# Host Cell Proteases: Cathepsins

10

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**Abstract**

Cathepsins are proteolytic enzymes with a broad spectrum of substrates. They are known to reside within endo-lysosomes where they acquire optimal conditions for proteolytic activity and substrate cleavage. However, cathepsins have been detected in locations other than the canonical compartments of the endocytotic pathway. They are often secreted from cells in either proteolytically inactive proform or as mature and active enzyme; this may happen in both physiological and pathological conditions. Moreover, cytosolic and nuclear forms of cathepsins have been described and are currently an emerging field of research aiming at understanding their functions in such unexpected cellular locations. This chapter summarizes the canonical pathways of biosynthesis and transport of cathepsins in healthy cells. We further describe how cathepsins can reach unexpected locations such as the extracellular space or the cytosol and the nuclear matrix. No matter where viruses and cathepsins encounter, several outcomes can be perceived. Thus, scenarios are discussed on how cathepsins may support virus entry into host cells, involve in viral fusion factor and polyprotein processing in different host cell compartments, or help in packaging of viral particles during maturation. It is of note to mention that this review is not meant to comprehensively cover the present literature on viruses encountering cathepsins but rather illustrates, on some representative examples, the possible roles of cathepsins in replication of viruses and in the course of disease.

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## 10.1 Biosynthesis and Default Trafficking Pathway

Cathepsins are synthesized as preproenzymes, whereby the signal peptide targets for entry of the nascent chains into the lumen of the rough endoplasmic reticulum (rER). The signal peptide (pre) is co-translationally cleaved off by signal peptidase upon entry into the ER lumen. The propeptides (pro) keep procathepsins in the zymogen form, that is, proteolytically inactive during their transport from the rER via the Golgi apparatus and the *trans*-Golgi network (TGN) to late endosomes (Fig. 10.1) (Brix et al. 2008, 2015; Erickson et al. 2013). Procathepsins need to pass the quality control of the rER before they become posttranslationally modified while traveling to the TGN. Interestingly, proper folding of cathepsins can be conveyed by their propeptides as some of these bear chaperone functions. The most important and best-studied posttranslational modification of cathepsins is mannose 6-phosphorylation. It is believed to occur in the cis-Golgi and to be required for recognition by cation-dependent mannose 6-phosphate receptors (CD-MPR) of the TGN, which sort the zymogens into clathrin-coated transport vesicles (Fig. 10.1) (De Duve and Wattiaux 1966; Kornfeld and Mellman 1989; von Figura 1991; Kornfeld 1992; Erickson et al. 2013; Brix et al. 2015). Upon arrival in late endosomes, the pH drops such that procathepsins dissociate from the sorting CD-MPR, which is recycled back to the TGN via retromer-coated vesicles. In late endosomes, procathepsins are proteolytically processed to acquire the mature and proteolytically active state (Fig. 10.1). Therefore, either asparaginyl endopeptidase (AEP), which is better known as legumain, or cathepsins themselves cleave procathepsins in *trans* such that the propeptides are removed and the mature forms are delivered for further functioning as soluble enzymes in the compartments of the endocytic pathway. Thus, proteolytic processing for maturation and activation of procathepsins takes place in the late endosome.

As indicated above, the molecular architecture of cathepsins features N-terminal signal peptides that are typically followed by inhibitory propeptides and the peptidase domains (Tables 10.1, 10.2 and 10.3). However, the mature, single-chain form of cathepsin B, in particular, can be processed further, thereby yielding a two-chain form that consists of a light and a heavy chain, which remain bound to each other by disulfide bonds (Mort and Buttle 1997). Both, single- and two-chain forms of cathepsin B are proteolytically active as hydrolases. The molecular architecture of procathepsin-activating AEP/legumain differs from that of cathepsins, in that the peptidase domain directly follows the signal peptide and the pro-domain is found at the C-terminus (Table 10.2). Legumain is further exceptional, because it acts not only as a peptidase but features also peptide ligase activity depending on the conditions it is exposed to (Dall and Brandstetter 2016).

## 10.2 Proteolytic Activity and Substrate Cleavage Preferences of Aspartic, Cysteine, and Serine Cathepsins

Cathepsins belong to either of three classes of proteolytic enzymes, namely, aspartic, cysteine, or serine proteases (Rawlings 2013; Rawlings et al. 2016). Hence, cathepsins are classified according to the amino acids of their active sites that are

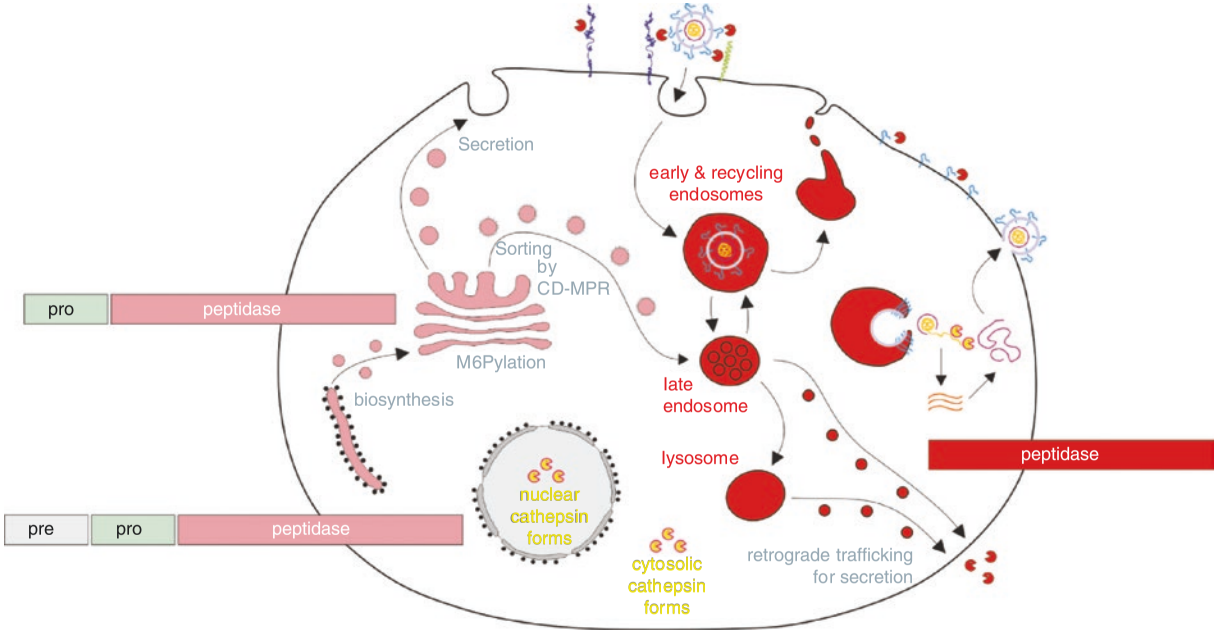


**Fig. 10.1** Canonical and noncanonical transport pathway of cathepsins explaining possible interactions with viruses. Biosynthesis as preprocathepsins (light red) is at the rough endoplasmic reticulum from where the zymogen forms are transported via the Golgi apparatus and the *trans*-Golgi network to late endosomes for proteolytic processing yielding mature cathepsins (red). Procathepsins can also follow the secretory pathway when skipping recognition by CD-MPR at the TGN (left). Upon secretion, the procathepsins can be recaptured with the help of CI-MPR (green) at the plasma membrane (top, center). Retrograde transport of vesicles containing cathepsins recruited out of late endosomes or lysosomes explains the secretion of mature cathepsins into the extracellular space (bottom, right). LDL receptor-related proteins (LRPs; blue) serve as alternative cell surface receptors binding pro- and mature cathepsins for localized pericellular action (top, center) or re-internalization and delivery to endo-lysosomes. Specific forms of cathepsins are also present in the cytosol and in the nuclear matrix (pink-yellow), where they are involved in processing of transcription factors, core, and linker histones, in part by being bound to DNA. Secreted, extracellular cathepsins can interact, in principle, with viruses (cyan-violet-dark yellow) before endocytic entry (top, center), especially when bound to cell surface receptors like the CI-MPR (green) or an LRP (blue). In addition, cathepsins in the pericellular space may interfere by proteolytically processing viral proteins or by involving in shedding of cellular constituents, such as the glycocalyx, thereby enhancing the likelihood of viral entry. Moreover, it is possible, in principle, that cytosolic cathepsins (right, middle) mediate viral polyprotein processing, a proposal that remains speculative at this point

**Table 10.1** Characteristics of aspartic cathepsins

| Cathepsin | Gene symbol | Catalytic type | Clan family subfamily | Uniprot accession number | Locus | Domain architecture (peptidase unit from aa to aa) length in aa | N-Glycosylation, predicted | Disulfide bonds, predicted | Endogenous inhibitor/s | Pathways KEGG |
|---|---|---|---|---|---|---|---|---|---|---|
| **Cathepsin D** Conner (2004) | *CTSD* | Asp | AA A1.009 | P07339 | 11p15.5 | Pre-pro-mature (66–410) 345 aa | 2 | 4 | Alpha-2-macroglobulin, pepstatin | **Lysosome** Tuberculosis |
| **Cathepsin E** Kay and Tatnell (2004) | *CTSE* | Asp | AA A1.010 | P14091 | 1q31 | Pre-pro-mature (61–396) 336 aa | 2 | 3 | Pepstatin | **Lysosome** |

Associations with human diseases: Cathepsin D: mutation in congenital neuronal ceroid lipofuscinosis (mutation in *CTSD*) (Siintola et al. 2006); genetic association with Alzheimer's disease (Davidson et al. 2006)
Data compilation based on MEROPS—the peptidase database—accessible at merops.sanger.ac.uk (Rawlings et al. 2016)

**Table 10.2** Characteristics of AEP/legumain and cysteine cathepsins

| Cathepsin or legumain | Gene symbol | Catalytic type | Clan family subfamily | Uniprot accession number | Locus | Domain architecture (peptidase unit from aa to aa) length in aa | N-Glycosylation, predicted | Disulfide bonds, predicted | Endogenous inhibitor/s | Pathways KEGG |
|---|---|---|---|---|---|---|---|---|---|---|
| **Asparaginyl endopeptidase (AEP)/legumain** (animal type (Chen et al. 1997) | LGMN | Cys | CD C13.004 | Q99538 | 14q32.1 | Pre-mature-pro (25–286) 262 aa | 4 | 0 | Cystatin C, cystatin E/M, cystatin F | Antigen processing and presentation **Lysosome** |
| **Cathepsin B1** (cathepsin B, cathepsin II) (Mort 2004) | *CTSB* | Cys | CA C01.060 | P07858 | 8p22 | Pre-pro-mature-pro and Pre-pro-LC-pro-HC-pro (80–331) 252 aa | 1 | 7 | Serpin SPI2, cystatin A, cystatin B, cystatin C, cystatin D, cystatin E/M, cystatin SN, alpha-2-macro-globulin, kininogen | Antigen processing and presentation **Lysosome** |
| **Cathepsin C** (dipeptidyl peptidase I) (Turk et al. 2004) | *CTSC* | Cys | CA C1.070 | P80067 | 11q14.1-q14.3 | Pre-pro-mature (229–459) 231 aa | 4 | 0 | Cystatin A, cystatin B, cystatin C, cystatin SN | **Lysosome** |

(continued)

**Table 10.2** (continued)

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|---|---|---|---|---|---|---|---|---|---|---|
| **Cathepsin F** (Bromme 2004a) | *CTSF* | Cys | CA C01.018 | Q9UBX1 | 11q13.1-q13.3 | Pre-pro-mature (270–484) 215 aa | 5 | 3 | | **Lysosome** |
| **Cathepsin H** (Kirschke 2004a) | *CTSH* | Cys | CA C01.040 | P09668 | 15q24-q25 | Pre-pro-mature (115–334) 220 aa | 1 | 4 | | **Lysosome** |
| **Cathepsin K** (cathepsin O, cathepsin O2) (Bromme 2004b) | *CTSK* | Cys | SA C01.036 | P43235 | 1q21 | Pre-pro-mature (115–329) 215 aa | 1 | 3 | SSCA1 (squamous cell carcinoma antigen 1), serpin B13, propeptides of cathepsins L, S, K, V | **Lysosome** Osteoclast differentiation Rheumatoid arthritis Toll-like receptor signaling pathway |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Cathepsin L** (Kirschke 2004b) | *CTSL1* | Cys | CA C01.032 | P07711 | 9q21-q22 | Pre-pro-mature (113–333) 221 aa | 2 | 3 | Protein C inhibitor, SSCA1, serpin B13, colligin 2, cystatin A, cystatin B, cystatin C, cystatin D, cystatin E/M, cystatin SN, histidine-rich glycoprotein inhibitor unit 1 (HRG), propeptides of cathepsins K, L, S, V, MHC II invariant chain p41 form, testican-1, alpha-2-macroglobulin, kininogen | Antigen processing and presentation **Lysosome** Phagosome Proteoglycans in cancer Rheumatoid arthritis |

(continued)

**Table 10.2** (continued)

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Cathepsin V** (cathepsin L2, cathepsin U) (Brömme et al. 1999) | *CTSL2* | Cys | CA C01.009 | O60911 | 9q22.2 | Pre-pro-mature (113–334) 222 aa | 2 | 3 | cystatin E/M, propeptide of cathepsin V | **Lysosome** |
| **Cathepsin O** (Velasco and Lopez-Otin 2004) | *CTSO* | Cys | CA C01.035 | P43234 | 4q31-q32 | Pre-pro-mature (103–321) 219 aa | 2 | 3 | | **Lysosome** |
| **Cathepsin S** (Kirschke 2004c) | *CTSS* | Cys | CA C01.034 | P25774 | 1q21 | Pre-pro-mature (114–331) 218 aa | 1 | 4 | SCCA1, cystatin A, cystatin B, cystatin C, cystatin D, propeptides of cathepsins K, L, S, V | Antigen processing and presentation **Lysosome** Phagosome Tuberculosis |
| **Cathepsin W** (Dalton and Brindley 2004) | *CTSW* | Cys | CA C01.037 | P56202 | 11q13.1 | Pre-pro-mature (127–364) 238 aa | 2 | 3 | | **Lysosome** |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Cathepsin X** (carboxypeptidase LB, cathepsin B2, cathepsin IV, cathepsin Y, cysteine-type carboxypeptidase) (Menard and Sulea 2004) | *CTSZ* | Cys | CA C01.013 | Q9UBR2 | 20q13 | Pre-pro-mature (57–302) 246 aa | 2 | 5 | | **Lysosome** |

Associations with human diseases: Cathepsin C: functional absence in Papillon-Lefevre syndrome (Hart et al. 1999, 2000; Toomes et al. 1999), Haim-Munk syndrome (mutation in CTSC) (Cury et al. 2005)
Cathepsin K: functional absence in pycnodysostosis (Gelb et al. 1996; Johnson et al. 1996; Hou et al. 1999)
Cathepsin V: involvement in *Myasthenia gravis* suggested (Tolosa et al. 2003)
Cathepsin S: involvement in immune responses (Driessen et al. 1999)
Cathepsin X/Z: involvement in immune response to *Helicobacter pylori* (Krueger et al. 2005; Obermajer et al. 2009)
Data compilation based on MEROPS—the peptidase database—accessible at merops.sanger.ac.uk (Rawlings et al. 2016)

**Table 10.3** Characteristics of serine cathepsins

| Cathepsin | Gene symbol | Catalytic type | Clan family subfamily | Uniprot accession number | Locus | Domain architecture (peptidase unit from aa to aa) length in aa | N-Glycosylation, predicted | Disulfide bonds, predicted | Endogenous inhibitor/s | Pathways KEGG |
|---|---|---|---|---|---|---|---|---|---|---|
| **Cathepsin A** (serine carboxypeptidase A, cathepsin I) (Pshezhetsky 2004) | *CTSA* | Ser | SC S10.002 | P10619 | 20q13.1 | Pre-pro-mature (39–480) 442 aa | 2 | 4 | | **Lysosome** Renin-angiotensin system |
| **Cathepsin G** (Salvesen 2004) | *CTSG* | Ser | PA S01.133 | P08311 | 14q11.2 | Pre-pro-mature (21–245) 225 aa | 1 | 3 | Tissue factor pathway inhibitor 1 (TFPI), alpha-1-peptidase inhibitor (serpin A1), alpha-1-antichymotrypsin (serpin A3), serpin B1, SCCA1, SCCA2, serpin B6, SLPI (secretory leukocyte peptidase inhibitor), alpha-2-macroglobulin, SPINK5 g.p. | Amoebiasis **Lysosome** Neuroactive ligand-receptor interaction Renin-angiotensin system Systemic lupus erythematosus |

Associations with human diseases: Cathepsin A: functional absence in galactosialidosis (Zhou et al. 1995)
Data compilation based on MEROPS—the peptidase database—accessible at merops.sanger.ac.uk (Rawlings et al. 2016)

responsible for catalytic hydrolysis of peptide bonds. Aspartic acid or cysteine residues are part of the catalytic dyad of the aspartic and cysteine cathepsins, respectively, while serine is part of a catalytic triad in which an additional residue is required to stabilize the oxyanion hole in the acyl intermediate upon interaction between the enzyme and its substrate. Aspartic cathepsins are acting as water nucleophiles, whereas cysteine and serine cathepsins act as protein nucleophiles (Baici et al. 2013). Hence, the nucleophile is provided by a side chain of an amino acid in the active site, namely, the sulfhydryl or the hydroxyl group of either cysteine or serine, respectively, in cysteine and serine cathepsins. For example, serine bears the nucleophilic hydroxyl group of serine cathepsins, while histidine acts as the general base. In addition, aspartate helps to orient the imidazolium ring of histidine such that it activates serine to perform the nucleophilic attack on the peptide bond of the substrate, whereby a temporary complex between the enzyme and its substrate forms, i.e., the acyl intermediate. This breaks down rapidly, resulting in protonation of the general base histidine. Subsequent hydrolysis of the scissile bond occurs when a water molecule enters. The reaction mechanism of peptide bond hydrolysis catalyzed by cathepsins therefore involves two substrates, the protein or peptide substrate and a water molecule, and two products are generated, namely, an N- and a C-terminal peptide product.

Most of the cathepsins act as endopeptidases (Fig. 10.2). However, the cysteine cathepsins B and X, the serine cathepsin A, and legumain are also acting as carboxypeptidases, whereas cysteine cathepsin H acts as an aminopeptidase and cysteine cathepsin C forms dimers acting as dipeptidyl peptidase (Fig. 10.2). Thus, cathepsins are mostly acting on their peptide or protein substrates as monomers, but some can dimerize or even multimerize, thereby eventually altering substrate specificity (see also below).

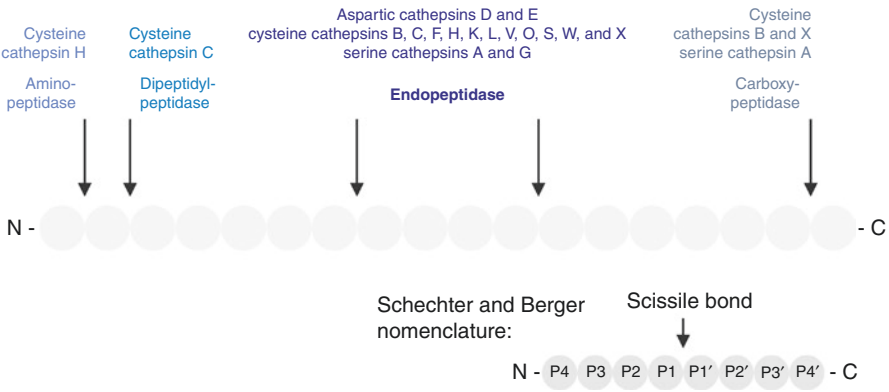


**Fig. 10.2** Schematic drawing depicting distinct cathepsins acting as endo- and exopeptidases on a hypothetical peptide substrate. Amino acids are represented as beads on a string from N- to C-terminus in the top panel. Cathepsins are listed according to their mechanism of substrate cleavage. Bottom panel denotes naming of amino acids on both sides of the scissile bond according to the Schechter and Berger nomenclature

Proteolytic activity and substrate specificity are typically determined with recombinant enzyme preparations in well-defined in vitro assays. According to the Schechter and Berger nomenclature (Schechter and Berger 1968), the amino acids next to the scissile bond toward the N-terminus of the substrate are named P1, P2, P3, and P4, while the C-terminal amino acid next to the scissile bond is named P1′, and the following amino acids of the substrate are referred to as P2′, P3′, and P4′, respectively. The substrate-binding pockets in the cathepsins are named accordingly as S1–S4 and S1′–S4′, respectively, as for every other peptidase. Standard assays to determine the activity of cathepsins are available, as are synthetic substrates, which are preferentially or specifically cleaved by the enzymes. Standard cathepsin activity assays mainly consider peptide cleavage at optimal conditions, whereby a reducing environment of acidic to slightly acidic pH—mimicking the conditions in the endo-lysosomal compartments—is considered as the main determinant. The reader is referred to the *Handbook of Proteolytic Enzymes* (Barrett 2004) and to the "MEROPS database" (www.merops.sanger.ac.uk) (Rawlings et al. 2016), which provides a comprehensive and excellent compendium of proteases, their substrates, and inhibitors, for further up-to-date information.

It should be noted that cathepsins vary in their substrate specificities, but many exhibit overlapping substrate cleavage preferences, making it sometimes difficult to distinguish cathepsin activities. This ambiguity in substrate specificity often complicates their specific inhibition. In addition, cathepsins are redundantly expressed (Brix et al. 2008; Reiser et al. 2010; Gansz et al. 2013; Sloane et al. 2013). Thus, a cathepsin can be upregulated to take over the function of a related enzyme, when the latter is eliminated by gene knockout or inhibited by pharmacological interventions. This phenomenon of redundant regulation of cathepsin expression is observed especially when covalent and irreversible instead of transient reversible inhibition is applied.

## 10.3 Endogenous Inhibitors of Cathepsins

Cathepsin activities are counterbalanced by endogenous inhibitors (Tables 10.1, 10.2 and 10.3). The aspartic cathepsins D and E, which are mostly detected in lysosomes, are inhibited by pepstatin (Dean 1979) or by a natural product isolated from the sea anemone *Actinia equina*, equistatin. This latter inhibitor interestingly also interacts with cysteine peptidases (Lenarcic et al. 1997; Lenarcic and Turk 1999) including the cysteine cathepsins that are found preferentially in endo-lysosomes but also frequently in extra- and pericellular locations. Furthermore, aberrant or alternative forms of cysteine cathepsins are detected in the cytosol, as well as the nuclear and mitochondrial matrices (Brix et al. 2015). Cytosolic cystatins A and B, also known as stefins A and B (Machleidt et al. 1983; Brzin et al. 1983), as well as secreted cystatins C, D, E/M, F, and SN (Barrett 1986; Sloane et al. 1990; Turk and Bode 1991; Alvarez-Fernandez et al. 1999; Abrahamson et al. 2003; Turk et al. 2008; Zeeuwen et al. 2009) serve as endogenous inhibitors of the cysteine cathepsins. In addition, alpha-2-macroglobulin has been identified as an inhibitor of cysteine cathepsins (Fritz 1979; Travis 1988). Cysteine cathepsins, in principle, can also be inhibited by serpins, which are cross-class inhibitors of

serine proteases (Silverman et al. 2001). Like the cystatins, serpins are present intra- and extracellularly. Serpins have a reactive center loop that serves as a bait for the attacking serine protease and, upon cleavage, remains bound by covalent linkage to the enzyme (Huntington 2006; Whisstock et al. 2010).

## 10.4 Tools to Study Expression, Trafficking, and Activities of Cathepsins

Numerous tools are available to study the cathepsins. Specific antibodies are used in immunostaining experiments for subsequent inspection by light and electron microscopy. Such strategies in using antibodies for the detection of cathepsins often bear specificity problems (Weber et al. 2015), because cathepsins are evolutionary conserved and show extensive sequence homologies.

Synthetic substrates, like chromogenic or fluorogenic peptides, are available for in vitro activity assays (see, MEROPS database for further details). Likewise, cyto- or histochemical approaches have been described, which employ synthetic cathepsin substrates that are converted into products less diffusible and readily detected by means of microscopy (Spiess et al. 1994; Brix et al. 1996). Enzymography in polyacrylamide gels is another means of determining multiple cathepsin activities at a time in cell or tissue lysates (Chen and Platt 2011; Platt et al. 2016).

A number of chimeric proteins consisting of cathepsins and fluorescent proteins have been generated which can be expressed in mammalian cells (for reviews, see Brix et al. 2008; Arampatzidou et al. 2011). Tagged cathepsins follow the regular transport pathways like the endogenous enzymes (Naganawa et al. 1999; Linke et al. 2002a, b; Arampatzidou et al. 2011; Frizler et al. 2013; Tamhane et al. 2015; Tamhane et al. 2016). Trafficking studies in living cells have been combined with co-localization experiments in which compartment-specific markers have been used to spot full-length or N-terminally truncated specific forms of cathepsins (see below) while they are on the move through the cell (Linke et al. 2002a; Zwicky et al. 2003; Müntener et al. 2004; Mayer et al. 2008). Intervention by either permanently blocking or transiently interfering with specific transport routes has been applied in order to investigate distinct enzyme trafficking in a variety of eukaryotic cells.

The most elegant way to visualize cathepsin activities "on the spot" and in a quantitative manner is by using so-called activity-based probes (ABPs) (Greenbaum et al. 2002; Jessani et al. 2004; Blum et al. 2005; Blum 2008; Edgington et al. 2011; Blais et al. 2012; Zou et al. 2012; Grzywa and Sieńczyk 2013; Sanman and Bogyo 2014). ABPs consist of a reactive functional group (so-called warhead) that binds to the target protease, a linker region that resembles a peptide substrate, and additional tagging groups. The tagging groups are typically fluorogenic, but biotin or iodinatable moieties have also been chemically engineered onto ABPs. Hence, a family or subfamilies of cathepsins are functionally addressed and become covalently tagged upon cleavage of the ABP's linker. ABPs are available as broad-spectrum probes used in approaching the proteolytic activities of aspartic, cysteine, and serine cathepsins. In addition, specific ABPs have been synthesized that can be cleaved by one or only few cathepsins. Moreover, ABPs with propeptide-mimicking features in the peptide

backbone have been used (Schaschke et al. 1998; Diederich et al. 2012). Such highly specific ABPs are, in principle, able to distinguish even closely related cathepsins.

The one-to-one binding principle of ABPs renders them powerful tools to quantify cathepsin activities. This is to say that most ABPs act as inhibitors that are attacked and cleaved in a substrate-like fashion, but remain bound to the targeted enzyme, thus forming an irreversible, covalent complex with the target protease. The concept of ABP labeling of cathepsins can be compared with the suicidal inhibition mechanism of the serpins on their target proteases (see above). The principle of specific and irreversible binding bears the advantage of rendering ABPs into quantitative probes that allow to detect only mature, proteolytically active cathepsins. However, the disadvantage of many ABPs is equally intrinsic, namely, they act as protease inhibitors and may eventually lead to pharmacological knockdown of proteolytic activities when applied to living cells.

The above sketched labeling options to visualize, localize, and quantify cathepsins can be and have been combined in all possible variations (Baruch et al. 2004; Blum et al. 2005, 2007; Brix and Jordans 2005; Blum 2008; Sadaghiani et al. 2007; Brix et al. 2008; Arampatzidou et al. 2011; Salpeter and Blum 2013). Such experiments have helped to uncover the transport pathways that are followed by pro- and mature cathepsins in different eukaryotic cells. Importantly, it is now state of the art to distinguish proteolytically active from inactive cathepsins. Moreover, it became clear that each and every cell type may use cathepsins strategically in its own, distinct way in support of the specific cell's function (Brix et al. 2008, 2015; Mohamed and Sloane 2006; Sloane et al. 2013; Weiss-Sadan et al. 2017). Hence, subcellular locations of cathepsin activities have been detected that encompass the expected canonical endo-lysosomal compartments. However, quite often locations of cathepsin activities were detected which were, by all means, unexpected. Thus, cathepsins exhibit an astonishing variety of transport pathways to reach numerous intra- and extracellular locations where the proteases may function in a spatially confined and temporally regulated manner (Mohamed and Sloane 2006; Gocheva and Joyce 2007; Brix et al. 2013, 2015; Akkari et al. 2016).

## 10.5 Unexpected Locations Reached by Cathepsins Are Explained by Noncanonical Trafficking

### 10.5.1 Alternative Pathways of Activation upon Secretion of Procathepsins

Procathepsins may skip recognition by the CD-MPR at the TGN and become further sorted and transported along the secretory pathway with destination to the cell surface for subsequent secretion into the extracellular space (Fig. 10.1, left transport route). Pericellularly, the procathepsins may become activated by soluble endopeptidases or through the action of plasma membrane-bound ectoenzymes. Yet another pathway is used in particular in cells of the innate immune system, like macrophages, that express the cation-independent mannose 6-phosphate receptor (CI-MPR, also referred to as IGFII/M6PR) (Mason et al. 1987; Pohlmann et al. 1995; Collette et al. 2004). This

cell surface receptor acts as a scavenger receptor and functions in rescuing faulty or excessively secreted procathepsins for subsequent internalization by endocytosis and sorting along the endocytic pathway. Hence, during their transport from the extracellular space via early endosomes to late endosomes (Fig. 10.1, right transport route), such secreted forms of cathepsins eventually are activated in the same compartment as those following the default transport pathway but delayed in time.

### 10.5.2 Secretory Lysosomes

Distinct cell types feature so-called secretory lysosomes (Andrews 2000; Brix et al. 2008). These are endocytic compartments, which resemble late endosomes and lysosomes in their characteristic biochemical composition. Hence, secretory lysosomes or cathepsins recruited from within late endosomes and lysosomes are transported in a retrograde fashion, such that the vesicles fuse with the plasma membrane, typically upon signaling. This alternative route of cathepsin transport (Fig. 10.1, bottom right transport route) is observed in a number of specific cell types and allows for secretion of proteolytically active cathepsins into the extracellular space (Linke et al. 2002a, b; Büth et al. 2004).

### 10.5.3 Cathepsins in Extracellular Locations and Bound to the Cell Surface

Cathepsins are present in the extracellular space as mature and active enzymes or as inactive proforms. While procathepsins that underwent mannose 6-phosphorylation bind to the CI-MPR (see above) (von Figura 1991; Erickson et al. 2013; Brix et al. 2015), mature cathepsins and procathepsins can also become bound by alpha-2-macroglobulin (Arkona and Wiederanders 1996; Peloille et al. 1997). Moreover, this protein serves as a regulator of a number of proteases, namely, it is an inhibitor of matrix metalloproteinases and cysteine cathepsins alike. Other receptors known to interact with secreted cathepsins are those of the diverse low-density lipoprotein (LDL) receptor-like protein (LRP) family of transmembrane proteins present at the cell surface of almost all cell types (Poller et al. 1995; Willnow et al. 1996; Herz and Strickland 2001). Megalin/gp330 is one of the LRPs that is supposed to mediate internalization and endo-lysosomal delivery of cathepsins, if previously secreted by mistake (Nielsen et al. 2007). Thus, LRPs function in a way similar to the CI-MPR but interact also with pro- and mature cathepsins lacking mannose 6-phosphorylation (Fig. 10.1, right transport route).

### 10.5.4 Regulation of Cathepsin Activities in the Extracellular Space

Pericellular and extracellular localization of procathepsins and mature cathepsins has been observed under both physiological and pathological conditions. For instance, cathepsin-mediated extracellular proteolysis has been shown to promote

cancer cell invasion and tumor progression through extracellular matrix (ECM) degradation (Rochefort et al. 2000; Joyce and Hanahan 2004; Liaudet-Coopman et al. 2006; Mohamed and Sloane 2006; Gocheva and Joyce 2007; Sloane et al. 2013). It remains to be seen, however, if the enhanced cathepsin secretion responsible for excessive ECM degradation is triggered by the acidic environment in tumor tissue or by other mechanisms (Cavallo-Medved and Sloane 2003).

Depending on the extracellular conditions, cathepsins may acquire altered or additional substrate preferences. For example, the cysteine cathepsin K is secreted under physiological conditions as an active enzyme and acts in the acidic resorption lacuna formed by osteoclasts during bone turnover (Gelb et al. 1996; Saftig et al. 1998; Rachner et al. 2011; Fonović and Turk 2014; Brömme et al. 2016). However, cathepsin K is also responsible for degradation of thyroglobulin in the extracellular thyroid follicle lumen, namely, at neutral pH and in oxidizing conditions (Tepel et al. 2000; Friedrichs et al. 2003; Jordans et al. 2009). Moreover, secreted cathepsin K monomers can interact with each other, thereby forming polymeric ringlike structures, depending on the molecular composition of the extracellular space (Li et al. 2002). It has been shown that ECM components like glycosaminoglycans and other constituents are essential as scaffolding factors supporting extracellular interactions of cathepsin K molecules (Li et al. 2004). Moreover, cathepsin K can be stabilized extracellularly by binding to cell surface molecules like clusterin (Novinec et al. 2012), which also interacts with protease receptors of the LRP family, e.g., megalin/gp330 (Lemansky et al. 1999). Importantly, the activity of this particular enzyme—cysteine cathepsin K—differs in monomeric and dimeric as well as in soluble and ECM- or membrane-bound form (Aguda et al. 2014). Hence, different substrates can be cleaved by the same cathepsin, and a given substrate can be processed differently by that enzyme, depending on whether the cathepsin is secreted as an active monomer or dimer, scaffolded by ECM constituents, or bound by cell surface receptors complexed with allosteric cofactors. It is important to note that cysteine cathepsin K is well-studied, in particular, because of its significance as an anti-osteoporotic drug target.

These examples highlight that more research is required to understand how the enzymatic activities of cathepsins are regulated, in particular, in unexpected locations. Thus, the well-accepted concept that cathepsins optimally cleave protein and peptide substrates at acidic pH in reducing environments, as found in endolysosomes, must be broadened. This is all the more important when cathepsin activities are investigated under conditions of cellular stress as is the case with viral infection.

### 10.5.5 Cytosolic and Nuclear Cathepsins

As detailed above, cathepsins belong to the obligate constituencies of the compartments of the endocytic pathway where they exert their functions most optimally. However, the phenomenon of leaky lysosomes has also been known for long. Cellular stress as is the case with cancer cells under prolonged drug treatment or UV

irradiation can cause leakiness of endo-lysosomal membranes. Mature cathepsins may then be released into the cytosol, where proteolytic activity is controlled by different means, such as endogenous inhibitors that specifically bind to the enzymes or other biochemical properties of the cytosol that are not optimal for cathepsin-mediated cleavage. However, when escaping these safeguarding measures by, for instance, inhibitor downregulation, proteolytically active cathepsins may be present in the cytosol for prolonged time intervals and induce apoptotic, necrotic, or pyroptotic cascades resulting in cell death (Turk et al. 2000; Luke et al. 2007; Turk and Turk 2009; Aits and Jäättelä 2013; Flütsch and Grütter 2013). Furthermore, cytosolic and nuclear cathepsins may modulate cell cycle progression (Goulet and Nepveu 2004; Brix et al. 2015; Tamhane et al. 2016).

### 10.5.6 Alternative Cathepsin Forms

N-terminally truncated forms of the canonical preprocathepsins translated from, e.g., alternative transcripts are believed to lack the signal peptide and parts of the propeptide (Mehtani et al. 1998; Zwicky et al. 2003; Müntener et al. 2004; Baici et al. 2006; Schilling et al. 2009; Tholen et al. 2014; Brix et al. 2015). They are therefore not targeted for entry into the ER lumen and will not follow the secretory pathway. Instead, N-terminally truncated cathepsins are retained in the cytosol and can even fold properly as they acquire a proteolytically active state in this unexpected location (Goulet and Nepveu 2004; Luke et al. 2007; Reiser et al. 2010; Tedelind et al. 2010). Some of these alternative and aberrant forms of the cathepsins occur even in the nuclear matrix (Fig. 10.1, center at bottom). The mechanism by which they are transported through the nuclear pore complexes is not known, because most endo-lysosomal enzymes (except AEP/legumain) lack a nuclear localization sequence (NLS). It is reasonable to assume, however, that such nuclear cathepsins, as well as the cytosolic forms, are involved in processing of transcription factors, core, and/or linker histones. In vitro experiments further revealed an important role of DNA as a potential scaffolding factor that interferes with serpin-mediated control of cathepsin activities in environments that mimic unexpected cellular locations like the nuclear matrix (Ong et al. 2007).

## 10.6 Cathepsins Meeting Viruses, Viruses Meeting Cathepsins

### 10.6.1 Transient Encounters when Traveling Along the Endocytic Pathway

Cathepsins are well known to process the spike proteins of SARS and MERS coronaviruses, thereby activating viral fusogens and enabling host cell entry from within endosomes (Millet and Whittaker 2015; Simmons et al. 2013; Heald-Sargent and Gallagher 2012). Thus, treatment options of preventing host cell infection with

SARS-CoV have been proposed that involve cysteine cathepsin L inhibitors (Tong 2006).

A very complex mechanism of proteolytic activation of the fusion proteins of henipaviruses (HNV) has been described as an essential prerequisite for infectivity and pathogenicity of these highly pathogenic paramyxoviruses (Weis and Maisner 2015). The non-fusogenic F0 protein of HNV is translated in host cells and is subsequently transported along the secretory pathway in its inactive form to the plasma membrane. Upon re-internalization and processing in recycling endosomes by cathepsins B and L (Pager and Dutch 2005; Meulendyke et al. 2005; Vogt et al. 2005; Pager et al. 2006; Diederich et al. 2005, 2012), activated F1/F2 complex travels back to the plasma membrane, where it is incorporated into budding virus particles, or mediates fusion of an infected cell with a neighboring cell. Hence, endosomal cysteine cathepsins B and L play an essential role in promoting spread of infection and formation of syncytia.

Thus, there are significant differences in cathepsin-mediated activation of viral fusion proteins: with HNV it occurs at a late stage of replication, whereas SARS and MERS coronaviruses are activated upon virus entry into host cells. Endosomal cysteine cathepsins B and L are also involved in the processing of the envelope glycoproteins of Marburg and Ebola viruses. Cathepsin cleavage enables the glycoprotein to interact with the Niemann-Pick disease type C1 (NDC-1) protein of the host cell, which is an essential step in filovirus entry (Hunt et al. 2012). Likewise, endosomal cathepsins are utilized by non-enveloped reoviruses for host cell entry. After removal of the outer capsid protein σ3 by cathepsins, the viral protein μ1 is exposed, which is a fusion protein, promoting endosomal membrane rupture (Danthi et al. 2010).

### 10.6.2 Altered Cathepsin Expression in Virus-Infected Cells

Virus infection may affect transcriptional regulation of cathepsin genes leading to disbalanced cell functions. In addition to the N-terminally truncated forms of cathepsins and those reaching the cytosol as full-length enzymes due to release from endo-lysosomes, it is conceivable that alternate cathepsin forms may derive from altered genes. These may result from gene mutations or chromosomal aberrations, as occurring in cancer cells, or due to upregulated translation of alternative transcripts. Viral oncogenes may affect amplification of cathepsin genes (Mohamed and Sloane 2006), and it was proposed that endogenous retroviruses or elements thereof may activate placenta-specific genes encoding cysteine cathepsins of mice (Rawn and Cross 2008).

Moreover, HIV-infected macrophages have been reported to upregulate both cytosolic cysteine protease inhibitor cystatin B (stefin B) and cysteine cathepsin B, believed to trigger neuronal cell death in HIV-1-associated neurocognitive disorder (HAND) (Rivera et al. 2014). Similarly, virus transformation of cultured cells in vitro is known to cause upregulation and secretion of the so-called major excreted protein (MEP), which was found in the secretion media of transformed fibroblasts and identified as proteolytically active cathepsin L (Mason et al. 1987; Rubin 2005).

Thus, viruses may not only affect transcriptional regulation of cathepsin genes but may thereby also cause mis-trafficking of the proteases. In hepatocellular carcinoma, caused by infection with hepatitis B and C viruses, altered cathepsin trafficking also results in their secretion. In this particular case of virus-induced liver cancer, the over-secretion of cathepsins is further complicated by the defective functioning of the IGFII/M6P receptor (CI-MPR) pathway, normally acting as a recapture mechanism for internalization of faulty and excessively secreted cathepsins (see above) (Scharf and Braulke 2003). Thus, in the absence of re-internalization cues, the cathepsins may be present in enhanced amounts at the cell surface of hepatocellular carcinoma cells.

The findings summarized above show that another, so far only rarely considered scenario may be likely. Namely, viruses may interact with cathepsins present in the extracellular space of, e.g., cancer cells, even before entering the host cell's endocytic compartments. In acknowledging that cathepsins may well act as proteolytic enzymes already in the pericellular space (see above), it becomes clear at this point that some viruses and their constituents can be processed, in principle, by extracellular cathepsins, that is, before actually entering the host cell by endocytosis (Fig. 10.1, top, center).

It has also to be mentioned in this context that extracellular cathepsin B-mediated shedding of constituents of the glycocalyx of endothelial cells was proposed as a process in support of infection with viruses causing hemorrhagic fevers (Becker et al. 2015). Likewise, cysteine cathepsins B-, L-, and S-mediated shedding of E-cadherin, an important cell-cell adhesion molecule, was suggested to cause epithelial cell damage, thereby promoting disease progression in patients with viral infections (Grabowska and Day 2012).

Taken together, these observations illustrate different mechanisms by which viruses may upregulate expression and stimulate secretion of cathepsins. They also suggest that cathepsins activate viruses not only in endocytotic compartments but also at the cell surface. Finally, these findings support the concept that cathepsins contribute to pathogenesis not only by activating the fusion capacity of viruses but also by other mechanisms promoting cell and tissue damage.

### 10.6.3 Endo-Lysosomal Cathepsins and the Immune Response to Viral Infections

Viruses entering host cells by endocytosis are known to trigger a Toll-like receptor (TLR)-mediated immune response which eventually leads to interferon-alpha production (Sun et al. 2010). Hence, viral nucleic acids are recognized as PAMPs (pathogen-associated molecular patterns) by proteolytically processed transmembrane pattern recognition receptors like TLR9. Proteolytic activation of TLR9 is catalyzed by endosomal AEP/legumain and cathepsins (Bauer 2013). Thus, cathepsins are also involved in the immune response to viral infection.

Cathepsin-mediated processing of viral proteins—typically protein fusogens—happens in early and recycling endosomes (see above), which are connected with

other compartments of the endocytic pathway, namely, also with late endosomes or multivesicular bodies (MVBs). MVBs are believed to serve as a place for generation of exosomes (Cocucci and Meldolesi 2015; Hurley 2015). In this regard, it is interesting to note that infection with the filoviruses EBOV and MARV involves the molecular machinery of the ESCRT pathway, which is required for MVB formation and which is important for virus replication, nucleocapsid formation, and maturation in a compartment positive for late endosomal markers (Dolnik et al. 2015).

Another vicious cycle is working in cross-presentation, which follows from initial phagocytosis of portions or entire virus-infected cells by dendritic cells (Rock and Shen 2005). These professional antigen-presenting cells depend on cysteine cathepsin S-mediated endosomal processing of internalized proteins, which then results in antigen presentation in the context of MHC class II. In the unfortunate case of endosomal processing of viral proteins, the resulting viral antigens may therefore be presented on the surface of dendritic cells via MHC class II, instead of MHC class I. When antigen presentation happens in the context of MHC class I, alerted cytotoxic T cells eliminate the virus-infected cells. Upon cross-presentation, however, tolerance may be a nonproductive outcome.

### Concluding Remarks

Finally, a chapter on cathepsins in a book on viruses cannot end without placing a special note in the context of therapeutic approaches aiming at eliminating viruses from the host. In particular, the retroviral HIV protease is important for maturation of the virus particles and, hence, targeted therapeutically (Moyle and Gazzard 1996; Cooper 2002). HIV proteinase structurally resembles the ubiquitously expressed aspartic cathepsin D, denoting the similarities between host cell's aspartic cathepsins and retroviral aspartic proteases, which are—in the case of HIV—successfully inhibited when approached by transition-state inhibitors. Therefore, and in conclusion, basic science researchers, virologists, and clinicians have learned a lot from the structural similarities of host cell cathepsins and viral proteases. We deduce that interactions between cell biologists and virologists bear more interesting facts to be gathered in the future. Moreover, many more potential therapeutic answers are to be developed from the encounters of cathepsins and the various viruses utilizing the proteases in their own favor. Hence, some of the comments in this chapter are meant in support of stimulating future discussions in the spirit of thinking "out of the box," and, like the cathepsins, following paths beyond the canonical pathways. In our opinion, this is an endeavor worth to be undertaken and continued in the future.

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