The role of autophagy in clearance of protein aggregates

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Abbreviations

AD	Alzheimer's disease
Alfy	Autophagy-linked FYVE protein
ALIS	Aggresome-like inducible structures
ALS	Amyotrophic lateral sclerosis
AML	Acute myeloid leukemia
AMPK	AMP-activated protein kinase
Ape1	Aminopeptidase I
APL	Acute promyelocytic leukemia
a-Syn	a-Synuclein
Atg	Autophagy-related genes
ATO	Arsenic trioxide
ATRA	All-trans retinoic acid
BafA1	BafilomycinA1
Bchs	Blue Cheese
BD	Binding domain
Bif-1	Bax-interacting factor 1
CMA	Chaperone-mediated autophagy
CML	Chronic myeloid leukemia
CNS	Central nervous system
COPI	Coat protein complex I
Cvt	Cytoplasm to vacoule targeting
DFCP1	Double FYVE domain-containing protein 1
EM	Electron microscopy
ER	Endoplasmic reticulum
ERGIC	ER-Golgi intermediate compartment
ESCRT	Endosomal sorting complex required for transport
GAP	GTPase-activating protein
GST	Gluthathione S-transferase
HD	Huntington's diseases
HDAC6	Histone deacetylase 6
HEK293	Human embryonic kidney 293
HOPS	Homotypic vacoule fusion and protein sorting
Htt	Huntingtin
IBMPFD	Paget disease of bone and frontotemporal dementia
IF	Immunofluorescence
IM	Isolation membrane
IP	Immunoprecipitation
KO	Knock out
LAMP2A	Lysosome-associated membrane protein type-2A
LC3	Light chain 3
LIR	LC3-interacting region
LLPs	Long-lived proteins
LMB	Leptomycin B
LRS	LC3-recognition sequence
mAtg9	Mammalian Atg9

3-MA	3‑Methyladenine
MBP	Maltose-binding protein
MEF	Mouse embyonic fibroblast
MTOC	Microtubule-organizing centre
MVB	Multivesicular body
NBs	Nuclear bodies
NBR1	Neighbour of BRCA1 gene 1
NES	Nuclear export sequence
NFκB	Nuclear factor-kappaB
PAS	Pre-autophagosomal structure
PB1	Phox and Bem 1
PDK1	Phosphoinositide-dependent protein kinase-1
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PI3P	Phosphatidylinositol-3-phosphate
PI3K	Phosphatidylinositol 3-kinase
PI3,4,5P3	Phosphatidylinositol3,4,5-trisphosphate
PKB	protein kinase B
PLZF	Promyelocytic leukemia zinc finger
PML	Promyelocytic leukemia protein
PolyQ	Polyglutamine
PX	Phox homology
RA	Retinoic acid
RARA	Retinoic acid receptor alpha
RISCs	RNA-induced silencing complexes
ROS	Oxygene species
SCA	Spinocerebellar ataxia
ShRNA	Short hairpin RNA
SiRNA	Small interfering RNA
S6K	Ribosomal subunit S6 kinase
SNARE	Soluble N-ethylmaleimide-sensitive fusion protein attachement receptors
SQSTM1	Sequestome 1
SUMO	Small ubiquitin-related modifier
TOR	Target of rapamycine
TORC1/2	TOR complex 1 or 2
TGN	Trans-Golgi network
TP53INP2	Tumor protein 53-induced nuclear protein 2
UAS	Upstream Activation Sequence
UBA	Ubiquitin-associated
ULK1	Unc-51-like kinase 1
UPS	Ubiquitin-proteasome system
UVRAG	Ultraviolet irradiation resistance-associated gene
VCP	Valosin-containing protein
VMP1	Vacuolar membrane protein 1
Wm	Wortmannin
XPO1	Exportin 1
Y2H	Yeast-two hybrid

List of papers

I Clausen TH, * Lamark T, * **Isakson P**, * Finley K, Larsen KB, Brech A, Øvervatn A, Stenmark H, Bjørkøy G, Simonsen A, Johansen T. (**2010**). p62/SQSTM1 and ALFY interact to facilitate the formation of p62 bodies/ALIS and their degradation by autophagy. *Autophagy*. Apr;6(3):330-44.

II Filimonenko M, * **Isakson P**, * Finley KD, Anderson M, Jeong H, Melia TJ, Bartlett BJ, Myers KM, Birkeland HC, Lamark T, Krainc D, Brech A, Stenmark H, Simonsen A, Yamamoto A. (**2010**). The selective macroautophagic degradation of aggregated proteins requires the PI3P-binding protein Alfy. *Mol Cell*. Apr 23;38(2):265-79.

III **Isakson P**, Bjørås M, Bøe SO, Simonsen A. (**2010**). Autophagy contributes to therapy-induced degradation of the PML/RARA oncoprotein. *Blood,* Sep 30;116(13):2324-31.

*These authors contributed equally to this work.

Introduction

In order to survive and maintain proper homeostasis, eukaryotic cells must continuously synthesize new proteins, as well as eliminate unwanted and excessive proteins. It therefore may be unsurprising that under disease conditions, a common cytopathological feature is the appearance of intracellular misfolded aggregate-prone proteins, reflecting an imbalance between protein synthesis and degradation and a disruption in homeostasis. In light of this, knowledge about the molecular mechanisms involved in protein aggregation and degradation is important in order to target various diseases with specific drugs. Autophagy has proven to be important for degradation of aggregate-prone proteins associated with cancer as well as neurodegenerative diseases. How protein disaggregation is achieved and whether survival is solely dependent on autophagy-mediated elimination of aggregates remain to be elucidated. In this thesis I will describe and discuss my contributions to this field of research.

Protein degradation in eukaryotes

The existence of proteolytic activities was detected in yeast as early as 1898 (Hahn, 1898). Later, the discovery of the acidic organelle, the lysosome, by de Duve and coworkers in the 1960s was a major breakthrough (De Duve, 1963). In the following years, it was observed that protein degradation can occur outside lysosomes in an energy-dependent process, (Etlinger and Goldberg, 1977) and the proteasome, found to be located in the cytoplasm and nucleus of all eurokaryotic cells, was later found to be responsible for this non-lysosomal protein degradation (Coux et al., 1996; Hilt and Wolf, 1996; Hochstrasser, 1995; Peters, 1994; Rivett, 1990; Rivett, 1993; Rubin and Finley, 1995). Proteasome-dependent degradation is responsible for selective removal of ubiquitinated, short-lived and aberrant proteins (Heinemeyer et al., 1991) and is therefore often referred to as the ubiquitin-proteasome system (UPS) (Fig.1). As many short-lived proteins have important regulatory functions, proteasome-mediated proteolysis plays a key role in various cellular processes like cell cycle regulation, gene expression and response to oxidative stress (Attaix et al., 2001; Hershko et al., 2000).

Unlike the UPS, autophagy is involved in the removal of long-lived proteins and was originally considered a bulk degradation mechanism responsible for protein turnover during periods of nutrient limitation (Klionsky and Emr, 2000). Although the UPS and autophagy

degrade cytoplasmic proteins under different conditions, both pathways have been implicated in the removal of incorrectly folded or damaged proteins and can target and degrade similar cargoes, such as the neuronal α-synuclein (Webb et al., 2003). Recently, ubiquitin has been implicated to also act as a signal for autophagy, further indicating that the activity of the UPS and autophagy might be connected (referred to as proteolytic cross-talk) (Pandey et al., 2007). For example, autophagy has been found to act as a compensatory degradation system when the UPS is blocked (Korolchuk et al., 2009). In contrast, blocking autophagy seems to inhibit the function of the UPS as well, through sequestration of ubiquitinated proteins by the autophagy receptor p62 (Rubinsztein et al., 2009). However, the mechanisms underlying this proteolytic cross-talk are not clear and much remains to be learned about the selectivity of a particular protein substrate for degradation in a particular pathway.

Autophagy

General function and mechanism

The term autophagy (*auto phagin* from greek meaning Self-Eating) was first introduced in the mid-sixties by Christian De Duve to describe digestion of endogenous material within cells. Autophagy is an evolutionarily conserved process in eukaryotes whereby intracellular cytoplasmic material is delivered to lysosomes for degradation. There are three major types of autophagy in eukaryotes: chaperone-mediated autophagy (CMA), microautophagy and macroautophagy (Fig.1).

Chaperone-mediated autophagy (CMA) has so far only been characterized in higher eukaryotes. It involves a direct translocation of cytosolic proteins across the lysosomal membrane. Proteins containing a pentapeptide motif (KFERQ or similar sequences) are recognized by Hsc70, which facilitates protein unfolding and delivery of the protein to the CMA receptor lysosome-associated membrane protein type-2A (LAMP2A), a lysosomal membrane protein. LAMP2A functions as a receptor and pore for translocation of the protein across the lysosomal membrane (Chiang et al., 1989; Cuervo, 2010; Cuervo and Dice, 1996; Cuervo et al., 1995; Dice et al., 1990).

Microautophagy, characterized mainly in yeast, is the process by which cytoplasmic material become sequestered through a direct invagination of the vacuole membrane (the

yeast equivalent of the lysosome) (Ahlberg and Glaumann, 1985; Xie and Klionsky, 2007). It is generally thought that microautophagy accounts for the basal rate of intracellular protein degradation in normal non-stimulated conditions (Cuervo and Dice, 1998), though later discoveries in yeast showed that this pathway can also be induced by various conditions (Dubouloz et al., 2005). Microautophagy has the capacity to sequester large structures such as entire organelles through both selective and non-selective mechanisms. Moreover, certain cargo, e.g. mitochondria can be degraded both by micro- and macroautophagy, but how this selectivity is regulated is not known (Lemasters, 2005). Very recently, a microautophagy-like process was characterized in eukaryotic cells (Sahu et al., 2011), involving delivery of cytosolic materials to the intraluminal vesicles of late endosome/multivesicular bodies (MVBs) in an ESCRT (Endosomal sorting complex required for transport)-dependent manner.

Macroautophagy, hereafter referred to simply as autophagy, is the best characterized form of autophagy. It was first identified and characterized in mammalian cells by electron microscopy (EM) studies (Seglen et al., 1986). This process involves nucleation of a membrane, named the phagophore or isolation membrane (IM), which expands to form a double-membrane vesicle called the autophagosome. The autophagosome either fuses directly with the lysosome or with endocytic vesicles, generating an amphisome, that eventually fuses with the lysosomal compartment resulting in the formation of an autolysosome where the sequestered material becomes degraded by lysosomal hydrolases.

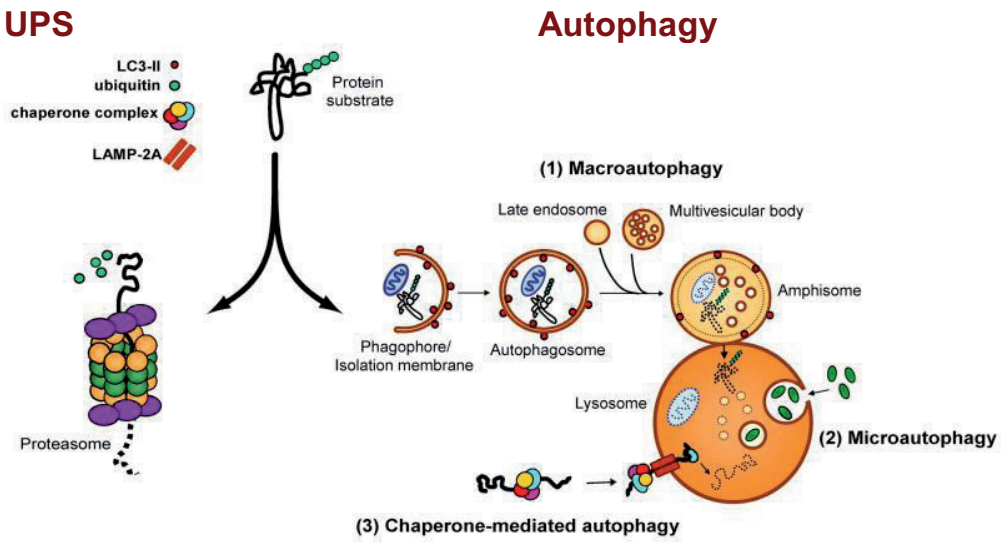

Fig.1. In eukaryotic cells, intracellular proteins can be degraded via two main proteolytic systems: the ubiquitin-proteasome system (UPS) and the lysosomal autophagy pathway. Delivery of cytoplasmic material to the lysosomes by autophagy can occur by three different pathways; (1) macroautophagy, which involves the sequesteration of cytoplasmic components by a membrane forming an autophagosome, which fuses with the lysosome (2) microautophagy, which invovles engulfement of small volumes of cytoplasma by a direct invagination of the lysosomal membrane (3) chaperone-mediated autophagy (CMA), a process by which soluble substrates associated with a specific chaperone complex are translocated into the lysosome through the LAMP-2A lysosome receptor. Proteins tagged with a polyubiquitin chain can be targeted by both the UPS and autophagy. Adapted from (Nedelsky et al., 2008).

Autophagy was initially characterized as a survival mechanism induced in response to nutrient deprivation (starvation), leading to production of metabolites required to synthesize essential molecules and ATP that are needed for cell survival (Kuma et al., 2004; Lum et al., 2005). Starvation-induced autophagy is considered a nonspecific process, involving random sequestation of cytoplasmic components (Kopitz et al., 1990). Autophagy is also induced by other physiological stimuli, such as growth factors and oxidative stress, as well as by pathogen invasion. However, autophagy can also proceed at basal levels, performing important quality control functions by selectively removing damaged organelles, pathogenic inclusions or invasive bacteria. Cargo-specific names have been given to describe these

various forms of selective autophagy (e.g. mitophagy and pexophagy to describe degradation of mitochondria and peroxisomes, respectively) as illustrated in Fig.2 and summarized in table 1 (Klionsky et al., 2007; Mizushima et al., 2008). The cytoplasm to vacoule targeting (Cvt) pathway (Fig.2, table1) is a form of selective autophagy, described in yeast, which involves delivery of lysosomal hydrolases to the yeast vacolue (Harding et al., 1995).

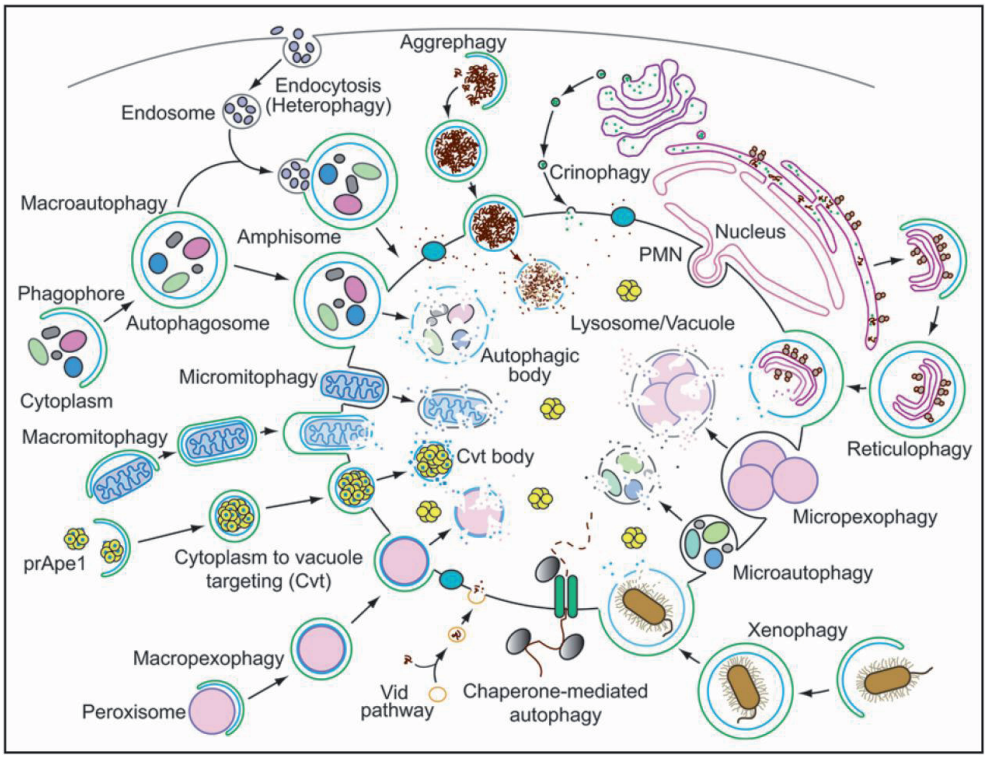

Fig.2. Summary of lysosomal degradative pathways of yeasts and higher eukaryotes, showing that a particular protein or organelle can be delivered to the vacoule/lysosome for final degradation by a variety of specific pathways. See also table 1 for details. Adapted from (Klionsky et al., 2007).

The identification of about 30 autophagy-related (Atg) genes in the last decade, using yeast genetic screens, have made it possible to elucidate the core molecular mechanisms of autophagy (Harding et al., 1995; Thumm et al., 1994; Tsukada and Ohsumi, 1993). Many of the Atg genes encode proteins that are required for autophagy and are conserved from yeast to

man (Klionsky et al., 2003), although there are some rare exceptions such as the mammalian-specific Atg101 which is not found in yeast (Hosokawa et al., 2009b). Most Atg proteins are localized to the spot(s) in the cytosol where the autophagosome forms, the pre-autophagosomal structure (PAS) in yeast and the expanding phagophores/IMs in mammals (Suzuki et al., 2001). The Atg proteins predominantly make up the core autophagic machinery which is required for autophagosome formation. These include four major multi-protein complexes: (1) the autophagy-specific class III phosphatidylinositol 3-kinase (PI3K)/Vps34 complex (containing the catalytic Vps34 and regulatory Vps15/p150 subunits, as well as Atg6/Beclin-1 and Atg14), (2) the Atg1/ULK-1 kinase complex (also including Atg13, FIP200, Atg101, Atg17, Atg29 and Atg31), (3) the Atg12 and Atg8 conjugation systems (Atg7-Atg10-Atg12-Atg5- Atg16 and Atg4-Atg7-Atg3-Atg8, respectively) and (4) the Atg9 cycling pathway. These protein complexes, as well as proteins needed for autophagosome maturation, involving fusion of the autophagosome with endocytic vesicles, are described in more detail below.

The discovery of the Atg genes initiated an exciting era in research and has provided a growing understanding of the complex process of autophagy and its role in various physological and pathological conditions, including starvation responses, anti-aging, immunity, differentiation, lipid metabolism, development and protection from cell death (Mizushima, 2005). Furthermore, autophagy is associated with various diseases, including cancer, neurodegeneration (clearance of intracellular aggregate-prone proteins,) and infectious diseses (removal of pathogens). However, there is still much to be learned about the mechanism underlying autophagy, a process that can be broken down into several steps; (A) induction, (B) nucleation, (C) expansion and (D) maturation. Our current understanding of each step will be described in more detail below.

A. Induction

Insufficient autophagy, as well as excessive levels may be harmful (Komatsu et al., 2007; Kuma et al., 2004), therefore it is unsuprising that autophagy is a tightly regulated process in all eukaryotes, likely involving a complex network of various stimulatory and inhibitory inputs. Autophagy can be induced by several conditions, including nutrient limitation (starvation), energy depletion and lack of growth factors (insulin/IGF) (Yang and Klionsky, 2010). It can also be induced by various cellular stressors as heat and oxidative stress. It is still unclear how many of the major signaling pathways that regulate autophagy directly influence the autophagy machinery, although it is clear that the mammalian target of rapamycin (m*TOR*) plays a major role in integrating various signals. Inhibition of mTOR leads to activation of autophagy likely through the release of mTOR-mediated inhibitory phosphorylation of Atg1 orthologues (ULK-1/2) in mammalian cells (Ganley et al., 2009; Hosokawa et al., 2009a; Jung et al., 2009). In addition, PI3Ks have been found to be important regulators of autophagy (Blommaart et al., 1995; Lindmo et al., 2008). The class I PI3K inhibits autophagy through activation of mTOR, whereas the activity of the class III PI3K/Vps34 is required for induction of autophagy (Lindmo and Stenmark, 2006). Identification of the inhibitory action of 3‑methyladenine (3-MA) (Seglen and Gordon, 1982), provided the first evidence for a regulatory effect of protein kinases and phosphatases in autophagy (Holen et al., 1992) and it was later found that 3-MA is a PI3K inhibitor (Lindmo and Stenmark, 2006).

1. Class I PI3K and mTOR signaling

mTOR is a nutrient and energy-sensing kinase that coordinates cell growth, cell-cycle progression and protein synthesis (Klionsky and Emr, 2000). mTOR is activated by signaling from the insulin receptor, insulin-receptor substrates 1 and 2 (IRS1/2), phosphoinositide-dependent protein kinase-1 (PDK1) and protein kinase B (PKB)/AKT (Zoncu et al., 2011) (Fig.3). mTOR activity is controlled by the heterodimer TSC complex, TSC1-TSC2, which acts as a GTPase-activating protein (GAP) for the small GTPase Rheb (Ras homolog) (Zoncu et al., 2011). mTOR is stimulated by the active GTP-bound form of Rheb, thus the TSC complex acts to inhibit mTOR function (Huang and Manning, 2008). Activation of insulin receptors activates PKB which phosphorylates and inhibits TSC1-TSC2 complex, leading to the activation of mTOR signaling and inhibition of autophagy (Meijer and Codogno, 2004).

TOR forms two functionally distinct protein complexes, TOR complex 1 and 2 (TORC1 and TORC2), where TORC1 is primary involved in regulation of autophagy (Loewith et al., 2002).

mTOR targets include a number of proteins of the translational machinery. In particular, phosphorylation and activation of 4E-BP1 and ribosomal subunit S6 kinase (S6K) are stimulated by serum, insulin and growth factors in an mTOR-dependent manner. p70S6K exerts a negative feedback on mTOR signalling by phosphorylating IRS1 to downregulate insulin signaling, leading to a decline of phosphatidylinositol 3,4,5P3 (PI3,4,5P3), an inhibitor of autophagy (Yang and Klionsky, 2010). This feedback regulation may ensure a basal level of autophagy that is important for homeostasis even under nutrient rich conditions (Klionsky et al., 2005).

Recently, mammalian Atg13, FIP200 (Atg17), ULK1 and ULK2 have been identified as direct targets of mTOR (Chan et al., 2009; Hara and Mizushima, 2009; Hara et al., 2008; Hosokawa et al., 2009a; Jung et al., 2009; Mizushima, 2010). The yeast Atg1 kinase and its mammalian homologue ULK1 function downstream of TOR1/mTOR to induce autophagosome formation. During nutrient-rich conditions, active mTOR is associated with the ULK1 complex and can thereby phosphorylate ULK1, FIP200 and Atg13, acting as a negative regulator of the ULK1 complex and autophagy. In contrast, absence of amino acids or treatment with the drug rapamycin (Jung et al., 2010) stimulate autophagy through inactivation of mTOR, and thereby activation of the Atg1/ULK1 complex leading to nucleation of autophagic membranes (Chan and Tooze, 2009). Recently, ULK1 was observed to become activated upon glucose starvation in an AMP-activated protein kinase (AMPK) dependent manner (Kim and Guan, 2011). Energy depletion leads to activation of AMPK, which mediates phosphorylation and activation of TSC1–TSC2, leading to inactivation of TOR and induction of autophagy. ATP has been shown to be required for both the autophagic sequestration step (Plomp et al., 1987) and for the fusion of autophagosomes with other organelles (Reunanen and Nykanen, 1988).

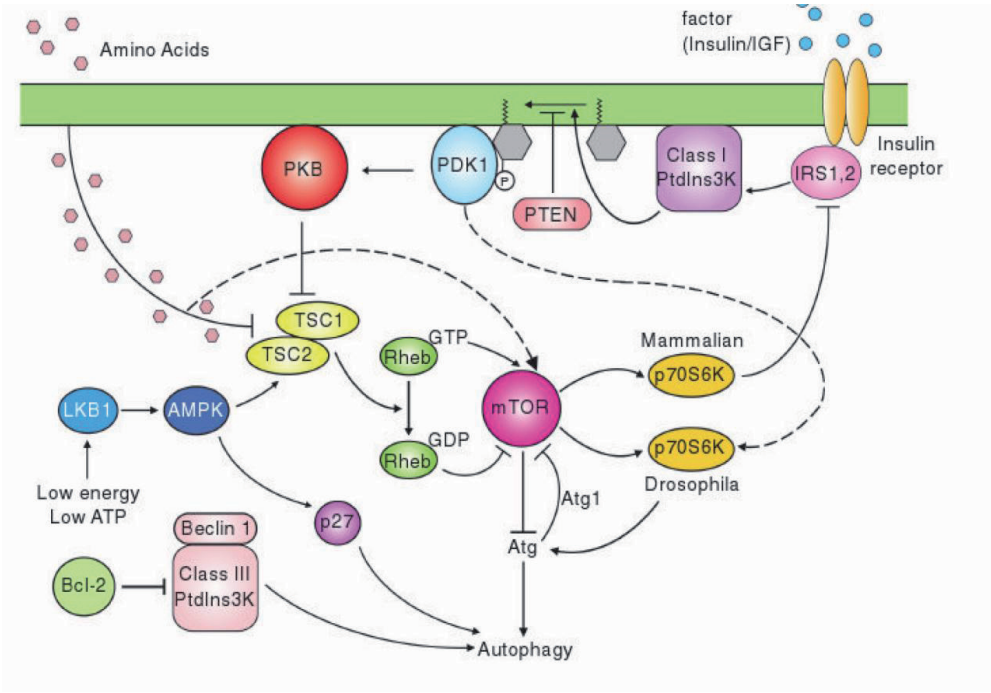

Fig.3. Regulation of autophagy in mammalian cells. Autophagy occurs at a basal level and can be induced in response to environmental signals including nutrient, energy deprivation and also microbial pathogens. Insulin regulates growth by binding to the insulin receptor, causing activation of class I PI3K signalling and phosphorylation of plasma membrane lipids. The regulation of autophagy is complex and far from understood. The best characterized regulatory pathway includes class I PI3K and TOR, which act to inhibit autophagy. The class III PI3K/Vps34 is needed for activation of autophagy. TOR activity is probably regulated in part through feedback loops to prevent insufficient or excessive autophagy. 3-methyladenine (3-MA) and wortmannin (Wm) also inhibit class I phosphatidylinositol 3-kinases (PI3K), but the overall effect of these compounds is a block in autophagy (because they inhibit the downstream class III PI3K that produces PI3P, which is needed for autophagy). Adapted from (Klionsky and Yang, 2009).

2. Clas III PI3K and Atg6/Beclin1 signaling

The induction and nucleation of autophagy additionally requires the class III PI3K, an ortholog of the only PI3K found in yeast, Vps34 (commonly referred to as Vps34 hereafter) (Odorizzi et al., 2000). In the context of autophagy, Vps34 forms two distinct complexes, complex I and II, where complex I functions in autophagosome formation, whereas complex II plays a role in autophagosome maturation and endocytosis (described later). The two

complexes have three common components, p150 (the homolog of yeast Vps15), Vps34 and Beclin-1 (the homolog of yeast Vps30/Atg6) (Kihara et al., 2001; Yue et al., 2003). In addition, complex I contains Atg14L (mammalian homologue of yeast Atg14), whereas yeast Vps 38 (and the putative mammalian homologue ultraviolet irradiation resistance-associated gene, UVRAG) is specific for complex II. In yeast, Atg14 can direct the Vps34 complex I to the PAS (Obara et al., 2006) and the mammalian ortholog Atg14L have been found to recruit at least a subset of Vps34 to the sites of autophagosome nucleation, called omegasomes, in the ER (Itakura et al., 2008; Matsunaga et al., 2010). The regulatory kinase Vps15/p150 is required for Vps34 membrane association and activity (Stack et al., 1995). The Vps34 complex I can be activated by various Beclin-1 interacting proteins, UVRAG, AMRA-1 and Bax-interacting factor 1 (Bif-1), and inhibited by another Beclin-1 interacting partner, Bcl-2 (B-cell lymphoma 2) (Itakura et al., 2008; Liang et al., 2006; Takahashi et al., 2007; Zhong et al., 2009). Interestingly, Vps34 can activate mTOR during amino acid rich condition leading to inhibiton of autophagy in mammalian cells (Byfield et al., 2005; Nobukuni et al., 2005). Thus, depending on its binding partners, Vps34 is subjected to different modes of regulation, leading to activation or inhibition of autophagy, but how this is regulated is not understood. Disruption of the association of Bcl-2 and Bcl-XL with Beclin-1, however, seems to be critical for the activation of the Vps34 complex I and stimulation of autophagy (Pattingre et al., 2005; Wei et al., 2008).

B. Nucleation

Ever since the discovery of autophagy, the precise origin of the autophagosomal membranes has been under intense debate. In mammalian cells, autophagy is initiated by the formation and elongation of the phagophore/isolation membrane (IM). In yeast, the IM arises from the PAS (Suzuki et al., 2001) whereas the IM in mammalian cells has been suggested to arise in different areas in the cytoplasm.

Four models have been proposed for the autophagosome formation: (1) *de novo* delivery of lipids either by lipid transfer proteins to sealed bilayers or to open bilayers that are stabillized by a putative capping protein; (2) vesicular trafficking through heterotypic vesicle fusion; (3) cisternal assembly through homotypic vesicle fusion; and (4) membrane remodelling/extension (Longatti and Tooze, 2009). Models 2 and 3 occur via a *maturation* process, involving vesicle-mediated transport of membrane from pre-existing membranes,

whereas model 4 propose that the IM is derived directly from a compartment, such as the endoplasmic reticulum (ER).

In the *maturation* model various candidates have been proposed to be the origin of the phagophore membrane including Golgi structures (Locke and Sykes, 1975), endocytic compartments (Dunn, 1990) and mitochondria (Luo et al., 2009). Recently, the plasma membrane was also proposed to contribute to the formation of the IM (Ravikumar et al., 2010).

Several recent studies provide good evidence for a role of the ER in autophagosome formation, suggesting that the IM originates from membranes of pre-existing organelles. Named after their shape, the omegasomes are PI3P-positive structures that form from the ER upon induction of autophagy. The PI3P effector DFCP1 (double FYVE domain-containing protein 1) localizes to these spots in the ER and is therefore used as an omegasome-marker. Omegasomes are also found to be positive for the core autophagy proteins Atg8 and Atg5, and newly formed autophagosomes seem to escape from these structures (Axe et al., 2008). It has also been shown that the ER has a similar thin type of membrane (6-7 nm) and a number of ER proteins have been identified both on the IM and the autophagosome (Ueno et al., 1991). Furthermore, studies by electron microscopic tomography have revealed that ER is associated with the IM in mammalian cells (Hayashi-Nishino et al., 2010; Yla-Anttila et al., 2009).

In mammalian cells, the two transmembrane proteins mAtg9 (Noda et al., 2000; Yamada et al., 2005) and vacuolar membrane protein 1 (VMP1) (Dusetti et al., 2002) are proposed to contribute to the formation of the autophagosomes.

VMP1 localizes predominantly to the ER. Overexpression of VMP1 was shown to induce autophagosome formation even under nutrient-rich conditions and this seems to depend on its binding to Beclin-1. Recently, a novel VMP1-interacting protein, called tumor protein 53-induced nuclear protein 2 (TP53INP2), was shown to be essential for autophagy (Nowak et al., 2009). It translocates from the nucleus to autophagosomes upon induction of autophagy, where it binds to one of the mammalian homologs of yeast Atg8, microtubuli-associated protein 1 (MAP1) light chain 3 (LC3). Therefore, this protein was proposed to act as a scaffolding protein recruiting other Atg proteins to the IM.

In yeast, Atg9 is transported to the PAS from a compartment in close proximity to the mitochondria (Mari et al., 2010) and as Atg9 is required for PAS formation, it is likely that it mediates transport of at least part of the lipids required to create this structure (Longatti and

Tooze, 2009; Nazarko et al., 2005; Reggiori et al., 2005b; Reggiori et al., 2004). The sorting mechanism for the shuttling of Atg9 from mitochondria to the PAS is unknown, but seems to require actin (Reggiori et al., 2005a). mAtg9 however cycles from the trans-Golgi network (TGN) to a peripheral Rab7-positive endosomal pool and is present on autophagosomes after starvation (Young et al., 2006), in a ULK1-dependent manner (Chan et al., 2007; Webber et al., 2007). The yeast ULK1 homologue, Atg1, is also responsible for the recruitment of other Atg proteins to the PAS (Cheong et al., 2008; Kawamata et al., 2008). The role of Atg1/ULKs in autophagy induction has not yet been properly characterized, however, the activity of ULK kinase increases during starvation, and kinase-dead mutants of ULK exert a dominant-negative effect on autophagosome formation (Hara et al., 2008). Atg13 is a phosphoprotein that becomes dephosphorylated when mTOR is inactivated and can bind to ULK1/2, which leads to their interaction with FIP200. It was observed that ULK and FIP200 localize to the IM, suggesting that the complex play an essential role in the early stages of autophagosome formation (Hara et al., 2008). Interestingly, ULK1/2 and mAtg13 has been observed to form a tight association with membranes (Chan et al., 2009).

The Vps34-associated protein BIF-1 has been shown to be required for autophagy and to localize to autophagic membranes. BIF-1 has a BAR domain, known to facilitate membrane curvature, suggesting that BIF-1 mediates bending of autophagic membranes (Itoh and De Camilli, 2006). Interestingly, BIF-1 was proposed to interact with mAtg9 (Takahashi et al., 2008b).

Vps34 phosphorylates phosphatidylinositol (PI) at the 3-position of the inositol ring to create phosphatidylinositol-3-phosphate (PI3P) (Lindmo and Stenmark, 2006; Simonsen and Tooze, 2009). The role of PI3P in autophagy is not clear, although it is likely to recruit PI3P-binding proteins that are important for autophagosome formation. PI3P has been found to recruit PX (phox homology) and FYVE (conserved in Fab1, YOTB, Vac1 and EEA1) domain-containing proteins such as Atg20, Atg24 and Atg13 (Gillooly et al., 2001; Nice et al., 2002; Obara et al., 2008a; Wishart et al., 2001). In yeast, PI3P is preferentially localized to the inner autophagosomal membrane (Obara et al., 2008a; Obara and Ohsumi, 2008), where it recruits Atg18 (orthologue of mammalian WIPI-1) and Atg2 (Obara et al., 2008b). It has been shown that Atg18 associates with PI3P directly (Obara et al., 2008b). Moreover, a siRNA screen revealed that the lipid phosphatase Jumpy affects autophagy (Vergne et al., 2009). Jumpy can dephosphorylate PI3P and thereby inhibit autophagy by acting at an early stage on autophagosome formation.

C. Expansion

The process of membrane elongation and completion to form the autophagosome requires the two ubiquitin-like proteins Atg12 and Atg8 and their conjugation systems (Fig.4) (Ichimura et al., 2000; Mizushima et al., 1998). Several Atg8 homologues exist in mammalian cells, generally divided in two families, the MAP1-LC3 family and the GABARAP family. MAP1-LC3B is the best studied homolog, and will be herein referred simply as LC3. Both conjugation systems are evolutionary conserved from yeast to humans. In brief, Atg12 and LC3 are activated by an E1-like enzyme (Atg7) and conjugated by an E2-like enzyme (Atg10 and Atg3, respectively) to Atg5 or phosphatidylethanolamine (PE), respectively (Mizushima et al., 1998; Ohsumi and Mizushima, 2004).

The Atg12-Atg5 conjugate associates with a small coiled-coil membrane-bound protein, Atg16L (an ortholog of yeast Atg16), to form an Atg12-Atg5-Atg16L complex (Mizushima et al., 2003). Atg16L directs the Atg12-Atg5 complex to the IM, and this complex has been proposed to work in an E3-like fashion for the conjugation of LC3 (Hanada et al., 2007; Mizushima et al., 2001). The Atg12–Atg5–Atg16L complex also determines the sites of LC3 lipidation (Fujita et al., 2008).

Before conjugation to PE, the carboxy-terminal residue of LC3 is cleaved off by the cystein protease Atg4, exposing a critical Glycine residue at the C terminus (Kirisako et al., 2000) which become covalently conjugated to PE (Ichimura et al., 2000). Soluble LC3 is called LC3-I whereas the membrane bound, autophagosome associated form is referred to as LC3-II. LC3-II is inserted into both leaflets of the forming autophagosome. Whereas LC3-II on the outer leaflet is retrieved through delipidation by Atg4, LC3-II on the inner leaflet remains bound to autophagic membranes throughout the pathway and thus serves as an important biomarker of autophagy (Kabeya et al., 2000; Kirisako et al., 2000; Klionsky et al., 2008). LC3 can mediate membrane tethering and may contribute to autophagosome membrane expansion. LC3 might also assist the final fusion to close the autophagosome, a poorly understood step (Nakatogawa et al., 2007).

Recently, autophagosome-like structures were found in Atg5 knock out mouse cells indicating that an Atg5- and Atg7-independent form of autophagy may exist (Nishida et al., 2009). Exposure to etoposide was an apparent trigger for this event. It is not known to what extent this form of autophagy contributes to protein catabolism under normal physiological situations, or during disease. Recently, mammalian Atg12 was also shown to conjugate to

Atg3, which is not involed in starvation-induced autophagy, but is rather important for regulation of mitochondrial homeostasis and cell death (Radoshevich et al., 2010).

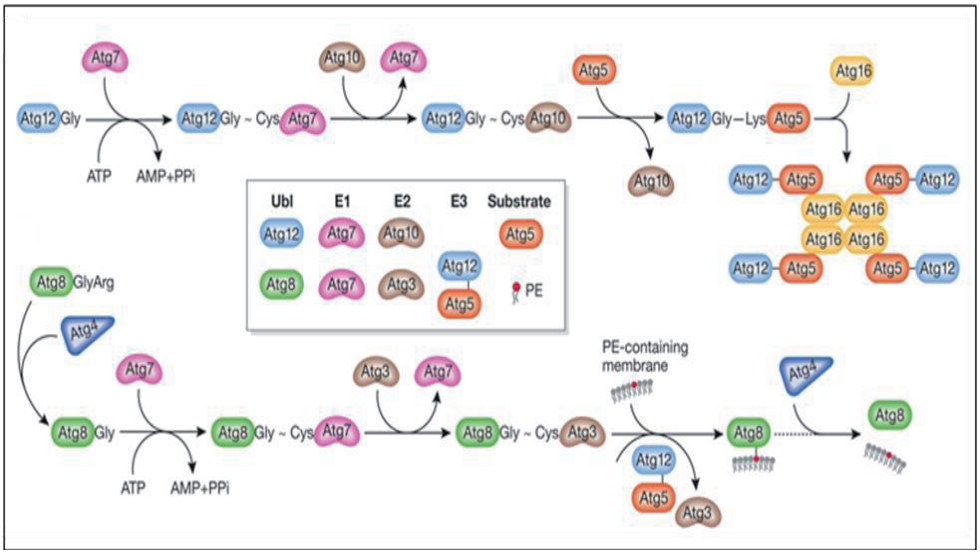

Fig.4. The Atg12 and Atg8 ubiquitin-like conjugation pathways are required for autophagosome formation. Atg4 encodes a cysteine protease that cleaves Atg8. Atg7 is similar to an E1-like protein, and Atg10 and Atg3 encode E2-like proteins. Atg5, Atg12 and Atg16 are physically associated with the isolation membrane, whereas Atg8 is directly conjugated to the lipid phosphatidylethanolamine (PE) that is inserted in the isolation membrane. Adapted from (Geng and Klionsky, 2008).

D. Maturation

After the final closure of the autophagosome, it matures by fusion with endocytic compartments, creating amphisomes (Gordon and Seglen, 1988), prior to fusion with lysosomes, creating the autolysosome in which the cargo is degraded (Fig.1) (Tooze et al., 1990). In yeast, the autophagosome fuses directly with the vacuole which indicates a higher complexity of this process in higher eukaryotes. Endocytosis is the process whereby extracellular material is internalized by an invagination of the plamsa membrane (Besterman and Low, 1983). Several factors have been reported to be important for the convergence of autophagic and endocytic vesicles, such as the coat protein complex I (COPI) and the endosomal sorting complex required for transport (ESCRT) (Filimonenko et al., 2007; Lee et

al., 2007; Razi et al., 2009; Rusten and Simonsen, 2008). COPI is found at early endosomes which function as a sorting station for endocytic cargo, whereas ESCRTs are required for formation of MVBs and sorting of endocytic cargo targeted for lysosomal degradation into MVBs (Simonsen and Tooze, 2009). As described above, the Vps34/class III PI3K complex II, containing UVRAG can also regulate the maturation of autophagosomes (Simonsen and Tooze, 2009). Recently, the endosomal PI3P 5-kinase PIKfyve has been shown to be involved in maturation (de Lartigue et al., 2009), indicating that also endocytic membrane lipids are important for proper autophagosome maturation.

In mammalian cells, the fusion of autophagosomes with lysosomes is facilitated by microtubules and seems to require dynein, structures which are not required for fusion of yeast autophagosomes with the vacuole (Aplin et al., 1992; Fass et al., 2006; Fengsrud et al., 1995; Kirisako et al., 1999; Kochl et al., 2006; Punnonen and Reunanen, 1990; Ravikumar et al., 2005; Webb et al., 2004). One possibility is the involvement of LC3 in this regulation, as it was originally identified as microtubule-associated protein light chain 3. Moreover, members of the Rab family of small GTPase, such as Rab7, Rab5 and Rab11, are involved in maturation of the autophagosome (Gutierrez et al., 2004; Stein et al., 2005). Interestingly, the Rab7-, PI3P- and LC3-binding protein FYCO1 was found to promote microtubule plus end-directed transport of autophagosomes (Pankiv et al., 2010a), thereby connecting transport of autophagosomes to the fusion with lysosomes.

The Class C Vps/HOPS (homotypic vacoule fusion and protein sorting) complex is known to regulate tethering and fusion of endosomes with the vacoule/lysosome (Haas et al., 1995; Seals et al., 2000) by serving as a guanine-nuclotide exchange factor (GFF) for Rab7 (Ostrowicz et al., 2008; Rieder and Emr, 1997; Wurmser et al., 2000). Recently, it was found that the mammalian HOPS complex binds to the complex II subunit UVRAG, which is a Beclin-1 binding protein (Liang et al., 2008) and colocalizes with Rab9 positive endosomes (Itakura et al., 2008). Moreover, SNARE (soluble N-ethylmaleimide-sensitive fusion protein attachement receptors) proteins have been found to be involved in the fusion process both in yeast and mammalian systems (Furuta et al., 2010; Ishihara et al., 2001; Kihara et al., 2001).

Selective autophagy

Autophagy was long considered to be a nonselective bulk protein degradation system, but recent work clearly indicates that it can also be a highly selective process. Selective autophagy relies upon specific cargo-recognizing autophagy receptors and adaptor proteins that link the cargo with the core autophagic machinery. Cargo-specific names have been given to describe the various types of selective autophagy, such as aggrephagy (aberrant protein aggregates and disease-related inclusions), mitophagy (mitochondria), pexophagy (peroxisomes) and xenophagy (invasive pathogenes) (Klionsky et al., 2007) (Table 1, Fig.2). Selective autophagy was first described in yeast, where it was named as the cytoplasm to vacoule targeting (Cvt) pathway. Selective autophagy is believed to perform a quality control function, and must therefore have the ability to distinguish its substrate, such as aggregation-prone proteins or dysfunctional mitochondria, from their normal counterparts. The signals involved in recognition of selective cargo for autophagy is largely unknown. The best studied example involves recognition of ubiquitinated cargo by the ubiqutin binding protein p62.

p62 can directly interact with both ubiquitin and LC3 (Ichimura et al., 2008; Komatsu et al., 2007; Pankiv et al., 2007) and thereby facilitate autophagic degradation of ubiquitinated cargo, such as intracellular bacteria (Zheng et al., 2009), protein aggregates (Bjorkoy et al., 2005), the midbody remnant formed after mitosis (Pohl and Jentsch, 2009), peroxisomes (Kim et al., 2008; Platta and Erdmann, 2007) and mitochondria (Geisler et al., 2010). In lines with these studies, many inclusions found in autophagy-deficient cells are positive for both ubiquitin and p62. Interestingly, loss of p62 combined with impaired autophagy greatly reduces the formation of ubiquitin inclusions in mice and flies (Komatsu et al., 2007; Nezis et al., 2008), indicating that p62 also plays a role in protein aggregate formation.

Other examples of autophagy receptors are NIX for mitochondrial clearance (Novak et al., 2010), NDP52 for cytosolic bacteria (Thurston et al., 2009) and NBR1(neighbour of BRCA1 gene 1) which is required for removal of ubiquitinated protein aggregates (Kirkin et al., 2009). NBR1 have a very similar domain structure as p62 and both are themselves substrates of autophagy and continuously degraded (Bjorkoy et al., 2005; Kirkin et al., 2009; Lamark et al., 2009; Pankiv et al., 2007). In yeast, the autophagy receptor Atg32 was recently found to target mitochondria for degradation by autophagy (Kanki et al., 2009; Okamoto et al., 2009). The autophagy receptors all contain two key domains: an LC3-interacting region (LIR) or LC3-recognition sequence (LRS) that allows interaction with Atg8 family members

(Atg8/LC3, GABARAP, GEC1/GABARAPL1 and GATE-16/GABARAPL2), and a cargo recognition domain, as for p62 an UBA (ubiquitin-associated) domain (Pankiv et al., 2007; Vadlamudi et al., 1996). The autophagy receptors might also interact with specificity adaptors, which function as scaffolding proteins that bring the cargo-receptor complex in contact with the core autophagic machinery to allow sequestration of the substrate. In addition to the autophagy receptors and specificity adaptors, selective autophagy in general relies on the same molecular core machinery as non-specific autophagy.

Aggrephagy	Selective macroautophagic sequestration of protein aggregates
Crinophagy	Is the "uptake" of secretory proteins in lysosomes, either by fusion of lysosomes and secretory vacuoles, or by translocation of secretory proteins from the trans-golgi network (TGN) to lysosomes (Ahlberg et al., 1987; Marzella et al., 1981).
Reticulophagy	Selective macroautophagy of endoplasmic reticulum (ER)
Pexophagy	Involves the sequestration and degradation of peroxisomes through macroautophagy or microautophagy. It has been identified in yeast but also observed in hepatocytes (Luiken et al., 1992; Sakai et al., 2006).
Xenophagy	When microbes (e.g., bacteria, fungi, parasites and/or viruses) are removed by selective macroautophagy
Vid pathway (Vacoule import and degradation)	Selective uptake of cytosolic fructose-1,6-biphosphatease, and possibly other proteins, within 30 nm single membrane vesicles, followed by fusion with the vacoule
Cytoplasm to vacoule targeting (Cvt)	Is a biosynthetic pathway in yeast that transports resident hydrolases aminopeptidase I (prApe1) to the vacoule through a selective macroautophagy-related process as illustrated in Fig.2. The phagophore assembly site (PAS) either becomes the sequestering vesicle or generates it. The precursor form (prApe1) forms oligomers in the cytosol, and is targeted through the action of a receptor, Atg19, and the adaptor or scaffold protein Atg11 to allow selective cargo recognition and packaging. The completed vesicle fuses with the vacuole, the yeast analogue of the mammalian lysosome (Klionsky et al., 2007).
Piecemeal microautophagy of the nucleus (PMN)	Intrusion of portions of the nucleus into the vacuole, by interaction between the vacoule membrane protein Vac8 and the outer-nuclear membrane protein Nvj1, followed by scission and degradation (Kvam and Goldfarb, 2007).

Table 1. Suggested definitions of selective types of autophagy (Klionsky et al., 2007) (Fig.2)

Aggrephagy

The term aggrephagy was coined by Seglen and co-workers to describe autophagy-mediated clearance of protein aggregates (Overbye et al., 2007). The ability of proteins to aggregate is a fundamental process through which proteins exert their normal function. However, the maturation of misfolded or unfolded protein into protein aggregates is also a cytophathological feature of many disorders. Generally, protein aggregation is caused by an abnormal protein conformation, leading to the formation of oligomeric intermediates (Merlini et al., 2001), which can further mature into small protein aggregates. These small protein aggregates can again form into a wide variety of structures (Dobson, 2003), termed histologically as intracellular inclusions, bodies, tangles or threads (Grune et al., 2004; Kopito, 2000). Larger cytoplasmic inclusions can evolve further and coalesce into an aggresome, a pericentriolar, membrane-free cytoplasmic inclusion formed specifically at the microtubule organizing center (MTOC) containing misfolded, ubiquitinated proteins caged within intermediate filaments such as vimentin or keratin (Johnston et al., 1998; Kopito, 2000). It has been proposed that the aggresome is a protective structure, formed to sequester proteins that cannot be degraded by the proteasome and packaged for degradation by autophagy (Johnston et al., 1998; Kopito, 2000). However, not all kinds of protein inclusions or aggresomes are degraded by autophagy. It was demonstrated that aggresomes generated in cells expressing mutant huntingtin or mutant tau, or co-expressing synphilin-1 and alpha-synuclein, were removed by autophagy, while inclusions produced in AIMP2 (p38)- or mutant desmin-expressing cells were resistant to autophagic clearance (Wong et al., 2008). Protein aggregates can form inside the cell as a result of various cellular stressors, such as abnormal protein expression, defective proteasomes, mutations, oxidative stress, aging or protein misfolding (Kopito, 2000).

Misfolded proteins generally become poly-ubiquitinated. Whereas such proteins are normally degraded by the UPS, aggregate-prone proteins may be poor substrates for proteasomal degradation as they are highly insoluble and too big to pass through the narrow barrel-shaped proteasome (Stefanis et al., 2001; Verhoef et al., 2002). K48-linked ubiquitin chains are a classical signal for degradation via the UPS and it has been suggested that autophagic subtrates are modified by K63-linked ubiquitin chains (Tan et al., 2008). In line with this, the autophagy receptors p62 and NBR1 have been shown to preferentially recognize K63-linked ubiquitin chains (Kirkin et al., 2009; Long et al., 2008; Wooten et al., 2008) and

inclusions labelled with K63-linked ubiquitin chains have been associated with autophagic degradation (Tan et al., 2008). Moreover, the ubiquitin-binding histone deacetylase 6 (HDAC6) is required to recruit ubiquitinated, misfolded proteins to the aggresome (Iwata et al., 2005b; Kawaguchi et al., 2003; Olzmann et al., 2007). In addition to ubiquitination, it was reported that acetylation of mutant aggregate-prone Huntingtin (Htt) enhances its degradation by autophagy and an acetylation-deficient mutant strongly accumulated in the cells (Jeong et al., 2009).

The molecular mechanisms of aggrephagy are reminiscent of the yeast Cvt pathway. The substrates preApe1 and Ams1 bind specifically to the receptor protein Atg19, which possesses a classical LIR (Noda et al., 2008). However, prior to binding to Atg8, the prApe1-Atg19 complex binds directly to the adaptor Atg11, which transports the complex to the PAS (Monastyrska and Klionsky, 2006). Atg11 also interacts with other core Atg proteins (He and Klionsky, 2006; Yorimitsu and Klionsky, 2005), suggesting that it acts as a scaffolding protein. Interestingly, Atg11 is also involved in other forms of selective autophagy, like mitophagy and pexophagy, but is not required for non-selective autophagy.

In paper I and II we show that the large protein Alfy (autophagy-linked FYVE protein) has a similar function to the specificity adaptor Atg11. Alfy is ubiquitously expressed and evolutionarily conserved. Alfy contains a PI3P-binding FYVE domain at its C-terminus which is preceded by five WD40 repeats and a PH-BEACH domain. Alfy was previously found to be recruited from the nucleus to cytoplasmic ubiquitin-positive structures under cellular stress such as starvation or exposure to proteasome inhibitors (Simonsen et al., 2004). Alfy interacts with the ubiquitin autophagy receptor p62, and possibly NBR1, and assists in specific degradation of ubiquitinated protein aggregates by recruiting Atg5 and LC3 to the complex, as well as PI3P-containing membranes (Paper I and II). Moreover, Alfy is not required for starvation-induced autophagy (Paper II) and is probably degraded by autophagy only when associated with p62-bodies or other types of aggregates (Paper I and II). *Drosophila* lacking the Alfy homologue *Blue Cheese* (*bchs*) has been shown to have a reduced life span and accumulate Ub-positive inclusions and display neurodegenration (Finley et al., 2003).

Ubiquitin-binding proteins in autophagy

p62 and NBR1

p62 also known as sequestesome 1 (SQSTM1) is a component of the ubiquitin-positive inclusion bodies found in some neurodegenerative and liver diseases (Kuusisto et al., 2001; Zatloukal et al., 2002). In addition to being a cargo receptor for protein aggregates, both p62 and NBR1 have been proposed to be required for the formation of ubiquitinated protein aggregates, also called p62 bodies, sequestosomes or aggresome-like inducible structures (ALIS) (Bjorkoy et al., 2005; Szeto et al., 2006) (Paper I). Accordingly, overexpression of p62 lead to accumulation of ubiquitinated protein aggregates (Bjorkoy et al., 2005; Seibenhener et al., 2004). There are two important studies indicating that p62 is crucial for formation of protein aggregates and their clearance by autophagy. First, the formation of aggresome-like inclusion bodies is significantly impaired in p62 deficient cells (Pankiv et al., 2007) (Paper I). Consistent with this, it was also demonstrated that large ubiquitin-positive protein aggregates, which accumulate in Atg7 knock-out (KO) mice or Atg8 mutant flies, no longer persist in the absence of p62 (Komatsu et al., 2007; Nezis et al., 2008). Secondly, p62 was found within double membrane vesicles by electron microscopy (EM) (Bjorkoy et al., 2005). Both p62 and NBR1 possess an oligomerization domain (PB1) through which they can homo- or hetero-oligomerize and thereby mediate formation of protein aggregates (Bjorkoy et al., 2005; Lelouard et al., 2002; Szeto et al., 2006). In addition, the PB1 domain of p62 enables it also to interact with the protein kinases PKCζ, PKCλ/ι, MEKK3 and MEK5 (Lamark et al., 2003; Nakamura et al., 2010; Sanchez et al., 1998; Wilson et al., 2003). NBR1 and p62 also have a ZZ Zinc finger domain, a C-terminal UBA domain and a LIR domain (Kirkin et al., 2009; Pankiv et al., 2007) and can therefore link ubiquitinated protein aggregates to the core autophagic machinery.

Recently, it was shown that localization of p62 and NBR1 to the autophagosome formation site requires their PB1 domain, but not their interaction with LC3 (Itakura and Mizushima, 2011). Moreover p62 colocalizes with early autophagy proteins, such as ULK1 and VMP1, suggesting that p62 and NBR may contribute to determine where the autophagosomes are nucleated. This raises the question of whether also Alfy is located there.

Interestingly, p62 interacts with the E3 ligase TRAF6 (Geetha and Wooten, 2002), and may also via its interaction with KEAP1 facilitate recruitment of the E3 ligase cullin 3 (Lau et al., 2010). Ub ligases are present in most protein inclusions and p62 might recruit E3 ligases to these structures to facilitate ubiquitination, leading to recruitment of more p62. Mutations

of the p62 UBA domain are associated with increased osteoclastogenesis in Paget disease of the bone(Duran et al., 2004; Kurihara et al., 2007; Yip et al., 2006). p62 can also act as regulator of the oxidative stress response. The level of p62 increases in response to oxidative stress (Nagaoka et al., 2004), which is thought to be a protective response against oxidative damage to the cell. Under normal conditions, the p62 level is low and Nrf2 is bound to KEAP1 and rapidly degraded by the proteasome. But upon oxidative stress, the level of p62 increases and it then binds to KEAP1, leading to dissociation of Nrf2, which translocates to the nucleus and stimulates an anti-oxidant response, including induced expression of p62, thus creating a feedback loop (Jain et al., 2010; Komatsu et al., 2010; Lau et al., 2010).

HDAC6

Unlike most members of the histone deacetylase (HDAC) family, HDAC6 is localized to the cytoplasm and contains an ubiquitin-binding domain (BUZ finger). It associates with both microtubules and the actin cytoskeleton (Gao et al., 2007; Hubbert et al., 2002; Kawaguchi et al., 2003; Matsuyama et al., 2002; Seigneurin-Berny et al., 2001; Zhang et al., 2003). Because it can bind both to ubiquitinated misfolded protein aggregates and to the microtubuli motor protein dynein it was proposed that HDAC6 can facilitate transport of aggregates to the MTOC to form the aggresome (Kawaguchi et al., 2003). Moreover, large aggresomes do not form in HDAC6 deficient cells, rather dispersed microaggregates throughout the cytoplasma are observed, suggesting a failure to transport the protein aggregates to the MTOC. The accumulation of these toxic species in the MTOC region facilitate their clearance by autophagy, as autophagic vesicles and lysosomes have been found concentrated around the aggresome (Iwata et al., 2005c; Lee et al., 2010). In line with this, ubiquitinated protein aggregates were observed in neurons of HDAC6 KO mice (Lee et al., 2010). This is in contrast to p62 deficient cells, where no protein microaggregates are observed (Komatsu et al., 2007). This suggests that p62 might act upstream to HDAC6 to concentrate misfolded proteins into aggregates, whereas HDAC6 directs their dynein-dependent transport to the aggresome. In addition, HDAC6 is proposed to be required for the maturation step of autophagy by recruiting a cortactin-dependent actin remodeling machiney (Lee et al., 2010). By activating cortactin via deacetylation (Zhang et al., 2007), it promotes the formation of an F-actin network that stimulates the fusion of autophagosomes with lysosomes (Lee et al., 2010).

HDAC6 exist in a complex with the ATPase p97, also called valosin-containing protein (VCP). Like HDAC6, this protein is also required for the formation of the aggresome and for autophagosome maturation. Mutations in these proteins are known to cause inclusion body myopathy associated with Paget`s disease of the bone and frontotemporal dementia (IBMPFD), a disease characterized by protein aggregate accumulation, neurodegeneration and muscle defects (Watts et al., 2004).

Autophagy and neurodegenerative diseases

Under normal conditions, autophagy is present at basal levels to maintain protein homeostasis. The demand for cellular quality control through autophagy is particularly important in post-mitotic cells, such as neurons and myocytes (Hara et al., 2006; Komatsu et al., 2007; Komatsu et al., 2005; Nakai et al., 2007) and dysfunctional autophagy has been linked to neuronal death in many neurodegenerative disorders. A major quality control function of autophagy in neurons involves the clearance of misfolded proteins which might become cytotoxic and cause neuronal dysfunction or death if not properly removed (Rubinsztein, 2006). Thus, enhancing autophagy to eliminiate protein aggregates would be a logical therapeutic approach in neurodegenerative disease. However, others argue that the soluble oligomers are more toxic and that sequestration of misfolded proteins into aggregates might prevent the misfolded proteins from harming the cell until they become degraded by autophagy or the proteasome (Arrasate et al., 2004; Szeto et al., 2006; Takahashi et al., 2008a; Tanaka et al., 2004).

There is growing evidence that autophagy has a protective role against neurodegeneration, but how autophagy can prevent neurodegeneration is not completely understood. Indeed, we know that autophagy has the capacity to selectively eliminate protein aggregates or inclusion bodies, via the adaptor proteins Alfy and p62 (Paper I and II). Moreover, proper turnover of p62 by autophagy is critical to prevent spontaneous aggregate formation (Komatsu et al., 2007). A role of autophagy in disorders such as Alzheimer's disease (AD; Nixon et al., 2005;Cataldo and nixon, 1990), Parkinson's disease (PD; Anglade et al., 1997), polyglutamine expansion disorders (e.g.Huntington's diseases,HD); Ravikumar et al., 2002) and in different forms of ataxia (Berger et al., 2006) has been described. Accumulation of intracellular protein aggregates are commonly observed in these diseases.

These aggregates consist of misfolded or aggregate-prone mutated versions of normal proteins, exemplified by the cytotoxic polyglutamine-expanded huntingtin (Htt) protein causing HD.

Polyglutamine neurodegenerative diseases

Several neurodegenerative diseases, commonly known as the polyglutamine (polyQ) expansion disorders, are caused by an expansion of a CAG trinucleotide repeat, encoding glutamine, in the disease-associated proteins. They are all progressive, typically beginning in adulthood and culminating in death over a 10 to 30 year period. Although quite different in their pathophysiology, the presence of ubiquitin-positive intra-nuclear and/or cytoplasmic aggregates is a hallmark of all polyQ diseases. The polyQ expansion leads to abnormal protein folding and conformation resulting in aggregation-prone proteins. In all polyQ diseases, except SCA6, the longer the polyglutamine tract, the more severe and the earlier age of disease onset. In general, a stretch of 37 glutamine repeats is non-pathogenic, but expansions larger than this are strongly associated with disease (Hughes and Olson, 2001). Furthermore, fragments of Htt containing a polyQ stretch of more than 40 repeats are insoluble whereas fragments carrying non-pathogenic repeat lengths are soluble in SDS (Gatchel and Zoghbi, 2005).

HD is the most common and best studied of the polyQ diseases. It is an autosomal dominant disease and is caused by a polyQ expansion in exon 1 of the gene encoding Htt. HD involves neuronal loss in the striatum and cortex leading to gradual loss of voluntary movement coordination and eventually death of the patient. Previous studies of aggregation have revealed that elimination of the accumulation-prone proteins permits symptomatic reversal in a HD mouse model (Yamamoto et al., 2000). The disappearance of the aggregates often correlates with regression of symptoms. Autophagy is specifically important for the degradation of aggregate-prone mutant Htt and not wild-type soluble Htt (Ravikumar et al., 2002; Yamamoto et al., 2006) and may help re-establish normal cellular function. Recent study has shown that the turnover of cytosolic components is impaired in HD cells (Martinez-Vicente et al., 2010). Furthermore, it has also been demonstrated that autophagy is essential for the elimination of cytoplasmic but not nuclear aggregated forms of mutant Htt and ataxin-1 (Iwata et al., 2005a).

Autophagy and cancer

Both non-selective and selective autophagy are important in human health and disease. It was only some years ago that scientists established a link between autophagy and disease, and cancer was one of the first diseases genetically linked to impaired autophagy. It was found that mice having only one copy of the *Beclin1* gene develop spontanous tumors (Liang et al., 1999; Yue et al., 2003). Moreover, monoallelic deletion of this gene occurs in 40-75% of human ovarian, breast and prostate cancers (Aita et al., 1999). These studies suggested that autophagy is a tumour suppressor pathway. By contrast, autophagy can also be deleterious, as when it is activated in more advanced stages of cancer to facilitate survival of cells in low-vascularized tumors (Mathew et al., 2007). This paradox can be explained by two hypotheses, first, in apoptosis-defective cells, when tumour cells cannot die by apoptosis upon exposure to metabolic stress, autophagy may prevent death by necrosis, a process that might enhance local inflammation and thereby increase tumour growth rate (Degenhardt et al., 2006). Second, stressed autophagy-defective tumor cells accumulate p62, damaged mitochondria, reactive oxygene species (ROS) and protein aggregates, which might cause DNA damage leading to oncogene activation and tumorigenesis (Mathew et al., 2007; Mathew et al., 2009). Thus, autophagy probably functions to prevent cancer initially, but once tumor develops, the cancer cells utilize autophagy for their own cytoprotection.

Acute promyelocytic leukemia

The disease acute promyelocytic leukemia (APL) was first identified in 1957 and is a distinct subtype of acute myeloid leukemia (AML), a cancer of the blood and bone marrow. It represents 10-15% of the AML cases and the median age of patients with APL is approximately 40 years, which is considerably younger than the other subtypes of AML (70 years). The majority of AML cases are associated with non-random chromosomal translocations that often result in gene rearrangements (Look, 1997). APL is charactarized by a specific chromosomal translocation, t(15;17)(q22;q11-12) involving the genes encoding the promyelocytic leukemia protein (PML) on chromosome 15 and that encoding retinoic acid receptor alpha (RARA) on chromosome 17 (Martens and Stunnenberg, 2010; Nasr et al., 2009). The PML/RARA fusion protein is a product of this translocation and exhibits a transcription and differentiation block at the promyelocytic stage of granulocytic maturation, leading to accumulation of abnormal promyelocytes in the bone marrow (Melnick and Licht,

1999). RARA is a retinoic acid (RA)-responsive transcription factor and the tumor supressor protein PML is known to form distinct nuclear foci referred to as PML-nuclear bodies (PML-NBs). A number of proteins involved in different cellular processes, such as transcription, DNA repair, cell cycle regulation and apoptosis localize to PML-NBs (Bernardi and Pandolfi, 2007; Dellaire and Bazett-Jones, 2004; Zhong et al., 2000). Moreover, misfolded proteins, as well as proteasomes, have been found to localize to PML-NBs (Fu et al., 2005; Rockel et al., 2005). In paper I we found that both p62 and Alfy are localized to PML-NBs, but the functional significance of this is not known.

Previous studies have shown that the differentiation block and the transcriptional repression induced by PML/RARA involve RARA homodimerization (Sternsdorf et al., 2006), PML sumoylation (Zhu et al., 2007), binding to the nuclear receptor RXR (Zeisig et al., 2007), and recruitment of the polycomb complex (Villa et al., 2007). The RARA/RXR complex binds DNA and recruit corepressor complexes leading to repressed transcription of its target genes (Nasr et al., 2009). Moreover, PML/RARA has been shown to also disrupt the PML-NBs (Dyck et al., 1994; Koken et al., 1994), raising the question whether other molecular mechanisms than transcriptional repression may be implicated in APL leukogenesis. Without treatment, APL is rapidly fatal, however with appropiate therapy it is the most frequently cureable subtype of adult AML (Parmar and Tallman, 2003). APL is sensitive to two clinically active therapies, all-*trans* retinoic acid (ATRA) and arsenic trioxide (ATO). ATRA-based treatment is commonly employed as the frontline therapy for APL patients, whereas ATO predominantly is being used for treatment of patients that have relapsed or that are irresponsive to ATRA (Nasr et al., 2009). Serveral lines of evidencde have shown that the synergistic effect of the ATRA/ATO combination for APL treatment strongly promote PML/RARA degradation and clinical remission, thus avoiding the need for chemotherapy (Estey, 2003; Ravandi et al., 2009; Warrell et al., 1993). Both agents represents major advances in the treatment of this disease and cause clinical remission by targeting PML/RARA-mediated transcription repression and protein stability through stimulating proteolytic degradation of the PML/RARA oncoprotein. ATRA targets both RARA and PML/RARA for degradation and induce activation of RARA responsive genes and granulocytic differentiation (Gianni et al., 2000; Liu et al., 2000). ATO also causes proteolytic degradation of PML, although probably by a different mechanism than ATRA, as well as disease remission by contacting a cysteine-rich motif present within the PML protein (Zhang et al., 2010). ATO treatment causes PML and PML/RARA sumoylation, ubiquitination and

proteolytic degradation (Lallemand-Breitenbach et al., 2008; Tatham et al., 2008). Recently, studies of PML/RARA catabolism revealed that degradation of this disease-related mutant protein is highly dependent on autophagy, in addition to the ubiquitin-proteasome system (Wang et al., 2011) (Paper III).

Aims of the study

The main purpose of this thesis has been to contribute to a better understanding of the function and dynamics of autophagy and to understand its relation to disease. We were particularly interested in investigating the role of Alfy in autophagy, and how autophagy contributes to degradation of protein aggregates. Because the autophagy receptor p62 was found to colocalize extensively with Alfy, we wanted to identify a possible functional interaction between these proteins. Finally, we asked whether aggregate-prone proteins associated with cancer also can be degraded by autophagy. The specific aims of the papers were as follows:

Paper I: **Investigation of a possible interaction between Alfy and p62 and how they contribute to formation and clearance of ubiquitinated protein aggregates.**

We had previously reported that Alfy, a PI3P-binding protein, is recruited from the nucleus to cytoplasmic ubiquitin-positive protein aggregates which also colocalize with the autophagic markers Atg5 and LC3 upon cellular stress. We then found that the ubiquitin-binding protein p62 colocalize extensively with Alfy and decided to investigate whether Alfy and p62 interact to facilitate the formation of ubiquitinated aggregates and their degradation by autophagy. Moreover, we asked whether p62 plays a role in the nucleocytoplasmic shuttling of Alfy.

Paper II: **To elucidate the specific role of Alfy in degradation of disease-associated protein aggregates by autophagy.**

Drosophila lacking the Alfy homologue *blue cheese* show a neurodegenerative phenotype with the presence of ubiquitin-positive aggregates in their brain. Having identified a link between Alfy and autophagy, we hypothesized that Alfy/*bchs* might be involved in autophagic clearance of aggregate-prone proteins. Using mouse and fly models of Huntington's disease we aimed at elucidating the mechanism(s) by which

Alfy mediates autophagic degradation of protein aggregates and to investigate whether Alfy functions both in aggrephagy and starvation-induced autophagy.

Paper III: To contribute to the understanding of the therapy-induced degradation of the oncoprotein PML/RARA.

Previous studies had indicated that the ubiquitin-proteasome pathway is the main mechanism involved in therapy-induced PML/RARA degradation. However, proteolytic degradation of PML/RARA by caspases, neutrophil elastase and lysosomal proteases had also been reported, suggesting the existence of multiple proteolytic pathways with a potential to target PML/RARA for degradation. The PML/RARA fusion protein is known to be prone to aggregation, a feature that makes it a good substrate for autophagic degradation and we therefore wanted to study a possible role of autophagy in PML/RARA catabolism.

Summary of included papers

Paper I. p62/SQSTM1 and Alfy interact to facilitate the formation of p62 bodies/ALIS and their degradation by autophagy

Accumulation of ubiquitinated protein aggregates in the cytoplasm and/or nuclear inclusions are hallmarks of several neurodegenrative disorders, as well as other human diseases including those affecting muscles, heart and liver. The Ub-binding protein p62 accumulates in Ub-positive inclusions in several diseases and it has recently become evident that p62 can target ubiquitinated aggregate-prone proteins for degradation by autophagy. p62 homopolymerizes through its PB1 domain and contains an LC3-interacting region (LIR), which explains how p62 can function as a cargo receptor facilitating the degradation of ubiquitinated protein aggregates by autophagy. From the literature it was known that p62 is required for the formation of ubiquitinated protein aggregates, also called p62 bodies, sequestomes or aggresome-like inducible structures (ALIS).

Alfy (autophagy linked FYVE protein) becomes recruited from the nucleus to cytoplasmic ubiquitinated protein aggregates and co-localizes with autophagic markers upon

cellular stress such as starvation. Both Alfy and p62 localize to PML-NBs, which, in addition to several other functions, have been found to contain misfolded proteins. In this work we wanted to deepen our molecular understanding of how p62 and Alfy interact to regulate formation and degradation of misfolded ubiquitinated protein aggregates.

Since the Alfy-positive bodies appear very similar to those formed by p62, we first asked if these structures are p62 bodies. Confocal immunofluorescence microscopy revealed that p62 is a major constituent of all Alfy positive cytoplasmic bodies. Furthermore, siRNA-mediated depletion of p62 severely inhibited the starvation-induced redistribution of Alfy from the nuclear region into cytoplasmic bodies. Moreover, Alfy and p62 accumulated in PML-NBs when nuclear export was blocked by the exportin-1 inhibitor leptomycin B (LMB). Interstingly, we observed that Alfy was not localized to PML-NBs in cells transfected with siRNA against p62 and vice versa, accumulation of p62 in NBs was strongly reduced in Alfy-depleted cells treated with LMB.

We next wanted to study whether Alfy, like p62 is required for the formation of cytoplasmic p62 bodies. Surprisingly, we observed a reduction of the number of p62 bodies in HeLa cells depleted of Alfy. Furthermore, western blot analysis revealed that the insoluble fraction of p62 was reduced after knockdown of Alfy, indicating that Alfy is important for aggregation of p62. Next, we studied redistribution of Alfy when p62 was ectopically expressed. The only region of p62 that appear to be required for cytoplasmic redistribution of endogenous Alfy was the the region of p62 that contains nuclear localization and nuclear export signals that are essential for shuttling of p62 between the cytoplasm and the nucleus. This suggest that p62 needs to enter the nucleus in order to redistribute Alfy. We also demonstrated that endogenous p62 and LC3 was efficiently co-immunoprecipitated with endogenous Alfy from HeLa cell lysates, indicating that these proteins are in the same complex in vivo. We next set out to map the regions in Alfy important for its interaction with p62 and recruitment into endogenous p62 bodies. Confocal immunofluorescence analysis revealed that only Alfy constructs containing the PH-BEACH domain of Alfy were efficiently recruited into p62 bodies. Moreover, pulldown assays established a direct interaction between this part of Alfy and p62. In line with these results we found that the Drosophila p62 homologue Ref(2)P accumulates in Ub inclusions in the brains of flies carrying mutations in the Alfy homologue Blue cheese (bchs), indicating that Alfy is required for formation of p62-bodies that become substrates for autophagy. Taken together, p62 and Alfy interact and colocalize both in cytoplasmic and nuclear protein bodies. p62 is required to recruit Alfy from

the nucleus to the cytoplasmic Ub-positive bodies and both proteins are required for formation of cytoplasmic bodies and their degradation by autophagy.

Paper II. The Selective Macroautophagic Degradation of Aggregated Proteins Requires the PI3P-Binding Protein Alfy

Aggregate-prone proteins are poor substrates for proteasomal degradation due to their insolubility and large size. Instead, such aggregate-prone proteins have been found to be degraded by autophagy. However, it was unclear whether their degradation is selective and the molecular machinery involved was not characterised. We hypothesised that Alfy is essential for the macroautophagy-mediated clearance of aggregation-prone proteins and that Alfy might be invovled in selective autophagy (aggrephagy).

The first approach towards this aim was to examine whether Htt inclusions could be found in autophagosomes. Using EM and biochemical analysis we detected inclusions within isolated autophagosome (AV) fractions from cells stably expressing an exon1 fragment of Htt containing a polyQ stretch of 103 fused to mCFP (Htt1 103Q-mCFP cells). Moreover, we show that Alfy colocalizes with Htt-polyQ inclusions and is found in the AV fraction. Alfy is required for autophagy-mediated clearance of these aggregated proteins; filter trap assays and immunofluorescence quantifications revealed that Alfy depletion significantly inhibited aggregate-clearance in two different tet-regulatable cell lines (HeLa and Neuro2a) expressing exon1-Htt with a pathogenic stretch of 65 or 103 glutamine repeats. We observed the same effect when autophagy was suppressed using siRNA against Atg5 or inhibited by 3-MA, confirming the involvement of autophagy in degradation of these inclusions.

To explore this further, we turned to a non-polyQ protein known to form cellular aggregates, α-synuclein (α-Syn), with and without the aggregation-prone mutation, A53T. Although the soluble form of α-Syn can be degraded by chaperone-mediated autophagy (Cuervo et al., 2004; Vogiatzi et al., 2008), its aggregated form may be degraded by macroautophagy (Sarkar et al., 2007). Consistent with the polyQ aggregate clearance data, filter trap analysis revealed that Alfy KD inhibited the ability of the cells to eliminate the SDS-insoluble α-Syn proteins, and co-IP analysis found that Alfy and Atg5 precipitated with the α–synuclein proteins. Therefore, Alfy is required for the removal of different types of protein aggregates.

To determine whether Alfy is required for general starvation-induced macroautophagy we examined the degradation of long-lived proteins (LLPs) in both HeLa and Neuro2a cells subjected to serum and amino acid withdrawal. We found that, as with treatment with 3-MA, knock down of Beclin-1 inhibited LLP degradation, but depletion of Alfy had no effect. The level of lipidated LC3-II and the amount of GFP-LC3 puncta formed in stably transfected Hek293 cells was also unaffected upon treatment with siRNA targeting Alfy. To further explore the role of Alfy in starvation-induced autophagy we examined autophagosome formation, using Lyso Tracker Red staining, in the larval fat body of *Drosophila* lacking the Alfy homologue *bchs*. Upon starvation WT and *bchs* mutants showed a similar increase in autolysosome formation, while larvae expressing a *dsAtg1-RNAi* construct in the fat body had significantly fewer red puncta. Taken together, Alfy/*bchs* are essential for the macroautophagy-mediated clearance of aggregation-prone proteins (aggrephagy), but are not required for starvation-induced autophagy.

We next sought to determine whether Alfy interacts with the polyQ inclusions. Immunoprecipitation of the htt polyQ protein revealed that endogenous Alfy, as well as both N- and C-terminal parts of Alfy interacts with the inclusions. Based on the findings in paper I, where we found that Alfy makes a complex with p62 and LC3, we wanted to examine whether Alfy interacts directly with any of the Atg proteins. Using various techniques, we found that Alfy interacts directly with Atg5 and that the C-terminal WD-40 repeat region of Alfy are required for this interaction. Furthermore, we could show that the complete complex containing Alfy, Atg5, p62, LC3-I and -II and full length mutant Htt was immunoprecipitated from HD patient lysates, but not from age-matched control patients. In a primary neuronal model of HD, we showed that over-expression of an Alfy C-terminal fragment led to a significant reduction of protein aggregates in the neurons. Similarly, we observed a neuroprotective effect of over-expressing full length *Bchs* or its C-terminal in a *Drosophila* eye model of polyglutamine toxicity, an effect which was abrogated when autophagy was inhibited by Atg8 RNAi.

Paper III. Autophagy contributes to therapy-induced degradation of the PML/RARA oncoprotein

Several pathways have been implicated in therapy-induced degradation of PML/RARA, including cleavage of the PML moiety by proteases and a SUMO-dependent ubiquitin-mediated proteasome degradation process. Knowing that PML/RARA is highly prone to aggregation, a feature that makes it a preferable substrate for autophagic degradation, we wanted to investigate whether autophagy contribute to the degradation of PML/RARA.

To test this, we monitored the expression levels of the oncoprotein in the APL cell line NB4, which contains the t(15;17) chromosomal translocation that results in expression of the fusion protein, under conditions that stimulate or inhibit autophagic degradation. Stimulation of autophagy by amino acid deprivation (starvation) caused a decrease in PML/RARA levels, whereas inhibition of this degradation pathway by 3-MA or Bafilomycin A1 (BafA1) resulted in accumulation of PML/ RARA. In line with these results we found that treatment with the mTOR inhibitor rapamycin increased autophagy-mediated degradation of PML/RARA. In order to further explore whether autophagy contributes to clearance of the oncoprotein, we used siRNA to supress the unc-51-like kinase 1 (ULK1), a protein kinase required for induction of autophagy, in HeLa cells that transiently expressed the fusion protein. Whereas PML/RARA levels decreased significantly in starved cells treated with control siRNA, ULK1-depleted cells displayed an accumulation of PML/RARA. Two clinically active therapies, all-*trans* retinoic acid (ATRA) and arsenic trioxide (ATO), cause clinical remission by stimulating proteolytic degradation PML/RARA. We therefore next confirmed the ability of ATRA and ATO to induce PML/RARA degradation, both alone and in combination. Interestingly this effect was considerably reduced in the presence of the autophagy inhibitor BafA1. We further found that the level of phosphorylated p70S6K decreased significantly in both ATRA- and ATO- treated cells, indicating that ATRA and ATO activate autophagy via an mTOR-dependent pathway. Finally, since APL therapy strongly associates with differentiation, we investigated whether autophagy plays a role in granulocytic differentiation of APL cells. Using flow cytometry we were able to monitor the expression of the granulocyte surface marker protein CD11b. Interestingly, we observed that inhibition of autophagy significantly prevented ATRA-induced differentiation of APL cells and that stimulation of autophagy by rapamycin promoted the differentiation of APL cells. Overall, our results identify autophagy as a new pathway involved in therapy-induced PML/RARA degradation and differentiation along the granulocytic linage.

Discussion

The papers included in this thesis contribute towards our understanding of the degradation of aggregate-prone proteins by autophagy. Dysfunctional autophagy has been linked to many diseases such as cancer and neurodegeneration. Nevertheless, several questions regarding the role of autophagy in disease remain to be answered. Through the work presented here we have attempted to shed light on some of these questions. We have investigated the mechanisms by which aggregate-prone proteins are targeted for autophagy and found that the proteins Alfy and p62 are important for this selective process by linking ubiquitinated proteins to the core autophagy machinery (Paper I and II). Moreover, we found that autophagy is involved in degradation of aggregate-prone proteins associated with neurodegenerative disease (e.g huntingtin inclusions, Paper II) and cancer (PML/RARA oncoprotein, Paper III), indicating an important role of this pathway in human health.

Autophagy and neurodegeneration

The intracellular accumulation and aggregation of proteins is a common theme across many age-related neurodegenerative diseases. These include Alzheimer's disease (AD), Huntington's disease (HD), taupathies, the spinocerebellar ataxia (SCA), Parkinson's disease (PD), Amyotrophic Lateral sclerosis (ALS) and prion disease (Yamamoto and Simonsen, 2010). Many of these neurodegenerative disorders are caused by mutations resulting in production of misfolded proteins that are specific for each disease. Typically, at an early stage of disease damaged proteins remain soluble and they can undergo proteolysis by the UPS when marked by ubiquitin. Therefore the ubiquitin-dependent degradation of misfolded proteins by proteasomes constitutes a critical part of a cytoprotective quality control machinery, as defects in this system lead to the accumulation of the misfolded proteins. However, at later stages of disease mutant proteins tend to form aggregates. Continous production of damaged proteins might exceed the capacity of the UPS or it is possible that a dysfunctional UPS plays an additional role in pathogenesis. Moreover, most large aggregate-prone proteins cannot be processed by the UPS due to the size of the narrow barrel of the proteasome where polypeptides are digested (Verhoef et al., 2002). In fact, protein aggregates can inhibit the UPS activity by clogging the proteasomes, resulting in further accumulation of

protein aggregates (Bennett et al., 2007; Snyder et al., 2003). Thus, autophagy may serve as a back-up mechanism allowing cells to get rid of toxic protein aggregates. In this regard α-synuclein can be degraded by both the UPS and CMA (Webb et al., 2003), but is removed by macroautophagy when it is mutated (Cuervo et al., 2004) (Paper II). Furthermore, the mutant variant of huntingtin (Htt) inhibits UPS function and induces autophagosome formation (Kegel et al., 2000; Venkatraman et al., 2004). Therefore degradation of protein aggregates by autophagy could have evolved as a mechanism to preserve the function of the UPS and several studies indicate that cells compensate for impairment of one form of proteolysis by elevating an alternate form of protein degradation (referred to as proteolytic cross-talk) (Pandey et al., 2007). The cross-talk between the UPS and autophagy seems to be both at the gene regulatory level and at the substrate selection level. At the gene regulatory level, some transcription factors (FoxO3 and Nrf2) activate genes involved in both UPS and autophagy. In addition to misfolded proteins, other substrates can be shared by the UPS and autophagy, including the ubiquitin-interacting proteins p62 and Ubiquillin-1 (Bjorkoy et al., 2005; Ichimura et al., 2008; Ko et al., 2004; Korolchuk et al., 2010; Lamark and Johansen, 2010; Pankiv et al., 2007; Rothenberg et al., 2010; Seibenhener et al., 2004; Wong and Cuervo, 2010). Moreover, the protective effect of the compensatory upregulation of autophagy in proteasome-inhibited cells has been suggested to depend on HDAC6 and p97/VCP (Ding and Yin, 2008; Pandey et al., 2007). During proteasomal impairment, p97/VCP and HDAC6 are responsible for the accumulation of misfolded proteins in aggresomes (Kawaguchi et al., 2003), which may allow them to be degraded more efficiently by autophagy (Iwata et al., 2005c). In contrast, inhibition of autophagy, leading to accumulation of p62, was shown to delay the delivery of ubiquitinated proteins to the proteasomes (Korolchuk et al., 2009), suggesting that these pathways not only have a compensatory effect on each other, but that impairement of one pathway can cause inhibition of the other degradative pathway. However, further studies are required to understand how this is regulated.

The presence of ubiquitinated inclusions in the nucleus and/or cytoplasma is a diagnostic hallmark of several neurodegenerative diseases. Unfortunately such inclusions are detected at a late stage of disease or post portem and their impact during early phases of the disease is poorly understood. Nonetheless, the elimination of such inclusions has become a major effort in therapeutic development, largely due to studies showing that symptomatic reversal in mouse models of neurodegenerative disease is tightly associated with clearance of the aggregated protein (DiFiglia et al., 2007; Lin et al., 2009; Mallucci et al., 2003; Regulier

et al., 2003; Southwell et al., 2009; Wang et al., 2008; Xia et al., 2004; Yamamoto et al., 2000; Zu et al., 2004). Unfortunately, these studies have not created a definitive link between protein aggregation and pathogenesis thus several key questions remain unanswered. For example, why protein aggregates form in the brain remains unclear. Equally debated is whether the inclusions are causative or crucial to the disease process or whether they are protective. Increasing evidence supports the notion that in general aggregates confer toxicity and disturb neuronal function by hampering axonal transport, synaptic integrity, transcriptional regulation and mitochondrial function. To understand the relationship between neuronal pathology and autophagy, protein aggregation was studied in conditional Atg7 and Atg5 knock-out mice (Hara et al., 2006; Komatsu et al., 2006). Interestingly, neuron-specific Atg7 and Atg5 gene deletion in mice evokes progressive motor deficit, neurodegeneration and formation of inclusion bodies, without corresponding defects in proteasome function, indicating that dysfuntional autophagy alone set off a neurodegenrative cascade. Similar results were obtained in cell culture studies and in *Drosophila* lacking core autophagy genes (Juhasz et al., 2007; Lindmo et al., 2008; Simonsen et al., 2008) (PaperII). These finding suggest that autophagy plays a pivotal role in preventing accumulation of protein inclusions causing neurodegeneration through its contribution to intracellular quality control and turnover of aggregate-prone proteins. Impairment of autophagy by inhibitors or by depletion of essential Atg genes also prevents clearance of aggregate-prone proteins. For example, the autophagy inhibitors BafA1 (inhibiting the lysosomal proton pump) or 3-MA (inhibiting PI3K and thus autophagosome formation) led to decreased clearance of aggregate-prone proteins such as mutant Htt or mutant forms of α-synuclein (causing familial PD) (Qin et al., 2003; Ravikumar et al., 2002; Webb et al., 2003) (Paper II). There is evidence that Htt aggreggates can be removed by autophagy despite activation of mTOR, which normally inhibits autophagy when active (Yamamoto et al., 2006). Although an mTOR-independent autophagy pathway clearly exist (Sarkar et al., 2005), mTOR signalling plays an important role in the disposal of protein aggregates by autophagy. Studies have found that mTOR becomes trapped in inclusion bodies, thereby promoting autophagy and clearance of toxic aggregates in a mouse HD model (Ravikumar et al., 2004). Moreover, the TOR inhibitor rapamycin was found to protect against the cytotoxic effects of different aggregate-prone proteins, including polyglutamine containing proteins and mutant Tau (Berger et al., 2006). Clearance of mutant Htt was also found to depend on expression of Atg6/Beclin 1 (Shibata et al., 2006; Yamamoto et al., 2000).

Degradation of aggregates by autophagy is somehow contradictory to the hypothesis claiming that generation of protein aggregates is a protective mechanism. However, it is still possible that autophagy can protect cells from toxic proteins at at least two levels; i) by degradation of misfolded proteins in soluble or oligomeric states and 2) by clearance of protein aggregates, which are themselves not toxic, but which might become toxic if not properly removed. Accordingly, it has been suggested that the primary target of autophagy seems to be diffuse cytosolic proteins, not inclusion bodies themselves, indicating that inclusion body formation in autophagy-deficient cells is a secondary event to impaired general protein turnover (Hara et al., 2006). In line with this theory, the cells have developed a selective pathway, aggrephagy (discussed in more detail below), to mediate clearance of such aggregate-prone proteins.

Conditional mouse models of HD (Yamamoto et al., 2000), prion disease (Mallucci et al., 2003) and SCA1 (Zu et al., 2004) have shown that elimination of the expression of the toxic protein leads to a concomitant clearance of accumulated proteins and symptomatic reversal. However, these observations do not clarify whether it is the aggregated form of the protein or the soluble form that contributes towards pathogenesis. Unfortunately examination of this question has been difficult to address, as smaller, more toxic subspecies may still exist even if inclusions are no longer visible. Moreover, if the efficacy of the conditional models lies on the elimination of the soluble protein, the clearance of the aggregated proteins may simply be a passive by product that does not impact the reversal of symptoms. Nonetheless, the correlation between the length of the polyglutamine repeat in mutant Htt, which in turn lead to greater protein aggregation, and the earlier onset of the associated disease (Perutz and Windle, 2001), strongly suggests that the aggregates may be the cytotoxic species. Moreover, in mammalian cell culture, there is a strong correlation between aggregate formation and cellular toxicity (Paper II). In many cases, the formation of aggregates precede cell death (Wyttenbach et al., 2000), although microscopic monitoring of survival of cells containing aggregates has suggested that large microscopically visible inclusions may be protective (Arrasate et al., 2004). It does not rule out the possibilty that the smaller oligomeric precursors of these aggregates, or the process of aggregation itself, may in fact be toxic.

If aggregate-removal can be beneficial, it is important to understand the mechanism invovled in clearance of these aggregate-prone proteins. In our study (Paper II) we used an aggregation-prone protein model of Htt; the protein fragment encoded by exon1 containing different polyQ repeat lengths, 25Q being non-aggregating or 65Q and 103Q forming aggregates. Aggregates were detected after transient tranfection or in HeLa and N2a cells with

inducible expression of Htt-25Q, -65Q or -103Q. Moreover, primary rat cortical neurons were transduced with lentivirus expressing Htt-72Q. In order to rule out over-expression artefacts, we also used fibroblasts obtained from control or HD patients in our study. We observed that Htt-65Q or -103Q inclusions were indeed sequstered by autophagic membranes, as they colocalized with autophagic markers (IF) and by EM were found to be surrounded by a double membrane. Moreover, inclusions were found in the autophagic vesicle fraction obtained by fractionation of cells expressing Htt-polyQ. Thus, our data strongly indicate that aggregation-prone protein inclusions can be found within autophagosomes. Whether entire protein aggregates are directly degraded by autophagy, or if autophagy rather degrades soluble aggregate precursors is still debated. Autophagy has also been implicated in the dissolution of larger neuronal inclusions into smaller aggregates (Rideout et al., 2004). We report that protein aggregates larger than 1 μm are observed inside autophagic membranes (Paper II), indicating that the autophagic machinery can degrade entire aggregates. However, the observed aggregate pool was heterogenic and whereas most of the smaller aggregates were surronded by a double membrane, most of the larger aggregates (larger than 1 μm) were membrane free. We and others have found that increased levels of autophagy is neuroprotective in *Drosophila* and mice HD models (Simonsen et al., 2008; Williams et al., 2006) (Paper II), and our data might suggest that the observed protective role of autophagy may be caused by degradation of smaller aggregates to eliminate their toxicity.

Supporting the idea of aggregates being toxic species which need to undergo proteolysis not to harm cellular function, the autophagy receptor p62 is found to be a component of protein aggregates associated with several neurodegenratative diseases (Gal et al., 2009; Holm et al., 2007). Interestinlgy, where inclusions found in autophagy-deficient cells are positive for both ubiquitin and p62, loss of p62 combined with a deficiency in autophagy greatly reduces the formation of ubiquitin inclusions in mice and flies (Komatsu et al., 2007; Nezis et al., 2008). These findings indicate that p62 is involved in formation of the observed aggregates. In line with the *in vivo* data, p62 has been shown to be required for the formation of ubiquitinated protein aggregates, also called p62 bodies, sequestosomes or ALIS (Bjorkoy et al., 2005; Szeto et al., 2006) (Paper I). These structures contain misfolded ubiquitinated proteins and can be experimentally induced by addition of the translational inhibitor puromycin (Lelouard et al., 2004) (Paper I). The role of p62 in formation of ubiquitin positive protein aggregates depends on its polymerization domain (PB1), through which it can homo- or heteropolymerize or interact with other binding partners, as well as its

C-terminal UBA domain, responsible for binding to ubiquitinated proteins. The protein NBR1 has a very similar domain structure to p62 and has also been found to mediate formation of protein aggregates and their degradation by autophagy. The latter requires their interaction with LC3 through a LIR (LC3 interaction) motif. In addition to p62, Alfy (Simonsen et al., 2004) was found to be required to recruit ubiquitinated proteins into aggregates that become degraded by autophagy (Paper I). However, we did not conclude whether the aggregates are sequestered by preexisting autophagic membranes or if the autophagic machinery is recruited to the aggregates to mediate local assembly of autophagic membranes around the aggregates. Alfy is a large (400kDa) scaffolding protein containing several domains which can facilitate recruitment of the autophagic machinery to the protein aggregates; a p62-interacting BEACH domain (Paper I), a PI3P-binding FYVE domain (Simonsen) and WD40-repeats engaged in interaction with Atg5 (Paper II). In paper I we show that p62 and Alfy interact and colocalize both in cytoplamsic and nuclear protein bodies (NBs). Both proteins cooperate to sequester ubiquitinated proteins into p62 bodies, but it is not clear how these proteins work together in this process. Aggregation of the cargo may, therefore, be one of the first steps toward degradation, and inhibition may confer toxicity because the protein can no longer be eliminated (Paper II). We observed an accumulation of the *Drosophila* p62 homologue Ref(2)P in ubiquitin positive inclusions in the brains of flies carrying mutations in the Alfy homologue *Blue cheese* (*bchs*), demonstrating that Alfy is required for the degradation of p62-associated ubiquitinated proteins *in vivo*. Interestingly, *bchs* did not accumulate in flies lacking the p62 homologue Ref(2)P, indicating that p62 might assist aggregate-prone proteins to form microaggregates that can be combined and deposited into larger aggregates by Alfy. The large size of Alfy suggests that it works as a major scaffolding protein involved in the assembly of p62 bodies, promoting the subsequent degradation of these protein aggregates by autophagy.

Selective autophagy - aggrephagy

Autophagy has generally been considered a non-selective degradative pathway activated by nutrient starvation to efficiently recycle cellular components and support cell survival until nutrients are replenished. It has, however, become evident that autophagy also proceeds at basal levels, and that it has an important quality control function by disposing of protein aggregates, damaged organelles and other substrates that are toxic or no longer needed (Klionsky et al., 2007). In contrast to the classical non-selective starvation-induced autophagy, this form of autophagy must have the ability to distingiush aberrant protein aggregates and damaged organelles from their normal counterparts. The characterization of the two cargo receptors, p62 (also called SQSTM1) and NBR1, has revealed that, similar to proteasome-mediated protein degradation, an ubiquitin-dependent system can confer the substrate specificity for autophagy (Kirkin et al., 2009; Pankiv et al., 2007). As mentioned above, p62 and NBR1 contain an LIR, mediating interaction with Atg8/LC3 family members (LC3/GABARAP/GATE-16) and a ubiquitin-binding UBA domain (Bjorkoy et al., 2005; Pankiv et al., 2007) and therefore have the ability to deliver the cargo to the autophagic machinery. In addition to being a cargo receptor for protein aggregates, p62 has been proposed to act as a cargo receptor for other ubiquitinated substrates, such as intracellular bacterial (Zheng et al., 2009), protein aggregates (Bjorkoy et al., 2005), the midbody remnant formed after mitosis (Pohl and Jentsch, 2009), peroxisomes (Kim and Guan, 2011; Platta and Erdmann, 2007), soluble protein (Gao et al., 2010; Kim et al., 2008) and mitochondria (Geisler et al., 2010).

In our studies we observed that p62 and NBR1 closely colocalize with Alfy on cytoplasmic ubiquitinated Htt polyQ aggregates and that p62 and Alfy are present in the same Htt-associated complex (Paper II). Importantly, we found that endogenous Alfy did not accumulate in aggregates formed by the point mutant of NBR1 (D50R) which is unable to interact with p62. This suggests that Alfy is recruited to protein aggregates via p62 in a NBR1-independent manner. Moreover, this complex also contains the autophagic markers LC3 and Atg5. Interestingly, Atg5 and LC3 did not co-immunoprecipitate with the aggregated Htt when Alfy was depleted. This suggests that aggregate-prone polyQ proteins can interact with p62 and/or NBR1, and that Alfy may target these proteins for degradation by autophagy. In line with this, Alfy depletion resulted in a significant decrease of aggregate clearance, both in HeLa and N2a cells expressing either Htt-Q65 or –Q103. The effect of Alfy depletion was

similar to cells treated with the autophagy inhibitor 3-MA or depleted of Atg5. Moreover, double knock down of Alfy and Atg5 did not result in further inhibition of clearance, suggesting that they function in a common autophagy pathway. The turnover of Htt inclusions was measured by the filter trap assay, a technique which can be used to observe accumulation of SDS-insoluble material (Wanker et al., 1999). In contrast, Alfy depletion had no effect on clearance of non-aggregated SDS soluble Htt-Q25 proteins, indicating that Alfy is specifically required for turnover of aggregate-prone Htt proteins. This is consistent with earlier observations in *Drosophila* lacking *bchs*. *Bchs* mutant flies are adult viable but have a reduced life span due to an accelerated accumulation of ubiquitin-positive inclusions and neuronal degeneration (Finley et al., 2003).

But how can Alfy contribute to aggregate clearance by autophagy? Alfy has the potential to bind to proteins via its C-terminal BEACH or WD40 repeats domains and to membrane lipids through its PI3P-binding FYVE-domain and possibly through the PH domain. Considering also the big size of this protein, Alfy probably acts as a scaffold protein for the autophagic machinery to build or target autophagosomal membrane onto the protein aggregates. Consistent with this, we found that Alfy directly interacts with Atg5 through its C-terminal WD40 repeats and that it can bind to Htt-PolyQ via its N-terminal part. However, we do not know whether Alfy can recognize the ubiquitinated aggregates directly or if it is recruited indirectly, e.g through its interaction with p62. Interestingly, depletion of Alfy prevented efficient recruitment of Atg5, as well as LC3, to the Htt inclusions. In GST-pulldown experiments we observed an interaction between the Alfy C terminus and Atg5, but not with LC3, suggesting that Alfy might aid the stabilty of the interaction between LC3 and p62 or that LC3 interacts through another region of Alfy. Atg5 has been shown to act as an ubiquitin E3-ligase-like protein for the lipid conjugation of LC3 (Hanada et al., 2007). Thus, Alfy may bring Atg5 to the inclusion to act as part of a greater E3 ligase-like complex for LC3, which allows formation of autophagosomal membrane around the inclusion. This scenario fits with the general concept of selective autophagy which implies that each cargo is specifically recognized followed by formation of the autophagic membrane closely to the target, thus excluding bulk cytoplasm.

We propose that Alfy is required for the autophagic elimination of aggregation-prone proteins (a process named aggrephagy), but not for autophagic degradation of bulk cytosol in response to starvation. We assessed the degradation of LLP, formation of LC3 puncta and LC3 lipidation under starvation conditions in cells depleted for Alfy and found no difference

from control cells. In contrast, general inhibition of autophagy by 3-MA or depletion of Beclin-1 or Atg7 inhibited induction of autophagy during nutrient deprivation, as analyzed by LLP degradation and decrease of GFP-LC3 positive autophagosomes, in line with previous studies (Shibata et al., 2006).

We have previously shown that Alfy is ubquitously expressed in mouse tissues, with the highest levels in brain (Simonsen et al., 2004). Consistent with this, *bchs* seems to be selectively expressed in *Drosophila* brain (Finley et al., 2003). This suggests that Alfy/*bchs* may be especially important in nondividing cells, in which removal of toxic aggregated proteins is essential for cell survival. As tissue expression levels of Alfy (highest in brain and lowest in liver) inversely correlate with responsiveness to starvation it is tempting to speculate that regulation of the Alfy level of might be a way for the cell to regulate starvation-induced autophagy versus aggrephagy (Yamamoto and Simonsen, 2011). Alfy would be an ideal candidate for such a regulator, as it shuttles between the nucleus and the cytoplasm. As Alfy interacts with the core autophagic machinery (Atg5 and PI3P), increased cytoplasmic Alfy levels may lead to toxicity under conditions where non-selective autophagy may be necessary, and keeping Alfy in the nucleus would be a way of avoiding this. In line with this hypothesis we find that Alfy is mostly localized in the nucleus under normal conditions and becomes recruited to the cytoplasm during conditions favoring formation of protein aggregates, e.g. puromycin treatment (paper I). Moreover, we find that p62 is required for Alfy to leave the nucleus (paper I), which could indicate that this autophagic receptor signals when aggrephagy should be activated.

It seems that the role of Alfy in aggregate clearance can be extended to other aggregating proteins as Alfy depletion also inhibits clearance of mutant α-synuclein A53T and co-IP experiments revealed that α-synuclein is detected in the same complex as Atg5 and Alfy. Furthermore, Alfy, as p62, seems to be involved in selective autophagy of other ubiquitinated structures, such as the midbody ring structure (Isakson et al, unpublished). Alfy is localized to the midbody region during cytokinesis and to ubiquitinated midbody remnants and is required for their proteolysis by autophagy. Moreover, we have evidence that Alfy is involved in autophagic clearance of the oncoprotein PML/RARA (Isakson, unpublished), and recently a role for p62 in PML/RARA degradation was also reported (Wang et al., 2011) (Paper III). The function of p62 and Alfy in selctive autophagy of ubiquitinated cargo is highly similar to the role of Atg19 and Atg11, respectively in the yeast Cvt pathway. In the Cvt pathway, the LIR-containing autophagy receptor Atg19 binds the cargo (the vacuolar

hydrolase aminopeptidase 1 (ApeI)), followed by interaction with the adaptor Atg11, which transports the complex to the pre-autophagosomal structure (PAS) to allow binding to Atg8 and formation of the autophagic membrane around the cargo.

Our data stronlgy indicates that Alfy contributes to aggrephagy. But since Alfy and selective autophagy normally exist, why are then aggregates formed in human disease? In physiological situations, it is important to maintain a balance between the level of newly sythesized protein and their degradation. It is tempting to suggest that increased levels of autophagy can diminish the level of misfolded protein and thereby have a protective effect in models of neurodegenerative diseases. Indeed, studies in *Drosophila* and mice models of HD have revealed that enhanced levels of autophagy promote aggregate clearance and reduced the disease-like symptoms (Lindmo et al., 2006; Ravikumar et al., 2004; Simonsen et al., 2008). Taking this into account, we speculated that by increasing Alfy expression levels we may increase aggregate clearance. Since Alfy is a very large protein it is difficult to achieve overexpression of the full-length protein. We therefore expressed different regions of Alfy and thus found that overexpression of the C-terminal part of Alfy, containing the WD40 repeats and the FYVE domain, had a neuroprotective effect in primary neurons expressing Htt-polyQ. Moreover, overexpression of either full length or a C-terminal part of *bchs* in a *Drosophila* eye model of polyglutamine toxicity was protective. Interstingly, when the WD40 repeats were mutated in that context, Alfy could no longer rescue aggregate clearance, indicating that this effect is due to its interaction with Atg5 and that the neuroprotective effect correlates with decreased amounts of protein aggregates. It will be very interesting to learn whether overexpression of Alfy in the brain of HD mouse models may increase the rate of aggregate clearance and prevent their accumulation and toxic effects. Such studies are currently in progress (Yamamoto, unpublished).

Autophagy in acute promyelocytic leukemia

Acute promyelocytic leukemia (APL) is a well-characterized subtype of myeloid leukemia (AML). The genetic hallmark of APL is a translocation t(15;17)(q22;q11-12), leading to the fusion of the gene encoding *promyelocytic leukemia* (*PML*) on chromosome 15 to the *retionic acid receptor alpha* (*RARA*) gene on chromosome 17, generating the *PML/RARA* fusion gene and protein (Nasr et al., 2009). PML/RARA is a transcriptional repressor that binds RARA response elements, resulting in both dominant-negative and gain-of-function properties. This leads to a differentiation block at the promyelocytic stage of granulocytic maturation and thereby an abnormal accumulation of immature granulocytes named promyelocytes in the bone marrow (Melnick and Licht, 1999). Although more than 97% of APL patients have the t(15;17) translocation, several rare variant translocations that always involve *RARA* are observed in the remaining APL patients. The most common is the t(11;17) translocation that fuses the promyelocytic leukemia zinc finger (*PLZF*) gene to *RARA* (Chen et al., 1993).

Three distinct proteasome-dependent pathways are known for therapy-induced PML/RARA degradation. The first one includes all trans-retinoic acid (ATRA)-induced cleavage by proteases of the PML moiety of PML/RARA (Nervi et al., 1998). The second is also ATRA-mediated and involves a direct activation of proteasome-dependent degradation of DNA-bound, hormone-activated RARA or PML/RARA (Zhu et al., 1999). Finally, arsenic trioxide (ATO) targets the PML part of the fusion protein and specifically induces a SUMO-dependent ubiquitin-mediated degradation process (Tatham et al., 2008). In paper III we identified autophagy as a new pathway for therapy-induced PML/RARA degradation. To study the PML/RARA catabolism we used the APL cell line NB4, which contains the t(15;17) chromosomal translocation which result in expression of the fusion protein (Lanotte et al., 1991). We found the PML/RARA fusion protein to be highly prone to aggregation as it was mainly found in the extraction fraction of 8M UREA. Other extraction buffers containing 0.5% Triton X-100 or 2% SDS were not efficient in extracting the fusions protein. Interestingly, both the normal PML and RARA were found to be considerably more soluble to extraction by Triton compared with PML/RARA, suggesting that the fusion protein is more aggregate-prone than its respective fusion partners expressed from non-rearranged alleles. As mentioned previously, abnormal or aggregation-prone proteins form aggregates that are preferable substrates for autophagy. Thus, other oncogenic fusion proteins may share similar features of poor solubility and may also be targets of autophagy.

We demonstrated that inhibition of autophagy in NB4 cells by inhibitors such as 3-MA or BafA1 resulted in an accumulation of PML/RARA, whereas activation of autophagy by starvation or treatment with the mTOR inhibitor rapamycin resulted in a decreased level of PML/RARA. This suggests that the basal turnover of this oncoprotein is regulated by autophagy. However, inhibitors such as 3-MA are not specific to autophagy, as it also interferes with other PI3K-dependent intracellular trafficking pathways (Mizushima, 2004; Punnonen et al., 1994). To specifically address the role of autophagy in degradation of PML/RARA, HeLa cells with ectopic expression of PML/RARA were treated with siRNA against ULK1, which also caused an accumulation of the oncoprotein, further indicating that PML/RARA is degraded by autophagy.

APL is a one of the rare malignancies that can be cured by targeted agents, without DNA-damaging chemotherapies (Estey et al., 2006; Ravandi et al., 2009; Sanz et al., 2009; Wang and Chen, 2008; Warrell et al., 1993). The two clinically active therapies, ATRA and ATO, both induce the degradation of the PML/RARA fusion protein and several studies have shown that a combination of ATRA and ATO has a synergistic effect on PML/RARA degradation (Kogan, 2009; Nasr et al., 2008; Tallman and Altman, 2009). Moreover, patients treated with a combination of ATRA and ATO have a more rapid and complete clearance of leukemia cells, and have a significantly longer period of relapse-free survival (Hu et al., 2009; Shen et al., 2004; Wang et al., 2004). Indeed, APL mouse models have shown synergistic effects of both drugs in prolonging survival and even cure leukemia (Jing et al., 2001; Lallemand-Breitenbach et al., 2005; Rego et al., 2000).

We confirmed that both ATRA and ATO induce PML/RARA degradation and have a synergistic effect (Paper III). Importantly, we observed that the ATRA and ATO induced PML/RARA proteolysis was considerably reduced in the presence of the autophagy inhibitor BafA1, indicating again that autophagy is required for effective PML/RARA clearance. Interestingly, both drugs were also found to induce autophagy, as measured by the level of the lipidated form of LC3 and by quantification of LC3 and p62 positive dots in NB4 cells upon treatments with ATRA and ATO.

mTOR is generally inactivated upon activation of autophagy and the phosphorylation state of its substrates can therefore be used to monitor the autophagic activity (Yang and Klionsky, 2010). We found that the level of p70S6K kinase (p-p70S6K) decreased significantly in both ATRA- and ATO- treated cells, indicating that ATRA and ATO activate autophagy via an mTOR-dependent pathway. This is consistent with previous findings

showing that blocking mTOR signaling, by the rapamycin analog RAD001, induce differentiation of APL cells (Nishioka et al., 2008). Moreover, PML is shown to suppress tumor progression via inhibition of mTOR (Bernardi et al., 2006; Bernardi et al., 2011). Thus, modulation of autophagy in APL could have important therapeutic consequences. Use of mTOR inhibitors may be a promising treatment strategy for individuals with a subset of human leukemia. This approach could be particularly promising in leukemias or sarcomas caused by fusion proteins. In fact, in solid tumors such as breast cancers, degradation of key transcritional activators such as estrogen receptor, have been of clinical use (Raina, 2004). However, autophagy can have a double-edged sword function in cancer and drug-induced autophagy induction should therefore be used with care. Induction of autophagy in tumor cells have been observed to promote tumor cell survival in response to starvation, hypoxia, oxidative damage or other stress (Shintani and Klionsky, 2004). In this case, inhibition of autophagy for example by specific gene inactivation might prevent tumorigenesis. Along this line, it was shown that the combination of a tyrosine kinase inhibitor with inihibitors of autophagy resulted in elimination of chronic myeloid leukemia (CML) stem cells (Bellodi et al., 2009).

Treatment-induced PML/RARA degradation allows differentiation and APL remission. It was therefore important to study if autophagy plays a role in the drug-induced differentiation. Although ATRA and ATO cooperate to induce PML/RARA degradation (Nasr et al., 2008; Quignon et al., 1997; Zhu et al., 2001) they do not synergize to induce differentiation (Chen et al., 1996; Chen et al., 1997; Shao et al., 1998) and only ATRA treatment has been found to stimulate terminal granulocytic differentiation. Interestingly, we observed that inhibition of autophagy significantly prevents ATRA-induced differentiation of APL cells and that stimulation of autophagy by rapamycin promotes the differentiation of APL cells. Contradictory to our study, it was reported that the mTOR/p70s6K pathway is activated by ATRA in NB4 cells (Lal et al., 2005). Activated mTOR is associated with the ULK1 complex leading to inhibition of autophagy. However, it has been shown that the differentiation-inducing effects of ATRA are not dependent on class I PI3K/mTOR signaling and that mTOR signaling rather plays a role in basal and ATRA induced cell survival mechanisms in APL (Billottet et al., 2009).

Our study also raises interesting questions as to the possible links between autophagy and PML, which is highly stress-sensitive and may also assemble into cytoplasmic bodies (Jul-Larsen et al., 2010). In addition to a role in leukemia, PML has also been linked to other

cancers (Gurrieri et al., 2004), and down regulation of PML in a number of cancers has been observed (Gurrieri et al., 2004; Zhang et al., 2003). The PML protein forms distinct nuclear foci referred to as PML nuclear bodies (NBs). Expression of the fusion protein PML/RARA leads to disruption of these structures formed by the normal PML protein in the nucleus (Melnick and Licht, 1999). Interestingly, in addition to misfolded proteins and PML/RARA, several proteins of the autophagic machinery, such as Beclin1 (Liang et al., 2001) and p62 (Pankiv et al., 2010b) are known to localize to PML-NBs and to undergo nucleocytoplasmic shuttling. However, very little is known about how this nucleocytoplasmic shuttling affects autophagy and it is not clear why these autophagy related proteins shuttle through the nucleus and localize to PML-NBs. Also Alfy is found in PML bodies in the nucleus and shuttle between the nucleus and the cytoplasm (Paper I). The fact that they all localizes to both nuclear and cytoplasmic aggregated proteins raise the question of whether Alfy and p62 are involved in degradation of nuclear aggregates. In the nucleus, p62 is proposed to recruit proteasomes to nuclear aggregates to facilitate their degradation (Pankiv et al., 2010b).

In paper II we found that localization of Alfy to cytoplasmic Htt inclusion involved recruitment of Alfy from the nucleus, as it was blocked by the nuclear export inhibitor, leptomycin B (LMB). Similar Alfy relocation can be initiated by other stress factors as starvation, resulting in localization of Alfy to Ub and p62-positive cytoplasmic bodies. As mentioned previously p62 and Alfy are required for the sequestration of misfolded proteins into p62 bodies and both proteins are involved in selective autophagy. In paper I we have shown that p62 is required to recruit Alfy from the nucleus to the cytoplam. It is therefore likely that p62 and/or Alfy are required to recruit PML/RARA from the nucleus to the cytoplasm. Another possibility is that Alfy and p62 might facilitate recognition of nuclear aggregates by the autophagic machinery when the nuclear envelope is disassembled during mitosis.

If and how nuclear aggregates are degraded by autophagy is currently not know. Autophagy has been shown to eliminate cytoplasmic polyglutamine-containing aggregates more efficiently than nuclear aggregates (Iwata et al., 2005a) and this differential efficiency of degradation could explain why nuclear aggregates are more cytotoxic than cytoplasmic ones. Post-translational modifications, like ubiquitination, acetylation and phosphorylation, have been shown to specifically enhance the degradation of the aggregate-prone proteins by autophagy. Huntingtin is also found in the nucleus, and aggregate-prone mutant versions of huntingtin must be exported to the cytoplasm to be degraded by autophagy. This might be

regulated by acetylation and phosphorylation of huntingtin (Jeong et al., 2009; Thompson et al., 2009). Acetylation of mutant aggregate-prone huntingtin located in the nucleus enhances its degradation by autophagy (Jeong et al., 2009). In contrast, mutant huntingtin that is resistant to acetylation strongly accumulates in the expressing cells. Interestingly, it has recently been shown that PML exists as an acetylated protein in HeLa cells (Hayakawa et al., 2008). Moreover, increased acetylation of PML is associated with increased sumoylation of the protein *in vitro* and *in vivo*. It will be interesting to learn whether the PML part of PML/RARA becomes acetylated, and if this facilitates its degradation by autophagy.

Experimental considerations

In this section I will discuss some of the advantages, pitfalls and limitations of the techniques used in this thesis.

Cell lines

Cultured human cells has been used to address several fundemental questions in cell biology which has led to the generation of a vast amount of knowledge. In this thesis we have used multiple cell lines. In paper I and II we used HeLa cells as our main experimental system. HeLa cells originate from cervical cancer cells taken from Henrietta Lacks, a woman of African American origin who was diagnosed with cancer in 1951, and were the first human cells to be continuously grown in cell culture. HeLa cells are extensively used in laboratories around the world, and display a number of useful properties as a laboratory model. They are robust and fast growing, expression and depletion of proteins by transfection can easily be performed, and in our specific context, their autophagosomal structure can easily be detected by microscopy. It is however worth noting that these cells have a non-human number of chromosomes (82 chromosomes).

In Paper III we used NB4 cells, a cell line derived from the bone marrow of a patient with acute promeolytic leucemia (APL) (Lanotte et al., 1991), to study PML/RARA catabolism. This cell line is characterized by the t(15;17) (q22;qll-12) chromosomal

translocation that result in expression of endogenous PML/RARA fusion protein. Moreover, it mimics several features of clinically isolated APL blast cells, including the ability to undergo ATRA-stimulated differentiation along the granulocyte linage. These features make this cell line a remarkable tool for leucemia studies. However, the NB4 cells have proven difficult to transfect. In order to deplete autophagy-specific genes we tried to transfect the NB4 cells using different liposome-based transfection reagents, as well as the Amaxa nucleofector, without success.

In paper II we used immortalized mouse embryonic fibroblast (MEF) cells, human embryonic kidney 293 (HEK293) cells and N2a cells (murine neuroblastoma cell line) to study the role of Alfy in autophagy. We used Atg5 knock-out MEFs (Kuma et al., 2004) which allowed us to analyse the effects of depletion of autophagy. These cells are better to use than siRNA against Atg5 because the siRNA-mediated Atg5 depletion is not always complete. However, one should consider that even though autophagy is inhibited in the Atg5 KO MEFs, depletion of Atg genes could lead to implications that are distinct from autophagy; for example, Atg5 has been implicated in cell death pathways (Luo and Rubinsztein, 2007). Moreover, the impairment of autophagy ultimately hinders flux through the UPS, the other major catabolic route in cells. Another problem with the knock-out cells is that they might upregulate a redundant pathway to compensate for the one lost. This is most often not seen in knock down situations where the effect exists for shorter time.

We also used HEK 293 cells stably expressing GFP-tagged LC3 (Chan et al., 2007). This cell line allowed us to monitor the level of autophagsome formation, analyse the autophagosome formation and determine colocalization of proteins with LC3. Although GFP-LC3 is a broadly used tool to visualize autophagy, caution should be used to misinterpret the results, as GFP-LC3 overexpression may cause artefacts. It was reported that overexpressed GFP-LC3 may form protein aggregates independent of autophagy, as GFP-LC3 positive structures were seen in autophagy-deficient cells (Kuma et al., 2007). A lipidation deficient mutant of LC3 (Tanida et al., 2008) and EM analysis can be used to avoid false results.

To study a neuronal disorder such as Huntingtons disease, we used cell lines expressing a shorter form of the mutant protein, Huntingtin (Htt). In paper II, we used HeLa and N2a cells with stable conditional expression of the exon1 of Htt witt either 25, 65 or 103 glutamin repeats, fused to mCFP (Htt-Q25/65/103-mCFP) (Yamamoto et al., 2006). The expression of the mutant protein is regulated by a Tet-off system, meaning that protein expression can be turned off by addition of tetracycline or the tetracycline analogue

Doxycyclin (Dox) in the media. Aggregates formed upon expression of Htt-Q65/103-mCFP are degraded in 5 days after Dox treatment (Yamamoto et al., 2006). This model system allowed us to specifically monitor aggregate clearance without interference of newly synthesized proteins. However, in physiological situations, proteins are continuously produced and an effect on their clearance will depend on the balance between newly sythesized protein and their degradation.

Transient transfection and overexpression of proteins

Transient expression of proteins in eukaryotic cells is a widely used tool to study their interaction partners, localisation and intracellular trafficking and this method has been used in all studies included in this thesis. Several advantages are offered by this technique. It allows the study of intracellular proteins whose endogenous amounts are below the detection level. Moreover, if the transiently expressed protein carry a tag (e.g GFP or myc) one can easily detect proteins when protein-specific antibodies are lacking. One can also analyse the effect of a deletion or point mutant of the protein of interest, exemplified by the transient expression of mCherry-p62ΔUBA lacking the UBA domain responsible for binding of p62 to ubiquitin (used in paper I). Use of this method made it possible to analyse aggregate clearance by a rescue assay where the disease-phenotype is reversed by reintroducing parts of the protein Alfy into the cells.

The primary rat cortical neurons were transduced with a lentiviral model that expresses an exon1 fragment of Htt with 72Q (exon1Htt72Q) tagged to GFP. By using this sytem we could facilitate a stable expression of Htt. Lentiviruses have been adapted as gene delivery vectors because of their ability to integrate into the genome of non-dividing cells. To obtain a lentivirus, several plasmids, such as transfer vector plasmid, the packaging plasmid and a plasmid with the heterologous envelope gene (ENV) of a different virus, are transfected into a packaging cell line (Amado and Chen, 1999). For safety reasons lentiviral vectors never carry the genes required for their replication. The lentiviral vector conatins sequences necessary for the vector to infect the target cell and for gene transfer. The viral genome in the form of RNA is reverse-transcribed when the virus enters the cell to produce DNA. The DNA is then inserted into the genome at a random position by the viral integrase enzyme. A problem with lentivirus is that it can disturb the function of cellular genes and lead to cancer.

Although a greatly appreciated technique, there are also disadvantages that should be concidered. The level of the expressed protein is generally higher than the endogenous protein, which may cause artefacts such as altered protein localization and protein aggregation. Thus, transient expression should be used with caution in the context of aggregate studies. In this study, when looking at overexpressed proteins by immunofluorescence, we tried to select cells which expressed moderate amounts of protein and which had a normal morphology.

Gene silencing

Small interfering RNA (siRNA) mediated downregulation of a specific protein is used throughout this thesis. Cells are transfected with 21 nucleotide long siRNA oligos, which assemble into endoribonuclease-containing complexes known as RNA-induced silencing complexes (RISCs), resulting in unwinding, cleavage and destruction of complementary RNA molecules (Filipowicz, 2005). This effect can also be achieved by introducing vectors expressing short hairpin RNA (shRNA) into cells. The vector normally contain of an U6 or H1 promoter to ensure that the shRNA is always expressed. A shRNA is a sequence of RNA that makes a tight hairpin turn that can be used to silence gene expression via RNA interference. In paper II, we used lentivirus sh-RNA to mediate a constitutive gene knock down of Alfy. The use of siRNA also has drawbacks, one problem is the possibility of off-target effects. The introduced siRNA may bind to a partially complementary mRNA molecules, leading to problems interpreting the results, and to potential toxicity. To validate our results we used more than one single oligo per gene in our experiments. To prove the specificity of the knockdown, protein expression levels and size were studied by Western blotting. The knockdown efficiency can vary from experiment to experiment and must therefore be checked each time. A complete gene silencing is difficult to achieve, but is required in some cases to inhibit the function of proteins that can sustain a cellular function even at very low expression levels (e.g. Atg5). In such cases, the use of cell lines derived from knockout animals is a better alternative and Atg5 KO MEFs were used in paper II. However, animal technology is costly and time consuming.

Drosophila model

The fruit fly *Drosophila melanogaster* has become a powerful genetic model organism to study the process of autophagy and its relation to disease since both cancer and neurodegenration can be modeled in this organism. The study of specific genes can be achieved either by using loss of function mutants or by overexpression of proteins using the UAS/Gal4 system. This techinque was used in paper II where we show that the Alfy homolog *Blue Cheese (bchs)* can rescue the neurodegenerative phenotype in a *Drosophila* eye model of polyglutamine toxicity (Kazemi-Esfarjani and Benzer, 2000). The system has two parts: the GAL4 gene, encoding the yeast transcription activator protein Gal4, and the UAS (Upstream Activation Sequence), a short section of the promoter region, to which Gal4 specifically binds to activate gene transcription. For overexpression studies the gene of interest is inserted behind an UAS sequence in a transposable "P"-element vector, followed by insertion into the genome and generation of an UAS-containing stock. Various promoter-specific Gal4 stocks exist, called *driver* lines, where Gal4 is specifically expressed in some cells or tissue (e.g in the eye retina in paper I or brain in paper II). This inducible system is adapted from yeast, which provides an advantage, as Gal4/UAS are not normally present in *Drosophila.* Thus, their expression does not interfere with other processes in the fly cells (Toba et al., 1999).

In paper II, we used an established fly model where polyQ127 is expressed specifically in the eye using the eye-specific driver GMR-Gal4. These flies have a recognizable disrupted eye phenotype with a reduced size, loss of pigmentation and neurotic patches corresponding to damaged photoreceptor cells of the ommatidia. By achieving co-expression of full length (*GMR-Gal4, UASpolyQ127, UAS-FL-Bchs*) or a C-terminal fragment of *bchs* (*GMR-Gal4, UAS-polyQ127, UAS-bchs-C1000*) we observed a reduction of these symptoms. Moreover, we could show that the *bchs*-mediated rescue of the polyQ-induced symptoms was autophagy dependent, as the symptoms were not reversed when autophagy was suppressed by co-expression of Atg8a RNAi (*GMR-Gal4, UAS-polyQ127, UAS-bchs-C1000, UAS-dsAtg8-RNAi*).

As an alternative approach to analyse neuronal dysfunction and clearance of aggregate-prone proteins we used differential detergent extraction of proteins from fly head extracts followed by Western blotting for insoluble ubiquitinated proteins (IUP). It has recently been shown that *Drosophila blue cheese* mutants show neural degeneration and accelerated formation of Ub-positive protein aggregates in the central nervous system (CSN)

(Finley et al., 2003). In paper I we show that the *Drosophila* p62 homolog Ref(2)P accumulates in flies carrying mutations in the Alfy homologue *blue cheese*.

The *Drosophila* larva fat body (equivalent to hepatic/adipose tissue in mammals) is an especially useful tissue in studying autophagy. Autophagy can be induced by starvation in early larval stages and is also induced developmentally in late larval stages (Scott et al., 2004). The fat body functions as the organism's nutrient sensor, and is responsible for providing nutrients to the organism during starvation and during metamorphosis (Aguila et al., 2007; Colombani et al., 2003; Geminard et al., 2009; Lee and Park, 2004; Liu et al., 2009). Fat body cells are large, making it easy to visualize sub-cellular compartments such as autophagosomes and lysosomes. Using flies expressing GFP-LC3 in the fat body, in combination with Lysotracker red, a red dye that labels acidic organelles, one can distinguish autophagosomes (green) from autolysosomes (yellow) and lysosomes (red). However, Lysotracker can also be used alone to measure the level of autophagy in the larval fat body, as large lysotracker-positive structures are induced upon induction of autophagy and not in flies expressing RNAi of Atg5 (Scott et al., 2004) or Atg1 (Scott et al., 2007). In paper II we show that starvation-induced autolysosomal formation is not dependent on Alfy, as the amount of red dots in the fat body of two different *Bchs* mutant was similar to wild type, whereas the lysotracker-red structures was depleted when Atg1 was supressed by expression of Atg1-RNAi using the fat body driver Cg.

Confocal microscopy

Immunofluorescence microscopy (IF) was used throughout this thesis to visualise the infrastructure of the cell, to study the localization of proteins involved in autophagy and to perform quantitative analyses. This method was also used for primarily measurments of p62 or/and LC3 positive dots per cell in different conditions that stimulate or inhibit autophagy. These studies are feasible using confocal microscopy, in contrast to the conventional fluorescent microscopy. While conventional microscopy illuminates the entire specimen, confocal laser-scanning microscopy only detects the fluorescence within the focal plane, excluding background information above and below the focal plane (Saggau, 2006). The image represents a thin cross-section of the specimen/cell, which allows the visualisation of proteins stained with fluorochrome-labeled antibodies or fused to intrinsic fluorescent

proteins like GFP. The localization of individual proteins in eukaryotic cells is often determined by IF, a procedure requiring fixation and permeabilization of the cells before antibodies can be applied. The high level of cytosolic protein following ectopic expression of a protein can cover the signal arising from the specific cellular structures like autophagosomes and protein aggregates. This can be avoided by depleting the cells of cytosol prior to fixation. Permeabilization (before or after fixation) is usually achieved by extracting membrane lipids with mild detergents like saponin, which preserve better the cellular architecture. Neverthless, resulting images might still contain some background staining that may interfere with the immunostaining proper. The antibody specificity is another issue to consider when detecting endogenous proteins, as the antibodiy may bind to other proteins. To control for this, staining of specific cellular structures should disappear when the protein of interest is depleted with siRNA. Analysis of the PML/RARA fusion protein was studied by an anti-RARA antibody in most of experiments in paper III. Using this antibody for IF makes it impossible to distinguish the normal RARA protein from the fusion protein. However, these problems could be solved with methods such as Western blot or ectopic expression of this oncoprotein.

One can also use dyes specific for various subcellular compartments, e.g.Draq5 which stains the nucleus (paper II) and Lysotracker, as described above. The method is dependent on high quality, thin-section confocal images in order to co-detect two, or even three (paper II) proteins. Excitation wavelengths of each fluorophore have been carefully defined and care has been taken to avoid bleed-through from one channel to another, by optimising the microscpy settings when producing multicoloured images. Such bleed-through could potentially cause false positives leading to the incorrect conclusion of a co-localization. There is also a risk of bleaching the fluorochrome and pictures should be taken before the sample is significantly bleached. When using confocal IF images for quantification of e.g. the number of spots, it is important to select the cells as randomly as possible and measure a large number of cells to increase the statistic strength of the results.

Electron microscopy

Morphological detection of autophagsomes by EM is the most reliable method for studing autophagy. It uses a beam of highly energetic electrons that enable the examination of objects on a very fine scale (nanometer level). The autophagosome appear as double membrane vesicle and generally contain cytoplasmic material, but as it can later fuse with endocytic

structures it can be difficult to distinguish the different types of autophagic and endocytic vesicles solely by morphology (Liou et al., 1997). Using immuno-EM, a technique which allows immunostaining with specific antibodies conjugated to gold particles of different sizes, we were able to study whether protein aggregates (HttQ65/103-mCFP labeled with anti-GFP antibodies) could be detected inside the autophagosomes (Paper II) and we could also determine the distribution of p62 in HeLa cells treated with puromycin (Paper I).

Degradation of long-lived proteins in response to amino acid deprivation

The ubiquitin-proteasome system is responsible for the highly selective degradation of short-lived proteins whereas autophagy is responsible for the turnover of long-lived proteins (LLPs). By measuring the rate of degradation of LLP one can monitor the rate at which proteins are transported via the autophagic pathway to the lysosomes (autophaic flux). The method is based on a pulse-chase approach, were cells are incubated in media containing isotopically labeled amino acid ($^{[14C]}$valine) to label all cellular proteins, then washed and chased to allow degradation of short-lived proteins. Finally, the cells are incubated in nutrient rich or deprived media in the prescence or absence of an autophagy inhibitor (e.g. 3-MA) for 4 hrs and the level of LLP degradation is measured by counting the amount of TCA-soluble (degraded material) vs TCA-precipitated (intact protein) radioactivity. The chase is done in the prescence of cold valine to prevent reutilization of the radiolabeled amino acids for protein synthesis.

Over all, protein turnover is not directly correlated with autophagy or lysosomal proteolysis; we therefore monitor LLP turnover during starvation induced in the prescence of 3-MA or compare with results in cells depleted for autophagy (Paper II). However, it shall be considered that amino acids can acts as inhibitors of autophagy and radioactive valine is therefore used, as this amino acid does not inhibit autophagy (van Sluijters et al., 2000).

Evaluation of aggregate clearance

The filter trap assay is widely used to evaluate the accumulation of insoluble protein aggregates in various diseases. The assay is based on the finding that the polyglutamine-containing protein aggregates are insoluble in SDS and are retained on a cellulose acetate membrane with pore size 0,2 μm, whereas the monomeric forms or microaggregates of the protein do not bind to this filter membrane (Scherzinger et al., 1997; Wanker et al., 1999).

We used this technique to determine whether Alfy is required for clearance of polyglutamine-containing protein aggregates (huntingtin and a-synuclein aggregates in paper II). The level of aggregates can be analysed by immunoblotting as a quantitative alternative to confocal microscopy. To ensure that the detected clearance is due to autophagy, we included inhibitors of autophagy or siRNAs targeting Atgs in our experiments. Additionally, we also checked that the proteasome activity was similar in all conditions to exclude activation of the UPS. This technique do not allow detection of inclusions smaller than 0,2 μm. However, microaggregates are also targeted by autophagy and therefore it is important to study their turnover in the context to Alfy. To solve this problem we used a PVDF and a cellulose acetate sandwich approach, which allow also smaller SDS-insoluble aggregates to be detected by the dot-blotter technique.

Protein-protein interaction

Protein-protein interactions form the basis of most cellular processes and many biochemical methods exist to detect such interactions. In this thesis we used various techniques, such as immunoprecipitation (IP), the yeast-two hybrid (Y2H) system and glutathione S-transferase (GST) pull down to reveal interactions between Alfy and p62 (Paper I) and Aly and Atg5 (paper II). Each approache has its own strengths and weaknesses, especially in variation of their sensitivity, efficiency, and rapidity. Thus, using a combination of these techniques gives more reliable results and decrease the possibility of mis-interpretation.

Pull-down assays are widely used for the detection of protein-protein interactions and exist in many variations. In general, the protein of interest is attached either covalently to a pre-activated resin or non-covalently through a high affinity interaction between the protein and the resin. The protein of interest is often expressed as a recombinant protein fused to gluthathione S-transferase (GST) or maltose-binding protein (MBP), as in paper I (interaction

between Alfy and p62) and paper II (interaction between Alfy and Atg5). The fact that two recombinant proteins interact strongly indicate that there is a direct interaction between these two proteins.

The Y2H system is used to investigate the interaction between artificial fusion proteins inside the nucleus of yeast. This approach can be used on a screening scale to identify potential interaction partners. It is based on activation of downstream reporter gene(s) by the binding of a transcription factor onto an upstream activating sequence (UAS). The transcription factor is split into two separate parts, called the binding domain (BD), which is responsible for binding to the UAS, and an activation domain (AD), which is responsible for gene activation. Two plasmids are used in this technique; one that expresses the BD fragment fused to a protein, commonly called the bait protein, while the other plasmid encodes the AD fragment fused onto another protein, the prey protein. If the bait and prey interact, the AD and BD of the transcription factor are indirectly connected, which activate the transcription of a reporter gene (LacZ or *HIS3*). We used the yeast reporter strain L40 (Vojtek et al., 1993) which was cotransformed with the BD-vector pLexA-hAtg5 and the AD-vector pGAD-Alfy (Paper II). A positive interaction was scored by measuring β-galactosidase activities, the product of the reporter gene LacZ. A weakness of this approach is the high number of false positives and negatives that can be observed. Another concern is that aberrant folding or post-translational modifications of the mammalian proteins can occur because they are expressed in yeast which can lead to false positives or negatives. Another problem that arised was that the bait vector pLexA-hAtg5 caused some gene activation in the absence of the AD-Alfy protein. To solve this problem we added 5 mM 3-amino-1,2,4-triazole (3-AT), a competitive inhibitor of the *HIS3*-gene product which provide a weaker sensitivity of the AD.

Co-immunoprecipitation is considered to be the gold standard assay for protein–protein interactions, as it can be performed with endogenous proteins within the cell. The protein of interest is isolated from a cell lysate with a specific antibody and putative interaction partners are identified by Western blotting. However, this method can not verify direct interactions and is not a screening approach.

Autophagic markers (LC3 and p62)

One important tool for assessing autophagy is the use of autophagic markers. LC3, a mammalian homologue of yeast Atg8, is the only known mammalian protein that associates

with the autophagic membranes throughout the pathway. The two forms of the protein, the cytosolic LC3-I (18 kDa) and the membrane-bound LC3-II (16 kDa) can easily be detected by Western blotting The amount of LC3-II and the LC3I/LC3II ratio correlates well with the number of autophagosomes (Kabeya et al., 2000). When working with LC3 one should know that the protein is easily degradaded during the preparation of the cell lysate and its therefore important to use protease inhibitors and keep the samples on ice. Use of higher concentration of tritonX-100 (2%) is necessary for its efficient extraction (Sou et al., 2006). We generally used 1% tritonX-100 in our lysis buffers and got well recognized bands, but addition of 0.05% SDS in our lysis buffer might have assisted that. LC3 itself is degraded by autophagy when the autophagosome fuses with the lysosome, so its overall protein level depends on the lysosomal turnover and autophagic activity. Thus, induction of autophagy by starvation will first cause increased LC3-II formation with subsequent decrease. LC3 II has a short half-life because autophagosomes are transient structures. In line with this, an LC3 blot can be difficult to interpret, but by comparing LC3-II levels in the presence or absence of lysosomal inhibitors (e.g. BafilomycinA1, BafA1) it is possible to get more accurate information about the autophagic flux. If autophagy is upregulated by a treatment, the LC3-II level is expected to be higher in the presence of lysosomal inhibitors than in control cells treated with the same inhibitor. In contrast, if the treatment inhibits autophagy then the level of LC3-II will be the same as in control cells in the presence of lysosomal inhibitors. In paper II we investigated whether Alfy is required for LC3 lipidation. LC3-II levels analysed from control and Alfy-depleted cells that were starved for 4 hrs in the presence of BafA1, showed that neither starvation-induced nor basal lipidation of LC3 was affected by Alfy depletion.

The cytosolic protein p62 is degraded by autophagy (Bjorkoy et al., 2005) and was therefore used as a biochemical marker for autophagic activity in this thesis. p62 binds to LC3 and to ubiquitinated cargo and thereby serves as a receptor for autophagy (Pankiv et al., 2007). p62 is also a common component of Ub-positive inclusions and various types of poly-ubiquitinated protein aggregates, including Lewy bodies (alfa-synuclein, PD), neurofibrillarity tangles (tau, AD) (Kuusisto et al., 2003; Nagaoka et al., 2004) and huntingtin aggregates (Htt, HD) as we also show in our study (Paper II). The p62 level is increased in cells depleted for autophagy, such as Atg5 knock out MEFs, compared to wild type MEFs. Another way to monitor autophagy is to use a double fluorescently-tagged (Cherry-GFP) LC3 or p62. The protein will be visualized as yellow (both green and red) in early autophagosomal structures,

while only red fluorescence will be detected when p62 reaches acidic compartments (late endosomes and/or lysosomes), as the mCherry tag is acid stable whereas GFP is quenched in the acidic enviroment. Using this method in paper I, we demonstrate that autophagic degradation of p62 is not dependent on Alfy. As expected, a construct lacking the LC3-interacting (LIR) motif, displayed yellow flourescence and this was also the only construct that did not accumulate in autophagic structures upon Baf A1 treatment. However, the level of p62 is also influenced independent of autophagy, for exemple by apoptosis or protesomal inhibition (Kuusisto et al., 2001). It is therefore important to evaluate p62 levels in combination with other methods to study autophagy.

Drugs and inhibitors

Inibitors and drugs are widely used in therapy to treat patients. Many of them have improved treatment of various disorders, however, a note of cation is that drugs may exhibit side-effects. A drug can interfere with another pathway (s) than expected and this can result in undesirable secondary effects. Therefore it is important to take a skeptical view of the drug of interest. In order to minimize the secondary effects it is important to determine the optimal dosage of the drug.

3-methyladenine blocks autophagic protein degradation (Seglen and Gordon, 1982) by inhibiting the PI3Ks (Petiot et al., 2000). However, it should be kept in mind that this inhibitor (Blommaart et al., 1997) also interferes with other PI3K-dependent intracellular trafficking pathways (Mizushima, 2004; Punnonen et al., 1994). We used BafA1 to inhibit autophagy in many experiments. This inhibitor prevents the autophagosome-lysosome fusion by blocking the H^+ vATPase proton pump (Ohkuma et al., 1993). Evaluation of LC3 levels in the prescence of BafA1 indicate whether autophagy is upregulated or disrupted (as mentioned in the section above).

Puromycin was used in paper I to study the formation of cytoplasmic bodies. Treatment with puromycin causes premature chain termination of amino acids during translation, resulting in a large increase of prematurely terminated misfolded proteins that transiently accumulate in cytoplasmic bodies (named p62 bodies or ALIS; aggresome-like inducible structures) (Lelouard et al., 2004; Szeto et al., 2006).

Leptomycin B blocks exportin 1 (XPO1), a protein required for nuclear export of proteins containing a nuclear export sequence (NES) (Kudo et al., 1999). Leptomycin B treatment made it possible for us to adress the localization of Alfy and p62 to PML nuclear bodies (NBs) (Paper I).

In paper III we used the drugs ATRA and ATO to determine whether autophagy contributes to ATRA and ATO–mediated PML/RARA degradation in the APL cell line NB4. ATRA is known to cause disease remission in APL patients by acting on the transcriptional activity and cause granulocyte differentiation of APL cells (Melnick and Licht, 1999; Nasr et al., 2009). ATO, another effective agent used to cure APL, induce disease regression mainly by causing degradation of PML/RARA (Nasr et al., 2009). Arsenic-mediated proteolysis involve small ubiquitin-like modifier (SUMO)–ylation of PML/RARA.

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Originals publications

I

II

III

Errata

Title page

University of Oslo, Institute of Basic Medical Sciences
Faculty of Medicine, University of Oslo

Change to

University of Oslo, Institute of Basic Medical Sciences
Faculty of Medicine

Page 53 (line 7-10)

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