

Chapter 3

Compartmentalization of Proteolysis

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3.1 Introduction

Compartmentalization of proteolysis is essential for homeostasis in all tissues and organs, not only at the level of communication of cells with their environment, but also at the sub-cellular level (Fig. 3.1). Within the context of intracellular compartmentalization, it is important to consider the definition of organelles as sub-cellular regions limited by biological membranes that form distinct compartments featuring specific biochemical environments (Fig. 3.2).

Compartmentalization, in a cellular context, distinguishes hydrophilic sub-cellular regions of defined properties that are separated from one another by hydrophobic membranes (Palade 1964, 1966; Clegg 1991; Lipowsky 1995). Proteolysis, the catalytic hydrolysis (and thus cleavage) of peptide bonds, was traditionally thought to require hydrophilic environments (Gerlt and Babbitt 2001). Lipid-enriched, hydrophobic conditions were not considered conducive to proteolytic catalysis where a molecule of water plays a central role (Chaps. 1 and 2; Brown et al. 2000). However, we have learned during the past decades that such (strict) proposals are too narrow and no longer fully valid (Weihofen and Martoglio 2003; Wolfe and Kopan 2004; Ha 2007). Undisputedly, evolution has found a huge variety of solutions to the demands of our body in modifying proteins—be they

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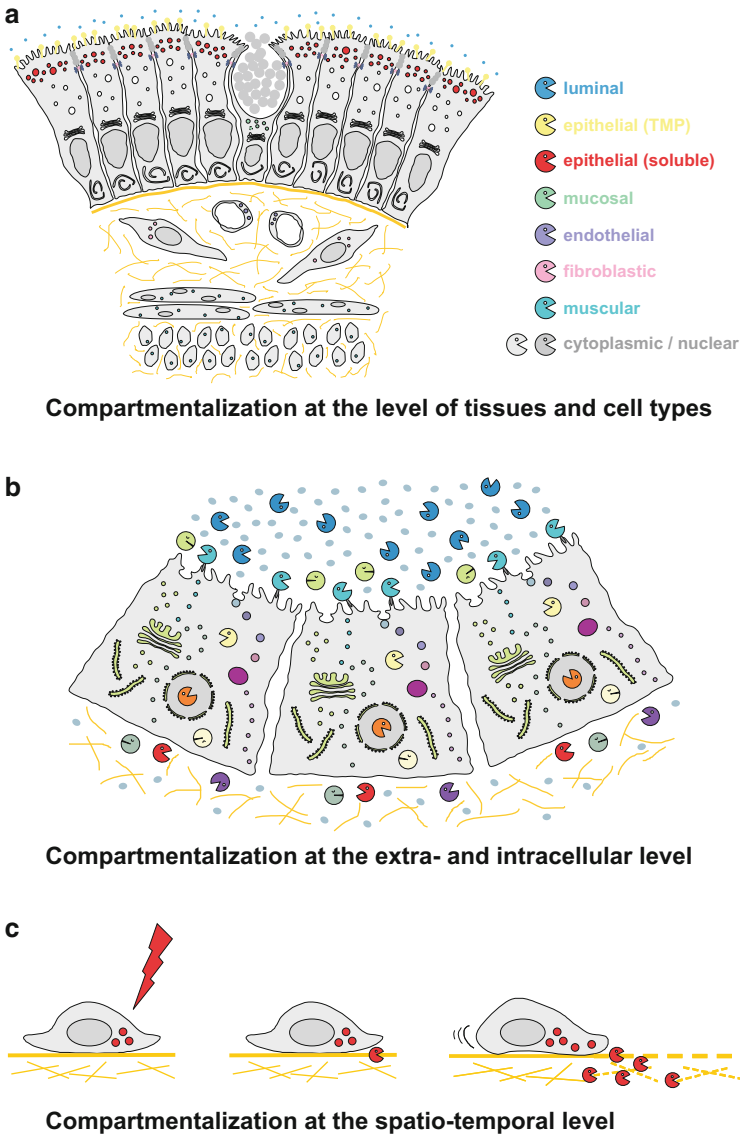


Fig. 3.1 Schematic representation of compartmentalized proteolysis. Proteases perform their tasks at different locations within cells or tissues. Functions of distinct proteolytic enzymes differ depending on the position they fill in the complex network of proteases and where those meet their natural substrates. Compartmentalization occurs at the level of tissues consisting of different cell types (**a**) and at the sub-cellular level (**b**). Regulation of proteolysis in space and time is possible by means of different distributions of proteases, their activators, their co-factors, their inhibitors and their substrates (**c**). Thus, compartmentalization of proteolysis is influenced by signalling events and thereby also depends on the cross-talk of cells with each other and their environment. Bolt signifies a signal from the environment

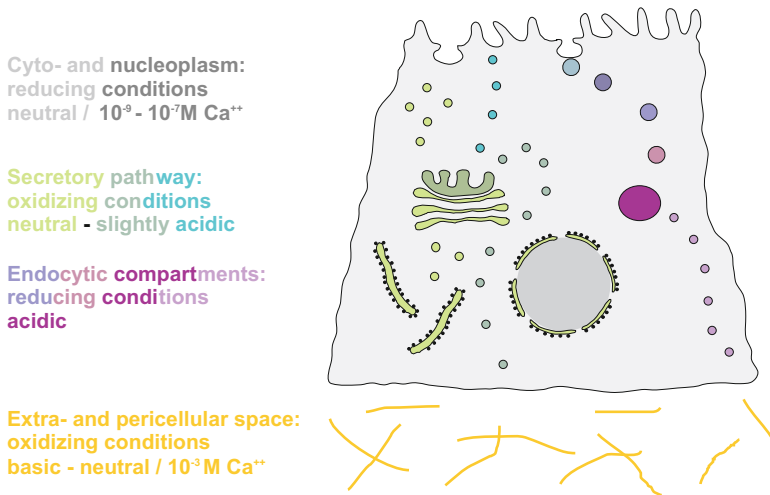


Fig. 3.2 Schematic representation of compartmentalization based on biochemical features of cellular organelles. Peptidases are faced with strikingly different biochemical conditions for substrate interaction and proteolytic cleavage depending on whether extra- or intracellular proteolysis takes place. The latter processes depend on where within the cellular compartments proteases engage in substrate cleavage; a number of examples of different biochemical conditions of proteolytic processing are depicted including oxidizing versus reducing, and acidic versus basic or neutral conditions. The ionic strength of certain compartments, e.g. the secretory granules, or the presence of metal ions are further well known factors contributing to the regulation of the activities of proteolytic enzymes

folded or unfolded, monomeric or multimeric—by protease-mediated hydrolysis that takes place in all possible environments including even the lipid bilayer itself (Chaps. 4 and 7–15; Fritz et al. 1973; Steiner et al. 1980; Fricker 1988; Travis and Fritz; 1991; Stubbs and Bode 1994; Bode et al. 1997; Chapman et al. 1997; Khan and James 1998; Galivan et al. 2000; Davies 2001; Chambers and Laurent 2002; Hooper 2002; Seiki 2002; Potempa et al. 2003; Schechter 2005; Arolas et al. 2007; Barrett and Rawlings 2007; Brix et al. 2008; Cobbe et al. 2009; Ovaere et al. 2009; Schweitzer and Naumann 2010).

Proteases were first defined by their presumably predominant functions as protein degrading enzymes. However, it has been conclusively shown that many proteases undertake a range of functions—and not simply as mere destructive enzymes. Although some proteases are essential in protein turnover, ensuring removal of unwanted or damaged proteins and producing pools of amino acids for *de novo* protein biosynthesis (Bonifacino and Weissman 1998; Reed 2003; Ciechanover and Iwai 2004), discrete cleavage or post-translational processing of target proteins is just as important. These processing events can promote protein folding, maturation, or achieve initiation, termination or modulation of protein functionality (Dobson 2003; Ciechanover 2005; Collins and Tansey 2006). They also engage in peptide processing, for example in antigen presentation, in the cellular defence of intruders and pathogens (Fineschi and Miller 1997; Nakagawa et al. 1998; Villadangos

et al. 1999; Riese and Chapman 2000; Trombetta and Mellman 2005; Herget and Tampe 2007; Watts 2012). Additionally, it is worthy to note that some proteases can also have *non-proteolytic* roles, a topic that requires additional attention as we attempt to grasp the complexity of the world of proteolytic enzymes beyond their active sites (Chaps. 2, 5, 6, and 8; Reudelhuber et al. 1998; Blasi and Carmeliet 2002; Friedl and Wolf 2003; Yu et al. 2006; Lamkanfi et al. 2007; Uddin et al. 2008; Strongin 2010; Bhat and Greer 2011; Kwak et al. 2011).

Proteases can act on substrates in a number of ways: in a simple one-to-one fashion, or to cut specific bonds in complex substrates consisting of several proteins, or to sever high molecular mass proteins containing multiple domains. More than one protease may cleave a specific peptide bond in any one substrate at a time, further adding to the complexity of proteolytic control. It may be of further relevance to note that proteases can act as single molecules or as oligomers (e.g. the 26S proteasome). Increasingly, we are finding evidence for the need for proteases to complex with activators or co-factors (Fig. 3.3). Although these co-factors can be other proteins, they may also comprise lipids (invadolysin, secretases, SPPases) (McHugh et al. 2004; Cobbe et al. 2009; Di Cara et al. 2013), carbohydrates (cathepsin K, ADAMs, ADAM-TSs) or even nucleic acids (truncated cathepsin V, separases) (Chaps. 1 and 2; Bode and Huber 1992, 2000; Uhlmann 2003; Ong et al. 2007; Sun et al. 2009). Proteases may position themselves in a particular cellular context by interacting with scaffolding factors, for example in apoptosis (Chap. 8; Seiki 2002; Schweitzer and Naumann 2010; Kersse et al. 2011), or they may traffic along traditional and novel, sometimes unexpected, transport pathways to reach the location required for proteolytic activity (e.g. endo-lysosomal enzymes, “alternative” secretion of some convertases) (Chap. 4; Linke et al. 2002a; Buth et al. 2004, 2007; Brix and Jordans 2005; Sloane et al. 2006; Brix et al. 2008; Sameni et al. 2009) (Figs. 3.2, 3.4, and 3.5). Proteolytic activity may be controlled by physical compartmentalization of the protease as well as the presence of specific endogenous inhibitor(s).

In this chapter, we highlight some of the facets of compartmentalization of proteolysis—without claiming to be comprehensive in discussing the unlimited variations on the theme of proteolytic activity. We will start by discussing the seemingly most obvious aspect of compartmentalization of proteolysis, that is, the action of proteases in different cellular compartments.

3.2 Sweet or Savoury—Salty or Tart: Biochemical Conditions of the Cleaving Environment

Evolution has given us proteases that are able to hydrolyze peptide bonds in a plethora of diverse biochemical conditions (Figs. 3.1 and 3.2). For example, calpain activity depends on free calcium concentrations ranging from 10^{-9} M in resting cells to 10^{-7} M in activated cells (Chap. 12; Vanlangenakker et al. 2008; Sorimachi et al. 2010), while other proteases like matrix metalloproteinases (MMPs) exhibit

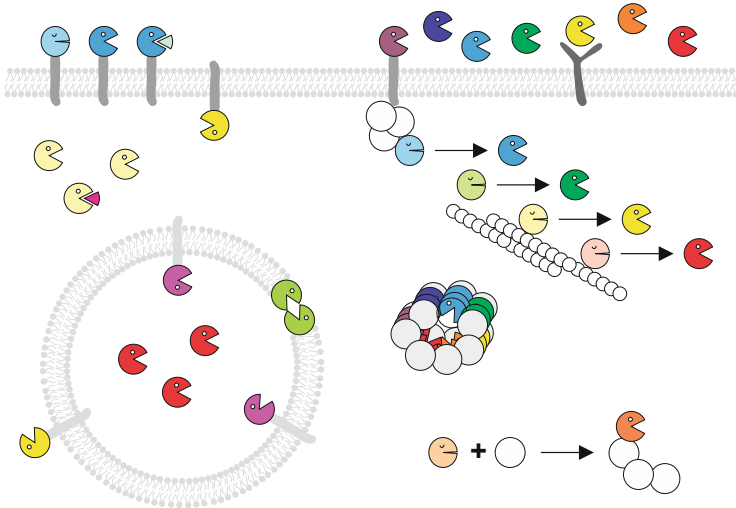
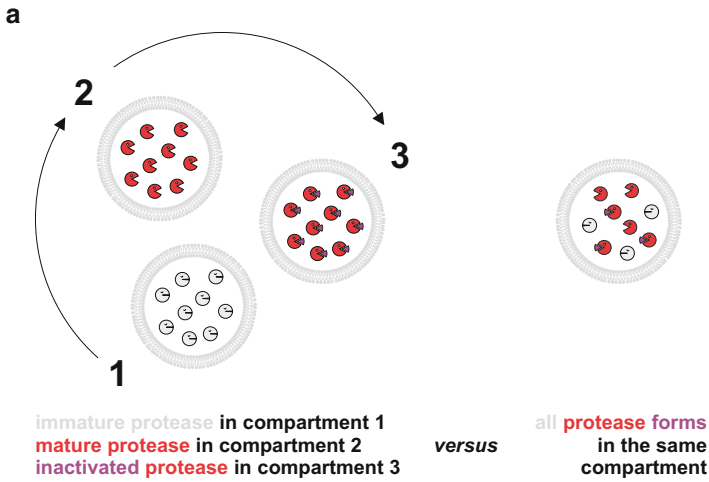


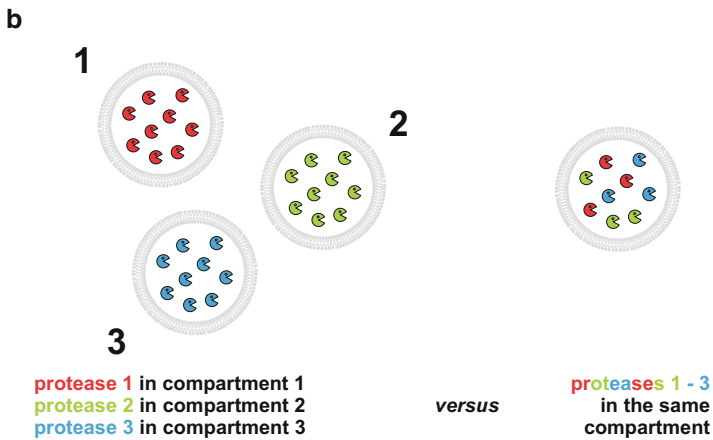
Fig. 3.3 Schematic representation of compartmentalization by lipid membranes, scaffolding factors, and complex formation. Proteases act as single molecules, in complexes, or in sequential modes. Proteolytic enzymes may be soluble, equipped with transmembrane domains, or interact with receptors and other scaffolding factors eventually initiating, terminating, or otherwise affecting the extent of substrate cleavage. Some proteases form multimeric complexes with other macromolecules such as other proteins, glycoproteins or proteoglycans in the extra- and pericellular space. In contrast, the formation of macromolecular assemblies within the cytosol results in substrate processing in e.g. the proteasome which provides a cleavage chamber with specific conditions, thus, separating the protease-substrate interaction from the surrounding cytosol without using the principle of compartmentalization by lipid membranes. Most proteolytic processes require hydrophilic conditions whereas others are enabled to take place near or at the level of lipid membranes for cleaving signal peptides or to carry out proteolysis within transmembrane domains

maximal activity in an extracellular environment characterized by 10^{-3} M free calcium. Furthermore, pH-values ranging from basic to highly acidic, and redox conditions from oxidizing (in the compartments of the secretory pathway and extracellular space) to reducing conditions (within endo-lysosomal compartments) are crucial determinants of protein folding and proteolytic activity (Chap. 2; Barrett and Kirschke 1981; Kirschke et al. 1995; McGrath 1999; Reinheckel et al. 2000; Pillay et al. 2002; Jordans et al. 2009; Zhou et al. 2010; Scott and Gruenberg 2011). Therefore, compartmentalization and complex formation not only generate efficient environments for proteolytic cleavage, but may modulate the local biochemical environment around an active protease.

One exquisite exemplification of this is in the proposed mechanism of Regulated Intramembrane Proteolysis (RIP) where proteases, like signal peptidases, sheddases, or the Alzheimer's Precursor Protein (APP)-cleaving secretases, acquire the ability to cleave near or within amphipathic, α -helical transmembrane domains (Chaps. 9 and 10; Annaert and De Strooper 1999; De Strooper and Annaert 2000; Steiner and Haass 2000; Urban et al. 2001; Urban and Freeman 2002; Ehrmann and Clausen 2004;



Compartmentalization by protease activities and functions



Compartmentalization by protease types

Fig. 3.4 Schematic representation of different principles of compartmentalization. **(a)** Proteolytic enzymes of mammalian cells are often synthesized as inactive precursors, the zymogen forms (1), that are matured by proteolytic processing or other means at the sub-cellular location where the active protease is required to function (2). In contrast, compartments equipped with inactivating factors such as endogenous protease inhibitors provide conditions that restrict proteolytic cleavages (3). Shuttling between the different compartments is realized by directed protein transport processes ensuring protease trafficking to the desired location for meeting with their substrates at a given time (*left panels*). In some cases, individual compartments house different forms of the same proteolytic enzymes which are regulated in their activity by interaction with activating or inhibiting factors to initiate or terminate proteolytic substrate cleavage (*right panel*). **(b)** Proteases belong to distinct protein families (for classification see, <http://merops.sanger.ac.uk>) which are sorted into different (*left*) or the same sub-cellular compartments of mammalian cells (*right*)

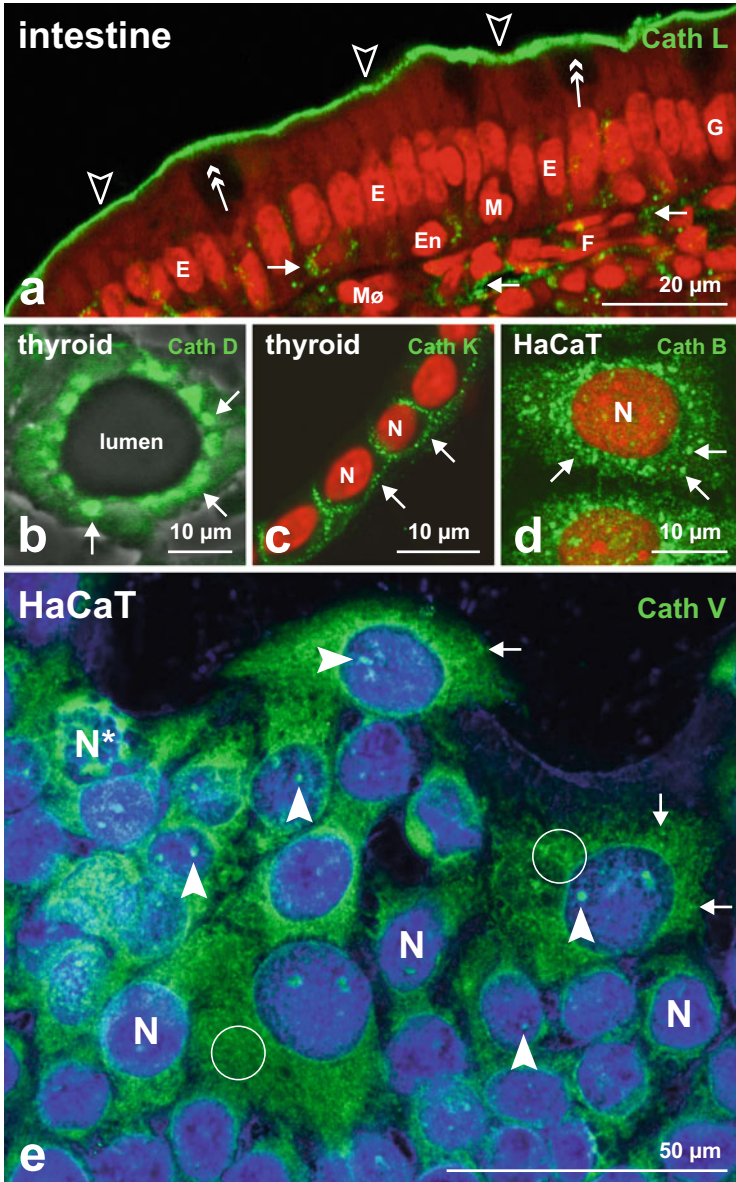


Fig. 3.5 Compartmentalization of cathepsins in mammalian cells. Confocal fluorescence micrographs of cryosections prepared from mouse tissue (a–c) and of cultured human cells (d and e) after immunolabelling with antibodies specific for the aspartic protease cathepsin D or the cysteine peptidases cathepsins B, K, L, or V, and fluorophore-conjugated secondary antibodies (green). Nuclei were counter-stained with Draq 5 (red in a, c, and d; blue in e) and tissue structures viewed in phase contrast (b); overlays of the single channels are displayed in false colours. Although all cathepsins depicted in this figure belong to the so-called “endo-lysosomal proteases”, their distribution patterns differ dramatically between the tissues and individual cell types. (a) Cathepsin L is detectable within vesicles gathering in the peri-nuclear regions of fibroblasts (F) and macrophages (Mø) of the lamina propria as well as of enteroendocrine cells (En) of the

Parkin et al. 2004; Selkoe and Wolfe 2007; Freeman 2008; Murphy 2009; Lichtenthaler et al. 2011). The proteases involved in RIP, represented by soluble and peripheral membrane proteins, are proposed to form channel-like cleaving environments by arranging into multimeric complexes with other proteins. In contrast, turnover of transmembrane proteins in general, which includes extensive cleavage of hydrophobic transmembrane domains in addition to more straightforward degradation of the hydrophilic luminal and cytosolic protein domains, is enabled by their extraction from within lipid-rich environments (membranes) through recruitment by the ESCRT (Endosomal Sorting Complex Required for Transport) machinery (Gu et al. 1997; Gruenberg 2001; Hicke and Dunn 2003; Gruenberg and Stenmark 2004; Piper and Katzmann 2007; van Meer and de Kroon 2011). A variety of hydrolytic enzymes present in the endocytic compartments, then degrade the transmembrane proteins in addition to their other substrates, such as membrane lipids (Chap. 4; Kirschke et al. 1995; McGrath 1999; Brix 2005).

Cleavage of peptide bonds in the extracellular milieu (Fig. 3.1) requires enzymes that thrive in carbohydrate- or glycosaminoglycan-rich environments; conditions of

Fig. 3.5 (continued) mucosal epithelium in mouse intestine (*arrows*). While this staining pattern is expected from the classical view on this member of the papain-like peptidase family, cathepsin L is almost absent from M-cells (M) and even secreted from the enterocytes (E) and goblet cells (G) of the mucosa (*double headed arrows*), from where it reaches the intestinal lumen and re-associates with the apical plasma membrane of the intestine epithelial cells (*open arrowheads*). These distribution patterns point to different functions that cathepsin L fulfils in the various cell types found in the intestinal mucosa. (**b** and **c**) Cathepsins D and K belong to different families of enzymes since they act as aspartic or cysteine peptidases, respectively. The epithelial cells of the thyroid gland sort both proteases into vesicles (*arrows*) that differ in size and are destined to either the apical (**b**) or the basolateral poles of thyroid epithelial cells depending on their physiological states (**c**). Both of these cathepsins together with a number of other related peptidases cleave the prohormone thyroglobulin by endo- and exopeptidatic modes, thereby liberating the thyroid hormones 3',3,5-triiodothyronine T3 and thyroxine T4. The precise cleavage patterns of the substrate depend on the position and activity of its processing enzyme in the various compartments of the endocytic pathway of thyrocytes and within the extracellular lumen of thyroid follicles. Thus, transport of proteases and substrate cleavage appear compartmentalized and tightly regulated in the polarized epithelial cells of the thyroid gland. (**d** and **e**) Human keratinocytes exhibit vesicular staining of the cysteine cathepsin B (*arrows* in **d**) whereas the cysteine cathepsin V is additionally and predominantly detected in an ER-like reticular pattern throughout the cytosol of HaCaT cells (*circles* in **e**). These localizations point to different maturation stages of the two cysteine cathepsins resulting from distinct transport routes under steady state conditions. Sub-compartmentalization is even more complex because molecular variants of cathepsin B and, even more prominent, cathepsin V variants lacking the signal peptides are detectable in the nuclei of proliferating keratinocytes (*arrowheads* in **e**). N denotes nuclei, and N* indicates an apoptotic cell in which the subcellular compartments appear condensed and structurally altered in comparison to the normal appearance of organelles in this keratinocyte cell line. For further details see references (Brix and Herzog 1994; Brix et al. 1996, 1997; Lemansky et al. 1998; Tepel et al. 2000; Linke et al. 2002a, b; Friedrichs et al. 2003; Buth et al. 2004, 2007; Mayer et al. 2006, 2008, 2009; Jordans et al. 2009; Vreemann et al. 2009; Tedelind et al. 2010, 2011; Arampatzidou et al. 2011a, 2012; Dauth et al. 2011b, 2012; Haugen et al. 2013; reviewed in Brix et al. 2001, 2008, 2011; Brix 2005; Brix and Jordans 2005; Arampatzidou et al. 2011b; Dauth et al. 2011a)

elevated negative charge and, hence, salt-‘enriching’ conditions (Jacques 1979; Bergers and Coussens 2000; Blobel 2000a; Rosenblum and Kozarich 2003; Stamenkovic 2003; Strongin 2010; Sato and Takino 2010; Zogg and Brandstetter 2011). Sequential cleavages mediated by cascades of proteases acting on one or several protein substrates (perhaps including the proteases themselves) in a tightly regulated manner seem to explain how compartmentalization is achieved even in the peri-cellular environment (Chaps. 13–15; Chapman et al. 1994; Owen and Campbell 1995; Holmbeck et al. 1999; Sternlicht and Werb 2001; Seiki 2002; Ellis 2003; Mott and Werb 2004; Sounni and Noel 2005; Munshi and Stack 2006), where lipid membranes are not the limiting structure of the reaction compartment, but rather serve as an organizing structural support. The solution of cells to such problems of focalized proteolytic events in the peri-cellular surrounding is often achieved by arranging for protease delivery to the cleavage environment in a sequential and spatially regulated manner (Basbaum and Werb 1996; Murphy and Gavrilovic 1999; Blobel 2000a; Hooper et al. 2001; Itoh 2006; Owen 2008; Vreemann et al. 2009; Friedl and Wolf 2009; Murphy and Nagase 2011; Pagano and Reboud-Ravaux 2011). Upon initiation via appropriate signals, fine-tuning the sequence of proteolysis occurs via interaction with partners comprising not only substrates but also other proteases, membrane receptors, and protease inhibitors (Chaps. 2 and 15; Brix et al. 2001; Schenk and Quaranta 2003; Dano et al. 2005; Jordans et al. 2009; Sun et al. 2009; Das et al. 2010) (Fig. 3.3).

Moreover, the chromatin environment of the nucleus also presents demanding conditions, characterized by extraordinarily long charged polymers such as DNA and RNA (negatively charged), and histones (positively charged). In addition, nucleic acids can provide scaffolding platforms for proteolysis (Ong et al. 2007) where the proteolytic enzymes feature positively-charged molecular surfaces to facilitate interaction with DNA as exemplified by separases to cleave the cohesin “glue” holding sister chromatids together (Heck 1997; Lamond and Earnshaw 1998; Nasmyth et al. 2000; Chapman 2004; Goulet et al. 2004; Goulet and Nepveu 2004; Ruchaud et al. 2007; Hudson et al. 2009; He et al. 2009; Sun et al. 2009; Yanagida 2009; Cauwe and Opendakker 2010).

These examples clearly illustrate that proteases are dependent on the properties of the cleavage environment and that proteases may react or be modulated by rapid and transient alterations of the biochemical conditions during signalling events (Squier 2006; Salvesen and Dixit 1997). However, proteases themselves may be regulated by proteolytic cleavage and by other potentially reversible post-translational modifications including acetylation, methylation, glycosylation, hydrocarbonylation (farnesylation, geranylation, myristoylation), oxidation, phosphorylation, sulfation, SUMOylation, or ubiquitylation (Chap. 5; Doucet et al. 2008). These findings have profound implications on our understanding of the role of proteolysis as, above all, proteases are the mediators of possibly the most important post-translational modification of proteins—cleavage! This is because proteolytic action is essentially non-reversible and therefore frequently represents ‘points of no return’ as exemplified by caspase activation during apoptosis (Chap. 8).

Thus, eukaryotic cells have devised an almost unlimited repertoire of proteases; including soluble enzymes, transmembrane and peripheral-membrane proteins, macromolecular assemblies in the cytosol (proteasomes) or in the extracellular environment (meprins, MMPs, plasmin/uPA/uPAR). These proteases have the ability to interact with a likewise versatile group of substrates ranging from dipeptides to supramolecular protein assemblies that can be modified by endo- or exo-peptidic cleavages (Chaps. 1, 2 and 5). The diversity of proteolytic enzymes is represented by some hundreds of molecules classified on the basis of the catalytic mechanism of action as aspartic, cysteine, glutamic, metallo, serine, or threonine proteases (Chap. 1; Barrett et al. 2001, 2003; Barrett 2004; Rawlings et al. 2004; Rawlings 2010). In addition, a number of peptidases have not yet been classified into any of the known enzyme families.

3.3 The Architectural Art of Compartmentalization of Proteolysis: Specified Rooms for Proteolytic Processing

Like a room plan that is defined when constructing a new house, the eukaryotic cell hosts a variety of compartments with specific, well-defined conditions for specific [proteolytic] activities.

The nucleus, like an office or a creativity centre, can be considered a compartment of planning where proteolytic processing of proteins is a rare event needed for reading and distribution of the blueprint dictating when proliferating cells progress through the cell cycle, or pause in G₀-phase in order to differentiate (Nasmyth et al. 2000; Pellman and Christman 2001; Goulet and Nepveu 2004). Therefore, histones, transcription factors, and structural proteins connecting sister chromatids are the main substrates known for nuclear proteases (Heck 1997; Goulet et al. 2004, 2007, 2008; Ong et al. 2007; Tedelind et al. 2010; Haugen et al. 2013). While more substrates will undoubtedly be identified in the future, it appears that protease activity in the nucleus of interphase cells is occasional and highly regulated. During mitosis, the nuclear envelope is disassembled (as a result of reversible phosphorylation cascades), and proteolysis follows the principles of the cytoplasm. Upon initiation of apoptosis by specific intrinsic or extrinsic factors, however, the nuclear envelope is also rearranged (with cleavage of nuclear lamins this time), eventually leading to programmed cell death (Wyllie et al. 1980).

The kitchen where meals are created can be compared with the centres of *de novo* protein biosynthesis such as the rough endoplasmic reticulum (rER), and the semi-autonomous organelles, mitochondria and plastids. Proteases of these compartments—perhaps with additional exceptions in the cytoplasm (see below)—are selective in their choice of substrates, i.e. they cut off short targeting sequences to ensure directionality of delivery of newly synthesized proteins (ER, mitochondria, plastids) and, together with chaperones, they sample and inspect protein domains for proper folding (cytosol, ER, mitochondria, plastids).

The proteases of the compartments of *de novo* protein biosynthesis are involved in maturation of newly synthesized proteins, and therefore enable limited proteolysis that is domain-driven, sequence-targeted and restrictive—rarely involving “arbitrary” cleavage along the length of the polypeptide chain. Only in conditions of overload or disease, resulting in massive misfolding of newly synthesized proteins, are the proteases of these compartments ‘up-regulated’ for enhanced protein processing. However, the cell will first take all measures possible to attempt enhanced folding or refolding assisted by chaperones that become up-regulated in the so-called Unfolded Protein Response (UPR). The misfolded proteins, however, like the kitchen garbage, are delivered to other compartments for storage and disposal in aggresomes and inclusion bodies (Kopito 2000; Singhvi and Garriga 2009). The improperly folded proteins will eventually be destined for degradation by proteasomes. This process, when involving the ER, is also known as ER-associated degradation (ERAD) during which newly synthesized but misfolded proteins of the ER lumen are retrotranslocated for degradation in cytoplasmic and ER-associated proteasomes (Wiertz et al. 1996a, b; Meusser et al. 2005; Romisch 2005).

The post-ER compartments of the secretory pathway including the intermediate compartment, Golgi apparatus, *trans*-Golgi network (TGN) and secretory vesicles are crucially important for modification, further maturation, sorting and packaging of proteins (Farquhar and Palade 1981, 1998; Varki 1993; Rothman and Wieland 1996; Kaiser et al. 2002). Like in a living room, molecules involved in post-translational modifications of pro-proteins (such as glycosylation, phosphorylation, sulfation, or proteolytic processing) gather to perform the critical cellular actions in preparation for extracellular secretion of proteins that frequently takes place in response to communication of cells with their extracellular environment (Docherty and Steiner 1982; Zhou et al. 1999). Well-studied proteases known for their significance as proprotein-processing enzymes are furin and other proprotein-convertases (PCs) (Seidah et al. 1991; Garten et al. 1994; Schaller and Ryan 1996; Nakayama 1997; Steiner 1998; Seidah and Prat 2002; Thomas 2002; Rockwell and Thorner 2004; Henrich et al. 2005; Scamuffa et al. 2006; Creemers and Khatib 2008; Seidah 2011). The PCs interact in a transient and selective manner with a huge variety of soluble proteins thereby processing the pro-forms of secretory proteins that include ECM constituents as well as peptide or protein hormones (Steiner 1969; Steiner et al. 1980; Seidah and Chretien 1999; Fu et al. 2008).

The major sorting steps take place at the level of the TGN, a compartment that resembles endo-lysosomes with respect to acidity, and where cargo loading and packaging into secretory vesicles or secretory granules and other Golgi-derived vesicles occurs. Thus, proteins en-route to endo-lysosomes including the zymogen [inactive] forms of proteases such as the asparaginyl endopeptidase (AEP/legumain) or the cathepsins are also transported along the secretory route to the TGN. From here, they reach their next sub-cellular destinations in a non-processed and proteolytically inactive proform (Chap. 4; Mort and Buttle 1997; Schaschke et al. 1998; Khan and James 1998; Linke et al. 2002a, b; Mach 2002; Wiederanders et al. 2003; Burden et al. 2007; Buttle 2007; Brix et al. 2008). Some endo-lysosomal proteases, like cathepsins B and L, are believed to become

sorted into and activated in secretory vesicles of pancreatic cells and in neurosecretory granules of pituitary cells (Tooze et al. 1991; Halangk et al. 2000; Hook et al. 2004, 2008; Meister et al. 2010; Funkelstein et al. 2010); these examples, however, are specific to certain cell types. Secretory granules and secretory vesicles of most eukaryotic cells may be considered as compartments where proteases that are almost ready to function (like chymase, trypsin, perforins) are stored together with a number of other secretory macromolecules in a highly concentrated and compacted fashion. Only very-limited proteolysis is taking place in these granules and vesicles, if at all (Gomez-Lazaro et al. 2010), and they may thus be considered analogous to hallways and corridors of houses that are used by both inhabitants and visitors simply for by-passing and through-traffic and possibly where coats are removed.

The dining room, designed for consumption of meals, serves as a reference point for the compartments of the endocytic pathway, i.e. endosomes and lysosomes (Gruenberg and Howell 1989; Schmid 1993; Gruenberg and Maxfield 1995; Mukherjee et al. 1997; Gu and Gruenberg 1999; Gagescu et al. 2000; Nichols and Lippincott-Schwartz 2001; Conner and Schmid 2003). A number of entry points exist for proteins reaching endo-lysosomes (Rechsteiner 1990; Seglen et al. 1996; Felberbaum-Corti et al. 2003; Lin et al. 2004; Klionsky 2007; Mizushima et al. 2008; Arias and Cuervo 2011; Chen and Klionsky 2011). Endo-lysosomes are multi-faceted and interchangeable sub-cellular compartments that perform numerous functions above their main catabolic tasks in the break-down of proteins and other internalized molecules (Brix 2005; Brix et al. 2008). Restricted and limited proteolysis in early endocytic compartments is extremely important in antigen processing for subsequent MHC class II dependent presentation (Riese and Chapman 2000). The early endosome is considered a ligand-unloading and a sorting station (not only for polarized epithelial cells), and it has receiving and distribution functions resembling those of the TGN in many respects (Howell et al. 1989; Sachse et al. 2002). Sorting may result in subsequent trafficking of proteins like receptors back to the plasma membrane for recycling, or further transport to the later endocytic compartments for transient storage or degradation (and thus, down-regulation) (Katzmann et al. 2002; Lin et al. 2004). The late endosome, however, is clearly the dining table where incoming proteins are taken apart and often broken down to the level of single amino acids. The menu (sequence) and the selection of substrates available on the dinner table will need one or many proteases to proteolytically process any given protein substrate (Tedelind et al. 2011). Thus, early, or possibly even all compartments along the endocytic pathway may be utilised—and, like the arrangement of our dinner tables, the number and types of cutlery will differ depending on the food served. The remnants of proteolytic processing will be stored in poorly accessible residual bodies often accumulating in lysosomes and in vacuoles [plant], which we may therefore compare with the trash bags of our house.

Peroxisomes of animal cells, glyoxysomes of plant cells, and the smooth ER of all eukaryotic cells are the major compartments for detoxification (Platta and Erdmann 2007) in which, in part, extremely oxidative environments enable

beta-oxidation which facilitates protein degradation. The composition of peroxisomes and glyoxysomes does not appear to contain proteases. Yet, occasionally, there have been reports of proteolytic enzymes like insulin-degrading enzyme (IDE) that are involved in the degradation of oxidized proteins within peroxisomes (Authier et al. 1996; Morita et al. 2000). However, these compartments are certainly not considered protein-processing compartments of major impact on cellular functions, so we may compare them to the trash compactor or garbage disposal of a house that dispose of contaminating and potentially toxic components.

3.4 Proteases Facing the Extracellular Space

The outside deck/terrace of eukaryotic cells is represented by the plasma membrane with a variety of cellular appendages like cilia, flagella, or microvilli reaching into the extracellular surroundings (Kenny and Maroux 1982). Like the plasma membrane itself, these appendages can carry a multitude of proteolytic enzymes anchored as transmembrane proteins or, if soluble, by binding to cell surface receptors (Chaps. 7, 9 and 11; Bode et al. 1996; Rosenblum and Kozarich 2003; Mentlein 2004; List et al. 2006; Bugge et al. 2007; Turner and Nalivaeva 2007; Lopez-Otin and Bond 2008; Szabo and Bugge 2008; Sohail et al. 2008; Sterchi et al. 2008; Choi et al. 2009; Ramsay et al. 2009; Yao 2010; Hendrickx et al. 2011). Such co-ordination of proteolytic activity in the peri-cellular environment is well known for important contributions in protein breakdown and peptide processing, in particular, at the apical plasma membrane domains of enterocytes (Chap. 11; Kenny and Maroux 1982; Bank et al. 2008; Matteucci and Giampietro 2009; Yu et al. 2010; Arampatzidou et al. 2011a, 2012). A classic example of this is in the most vital event of fertilization, where proteases are secreted from within the sperm head acrosome for degradation and modification of the protective vitellin layer surrounding the oocyte (Blobel 2000b). The oocyte is eventually reached by the sperm for subsequent fusion and zygote formation through explosive, actin polymerization-driven extrusion of microvillus-like extensions.

In addition to outward-looking appendages of cells, invaginations of the plasma membrane also play a role in the dynamics of proteolysis. Some invaginations form during internalization of molecules destined to reach the compartments of the endocytic pathway. Others, like caveolae (little flask-like caves), seem to be less well connected to the cellular interior, but provide a specific biochemical environment in which protein processing is facilitated (Simionescu et al. 1972; Hajjar and Acharya 2000; Predescu et al. 2001; Kim and Hajjar 2002; Pelkmans and Helenius 2002; Navarro et al. 2004). Thus, plasma membrane indentations—caveolae and other non-caveolin coated microdomains—can provide areas of concentrated cell surface receptors for restricting and focalizing proteases to specified sub-domains of the cell surface (Owen and Campbell 1995; Mai et al. 2000; Cavallo-Medved and Sloane 2003; Gumy et al. 2010).

Invadopodia may be analogous to caveolae in their protease concentrating roles but are constructed so as to extend finger-like into the extracellular matrix (Chen 1996; Murphy and Gavrilovic 1999; Linder 2007; O'Brien and O'Connor 2008; Frittoli et al. 2011). Invadopodia have become prominently known for their distinctive composition and functional duality for being involved in both cell adhesion and extracellular matrix degradation through associated proteases. Hence, delicate and rapidly interchangeable cellular extensions that protrude and communicate with the surrounding environment like an easily remodelled patio may also contribute in multiple ways to peri- and extra-cellular proteolytic processes (Woodward et al. 2007; Brix et al. 2008; Korkmaz et al. 2008; Pham 2008; Stetler-Stevenson 2008; Sato and Takino 2010). The proteases found in such exposed positions are usually characterized by extended extracellular domains which are often heavily glycosylated to promote stability against proteolytic attacks, and like their soluble relatives, provide many platforms and binding sites for interaction with other macromolecules or regulatory factors (Gahmberg and Tolvanen 1996; Manon-Jensen et al. 2010; Cawston and Young 2010).

The extracellular space itself, the garden, also houses a myriad of proteases that are derived from the many secretions of different cells in a tissue (Chaps. 6, 13–15; Andrews 2000; Overall and Blobel 2007). Proteolytic enzymes in the extracellular space may either be secreted in active form or remain latent until activation triggers other proteolytic enzymes to convert them into active proteases that often interact with each other to cleave protein substrates by sequential modes (Brix and Herzog 1994; Brix et al. 1996, 2001; Tepel et al. 2000; Linke et al. 2002a; Mayer et al. 2009; Vreemann et al. 2009; Tedelind et al. 2011). The extracellular space can therefore be considered a rich reservoir of proteolytic enzymes that are not only crucial for tissue remodelling but, in part, also contribute to organization of extracellular matrix components, a function that is crucial during morphogenesis in embryonic development (using ADAMs [A Disintegrin And Metalloprotease], ADAM-TSs) (Chap. 9; White 2003; Noel et al. 2004; Apte 2004; Edwards et al. 2008; Kveiborg et al. 2008; Reiss and Saftig 2009; van Goor et al. 2009; Dikic and Schmidt 2010; Urban 2010; Saftig and Reiss 2011).

3.5 Democracy as an Answer for Radical Decision-Making Processes

Radical decision-making during peptide bond cleavage may be seen as the hallmark of proteolytic activity, yet proteases can hardly be thought of as dictators. Often proteases act in proteolytic networks in which it is not the individual enzyme that counts (Chaps. 2 and 5). Collaboration, including finely-tuned and highly-regulated actions amongst several proteases, is instrumental in paving the way to success. Clearly, cleaving substrates at the right time and place, and with the desired pace and the required specificity is crucial (Liu et al. 1999; Kidd et al. 2001; Rao 2003;

Baruch et al. 2004; Joyce and Hanahan 2004; Blum et al. 2005; Brix and Jordans 2005; Carlson and Cravatt 2007; Gocheva and Joyce 2007; Victor and Sloane 2007; Blum 2008; Brix et al. 2008; Jedeszko et al. 2008; Paulick and Bogyo 2008). We shall discuss these aspects of sub-compartmentalization by looking in more detail at the specialists. These include the endo-lysosomal proteases, which come in a most astounding array and act, not exclusively but optimally in endosomes and lysosomes of mammalian cells (Chap. 4; Kirschke et al. 1995; Chapman et al. 1997; McGrath 1999; Turk et al. 2001; Brix 2005; Mohamed and Sloane 2006; Brix et al. 2008; Reiser et al. 2010) (Fig. 3.5).

The compartments of the endocytic pathway contains a wide range of protein processing and degrading enzymes including the cysteine peptidase AEP/legumain that engages in proteolytic maturation of the proforms of other endo-lysosomal proteases (Barrett and Rawlings 2001; Ishidoh and Kominami 2002; Watts et al. 2005; Haugen et al. 2013), aspartic cathepsins D and E (Barrett 1979; Yamamoto 1995; Dunn et al. 1998; Tatnell et al. 1998; Rochefort et al. 2000; Tsukuba et al. 2000; Dash et al. 2003; Nakanishi 2003; Liaudet-Coopman et al. 2006; Zaidi et al. 2008; Hook et al. 2008; Nicotra et al. 2010), the serine cathepsins A and G (Travis 1988; Hiraiwa 1999; Pham 2006; Caughey 2007; Korkmaz et al. 2008; Meyer-Hoffert 2009; Kessenbrock et al. 2011), and the most versatile group of the cysteine cathepsins B, C, F, H, K, L, O, S, V, W, and X/Z in man (Chap. 6; Kirschke et al. 1995; McGrath 1999; Turk et al. 2001; Greenbaum et al. 2002; Brix 2005; Sloane et al. 2005; Mohamed and Sloane 2006; Gocheva and Joyce 2007; Brix et al. 2008; Reiser et al. 2010). Rodents express even more (placental) cysteine cathepsins (Sol-Church et al. 2002) and protozoa like *Tetrahymena* are clearly very extreme examples with dozens of these proteolytic enzymes. The cathepsins that can be found in endo-lysosomes of every eukaryotic cell are the cathepsins B, D, G, H, and L (Turk et al. 2000; Reinheckel et al. 2001; Vasiljeva et al. 2007; Brix et al. 2008; Reiser et al. 2010), while other cathepsins may be present only in certain cell types that are facing very specific challenges, e.g. osteoclasts express high levels of cathepsin K to facilitate bone matrix turnover (Bromme and Okamoto 1995; Saftig et al. 1998; Tepel et al. 2000; Lecaille et al. 2003; Desmarais et al. 2009; Podgorski 2009; Rachner et al. 2011). Nonetheless, all of these proteolytic enzymes can process their substrates by limited or by full proteolysis (Brix and Herzog 1994; Brix et al. 1996, 2001; Friedrichs et al. 2003; Dauth et al. 2011a, b).

Substrates reach the endocytic compartments not only by internalization from the extracellular space, but also by direct entry from the cytosol via chaperone-mediated or “classic/conventional” autophagy (Dice 2007). The outcome of substrate proteolysis by endo-lysosomal proteases depends on whether individual or multiple proteases act on the substrate with endo- and/or exo-peptidase modes of cleavage (Chaps. 1, 2, 6, 15; Barrett and Kirschke 1981; Kirschke et al. 1995; McGrath 1999; Tepel et al. 2000; Jordans et al. 2009). Moreover, proteolytic processing in endocytic compartments is aided by increasingly reductive conditions that support protein unfolding (Pillay et al. 2002; Jordans et al. 2009; Scott and Gruenberg 2011). In general, the hydrolytic enzymes of endo-lysosomes will team

up so that almost all protein substrates with their many other, non-proteolytic post-translational modifications can be handled very efficiently. Interestingly enough, endo-lysosomal proteolytic enzymes are sometimes selective in their substrate choice, whereas others are less selective and cleave almost any protein substrate. The pH optimum of substrate cleavage by endo-lysosomal proteolytic enzymes spans an astonishingly wide range from neutral pH-values for cathepsin S down to the most acidic pH-values for cathepsin D (Takahashi and Tang 1981; Kirschke and Wiederanders 1994). Hence, protein processing and degradation by endo-lysosomal proteases is already initiated in the peri-cellular vicinity of specialized cells that secrete these enzymes in a regulated or non-regulated manner, and, in general, the activity of endo-lysosomal proteases is available in all compartments of the endocytic pathway (Brix and Herzog 1994; Brix et al. 2008). An example of this kind can be found in the thyroid gland, where a huge protein substrate (thyroglobulin) and its covalently cross-linked supramolecular assemblies are handled by the cathepsins B, K, L, and S acting in a temporal- and spatially-regulated, sequential manner for proteolytic liberation of iodinated thyronines from within the polypeptide chain (Brix et al. 1996, 2001; Friedrichs et al. 2003; Jordans et al. 2009; Dauth et al. 2011a).

The cocktail of proteases that is present in one or another sub-compartment of the endocytic pathway will determine the extent of protein substrate cleavages. Numerous questions therefore arise: what is an endosome and what is a lysosome? What governs or triggers transport of endo-lysosomal proteases into the interchangeable compartments of the endocytic pathway? And what are the targeting sequences and which mechanisms explain why some compartments retain fewer, while others contain more, of these proteolytic enzymes? The answers to these questions are partially derived from the study of mannose 6-phosphorylation of the pro-forms of endo-lysosomal proteases (Kornfeld 1992; Peters and von Figura 1994; Bresciani and von Figura 1996). This post-translational modification is maintained in the compartments of the secretory pathway, but partially also persists in the extracellular space. The well known cation-dependent and -independent mannose 6-phosphate receptors can deal with sorting at the level of the TGN, or with re-internalization should pro-forms become secreted (Peters et al. 1990; Hille et al. 1992; Koster et al. 1993; McIntyre et al. 1994; Pohlmann et al. 1995). However, this elegant molecular mechanism of endo-lysosomal enzyme trafficking is not realized in all cell types (Ludwig et al. 1994; Pohlmann et al. 1995; Tanaka et al. 2000). Therefore, the answers to these questions will not be universal, but vary from cell type to cell type (Linke et al. 2002a, b; Brix 2005; Brix and Jordans 2005; Brix et al. 2008; Tedelind et al. 2011).

3.6 Debating Clubs: Proteases and Their Inhibitors

Proteolysis is a result of balancing proteolytic and anti-proteolytic factors (Chaps. 6 and 15; Turk and Bode 1991; Basbaum and Werb 1996; Turk et al. 1997; Matrisian 1999; Coussens et al. 2002; Abrahamson et al. 2003; Turk et al. 2003; Kaiserman et al. 2006; Mohamed and Sloane 2006; Lopez-Otin and Matrisian 2007; Vasiljeva et al. 2007; Scott and Taggart 2010; Reiser et al. 2010). While this notion may have been formulated early on in the examination of proteolysis, it still remains an apt proposal. However, the temporal and spatial regulation of proteolysis must be considered as equally important to the balance with anti-proteolytic factors, if not more. Hence, a well orchestrated debating event between substrates, proteases, and their inhibitors is required for a given cellular process to dictate the final outcome of proteolytic cleavage. So far, many more investigations exist on the proposal of this house: “how and when and where to *start* proteolysis”, rather than on: “how and when and where to *halt* the event”. However, signalling may intervene (Mackie et al. 2002; Ichihara et al. 2006; Mockaitis and Estelle 2008; Murphy et al. 2009; Smith and Marshall 2010) and continued proteolysis may be required in some instances. Control is essential as if proteolysis is exceeded, it may lead to severe disease and eventual death. Therefore, inhibitors of proteases, that typically act intracellularly, are highly concentrated in extracellular fluids for prevention of premature proteolysis around a cell or tissue should the proteases happen to escape from cells in unplanned events (Travis and Salvesen 1983; Turk and Bode 1991; Nagase et al. 1996; Bode et al. 1999; Deveraux et al. 1999; Silverman et al. 2001; Salvesen and Duckett 2002; Abrahamson et al. 2003; Whisstock and Bottomley 2008; Drag and Salvesen 2010). Thus, termination of proteolytic cleavage is as important as its initiation and the Master of Ceremony that determines the sequence and types of proteolytic events taking place must consider protease inhibitors as essential factors in the compartmentalization of proteolysis.

Again, endo-lysosomal enzymes represent good examples for illustrating such control mechanisms by their endogenous inhibitors. Endo-lysosomal enzymes can become extremely dangerous for other cellular compartments or even for the entire cell if released into the extracellular space in an uncontrolled fashion. Therefore, endo-lysosomal proteolytic enzymes must be transported in a defined manner and premature activation is further shielded by potent 60–100 residue inhibitory N-terminal pro-peptide domains as in the case of cysteine cathepsin proteases (Chap. 4; Mach et al. 1994; Mach 2002; Wiederanders et al. 2003). These domains have two functions; firstly, they adopt their own clearly defined secondary structure which acts as a folding chaperone to facilitate the larger catalytic domain to fold. Secondly, the pro-peptide binds tightly across the active site of the catalytic domain in reverse orientation to normal substrates, thus acting as competitive inhibitors. This tight binding is pH dependent and as the inactive zymogen species is transported to the lysosomes, the propeptide will dissociate from the catalytic domain in the acidified lysosomal lumen, leaving the active site exposed and active and results in the irreversible proteolytic removal of the propeptide domain leaving

the mature active protease domain (Pungercar et al. 2009; Linke et al. 2002a, b). However, despite this understanding of the role of the pro-peptide, the recent studies highlighting the presence of cysteine cathepsins in the nucleus, a consequence of downstream translational initiation (Chapman 2004; Goulet and Nepveu 2004; Goulet et al. 2004; Tedelind et al. 2010), highlights that these control mechanisms for the pro-peptide require further supplementation by the presence of inhibitors such as Stefin B in the nucleus (Ong et al. 2007; Ceru et al. 2010).

Cystatins and stefins are the most powerful counter-players of some of the endo-lysosomal proteases (Turk and Bode 1991; Turk et al. 1997, 2003; Abrahamson et al. 2003). Some families of these protease inhibitors reside within the cytosol whereas others are secreted into the extracellular space for abundant presence in extracellular fluids of tissues and body organs. Hence, the cysteine peptidase inhibitors are separated as safe-guarding molecules from the endo-lysosomal cysteine proteases by a membrane, the endo-lysosomal membrane and/or the plasma membrane. Very similar strategies are realized for serpins that inhibit both serine- and cysteine peptidases and which are abundantly present in the cyto- and nucleoplasm of eukaryotic cells (Silverman et al. 2001; Whisstock and Bottomley 2006, 2008). The aspartic peptidases of endo-lysosomes are kept in control by another group of cross-class inhibitors, the thyrupins, which also interfere with cysteine peptidase activities (Chap. 2; Lenarcic and Bevec 1998; Novinec et al. 2006). Another regulatory mechanism of keeping the team of endo-lysosomal proteases well within their borders is reflected by the relatively strict pH-requirements for both proteolytic activity and stability of these enzymes, thereby explaining why many members of this large group of proteases can be kept in check, simply by compartmentalization to specific cellular regions of defined biochemical conditions (Mort et al. 1984; Mort and Buttle 1997; Jordans et al. 2009).

MMPs, meprins/BMPs, ADAMs and ADAM-TSs are best known for their proteolytic activities exerted in the extra- and peri-cellular space (Chaps. 7, 9, 11, 13–15; Brinckerhoff and Matrisian 2002; Coussens et al. 2002; Norman et al. 2003; Edwards et al. 2008; Reiss and Saftig 2009; Rosenberg 2009; Tallant et al. 2010). These enzymes are either soluble or transmembrane proteins. However, many act in sequence thereby establishing proteolytic cascades in which one metalloprotease activates the next (Chakraborti et al. 2003). These cascades of proteolytic activation often take place at the cell surface with the plasma membrane and its receptors serving as scaffolding support. Another excellent example of a similar kind is provided by the urokinase-type plasminogen activator (uPA) that interacts with the plasminogen activator receptor (uPAR), a transmembrane protein of the plasma membrane (Magdolen et al. 2000; Blasi and Carmeliet 2002; Behrendt 2004; Mondino and Blasi 2004; Shi and Stack 2007). Moreover, in a number of proteolytic processes that involve MMPs and PAs (Plasminogen Activators), the endogenous protease inhibitors TIMPs (tissue inhibitors of MMPs) and PAIs (PA inhibitors) become part of the proteolytic cascade and therefore fine-tune and regulate the pericellular activities of these proteolytic enzymes (Bode and Renatus 1997; Bode and Maskos 2001, 2003; Stetler-Stevenson 2008; Brew and Nagase 2010). Final termination of such proteolytic events that can involve multiple members of the same and

also of related and unrelated protease families is considered to occur by down-regulation (Jiang et al. 2001), i.e. degradation by endo-lysosomal pathways.

3.7 Speed-Dating of Proteolytic Enzymes and Their Substrates

So far we have not described one of the most intriguing examples of sub-compartmentalization of proteolysis and of processivity of proteolytic cleavage—the processing of linear polypeptides by the very well organized protease-machines of the cytosol, the proteasomes (Groll and Clausen 2003).

The cytoplasm, as the largest sub-compartment of eukaryotic cells, bears a variety of complex protein-processing assemblies (De Mot et al. 1999; Brandstetter et al. 2001; Rosenblum and Kozarich 2003). The best understood of such cytoplasmic protease assemblies is the 26S proteasome that can function in cleaving a variety of different peptide-bonds, and which can even be considered a protein processing machine when acting in conjunction with 19S cap-structures that comprise energy-dependent unfolding factors (Wolf and Hilt 2004; Vierstra 2009; Stadtmueller and Hill 2011). The structure of the yeast proteasome has been determined in atomic detail to a resolution of 2.4 Å by X-ray crystallography which also discovered that the proteasome belongs to a new class of proteases, the threonine peptidases (Groll et al. 1997). These astonishingly versatile, and, in cellular immune response reactions, highly adaptable (Groettrup et al. 1996, 2001a, b, 2010; van den Eynde and Morel 2001; Kloetzel and Ossendorp 2004; Rivett and Hearn 2004; Driscoll and Dechowdhury 2010) protease complexes provide a hydrolytic environment with unique properties in that four heptameric rings assemble to form a cleavage chamber (Groll and Clausen 2003; Stadtmueller and Hill 2011) that concentrates, guides and shields protease substrates from surrounding influences without being enclosed by a lipid membrane. The fully functional proteasome is already the 20S assembly which is complemented by two 19S regulatory caps, one at each end to prevent inappropriate degradation in the larger 26S proteasome.

The precise localization of proteasomes is still a matter of debate (Rivett 1998; Brooks et al. 2000a, b; Wojcik and DeMartino 2003). While their cytoplasmic location is undisputed, it is less clear under which circumstances—and for which reasons, proteasomes may be detected in the nucleus. Proteasomes are often associated with the cytosolic leaflets of the ER membrane or the nuclear envelope where they engage in ERAD, the ER-associated degradation of misfolded protein intermediates that are destined for destruction in order to protect the cell from an overload and accumulation of unfolded proteins (Wiertz et al. 1996a, b). Likewise, proteasomes are detectable as constituents of the so-called aggresomes, non-membrane enclosed regions of the cytoplasm of cells that harbour inclusions of misfolded proteins resulting from protein over-expression (Kopito 2000). In all

these latter cases, protein substrates may or may not require ubiquitinylation as a proteasome-targeting signal (Pickart 1997; Schwartz and Hochstrasser 2003; Ciechanover 2003; Tai and Schuman 2008).

The proteasome is thus a beautiful example where evolution has devised a complex protein assembly employing a variety of proteolytic activities to cleave substrates in a more or less specific fashion. The result may be release of peptides of defined length, suitable to enter the ER lumen via TAP-transporter for MHC class I dependent antigen presentation of virus-derived peptides (Abele and Tampe 2006, 2009; Hansen and Bouvier 2009), or tailor-made for further destruction by other proteolytic enzymes of the cytosol that feature amino- or carboxypeptidase activities (Gomis-Ruth 2008; Gomis-Ruth et al. 2012) in order to replenish the cellular pool of free amino acids.

3.8 How to Organise Proteolytic Actions in Busy Times Like Rapid Cell Cycle Progression, Remodelling of Cellular Components, or Cell Death

The cytoplasm is already considered as a compartment of *de novo* protein biosynthesis. However, it is also the cellular compartment of protein maintenance and turnover in response to signalling or mechanical damage of cytoskeletal proteins (Chap. 12; Goldberg and Dice 1974; Dice and Walker 1979; Dice 1987, 1990; Olson and Dice 1989; Rechsteiner and Hill 2005). It is also well understood that cell cycle progression depends on cyclic degradation and reformation of cyclins. Moreover, the cytoplasm contains many scaffolding factors, proteins and ribonucleoproteins, that have the ability to build and organize larger assemblies of macromolecules, such as the apoptosome that initiates a sequence of dramatic proteolytic events eventually leading to cell death in a tightly regulated and programmed manner (Chap. 8; Tschopp et al. 2003; Vanlangenakker et al. 2008; Declercq et al. 2009; Pop and Salvesen 2009; Vandenabeele et al. 2010; Krysko et al. 2011). Hence, while the cytoplasm may be the largest compartment of eukaryotic cells, it has established unique strategies to sub-compartmentalize proteolytic actions to certain required areas. As calcium waves emerging from the sperm entry point specify polarity of the zygote, and thus the body axis at the earliest stage of development, the cytoplasm of eukaryotic cells has found intriguing and elegant solutions to the primary question of this chapter, compartmentalization of proteolysis.

Besides proteasomes and a number of other proteolytic enzymes that are important contributors to guarantee the pool of free amino acids, other proteases are found abundantly in the cytosol: the procaspases (Earnshaw et al. 1999; Riedl and Shi 2004; He et al. 2009; Pop and Salvesen 2009; Drag and Salvesen 2010) and the calpains (Sorimachi et al. 1997, 2010; Reverter et al. 2001; Storr et al. 2011). Procaspases are cysteine peptidases cleaving after aspartic acid upon their

proteolytic activation in the initiation of apoptotic pathways leading to programmed cell death (Alnemri et al. 1996). The assembly of caspases in apoptosomes is triggered by a variety of intrinsic and extrinsic signalling pathways (Wyllie et al. 1980; Budihardjo et al. 1999; Song and Steller 1999; Salvesen 2002; Riedl and Salvesen 2007; Kersse et al. 2011). Again, a sub-compartmentalization of their proteolytic activities can be conceived from the formation of restricted, caspase-enriched cytoplasmic areas where scaffolding factors serve as the platforms for apoptosome formation, and, as in the case of proteasomes, without the help of lipid membranes. Calpains (also cysteine peptidases) represent yet another solution to restricting proteolytic activities, as they are dependent on cytosolic free calcium in the range of nanomolar concentrations that exist only transiently in response to activation of cells via e.g. protein kinase C-mediated signalling (Sato and Kawashima 2001). Calpains are known to be involved in cytoskeletal protein remodelling in skeletal muscle cells under conditions of excessive contractile activity that may cause micro-damage to the muscle fibers (Chap. 12; Koohmaria 1992).

Besides the above described mechanisms of restricting proteolytic activity of cytoplasmic proteases to certain areas of the cytoplasm, a number of 'safe-guarding' protease inhibitors can be found in certain areas of the cytoplasm as well (Turk et al. 2003; Kaiserman et al. 2006) (see also above). First, and foremost, the serpins which act as cross-class inhibitors of both serine and cysteine peptidases are found (Blasi 1993; Smirnova et al. 1994; Brunner and Preissner 1994; Pappot et al. 1995; Bailey et al. 2006; Kaiserman et al. 2006; Izuhara et al. 2008). Secondly, the cystatins are localised not only in all body fluids and in endocytic compartments, but other members of this inhibitor family are also present in the cytoplasm (Travis and Salvesen 1983; Turk and Bode 1991; Bode et al. 1999; Deveraux et al. 1999; Silverman et al. 2001; Abrahamson et al. 2003; Whisstock and Bottomley 2008). Cystatins are believed to protect cells from sudden death by inhibiting cysteine peptidases which not only reside and act normally in the cytoplasm, but which may become enriched in the cytosol if endo-lysosomes suffer membrane leakage or rupture (Chap. 8; Nixon and Cataldo 1993; McNeil and Steinhardt 1997; Gerasimenko et al. 2001; Degli Esposti 2008; Ivanova et al. 2008; Turk and Turk 2009). Thus, eukaryotic cells can further transiently modulate proteolytic activity through the expression and localisation of cognate inhibitors.

3.9 Themes Emerging from the Discovery of the New Kids on the Block

The role for selected proteolysis in ubiquitin biology is an area that is fast developing. Ubiquitin (and indeed related peptides such as SUMO, ISG15) is post-translationally added to proteins by E3 ubiquitin ligase complexes (Pickart and

Eddins 2004). This modification of a specific protein can have a broad range of effects, from simple targeting of a protein for degradation, to modification of its activity in a manner not dissimilar from the role of phosphorylation throughout biological networks. Ubiquitination forms a key signaling role in processes such as cell cycle regulation (Song and Rape 2008), enzyme activation/inactivation (Adhikari et al. 2007) and DNA repair (Kennedy and D'Andrea 2005).

Ubiquitin is a 76 residue peptide, the free C-terminus of which is conjugated onto lysine side chain amino groups on target proteins forming a pseudo-peptide bond (Pickart 2001). However, the form of this ubiquitination can vary substantially from a simple mono-ubiquitination to addition of a number of ubiquitins (poly-ubiquitination). This post-translational modification is further complicated in that ubiquitin itself has a number of lysine residues and a branched poly-ubiquitin can be created through these points—each of which has its own distinctive conformation and role (K48 branched polymers labels proteins for degeneration and K63 polymers activate target proteins) (Pickart and Fushman 2004).

Key to the role of ubiquitination signaling is the fact that it is reversible and removal of ubiquitin is coordinated by proteases called deubiquitinating enzymes (DUBs). To date, almost 100 DUB proteases have been identified, belonging to cysteine and metalloprotease families (Reyes-Turcu et al. 2009). However, it is interesting to note that no endogenous inhibitors of these proteases have been identified to date and control of their activity appears to use other mechanisms including transcriptional control, substrate recognition and physical compartmentalization (Zhao et al. 2008).

In addition to these mechanisms of control, within the cytoplasm where the majority of these proteases are located, a major source of control is the presence of binding partners and adaptor proteins. Indeed, many of the DUB proteases contain other domains in addition to their catalytic core to facilitate binding to other proteins, and in particular scaffold proteins where it is thought that the DUB forms part of a multi-protein complex (often with the cognate E3 ubiquitin ligase complex), coordinating its activity and substrate specificity (Marfany and Denuc 2008). In this regard, it is perhaps particularly striking that invadolysin, a metalloprotease coordinating mitotic progression, has recently been found to interact genetically with non-stop, a DUB (MM Heck, personal communication).

3.10 And Now, We Retire to the Study

We conclude that we have only glimpsed the tip of the ice-berg. Many more intriguing features of how proteolysis is compartmentalized in the realm of the cell are to be discovered, understood and set into context before we will fully grasp the complexity of proteolytic enzymes and their compartmentalization principles. Faulty localization, delayed or too early, too fast or too slow, extensive or non-productive interactions of proteases, substrates, inhibitors and other factors regulating proteolysis will result in dramatic effects to the cell—and likely in

diseases detrimental to the functioning of an organism. Understanding the regulation of intra- and extra-cellular proteolysis will be an important step to assess the physiology of eu- and prokaryotic cells. In turn, this understanding will be crucial to clarifying the routes leading to disease, and ultimately, shed light on potential therapeutic pathways.

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