Chapter 6
Exploring Systemic Functions of Lysosomal Proteases: The Perspective of Genetically Modified Mouse Models

Martina Gansz, Ursula Kern, Christoph Peters, and Thomas Reinheckel

6.1 Introduction

The development of techniques for random and targeted mutagenesis of the mouse genome represents a milestone for the functional analysis of genes in the context of a living organism (Capecchi 2005; Gondo et al. 2009). The investigation of proteases employing genetically engineered mice (GEM) started soon after the methods for genome modification had been inaugurated in the second half of the 1980ies (Frels et al. 1985; Thomas and Capecchi 1987). The first mouse line with targeted inactivation of a gene for a lysosomal peptidase was that for the aspartic endoprotease cathepsin D (Saftig et al. 1995), with more 'knock-out' mouse lines for the aspartic and cysteine cathepsins to follow (Buhling et al. 2011; D'Angelo et al. 2010; Halangk et al. 2000; Nakagawa et al. 1999; Ondr and Pham 2004; Pham and Ley 1999; Roth et al. 2000; Saftig et al. 1998; Sevenich et al. 2010; Tang et al. 2006; Tsukuba et al. 2003). However, it soon became evident that generation of protease GEM does not only require mastering the introduction of the genome modification into the mouse germ line—it also required systematic phenotyping of

M. Gansz
Institute of Molecular Medicine and Cell Research and Faculty of Biology, Albert-Ludwigs-University Freiburg, Freiburg, Germany

U. Kern
Institue of Molecular Medicine and Cell Research, Spemann Graduate School of Biology and Medicine (SGBM) and Faculty of Biology, Albert-Ludwigs-University Freiburg, Freiburg, Germany

C. Peters
Institue of Molecular Medicine and Cell Research, and BIOSS Centre for Biological Signalling Studies, Albert-Ludwigs-University Freiburg, Freiburg, Germany

T. Reinheckel (✉)
Institute of Molecular Medicine and Cell Research and BIOSS Centre for Biological Signalling Studies, Albert-Ludwigs-University Freiburg, Stefan Meier Str. 17, Freiburg 79104, Germany
e-mail: thomas.reinheckel@uniklinik-freiburg.de

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the GEM on molecular, biochemical, cellular, and tissue levels as well as functional analysis of the organ systems. In 2001 we published a review entitled 'Towards Specific Functions of Lysosomal Cysteine Peptidases: Phenotypes of mice deficient for Cathepsin B or Cathepsin L', which summarized the data that had emerged from the analysis of GEM with loss of function mutations of these cysteine cathepsins (Reinheckel et al. 2001). The paper was discussing the periodic hair loss and the epidermal hyperproliferation of cathepsin L deficient mice, the role of cathepsins in MHC class II mediated antigen presentation, the role of cathepsin B in the early pathogenesis of acute pancreatitis, and the involvement of cathepsin B in cell death processes. To date one can state that much progress in understanding the cathepsin functions in all the topics mentioned above has been obtained during the past decade. This has been addressed by a number of recent in-depth reviews (Brix et al. 2008; Hsing and Rudensky 2005; Reiser et al. 2010; Turk and Turk 2009; Zeeuwen et al. 2009). Over the past 10 years of 'cathepsin mouse genetics' a clear trend extending the analysis of GEM with a single gene mutation to analysis of double- and triple mutants that have deficiencies for multiple proteases, express the human enzyme in the context of the respective gene knock-out, or carry genetic modifications of protease genes simultaneously with their inhibitors, emerged. This enabled stringent gain- and loss-of-function studies of lysosomal proteases and their inhibitors in a complex living system, i.e. *mus musculus*. Here we illustrate these developments by addressing four areas in which considerable progress has been made by employing GEM: Firstly we will revisit the role of endolysosomal cysteine proteases and their inhibitors in skin homeostasis and skin cancer. Secondly, the involvement of cathepsins in atherosclerosis and a previously unknown role of cathepsin L in the heart will be discussed. Finally, we focus on the interplay of cathepsins and their inhibitors in neurodegeneration. Throughout these diverse subjects we show how the normal proteolytic balance has been disturbed by the genetic alternations introduced in the GEM.

6.2 Cathepsin L in Epidermal Homeostasis and Regulation of Hair Cycling

6.2.1 Cathepsin L Deficiency as Molecular Cause of the Furless Phenotype

The balance of proteolytic activity is detrimental for skin and hair follicle homeostasis (Zeeuwen et al. 2009). A well known example is the complex phenotype of periodical hair loss and epidermal hyperproliferation of cathepsin L deficient mice (Roth et al. 2000). This skin phenotype is identical to the classical spontaneous mouse mutant *furless* (*fs*) (Green 1954) and the radiation induced mutation nackt (*nkt*) (Benavides et al. 1999). By genetic complementation experiments it turned out that *furless* and *nkt* are caused by a point mutation and a deletion of the cathepsin L gene, respectively (Benavides et al. 2001; Roth et al. 2000).

How the loss of cathepsin L disturbs the differentiation and cycling of the hair follicle is still not fully understood. However, the cell type in which cathepsin L is critically required to maintain regular hair growth has been identified by crossing transgenic mice that expressed mouse cathepsin L (Ctsl) under control of the keratin 14 promotor (K14) with cathepsin L deficient ($Ctsl^{-/-}$) mice. The resulting K14-Ctsl/$Ctsl^{-/-}$ mice express the protease only in stratified epithelia, i.e. in keratinocytes of the epidermis and hair follicles, but not in the mesenchymal parts of the skin (Reinheckel et al. 2005). The result of this genetic experiment was a rescue of the *furless* phenotype indicating a keratinocyte specific function of mouse cathepsin L. Notably, GEM expressing human cathepsin V, the orthologue to mouse cathepsin L, controlled by the K14-promotor and crossed to cathepsin L null mice showed the same normalization of the fur suggesting identical functions of mouse cathepsin L and human cathepsin V in keratinocytes (Hagemann et al. 2004). Human cathepsin V and the highly homologous 'classical' human cathepsin L are both expressed in human skin keratinocytes (Bernard et al. 2003; Zeeuwen et al. 2007). Interestingly, crossing mice transgenic for human cathepsin L with the cathepsin L knock-out did not affect the periodic hair loss (Sevenich et al. 2006). However, these GEM express human cathepsin L not controlled by the K14-promotor but regulated by the genuine human cathepsin L promoter elements (Houseweart et al. 2003). Although these GEM express active human cathepsin L in various organs such as kidneys, heart, and brain we were not able to detect the human cathepsin L in the epidermis of these mice (TR unpublished data). Hence, the functional analysis of human cathepsin L in mouse epidermis requires further investigation.

On cellular level the disturbed hair cycle in cathepsin L deficient mice is caused by impaired apoptosis and increased proliferation of hair follicle keratinocytes during the physiological regression phase (the catagen) of the hair follicle (Tobin et al. 2002). The prolonged catagen results in profoundly disturbed differentiation of the hair follicle inner root sheet that normally anchors the hair shaft. Loss of this anchor in cathepsin L deficient mice causes the typical periodic hair loss at the end of the catagen and during the resting phase (telogen) of the hair cycle. The massive regression of the hair follicle in the catagen is required for anchoring of the hair shaft and failure to regress properly leads to the abnormal shedding observed in cathepsin L deficient mice. In this regard, trichohyalin the major structural protein of the inner root sheet, which is subjected to protease dependent processing, is a possible substrate protein of cathepsin L (Tobin et al. 2002). Trichohyalin is associated with filaggrin, another structural protein expressed in the inner root sheet and in differentiated epidermal keratinocytes. The final step in processing of filaggrin is impaired in the cathepsin L deficient nkt mice (Benavides et al. 2002). Since hair follicles are extremely difficult to address by biochemical methods the detailed analysis of the proteolytic system of these 'mini-organs' remains a considerable challenge. However, novel generations of GEM provide excellent tools to address these fascinating biological questions (Rendl et al. 2005).

6.2.2 Cathepsin L Regulating Epidermal Proliferation and Carcinogenesis

In addition to the hair loss phenotype, cathepsin L deficiency causes hyperproliferation of basal keratinocytes resulting in epidermal hyperproliferation, acanthosis, and hyperkeratosis (Roth et al. 2000). This epidermal phenotype of cathepsin L knock-out mice is very likely to be caused by a keratinocyte specific defect, because keratin 14 promoter controlled expression of mouse cathepsin L or human cathepsin V in Ctsl-null mice results in a normal state of epidermal proliferation and histology (Hagemann et al. 2004; Reinheckel et al. 2005). In primary mouse keratinocyte cultures the lack of cathepsin L resulted in increased recycling of the epidermal growth factor (EGF) and higher levels of the EGF-receptor (Reinheckel et al. 2005). In consequence of this impaired growth signal termination, cathepsin L deficient keratinocytes show higher proliferation rates in response to EGF as compared to wild-type keratinocytes. Work with keratinocyte conditioned media showed that this concept is not restricted to EGF but seems to apply for various proliferative signals (Dennemarker et al. 2010a; Reinheckel et al. 2005). The relatively mild epidermal phenotype of cathepsin L null mice is exaggerated in the context of skin cancer as shown in transgenic K14-HPV16 skin cancer mice crossed to congenic cathepsin L knock-out mice (Dennemarker et al. 2010a). The K14-HPV16/*Ctsl*$^{-/-}$ mice showed early tumor onset, anaplastic squamous cell cancers, and a highly increased frequency of lymph node metastasis. On molecular level the concentration of active GTP-Ras was increased in cathepsin L deficient epidermis. Furthermore there was an increased activation of the protein kinase B/Akt and mitogen-activated protein kinase pathways in cathepsin L knock-out cells, especially if treated with media conditioned by cathepsin L deficient keratinocytes (Dennemarker et al. 2010a). Taken together, cathepsin L deficiency seems to result in sustained oncogenic signaling in keratinocytes due to impaired termination of growth factor stimulation. In line with these findings, the formation of chemically (DMBA/TPA) induced skin cancer is enhanced in the radiation-induced cathepsin L mutant *nkt* (Benavides et al. 2011). Moreover, transgenic expression of the human cathepsin L inhibitor hurpin in mice increased their susceptibility to develop chemically induced skin carcinomas and reduced apoptosis in UVB-irradiated skin (Walz et al. 2007).

However, cathepsins are often thought to act as tumor promoters by enhancing invasiveness and angiogenesis facilitated by degradation of extracellular matrix components and processing of proangiogenic peptides (Mason and Joyce 2011; Mohamed and Sloane 2006). In support of this idea the depletion of one of the extracellular inhibitors of cysteine cathepsins, i.e. cystatin C, in the K14-HPV16 model leads to an accelerated tumor phenotype with concomitant increase in general cathepsin activity (Yu et al. 2010). In this work, the facilitated tumor progression is ascribed to enhanced proliferation and reduction of cell death as well as improved angiogenesis enabled by increased production of angiogenic peptides. These contradictory observations obtained by cathepsin L deficient and

cystatin C deficient mice may be explained as the ablation of cystatin C increases extracellular activity of cysteine cathepsins whereas in the critical function of cathepsin L in keratinocytes appears to be in growth factor termination within the endolysosome. Regarding the widely discussed issue of cathepsin inhibition as potential cancer therapy, it is important to realize that cathepsin L is a multifunctional protease whose inhibition has the potential to promote cancer rather than blocking it.

6.2.3 *Balanced Cathepsin Activity Required for Skin Barrier Function*

The formation of the stratum corneum is essential for the barrier function of the skin protecting against mechanical stress, infections and water loss (Proksch et al. 2008). The process of cornification, as the final step in epidermal differentiation, is mainly fulfilled by protein- and lipid-crosslinking that is carried out by transglutaminases. Ichtyosis is the general term for disturbances in cornification and is caused by different genetic defects affecting either structural proteins like keratin or the transglutaminases. Additionally, the balance between protease activity and inhibition turned out to be important in the cornification process; not at least because transglutaminases need to be activated by limited proteolysis (Zeeuwen et al. 2009).

Mice that are deficient for cystatin M/E, an extracellular inhibitor of cysteine proteases, show severe defects in cornification and desquamation of the epidermis and die shortly after birth due to dehydration (Zeeuwen et al. 2002). This was at first ascribed to overshooting activity of transglutaminase-3 due to deregulated activation by the cysteine protease legumain. However, it turned out that cystatin M/E has an additional protease binding site recognizing cathepsin L (Cheng et al. 2006). Impressively, cystatin M/E deficient mice crossed with $Ctsl^{-/-}$ mice do no longer develop ichtyosis and are viable, while the ablation of legumain or transglutaminase-3 did not rescue the phenotype of cystatin M/E deficient mice (Zeeuwen et al. 2011). Hence excessive cathepsin L activity appears to be the key pathomechanism of ichtyosis in cystatin M/E deficient mice. The increase in cathepsin L due to loss of cystatin M/E leads to accelerated activation of cathepsin D which in turn might overactivate transglutaminase-1. Although the cystatin M/E-Ctsl double deficient mice are rescued from ichtyosis, skin and hair homeostasis is not restored completely as these mice show in addition to the periodical hair loss of cathepsin L deficient mice a complete loss of fur after 2–3 hair cycles (Zeeuwen et al. 2011). In these mice the dermal connective tissue strongly resembles fibrotic scar tissue with parallel organization of collagen fibers. Moreover, cystatin M/E deficient mice expressing cathepsin L from one allele develop inflammatory alterations of the eye cornea. This eye phenotype, which is 100 % persistent, is again caused by cathepsin L dependent imbalanced proteolysis as this is not observed in the double deficient animals. An ablation of cystatin B (also named stefin B), which causes the Unverricht-Lundborg epilepsy (EPM1), leads to a

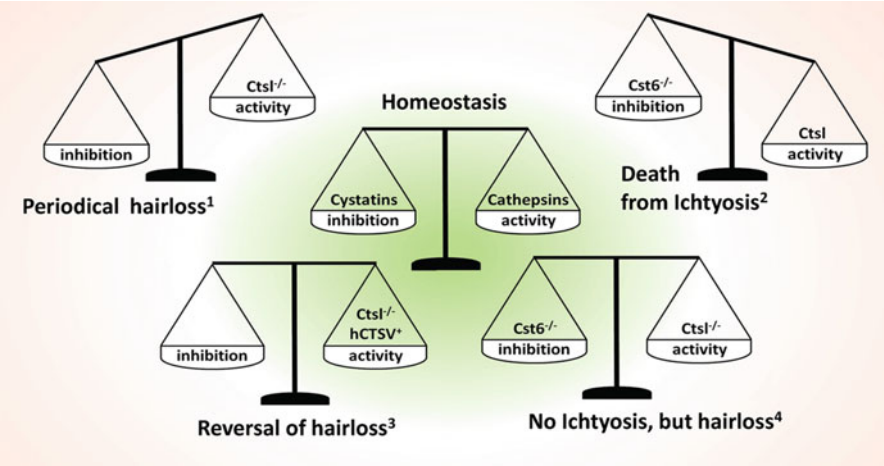

Fig. 6.1 The balance in proteolytic activity of cathepsin L is essential for skin and hair homeostasis. As soon as this balance is disturbed by a deficiency in cathepsin L ($Ctsl^{-/-}$) or its inhibitor cystatin M/E ($Cst6^{-/-}$) functions in hair and skin development and maintenance are severely impaired. The combination of both deficiencies or the expression of a human cathepsin L homologue ($hCTSV^{+}$) redresses the proteolytic balance which manifests in moderate or completely reversed phenotypes. (1)—Roth et al. (2000); (2)—Zeeuwen et al. (2002); (3)—Hagemann et al. (2004); (4)—Zeeuwen et al. (2010)

comparable eye phenotype (Pennacchio et al. 1998). However, the responsible protease in this case was has not been identified yet, because the phenotype was not rescued by additional ablation of cathepsin L (Houseweart et al. 2003). Figure 6.1 summarizes the GEM findings regarding the role of cathepsin L in epidermis and hair follicles. Although there are many unsolved questions regarding the biochemical and cellular mechanisms of cathepsin L function, the genetic evidence strongly suggests that tightly balanced cathepsin L activity is essential for epidermal homeostasis.

6.3 Cysteine Cathepsins and Their Inhibitors in the Cardiovascular System

6.3.1 Atherosclerosis and Abdominal Aortic Aneurysm

Atherosclerosis and abdominal aortic aneurysm are life threatening inflammatory diseases of blood vessel walls. They involve major remodeling of the extracellular matrix in different layers of vessel walls (Garcia-Touchard et al. 2005). Collagens and elastin are the most abundant macromolecular constituents of the extracellular matrix of the intima and media layers. Elevated collagenolytic and elastinolytic activities have been detected in atherosclerotic vessels. They are involved in the

reduction of collagens and elastin in the final stages of both diseases ultimately resulting in rupture of atherosclerotic plaques with e.g. subsequent thrombosis of coronary arteries and myocardial infarction or rupture of the entire aortic wall in the case of abdominal aortic aneurysm. Multiple proteolytic enzymes belonging to the classes of matrix metallo-, serine- and cysteine proteases contribute to the extracellular degradation of collagens and elastin in the course of these devastating pathophysiological processes (Newby 2005; Nicholl et al. 2006; Sukhova and Shi 2006; Sukhova et al. 1998; Sun et al. 2009, 2011). Since the contribution of the metallo- and serine-proteases are not within the immediate scope of this chapter and have been reviewed recently, we are going to focus on the role of cysteine proteases and their inhibitors. Enzymatic activities of three major collagenolytic and elastinolytic cysteine cathepsins S, K and L have been shown to be significantly elevated in atherosclerotic as well as aneurismal lesions in humans (Sukhova et al. 1998). On one hand this is due to an elevated expression of these enzymes under these pathological conditions but on the other hand a significant reduction of the expression of their major inhibitor cystatin C has been described contributing to the imbalance towards enhanced collagenolytic and elastinolytic activities in the diseased tissue (Shi et al. 1999; Sukhova and Shi 2006). For experimental evaluation GEMs developing diet-induced atherosclerosis after consuming a 'Western' high-cholesterol diet were made deficient for one of the three cysteine cathepsins S, K or L by crossing with the respective knock-out strains. In the absence of any one of these three cathepsins an attenuation of the developing atherosclerosis has been observed (Kitamoto et al. 2007; Lutgens et al. 2006; Sukhova et al. 2003). On the other hand knocking out cystatin C—a major extracellular inhibitor of cysteine cathepsins—in the atherosclerosis model and thereby elevating the extracellular activities of the relevant cysteine cathepsins results in a significant reduction of the elastin content of the vessel media and an increased collagen as well as smooth muscle cell content (Sukhova et al. 2005). In the course of the development of atherosclerotic and aneurismal lesions elevated numbers of different cell types, e.g. endothelial cells, smooth muscle cells and macrophages have been described in and around these lesions. Elevated levels of one of the most potent elastinolytic and collagenolytic enzymes, cathepsin L, have been detected in these cells (Liu et al. 2006).

Macrophages receive increasing attention as key players in the pathogenesis of atherosclerosis and possibly target cells for therapy (Wilson et al. 2009). Macrophages may also be the main source for proteolytic enzymes within the atherosclerotic lesions. Recent evidence on an exciting intracellular function of cathepsins added to the picture. Macrophages deficient for cathepsin B or cathepsin L show significantly reduced inflammasome activation and secretion of the proinflammatory interleukine-1β after phagocytosis of crystalline cholesterol (Duewell et al. 2010). As shown also for other crystalline agents, the cathepsins enter the cytosol caused by mechanical disruption of the phagolysosomal membrane and activate the NALP3 inflammasome by a yet unknown mechanism (Halle et al. 2008; Hornung et al. 2008). Subsequently activated caspase 1, as part of the inflammasome complex, cleaves pro- interleukine-1β to produce its active form that

attracts further immune cells; thus enhancing inflammation in the atherosclerotic vessel wall.

Together these data suggest that cysteine cathepsins in vessel walls and inflammatory cells aggravate the course of atherosclerosis as well as abdominal aortic aneurysm.

6.3.2 Cardiac Homeostasis and Cardiomyopathy

Upon necropsy of 1-year-old cathepsin L deficient mice enlarged hearts have been found. A systematic investigation of this phenotype revealed that the relative heart weight of cathepsin L deficient mice of this age group is elevated in comparison to wild type controls and that approximately 25 % of these animals presented with grossly enlarged hearts closely resembling human late onset dilated cardiomyopathy. Echocardiographic investigations confirmed an increase in size of the left ventricle, which was accompanied by an enlargement of the left atrium in the severe cases. Functionally, the fractional shortening—a measure of the contractility of the left ventricle—was significantly reduced in mutant mice. In the severely dilated hearts the pressure gradient across the aortic and mitral valves was elevated more than threefold in comparison to the respective controls. In these dilated hearts valve insufficiencies and changes in electrophysiology characteristic of clinical cardiomyopathies were seen. However, between weaning and 12 months of age the mortality of cathepsin L deficient animals was not elevated in comparison to wild type controls (Petermann et al. 2006; Stypmann et al. 2002). On the microscopic level these pathologies are reflected by a progressive and extensive interstitial fibrosis of the myocardium first observed at an age of 4 months. Cardiomyocytes presented with an elevated number of pleomorphic nuclei characteristic of cardiomyopathies. Infiltration of immune cells into the myocardium was not observed. Ultrastructurally, cathepsin L deficient cardiomyocytes revealed numerous vacuoles and end-stage lysosomes—also termed residual bodies—not observed in age-matched controls as early as 3 days post partum. The enlargement of the endosomal/lysosomal compartment in cardiomyocytes devoid of cathepsin L was also reflected by an elevated staining with an acidophilic dye exhibiting a patchy pattern of the lysosomes as compared to a reticular staining in wild type controls (Petermann et al. 2006). The early appearance of these alterations points at an essential role of cathepsin L in the endosomal/lysosomal compartment of cardiomyocytes. The alterations of the endosomal/lysosomal compartment in cardiomyocytes, which were also found in thyroid epithelial cells and keratinocytes of cathepsin L deficient animals, are characteristic of a defective macroautophagy (Friedrichs et al. 2003; Tobin et al. 2002). Further studies of $Ctsl^{-/-}$ mouse embryonic fibroblasts expressing the autophagy marker GFP-LC3 as a model system revealed no defects in the initiation of autophagy, formation of autophagosomes and fusion of these structures with lysosomes but rather a defect in degradation of contents of the autophagolysosomes in the absence of cathepsin L resulting in an enlargement and accumulations of the organelles as observed

(Dennemarker et al. 2010b). Notably, the cardiac phenotype of cathepsin L deficient mice was rescued by cardiomyocyte-specific expression of cathepsin L under control of the myosin heavy chain promoter in otherwise cathepsin L deficient mice (Spira et al. 2007). The heart weight and the size of the left ventricle were normal in the latter animals. Atrial enlargement was not observed and fractional shortening had improved. Expression of the natriuretic peptide, ANP, a marker of sheer stress in the myocardial wall, which is elevated in cathepsin L deficient mice, was normalized in these rescue transgenic animals indicating normalized heart function. The interstitial fibrosis, however, was not rescued (Spira et al. 2007). Interestingly, in transgenic mice constitutively overexpressing human cathepsin L in cardiomyocytes cardiac hypertrophy induced by aortic banding was reduced in comparison to wild type controls due to an attenuation of the Akt/GSK3β cascade (Tang et al. 2009).

Taken together, late onset dilated cardiomyopathy in mice can be caused by a deficiency of the lysosomal endoproteinase cathepsin L triggering a defect in macroautophagy with disturbed degradation of the autophagolysosomal content in cardiomyocytes.

6.4 Cathepsins in Lysosomal Neurodegenerative Disorders

Neurodegenerative disorders are often caused by altered turnover of proteins that form plaques and fibrils. Genetic defects in lysosomal catabolic enzymes cause lysosomal storage disorders and may also lead to pathogenic protein accumulation in the brain (Bellettato and Scarpa 2010; Neufeld 1991). As discussed below, the aspartic protease cathepsin D and the combined deficiency of the cysteine cathepsins B and L have been associated with neurodegeneration mainly connected with their role in bulk protein degradation and cell death (Fig. 6.2).

6.4.1 Cathepsins in Neuronal Storage Disorders

Cathepsin D deficient mice die approximately 26 days after birth (Saftig et al. 1995). Undigested lipoprotein, mainly the subunit c of mitochondrial ATP synthase accumulates within lysosomes in affected neurons and a neurodegenerative phenotype similar to neuronal ceroid lipofuscinosis (NCL) was described in the cathepsin D deficient mouse brain (Koike et al. 2000, 2005). Interestingly, several spontaneous cathepsin D mutations in sheep and bulldog that show an NCL-like disease phenotype have been identified (Awano et al. 2006; Tyynela et al. 2000). Most importantly mutations in the human cathepsin D gene are the cause for a severe juvenile form of NCL (Siintola et al. 2006; Steinfeld et al. 2006). Hence, cathepsin D is essential for neuronal homeostasis in the mammalian organism. In contrast, mice lacking either cathepsin B or L do not show any spontaneous neurological phenotypes. However, combined deficiency is lethal and leads to rapid loss of cerebral cortical neurons and cerebellar Purkinje and granule cells

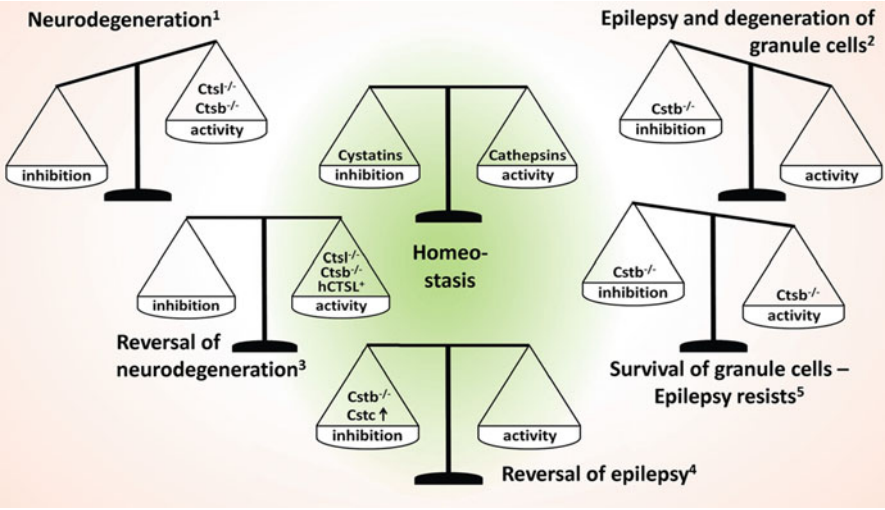

Fig. 6.2 Cellular homeostasis of neurons depends on the balance in proteolytic activity of cysteine cathepsins and their inhibitors. In case of Cathepsin L and B double deficient mice ($Ctsl^{-/-}$; $Ctsb^{-/-}$) the lack protease activity leads to neurodegeneration which is reversed if the human cathepsin L homologue ($hCTSL^{+}$) is expressed. Epilepsy and degeneration of granule cells is caused by an increase in proteolytic activity due to depletion of the cathepsin inhibitor cystatin B ($Cstb^{-/-}$). This phenotype is ameliorated by an additional knock out of cathepsin B and completely reversed by overexpression of cystatin C (Cstc). (1)—Felbor et al. (2002); (2)—Pennacchio et al. (1998); (3)—Sevenich et al. (2006); (4)—Kaur et al. (2010); (5)—Houseweart et al. (2003)

(Felbor et al. 2002). Again neurons show substantial accumulation of lysosomes and double-membrane vesicles in the perikarya of neurons and uncommon swelling of axons. In how far the composition of the accumulated material within the vesicles differs from those observed in cathepsin D deficiency is still not fully understood. The comparison of brain lysosomes of cathepsin B/L double deficient mice with wild-type controls by proteome analysis revealed increased abundance of proteins either associated with lysosomal recycling or neural growth (Stahl et al. 2007). This suggests a potential role for cathepsin B and L in recycling during postnatal neuronal development, which may influence axon outgrowth and synapse formation. These functions are shared by cathepsin B and L which is a typical example for the functional redundancy among cysteine cathepsins. Accordingly, human cathepsin L is able to overtake brain associated functions of murine cathepsin L and B, as transgene expression in double deficient mice prevents neurodegeneration (Sevenich et al. 2006).

6.4.2 Cathepsins as Executor Proteases in Neuronal Macroautophagy

In the current understanding of how cells are protected from aggregate formation the induction and efficient turnover of autophagosomes is essential (Banerjee et al. 2010). Degradation of aggregate-prone proteins and damaged organelles via the autophagy-lysosomal pathway is possibly even more efficient than via the ubiquitin-proteasome pathway (Korolchuk et al. 2010). In several common neurodegenerative disorders the neurons show an accumulation of autophagosomes, which is most likely due to impaired turnover, rather than enforced induction of autophagy. Thereby a mechanism which is carried out to protect the cell in the end promotes neuronal cell death due to accumulation of autophagosomes (overview in Banerjee et al. 2010). This concept has been proven in Atg-7 deficient autophagy impaired mice that die from massive loss of cerebral and cerebellar neurons (Komatsu et al. 2006). Detailed investigation of the accumulated vesicles in brains of cathepsin D single deficient and cathepsin B and L double deficient mice revealed that these carry the autophagosome marker LC3 (Koike et al. 2005). The processed membrane-bound version of LC3, LC-3 II, is highly increased in cathepsin deficient brains and correlates with disease progression which is a clear sign for massive autophagy. Defects in turnover of autophago-lysosomes have been described even in cathepsin L single deficient mice (Dennemarker et al. 2010b). It turned out that neither the initiation of autophagy nor formation of autophagolysosomes is impaired. This implies a non redundant function of cathepsin L in the final degradation process of the incorporated material. Is cathepsin L missing the digestion cannot be completed and these autophagolysosomes accumulate. In conclusion neurodegenerative disorders induced by loss of lysosomal enzymes may be due to insufficient and deregulated autophagy. This concept may be extended to other forms of NCL as autophagy is also accelerated in initiation but incomplete in CLN3 mutated mice, a mouse model for juvenile NCL (Cao et al. 2006). In general enforcing autophagy as a neuroprotective program could be a therapeutic approach in several common neurodegenerative disorders. This may be achieved by improving cathepsin function in the autophagy-lysosomal pathway. This has been shown in mice transgenic for the human amyloid precursor protein as the accumulation of this aggregate-prone protein in autophagosomes is rescued by enhancement of lysosomal cathepsin activity by depletion of cystatin B (Yang et al. 2011).

6.4.3 Cystatin B a Critical Regulator of Neuronal Proteolytic Balance

As mentioned above, cystatin B depletion can be neuroprotective in some conditions. However, the cystatin B example illustrates delicate the balance of proteases and their inhibitors as loss of cystatin B causes the familial Unverricht-Lundborg epilepsy (Lalioti et al. 1997; Pennacchio et al. 1996). Cystatin B knock-out mice also suffer from seizures and ataxia (Pennacchio et al. 1998). In these mice

lots of granule cells in the cerebellum die by apoptosis, which is most likely the reason for the neurological phenotype. There is no accumulation of vesicles or plaques found in this kind of disease. To investigate whether the deregulated activity of cathepsins is responsible for this phenotype, cathepsins B, L or S have been deleted in cystatin B deficient mice (Houseweart et al. 2003). Dying of granule cells in the cerebellum was remarkably reduced by ablation of cathepsin B, whereas deficiency in cathepsin L or S did not affect cell death (Houseweart et al. 2003). However, also ablation of cathepsin B did not rescue the phenotype of seizures and ataxia indicating that either other proteases compensate for cathepsin B function or other targets of cystatin B contribute to disease progression. Accordingly, the overexpression of cystatin C, another cysteine protease inhibitor, is able to rescue the phenotype of cystatin B deficiency, most likely because it targets more than one protease and is therefore not affected by compensatory effects (Kaur et al. 2010; Lieuallen et al. 2001).

Figure 6.2 summarizes the findings on lysosomal proteases and their endogenous inhibitors, namely cystatin B, in the nervous system of protease or inhibitor deficient GEM. Again, cellular homeostasis depends on the fine tuned balance of the proteolytic system.

6.5 Conclusions

The—still incomplete—list of examples presented in the previous sections illustrates how GEM models have been extensively employed in order to study lysosomal proteases and their inhibitors in a complex mammalian organism. What are the most important general lessons learned from these studies? Firstly, there are highly conserved *in vivo* functions of the orthologous mouse and human cathepsins illustrated by the rescue of knock-out phenotypes by expression of the respective human cathepsin orthologue. Secondly, the phenotypic effects caused by genetic deletion or a transgenic expression of cathepsins or their endogenous inhibitors are strikingly dependent on the cell-, tissue- and process-context. This is even true for the ubiquitously expressed proteins, such as cathepsins B and L or the cystatins B and C. Finally, these recent investigations provided strong *in vivo* evidence for the intriguing balance of proteolytic and anti-proteolytic processes. This demonstrates that cathepsins are embedded in a complex proteolytic network as a part of the entire signaling network of cells and tissues. In future, tools and methods of systems biology will help us to further understand these interactions.

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