Proteomic Studies of Hepatocytic Autophagosomes

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Oslo, Norway 2007

Series of dissertations submitted to the
Faculty of Mathematics and Natural Sciences, University of Oslo
Nr. 682

ISSN 1501-7710

Cover: Inger Sandved Anfinsen.
Printed in Norway: AiT e-dit AS, Oslo, 2007.

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ACKNOWLEDGEMENT

When Gaius Julius Caesar was 32 years, according to historian Suetonius, he went to the forum in the town of which he currently was quaestor, where there was an equestrian sculpture of Alexander the Great. He realised with dissatisfaction he was now at an age when Alexander had the world at his feet, while he had achieved comparatively little. Although my aims are vastly more modest, *I* have reached a milestone of which I am proud at the same age. This effort has not come without the important contribution of many people, whom I duly thank for their involvement:

I would primarily thank my supervisor professor Per O. Seglen for inspiration, tutelage and for always being open to discussion. He has been a maverick in cell science for decades, Seglen is an irreplaceable and vast source of knowledge and his understanding of science is near unrivalled.

I am utmost grateful to The Norwegian Cancer Society for financial support in the period 2002-2005, The Research Foundation of the Radium Hospital in 2006 and finally South-Eastern Norway Regional Health Authority for 2007. I take pride in being able to work at the renowned Institute for Cancer Research during my Ph.D. - vocation.

The colleagues at the Cell Biology Department have been instrumental for me by providing a good working environment and structural framework, special thanks are to be given to Head of Department professor Erik Boye, Jorun Heitmann and co-supervisor professor Kirsten Skarstad for administrative help, optimism and encouragement.

I would also like to express my gratitude towards my closest co-workers in the Seglen-group. Frank Sætre, Linda Korseberg Hagen, Tonje Asmyhr and Abir Ahmad have been supplying technical assistance *par excellence* as well as refined theoretical knowledge. My co-authors Marianne Lunde Sneve and Dr. Monica Fengsrud are to be thanked for contributing with a plethora of scientific as well as practical advice. Dr. Hamid Samari's constant supply of discussion themes, on and off science, has been very much treasured. Svein-Ole Mikalsen is to be greatly complimented on accommodating discussion and technical recommendations. All present and past group-members have been important to create a great working atmosphere, of which I am appreciative.

My family and friends have been a valuable support in these years with their earnest curiosity and enthusiasm. The friendly competition among the Øverbye brothers

has also contributed to my progression, furthermore, my mom and dad shall be thanked in particular for being certain that I could fulfil my potential, when I was in doubt.

Thank you my binary stars Selma and Synne for always reminding me that the world is heliocentric. Thanks to Gøril, my *belle*, for putting up with my frequent absence, absent-mindedness and for sharing ups and downs and, well, for everything. I told you I'd be done one day.

Anders Øverbye
Oslo, October 2007

List of abbreviations

3-MA	3-methyl adenine
AD	Alzheimer's disease
ADP	adenosine diphosphate
AICAR	5'amino-4-imidazolecarboxamide riboside
Akt/PKB	akt/ protein kinase B
AMBRA1	activating molecule in Beclin-1 regulated autophagy
AMP	adenosine monophosphate
AMPK	5'AMP-activated protein kinase
Ape1	aminopeptidase1
AQUA	absolute quantification method
ATG	autophagy related gene
ATP	adenosine triphosphate
BHMT	betaine:homocysteine methyltransferase
CAD	collisionally activated decomposition
CaMKII	calmodulin-dependent kinase II
CHIP	carboxy-terminal end of HSC70 interacting protein
CID	collision induced dissociation
CMA	chaperone mediated autophagy
CVT	cytoplasm to vacuole
DAMP	N-(3-[2,4-dinitrophenyl]-amino) propyl-1-N (3-aminopropyl-methylamine) dihydrochloride
DAPK	death-associated protein kinase
DIGE	differential gel electrophoresis
ECD	electron capture dissociation
EEA1	early endosome antigen 1
eIF2a	eukaryotic initiation factor 2 alpha
eIF4E	eukaryotic initiation factor 4E
ER	endoplasmic reticulum
ERK	extracellular regulated kinase
ESI	electron spray ionization
ETD	electron transfer dissociation
FTICR	Fourier transform ion cyclotron resonance
GFP	green fluorescence protein
GDP	guanosine triphosphate
GMP	guanosine monophosphate

GTP	guanosine diphosphate
HD	Huntington's disease
HDAC6	histone deacetylase 6
HSC70	heat shock cognate protein of 70 kilodalton
HSP90	heat shock protein of 90 kilodalton
HUPO	Human Proteome Organization
ICAT	isotope-coded affinity tags
IFN	interferon gamma
IT	ion trap
kDa	kilodalton
LAMP2	lysosome-associated membrane protein 2
LC	liquid chromatography
LC3	microtubule associated protein 1 **light chain 3**
M6PR	mannose-6-phosphate receptor
MALDI	matrix-assisted laser desorption/ionisation
MDC	monodansylcadaverine
MEF	mouse embryonic fibroblasts
MPT	mitochondrial permeability transition
mRFP	monomeric red fluorescence protein
MS	mass spectrometer
MS/MS	tandem mass spectrometry or mass spectrometer
mTOR	mammalian target of rapamycin
mTORC1/2	mammalian TOR complex 1/2
NV	nucleus-vacuole (NV)
p62/ SQSTM1	Sequestsome 1/ 62 kd protein ubiquitin-like
PAS	preautophagosomal structure, phagophore assembly site
PCD	programmed cell death
PD	Parkinson's disease
PDK1/2	PIP3-dependent kinase 1-2
PE	phosphatidylethanolamine
PEBP	phosphatidylethanolamine-binding protein
PI	phosphoinositide
PI3K	phosphotidyl-3'OH kinase
PIP3	phosphatidylinositol 3-phosphate
PKC	protein kinase C
PMN	piecemeal microautophagy of the nucleus
PP1	protein phosphatase 1
PP2A/B	protein phosphatase 2A/2B
PtdIns	phosphatidylinositol

PTEN	phosphatase and Tensin homolog
Q	quadrupole
Rheb	Ras homolog enriched in brain
ROS	reactive oxygen species
S6K	ribosomal protein S6 kinase of 70 kd
SAPK	stress-activated protein kinase
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEK1	SAPK/ERK kinase
SELDI	surface-enhanced laser desorption/ ionization
SILAC	stable isotope labeling of amino acids in cell culture
siRNA	small interfering RNA
SOD1	superoxide dismutase (Cu/Zn)
SRM	selected reaction monitoring
ssDNA	single stranded DNA
TGN	trans-Golgi network
TNF	tumor necrosis factor alpha
TOF	time of flight
TOR	target of rapamycin
TORC1/2	TOR complex 1 /2
TSC1/2	tuberous sclerosis complex 1 /2
ULK1	unc51-like 1 protein /hsAtg1
UPR	unfolded protein response
UPS	ubiquitin-proteasomal system
UVRAG	UV-resistance associated gene
VID	vacuolar import and degradation
VPS	vacuolar protein sorting
ZMP	5-aminoimidazole-4-carboxamide ribotide

Table of contents

LIST OF INCLUDED PAPERS

This thesis is based on the following original papers. In the text they will be referred to by Roman numerals.

I. Lunde Sneve, M., **Øverbye A.**, Fengsrud M., and Seglen P.O.(2005). Comigration of two autophagosome-associated dehydrogenases on two-dimensional polyacrylamide gels. *Autophagy.* 1:157-162.

II. **Øverbye, A**., Fengsrud M., and Seglen P.O. (2007). Proteomic analysis of membrane-associated proteins from rat liver autophagosomes. *Autophagy.* 3:300-322.

III. **Øverbye, A**. and Seglen P.O. Selective Binding of Betaine:Homocysteine Methyltransferase Fragments to Autophagic Membranes. *Manuscript*.

IV. **Øverbye, A**. and Seglen P.O. Phosphorylated and Non-phosphorylated Forms of Catechol *O*-Methyltransferase in Rat Tissues. *Manuscript.*

SUMMARY OF PAPERS

I: Comigration of two autophagosome-associated dehydrogenases on two-dimensional polyacrylamide gels

In this paper, two-dimensional immunoblotting was used to study variants of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a largely cytosolic enzyme previously found to be represented by an N-truncated variant in autophagic membranes from rat hepatocytes (Fengsrud, M. *et al.* 2000, Biochem. J. **352**, 773-781). With wide-range isoelectric focusing (pH 3-10), immunostaining of 5-6 putative GAPDH forms could be observed. These included full-length forms (37 kDa), partly associated with cytoplasmic membranes, as well as short, presumably truncated forms (35 kDa) partly, but specifically, associated with the membranes of autophagosomes and lysosomes. Upon attempting to verify the enzyme identity by MALDI-TOF MS-based tryptic fingerprinting, two of the full-length forms could be identified as GAPDH, but the major short form was, surprisingly, identified as a different enzyme, 3-α–hydroxysteriod dehydrogenase (HSD). To improve the spot resolution, we switched to narrow-range (pH 6-9) isoelectric focusing, and used immunoblotting with an HSD-specific antibody. The antibody identified four HSD variants, subsequently verified by MALDI-TOF tryptic fingerprinting, all with a molecular mass of 35 kDa (but differing in pI) and all showing an autophagosomal membrane enrichment. In addition, the antibody produced a nonspecific immunostaining of three spots at 44 kDa, identified by MALDI as variants of isocitrate dehydrogenase. One of the HSD variants still coincided with the major 35 kDa GAPDH variant, and would probably have contributed significantly to the autophagosomally enriched, silver-stained protein spot previously ascribed to GAPDH. The fact that several HSD variants were binding selectively to autophagic membranes would be consistent with a function for this enzyme in autophagic degradation.

II: Proteomic analysis of autophagosomal membrane proteins

Since autophagosomes are essentially a package of cytoplasm surrounded by a specific autophagic delimiting membrane, proteins selectively associated with the latter should be recognizable by their higher abundance in autophagosomal membrane preparations than in membranes from whole cytoplasm. To obtain a general overview of such proteins, we took a proteomic approach, using narrow-range two-dimensional gel electrophoresis to separate proteins in the sedimentable (membrane-containing) fraction from frozen-thawed autophagosomes and compare them with proteins in the sedimentable fraction from frozen/thawed cytoplasm. 1,570 distinct protein spots were detected by silver staining, including 67 spots that were significantly, at least twice enhanced in the autophagosomal membranes. By MALDI-TOF mass spectrometric peptide mass fingerprinting we could identify 58 protein variants, including 11 proteins that were enriched $\geq$20x, corresponding to a virtually exclusive autophagic membrane localization (i.e., in the autophagosomal delimiting membrane). Examples of such proteins were a small form of heat shock cognate protein 70 (Hsc70), phosphatidylethanolamine-binding protein (PEBP), catechol-*O*-methyltransferase (COMT), betaine:homocysteine methyltransferase (BHMT), selenium-binding protein 2 and several peroxiredoxins. The autophagic membrane-association of several chaperonins and proteins involved in methylation, redox reactions and drug metabolism might suggest a role as binding proteins in a selective scavenging of unfolded, hypermethylated, oxidized, drug-adducted or otherwise denatured proteins during the sequestration of cytoplasm by the phagophores.

III: Selective Binding of Betaine:Homocysteine Methyltransferase Fragments to Autophagic Membranes

The presence of several forms and assumed fragments of betaine:homocysteine methyltransferase in our detailed study of autophagic membrane-associated proteins in paper II compelled us to investigate this enzyme further. Using a specific antibody against the N-terminal peptide of full-length BHMT, we detected several additional BHMT-derived polypeptides, the identities of which were verified by MALDI-TOF and ion trap mass spectrometry. A 17-kDa BHMT fragment turned out to be a complete *in*

vitro artifact, generated by proteolytic cleavage even in SDS-containing lysates. A 10-kDa BHMT fragment was present in intact cells, but additionally both generated and degraded *in vitro*. Larger fragments at 27, 30 and 33 kDa were relatively stable *in vitro*, but could be slowly degraded upon disruption of lysosomes in the absence of cytosol or in the presence of SDS. All cleavages were apparently enzymatic as indicated by the suppressive effects of various proteinase inhibitors, and involved both leupeptin-resistant and leupeptin-sensitive proteinase activities, the latter probably being ascribable to released lysosomal cathepsins.

The small, 10-kDa BHMT form (p10) was characterized by MALDI-TOF and ion trap mass spectrometry as an N-acetylated, N-terminal BHMT fragment with additional modifications (acetylation or trimethylation) at several internal lysines. This fragment was found to accumulate in leupeptin-treated hepatocytes over a period of 4 h, probably by intralysosomal entrapment, and could possibly serve as an indicator of autophagic-lysosomal flux detectable in whole cells. In purified autophagosomes, a substantial fraction of the p10 protein as well as of the larger p27, p30 and p33 BHMT fragments were found to be associated with autophagosomal membranes, whereas in preparations from whole cells they were almost exclusively soluble. This selective binding of protein fragments to autophagic membranes could indicate an active role of autophagy in the specific elimination of these and other abnormal cellular proteins.

IV: Phosphorylated and Non-phosphorylated Forms of Catechol O-Methyltransferase in Rat Tissues

In paper II we demonstrated the existence of two autophagosomally enriched variants of catechol-*O*-methyltransferase (COMT), one of which was found to be phosphorylated. In paper IV we have investigated this modification in more detail, extending the scope to several rat tissues. The two well-known isoforms of COMT, S-COMT ("soluble") and MB-COMT ("membrane-bound") were detected in many tissues by our novel, domestic antibody, but subcellular fractionation revealed that S-COMT was often membrane-bound and MB-COMT often soluble, suggesting that their names should be changed. In rat liver, where COMT is most abundant, two-dimensional immunoblotting revealed seven different spots, all identified as COMT by a combination of mass spectrometric techniques. Three of the five S-COMT variants could be shown to

be N-acetylated, and the two most acidic forms were found to be phosphorylated in their C-terminal tail at Ser260. No modifications were found in the two MB-COMT variants. The most acidic S-COMT variant was entirely liver-specific; other tissues expressed a limited, but relatively tissue-specific set of COMT forms. Phosphorylated COMT was thus found to be present in liver, brain, thymus and kidney. Given the key role of COMT in the metabolism of natural and pharmacological catecholamines, the discovery of COMT phosphorylation, and hopefully the future elucidation of its regulatory significance, should be of considerable medical interest.

INTRODUCTION

The work presented in this thesis relies on two defined research fields in life science: autophagy and proteomics. While the former describes a cell biological phenomenon, the latter can be considered a tool with which a greater understanding of cellular mechanisms can be achieved. The preface of the thesis will aim at providing an introduction to both areas of research, with focus on recent development.

AUTOPHAGY

The main mechanism of *autophagy* is the sequestering of cytoplasmic material, including organelles, by an isolating membranous cisternal organelle and delivery of the material to the lysosome for degradation. The membranous organelle, which is termed a *phagophore* at the sequestration step, matures into a vacuole named an *autophagosome* upon completed engulfment. This autophagic organelle can be considered a transportation vehicle which in turn fuses with other vacuoles, initially with the endosome to form an *amphisome*. Next, the amphisome fuses with a *lysosome*, delivering the sequestered material inside. An autophagosome may also fuse directly with a lysosome (see Fig. 2 and see below for detailed description). In the lysosome the contents of the autophagosome are degraded by acidic hydrolases. Autophagy is thus a lysosomal degradation process.

This system is complemented by non-lysosomal degradation, which is dominated by the ubiquitin-proteasomal system (UPS). In short, this system takes care of short-lived proteins as well as abnormal and misfolded proteins by tagging them with chains of ubiquitin, an 8 kDa polypeptide. The tagged proteins are then tethered and degraded by proteasomes residing in the cytoplasm. The proteasome is a multienzyme complex consisting of several subunits that dismantle proteins (Hochstrasser, 1995; Kvam and Goldfarb, 2007). Lysosomal and non-lysosomal degradation have been regarded as completely separated systems, but new insights into these mechanisms indicate a closer interrelationship, as seen in several pathological disorders, where one system can compensate for malfunction of the other (see below; Wooten et al., 2006).

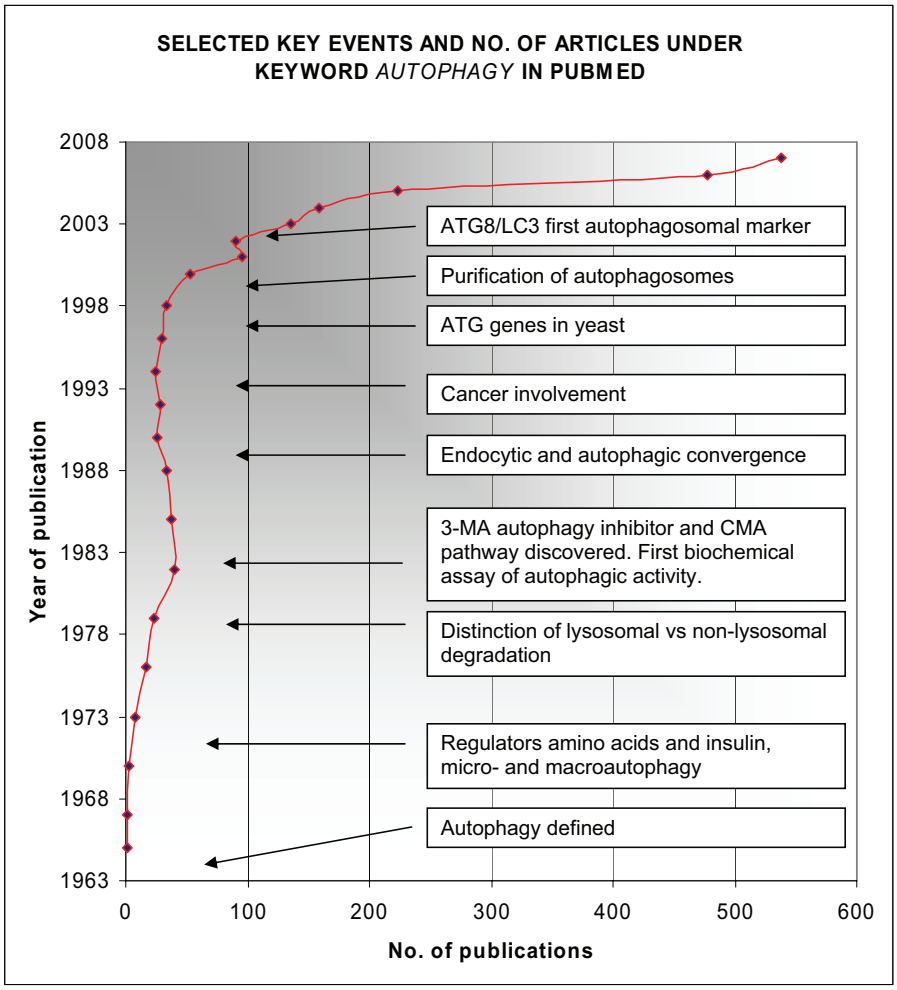

Figure 1. Progression of autophagy-related research. Indications of expansion in the field by number of articles published per year 1963-2007, some key events displayed.

The pioneering work of Christian De Duve who characterized the lysosome and invented the term autophagy (origin: *Greek* auto – *self,* phagein – *to consume/eat)* (de Duve and Wattiaux, 1966; de Duve, 1963), initiated a series of studies where biochemical tools in addition to the usage of electron microscopy were used extensively to examine the mechanisms of this process. Over the years many milestones have been reached, and a recent expansive growing interest and knowledge of the field has been witnessed (see

Fig. 1), along with numerous tools to dissect the molecular machinery (Klionsky et al., 2007b).

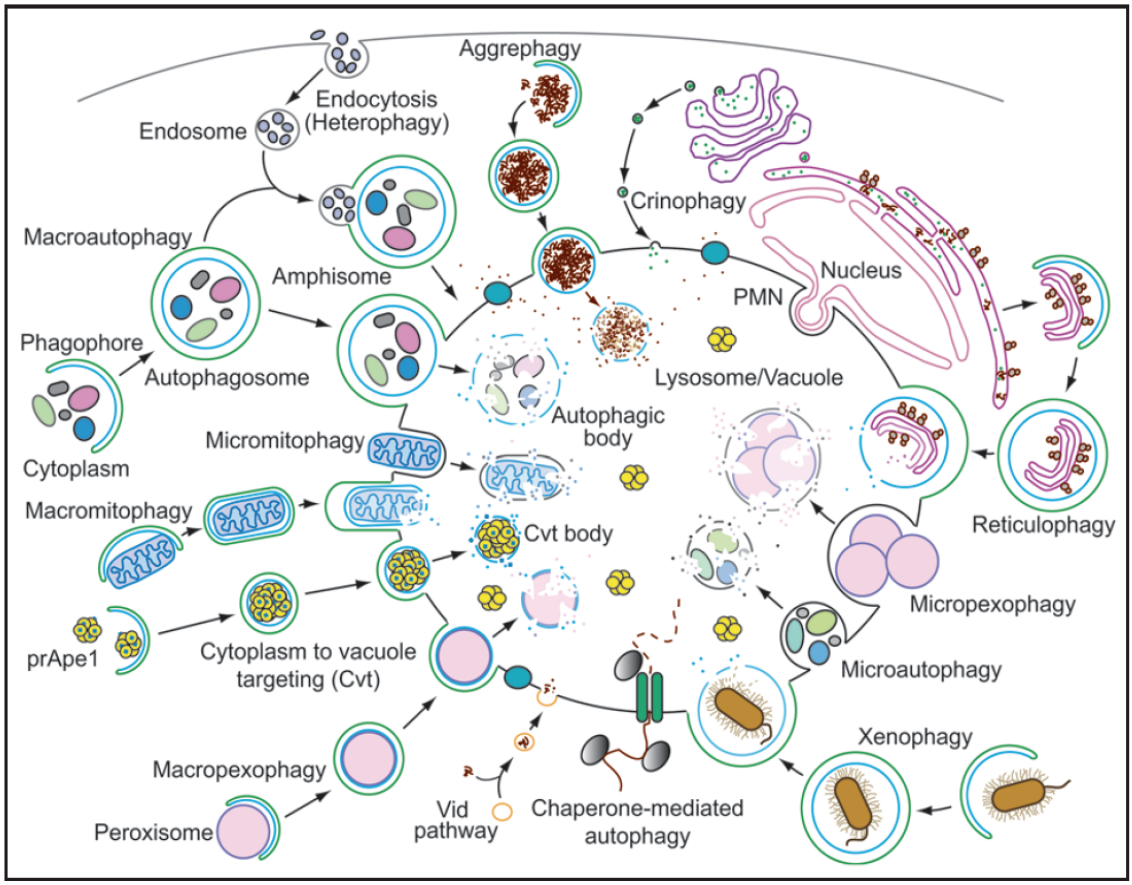

Figure 2. Summary of lysosomal degradation pathwats. Refer to text for details. From (Klionsky et al., 2007a).

AUTOPHAGIC PROCESSES

MACROAUTOPHAGY

Autophagy has been studied in many eukaryotes from yeast to humans, and is in many aspects similar in all cell types, but with several differences. A plethora of definitions and terms are related to this field (for review: see Klionsky et al., 2007a), and this thesis will aim at a brief description. As originally defined, autophagy (now often termed **macroautophagy**) is a non-selective process in nature, engulfing apparently random portions of the cytoplasm for degradation, as a response to various mediators, especially amino acid deprivation (see below).

MICROAUTOPHAGY

Several minor versions of the autophagic processes exist (see Fig. 2). *Microautophagy* is the term coined for the direct uptake of cytoplasm by the lysosomal membrane from surrounding cytoplasm (Ahlberg et al., 1982). It differs from macroautophagy in two manners: 1) the site of sequestration is the degradative organelle and 2) this sequestration is performed by invagination or protrusion of the lysosomal membrane. The enclosed material is then encapsulated in a mono-layered membrane vacuole inside the lysosome and degraded in the same manner as for macroautophagy (Wang and Klionsky, 2003). The function of microautophagy remains elusive, but perhaps it plays a role in turnover of long-lived proteins. Morphometric studies on long-term starvation (48 hrs) in rat hepatocytes indicate an association between decline of microautophagic vesicles and basal proteolytic turnover (Mortimore et al., 1983). Simultaneous measurement of macroautophagy revealed no change in same time period.

VARIANTS OF MICROAUTOPHAGY

A related mechanism is *piecemeal microautophagy of the nucleus* (PMN), found in *Saccharomyces cerevisiae,* a process differing from of functional macroautophagy in that it does not require functional Atg8 (Roberts et al., 2003). PMN is a constitutive process but stimulated to high levels by starvation or rapamycin and active under cell division. PMN occurs at nucleus-vacuole (NV) junctions formed by interactions between the vacuole membrane protein Vac8 and the outer-nuclear membrane protein Nvj1, and results in the pinching off of nonessential portions of the nucleus for degradation (see

Fig. 3; Kvam and Goldfarb, 2007). There has also been a report on complete autophagic sequestration of the nucleus in murine epithelial cells (Kovacs et al., 2000).

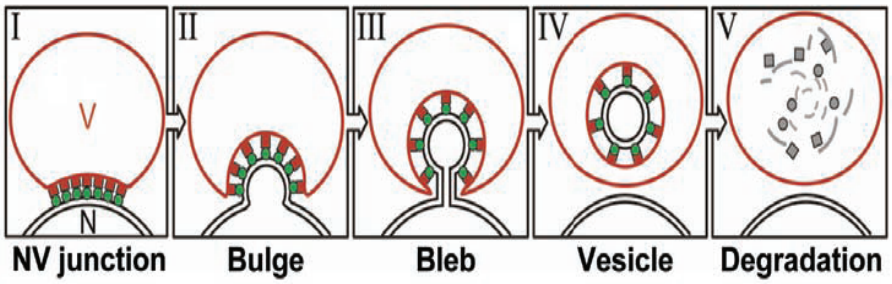

Figure 3: Five steps in piecemeal microautophagy of the nucleus machinery. Red squares Vac8, green squares Nvj1. N = nucleus, V = vacuole. From (Kvam and Goldfarb, 2007)

Recent inquiries concerning the fate of different cellular organelles have unraveled several specific targets for autophagy. In the methylotropic yeast *Pichia pastoris*, there has been found a subset of genes responsible for specific sequestration of peroxisomes, a process called *pexophagy,* of which there exist two sub-variants: *micro- and macropexophagy* – also seen in liver (Luiken et al., 1992).

Similarly, depolarized mitochondria undergoing mitochondrial permeability transition (MPT) seem to be subject to selective autophagy. This process, called *mitophagy,* also comes in two varieties, micro- and macromitophagy, as indicated on figure 2 (Lemasters, 2005). Also larger cellular objects appear able to elicit a specific autophagic response, such as smooth endoplasmic reticulum (ER), that has been observed to be enriched in autophagic vacuoles induced by phenobarbital-treatment (Bolender and Weibel, 1973). Additional data concerning autophagic sequestration of ER include a report on multiple rough ER cisternae concentrated in autophagic vacuoles during the unfolded protein response (UPR; Bernales et al., 2007), in sum justifying the term *reticulophagy* (Klionsky et al., 2007a).

CRINOPHAGY

Secretory protein granules formed in excess from the trans-Golgi network (TGN) have also been reported to be subject for lysosomal degradation (Glaumann et al., 1981), this

process is called *crinophagy* (*Greek:* crino- *exude*). The process depends on fusion of secretory vacuoles with the lysosome and delivery of internal proteins and peptides for degradation.

AGGREPHAGY

Aggrephagy, a novel term introduced in Paper II, refers to the concept of selective degradation of aggregates, preferentially of proteins (Øverbye et al., 2007). Aggregates could form inside the cell after various events, such as misfolding, defective proteasomes, aberrant protein expression, oxidative stress, mutations or ageing (Kopito, 2000). Many of these are involved in the pathogenesis of several diseases (see separate section below) such as huntingtin and presenilin 1, and evidence exist for clearance of these aberrant aggregates through autophagy (Rubinsztein et al., 2005). Aggresomes, the organelles

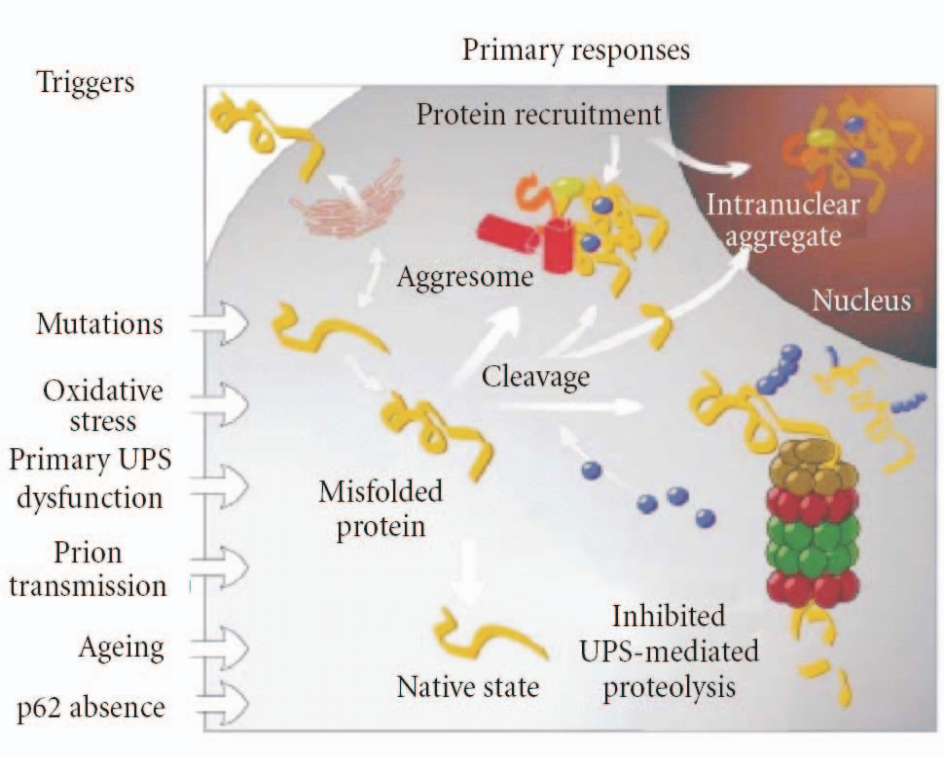

Figure 4. Formation of protein aggregates. Blue dots indicate ubiquitin. Yellow ribbon proteins. From (Wooten et al., 2006).

functioning as a collection site for aggregates in the cell are enveloped by a similar double-layered membranes as are the autophagosomes, and may thus fuse directly with lysosomes for degradation (Kopito, 2000; see Fig. 4).

XENOPHAGY

Selective autophagy may play a role in a protective response to invasion of foreign bodies as several *in vitro* studies report on the sequestering of pathogenic bacteria and viruses that have escaped phagosomes by autophagosomes (Gutierrez et al., 2004a; Deretic et al., 2006). This process has recently been termed *xenophagy* (Levine, 2005). An example is the uptake and degradation of group A *Streptococcus* that have escaped from phagosomes into the cytosol in HeLa cells or fibroblasts, a process that was shown to be blocked by Atg5 knockout (Nakagawa et al., 2004). With electron microscopy it has been demonstrated that the bacterium *Rickettsia conorii* was present in autophagosomal structures (Walker et al., 1997). Proinflammatory cytokines lead to increased macroautophagy and Rickettsia clearance from the cytoplasm. The metabolic inhibition of *Listeria monocytogenes* after phagosome lysis renders these bacteria susceptible for macroautophagy and lysosomal clearance (Rich et al., 2003). *Mycobacterium tuberculosis* was shown to be cleared from mouse macrophages and a human macrophage cell line by macroautophagy (Gutierrez et al., 2004a; Singh et al., 2006). Pharmacological inhibitors of macroautophagy inhibited starvation-induced clearance of a variant of *M. tuberculosis,* bovine BCG. Recently, parasites such as *Toxoplasma gondii* were added to the list of invading pathogens targeted by autophagy (Andrade et al., 2006). Clearance of tachyzoites from *T. gondii* upon macrophage activation could be stalled by PI3K-inhibitors and siRNA silencing of Beclin-1 (Ling et al., 2006).

Evidence that certain single-stranded viruses subvert the autophagosomes and use exocytosis from the amphisome to deliver mature viruses out of the cell has been produced (Kirkegaard et al., 2004). Furthermore, coronavirus and other viruses replicate on the surface of autophagosomal membranes (Jespersen et al., 1999; Prentice et al., 2004). These viruses appear to lock the fusion of autophagosomes, which carry their replication complexes, with lysosomes. For poliovirus and rhinoviruses this autophagy regulation is apparently mediated by the 2BC and 3A proteins, which are sufficient to induce the accumulation of autophagosomes (Jackson et al., 2005).

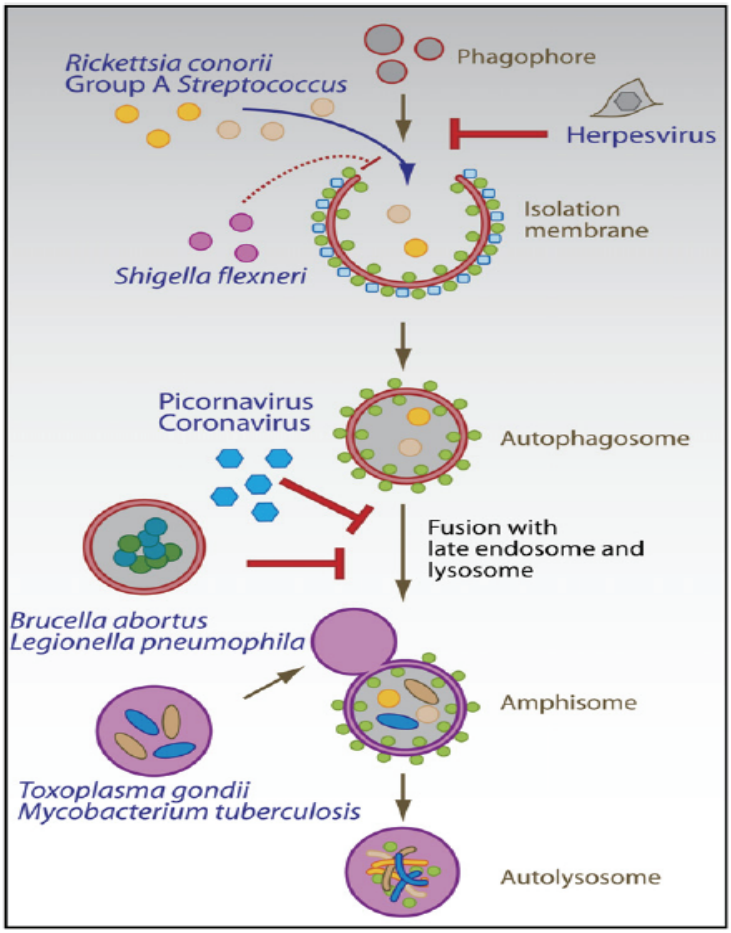

Figure 5. Different fates: Autophagic response to pathogenic invasion. Viruses, bacteria and parasites are either cleared or interfere with their own destruction by autophagy. From (Schmid and Munz, 2007).

CHAPERONE-MEDIATED AUTOPHAGY

Chaperone-mediated autophagy (CMA) describes a variant of lysosomal degradation where the protein cargo is recognized by a specific motif (KFERQ and related sequences) (Backer et al., 1983; Dice et al., 1990) and taken up across the lysosomal membrane. This

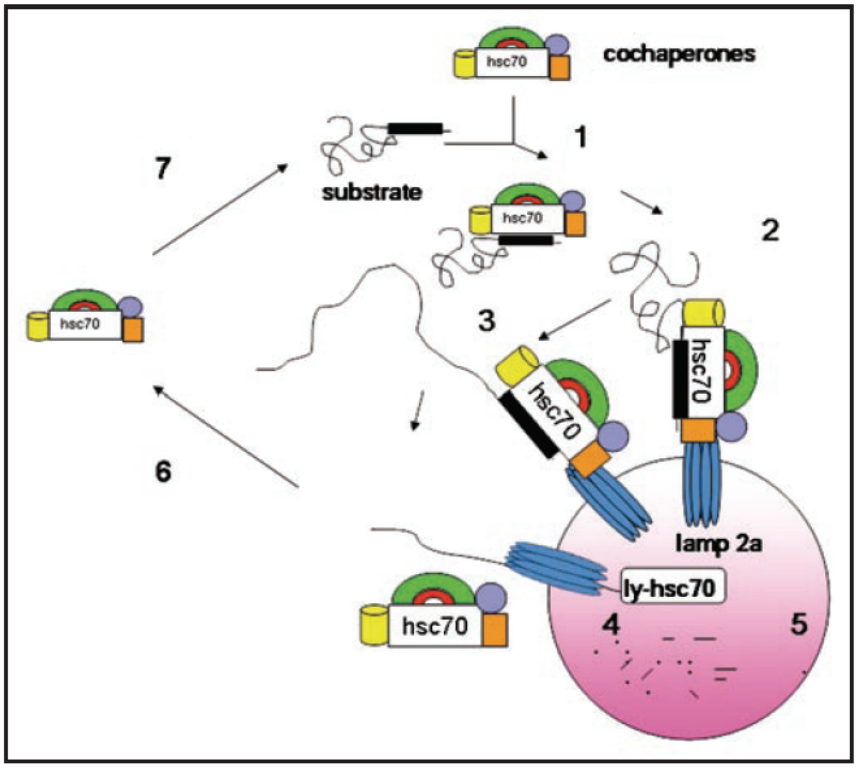

Figure 6. Chaperone-mediated autophagy.

1) Binding of HSC-70 complex to substrate, 2) translocation of complex to lysosome and binding to LAMP2A, 3) unfolding of substrate, 4) transfer of substrate across lysosomal membrane, 5) substrate degradation in lysosome 6) HSC70-complex recycles from lysosome and 7) may bind another KFERQ-motif substrate. From (Dice, 2007).

sequence is present in about 30% of cytosolic proteins (Chiang and Dice, 1988). It is also believed that certain post-translational modifications can generate similar motifs in proteins, extending the potential targets for CMA (Gracy et al., 1998). This process needs and is closely regulated by heat shock cognate protein of 70 kDa (HSC70), a molecular chaperonin (Chiang et al., 1989), in addition to secondary chaperones and a lysosomal receptor, lysosome associated membrane protein 2a (LAMP2a; Cuervo and Dice, 2000). HSC70 is a constitutively expressed heat shock protein and needs to be present both on the cytoplasmic and the lysosomal side of the lysosome. In order to transport target proteins across the membrane, HSC70 is attended by HSP40 which stimulates the former protein's ATPase activity, and heat shock protein interacting protein (HIP) initiates binding between HSP40, HSC70 and target protein (Suh et al., 1999; Hohfeld et al., 1995). Heat shock protein organizer protein (HOP) mediates binding between HSC70 and heat shock protein of 90 KDa (HSP90; Demand et al., 1998). HSP90 is necessary to supervise the folding and unfolding of target proteins, which seems to be the key function for the whole complex prior to translocation (Richter and Buchner, 2006). Finally, the interactions are regulated by Bcl2-associated athanogene-1 (BAG-1; Luders et al., 2000). When this complex approaches the lysosomal membrane, it binds to a homomultimeric complex of LAMP2a. This binding is the rate-limiting step in CMA, and is regulated by recruiting LAMP2a locally from lipid microdomains situated on the lysosomal membrane (Kaushik et al., 2006). LAMP2a is the only isoform of three LAMP2-proteins (a, b, c) involved in CMA. All forms are heavily glucosylated on the lysosomal side of the membrane.

In the lumen of lysosome, a more acidic variant of HSC70 (Ly-HSC70) awaits the cargo, and helps to translocate the, by now, unfolded target protein (Agarraberes et al., 1997). Ly-HSC70 has to be present in the lysosomes to make them CMA-competent. How this is achieved is unclear, although is has been speculated that macroautophagy delivers HSC70 to the lysosome for this end (Massey et al., 2006).

THE VID AND CVT PATHWAYS

Two pathways related to autophagy share many of its characteristics, namely the VID (vacuolar import and degradation) and the CVT (cytoplasm-to-vacuole) pathways present in yeast (see Fig. 2). VID has been demonstrated by the involvement in the specific

sequestration of the energy-regulating enzymes 1,6-fructose bisphosphatase and malate dehydrogenase in a small (30 nm) vesicle that fuses with the yeast vacuole. The VID process is triggered by a shift in glucose conditions (Shieh et al., 2001). In the CVT pathway hydrolases required for vacuolar function, such as aminopeptidase 1 (Ape1) and alpha-mannosidase are sequestered in double membrane-delimited vesicles (Huang and Klionsky, 2002; Abeliovich and Klionsky, 2001). Many proteins determined to be vital for autophagy in yeast also exert function in the CVT pathway (see table 1). A distinction between autophagy and CVT is the requirement for nutrients: autophagy is starvation-induced, while the CVT pathway is functional in rich medium.

AUTOPHAGY-RELATED GENES

The advent of genetic manipulation allowed the identification of several *autophagy related genes* (common nomenclature ATG) necessary for autophagosome formation in yeast (Tsukada and Ohsumi, 1993; Thumm et al., 1994), of these many have known mammalian orthologs (see Table 1). Prominent among these proteins is Atg1, a kinase residing in a complex also consisting of Atg11, Atg17, Atg 20 and Atg24. It works as a sensor of amino acid starvation or rapamycin-treatment through the binding of a Vac8-Atg13 dimer to Atg1 for activation (see Fig. 7A). This interaction is regulated by TOR: when active (in the presence of amino acids and the absence of rapamycin), this kinase leads to a multiphosphorylated Atg13. This in turn disables binding to Atg1 thus inhibiting autophagy (Kamada et al., 2000).

Formation of phosphatidylinositol 3-phosphate is another essential step, involved in phagophore formation, and performed by a lipid kinase (see Fig. 7B). In *Saccharomyces cerevisiae*, this kinase activity resides in a complex composed of Atg6, Atg14, Vps15 (an activator of Vps34) and the class III PI-3 kinase, Vps34. Atg14 serves as a bridge between Atg6 and Vps34. The presence of Atg9 appears also to be an essential component at the cytoplasmic membrane for Vps34 activation (Young et al., 2006). The mammalian counterpart of the PI3K class III complex consist of hVps34, Beclin-1 (Atg6), p70 (Atg14) and p150 (Vps15). New insights in mammalian autophagy

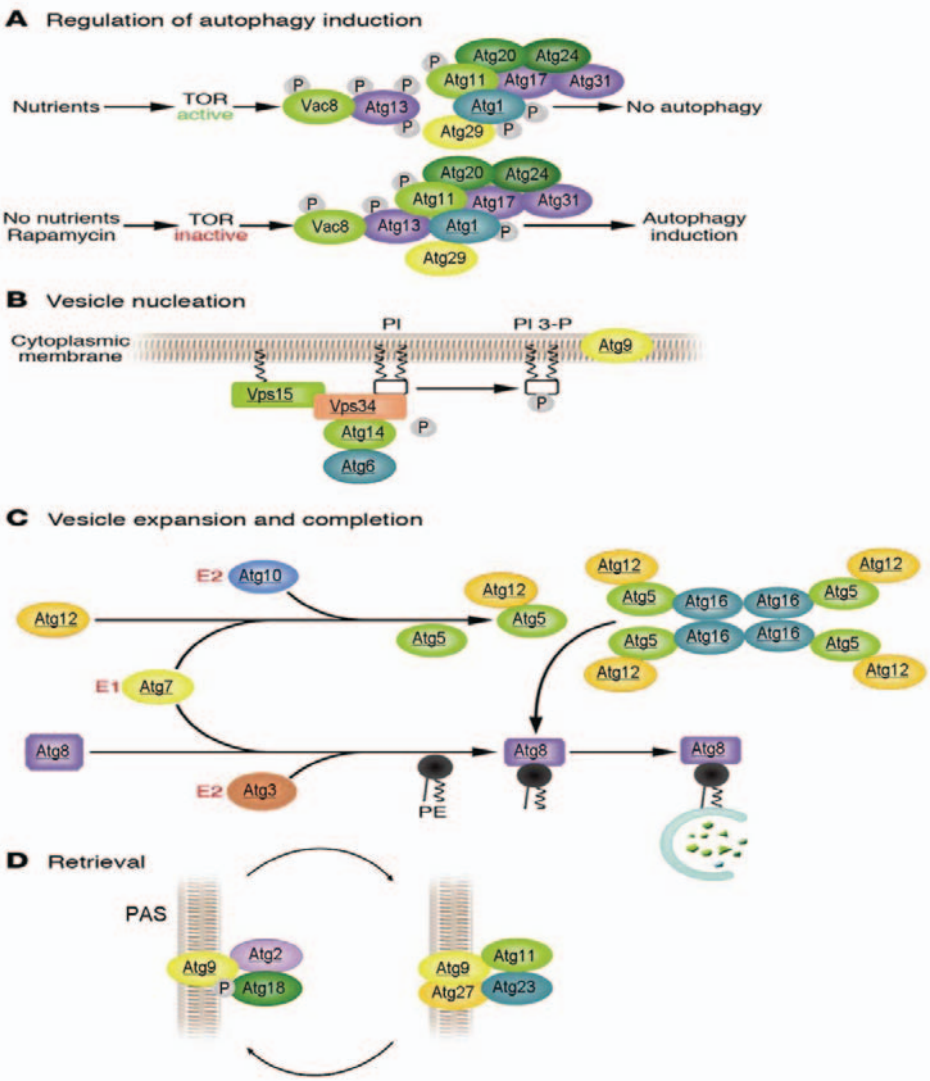

Figure 7 A-D: Atg complexes involved in autophagy. Underlined proteins indicate known orthologues in higher eukaryotes. Adapted from (Levine and Yuan, 2005).

have revealed two activating partners for Beclin-1: UVRAG and Ambra1 (Liang et al., 2006), the latter will be discussed below.

Two conjugates mechanistically related to the ubiquitin system have been shown to be required for autophagic vacuole formation: the Atg12-Atg5-Atg16 complex and the Atg8-PE conjugate (Ichimura et al., 2000; Ohsumi, 2001; see Fig 7C). Atg12, a ubiquitin-like protein, is activated by Atg7 in an E1-like manner and transferred to Atg10, which functions as an E2-like enzyme (Mizushima et al., 1998). Atg12 then binds to Atg5 through its C-terminus, establishing a stable conjugate. Atg5 may later bind to Atg16 to form the Atg12-Atg5-Atg16 complex (Mizushima et al., 1999). Atg8, another ubiquitin-like protein, is also activated by Atg7, after initial proteolytic processing by Atg4. In this case Atg3 acts as the E2-like enzyme that transfers the phospholipid phosphatidylethanolamine (PE) to the C-terminal glycine to form Atg8-PE (Kabeya et al., 2002). The Atg12-Atg5-Atg16 complex appears as a tetramer that seems to be essential for phagophore expansion and necessary for recruitment of Atg8-PE to the phagophore (Tanida et al., 2004; see also below).

A fourth functional group of Atgs involved in the autophagic machinery is the Atg9 –associated protein complex (see Fig. 7D). In yeast, Atg9 retrograde cycling between the phagophore assembly site (PAS) and mitochondria or peripheral pool found in proximity to ER is dependent on Atg2 and Atg18 as well as Atg23. These proteins are in turn recruited by Atg1 and the Vps34 complexes (Reggiori et al., 2004). In the absence of these protein complexes Atg9 is present at PAS; when activated, Atg9 redistributes to the peripheral pool. In mammalian cells, the hypothesis presented by Dr. Tooze and her coworkers explains a reversed, but analogous system (Chan et al., 2007; Webber et al., 2007). Mammalian Atg9 (mAtg9) cycles from the trans-Golgi network (TGN) to a peripheral Rab7-positive endosomal pool upon starvation. This translocation is hindered by knockout of the mammalian Atg1 analogue ULK1 and resembles shuttling of cation-independent mannose 6-phosphate receptor (CI-MPR). This may indicate that TGN is in close proximity to the mammalian equivalent of PAS.

Atg8 has four known mammalian homologues, microtubule-associated protein 1 light chain 3 (MAP1-**LC3**; Kabeya et al., 2000), GABA-receptor associated protein (GABARAP; Wang et al., 1999), Golgi apparatus ATPase enhancer of 16 kd (GATE-16; Legesse-Miller et al., 2000) and ATG8L (Tanida et al., 2006). Only LC3 has been

convincingly associated with autophagy. LC3 appears in two forms, I & II, migrating as an 18 and 16 kDa protein in a SDS-PAGE gel respectively: LC3-I is predominantly cytosolic, while LC3-II is PE-bound and associated with membranes. LC3-II is to date the only autophagosome-specific protein known and in contrast to the Atg16L complex, does not disengage the completed vacuole. Therefore, it is the most commonly used marker for autophagosome formation and monitoring of autophagic flux, especially as a genetically modified fluorescent protein by addition of a GFP-tag. Recently, a novel way of monitoring autophagosomal maturation was introduced by the Yoshimori laboratory, where a tandem fluorescence tagged mRFP-GFP-LC3 was used as a marker (Kimura et al., 2007). This approach has the advantage of informing the researcher of the lysosomal fusion step, where the GFP-tag will be extinguished by the acidic environment, in contrast to mRFP-LC3. A possible error of monitoring autophagic flux by GFP-LC3 staining was demonstrated where overexpression of transfected LC3 led to incorporation of LC3 in accumulating protein aggregates in cells with non-existent autophagy (Kuma et al., 2007). These studies should contribute to a more careful interpretation of autophagy measured by LC3-accumulation alone.

The Atg12-Atg5-Atg16L complex is also present in mammals, although Atg16L is considerably larger than yeast Atg16 (63 kDa vs 17 kDa) and has in addition a WD-repeat domain indicating putative protein-protein interaction not seen in yeast (Mizushima et al., 2003). The mammalian complex encompasses an 400 kDa tetramer or 800 kDa octamer. Both Atg12 and Atg5 are essential for autophagy in higher eukaryotes: newly-born Atg5 null mice develop severe malfunctions and die, indicating the importance of functional autophagy in neonatal mammals (Kuma et al., 2004).

Yeast	*Human /Mouse*	*Pathway*	*Function*
Atg1	ULK1	BOTH	Protein kinase, PAS localization
Atg2		AUT	Peripheral membrane protein, Atg9 interaction
Atg3	HsAtg3	AUT	E2-like protein for Atg8/LC3
Atg4	HsAtg4A/ B/ C/ D	AUT	Cysteine protease for GATE-16 (A), LC3 (B), GABARAP (B) and delipidation enzyme for Atg8-homologs.
Atg5	HsAtg5	AUT	Ubiquitin-like protein, interacts with Atg12, PAS localization
Atg6	Beclin-1	BOTH	Tumor suppressor, PI3K complex
Atg7	HsAtg7	AUT	E1-like protein for Atg8/LC3 and Atg12
Atg8	MAPLC3/ GABARAP/ GATE-16/ ATG8L	BOTH	Modifier for autophagosomal membrane, PE-conjugated
Atg9	HsAtg9	BOTH	Membrane protein, PAS localization
Atg10	mAtg10	AUT	E2-like protein for Atg12
Atg11		BOTH	Cargo recognition
Atg12	hAtg12	AUT	Interacts with Atg5, PAS localization
Atg13		AUT	Interacts with Atg1, hyperphosphorylated under normal conditions
Atg14	p70	BOTH	PI3K complex
Atg15		?	Putative lipase, autophagosome breakdown
Atg16	Atg16L	AUT	Binds Atg12-Atg5, PAS localization
Atg17		BOTH	Interacts with Atg1
Atg18	WIPI49	BOTH	Peripheral membrane protein, Atg2 interaction
Atg19		CVT	Cargo receptor
Atg20		BOTH	Peripheral membrane protein, PAS localization, PX domain
Atg21		CVT	Atg8 recruitment
Atg22		AUT	Integral membrane protein, efflux from lysosome, permease
Atg23		BOTH	Peripheral membrane protein, Atg9 interaction, PAS localization
Atg24		CVT	Sorting nexin protein, pexophagy and CVT pathway
Atg25		Pexopagy	Pexophagy
Atg26		?	Peripheral membrane protein
Atg27		AUT	Atg9 interaction, PI3K-complex
Atg28		Pexophagy	Pexophagy
Atg29		AUT	PAS localization
Atg31		AUT	Atg17 interaction

Table 1: AUT-CVT genes comparison (adapted from (Klionsky et al., 2003), (Klionsky and Emr, 2000); (Mizushima et al., 2002); (Tanida et al., 2004) (additional references, Atg27 (Yen et al., 2007); Atg28 (Yen and Klionsky, 2007; Stasyk et al., 2006); Atg29 (Kawamata et al., 2005); Atg31 (Kabeya et al., 2007); (http://GO db.yeastgenome.org, accessed 2007-07-04)).

Another mammalian ortholog of autophagy-related genes deserves closer description. The mammalian gene Beclin-1 (which corresponds to yeast Atg6) was originally identified as a Bcl-2-binding protein from yeast two-hybrid experiments (Aita et al., 1999). Beclin-1 can complement autophagy in autophagy-defective yeast with Atg6 deficiency, and promotes autophagy in human breast carcinoma cells, MCF-7 (Liang et al., 1999). Interestingly, the anti-apoptotic protein Bcl-2 inhibits starvation-induced autophagy as a function of its direct interaction with Beclin-1. In yeast, mammalian cells and *in vivo* models, Bcl-2 effectively inhibits Beclin-1-mediated autophagy. Furthermore Bcl-2 mutants lacking the Beclin-1-binding domain also lose their autophagy-inhibitory effect (Pattingre et al., 2005).

THE AUTOPHAGIC ORGANELLES

THE PHAGOPHORE

The *phagophore*, sometimes referred to as the isolation membrane, is the initial organelle in the autophagosomal-lysosomal pathway (Seglen, 1987). It appears to be a flattened membrane cistern which expands to envelop cytoplasmic material until it closes to form an autophagosome. In yeast there is evidence for these organelles to be located at the so-called pre-autophagosomal structure (PAS), also dubbed phagophore assembly site (Suzuki et al., 2001), and believed to be a processing plant for phagophores. In this region the majority of the Atg-proteins necessary for autophagy are found. The phagophore itself contains different protein complexes which reside at the organelle, such as the Atg8-PE conjugate and Atg16-12-5 complex. The latter of these is localizing to the extreme ends of the organelle at the time of sequestration (Kuma et al., 2002).

The origin of the phagophore membrane is a topic of extensive debate. It may materialize either by reuse of preexisting membrane material or by *de novo* synthesis. Early suggestions included the plasma membrane (Oledzka-Slotwinska and Desmet, 1969), Golgi-apparatus (Locke and Sykes, 1975) and lysosomes (Sakai et al., 1989), in addition to compartments from the endocytic pathways (Dunn, 1990) as source of the isolation membrane. These postulates have been mostly disregarded due to the morphological character of the phagophore as well as the absence of common marker proteins from these membranes. In fact, proteins seem to be rather scarce on the outer membrane surface, the cisternal membrane also appears to be rich in unsaturated fatty acids as detected by electron microscopy, and confirmed by osmium staining (Reunanen et al., 1985; Fengsrud et al., 2000). Protein markers common in endoplasmic reticulum (ER) found in autophagosomal structures indicate a stronger candidacy for this cisternal cytoplasmic organelle (Novikoff and Shin, 1978; Reunanen and Hirsimäki, 1983), and evidence for cycling of the membrane protein Atg9 from ER to PAS in yeast supports this explanation (Reggiori et al., 2005). However, in accordance with other studies with early autophagic structures (Reunanen et al., 1985; Furuno et al., 1990) purified autophagosomes from rat hepatocytes lack any enrichment of ER markers (Strømhaug et al., 1998). In mammals, the reported ULK1- and starvation-dependent trafficking of mAtg9 between TGN and endosomes indicate the trans-Golgi network as a supplier of

membranes to PAS (Köchl et al., 2006). The presence of the mammalian homologue of Atg18, WIPI49, on TGN gives additional support to this theory (Jeffries et al., 2004).

Induced autophagy in yeast involves the new-synthesis of proteins necessary for phagophore formation: the eukaryotic initiation factor 2α (eIF2 α) kinase Gcn2 and its downstream target Gcn4, a transcriptional transactivator of autophagy genes, turn on autophagy during nutrient depletion (Tallóczy et al., 2002; Takeshige et al., 1992). In contrast, cycloheximide does not impair autophagosome formation in mammalian cells, indicating a constitutive pool of available phagophores (Kovács and Seglen, 1981). Mammalian autophagy can proceed for several hours without the need for protein synthesis (Lawrence and Brown, 1993) suggesting an extensive reutilization of phagophores and associated proteins for sequestration.

THE AUTOPHAGOSOME

Upon completion the *autophagosome* appears as a vacuole delimited by a distinct double- (sometimes multi-) layered membrane, and containing a heterogeneous mixture of

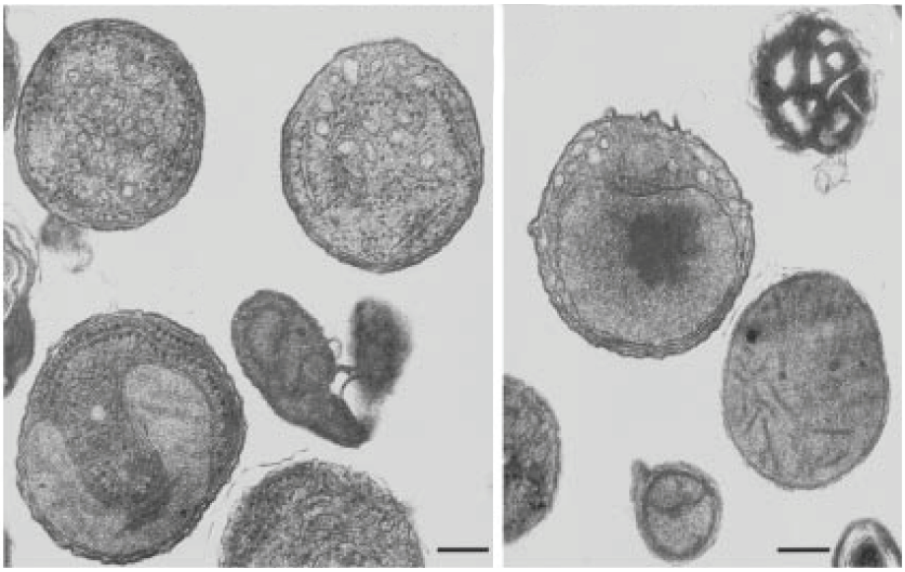

Figure 8. Autophagosomes. Electron microscopic images of purified isolated autophagosomes containing varius subcellular components. From (Strømhaug et al., 1998).

cytoplasmic material, including organelles, proteins and various cellular components (Seglen, 1982). It has been estimated that 40 % of the protein in a cell can be devoured by autophagy under starvation conditions. The vacuole is usually about 200 nm in diameter, but can be substantially larger, as in case of the 1000 nm autophagosomes detected in neurons containing aggregates, and for xenophagic purposes (Rubinsztein et al., 2005). It is best characterized by electron microscopy, where an autophagosome will appear as a membrane-inclusion of cytoplasm, and due to the ideally non-specific nature of uptake, the inside would not differ substantially from the outside (Fengsrud et al., 2000). Simultaneously, it should be devoid of markers that are specific for later vacuoles in the lysosomal pathways as mentioned below. The half-life of the autophagosomes has been estimated to 8-10 minutes (Pfeifer, 1978).

THE AMPHISOME

After completion the autophagosome may suffer different fates. The usual route leads to a convergence with the endocytic pathway in the manner of fusion with an endosome, this new union have received the term *amphisome* [amphi- both] (Gordon and Seglen, 1988). This prelysosomal meeting point has been demonstrated by both biochemical means, as well as by cryo-electron microscopy (Tooze et al., 1990). The joint vacuole itself has been isolated and described by Berg and co-workers, who also indicate that both early and late endosomes may fuse with autophagosomes (Berg et al., 1998). This important distinction between autophagosomes and amphisomes can be made biochemically by checking for the presence of endocytic markers such as early endosomal antigen 1 (EEA1), asialoglycoprotein receptor (ASGPR) or mannose 6-phosphate receptor (M6PR) and the absence of lysosomal marker exemplified by LAMP1 and cathepsin B and D (Fengsrud et al., 2000). Noteworthy is also the fact that yeast lacks the amphisome altogether, implying 1) more complex functions of the autophagic pathways in higher eukaryotes or 2) inability of yeast genetic assays to also identify key proteins needed for amphisome formation, although autophagosome-endomsome fusion has been investigated in mammalian cells (Berg et al., 1998). The subsequent fusion of amphisomes with lysosomes apparently requires the small ras-like GTPases rab5 and 7; knockdown of these proteins leads to amphisome accumulation (Jäger et al., 2004; Gutierrez et al., 2004b).

THE LYSOSOME

The end of all things, at least for the degradation pathway here described, is the *lysosome*, originally discovered by de Duve in 1955 (de Duve et al., 1955). Lysosomes contain various enzymes to digest organelles, food particles, and engulfed pathogenic entities. They maintain an acidic interior (pH 4-5), optimizing the conditions for acidic hydrolases, by pumping protons from the cytosol into the lysosome by ATPases and chloride-ion channels. The low pH requirement of the lysosomal enzymes protects the cytosol in case of membrane disruption as the degradative enzymes will have suboptimal function in the more alkaline environment of the cytosol (pH 7.0); furthermore, these enzymes are kept in check by a number of cytosolic inhibitors. To distinguish between the origins of the material delivered for degradation, it has been common to denote the *autolysosome* as the organelle that is a merger of an autophagosome/amphisome and a lysosome, thus containing autophagocytosed material internally. In contrast a lysosomal organelle resulting from a union of an endosome and a lysosome is called a *heterolysosome*, evading the autophagic pathway and having exogenous material inside (*auto* = self, *hetero* = unlike (i.e. not self)).

There are two main ways to impair lysosomal degradation, either by inhibiting the proteases inside with for instance pepstatin A, leupeptin, or E64d (Seglen et al., 1979), or by neutralizing the intralysosomal acidic environment. The latter can be achieved by blocking the proton pump with a specific inhibitor of H+ vATPase, such as bafilomycin A (Ohkuma et al., 1993), vanadate (Fosse et al., 1995) or nigericin (Poole et al., 1972), or by adding weak bases that are able to cross the membrane, such as ammonia, increasing the pH inside the lysosomes (Seglen and Gordon, 1980).

The enzymes in the lysosomes are produced in the ER. They receive a M6PR tag on their way through the Golgi and are activated when reaching the inside of the lysosome. In yeast, the corresponding organelle is simply named the vacuole. It is on the receiving end of the CVT pathway that shares many of the genes of autophagy, most prominently the Vps34 kinase complex, where Atg14 is replaced by Vps38 (see table 1).

THE CYTOSKELETON

Transport in the cell of the various autophagic organelles and their fusion events are closely related to cytoskeletal network. It has been shown that different cytoskeleton inhibitors have diverse effect on autophagy, such as the microtubule disruptive compounds vinblastine and nocodazole that induce accumulation of autophagosomes in isolated rat liver cells by disabling their fusion with lysosomes or endosomes (Blankson et al., 1995; Kovács et al., 1982). However, these inhibitors have little or no effect on sequestration. The vinblastine effect was not dependent on nutrients nor on mTOR activity (see below), but required Atg5 and Atg6 (Köchl et al., 2006). The microfilament inhibitor cytochalasin D did not effect autophagy in hepatocytes (Blankson et al., 1995), but reportedly did so in kidney cell lines (Aplin et al., 1992). Another toxin, okadaic acid, has been shown to disrupt the intermediate filament keratin network, and to completely suppress autophagy in hepatocytes, without affecting the microtubular or microfilamentous networks. Furthermore, the resulting overphosphorylation undid the function of the cytoskeletal cross-linker plectin (Ruud Larsen et al., 2002). Additionally, autophagosomes are practically devoid of cytoskeletal proteins such as tubulin, plektin, keratin and actin, in an otherwise bulk sequestration process, supporting a role for the cytoskeleton as an external scaffold for the phagophore that is removed after completion of the sequestration (Strømhaug et al., 1998; Ruud Larsen et al., 2002).

The association of several autophagy-related proteins with microtubuli, e.g. LC3, BHMT (Sandu et al., 2000), and Atg4 (Lang et al., 1998), hints at a central autophagic function of these transportation structures. Knockout of HDAC6, a histone deacetylase, that is actually more specific for microtubules and dynein (Rubinsztein, 2007), leads to impaired fusion of autophagosomes; furthermore, overexpression of HDAC6 rescued cells from the toxicity related to proteasome inhibition (Iwata et al., 2005). It has been shown recently that HDAC6 also bind to polyubiquitinated proteins and may augment UPS-failure as demonstrated in a *D. melanogaster* spinal muscular atrophy (Pandey et al., 2007b). These data introduce this enzyme as a link between the main protein degradation pathways for misfolded proteins, revealing another piece of the puzzle for understanding the role of cytoskeletal networks in autophagic degradation (Pandey et al., 2007a).

Yet another protein related to ubiquitin is sequestosome 1/ ubiquitin-binding protein p62 (SQSTM1/ p62), a protein found in inclusion bodies containing

polyubiquitinated protein aggregates (Zatloukal et al., 2002). In diseased brain tissues it is found in Lewy bodies, PD neurofibrillar tangles and huntingtin aggregates (Kuusisto et al., 2001; Nagaoka et al., 2004; Kuusisto et al., 2003), in liver in Mallory bodies and associated with steatohepatatis and hyaline bodies in hepatomas (Zatloukal et al., 2002), as well as in aggregates in aggressive variants of breast cancer (Rolland et al., 2007). Recent discoveries indicate the appearance of p62 in aggresome-like induced structures (ALIS), which are the result of stressors commonly associated with induced autophagy, such as amino acid deprivation, oxidative stress and puromycin (misfolding inducer) treatment (Szeto et al., 2006). Johansen and coworkers discovered that p62 binds directly to LC3 through an N-terminal domain also necessary for ubiquitin-binding, and this interaction in crucial for degradation of p62-positive inclusion bodies by autophagy. They were also able to determine p62 binding partners by LC-MS-MS, identifying MAP1B as its partner for MAP1-LC3B binding (Pankiv et al., 2007; Bjorkoy et al., 2005). This finding correlates with another study where p62 levels was found to increase in Purkinje cells treated with autophagy inhibitors, leading to the suggestion of using p62 as a novel indicator for autophagy impairment (Wang et al., 2006b; Yue, 2007).

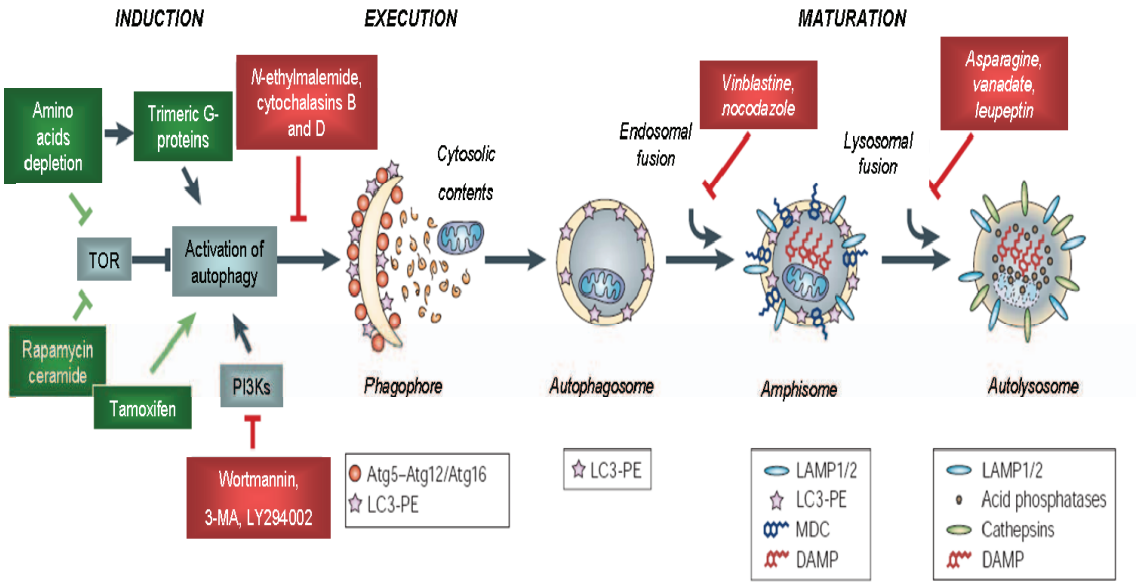

Figure 9: Regulators of different steps of autophagy. Green arrows and boxes indicate positive regulation, red arrows and boxes negative regulation. DAMP and MDC = staining agents. Refer to text for details. Adapted from (Kirkegaard et al., 2004).

AUTOPHAGY-REGULATING CONDITIONS

AMINO ACIDS AND HORMONES

There is evidence for autophagy performing a constitutive background clearing of proteins (Komatsu et al., 2005), but in addition it is highly induced by several factors, and inhibited by another set of factors. Central to regulation of autophagy are the *amino acids*, removal of which induces the process, i.e. as a starvation response (Mortimore and Schworer, 1977). Specific studies have been done to reveal that amino acids differ in the induction effect of their removal. Leucine has the highest inhibitory impact, but also phenylalanine, tyrosine, tryptophan, histidine, proline, methionine and glutamine may inhibit protein degradation (Grinde and Seglen, 1981; Seglen et al., 1980). Asparagine has been found to cause no change in autophagosome formation, but exerts its effect by blocking later fusion steps (Hoyvik et al., 1991, see Fig. 9). The important hormones insulin and glucagon modulate the amino acid effect, potentiating and antagonizing it, respectively (Kovács et al., 1989; Mortimore et al., 1987). However the added effect of these hormones is only seen at intermediate amino acid levels, neither insulin nor

glucagon exerting any influence when autophagy rates are very low or high (Schworer and Mortimore, 1979),(Mortimore et al., 1989). As amino acids are one of the end products of autophagic degradation, feedback-inhibition of the process is a logical mechanism, but the exact molecular means of this inhibition is not well understood. It has been suggested that the mechanism includes an extracellular amino acid receptor, such as for leucine (Miotto et al., 1992), but this remains disputed since amino acid transport across plasma membrane was shown to be necessary for the amino acid effect (Van Sluijters et al., 2000; Luiken et al., 1996). The target for amino acids inside the cell in relation to autophagy is primarily the protein kinase mTOR, possibly through involvement of Rheb-binding (Avruch et al., 2006, see below, Fig. 11). Insulin, a polypeptide hormone product of the pancreas in mammals, has its effect regulated through the specific insulin receptors at the cell surface, initiating a signal cascade through insulin receptor substrate (IRS), phosphatidylinositol- 3 kinase (PI3K) and protein kinase B (Akt/PKB; see below, Fig. 11).

CALCIUM

Calcium, a critically important ion in biological regulation appears to play a role in autophagy. Experiments with Ca^{2+} ionophores and chelators revealed dependency on the presence of Ca intracisternally, this observation was also strengthened by the use of the S/ER Ca^{2+} ATPase (SERCA) inhibitor thapsigargin (TG; Gordon et al., 1993). However, recent studies gainsay these findings by showing data that indicate autophagic stimulation by the same compound, though activation of the CaMKKbeta-pathway and mTOR inhibition (Mijaljica et al., 2007; Hoyer-Hansen et al., 2007). However, these latter findings are contradicted by an even more recent paper on the effect of S/ER Ca^{2+} stores in cardiac myocytes and its necessity for autophagy mediated by the S/ER variants of Bcl-2 or Bcl-X_L, vital Beclin-1 binding partners and regulators (Brady et al., 2007). Kroemer and co-workers have presented data on ER-stress in relation to autophagy, where the second messenger inositol 1,4,5-triphosphate (IP_3) acts on calcium-channels to inhibit starvation-induced, but not rapamycin-induced autophagy (Criollo et al., 2007b; Criollo et al., 2007a). It appears that the effect of IP_3 on autophagy is mediated through the 1,4,5 triphosphate receptor (IP_3R) and its ability to bind Bcl-2, since xestospongin B,

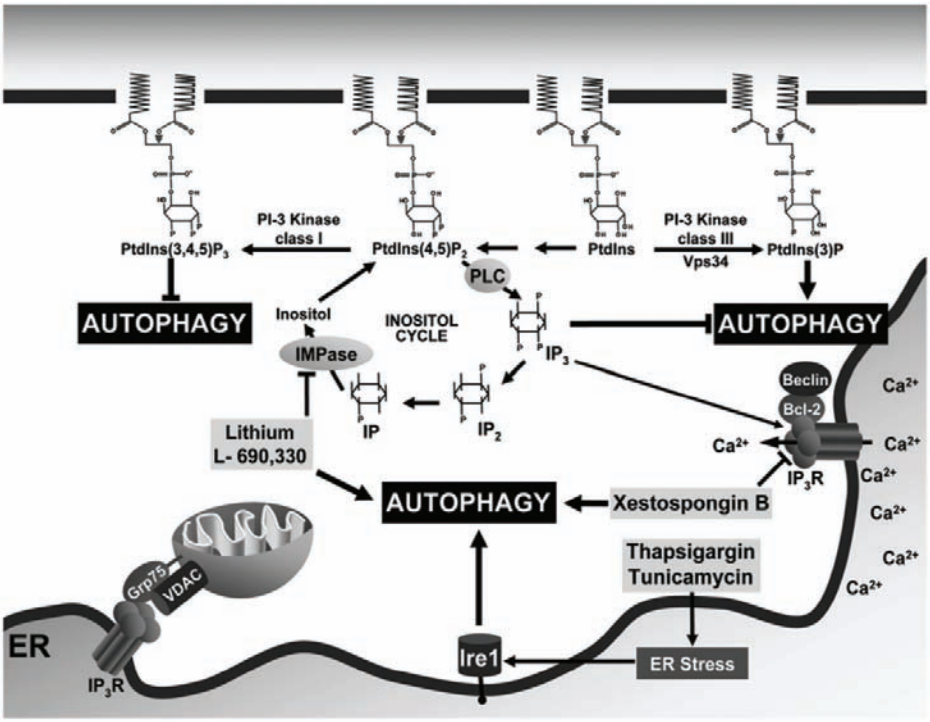

Figure 10. Regulation of autophagy by inositol derivatives. Description of the PtdIns pathway and involvement of PI3Ks class I and III in opposite roles in autophagy. PtdIns(4,5)P_2 processed by phospolipase C (PLC) releases IP_3, which in turn acts on its Ca^{2+}-regulating receptor IP_3R on the ER membrane. TG and tunicamycin leads to Ire1-mediated ER-stress and induced autophagy. IP3R inhibited by xestospongin B changes its interaction with Bcl-2, which in turn make more Beclin-1 available for autophagy activation independent of calcium stores. From (Criollo et al 2007).

an IP_3R antagonist, does not affect Ca^{2+} - concentrations in ER or cytosol while autophagy is induced (see Fig. 10).

LIPIDS AND LIPID-DERIVATIVES

The double-layered delimiting membrane of autophagic vacuoles depends on the correct balance of *lipids* present in the cell and the recruitment of phospholipid components. Phosphoinositides (PI), phosphorylated derivatives of the membrane lipid

phosphatidylinositol (PtdIns), regulate membrane trafficking, cytoskeleton function, and receptor signaling by recruiting of protein complexes to specific membranes in a reversible manner (Gillooly et al., 2001). PI3K is tied to regulation of autophagosome formation, since prominent inhibitors of the two classes of these kinases, 3-methyladenine (Seglen and Gordon, 1982) and wortmannin (Ui et al., 1995) also lead to autophagic arrest. The regulation of autophagy in mammalian cells occurs via two PtdIns 3-kinase complexes, class I and class III. The class III enzyme that generates phosphatidylinositol-3-phosphate (PtdIns(3)P) is similar to yeast Vps34 and exerts a stimulatory function on autophagy. Adversely, class I enzyme, which converts PtdIns(4,5)P_2 to PtdIns(3,4,5)P_3 at the plasma membrane, has an inhibitory effect on autophagy (Petiot et al., 2003; Petiot et al., 2000). This latter enzyme is part of a signaling cascade that activates mTOR (see below) and other kinases in the Akt/PKB pathways. The function of PtdIns(3)P in mammalian cells is not clear, although it is likely to have a role similar to that in yeast; however, the Atg20, Atg21, Atg24, and Atg27 proteins, which appear to bind this phosphoinositide, do not have known homologs in higher eukaryotes. Atg18 is the only PtdIns(3)P-binding protein with a mammalian homolog WIPI49 (Proikas-Cezanne et al., 2004). The formed phosphoinositide presumably recruits proteins containing FYVE or PX motifs required for autophagosome formation (Gillooly et al., 2001; Wishart et al., 2001). An example of a FYVE-domain protein connected to autophagy regulation is Alfy, shown to be associated with membranes and ubiquitin-positive aggregates (Simonsen et al., 2004).

RAPAMYCIN AND TOR KINASE

Rapamycin, also known as sirolimus, is a macrolide drug that works as a potent inducer of autophagy through binding of FK506-binding protein 12 (FKB12; Cutler et al., 1999), and sequential inhibition of its target protein: a serine/threonine protein kinase called TOR (target of rapamycin), structurally related to phosphatidylinositol kinase. Among several other key functions, activated TOR is a prominent inhibitor of autophagy. In addition to rapamycin, TOR is inactivated under amino acid-deficient conditions, resulting in enhanced autophagic activity. Under amino acid-rich conditions, however, TOR is active and autophagy is inhibited (Van Sluijters et al., 2000). One of the known effects on the molecular machinery of autophagy is the TOR-dependent phosphorylation

of Atg13 (see above Fig. 7A). TOR is conserved in mammalian cells (as mTOR) and both yeast and mammals (m)TOR is part of two complexes known as (m)TORC1 and 2 (see Fig. 11). Mammalian mTORC1 complex consist of the additional proteins Raptor and mLST8, while mTORC2 has Rictor, mLST8 and mSin1 as extra components (Nojima et al., 2003; Wullschleger et al., 2005; Wullschleger et al., 2006).

The major difference between the two complexes is that mTORC1 is rapamycin-sensitive, while mTORC2 does not respond to rapamycin. Additional stimuli for both complexes include stress, growth factors, insulin and phosphatidic acid. mTORC1 has several known protein phosphorylation targets including p70 ribosomal protein S6 kinase

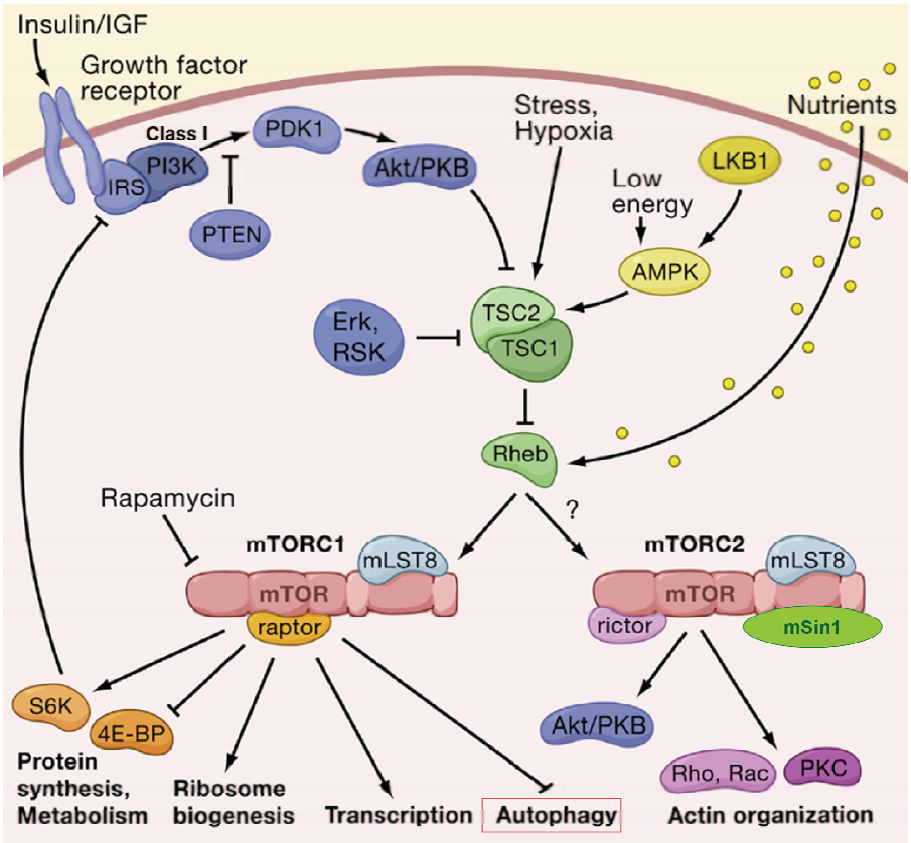

Figure 11: mTORC1 and 2 pathways. Refer to text for details. Adapted from (Wullschleger et al., 2006).

(S6K) and eukaryotic initiation factor 4E binding protein 1 (4E-BP1; Hay and Sonenberg, 2004). Although not unequivocally proven, the negative regulatory cascade upstream of mTOR includes class I PtdIns 3-kinase, PDK1 and Akt/PKB, while the phosphatase PTEN acts antagonistically to the PtdIns 3-kinase to induce autophagy (Arico et al., 2001). Akt/PKB in turn, inhibits the tuberous sclerosis complex TSC1/2, a complex also activated by the AMPK-cascade (for AMPK, see below). Finally, this complex, composed of proteins hamartin and tuberin, exhibits GTPase-activating properties to alter the small GTPase Rheb by changing its binding partner from GTP to GDP (Tee et al., 2003).

Rheb, first identified as a Ras homologue enriched in brain, is a G-protein involved in autophagy regulation by its activation of mTOR: it is potentially necessary for both mTORC complexes. Rheb expression is elevated in many tumor cells, and it may be the critical target by which farnesyl transferase inhibitors mediate their anti-tumor activity (Basso et al., 2005). mTORC2 appear to have a role in cytoskeleton regulation, especially during cell growth, through its stimulation of F-actin stress fibers, paxillin, Rho, Rac, cdc42 and protein kinase C (PKCα, Sarbassov et al., 2004). This complex also appears to be responsible for Akt/PKB activation, although through a different phosphorylation site than PDK1, indicating PDK2-properties for mTORC2 (Sarbassov et al., 2005).

Heterotrimeric guanine nucleotide-binding proteins (*G-proteins)* are molecules involved in signal transduction in general, but also specifically in modifying autophagy (Ogier-Denis et al., 1996). New evidence suggests that the pertussis toxin-sensitive alpha subunit of variant G_{i3} specifically is required for the insulin-mediated regulation of a autophagy and furthermore hints at a previously unrecognized function for $G\alpha_{i3}$ on autophagosomal membranes as a go-between with mTOR, thus implying a cross-talk between these related mediators of autophagy (Gohla et al., 2007). Autophagy was found to be stimulated by a Gα-interacting protein (GAIP) which promoted the hydrolysis of $G\alpha_{i3}$-bound GTP (Ogier-Denis et al., 1997). Another protein, AGS3, prevents dissociation of GDP from $G\alpha_{i3}$ again leading to stimulated autophagy (Meijer and Codogno, 2004).

The monomeric GTP-binding protein rab7 is believed to be located to delimiting membranes of amphisomes and autolysosomes, and has a late endosomal origin.

Furthermore, overexpression of a dominant negative form of rab7 protein led to the inability of autophagosomes to fuse with lysosomes (Kimura et al., 2007), indicating a crucial role for rab7 in this mechanism. The related protein Ras is also involved in autophagic regulation, by its position upstream of ERK1/2, which inhibits TSC1/2.

SPHINGOLIPIDS

Ceramide is a sphingolipid signal molecule with an essential role in cell growth, cell death, proliferation, and stress (Levade et al., 2002). Ceramide was first implicated as being a signal for programmed cell death when patients with the genetic disorder Niemann-Pick disease were found to have certain cell types that were resistant to apoptosis (Merrill 2006). Studies have indicated several targets for ceramide, including PP1, PP2A, PKC and cathepsin D (Pettus et al., 2002; Kashiwagi et al., 2002; Kajimoto et al., 2004; Heinrich et al., 1999), proteins that are activated subsequent to ceramide stimulation. In human colon cancer cells, C2-ceramide, a membrane-permeable ceramide variant, stimulated autophagy, probably through interference with class I PI3K activation and Akt/PKB-phosphorylation (Scarlatti et al., 2004). It was also shown to induce increased levels of Beclin-1. Furthermore, ceramide is believed to mediate tamoxifen-dependent accumulation of autophagic vacuoles in the human breast cancer MCF-7 cells. Ceramide also enhance transcription of BNIP3, a cell death factor protein found in mitochondria inducing autophagic cell death (Daido et al., 2004).

The related product sphingosine-1 phosphate created from sphingosine (deacylated ceramide) by sphingosine kinase 1 (SK1) has similar effects on autophagy as seen by apparent inhibition of mTOR and increased levels of Beclin-1 (Lavieu et al., 2006). The autophagic effects of S1P are, however, milder than for ceramide, and appear at a later stage in the signaling cascade, indicating different roles in regulating cell survival by autophagy for these two second messengers.

ENERGY

Energy in the form of ATP is, as for almost all cellular processes, also required for the autophagic process, at the initial sequestration step, for fusion events and for lysosomal proton pumping (Plomp et al., 1987; Plomp et al., 1989). By external stimuli that deplete ATP stores, the ratio between AMP and ATP increases, which leads to repressed autophagy, possibly due to the activation of energy-sensing AMP-activated protein kinase (AMPK) (Samari and Seglen, 1998). AMPK is a heterotrimeric serine/threonine kinase activated by LKB1-effected phosphorylation (Baas et al., 2003), and/or by adenosine monophosphate, its allosteric activator. Its main function is to gauge the

energy conditions in the cell through binding of AMP, and correspondingly down-regulating ATP-dependent metabolic processes to promote short-term cell survival. The ability of adenosine and AMP analogues, AICAR and ZMP respectively, to inhibit autophagy supports the involvement of AMPK (Møller et al., 2004b). Furthermore, the differentiated antagonistic effects of the flavonoid naringin against a spectrum of autophagy-inhibiting toxins as well as against AMP/ZMP, exactly parallels its antagonism of AMPK inhibition.

Ambient temperature is also a potent regulator of the autophagy, a total lack of autophagosomes being seen in isolated hepatocytes at 20˚ C (Gordon et al., 1987).

PROTEIN KINASES

Ample evidence for the impact of phosphorylation states on autophagy exist, in addition to the already mentioned AMPK and mTOR. S6K1 is another kinase that exerts an inhibitory effect on autophagic sequestration (Blommaart et al., 1995). This serine/threonine kinase, originating from a single gene encoding two variants, p85 and p70, the former including a nuclear localization signal, the latter predominantly cytoplasmic, is also known to be involved in cell growth. p70S6K is activated through sequential phosphorylations in the tail-region mediated by PI3K (Pullen et al., 1998), and additional phosphorylation events mediated by PDK and mTOR which promotes phosphorylation at the key site threonine-389 (Dennis et al., 2001). Since administration of an autophagy-repressing amino acid mixture also led to S6 phosphorylation, a target for S6K, a hypothesis that S6 was directing amino-acid-dependent inhibition of autophagy was presented (Blommaart et al., 1995). This is in accordance with the effect of rapamycin on both pathways in yeast (Noda and Ohsumi, 1998), but not with evidence from hepatocytes where rapamycin fails to work antagonistically on amino acid inhibition under certain conditions (Kanazawa et al., 2004). Recent studies have shown that autophagy-suppressive agents such as okadaic acid, AICAR and amino acids all led to phosphorylation of the tail region of S6K, but not S6 phosphorylation, indicating a relationship between autophagy and S6K, rather than S6 (Møller et al., 2004a). Further evidence for S6K involvement in autophagy emerges from a study where ATG1 was shown to regulate S6K by interfering with Thr389 activation site phosphorylation in *D.*

*melanogaste*r (Lee et al., 2007). Overexpression of ATG1 similarly inhibited S6K and was associated with stimulated autophagy, and cell growth arrest.

Algal toxins inhibit protein phosphatase type 2A, resulting in hyperphosphorylation and consequent inhibition of autophagy, indicating a positive effect of said phosphatase (Holen et al., 1993). Interestingly, the flavonoid naringin antagonized the okadaic effect and restored autophagic rates (Gordon et al., 1995). Other kinases may influence the process in the opposite direction, such as cyclin-dependent kinases: for example, the CDK inhibitor olomoucine was found to inhibit autophagy (Strømhaug et al., 1997). Furthermore, an involvement of Ca^{2+}/calmodulin-dependent kinase II (CaMKII; Holen et al., 1992) was indicated by the effect of KN62 and other CaMKII inhibitors, which canceled the autophagy-suppressive effect of okadaic acid (Holen et al., 1993).

Stress-activated kinases (SAPK) and mitogen-activated kinases (MAPK) are classes of protein kinases, among which ERK1/2 (extracellular signal-regulated kinase 1/2), SEK-1 (SAPK/ERK kinase) and JNK (c-Jun N-terminal kinase) seem to play a role in autophagy regulation. ERK1/2 is a part of the Ras/Raf-1/MEK/ERK cascade that seems to have a role in regulation of TSC1/2 (see Fig. 11). Reports on increased activity of this cascade is related to induction of autophagy in several human carcinomas; additionally, it seems that ERK1/2 interact with the previously mentioned GAIP to regulate autophagosome formation (Ogier-Denis et al., 2000). SEK-1 and JNK were both found to be activated in an okadaic acid-dependent and naringin-sensitive manner in rat hepatocytes, indicating the involvement of an AMPK - SEK/JNK-cascade in mediating toxin-repressive effects on autophagy (Møller et al., 2004a).

CALPAIN

The endopeptidase family of calpains consists of calcium-dependent proteins that are involved in cytosolic proteolysis. The catalytic subunit of 80 kDa joins with a regulatory subunit of 30 kDa to form a heterodimer, of which there exist several ubiquitous as well as tissue-specific variants (Goll et al., 2003). Calpains regulate protein activity by proteolytic processing. Their major function is exerted at the plasma membrane, to which they are recruited after Ca^{2+} activation in the presence of inositol-phospholipids (Saido et al., 1992). Substrates of calpain are numerous, including plasma membrane Ca^{2+}-ATPase

(Brown and Dean, 2007), PEBP (Chen et al., 2006), Atg5 (Yousefi et al., 2006) and possibly other autophagy-related proteins, since autophagy was non-existent in a calpain-knockout model of mouse embryonic fibroblasts (MEF, Demarchi et al., 2006). In cell lines overexpressing Atg5, its processing by calpain appears to work as a switch to programmed cell death, as demonstrated by an N-terminally processed form of Atg5 detected in mitochondria of apoptotic cells together with Bcl-X_L (Yousefi et al., 2006).

OXIDATIVE CONDITIONS

Reactive oxygen species (ROS) occur in the cell spontaneously or as a result of environmental influence, and may have deleterious effects on the cell. Reported stimuli of ROS formation include mitochondrial malfunction, ER stress and tumor necrosis factor stimulation (Kamata et al., 2005). ROS are highly reactive molecules against which cells have developed defense mechanisms, such as the ubiquitin-proteasomal pathway, but also autophagy, that remove damaged mitochondria and oxidized proteins. It seems that mildly oxidized proteins are targets for CMA (Kiffin et al., 2004), while cells under more severe oxidative stress accumulate damaged proteins prone to degradation through macroautophagy (Xiong et al., 2007). Oxidation may also be responsible for signaling events, the activity of several enzymes being modulated through oxidation of cysteines in their active site (Rhee et al., 2005). In a recent study, low levels of hydrogen peroxide (H_20_2) were produced in mitochondria at the early stages of starvation-induced autophagy in a class III PI3K-dependent manner (Scherz-Shouval et al., 2007). By adding antioxidants, autophagy was inhibited, indicating the need for ROS in autophagic initiation. Furthermore, the cysteine protease hAtg4, required for the inactivation and delipidation of Atg8 homologues, appeared to be the target for ROS. The active site consisting of a cysteine at position 81 in hAtg4A was oxidized, thus inactivating the enzyme, and increased lipidation of mammalian Atg8s was seen *in vitro* (Scherz-Shouval et al., 2007). In conclusion, this could mean that ROS may also work as a positive regulator of autophagy. Additional studies on rapamycin-induced autophagy and mitochondria in yeast show similar result, where ROS were observed quickly after autophagic induction in the cell together with oxidation of mitochondrial lipids. By adding resveratrol, an antioxidant found in grapes, the induced autophagy of cytosolic proteins as well as mitochondria was stalled (Kissova et al., 2006). Also, since damaged

mitochondria and peroxisomes produce more ROS than normal ones, it has been extrapolated that the selective sequestration of these organelles by autophagy may be initiated by hydrogen peroxide in a similar fashion (Liu and Lenardo, 2007).

AUTOPHAGY AND DISEASE

A viable autophagy is most important for the cell (and organism) as a maintainer of homeostasis. As the stimulated autophagic pathway can dispose of up to 4 % of the cellular proteins per hour, and may account for as much as 80 % of the total protein turnover, an impaired autophagy will have major consequences for the cell. In addition, the degradation of RNA and lipids will also contribute to the functional role of this pathway. On the tissue level, it has been shown that liver size may vary greatly in the absence or presence of nutrients, and autophagy is assumed to be a major contributor to this effect (Millward, 1980; Mortimore et al., 1987). For unicellular organisms, such as *S. cerevisiae*, a defect in autophagy will lead to rapid death under starvation conditions (Kametaka et al., 1998). For many disease-associated autophagic mechanisms, there is a balance between too much and too little activity, where one condition could be as pathologic as the other (see also Fig. 12). For instance, the lysosomal glycogen-storage disorder Pompe disease (Engel, 1970; Sharma et al., 2005), characterized by an accumulation of autolysosomes in muscle, seems to be caused by both a malfunction in lysosomal degradation and elevated autophagic activity (Raben et al., 2007).

The question of whether autophagy is involved in normal development is addressed in a recent study showing the involvement of autophagy in the clearance of dead cells in the early mouse embryo (Qu et al., 2007). Furthermore, embryos that contain a mutation to Beclin-1 die early during embryogenesis, exhibiting a developmental delay (Yue et al., 2003) whereas Atg5- or Atg7-deficient mice survive embryogenesis but suffer from nutrient and energy insufficiency soon after birth (Kuma et al., 2004; Komatsu et al., 2005). To elucidate the mechanism by which Beclin-1 is regulated during early stages of development, a gene-trap approach have been used to identify modulators, uncovering a new protein, called Ambra1, responsible for triggering Beclin-1–dependent autophagic response during embryogenesis to avoid neural tube defects (Maria et al., 2007).

The great surge in research on autophagy has been spearheaded by its emerging importance in the pathology of serious human diseases. The evidence for suppressed protein degradtion in **cancer** cells, and thus accelerated growth, indicates the possible

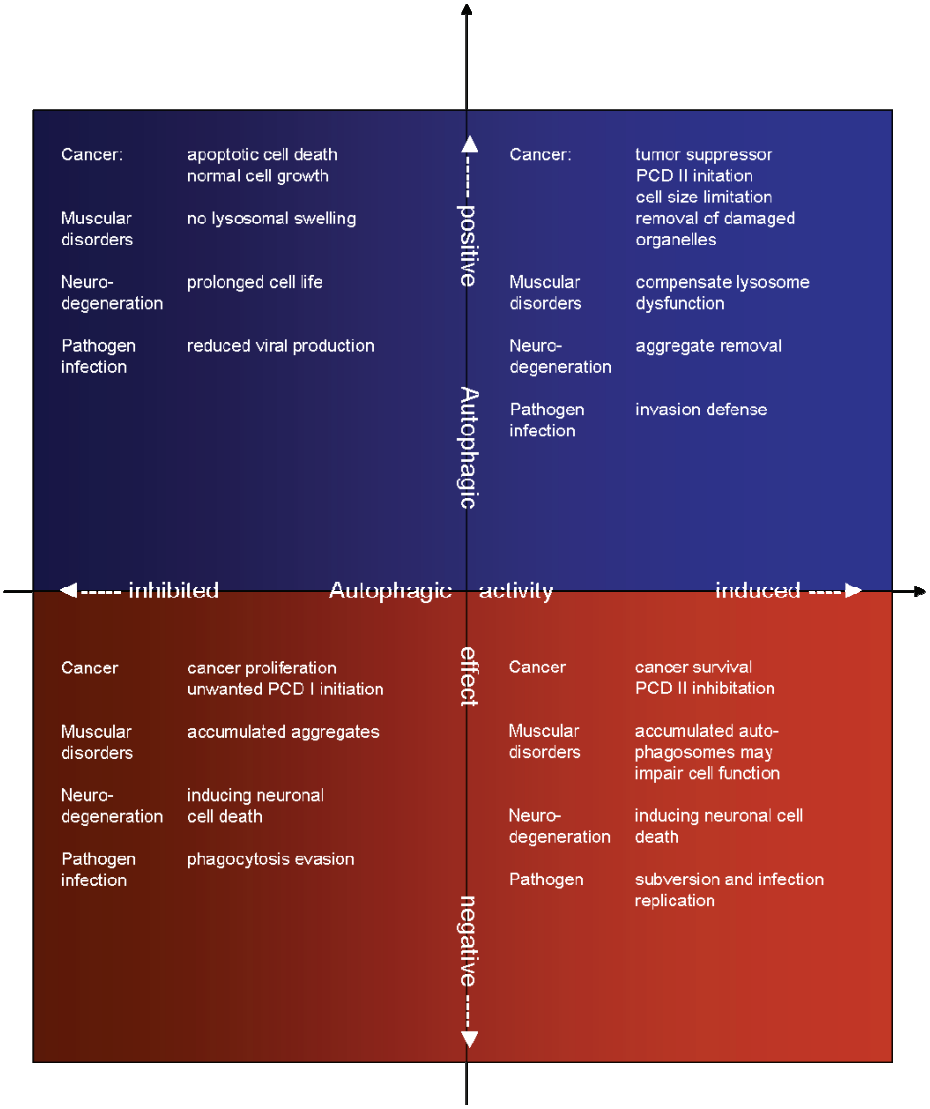

Figure 12. Possible effects of autophagy on certain diseases. Red area encompasses negative effects, blue area positive effects. Right/light side increased/induced autophagy, left/dark side repressed/inhibited autophagy. Adapted from (Shintani and Klionsky, 2004).

therapeutic value of autophagy regulation (Gunn et al., 1977). New developing strategies involve autophagy or lysosome inhibitors as adjuvants to chemotherapy, for example the use of chloroquine, an anti-malaria drug that disrupt lysosomal pH, which was shown to augment therapy of lymphomas (Amaravadi et al., 2007). In cells with defective apoptosis, autophagy inhibitors could sensitize cells to therapy otherwise rendered ineffective due to autophagy-driven cell survival (Jin and White, 2007). Furthermore, ionizing radiation aimed at killing cancer cells leads to abnormal proteins believed to be removed by an active autophagy (Paglin et al., 2001), in which case a supplementary inhibition of autophagy could be therapeutically beneficiary.

The basic tumour-suppressive role of autophagy was first demonstrated by the ability of preneoplastic and neoplastic liver cells, which have a low autophagic activity, to escape autophagic (3-MA-sensitive) cell death caused by amino acid starvation (Schwarze and Seglen, 1985). More recently, an overexpression of Beclin-1 (hAtg6) that led to increased autophagy was shown to be accompanied by a reduced tumorigenicity (Liang et al., 1998; Aita et al., 1999; Liang et al., 1999). The interaction between Bcl-2, an antiapoptotic pro-oncogene, and Beclin-1 might be at the core of the relationship between cancer and autophagy (Pattingre and Levine, 2006). A specific binding domain for Bcl-2 in Beclin-1 hinders the latter protein from taking part in autophagic stimulation as a part of the PI3K complex (Pattingre et al., 2005). There is also evidence for other known tumor suppressors which stimulate autophagy, e.g. PTEN (Arico et al., 2001), DAPk (Gozuacik and Kimchi, 2004) and p53 (Feng et al., 2005), and vice versa, oncogenes that suppress autophagy, such as mTOR (Feng et al., 2005) and class I PI3K (Petiot et al., 2000; Arico et al., 2001). It should, however, be stressed that some basic autophagic activity may be needed even by cancer cells, e.g., for survival under extreme nutrient deprivation (Thompson et al., 2005).

In addition to the elimination or support of intracellular pathogens discusses on page 12, autophagy may play a role in the adaptive **immune response.** The innate and adaptive immune system apparently triggers autophagy by producing tumor necrosis factor (TNF) alpha and interferon gamma (IFN, Gutierrez et al., 2004a). It has been shown that autophagic digestion of endogenously synthesized cytosolic proteins enables their processing for MHC II presentation thus connecting autophagy with adaptive immunity (Paludan et al., 2005; Dengjel et al., 2005). Apparently, the antigens are

presented on the cell surface after exocytosis from amphisomes (Levine and Deretic, 2007). CMA seems to be involved in the presentation of endogenous peptides for MHC class II (Zhou et al., 2005).

A protective effect against viruses by the eIF2a pathway, which induces autophagy has also been observed (Tallóczy et al., 2002). In contrast, some recent studies point to an autophagy-subversive mechanism of ssDNA viruses such as polio virus. Infected cells display vesicles similar to double-membrane delimited autophagosomes, and in these cells siRNA against LC3 and Atg12 reduced both intra- and extra-cellular viral yields (Kirkegaard and Jackson, 2005). Apparently, the poliovirus proteins 2BC and 3A which co-localize with LC3 induce autophagosome-like vesicles to form RNA replication platforms.

The turnover of cytosolic proteins may also prevent accumulation of toxic **aggregate-prone proteins**. Post-mitotic cells such as neurons are particularly susceptible to disruptions in basal homeostasis caused by such aggregates. Aggregation of proteins can be elicited in cells by a number of stimuli (see Fig. 4), some of which are also inducers of autophagy. A role for autophagy has been discovered in Alzheimer's disease (AD; Nixon et al., 2005; Cataldo and Nixon, 1990), Parkinson's disease (PD; Anglade et al., 1997), and in the degradation of proteins with polyglutamine expansions characteristic of Huntington's disease (HD; Ravikumar et al., 2002), and different forms of ataxia (Berger et al., 2006). In amyotrophic lateral sclerosis the anti-oxidant protein (Cu/Zn) superoxide dismutase (SOD1) is mutated and develops toxic aggregates. Monomers of mutated SOD1 are bound to and inhibit the proteasome after ubiquitinylation through the mediation of CHIP (Carboxy-terminal end of HSC70 interacting protein), a cochaperone for HSC70 (Huang and Klionsky, 2002; Urushitani et al., 2004).

Since the UPS may, furthermore, be impaired by protein aggregates (Hyun et al., 2003; Bence et al., 2001), it could be that autophagy serves as a mechanism that cells hold in reserve for the removal of protein aggregates. In this regard α-synuclein can be degraded by both UPS and autophagy (Webb et al., 2003) unless it is mutated (Cuervo et al., 2004; see Fig. 13). So can wild-type huntingtin, but the polyQ expansive variant inhibits UPS function and induces autophagosome formation (Venkatraman et al., 2004; Kegel et al., 2000). Therefore, as aggregates arise, cells could degrade these proteins by

autophagy while preserving the function of the UPS. Several studies now indicate that cells may attempt to compensate for impairments in one form of proteolysis (UPS) by dramatically elevating an alternate form of protein degradation (autophagy) (Keller et al., 2004; Pandey et al., 2007a).

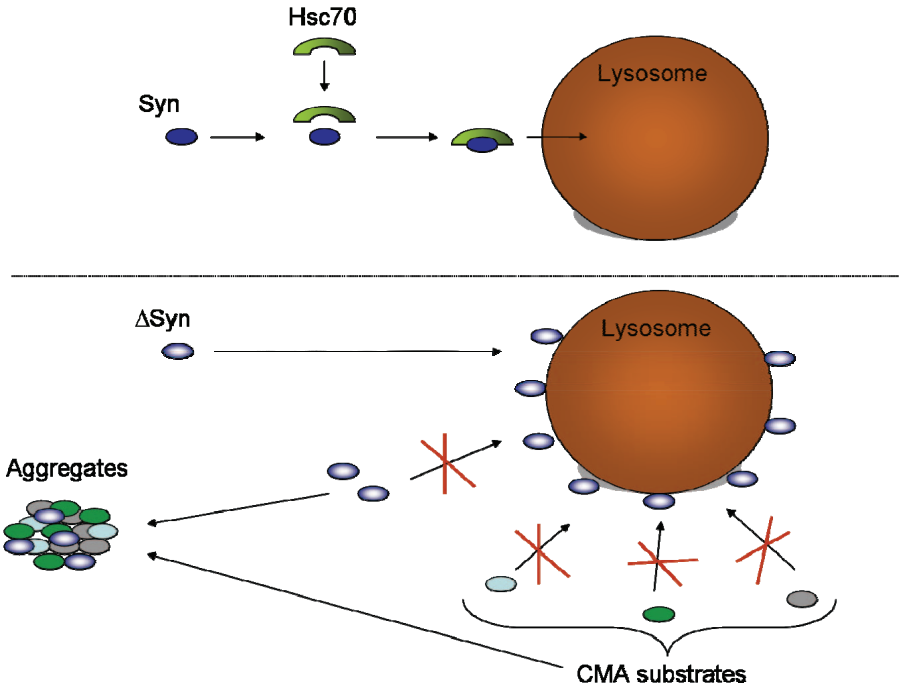

Figure 13. Effect of mutant α-synuclein on chaperone-mediated autophagy. Normal α–synuclein (Syn) is targeted by Hsc70 and delivered to the lysosome through CMA. A mutant form of α–synuclein (ΔSyn), however, binds to lysosome surface and blocks other CMA substrates leading to protein aggregates. Adapted from (Cuervo et al., 2004).

PROTEOMICS

Proteomics encompasses the large-scale identification and study of proteins, particularly their structures and functions. Proteins are vital parts of living organisms, as they are the main components of the physiological pathways of cells. The term "proteomics" was coined to make an analogy with genomics, the study of the genes. The word "proteome" is a portmanteau of "*prote*in" and "gen*ome*". The proteome of an organism is the set of proteins produced by it during its life cycle, or during a distinct internal or external condition.

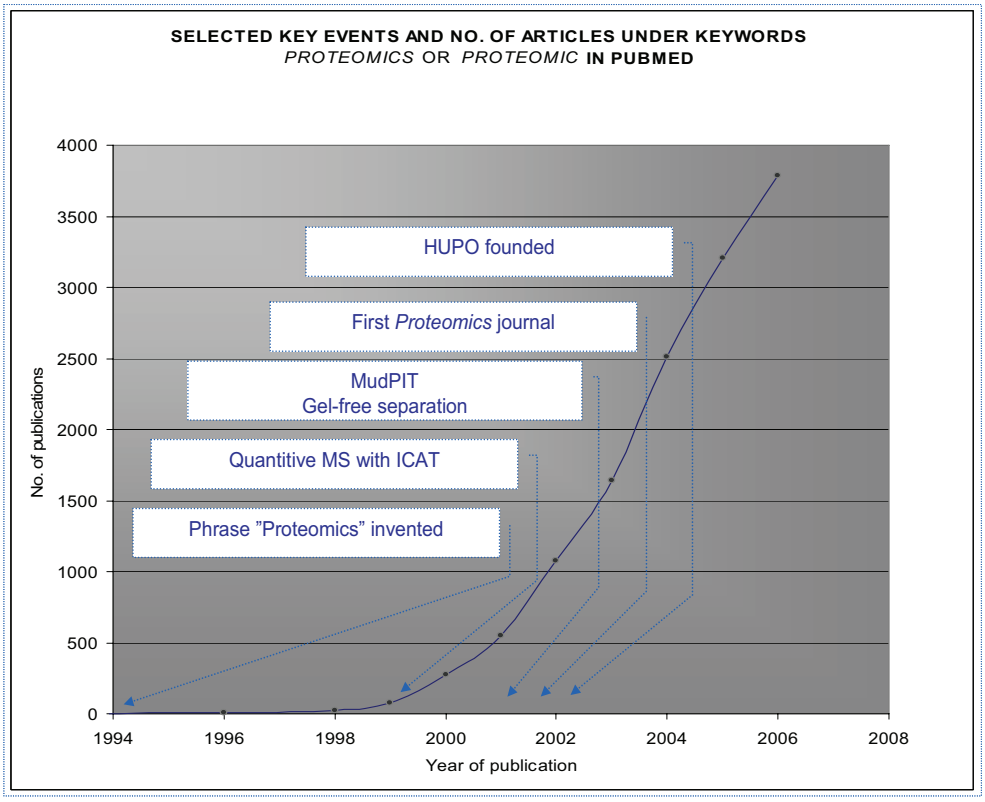

Figure 14. Progression of proteomic research. Indications of expansion in the field by number of articles published per year 1994-2006, some key events displayed.

Proteomics is often considered the next step in the study of biological systems, after genomics. However, it is much more complicated than genomics, mostly because while an organism's genome is rather constant, a proteome differs from cell to cell and constantly changes through its biochemical interactions with the genome and the environment. An organism has radically different protein expression in different parts of its body, different stages of its life cycle and different environmental conditions. Another major difficulty is the complexity of proteins relative to nucleic acids, by way of modifications which may occur on many amino acids.

Although protein analysis and mass spectrometry are well-established fields, it is just over a decade since the first pure proteomics article was published. Since then, a steady increase in research articles on proteomics has been witnessed: from less than a score in 1996 to almost 4000, outpacing the growth in the autophagy field (see Fig. 14). Scientists are very interested in proteomics because it gives a much better understanding of an organism than genomics. First, the level of transcription of a gene gives only a rough estimate of its level of expression into a protein. An mRNA produced in abundance may be degraded rapidly or translated inefficiently, resulting in a small amount of protein. Second, many proteins experience post-translational modification that profoundly affect their activities; for example some proteins are not active until they become phosphorylated. Methods such as phosphoproteomics and glycoproteomics are specialized subfields used to study post-translational modifications. Third, many transcripts give rise to more than one protein, through alternative splicing or alternative post-translational modifications. Finally, many proteins form complexes with other proteins or RNA molecules, and only function in the presence of these other molecules.

At the time of the completion of a rough draft of the human genome, many researchers started to look at how genes and proteins interact to form other proteins. A unexpected finding of the Human Genome Project is that there are far fewer protein-coding genes in the human genome than proteins in the human proteome (an estimate of 20,000 - 25,000 genes versus about 1,000,000 proteins, Lander et al., 2001; Venter et al., 2001). The protein diversity is thought to be due to alternative splicing, controlled protein degradation and ~200 different post-translational modification of proteins – occurring with a much greater variety than previously thought (Tsur et al., 2005). The discrepancy

implies that protein diversity cannot be fully characterized by gene expression analysis, thus proteomics is useful for characterizing cells and tissues.

HUPO

An international collaboration aiming at coordinating proteome research is headed by the Human Proteome Organization (HUPO: http:// www.hupo.org). It was launched to '*foster... international proteomic initiatives to better understand human diseases'*. The main focus for HUPO so far has been to standardize protocols for sample handling and data analysis. These steps are crucial to have under control when aiming to compare results on a world-wide basis. On a larger timescale, the goal for HUPO is to map all proteins in the human body. Specific emphasis has been put on a few select areas by initiating a Human Liver Proteome Project (http://www.hlpp.org), a Human Brain Proteome Project (http://www.hbpp.org) and a Plasma Proteome Project (http://www.ppp.org). In addition HUPO is engaged in establishing PSI- Proteomics Standard Initiative (Hermjakob et al., 2004; Orchard et al., 2003), as well as the Human Antibody Initiative (HAI), which include the Human Protein Atlas (http://www.proteinatlas.org; Uhlen et al., 2005). All research is available for scientists through internet connections and is free of charge.

MASS SPECTROMETERS

Mass spectrometry has since its infancy a century ago grown from a specialized field for isotope determination to a widely used tool for research and development in biomedicine, chemistry and physics (Thomson, 1912). It relies on separation of molecules by mass and has had many different manifestations during its development. As an instrument it consists of five parts; 1) an inlet system, 2) an ion source, 3) a mass analyzer, 4) a detector and 5) a recording system (Fig. 15). A breakthrough was the coupling of a mass spectrometer (MS) to a gas chromatograph in 1957, but even greater usage within the biological world was obtained by the discovery of new ionization techniques in the 1980s by Hillenkamp and Karas (Hillenkamp and Karas, 1990), Tanaka (Hirayama et al., 1987), and Fenn (Fenn et al., 1989). This discovery was rapidly having such a great impact that the latter two of the inventors received the Nobel Prize in Chemistry in 2002. Electronspray ionization (ESI; Fig. 16) and matrix-assisted laser desorption-ionization (MALDI; Fig. 16) enabled the vaporization of biomolecules such as proteins and peptides. For the ionized gas molecules to be analyzed they must be subjected to a magnetic or electric field which separates them according to their specific mass per charge ratio (m/z) in a mass analyzer. The development in mass analyzers would also influence the use of mass spectrometers within protein chemistry. The key parameters for mass analyzers in proteomics are sensitivity, resolution, and mass accuracy. The current

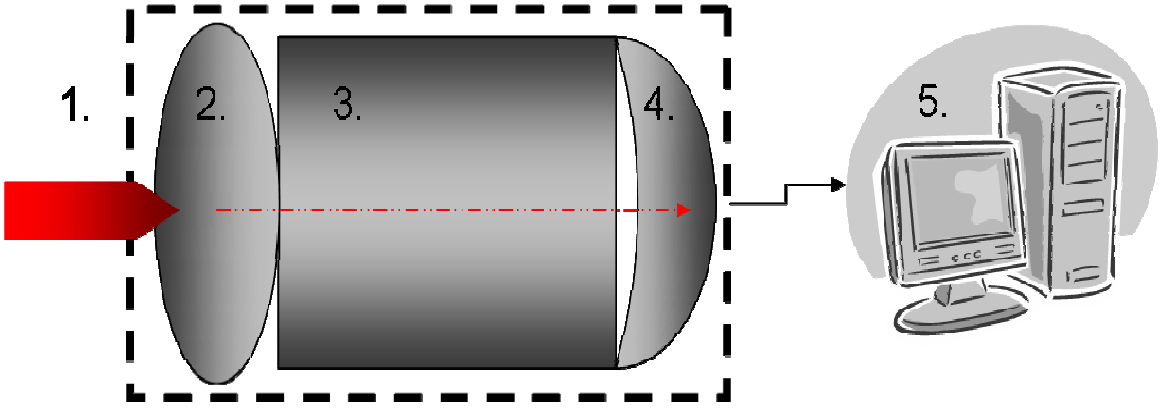

Figure 15. Schematics of a mass spectrometer. 1) Inlet, 2) ion source, 3) mass analysator, 4) detector, 5) recording system/ data analysis. Dotted black line indicates boundaries for mass spectrometer proper, and may be under vacuum conditions, red dotted line represents the trajectory of an ion.

instruments commonly used which fulfill these rigid criteria are ion trap (IT), time-of-flight (TOF), quadrupole (Q) and Fourier transform ion cyclotron resonance (FTICR) instruments. These can also be combined in sequence to expand the possibilities of the instrument, such as QTOF, QIT (another Nobel Prize-winning invention: Paul and Dehmelt in 1989), QQQ, IT-FT and TOFTOF (see Fig. 16 a-f).

In a TOF mass spectrometer, the ions can be deflected with an electrostatic reflector that also focuses the ion beam. Thus, the masses of the ions reaching the second detector can be determined with high precision and these masses can reveal the exact chemical compositions of the peptides, and therefore their identities. Accuracy and sensitivity are the strongest hallmarks for new TOF-instruments.

In a linear quadrupole mass filter, certain combinations of alternating current (AC) and direct current (DC) voltages allow an ion to have a stable trajectory; ions pass through the filter and strike a detector. In the QIT-MS, those ions with stable trajectories are trapped in the volume encompassed by the electrodes. The donut-shaped ring electrode takes the place of one pair of poles. The end-caps replace the other pair of poles. The dimensions of the QIT-MS are described in terms of the distance from the center of the trap to the closest point on each of these electrodes. Although the specifications of commercial QITs are significantly lower than those for an FT-ICR-MS, the QIT-MS is mechanically and operationally much simpler. In addition to the inherent advantages of a trapping instrument, the QIT-MS also boasts relatively simple vacuum requirements, a small footprint, fast analysis, and ruggedness, and it is relatively inexpensive. Furthermore, an ion trap is able to do several MS-stages up to MS^8 (Jin & Glish 1998). New developments include the advent of high capacity three-dimensional and linear versions, as well as the Orbitrap, which have the advantages of the ion trap with the accuracy typical of a TOF-instrument (Schwartz et al., 2002; Hu et al., 2005).

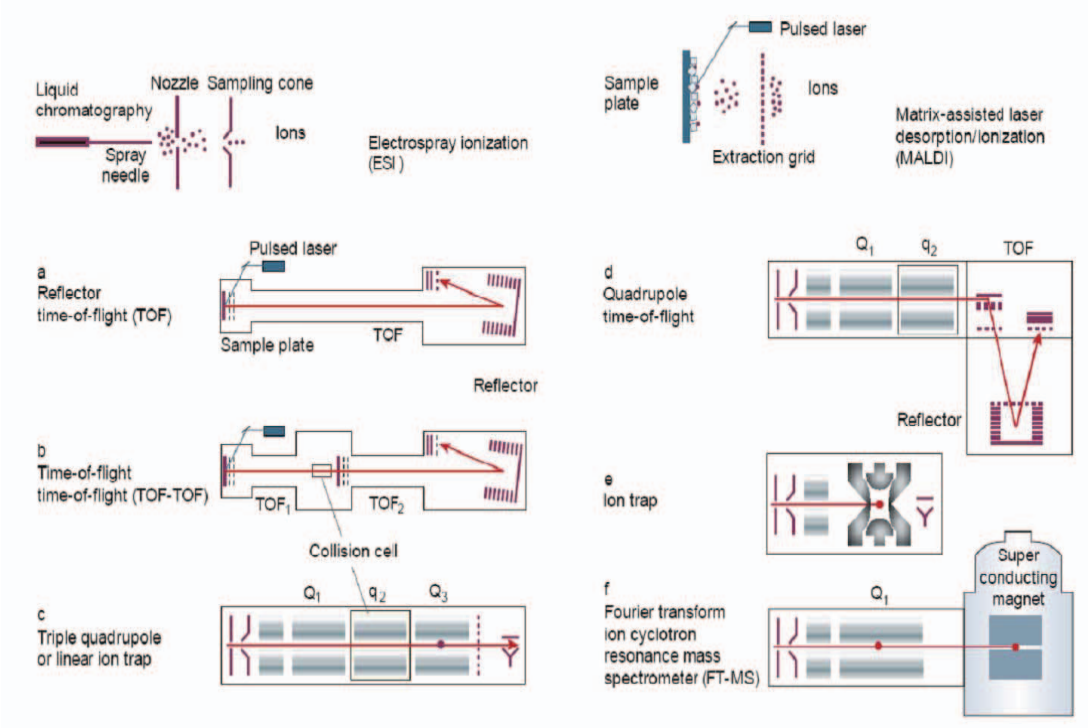

Figure 16 (a-f). Variants of ion sources and mass analyzers. From (Aebersold and Mann, 2003).

PROTEIN CHARACTERIZATION

Current research in proteomics requires first that proteins be resolved, sometimes on a massive scale. Protein separation can be performed using two-dimensional gel electrophoresis which usually separates proteins first by isoelectric focusing and then by molecular weight. Protein spots in a gel can be visualized using a variety of chemical stains or fluorescent markers and quantified by the intensity of their stain (see Fig. 17). Once proteins are separated and quantified, they are identified. Individual spots are cut out of the gel and cleaved into peptides with proteolytic enzymes. These peptides can then be identified by mass spectrometry through peptide mass fingerprinting (PMF). Due to its simplicity, mass accuracy and resolution, MALDI-TOF MS is the most favored

instrument for PMF identification of proteins. The mass list generated from the m/z ratios of peptides in the spectrum of a digested protein is compared with a theoretical database of proteins that have been treated with the same proteinase *in silico*. With high mass

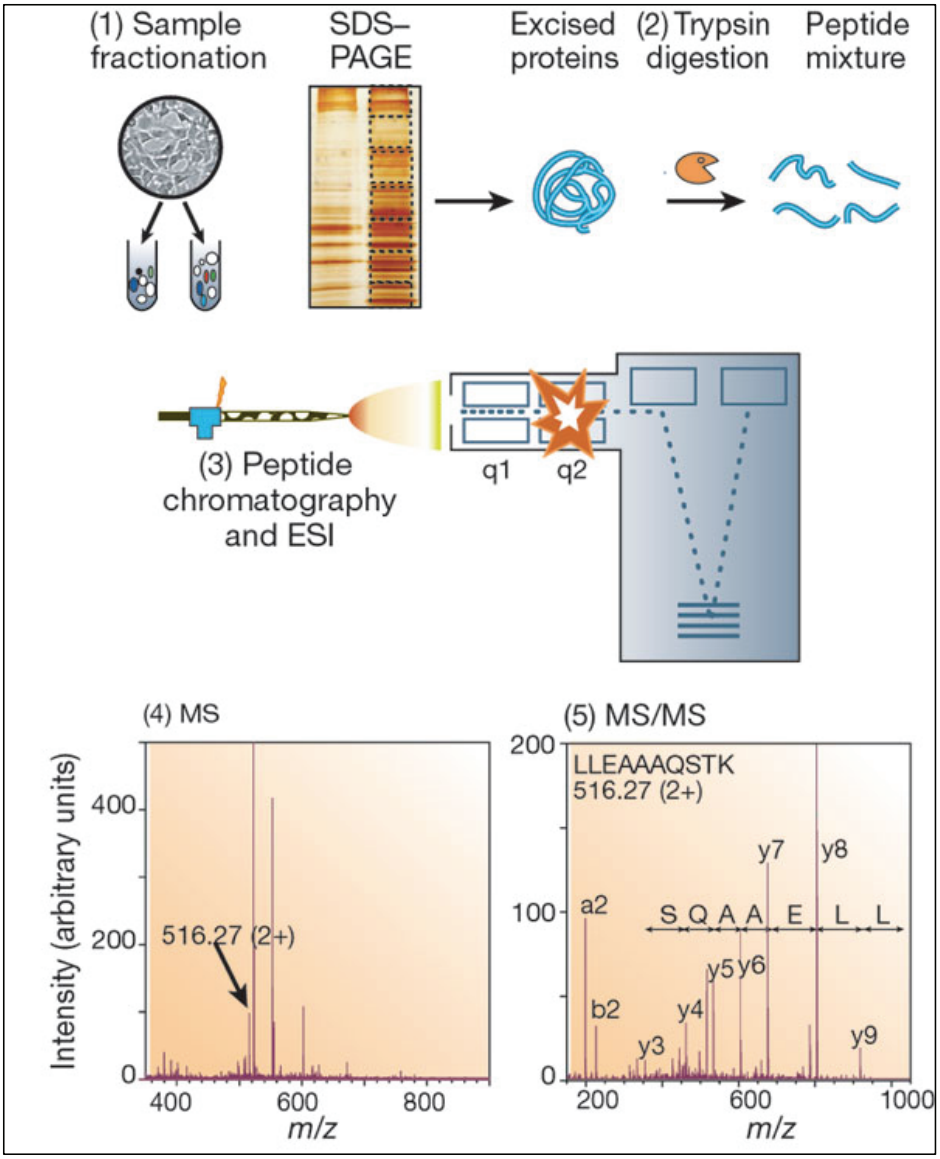

Figure 17. Schematic procedure for identification of proteins by LC-ESI-QTOF mass spectrometry. 1) Subcellular fractionation, and protein separation by SDS—PAGE, 2) Extraction and proteinase treatment to produce peptides, 3) LC MS of tryptic peptides, 4) Selection of parent ion that is processed by CID 5) to get peptide sequence information leading to proptein identification. From (Aebersold and Mann, 2003).

accuracy this procedure enables valid identification of a few proteins in a spot or simple mixture, especially if the organisms have a complete protein database or genome sequence. For more complex protein identification, a tandem mass spectrometer is used, with a sequence of mass analyzers (see Fig. 16). The peptides detected with the first MS are isolated /selected one by one and subjected to collision-induced dissociation (CID- or collisionally activated decomposition -CAD) before entering a new MS, which measures the resulting fragments in a MS/MS spectrum.

This usually leads to peptide backbone cleavage, and sequence information at the amino acid level, giving greater confidence in protein identification (see Fig. 18). Both methods rely on different search algorithms for verifying results and bioinformatics tools with inherent strengths and weaknesses, for instance the popular Mascot search engine from Matrix Science (http://www.matrixscience.com) and ProFound at The Rockefeller University (http://prowl.rockefeller.edu). Another fragmentation technique unique for ion traps called electron transfer dissociation (ETD) which uses fluoranthene anions to

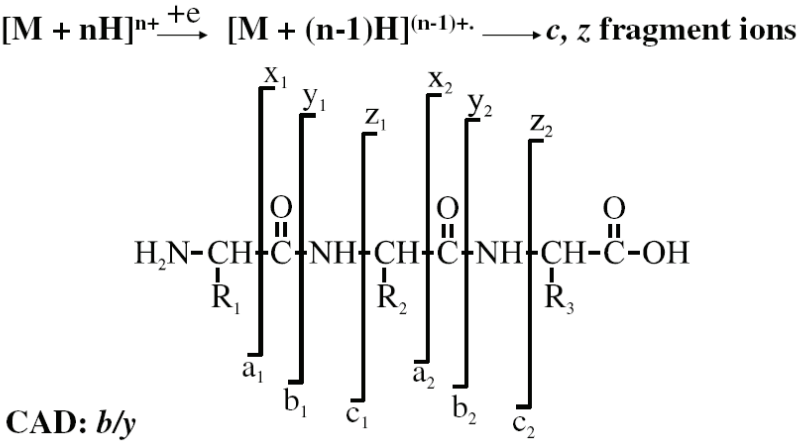

Figure 18: Fragmentation of peptides by tandem mass spectrometry. A charged peptide ion collides with an inert gas and is sequentially cleaved along the peptide backbone. Nomenclature starts with labeling of the cleaved fragments from the amino-terminal end, x,y,z with the charge on the C-terminal fragment, a,b,c with charge retained on the N-terminal fragment. CAD/CID preferentially produces to b/y-ions, ETD with c/z –fragment ion pairs. From (Mikesh et al., 2006).

transfer charged groups in gas phase (Mikesh et al., 2006) results in softer cleavages for labile PTMs, as demonstrated for glycoproteomics (Catalina et al., 2007).

METHODICAL CHALLENGES

One of the main limitations for a comprehensive proteomic analysis has its origin in the vast quantitative differences of the proteins themselves. Most techniques only allow the study of the most abundant proteins, as was illustrated by a systematic 2D-gel analysis of *S. cerevisiae,* where repeated runs revealed that the same 1500 proteins constituting of the major protein mass in the yeast cell would be detected by different basic methods (Gygi et al., 2000). Another demonstration of the concern with differences in abundance appeared in a 3-dimensional separation of rat liver proteins, where only highly expressed proteins were seen (Stevanovic and Bohley, 2001).

PROTEIN SEPARATION

The dynamic range of proteins found in cells and body fluids pleads for a higher order of separation, for instance, the abundance of plasma proteins range from 2 pg/ml (interleukin-6) to 50 mg/ml (albumin) (Lai et al., 2002; Ritchie et al., 1999). All physico-chemical properties of proteins can be used to separate them, preferably in an orthogonal manner. Common preparative separation methods utilized are high performance liquid phase chromatography (HPLC), such as weak or strong cation or anion exchange, hydrophobic interaction and reverse phase (RP) chromatography, as well as capillary electrophoresis, liquid phase isoelectric focusing (LP-IEF) and free-flow electrophoresis (Righetti et al., 2003). Many of these can be combined to further separate complex samples, enabling the discovery of less abundant protein variants, i.e. LP-IEF followed by strong cation exchange and RP-HPLC. It is important that the characteristics of the proteins used for separation display little overlap between each other to maximize separation effect. Another intriguing technique developed in Ghent is the COFRADIC method, where diagonal electrophoresis is applied to simplify complex mixtures (Gevaert et al., 2003; Sandra et al., 2007). This is done by performing a primary HPLC run of a complex mixture of tryptic peptides that is subsequently chemically altered at selected amino acids side chains. The changed characteristics of these peptides makes them shift

retention time when a second, identical HPLC run is performed, enabling the sorting out of modified peptides.

QUANTIFICATION

Quantification of proteins and peptides in complex samples is another potential pitfall of proteomic analysis. Two-dimensional (2-D) gel electrophoresis has been a historically important and currently widely used approach for high-throughput mass spectrometry-based quantification of proteins. By comparing the intensities of stained spots on 2-D gels, the relative amounts of specific proteins can be obtained (Lilley et al., 2002). Its use was further enhanced by introducing fluorescent staining dyes (DIGE), which made comparison easier and less time-consuming, although at a higher cost (Unlu et al., 1997).

Quantification at a larger scale can only be done with mass spectrometers, but there have been obstacles to climb before this can be achieved. Firstly, the ionization techniques are still not completely understood with regards to relationship between analyte inserted and signal measured. Secondly, there is a selectivity in which peptides become ions, and this will be reflected by poor relative quantification (Aebersold and Mann, 2003). The preferred way to overcome this is to apply a stable isotope to half the pool of samples.

This method makes use of the ability of a pair of chemically identical species with different molecular weights due to stable isotopes to be differentiated in a mass spectrometer, an example is isotope-coded affinity tags (ICAT; Gygi et al., 1999; see Fig. 19) and AQUA (Conrads et al., 2002). The ratio of the signals between the isotopes will reflect the relative amount of each of the samples. Different types of isotope-tagging of proteins are specific for sulfhydryl (Gygi et al., 1999), and amino acids (Munchbach et al., 2000). An assay for labeling in vitro have been developed: stable isotope labeling with amino acids in cell culture (SILAC), which incorporates heavy isotopes of carbon to specific amino acids in one set of samples (Ong et al., 2003). SILAC used in studies on Jurkat T-cells stimulated with CD95/Fas/Apo-1 to induce apoptosis demonstrated the efficacy of this technique when compared to silver-stained two-dimensional gels (Thiede et al., 2006).

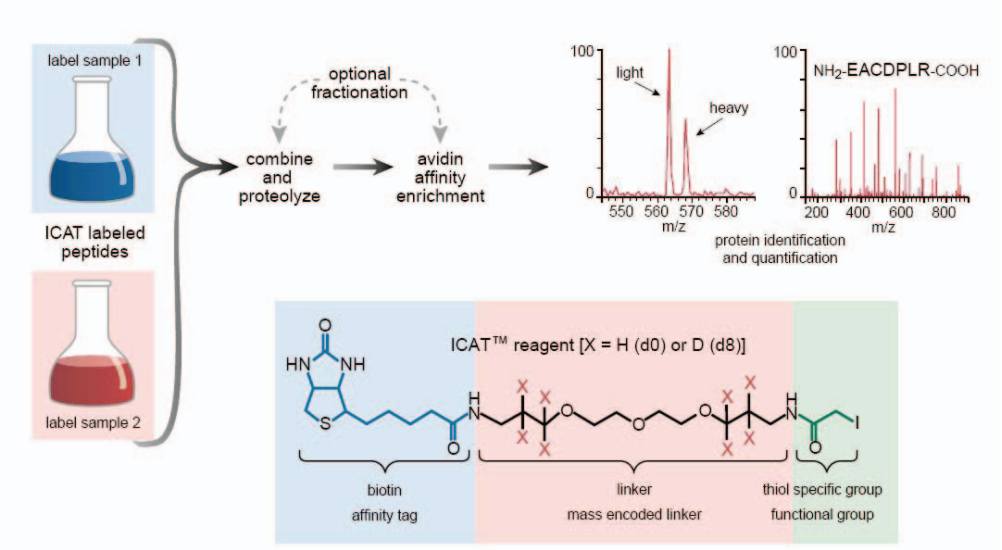

Figure 19. Quantification with ICAT. Two sets of samples are labeled with different isotopes, typically d0 and d8, the latter has 8 hydrogens exchanged for the one mass unit heavier isotope deuterium. After processing, a difference in m/z value of 8 Da can be detected in the mass spectrum and the corresponding sample peaks will have a ratio reflecting the quantitative relationship between the proteins in the samples. ICAT is a biotin-tagged patented reagent.

A alternative method is AQUA that aim at delivering absolute quantization based on synthetic internal peptide standards and selective reaction monitoring (SRM) mass spectrometer analysis (Gerber et al., 2003; Stemmann et al., 2001). The suitability of the method depend on criteria for sample preparation, amount and ease-of-use, for instance the ICAT design only enables pair-wise comparison, while AQUA relies on synthesizing peptides identical or similar to analyte. Hence, quantification of a protein mixture is only possible with preparation and beforehand knowledge of proteins of interest.

Emerging techniques combined with more sensitive instruments have changed the concept of quantification of large-scale proteomics studies, as demonstrated by a serum procedure developed at NIH Bethesda including FT instrumentation, standardized LC-set-up, abundant protein removal and most importantly better normalization algorithms (Wang et al., 2006a), the advent of label-free quantitative proteomics may be near.

Additional assurance might be found in utilizing evidence-based peptide libraries (Roy and Becker, 2007) that could nearly eliminate false positives that have been a pitfall of proteomics on a large scale.

DATA ANALYSIS

The staggering amount of data generated from a proteomic experiment is yet another potentially problem. To handle the output in a stringent and reliable way is crucial to proteomic research, and necessitates bioinformatics knowledge. The main problem with CID-generated peptide sequences is the occurrence of false positives and how to eliminate them without manual handling. Thus, it is important that computer programs use robust and transparent statistical principles to estimate accurate probabilities indicating the likelihood for the presence of a peptide or protein in the sample, such as evaluated for the yeast proteome (Keller et al., 2002; Peng et al., 2003). New standards are emerging for acceptable protein identifications- and m/z values are expected to be presented for external evaluation by peer-reviewer in the event of a publication of mass spectra data and stored in a public accessible database called PRIDE (http://www.ebi.ac.uk/pride; Martens et al., 2005).

TOP-DOWN PROTEOMICS

Analyzing the proteome by digesting the proteins into peptides and subsequential fractionation and identification is termed “bottom-up” approach. In many cases, a “top-down”- approach is wanted, due to the properties of intact proteins is closer to a real situation. A major limitation of top-down methods is that inefficient fragmentation of large analytes by CID often precludes protein identification, and most studies so far have been restricted to high p*I* and/ or low-mass proteins (Louie et al., 1996; Krishnamurthy et al., 1999). However, recent electron capture dissociation (ECD) or electron transfer dissociation (ETD) methods (see Fig. 18), when combined with high-resolution MS (FTICR MS) show promise in alleviating this problem (Lee et al., 2002; Macek et al., 2006). Fragmentation involves electron capture by the analyte followed by free-radical-based backbone cleavage, which is largely sequence-independent. A recent top-down study reported 101 protein identifications from bacterial whole-cell lysates (Patrie et al., 2006), and an analysis of histones illustrates the power of this approach for revealing

complex modifications of proteins and combinatorial regulation of modification sites (Beck et al., 2006).

PROTEOMICS AND DISEASE

One of the most promising developments to come from the study of human genes and proteins has been the identification of potential new drugs for the treatment of disease. Because most drug targets are proteins, an increased knowledge through studies will enable drug discovery, development and clinical practice. The detection of protein profiles associated with disease have been used since the advent of applying two-dimensional gel mapping on clinical samples (Tracy et al., 1982), but the inauguration of rapid mass spectrometers in the field can resolve tens of thousands protein and peptides in disease tissue, for instance cancer material, which could result in better and more rapid diagnosis (Petricoin et al., 2002).

DNA microchips have been used for the past decade (McNeish et al., 2004) and have given valuable insight in disease states on a transcriptional level. Although a recent study indicates good correlation between mRNA expression levels and proteins ($r = 0.71$), there is a consensus that protein microarray chips will give more accurate information of diseases states than genetic microarrays, as well as enabling profiling DNA-empty fluids such as plasma and urine (Shankavaram et al., 2007).

One of the first *protein profiling* of disease tissue using protein microchips was reported by Knezevic and co-workers who analysed tissue from squamous cell carcinomas of the oral cavity on an antibody microarray (Knezevic et al., 2001). Here they used laser-capture micro-dissection to obtain total protein from microscopic subcellular populations, and could demonstrate that expression patterns of several proteins changed with tumor progression in a controlled study. Recently, several other groups also report the use of protein microchips for profiling studies, including tissues such as prostate epithelium (Paweletz et al., 2001), skeletal muscle (Schulz et al., 2006) and the use of surface-enhanced laser desorption/ ionization (SELDI) for pancreatic adenocarcinomas (Melle et al., 2007). SELDI is a technique based on MALDI-principle that uses a target modified to achieve biochemical affinity with the analyte compound, enabling specific binding of substrate of interest (Wright et al., 1999).

Yet another option for protein profiling is scanning of tissues-cross sections called *imaging mass spectrometry*. It involves using laser-thin cryo-sections coated with matrix for MALDI MS analysis and measuring m/z values across the sample to obtain a typical fingerprint- a three-dimensional image of protein location of the tissue at various localisations (Stoeckli et al., 2001). These methods have now become more standardized (Altelaar et al., 2007), and applications range from brain tissue of PD patients (Pierson et al., 2004) to MPTP lesions from mice (Skold et al., 2006).

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