

## Review

# The lysosome: from waste bag to potential therapeutic target

Hanna Appelqvist<sup>1</sup>, Petra Wäster<sup>1</sup>, Katarina Kågedal<sup>1</sup>, and Karin Öllinger<sup>1,2,\*</sup>

<sup>1</sup> Experimental Pathology, Department of Clinical and Experimental Medicine, Faculty of Health Sciences, Linköping University, Linköping, Sweden

<sup>2</sup> Department of Clinical Pathology and Clinical Genetics, County Council of Östergötland, Linköping, Sweden

\* Correspondence to: Karin Öllinger, Tel: +46 10 1033261; Fax: +46 10 1031529; E-mail: karin.ollinger@liu.se

**Lysosomes are ubiquitous membrane-bound intracellular organelles with an acidic interior. They are central for degradation and recycling of macromolecules delivered by endocytosis, phagocytosis, and autophagy. In contrast to the rather simplified view of lysosomes as waste bags, nowadays lysosomes are recognized as advanced organelles involved in many cellular processes and are considered crucial regulators of cell homeostasis. The function of lysosomes is critically dependent on soluble lysosomal hydrolases (e.g. cathepsins) as well as lysosomal membrane proteins (e.g. lysosome-associated membrane proteins). This review focuses on lysosomal involvement in digestion of intra- and extracellular material, plasma membrane repair, cholesterol homeostasis, and cell death. Regulation of lysosomal biogenesis and function via the transcription factor EB (TFEB) will also be discussed. In addition, lysosomal contribution to diseases, including lysosomal storage disorders, neurodegenerative disorders, cancer, and cardiovascular diseases, is presented.**

**Keywords:** degradation, apoptosis, lysosomal membrane permeabilization, exocytosis, cholesterol

### Introduction

Lysosomes are the major digestive compartment within cells and originally described in the 1950s by Christian de Duve (Appelmans et al., 1955; de Duve, 1959), a finding that yielded the Nobel Prize. Lysosomes were long regarded as simple waste bags, but are now known as advanced organelles that are involved in many cellular processes and are considered crucial regulators of cell homeostasis (Figure 1). In this review lysosomal involvement in degradation of intra- and extracellular material, plasma membrane repair, cholesterol homeostasis, and cell death will be discussed. In addition, lysosomes and their contribution in the pathogenesis of diseases such as storage disorders, cancer, neurodegenerative disorders, and cardiovascular diseases will be highlighted. Bone remodeling, antigen presentation, and pathogenic defense are beyond the scope of this article and we refer to other recent reviews for information regarding lysosomal contribution in these processes (Soldati and Neyrolles, 2012; Watts, 2012; Zhao, 2012).

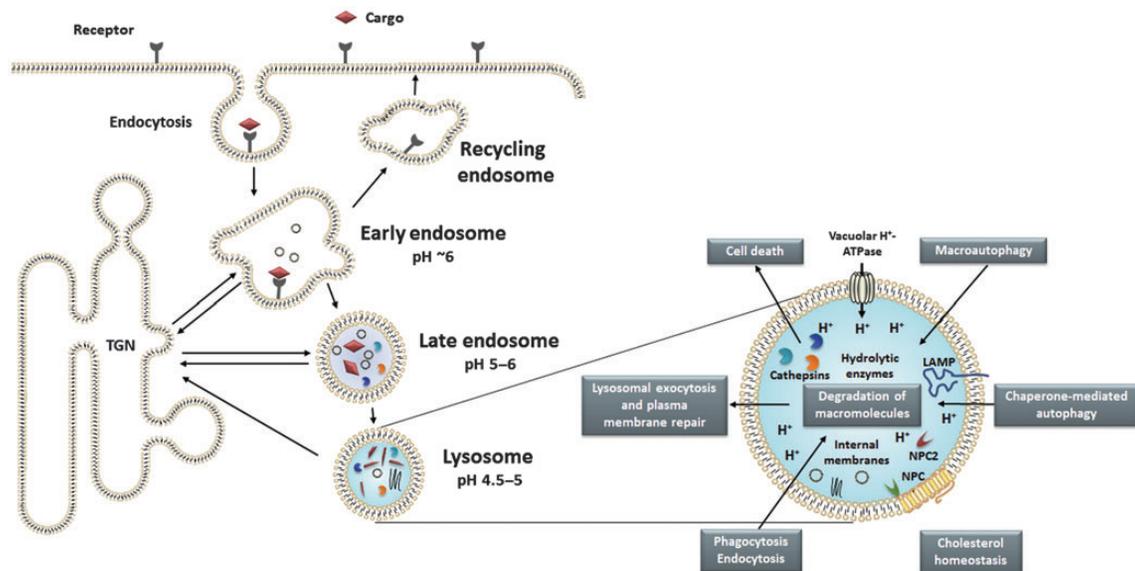
### Characteristics of lysosomes

The lysosome is an intracellular organelle with an acid interior, which contains acidic hydrolases and specific membrane proteins, but lacks the mannose-6-phosphate receptor (M6PR). The latter is being used as criteria to discriminate lysosomes from late endosomes. Lysosomes share these properties with cell type-specific compartments collectively referred to as lysosome-related

organelles, which include melanosomes in melanocytes, lytic granules in lymphocytes, delta granules in platelets, lamellar bodies in lung epithelial cells, and other variants of acidic granules (Huizing et al., 2008).

Lysosomes are found in virtually all eukaryotic cells. Ultrastructurally they appear as dense bodies in the cytosol, often in a perinuclear pattern. The shape of the lysosomes varies from spherical to sometimes tubular. Their size differs depending on cell type, and in most cells lysosomes are typically  $>1\ \mu\text{m}$ , although in some cells (e.g. macrophages) the diameter can exceed several microns (Lüllmann-Rauch, 2005). The size and number can increase drastically, for example as a result of accumulation of undigested material.

Lysosomes are limited by a single 7–10 nm phospholipid-bilayer (Safitig et al., 2010). A unique feature of the lysosomal membrane is its high carbohydrate content, due to heavily glycosylated lysosomal membrane proteins. There are 25 lysosomal membrane proteins identified (Lübke et al., 2009), where the most abundant are lysosome-associated membrane protein (LAMP)-1 and -2, lysosomal integral membrane protein (LIMP)-2, and CD63 (Eskelinen et al., 2003). Glycosylations at their luminal domains form a glycocalyx, which is suggested to protect the membrane from the lytic enzymes within the lysosome (Granger et al., 1990). In addition to the limiting membrane, lysosomes contain intralysosomal membranes, which represent the main site of membrane degradation within this organelle (Schulze et al., 2009). The inner membranes are rich in the phospholipid bis(monoacylglycerol)-phosphate (BMP), also known as lyso-bis-phosphatidic acid (LBPA), which is



**Figure 1** The endolysosomal system. Lysosomes represent the terminal for the degradative endocytic pathway, beginning with early endosomes budding from the plasma membrane. In early endosomes, cargo is either sorted into the recycling pathway back to the cell surface via a recycling endosome or retained for degradation. Through gradual maturation, the early endosome is transformed into a late endosome containing less cargo destined for recycling and higher amount of lysosomal proteins. The formation of intraluminal vesicles (ILVs) starts in early endosomes, and peaks in late endosomes. Internal membranes in lysosomes are mostly seen as lamellar membranes, but some ILVs can be present. To the right is a magnification of a lysosome, showing its critical involvement in many cellular processes, including endocytosis, autophagy, plasma membrane repair, cell death, and cholesterol homeostasis. LAMP, lysosome-associated membrane protein; NPC, Niemann–Pick type C protein; TGN, trans-Golgi network

exclusively found in lysosomes and late endosomes (Kobayashi et al., 1998).

Lysosomes contain up to 600  $\mu\text{M}$  calcium (Christensen et al., 2002; Lloyd-Evans et al., 2008), not dissimilar from the concentration described for the classic calcium storage organelle, the endoplasmic reticulum (Bygrave and Benedetti, 1996). Within the endolysosomal system calcium is important for maintaining normal trafficking, recycling, and vesicular fusion events (Lloyd-Evans and Platt, 2011). Lysosomal calcium can be released by one of the most potent intracellular calcium-releasing second messengers nicotinic acid adenine dinucleotide phosphate (NAADP) (Churchill et al., 2002), via its action on the two-pore channel (TPC) family.

The molecular machinery behind lysosomal biogenesis and the regulation thereof have long been unclear, but were recently revealed by the discovery of a specific gene network, which was given the name coordinated lysosomal expression and regulation (CLEAR). Many genes encoding lysosomal proteins harbor a CLEAR sequence (GTCACGTGAC) near the transcription start site (Sardiello et al., 2009). The transcription factor EB (TFEB) can enter the nucleus and bind to the CLEAR elements, thereby inducing gene transcription. There are  $\sim 500$  direct TFEB target genes, including genes involved in lysosomal biogenesis and autophagy (Palmieri et al., 2011; Settembre et al., 2011). TFEB is considered as a master regulator of lysosomal function and coordinates the function of the lysosomal network in order to meet the cellular need of degradation capacity.

### Lysosomal function essential for homeostasis

#### Degradation of macromolecules

The Greek word lysosome means digestive body, and in accordance, a well-known function of lysosomes is degradation. In the cell

there are two major degradation routes: the lysosomal network and the ubiquitin-proteasome system. While the proteasome is largely handling degradation of short-lived intracellular proteins, lysosomes are degrading all kinds of macromolecules of intra- or extracellular origin. Intracellular components intended for lysosomal degradation reach the lysosomes by different forms of autophagy, whereas exogenous material ingested by endocytosis passes through the endocytic compartment before reaching the lysosomes. The  $\sim 60$  resident hydrolases (including proteases, peptidases, phosphatases, nucleases, glycosidases, sulfatases, and lipases) have different target substrates, and their collective action permits the degradation of all types of macromolecules (Bainton, 1981). The end products of lysosomal digestion are reused by the cell after diffusion or carrier-mediated transport through the lysosomal membrane (Lloyd, 1996).

Among the lysosomal hydrolases, the best known are the cathepsin family of proteases. A number of human cathepsins have been identified and are categorized into three distinct groups based on the amino acid found in the active site; serine (A and G), cysteine (B, C, F, H, K, L, O, S, V, W, and X), and aspartic cathepsins (D and E). The aspartic cathepsin D and some of the cysteine cathepsins, including cathepsins B, C, H, and L, are ubiquitous and among the most abundant lysosomal proteases (Rossi et al., 2004). The acidic environment of the lysosomal lumen (pH 4.5–5.0) facilitates the degradation process by loosening the structures of macromolecules and is optimal for the activity of lysosomal hydrolases (Coffey and De Duve, 1968; Ohkuma and Poole, 1978). The characteristic acidic pH of lysosomes is a result of the action of the vacuolar  $\text{H}^+$ -ATPase (Ohkuma et al., 1982), a transmembrane multimeric protein complex. The vacuolar  $\text{H}^+$ -ATPase uses energy from ATP

hydrolysis to pump protons from the cytosol against their electrochemical gradient into the lysosomal lumen (Mindell, 2012).

### *Endocytosis*

Endocytosis is a process by which cells internalize the plasma membrane along with cell surface receptors and soluble molecules. Cells have multiple mechanisms for endocytosis, including clathrin-dependent and -independent routes. Lysosomes represent the terminal station for the degradative endocytic pathway, which starts at the plasma membrane (Figure 1). The cargo first arrives at the early endosome, which is the main sorting station in the endocytic pathway. The majority of cargo, including most receptors, is returned to the plasma membrane via the recycling endosomes (Huotari and Helenius, 2011). It has been estimated that 50% of the surface area of the plasma membrane is cycled in and out of a typical mammalian cell every hour (Steinman et al., 1983). Cargo destined for degradation is retained in the early endosome, which, through a process involving exchange of material and multiple fusion events, converts into a late endosome. Late endosomes are spherical and contain lysosomal membrane proteins (such as LAMPs) and acid hydrolases. In late endosomes, cargos undergo further sorting and are transported to other organelles such as trans-Golgi network (TGN; Figure 1). Trafficking between TGN and endosomes is a continuously ongoing process that is responsible for the removal of endosomal components and the delivery of lysosomal components (Huotari and Helenius, 2011). Newly synthesized enzymes are delivered to the appropriate endolysosomal compartment from the TGN network, and components are returned to the TGN for reuse. Lysosomes receive cargo from late endosomes; in addition, new lysosomal hydrolases and membrane proteins from the TGN are also transferred to the lysosomes. The influx of new components is essential, and without incoming endosomal traffic, lysosomes lose their intactness, acidity, and perinuclear localization (Bucci et al., 2000).

One of the characteristic features of endosomes is the accumulation of internal membranes within the lumen of the organelle (Kobayashi et al., 1998). Intraluminal vesicles (ILVs) are formed from the limiting membrane in the endocytic pathway, and their presence is essential for efficient cargo sorting (Doyotte et al., 2005; Woodman and Futter, 2008). In the early endosomes, the formation of ILVs begins, and in the late endosomes, proteins are sorted between the limiting membranes and ILVs. Due to the high content of ILVs, late endosomes are sometimes referred to as multivesicular bodies. Via the generation of ILVs, lipids and membranes are delivered to lysosomes in a form that is easily accessible to lysosomal hydrolases. In contrast to the limiting membrane, the membrane of ILVs has no protective coat of glycosylated proteins (Huotari and Helenius, 2011). The lipid composition of the ILV membrane is also different and contains more cholesterol and BMP (Kobayashi et al., 1998; Möbius et al., 2003). As presented in Figure 1, lysosomes contain fewer ILVs, and luminal lipids are observed as multilamellar membrane whorls.

The maturation process from early endosome to lysosome takes ~40 min. During this time, the vesicle undergoes a multitude of changes, including exchange of membrane components, movement to the perinuclear area, formation of ILVs, a decrease in luminal pH, acquisition of lysosomal components, and changes in morphology by which the tubular extensions of early endosomes

are lost (Huotari and Helenius, 2011). The low pH within lysosomes provides a better milieu for the acid hydrolases, but it is also essential for membrane trafficking and sorting of cargo.

The widely used distinction between early endosomes, late endosomes, and lysosomes simplifies the complexity of the endocytic pathway. There is a continuous exchange of content between the intermediates in the endocytic pathway, and therefore it is difficult to identify markers that specifically label a single organelle. However, early endosomal antigen 1 (EEA1) and Rab5 are widely used as markers of early endosomes (Mu et al., 1995). Late endosomes and lysosomes have an overlapping molecular content, including LAMPs and acid hydrolases. However, lysosomes can be distinguished from late endosomes by their lack of M6P receptors (Brown et al., 1986).

### *Autophagy*

During autophagy, cytoplasmic components, damaged proteins, and entire organelles are degraded and recycled to generate building blocks for anabolic processes. Autophagy in mammalian cells can be divided into chaperone-mediated autophagy, microautophagy, and macroautophagy. Chaperone-mediated autophagy is a process by which cytosolic proteins harboring specific recognition motifs are delivered to the lysosomes via the action of a chaperone and the lysosomal receptor LAMP-2A (Dice, 1990; Cuervo and Dice, 1996; Kaushik and Cuervo, 2012). Microautophagy involves direct engulfment of cytoplasmic cargo at the limiting lysosomal membrane (Li et al., 2012). During macroautophagy, sequestration of a small portion of the cytoplasm, including soluble materials and organelles, within a newly generated double membrane called the isolation membrane (or phagophore) results in the formation of an autophagosome (Burman and Ktistakis, 2010). Autophagosomes fuse with lysosomes for the degradation and recycling of their contents. This secures the supply of building blocks in the cell during starvation and permits the disposal of unneeded or non-functional organelles (Mizushima and Komatsu, 2011). Macroautophagy is thought to be the major type of autophagy, and it has been more extensively studied than microautophagy and chaperone-mediated autophagy. The mammalian target of rapamycin (mTOR) is central for regulation of the autophagic activity and senses the energy status by integrating signals from, for example, growth factors, amino acids, and glucose (Efeyan et al., 2012). Starvation is a potent inducer of autophagy and is due to inhibition of mTOR complex 1. Lysosomes change their intracellular distribution in response to nutrient availability, from peripheral location at nutrient-rich conditions to perinuclear clustering during starvation (Korolchuk et al., 2011). Under conditions of nutrient sufficiency mTOR is associated with the lysosomal membrane and interacts with TFEB to prevent its nuclear translocation. In response to impaired cellular energy status mTOR dependent phosphorylation of TFEB is interrupted allowing transcriptional regulation of genes encoding proteins needed for autophagosome formation and increased autophagic flux (Roczniak-Ferguson et al., 2012).

There is normally a basal rate of autophagy in cells to maintain homeostasis, but it can be strongly induced to protect cells under various physiological stresses, such as nutrient depletion and the presence of aggregated proteins (Mizushima and Komatsu, 2011). In mammalian cells, cell death is often associated with

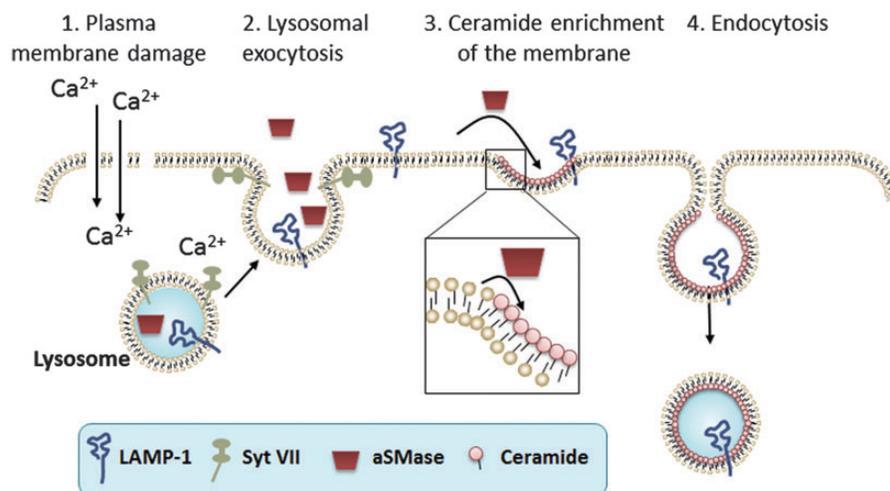
autophagic vacuolization, leading to the conclusion that autophagic cell death was a third cell death modality (Bursch et al., 2000). However, the presence of autophagic vesicles does not necessarily indicate that cell death is mediated by autophagy. Accumulating evidence suggests that autophagic cell death is usually an attempt of the damaged cell to adapt to stress rather than a mechanism to execute cell death (Shen et al., 2012).

#### Lysosomal exocytosis

Lysosomes are involved in a secretory pathway known as lysosomal exocytosis. Initially, lysosomal exocytosis was thought to be limited to specialized secretory cells, but this process seems to occur in all cell types (Rodríguez et al., 1997). Lysosomal exocytosis is a two-step process. First, lysosomes relocate from their perinuclear localization to the close vicinity of the plasma membrane (Jaiswal et al., 2002), where they fuse with each other. This process is followed by lysosomal fusion with the plasma membrane, which occurs in response to an increased intracellular concentration of calcium (Rodríguez et al., 1997). Lysosomal exocytosis plays a major role in important processes such as immune responses, bone resorption, cell signaling, and plasma membrane repair (Andrews, 2000, 2005). Lysosomes contain abundant amounts of ATP, which can be released to the extracellular space during lysosomal exocytosis (Zhang et al., 2007). Such extracellular ATP participates in communication that is mediated by a range of different ATP binding receptors (Ralevic and Burnstock, 1998). Interestingly, ATP can also induce lysosomal exocytosis, enabling intercellular signaling (Zhang et al., 2007).

Plasma membrane injury is a frequent event in mammalian cells, especially in cells that operate under conditions of mechanical stress, such as in muscles and skin (McNeil and Khakee, 1992). The permeability barrier can also be breached, for example, by pathogens gaining access to host cells by secreting pore-forming

toxins (Gonzalez et al., 2008). We have recently found that lysosomal exocytosis is involved in the repair of UVA irradiation-induced plasma membrane damage (unpublished data). During plasma membrane damage, restoration of plasma membrane integrity is essential for the survival of the cell and it is restored within seconds up to a minute, depending on the damage (Deleze, 1970; Steinhardt et al., 1994). As shown in Figure 2, the plasma membrane damage results in calcium influx. Calcium binds to synaptotagmin VII (Syt VII) at the lysosomal membrane and facilitates its interaction with soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), which are essential for membrane fusion (Martinez et al., 2000; Reddy et al., 2001; Andrews and Chakrabarti, 2005). The importance of Syt VII for efficient plasma membrane repair was highlighted in experiments in which Syt VII inhibition prevented membrane resealing (Martinez et al., 2000; Reddy et al., 2001). The lysosomal translocation results in the formation of a lysosomal patch that eventually fuses with the plasma membrane and restores plasma membrane integrity (McNeil, 2002). As a direct consequence of lysosomal exocytosis, lysosomal enzymes are released extracellularly, and the luminal part of LAMP-1 appears at the plasma membrane (Rodríguez et al., 1997). The lesion formed in the plasma membrane is removed by endocytosis to promote wound resealing (Idone et al., 2008). The endocytosis process is dependent on the action of acid sphingomyelinase (aSMase), which is released extracellularly during lysosomal exocytosis (Tam et al., 2010). The aSMase processes sphingomyelin to generate ceramide, which is believed to play an important role in the stress response. A high membrane ceramide content results in an inward bending of the membrane, which facilitates endocytosis (Holopainen et al., 2000). Moreover, ceramide-enriched rafts form signal transduction platforms that are involved in processes such as apoptosis signaling (Corre et al., 2010). Of note, impaired resealing is also observed in



**Figure 2** Plasma membrane repair by lysosomal exocytosis. Damage to the plasma membrane results in calcium influx into the cell (1), which triggers a repair process involving lysosomal exocytosis. Lysosomes are translocated to the periphery, where they form a patch that fuses with the plasma membrane in a calcium-dependent manner (2). Lysosomal exocytosis is dependent on the presence of the calcium sensing synaptotagmin VII (Syt VII), which is located at the lysosomal membrane. As a result of exocytosis, the luminal part of LAMP-1 appears at the plasma membrane. In addition, lysosomal exocytosis results in the extracellular release of lysosomal enzymes, including acid sphingomyelinase (aSMase). At the outer leaflet of the plasma membrane, aSMase converts sphingomyelin into ceramide (3). A high membrane ceramide content results in an inward bending of the membrane, which facilitates endocytosis (4). Thereby, the lesion formed in the plasma membrane is removed and the plasma membrane is restored.

cells microinjected with antibodies against the cytosolic tail of LAMP-1 (Reddy et al., 2001), suggesting an unidentified function of LAMP-1 during lysosomal exocytosis.

#### *Cholesterol homeostasis*

Cholesterol is an essential structural element of cellular membranes as well as a precursor for the synthesis of steroid hormones, bile acids, and lipoproteins. There are marked asymmetries in cholesterol concentration among intracellular membranes. Recycling endosomes, Golgi membranes, and the membranes of ILVs are cholesterol rich, whereas the ER, late endosomes, and lysosomes contain less cholesterol (Maxfield and Wustner, 2002; Möbius et al., 2003). The majority of cholesterol (up to 80%) is found in the plasma membrane, where it constitutes ~40% of total lipids (Lange et al., 1989; Maxfield and Wustner, 2002). Cholesterol participates in the formation of lipid rafts, which provide a special platform for proteins engaged in important cellular processes, such as signal transduction or vesicular transport.

In addition to *de novo* cholesterol synthesis in the ER, the uptake of low-density lipoprotein (LDL) via receptor-mediated endocytosis is an important route for cholesterol entry into the cell (Brown and Goldstein, 1986). LDL-derived cholesterol esters are transported to the lysosomes, where the action of acid lipase liberates free unesterified cholesterol. After being released from lysosomes, free cholesterol is transported to other cellular sites such as the Golgi, plasma membrane, and ER. When the free cholesterol level is too high, acyl-coenzyme A: cholesterol acyltransferase (ACAT), an ER resident enzyme, converts it into cholesterol esters, which are stored in cytoplasmic lipid droplets.

Two proteins present in the endolysosomal compartment have been shown to be necessary for cholesterol efflux from this organelle: the Niemann–Pick disease type C 1 (NPC1) and NPC2 proteins. NPC1 is a large 13-transmembrane protein that is localized to the limiting membranes of endosomes and lysosomes, while NPC2 is a small soluble protein found in the lysosomal lumen (Carstea et al., 1997; Neufeld et al., 1999). The correct functioning of both of these proteins is required for cholesterol efflux from the endolysosomal compartment; however, the mechanism by which these proteins operate remains to be elucidated. In the predominant theory, the soluble protein NPC2 binds free cholesterol in the lysosomal lumen and transports it to NPC1 in the limiting membrane (Kwon et al., 2009). Mutations in the NPC1 or NPC2 protein result in the lysosomal storage disease Niemann–Pick type C, characterized by lysosomal accumulation of sphingosine, glycosphingolipids, sphingomyelin, and cholesterol (Vanier, 2010).

#### **Lysosomal participation in cell death signaling**

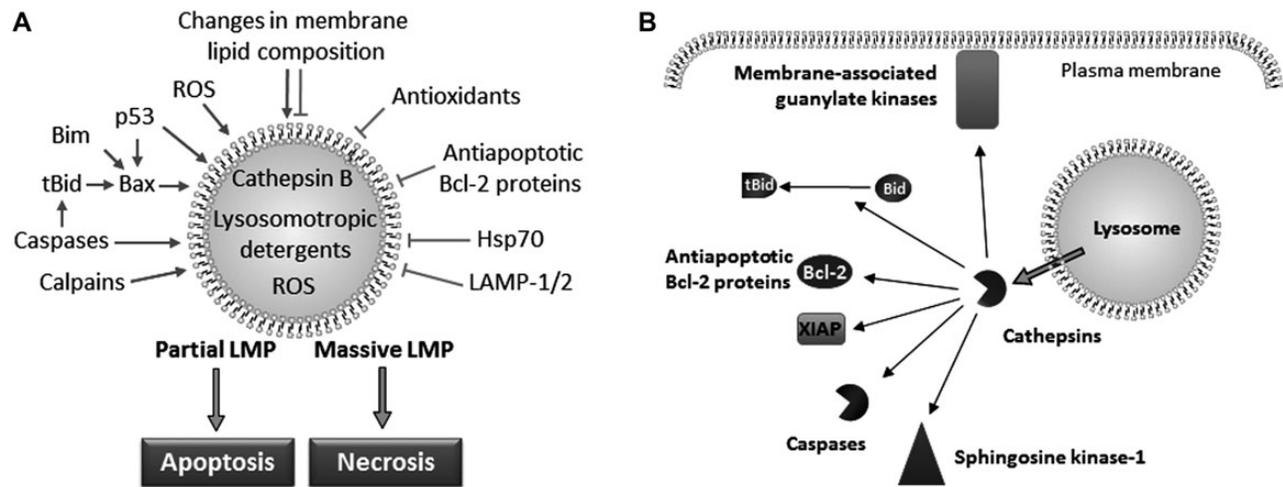
Due to their high hydrolase content, lysosomes are potentially harmful to the cell. Damage to the lysosomal membrane results in leakage of lysosomal content to the cytosol. Lysosomes were referred to as ‘suicide bags’ by Christian de Duve because partial permeabilization of the membrane induces apoptosis and massive lysosomal rupture induces necrosis (de Duve, 1959; Turk and Turk, 2009). The executors of lysosome-mediated apoptosis are not the lysosomes themselves but their hydrolases, more specifically, the cathepsins. A critical step in the mediation of apoptotic signaling by cathepsins is the release of cathepsins to the cytosol, a process known as lysosomal membrane permeabilization (LMP).

The importance of the cytosolic location of cathepsins for their pro-apoptotic function is highlighted by studies in which microinjection of cathepsins into the cytosol was sufficient to induce apoptosis (Roberg et al., 2002; Bivik et al., 2006; Schestkova et al., 2007). Depending on the lethal stimuli and the cell type, LMP can be a triggering event that is critical for activation of the signaling cascade, or it can occur later in the apoptotic process and contribute to amplification of the death signal. Signaling after LMP often involves activation of the caspase cascade, usually via the intrinsic pathway; however, cathepsins can also mediate cell death in a caspase-independent manner (Broker et al., 2004). Cathepsin B induces typical apoptosis-associated changes, including chromatin condensation, DNA fragmentation, phosphatidylserine exposure, and plasma membrane blebbing (Vancompernelle et al., 1998; Foghsgaard et al., 2001). Thus, cathepsins have the ability to participate in both the initiation and execution phases of apoptosis. Part from cathepsins, lysosomal calcium contributes to typical apoptotic changes. Externalization of phosphatidylserine, which serves as a recognition marker of apoptotic cells and enables efficient phagocytosis, is dependent on the release of calcium from the lysosomal compartment (Mirnikjoo et al., 2009).

Evidence supporting the role of lysosomes as cell death mediators comes from studies of compounds that directly target the lysosomes and affect the integrity of their membranes, such as lysosomotropic detergents. However, LMP is also an active contributor to apoptosis signaling induced by more classical apoptosis stimuli, such as ligation of death receptors, p53 activation, UV irradiation, growth factor deprivation, and oxidative stress (Roberg and Öllinger, 1998; Brunk and Svensson, 1999; Gucciardi et al., 2000; Yuan et al., 2002; Bivik et al., 2006). Although experimental data obtained in recent decades robustly illustrate the important and active contribution of lysosomes to cell death induced by a wide variety of stimuli, *in vivo* evidence for lysosome-mediated cell death has been sparse. Thus, critics have suggested that the lysosomal death pathway only occurs *in vitro* or under pathological conditions. However, a lysosome-mediated cell death pathway was recently shown to be active in the regression of the mammary gland after lactation (Kreuzaler et al., 2011). In addition, the expression level of cathepsin D is increased and has been functionally implicated in physiological cell death during embryonic development (Zuzarte-Luis et al., 2007). Thus, lysosomes and cathepsins are also active in cell death signaling under physiological conditions. In yeasts, the cathepsin D orthologue Pep4p is released from the vacuole, an analog of the lysosome, to the cytosol as a response to apoptotic stimuli, similar to the release of cathepsin D in mammalian cells (Pereira et al., 2010). These results suggest that the release of proteases from membrane-bound acidic organelles is an evolutionarily conserved cell death mechanism, indicating the crucial function of lysosomes in cell death signaling.

#### *Mechanisms of LMP*

The mechanism by which cathepsins escape the lysosomal compartment during LMP remains elusive, but several mechanisms have been proposed. Multiple mechanisms may exist that are employed differentially, depending on the inducing stimuli and cell type (Figure 3). The simultaneous release of cathepsins, protons, and lysosomal dyes suggests a nonspecific release mechanism, such as pore formation or limited membrane damage. The



**Figure 3** Lysosomal membrane permeabilization and cathepsin substrates in the cytosol. **(A)** Regulation of lysosomal membrane permeabilization (LMP). The machinery for LMP remains elusive, but several mechanisms have been proposed, and likely multiple mechanisms exist (Johansson et al., 2010). The relative contributions of the factors probably depend on the cell type and the apoptotic stimuli. As for mitochondrial membrane permeabilization, the Bcl-2 protein family is suggested to participate in the regulation of LMP (Kågedal et al., 2005; Werneburg et al., 2007). p53 has also been shown to trigger LMP in both transcription-dependent and -independent manners (Yuan et al., 2002; Li et al., 2007; Wäster and Öllinger, 2009). In addition, proteases, including caspases, calpains and cathepsins, promote the release of lysosomal constituents (Yamashima et al., 1996; Guicciardi et al., 2000; Werneburg et al., 2002; Eriksson et al., 2013). Oxidative stress and reactive oxygen species (ROS) destabilize the lysosomal membrane through lipid peroxidation (Persson et al., 2003), an effect that can be neutralized by antioxidants (Roberg and Öllinger, 1998). There are some proteins that function as safeguards of lysosomal integrity, including LAMP-1 and -2 and heat shock protein 70 (Hsp70) (Nylandsted et al., 2004; Bivik et al., 2007; Fehrenbacher et al., 2008; Kirkegaard et al., 2010). Changes in membrane lipid composition include membrane destabilizing factors, such as phospholipase A2 and sphingosine, as well as protecting factors, including cholesterol and sphingomyelin (Jäättelä et al., 1995; Kågedal et al., 2001; Caruso et al., 2005; Appelqvist et al., 2011). A regulated and partial destabilization of the lysosomal membrane results in apoptotic signaling, while total lysosomal rupture induces necrosis. **(B)** Proposed cytosolic cathepsin substrates during apoptosis. After release from lysosomes following LMP, cathepsins perform their pro-apoptotic function in the cytosol. Direct activation of caspases has been suggested (Ishisaka et al., 1998; Katunuma et al., 2001; Conus et al., 2008), but generally caspases are poor substrates for cathepsins (Cirman et al., 2004). In general, the action of identified cathepsin substrates results in the engagement of the mitochondrial pathway to apoptosis. Bid, a pro-apoptotic Bcl-2 family member, is the most well-studied cathepsin substrate (Cirman et al., 2004; Heinrich et al., 2004; Appelqvist et al., 2012a). Anti-apoptotic Bcl-2 family protein can be cleaved and inactivated by cytosolic cathepsins (Droga-Mazovec et al., 2008). However, the cathepsin-mediated cleavage of X-linked inhibitor of apoptosis (XIAP) suggests that cathepsins also participate in apoptosis signaling downstream of mitochondrial outer membrane permeabilization (Droga-Mazovec et al., 2008). Membrane-associated guanylate kinases that are involved in cell–cell contact, and sphingosine-1 kinase, whose activity promotes proliferation and survival, are other proposed cathepsin substrates (Taha et al., 2005; Ivanova et al., 2011).

release of lysosomal content has been suggested to be size-selective, as small (up to 40 kDa) but not large (over 70 kDa) FITC-dextran complexes are released from lysosomes during apoptosis (Bidère et al., 2003). However, the size selectivity does not apply to all cell death scenarios because the 150 kDa lysosomal hydrolase  $\beta$ -*N*-acetylglucosaminidase ( $\beta$ -NAG) has been shown to be released during LMP under certain experimental conditions (Nylandsted et al., 2004; Kågedal et al., 2005; Blomgran et al., 2007).

Obviously, the constituents of the lysosomal membrane are essential for maintaining the integrity of the organelle. Indeed, the lysosomal cholesterol content regulates the stability of lysosomes and the cell death sensitivity (Appelqvist et al., 2011, 2012b; Reinert et al., 2011). Damage to the membrane components or alterations in membrane structure and fluidity can affect lysosomal stability. There are reports suggesting that lysosomes differ in their vulnerability to LMP. For example, increased size and high lysosomal iron content can make lysosomes more prone to losing their

integrity (Abok et al., 1983; Ono et al., 2003; Persson et al., 2003). For a comprehensive description of potential mediators of LMP we refer to our review article, ‘Regulation of apoptosis-associated lysosomal membrane permeabilization’ (Johansson et al., 2010).

#### Functions of cathepsins in the cytosol

The involvement of lysosomal cathepsins in apoptosis has been demonstrated in various cellular models by both genetic manipulation and pharmacological inhibitors. The presence of endogenous cysteine cathepsin inhibitors in the cytosol can suppress the activity of accidentally released cathepsins (Brzin et al., 1983). In addition, an important regulatory mechanism seems to be pH-dependent proteolysis. Cathepsins are highly active at acidic pH but are inactivated at the neutral pH of the cytosol, due to irreversible unfolding (cysteine cathepsins) or reversible deprotonation of the active site aspartates (cathepsin D) (Turk et al., 1995; Lee et al., 1998). However, cathepsins can be stabilized by substrate binding, and some cathepsins can retain their proteolytic

activity at neutral pH for several hours, allowing transient activity in the cytosol (Kirschke et al., 1989). Another explanation for the preserved proteolytic action of cathepsins is cytosolic acidification, which have been described in a number of experimental systems (Gottlieb et al., 1995; Matsuyama et al., 2000; Nilsson et al., 2006; Appelqvist et al., 2012a). The abundant cysteine cathepsins B and L and the aspartate cathepsin D have been most studied with respect to their roles in apoptosis signaling.

In contrast to caspases, which require activation to promote apoptosis, cathepsins are already active when released from the lysosome following LMP. In the cytosol, cathepsins process other proteins to promote death signaling. In contrast to caspases, which have hundreds of identified substrates, only a small number of apoptosis-associated cathepsin substrates have been identified (Figure 3). The most studied cathepsin substrate is Bid. Multiple cysteine cathepsins (including cathepsin B, D, H, K, L, and S) activate Bid (Cirman et al., 2004; Heinrich et al., 2004; Caruso et al., 2006; Appelqvist et al., 2012a). In cells lacking the endogenous cysteine cathepsin inhibitor, stefin B, neuronal apoptosis cannot be prevented by ablation of Bid, which suggested the existence of other cathepsin substrates (Houseweart et al., 2003). This result led to the discovery of anti-apoptotic Bcl-2 proteins as cathepsin substrates (Droga-Mazovec et al., 2008). Engagement of the mitochondrial pathway is a common event downstream of LMP, as evidenced by the finding that cell death is absent in Bax/Bak double-deficient mice after LMP (Boya et al., 2003). Moreover, mimicking LMP by microinjection of cathepsin D directly into the cytosol induces cytochrome *c* release (Roberg et al., 2002). XIAP is degraded by cysteine cathepsins, indicating that cathepsins also participate in apoptosis signaling downstream of mitochondria (Droga-Mazovec et al., 2008). One report suggests that membrane-associated guanylate kinases, which are involved in cell–cell contacts, are cathepsin substrates (Ivanova et al., 2011). Another target of cathepsins is sphingosine kinase-1, the normal activity of which promotes proliferation and survival (Taha et al., 2005). Direct cleavage and activation of caspase-3 by lysosomal proteases has been suggested (Ishisaka et al., 1998; Katunuma et al., 2001), but in general, caspases are poor substrates for most cathepsins (Cirman et al., 2004). However, caspase-8 has been demonstrated to be activated by cathepsin D during neutrophil apoptosis (Conus et al., 2008). Also 14-3-3, a proposed inhibitor of the pro-apoptotic protein Bax, is a cathepsin D substrate during apoptosis; however, its effect is of minor importance during the initiation phase of apoptosis (Appelqvist et al., 2012a).

### Lysosomes in disease

As many cellular functions involve the lysosomal compartment, lysosomal disturbance has a profound impact on homeostasis. Therefore, it is not unexpected that lysosomal dysfunction causes and contributes to many diseases. Lysosomes have a central role in lysosomal storage disorders, but increasing evidence indicates that lysosomes are involved also in more widespread diseases, such as cancer, Alzheimer's disease, and amyotrophic lateral sclerosis (Nixon et al., 2008; Kirkegaard and Jättilä, 2009; Otomo et al., 2012). Due to the essential role of lysosomes in autophagy, lysosomal dysfunction impairs this process,

thereby contributing to disease (Levine and Kroemer, 2008).

### Lysosomal storage disorders

Lysosomal storage diseases represent a class of inborn pathologies characterized by the accumulation of material in lysosomes. These conditions are caused by the absence or reduced activity of lysosomal proteins, which results in the lysosomal accumulation of substances dependent on these particular proteins (Bellettato and Scarpa, 2010). Often, this material will be stored because digestion is impaired due to enzyme deficiency, but disease can also arise when transport out of the lysosomal compartment is compromised. Over 50 human lysosomal storage conditions have been recognized, and although individually rare, their combined prevalence is ~1 in 8000 births (Poupetova et al., 2010). The massive accumulation of substances affects the function of lysosomes and other organelles, resulting in secondary changes, such as impairment of autophagy, mitochondrial dysfunction, and inflammation. Altered calcium homeostasis is a common pathological feature in these disorders; however, the mechanism leading to defective calcium signaling differs. In NPC cells the lysosomal calcium store is reduced to ~30% due to sphingosine storage, and hence lysosomal calcium release is decreased (Lloyd-Evans et al., 2008). In contrast, in Mucopolidiosis type IV (caused by mutations in MCOLN1 gene, which encodes the ion channel mucolipin 1), calcium release is increased resulting in enhanced fusion within the endocytic pathway (Morgan et al., 2011). Lysosomal storage disorders frequently involve the central nervous system, where neuronal dysfunction or loss results in mental retardation, progressive motor degeneration, and premature death. For detailed information about such diseases, their clinical symptoms, the pathological changes as well as the therapeutic options available, we refer to the following articles (Ballabio and Gieselmann, 2009; Bellettato and Scarpa, 2010; Parkinson-Lawrence et al., 2010; Platt et al., 2012; van Gelder et al., 2012).

Interestingly, the TFEB pathway is activated under lysosomal storage conditions and might be responsible for the increased size of the lysosomal compartment that is present in these disorders (Sardiello et al., 2009). In addition, enhanced clearance of stored material was observed as a consequence of TFEB overexpression, suggesting that activation of the TFEB/CLEAR network could restore the cellular defect in many lysosomal storage disorders (Sardiello et al., 2009).

### Neurodegenerative disorders

Many of the secondary pathological changes (e.g. brain inflammation, alteration of intracellular trafficking, and impairment of autophagy) linked to lysosomal storage disorders are also observed in adult neurodegenerative disorders, such as Alzheimer's, Parkinson's, and Huntington's diseases (Bellettato and Scarpa, 2010). Even if lysosomal disturbances are not a direct cause of the disease, neurodegeneration associated with lysosomal dysfunction and defective autophagy is well documented (Nixon et al., 2008; Cheung and Ip, 2011). Correct autophagic function is essential for cells, particularly for neurons, which rely on autophagy for survival, and the inactivation of crucial autophagy genes in mice results in severe neurodegeneration (Hara et al., 2006; Komatsu et al., 2006). Alterations of the lysosomal compartment are particularly evident and closely linked to Alzheimer's pathology (Nixon and

Yang, 2011). Both disruption of substrate proteolysis within autophagolysosomes or defect lysosomal fusion has been suggested to contribute to autophagy failure in Alzheimer's disease (Lee et al., 2010; Coen et al., 2012).

Independent of the primary defect, disturbances in lysosomal function seem to have more severe consequences in the central nervous system than in other parts of the body. The reason for the selective vulnerability of neurons is unknown but is suggested to be due to (i) their limited regenerative potential, (ii) their status as postmitotic cells, which are unable to dilute undigested material by cell division, (iii) the lack of compensatory metabolic pathways and (iv) their dependence on efficient intracellular trafficking to connect the distal parts with the cell body (Bellettato and Scarpa, 2010).

Because lysosomal dysfunction is associated with many degenerative disorders, therapeutic interventions aiming at restoring lysosomal function may be useful for the treatment of diseases such as Alzheimer's and Parkinson's. Indeed, the restoration of lysosomal proteolysis and autophagy efficiency in mouse models of Alzheimer's disease has yielded promising therapeutic effects on neuronal function and cognitive performance (Pickford et al., 2008; Caccamo et al., 2010; Yang et al., 2011; Steele et al., 2012).

#### Cancer

Rapidly dividing cells, such as cancer cells, are highly dependent on effective lysosomal function, and dramatic changes in lysosomal volume, composition, and subcellular localization occur during transformation and cancer progression (Kirkegaard and Jäättelä, 2009; Kallunki et al., 2013). In a wide variety of cancers, cathepsins are highly upregulated and mislocalization during neoplastic progression results in secretion of both active and inactive forms of cathepsins (Palermo and Joyce, 2008; Kallunki et al., 2013). Secretion of proteolytically active cathepsins to the extracellular space might stimulate angiogenesis, tumor growth, and invasion (Gocheva et al., 2006). Cathepsin D is suggested to facilitate the extracellular activity of cathepsins by proteolysis of cystatin C, an endogenous inhibitor of cysteine cathepsins (Laurent-Matha et al., 2012). In addition, secretion of lysosomal glycosidases such as  $\beta$ -NAG facilitates extracellular matrix (ECM) degradation (Ramessur et al., 2010). The M6PR (also known as insulin-like growth factor II receptor) targets secreted lysosomal enzymes and it is frequently mutated or downregulated in a wide range of malignant tumors, which results in increased secretion of lysosomal enzymes. Forced expression of M6PR has been shown to reduce the tumorigenicity and invasive potential of the tumor cells by restoring the intracellular transport of cathepsins to lysosomes (Puxbaum et al., 2012).

Cancer cells often display p53 mutations that are believed to contribute to treatment resistance. Interestingly, a small molecule screen revealed that a majority of compounds capable of inducing cell death independent of p53 did so by triggering LMP and cathepsin-mediated killing of tumor cells (Erdal et al., 2005). This finding suggests that cancer cells insensitive to traditional therapies may be killed by agents that trigger the lysosomal cell death pathway. Indeed, oncogene-driven transformation is associated with increased cathepsin expression and higher sensitivity toward lysosome-mediated cell death (Fehrenbacher et al., 2004). Cathepsin-mediated degradation of LAMPs may contribute to increased lysosomal instability (Fehrenbacher et al., 2008).

Inhibition of aSMase also destabilizes the lysosomal membrane (Groth-Pedersen and Jäättelä, 2013). Interestingly, the expression of aSMase is significantly reduced in multiple carcinoma, which might contribute to lysosomal destabilization (Kallunki et al., 2013). However, reduced expression of aSMase also reduce the production of the pro-apoptotic lipid ceramide that might be interpreted as an anti-apoptotic adaptation in order to avoid cell death (Savic and Schuchman, 2013).

Thus, transformation-associated changes in the lysosomal compartment have opposing effects in cancer cells because they, on the one hand, increase the tumorigenic potential and simultaneously sensitize the cells to lysosome destabilization and cell death. Increasing our understanding of these changes will hopefully result in novel strategies to target the lysosomal compartment for use in future cancer therapies.

#### Cardiovascular diseases

Cathepsins contribute to the development and progression of cardiovascular diseases, including atherosclerosis and aneurysm formation, by influencing ECM turnover, inflammation, and apoptosis (Lutgens et al., 2007). ECM proteins such as elastin and collagens contribute to the structural integrity of the vascular wall and remodeling of ECM is one of the underlying mechanisms in cardiovascular diseases, as it influences the migration and proliferation of cells within the plaque. While normal arteries express little or no cathepsins, these proteins are abundantly expressed and secreted in atherosclerotic vessels (Sukhova et al., 1998; Jormsjö et al., 2002; Li and Yuan, 2004; Liu et al., 2006a). Inflammatory cytokines, involved in atherosclerotic disease, augment cathepsin expression and activity resulting in ECM proteolysis (Sukhova et al., 1998; Cheng et al., 2006; Liu et al., 2006a). Both human and animal studies demonstrate that especially cysteine cathepsins K, L, and S contribute to the formation and progression of atherosclerotic plaques and these proteases might also influence the stability of advanced plaques (Lutgens et al., 2006; Rodgers et al., 2006; Li et al., 2009; Cheng et al., 2011). In addition, cathepsins have been suggested to influence atherosclerosis by affecting lipid metabolism, for example by degrading lipoproteins (Lutgens et al., 2007).

The effect of cathepsins in atherosclerotic lesions is balanced by the expression of their endogenous inhibitor cystatin C. In addition to the increased expression of cathepsins, cystatin C levels have been shown to be reduced in atherosclerotic and aneurysmal lesions (Shi et al., 1999), further contributing to ECM degradation. In atherosclerotic mice deficient in cystatin C, the degradation of elastic lamina is increased and larger plaque formation found (Bengtsson et al., 2005). As cathepsins and cystatin C demonstrate differential expression at different stages of atherosclerosis, their serum levels are promising biomarkers for cardiovascular disease (Liu et al., 2006b).

ECM degradation and macrophage apoptosis enhance lesion vulnerability, resulting in plaque rupture and thrombus formation. LMP-dependent apoptosis can be initiated in macrophages by lipids, including oxidized lipoprotein particles and oxysterols. The mechanism involves LMP and relocation of cathepsins, followed by activation of the caspase cascade (Li et al., 1998; Yuan et al., 2000). Interestingly, endogenously formed cholesterol crystals induce acute inflammation, which also involves phagolysosomal

damage. This inflammation response is impaired in cells from cathepsin B- and cathepsin L-deficient mice (Duell et al., 2010), which further emphasizes the role of lysosomes in the process.

### Future targeting of lysosomes as therapeutic intervention?

The disease mechanisms are still, to a large extent, unclear for lysosomal storage disorders, neurodegenerative diseases, cardiovascular diseases, and cancer, which impedes the development of effective treatments. However, during the last decades the functions and implication of lysosomes in the pathology of these disorders have become evident, allowing new therapeutic opportunities.

Altered degradation capacity of lysosomes has been suggested to affect brain function during normal aging as well as in age-related diseases. In addition, lysosomal storage disorders share some cellular changes with protein accumulation diseases, like Alzheimer's, Parkinson's, and Huntington's, suggesting that lysosomal disturbances are involved. Therefore it has been suggested that the progression of protein accumulation diseases and lysosomal storage disorders may be retarded or reversed by positive modulation of the lysosomal system (Bahr et al., 2012). Z-Phe-Ala-diazomethylketone (PADK) is a mild inhibitor of cathepsin B and L, which at low concentration enhance hydrolase synthesis and maturation, thereby increasing the clearance capacity of lysosomes (Bendiske and Bahr, 2003). In a transgenic mouse model of Alzheimer's disease, systemic administration of PADK increased the expression and activity of cathepsins B, and PADK treatment resulted in clearance of intracellular amyloid beta and reduced extracellular deposits (Butler et al., 2011). These examples support the idea that lysosomal enhancement by small molecules can be used to remove toxic deposits and to retard the progression of devastating neurodegenerative diseases. Lysosomal degradation can also be enhanced by influencing the intralysosomal ion concentration. The acidic pH of the lysosomal compartment is essential for the correct functioning of this organelle. Cargo release, hydrolase maturation, degradation, autophagy and intracellular trafficking are all dependent on a low pH (Marshansky and Futai, 2008; Sobota et al., 2009). In disorders with an aberrant lysosomal pH, restoration of lysosomal acidification by therapeutic intervention could represent an efficient way to promote the degradation and clearance of accumulating macromolecules. Presenilin-1, a protein related to Alzheimer's disease, regulates the trafficking of the vacuolar H<sup>+</sup>-ATPase to the lysosomes. Accordingly, mutations within presenilin-1, which is one of the major risk factors for familial Alzheimer's disease, resulted in aberrant lysosomal pH, defective cathepsin activation, and faulty degradation that could be attributed to disrupted vacuolar H<sup>+</sup>-ATPase trafficking (Lee et al., 2010). These findings have recently been challenged and presenilin mutations were instead suggested to cause lysosomal dysfunction by altering lysosomal calcium homeostasis, thereby impairing lysosome fusion (Coen et al., 2012). Modulation of cellular calcium may therefore be another efficient therapeutic strategy. Indeed, compensating for the reduced lysosomal calcium levels in the NPC disease by elevating cytosolic calcium reversed the disease phenotype and prolonged survival of NPC mice (Lloyd-Evans et al., 2008). Some lysosomal storage disorders have also been associated with lysosomal alkalization (Bach et al., 1999; Pearce et al., 1999). This is, however, not a

general characteristic of lysosomal storage disorders because the lysosomal pH is not elevated in NPC disease (Lloyd-Evans et al., 2008; Elrick et al., 2012).

The search for therapies that reduce lysosomal storage is a continuous ongoing project. Cyclodextrin derivatives have been used to deplete cholesterol from cells and this is believed to be due to the solubilization of cholesterol within the interior of the cyclodextrin molecules. Unexpectedly, a variant of cyclodextrin incapable of solubilizing cholesterol was as effective at decreasing cholesterol load as its normal counterpart (Ramirez et al., 2011). A possible explanation for this effect is the finding that hydroxypropyl- $\beta$ -cyclodextrin reduces cholesterol storage in NPC1<sup>-/-</sup> mice by inducing lysosomal exocytosis (Chen et al., 2010). TFEB overexpression has been shown to reverse pathologic lysosomal storage by stimulating lysosomal exocytosis (Medina et al., 2011). In support of such clearance mechanisms operating *in vivo*, patients with lysosomal storage disorder secrete the storage products into the urine (Whitley et al., 1989; Wisniewski et al., 1994). However, inducing exocytosis as a therapeutic mechanism is a major concern because the potentially toxic stored contents might not be adequately cleared after release to the extracellular space in the brain (Schultz et al., 2011). Another way to induce the clearance of accumulated material is the induction of autophagy, which has proven beneficial as a therapeutic strategy in neurodegenerative disorders associated with protein aggregation (Harris and Rubinsztein, 2011). A prerequisite for a favorable effect of autophagic induction is a functional lysosomal compartment capable of cargo degradation.

An opposite intervention in the autophagic process, impairment of autophagy, is being investigated as a therapeutic approach to sensitize cancer cells to apoptosis-inducing agents. In general, autophagy functions as a protective mechanism that counteracts apoptosis by providing the cell with energy and building blocks as well as disposal of damaged and non-functional mitochondria, which are a source of reactive oxygen species (ROS) (Lee et al., 2012). Destabilization of the lysosomes by lysosomotropic detergents could be a promising strategy because destabilization would not only impair autophagy, but also promote tumor cell apoptosis through the release of cathepsins, thereby activating the lysosomal cell death pathway.

The significant pro-neoplastic properties of cathepsins, especially when secreted extracellularly, make them interesting targets for cancer therapy (Groth-Pedersen and Jäättelä, 2013; Kallunki et al., 2013). Cathepsin inhibition could be beneficial in cancer treatment; however, multiple cathepsins must be inhibited, due to their redundant function. Inhibition of lysosomal exocytosis may be a better strategy. Liu et al. (2012) showed that if the spontaneous release of proteases from lysosomes was prevented by the inhibition of exocytosis, the invasiveness of glioma cells was inhibited. Moreover, lysosomal function heavily depends on lysosomal pH. By inhibiting the vacuolar ATPase, invasion of human prostate cancer cells was prevented (Michel et al., 2013). In six different head and neck squamous cell carcinoma cell lines, sensitivity to cisplatin was correlated to lysosomal pH (Nilsson et al., 2010). Consequently, cellular sensitivity to cisplatin was increased by lowering the lysosomal pH by treatment with trichostatin A (Eriksson et al., 2013).

Understanding physiological processes in biochemical and molecular details not only offers insight into disease pathogenesis, but also permits the development of new diagnostic and prognostic tools, as well as the design of novel therapeutic compounds. Lysosomes are key components of many cellular processes, which make them attractive therapeutic targets. Future strategies to manipulate lysosomal function might be of great benefit for common diseases such as cardiovascular diseases, cancer, and neurodegenerative disorders.

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