tissues, like the gastrointestinal tract, that would be primarily influenced by orally administered therapeutics.

5.6 Impact of cathepsin K-deficiency on intercellular junctions of the colon

The results of chapter 4.4 demonstrated that cathepsin K-deficiency is concomitant with altered protein levels of ECM components, like collagen IV and laminin. In the same study we also investigated the effects of this deficiency on protein constituents of junctional complexes because cathepsins are known to exert profound influence on the expression and distribution of intercellular junctions [Gocheva et al., 2006; Boudreau et al., 2007]. Our findings revealed that absence of cathepsin K resulted in elevated levels of the adherens junction protein E-cadherin. In addition, mislocalization of occludin was observed in the colon of *Ctsk*$^{-/-}$ mice since this tight junction protein was found throughout the intestinal epithelial cells and away from their apical poles.

Our observations are consistent with other studies that have demonstrated such localization shifts of tight junction proteins associated with altered activities of cysteine cathepsins. In particular, absence of cathepsin L was shown to promote distribution of claudin-1 in the nucleus of intestinal epithelial cells [Boudreau et al., 2007]. Moreover, in a mouse model of intestinal manipulation mimicking the human condition of postoperative ileus, non-physiological release of cathepsin B resulted in damage of the cell junction proteins E-cadherin and claudin-1 [Vreemann et al., 2009]. The question that remains elusive is whether cathepsins are directly involved in degradation of intestinal intercellular junctions *in vivo*. *In vitro* studies have shown that specific cysteine cathepsins, namely cathepsins B, L, and S are able to act on E-cadherin as a substrate [Gocheva et al., 2006]. We now need to

investigate the ability of cathepsin K to degrade E-cadherin, a finding that could then explain the elevated levels of E-cadherin in the colon of *Ctsk*$^{-/-}$ mice.

It is well established that cell-cell and cell-matrix interactions have pivotal roles in a variety of processes such as adhesion, proliferation, differentiation, and migration and they are important players in maintaining intestinal barrier function and integrity. Therefore, alterations in the expression and localization patterns of ECM and junctional complex proteins are often associated with pathologies of the intestinal tract [Gibson et al., 1995; Hanby et al., 1996; Beaulieu, 1997; Karayiannakis et al., 1998; Schmitz et al., 1999; Gassler et al., 2001]. The involvement of cysteine cathepsins in turnover of ECM components and junctional proteins highlights the importance of these proteolytic enzymes in intestinal homeostasis and stability.

5.7 Intestinal mucus organization and growth in cathepsin K-deficient mice

The intestinal barrier function is crucial for the organism and impairment of this barrier can lead to pathological conditions of the intestine such as inflammatory bowel disease [Einerhand et al., 2002; Hansson, 2011]. The mucus layer covering the intestinal epithelial cells and protecting them from the luminal content has profound roles in regulating intestinal barrier function. Goblet cells are the intestinal cells responsible for production and secretion of Muc2 which is the major component of the mucus layers [Johansson et al., 2011b]. Recent studies demonstrated the presence of cathepsin K in murine goblet cells and colonic mucus layer [Sina et al., 2012]. Similar localization patterns were also observed in previous studies of our group that revealed localization and secretion of cathepsins K and L from human intestinal goblet cells and their re-association with the apical plasma membrane domains [Mayer et al., 2006]. Moreover, extracellular cathepsin K was recently identified as an antibacterial factor with anti-inflammatory potential that can ameliorate the severity of chronic DSS-induced mouse colitis [Sina et al., 2012].

Based on these observations we wanted to investigate whether cathepsin K-deficiency had an impact on structural and functional properties of intestinal mucus. In the study described in chapter 4.4 we used a newly established *ex vivo* method [Gustafsson et al., 2011] for measuring murine intestinal mucus thickness and growth. Our results revealed that the organization and growth properties of intestinal mucus are comparable in *Ctsk*$^{-/-}$ and WT mice without major differences. Moreover, immunofluorescence analysis of Muc2 in intestinal cryosections of WT and *Ctsk*$^{-/-}$ mice did not show any alterations in the expression and localization pattern of this mucin. However, since other cysteine cathepsins were found to be up-regulated in the colon of *Ctsk*$^{-/-}$ mice we speculate that compensatory processes might be involved that could explain the lack of mucus alterations. Such compensatory functions could be suggested for cathepsin L because this protease was found like cathepsin K in human goblet cells [Mayer et al., 2006] and in our study it was dramatically increased in the colon of

Ctsk$^{-/-}$ mice. Therefore, further research is required with a larger set of cathepsin-deficient mice and ideally with the double-deficient *Ctsk*$^{-/-}$/*Ctsl*$^{-/-}$ before we can exclude involvement of cathepsin K in mucus processing.

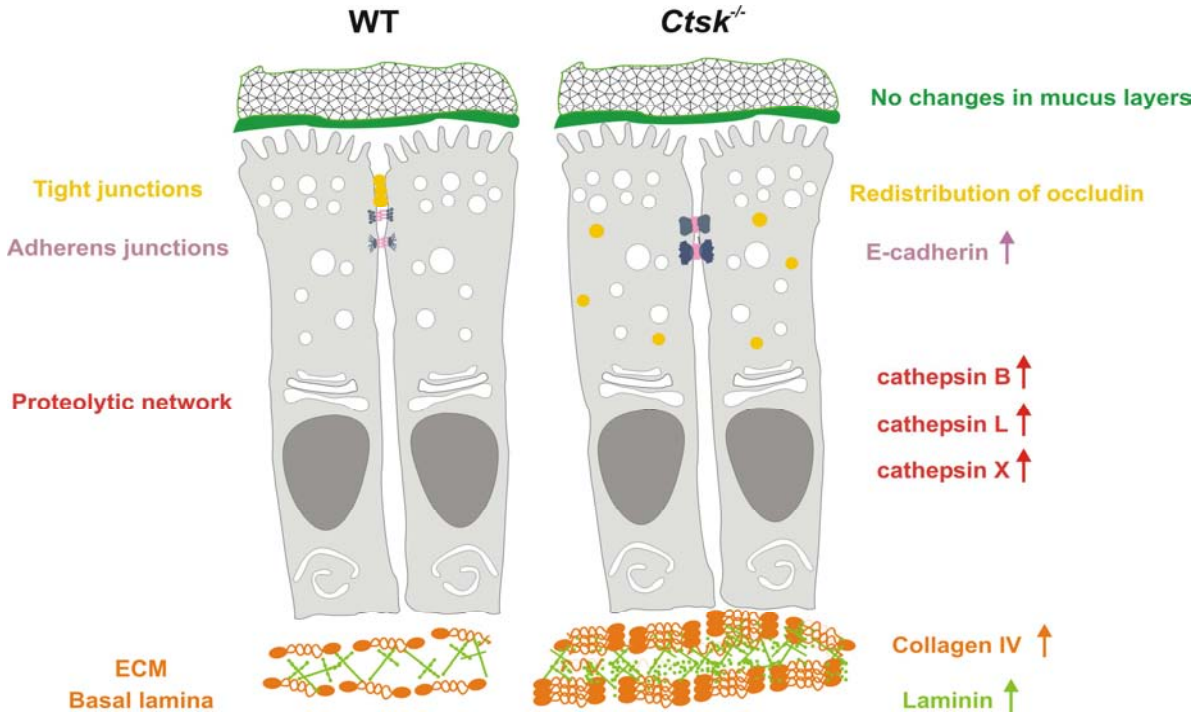

Figure 10: Schematic overview summarizing the alterations observed in the colon of cathepsin K-deficient mice. Our studies revealed alterations in the proteolytic network of *Ctsk*$^{-/-}$ mice when compared to WT, characterized by elevated levels of cathepsins B, L, and X. In addition, absence of cathepsin K had an impact on tight and adherens junctions. More precisely, in the colon of *Ctsk*$^{-/-}$ mice we observed redistribution of occludin that was found throughout the cell and not in the tight junctions. Moreover, E-cadherin protein levels were elevated in *Ctsk*$^{-/-}$ mice, although without changes in its localization pattern. ECM and basal lamina constituents were also influenced by cathepsin K-deficiency since we observed elevated levels of collagen IV and laminin in the colon of *Ctsk*$^{-/-}$ mice.

5.8 Differences in morphology, function, and proteolytic activity along the gastrointestinal tract

The multifunctional gastrointestinal tract is composed of different parts that exhibit characteristic structural and morphological features. The individual intestinal parts are involved in a variety of processes including digestion, absorption of water and nutrients, storage of waste products, and immune defence [Feldman and Schiller, 1997; Schneeman, 2002; Alberts, 2002]. Because of these established differences observed along the length of the gastrointestinal tract, our hypothesis was that the various parts should possess further unique characteristics that have not been reported yet. Therefore, we chose to investigate the expression and localization patterns of cysteine cathepsins in each part of the small and large intestine separately.

The results outlined in chapter 4.5 confirmed our initial hypothesis since the protein levels of cathepsins B and X were different among the intestinal parts. Interestingly, cathepsin B protein levels were highest in duodenum and lowest in jejunum while the opposite expression pattern was observed for cathepsin X that exhibited highest protein levels in jejunum and lowest in duodenum. Apart from variable protein levels, we also showed segment-specific proteolytic activity. By using a cathepsin B-specific ABP we demonstrated highest proteolytic activity of cathepsin B in mouse jejunum while in the other segments we observed less activity. Our results report for the first time that the localization and distribution of specific proteases vary significantly among the intestinal parts, suggesting different functions of these enzymes along the gastrointestinal tract. This variable proteolytic network found in each intestinal segment could also explain the segment-specific compensatory effects of cathepsin X observed in the intestine of *Ctsb*$^{-/-}$ mice. The notion of an important role of cysteine cathepsins in ECM remodeling prompted us to address the status of collagen IV in each intestinal part. Our findings indicated segment-specific expression pattern of collagen IV that exhibited higher protein levels in duodenum when compared to jejunum, ileum, and colon. We suggest that the observed differences in collagen IV protein levels are associated with specific requirements of ECM composition that is crucial for the structural and functional properties of each intestinal part.

Overall, our findings presented in chapter 4.5 highlight the major differences along the gastrointestinal tract in terms of morphology, structure, function, and proteolytic activity. This study also emphasizes the importance and necessity to consider the intestinal tract not as an entity but rather as a set of diverse tissue parts exhibiting unique properties and characteristic features. This last notion is particularly important for development of orally administered drugs that would probably have different outcomes on the various intestinal parts.

6 Conclusion

Overall, our findings suggest that cysteine cathepsins exert diverse tasks in the gastrointestinal tract that are important for proper structural and functional states of this tissue. Cathepsin-deficient mice appear to be a suitable *in vivo* model for elucidating the contributions of multifunctional cysteine cathepsins in tissue homeostasis. Our studies revealed that cathepsin K is involved in turnover of ECM components and proteins of junctional complexes in mouse colon since *Ctsk*$^{-/-}$ mice exhibited major alterations in the expression and localization patterns of these molecules. Moreover, we showed that each part of the intestinal tract has unique properties and is characterized by distinct proteolytic profiles that contribute to the development of segment-specific compensatory mechanisms. Taken together, these observations highlight the complexity of proteolytic networks found in diverse tissues and suggest that special caution is required regarding inhibitor-based treatments.

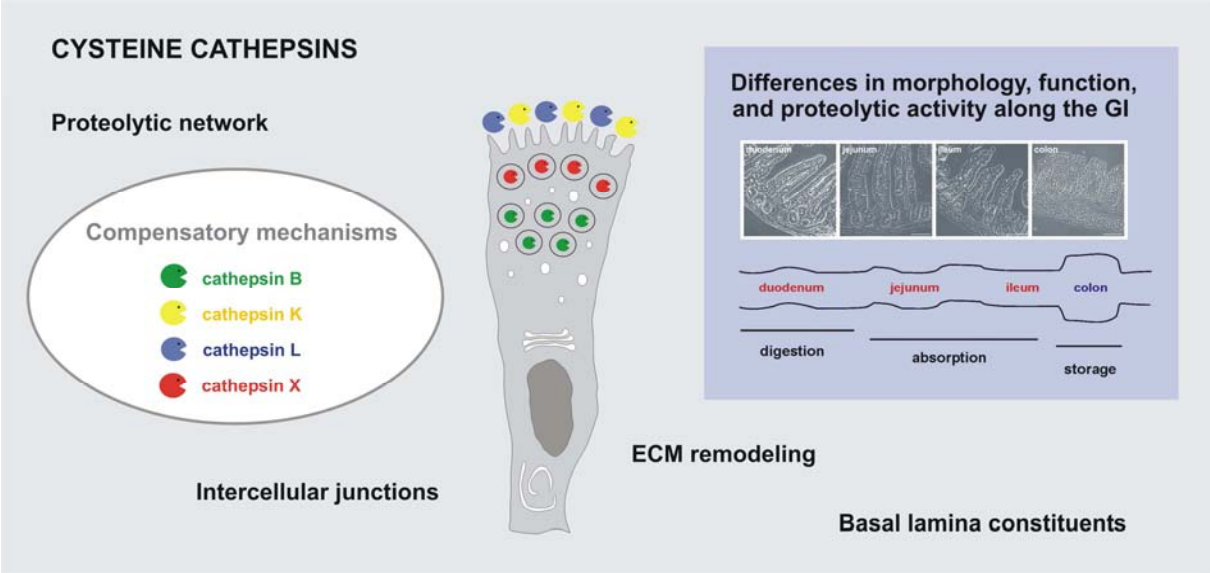

Figure 11: Schematic overview summarizing the involvement of cysteine cathepsins in intestinal homeostasis.

7 Perspectives

Our studies on cathepsin-deficient mice enabled us to gain further knowledge regarding the role of cysteine cathepsins in intestinal homeostasis. Our results on *Ctsk*$^{-/-}$ mice revealed involvement of cathepsin K in regulation of cell-cell and cell-matrix interactions through profound roles in turnover of ECM and intercellular junction proteins. However, since the protein levels of other cysteine cathepsins were found to be elevated in the colon of *Ctsk*$^{-/-}$ mice it remains questionable whether the observed alterations are due to direct actions of cathepsin K or if they occur through indirect mechanisms. For example one could speculate that maybe one of the elevated enzymes, namely cathepsins B, L, and X is responsible for the described changes in the intestine of *Ctsk*$^{-/-}$ mice or even another protease that has not been analyzed yet. Therefore, further studies should be performed with a larger set of cathepsin-deficient animals and in particular with double-deficiencies in order to maximize the chances to detect alterations and phenotypes that could be rescued by compensatory phenomena. An interesting model system would be the double-deficient *Ctsk*$^{-/-}$/*Ctsl*$^{-/-}$ mouse since cathepsins K and L have been shown to exhibit reciprocal compensation in various tissues, including the thyroid gland [Friedrichs et al., 2003]. Moreover, since both these proteases were found localized and secreted by goblet cells [Mayer et al., 2006], the *Ctsk*$^{-/-}$/*Ctsl*$^{-/-}$ mouse model would be ideal for analysis of intestinal mucus properties. Although our current results did not show alterations in assembly and composition of colonic mucus layers in *Ctsk*$^{-/-}$ mice we still can not exclude participation of cathepsin K in mucus processing before evaluating results from studies on more cathepsin-deficient animals.

Furthermore, it would be important to determine whether the elevated protein levels of cysteine cathepsins found in the colon of *Ctsk*$^{-/-}$ mice are concomitant with higher proteolytic activity. It is possible that despite the higher protein levels, the activities of these proteases are not altered due to regulatory mechanisms that could include up-regulation of endogenous inhibitors. Thus, one should also investigate the status of endogenous inhibitors of cysteine cathepsins, such as cystatins and stefins in the intestine of cathepsin-deficient mice. Additionally, studies at the mRNA level could be performed to find out if the increased protein levels of cysteine cathepsins depend on impaired transcriptional regulation. Likewise, such studies would be beneficial also for detecting regulatory mechanisms along the length of the gastrointestinal tract by comparing the individual segments of the small and large intestine. It would be important to further elucidate the proteolytic profiles found in each intestinal part and this could be achieved by performing CLIP chip microarray analysis. The CLIP chip is the only available microarray that allows to study exclusively the mouse degradome, meaning the whole set of proteases and their inhibitors at the transcript level [Kappelhoff and Overall, 2007]. Moreover, this microarray represents a valuable tool to compare the degradome between WT and various cathepsin-deficient mice as it has been shown by

studies conducted in our group [Vreemann, 2009]. However, one should keep in mind that the results obtained from such analysis refer only to transcriptional levels and do not apply to protein levels. Hence, it becomes clear that multiple approaches must be followed in order to better understand the complex proteolytic networks and their interactions.

Cysteine cathepsins exhibit interesting potentials as drug targets because they are associated with diverse pathologies and disorders [Mohamed and Sloane, 2006; Vasiljeva et al., 2007; Brix et al., 2008; Reiser et al., 2010]. Several therapeutic strategies are based on protease inhibitors and the cathepsin K inhibitors for osteoporosis treatment are currently in clinical trials and have gained significant attention [Turk, 2006; Vasiljeva et al., 2007; Stoch and Wagner, 2008; Gauthier et al., 2008]. Further research and deeper insights are required in order to better predict the outcomes of cathepsin inhibition and determine whether the inhibitor-based treatments are safe for the patients. Towards this direction, a suitable approach would be to treat WT mice with protease inhibitors and investigate the effects of proteolytic inhibition *in vivo*. Although cathepsin-deficient mice offer an excellent model to uncover roles of cysteine cathepsins in various tissues they do not represent ideal systems for predicting the effects of inhibitor treatment. That is because the cathepsin-deficient animals lack specific cathepsins already during development, a condition completely different from temporarily protease inhibition at later adult stages. It is important to note, however, that even in the case of treating WT mice with protease inhibitors one should always keep in mind the major differences in proteolytic networks between human and mouse species. In other words, *in vivo* studies with mouse models can offer us indications and not conclusions regarding the roles of proteases in health and disease.

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9 Statutory Declaration

I, **Maria Arampatzidou**, hereby declare that I have written this PhD thesis independently, unless where clearly stated otherwise. I have used only the sources, the data, and the support I have clearly mentioned. This PhD thesis has not been submitted for conferral of degree elsewhere.

Bremen, 4th of June 2012

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Maria Arampatzidou