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Regulation and function of autophagy in stress resistance and under pathophysiological conditions involves the co-chaperone BAG3

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Hiermit versichere ich, die vorliegende Dissertation selbständig verfasst und ausschließlich unter Verwendung der angegebenen Quellen und Hilfsmittel angefertigt zu haben.

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Vanessa Felzen

„Der Geist ist die Quelle aller Vewirrung"

Buddha

In Gedenken an Oma

Danksagung

Table of content

A. **Summary** **1**

B. **Introduction** **2**

B.1. Protein degradation pathways 2

B.1.1. The ubiquitin/proteasome system 3

B.1.2. Autophagy 4

B.1.2.1. Autophagy Initiation by mTOR and AMPK 6

B.1.2.2. Regulation of autophagy nucleation by the PI3K/BECN1 complex 8

B.1.2.3. Two ubiquitin-like conjugation systems directing autophagosome maturation 9

B.1.2.4. Selective Autophagy 11

B.1.3. HSC/HSP70 chaperones and their co-chaperones of the BAG protein family 13

B.1.4. Autophagy function in pathophysiology 19

B.1.4.1. Autophagy in breast cancer 20

B.1.4.2. Autophagy in neurons and neurodegenerative disease 21

B.2. Aim of the project: 24

C. **Results** **25**

C.1. Impact of autophagy in estrogen receptor-dependent stress resistance and breast cancer 25

C.1.1. Regulation of caspase 3 and BAG proteins in stress-resistant cancer cells 25

C.1.1.1. Increased survival of ERα expressing cells 26

C.1.1.2. Increased expression of BAG1 and BAG3 and reduction of Caspase 3-specific BAG3-cleavage products in the presence of ERα 29

C.1.2. The regulation of autophagy in stress-resistant cancer cell 32

C.1.2.1. Estrogen receptor expression differentially regulates transcription of autophagy-related genes. 32

C.1.2.2. Estrogen receptor α induces non-canonical autophagic activity 36

C.1.2.3. Increased autophagic flux is independent of ERE-mediated transcriptional activity of estrogen receptor α 46

C.1.2.4. Inhibition of autophagy reduces resistance of estrogen receptor α expressing cells to oxidative stress. 48

C.1.2.5. Knockdown of BAG3 reduces autophagic flux and contributes to a partial reversal of stress resistance in ERα-expressing cells. 50

C.2. The role of the autophagic protein degradation system in neuronal stress adaptation 55

C.2.1. Autophagic flux and autophagy is increased in hippocampal cells adapted to oxidative stress 55

C.2.1.1. BAG3 expression is increased in hippocampal cells exposed to chronic oxidative stress....57
C.2.1.2. The co-chaperone BAG1L is downregulated in resistant cells....57
C.2.1.3. Autopagy induction is independent of canonical mTOR regulation, Atg7 and Atg4 protein expression but displays a BECN1 protein upregulation....58
C.2.1.4. Lysosomal rearrangement in hippocampal cells adapted to oxidative stress is induced by BAG3 overexpression....61
C.2.1.5. Proteinhomeostasis is altered after traumatic brain injury (TBI) *in vivo* 62
D. **Discussion 66**
D.1. ERα regulates apoptosis and autophagy providing cytoprotection 66
D.1.1. ERα expression leads to the repression of caspase 3 and high levels of BAG1 and BAG3 on protein level....66
D.1.2. Impact of autophagy in estrogen receptor-dependent stress resistance and breast cancer 67
D.1.2.1. ERα induces a non-canonical autophagy pathway....70
D.1.2.2. ...independent of AMPK signaling and mTOR 71
D.1.2.3. Non canonical autophagy depends on BAG3, is regulated by DRAM1 and occurs with high levels of WIPI1, a known interactor of ERs....74
D.1.2.4. BAG3 is a novel mediator of ERα-induced autophagy and triggers oxidative stress resistance 75
D.1.2.5. Non-canonical autophagy markers, including BAG3 are novel targets for therapy of ER-postive breast cancer 78
D.2. Neuronal autophagy in oxidative stress conditions 80
D.2.1. Oxdative stress induced autophagy with high levels of BAG3 is continually mTOR independent: a general mechanism in BAG3-mediated autophagy?! 80
D.2.2. Beclin1 is strongly regulated in hippocampal cells and might contribute to cytoprotective autophagy 81
D.2.3. BAG3 induces a rearrangement of the lysosomal compartment 82
D.2.4. A switch from BAG1-proteasomal degradation to BAG3-mediated selective autophagy: the way to oxidative stress resistance in neurons in vitro...and in vivo? 82
E. **Materials and methods 87**
E.1. Cell culture: 87
E.2. Transfections: 87
E.3. Western blot analysis: 88
E.4. Immunocytochemistry: 89
E.5. Antibodies: 90
E.6. Transmission electron microscopy/ Glutaraldehyd-Osmium-fixation: 90
E.7. Human Breast cancer tissue: 90

E.8. PCR, Reverse Transcription PCR and quantitative real-time PCR: 91
E.9. ERE-Luciferase-Assay:....92
E.10. Survival experiments by Fluorescence Activated Cell Sorting (FACS): 92
E.11. Animal experiments 92
E.11.1. LC3EGFP mice....93
E.11.2. Statistical analysis: 94
F. **References 97**
G. **Appendix 111**
G.1. Publication output....111
G.1.1. Publications in journals....111
G.1.2. Distribution to Meetings 111
G.2. curriculum vitae 112
G.3. Abbreviations....113
G.4. Results of the autophagy gene array....115

A. Summary

Autophagy is a dynamic cellular process, which is indispensable to maintain protein homeostasis by degradation of unfolded, misfolded or aggregated proteins. Hence, it was reported that onset as well as progression of different diseases are regulated by autophagy. For instance autophagy facilitates stress resistance in cancer cells and is found to be activated by oxidative stress in neuronal cells. Recently, a new selective pathway of autophagy has been described: BAG3-mediated selective macroautophagy, which was also found to be induced in the paradigm of oxidative stress suggesting a role for cytoprotection. In this thesis autophagy was investigated in two different models of stress resistance *in vitro* and *in vivo* in order to analyze a potential role of autophagy in cytoprotection and the genesis of stress resistance, and subsequent to get further insights about BAG3 respectively.

The first part of the thesis was based on ERα-positive breast cancer cells, characterized by its resistance to different stressors. It is well known, that breast cancer cells can develop endocrine resistance and resistance to anti-hormone therapy and this can be facilitated via the autophagy pathway, but so far the description of a detailed autophagy expression profile of ERα-positive cancer cells is missing. In this work especially ERα expressing cells have been found to be highly resistant to oxidative stress. Furthermore we could show that ERα-expressing cells have a higher autophagic activity than cells expressing ERβ and cells lacking ER expression. Additionally, for autophagy-related gene expression we describe an ERα-specific "*autophagy-footprint*" that is fundamentally different to tumor cells expressing ERβ or lacking ER expression. This newly described ERα-mediated and estrogen response element (ERE)-independent non-canonical autophagy pathway, which involves the function of the co-chaperone BAG3, is independent of classical mammalian target of rapamycin (mTOR) and Phosphatidylinositol 3 Kinase (PI3K) signaling networks and provides stress resistance in the employed model systems. Strikingly we detected higher autophagy markers LC3, p62/SQSTM and BAG3 in ERα positive breast cancer tissues supporting our *in vitro* findings. Altogether, our study uncovers a novel non-canonical autophagy pathway that might be an interesting target for approaches of personalized medicine and treatment of ERα-positive breast cancer cells that do not respond to anti-hormone therapy and classical autophagy inhibitors.

The second study of this work concentrates on neuronal autophagy in the context of oxidative stress resistance. Neurons are highly vulnerable to disturbed proteostasis in particular as they are post-mitotic and develop neurodegenerative disease if become unbalanced. Therefore, autophagy might come into play to keep protein quality control (PQC) in stress conditions. The focus in this section was an *in vitro* model of hippocampal cells adapted to oxidative stress and was transferred to an *in vivo* model for traumatic brain injury, where oxidative stress is causatively linked to the pathophysiologic outcome. Hippocampal cells exposed to oxidative stress revealed autophagy and BAG3 strongly regulated whereas proteasomal degradation-related BAG1 was downregulated. Furthermore autophagy induction was again found to be independent on mTOR, but includes BECN1 regulation, underlining the function for BAG3-mediated autophagy rather in PQC then for nutrient supply. Additionally we could also transfer our results in an *in vivo* mouse model for traumatic brain injury, called controlled cortical impact (CCI), where we detected high levels of BAG3 and autophagy in ipsilateral brain areas after head trauma, where it might diminish proteotoxic stress and facilitates survival.

These findings indicate that BAG3-mediated non-canonical autophagy may act as a general adaptation and cytoprotective mechanism that point to a major role of the process in pathophysiological conditions. Thinking of new treatment approaches BAG3 is a promising target to inhibit autophagy in cancer cells, but on the other hand to induce autophagy in neuronal cells coping with neurodegenerative disorders.

B. Introduction

Vertebrates are based on complex organs, defined by various cell types, which enable, with their interaction and their complex organization, life.

Essential molecular components of cellular life are proteins, which direct various cell functions. The protein entirety, termed the proteome, has to be kept tightly balanced by protein homeostasis (proteostasis). Proteostasis maintains the integrity of the proteome under physiological as well as stress conditions. Therefore, fine-tuned protein quality control (PQC) pathways have evolved that regulate the balance between synthesis, folding, refolding and degradation of the proteins (Balch, 2008).

PQC is supported by a network of molecular chaperones, involved in protein folding and protein degradation pathways: the ubiquitin-proteasom-system (UPS) and the autophagosomal-lysosomal pathway (Hipp et al., 2014).

The evolutionary conserved eukaryotic lysosomal degradation pathways, called autophagy, are one of the main mechanisms supporting cellular proteostasis by clearance of aggregation-prone proteins and defective organelles. Recently, dysfunctions in the autophagic pathways have been implicated in different pathophysiological processes ranging from neurodegenerative disease to cancer (Jiang and Mizushima, 2014). Clarifying the role of autophagy in more detail is of great relevance in understanding the mechanisms behind the appearance of pathophysiological states.

B.1. **Protein degradation pathways**

Protein homeostasis is only guaranteed when there is a balance between building and degradation of proteins. Two main protein degradation pathways have evolved within the cell: the ubiquitin/proteasome system on the one hand and autophagy on the other. These two pathways differ in their structure as well as in their substrates and mechanism, both are essential for the cell.

B.1.1. The ubiquitin/proteasome system

The proteasome is a 2000 kDA complex consisting of two 19S (Svedberg) regulatory lid complexes and a 20S central proteolytic complex. The barrel-shaped center is composed of four rings consisting of two outer α-domains and two inner β-subunits. The 19S-subunits unfold the tertiary structure of the substrate under ATP hydrolysis, enabling the substrate to enter the central complex of the proteasome. The two inner β-rings show proteolytic activity and cleave the substrate into short polypeptides, which are released into the cytoplasm (Groll et al., 1997; Hendil et al., 2009; Murata et al., 2009).

Polyubiquitination of a given substrate acts as a signal for proteasomal degradation and is recognized by the two outer lid complexes. Ubiquitin-receptor proteins are also able to bind proteasomal substrates and get recognized by the lid complex of the proteasome themselves via their ubiquitin-like domains (UBL). Their substrate is then transferred to the proteasomal machinery and the receptor proteins get released after binding (Elsasser and Finley, 2005). Favored substrates of the proteasome are short half-life proteins whereas long half-life proteins are thought to be predominantly degraded by autophagy. Meanwhile under certain conditions reciprocal compensations between the proteasome and autophagy have been demonstrated and imply that the substrate principle is less rigid than once thought (Cuervo, 1998; Fuertes, 2003; Gamerdinger et al., 2009a)

Ubiquitin is an 8 kilo-Dalton (kDa) highly conserved and ubiquitously expressed protein (Kirkin and Dikic, 2007). Ubiquitin can bind directly to proteins through a glycine residue at its C-terminus. This reaction is catalyzed by a cascade of enzymes, activating (E1), conjugating (E2) and ligating (E3) ubiquitin to its target protein (Sowa and Harper, 2006). Polyubiquitin chains can be formed by isopeptide linkages at the seven different Lysine residues of Ubiquitin, whereby another ubiquitin is linked through its glycine at the C-terminal end of the molecule. Polyubiquitin chains of at least four ubiquitin units linked to a protein are sufficient to start sensing degradation (Thrower et al., 2000). Polyubiquitination is triggering the degradation of proteins through different pathways whereas K48-linked-polyubiquitination triggers proteasomal degradation and K63-linked-polyubiquitinated proteins are preferred to be degraded via the autophagosomal pathway (Tan et al., 2008; Thrower et al., 2000).

B.1.2. Autophagy

The word autophagy has its roots in the ancient Greek language meaning self-eating. In 1963, Christian de Duve used it for the first time in a biological context describing single or double membraned vesicles digesting cytoplasmic material (De Duve, 1963; Ravikumar et al., 2010a). Today, the term autophagy summarizes a set of evolutionary conserved lysosomal degradation pathways in which cytoplasmic components, including whole organelles (mitochondria, peroxisomes and endoplasmic reticulum) and macromolecules (proteins, glycogens, lipids and nucleotides) are transported to the lysosomes and get cleared by lysosomal hydrolases. Lysosomes are acidic organelles with pH 4.5-4.8 that provide proteases, carbohydrases, lipases and nucleases, which are all digestive enzymes that are distinguished by their acidic work optimum. Autophagy occurs at basal level but can be stimulated by different stressors like starvation, oxidative stress and pharmacological compounds (Mizushima et al., 2008). It was believed that the autophagosomal process is mainly unselective for a long time, but recent studies revealed that autophagy is not just constituted of bulk degradation but also has very selective characteristics (Sica et al., 2015). How and which signals exactly trigger selective or bulk degradation exactly, is still up for debate. Increasingly different roles of autophagy have been encountered qualifying it as a main pathway for maintaining cellular homeostasis. Under nutrient deprivation autophagy liberates energy supply through the increased degradation of proteins and lipids (Klionsky and Codogno, 2013). Caloric restricted rats show increased autophagy and furthermore extended life span (Donati et al., 2001). Immune reactions can also be supported by autophagy through restricting the propagation of pathogens. For instance *Salmonella enterica* gets enclosed by autophagosomes and degraded by lysosomes (Wild et al., 2011) (Levine and Deretic, 2007). Protein homeostasis is furthermore supported by autophagy through its capacity to diminish misfolded proteins as well as damaged organelles, which occur in cell stress situations in particular (Rubinsztein, 2010). Hence, these characteristics of autophagy are crucial adaptation processes for the cell to guarantee survival.

Three distinct types of autophagy have been specified: macroautophagy, microautophagy and chaperone-mediated autophagy. In the case of chaperone-mediated autophagy substrates are tagged by a pentapeptide motif with a KFERQ consensus sequence, which is recognized by the heat shock protein 70 (HSC70). HSC70-cargo

complex binds to the lysosomal membrane-associated receptor (LAMP2A), that directs unfolding and translocation across the lysosomal membrane (Cuervo and Wong, 2014). Microautophagy is the direct engulfment of cytoplasmic components by lysosomes. The substrate can be trapped in a boundary membrane by autophagic tubes, followed by the direct invagination through the lysosomal membrane (Benbrook and Long, 2012; Li et al., 2012). The most extensively studied of the three autophagy forms is called macroautophagy, which is described in detail in the next sections because of its great relevance in this thesis.

In the case of macroautophagy, hereafter referred to as autophagy, double-membraned vesicles, called autophagosomes, are formed. Autophagosomes engulf cytoplasmatic organelles and proteins and deliver them to lysosomes for degradation. For the two characteristics of recycling to guarantee energy supply and degradation to support PQC, autophagy plays an essential role in maintaining homeostasis (Tsukada and Ohsumi, 1993) (Mizushima and Komatsu, 2011). Autophagy responds to environmental cues via a variety of factors that mainly belong to homologues of autophagy-related genes (ATG's). ATG`s where originally identified in yeast (Ravikumar et al., 2010c) but ATG orthologs were found also in mammals and display similar roles. ATGs and homologs have been grouped according to their function in the autophagy pathway. The autophagy pathway could be subdivided roughly into four steps: 1. autophagy initiation 2. nucleation of a phagophore 3. elongation of the autophagosome 4. maturation and fusion with the lysosome (autolysosome), which are tightly regulated and initiated by the cross talk of several key players.

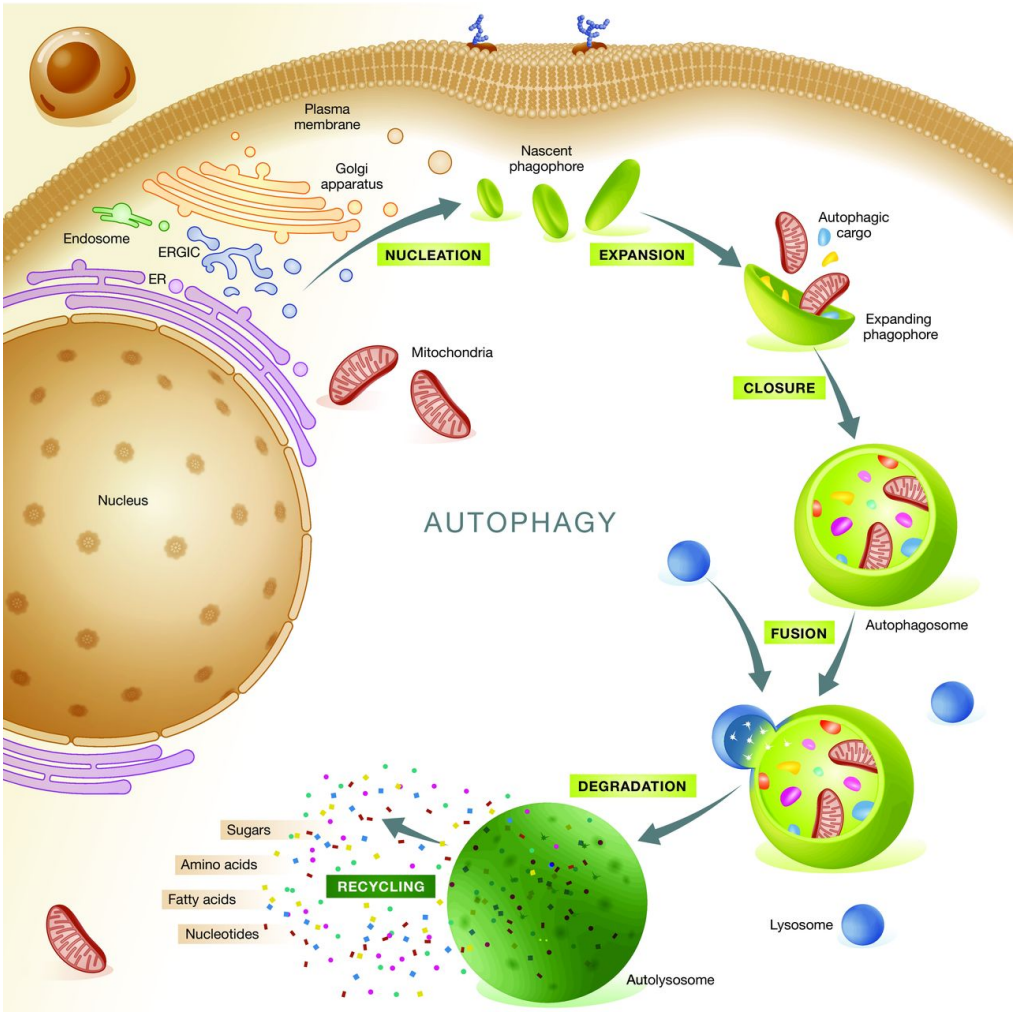

Figure 1: **Overview on macroautophagy:** In macroautophagy out of a phagophore a double membraned vesicle is formed (nucleation), called autophagosome, which engulfs defect organelles and proteins(Expansion; Closure). Furthermore the autophagsomes fuse with lysosomes (Fusion) to form an autophagolysosome where the cargo gets degraded by lysosomal hydrolases (Degradation) and the resulting macromolecules are released back into the cytosol by permeases. (Galluzzi et al 2015)

B.1.2.1. Autophagy Initiation by mTOR and AMPK

Two upstream major regulators in the control of autophagy are the mammalian target of rapamycin complex 1 (mTORC1) that negatively regulates autophagic activity and the AMP activated protein kinase (AMPK) which positively stimulates the autophagy pathway. The Serin/Threonin kinase mTOR is acting in complex either with the proteins Raptor or Rictor. The complex important for autophagy exists of Raptor and mTOR, termed mTOR complex 1 (mTORC1) (Rosner et al., 2010). If nutrient supply is sufficient, AMPK is inactive, whereas mTORC1 is hyperactivated and inhibits in turn autophagy (Figure2). mTORC1 acts by inactivating the ULK-complex (UNC-51-like kinase 1(ULK-1) and ATG13) a downstream activator of autophagy (Hosokawa et al., 2009). Under nutrient deprivation and other autophagy favoring conditions, AMPK gets activated and

phosphorylates tuberous sclerosis 2 (TSC2) as well as Raptor, two inhibitors of mTORC1 (Inoki et al., 2002; Kim et al., 2011). Thereby, the interaction of mTORC1 (mTOR/Raptor) and ULK1 is decreased and the interaction of ULK-1 and AMPK takes place, in which AMPK catalyzes the activating phosphorylation of ULK1 (Egan et al., 2011) and autophagy gets finally activated. Beside this, AMPK can further induce autophagy by the phosphorylation of beclin1 (BECN1) a main activating component of the autophagy machinery (Chang and Neufeld, 2009; Russell et al., 2013).

The ULK1 complex consists of ULK-1 and ATG13, ATG101 and Fak family kinase-interacting protein 200kDa (FIP200). If activated, this complex mediates the induction of autophagosome formation by interacting with the class III phosphatidylinositol 3-kinase (PI3K)/BECN1 complex (PI3KC) that is required for nucleation of the autophagosomal membrane (phagophore).

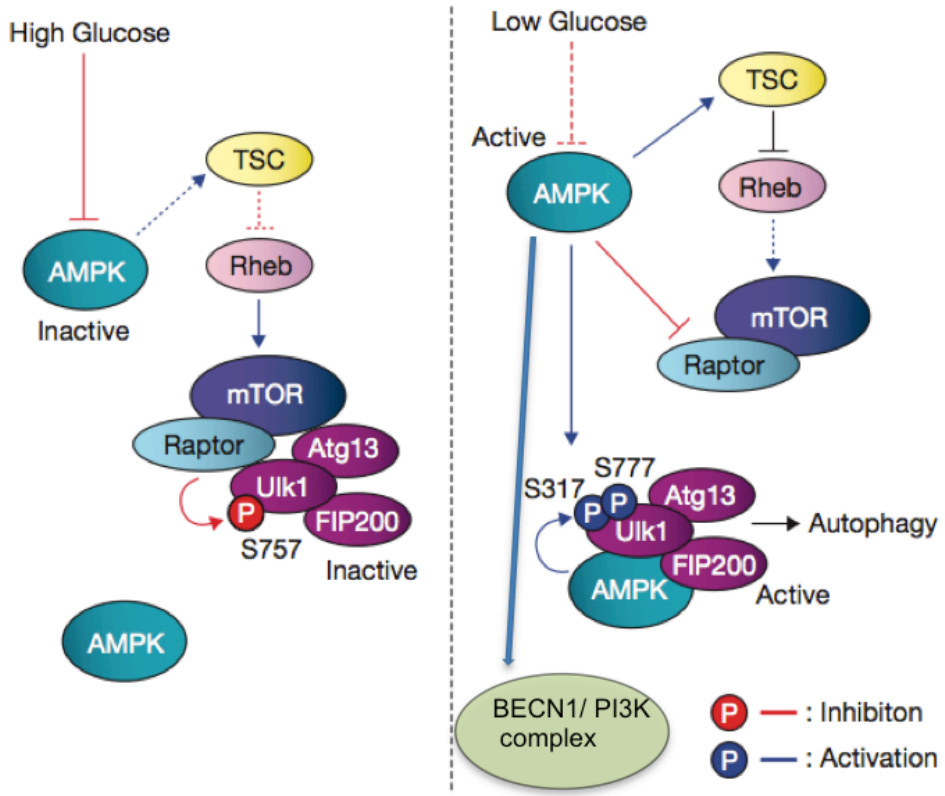

Figure2: mTORC1 (mTOR/Raptor-complex) and AMPK are main regulators of autophagy induction. Left site: AMPK is inactive when glucose is sufficiently present wheras mTORC1 is in its active form. In this situation mTORC1 phosphorylates ULK1 on Ser 757, which prevents the interaction of ULK1 with the AMPK. Right site: under nutrient restriction AMPK is active and phosphorylates TSC2 and Raptor, which leads to the inhibition of mTORC1. Accordingly phosphorylation of ULK1 on Ser 757 decreases and Ulk1 is

activated for the interaction with AMPK that phosphorylates Ulk1 on Ser 317 and Ser 777. ULK1 activation is followed by the initiation of autophagic machinery. Furthermore autophagy induction is induced by the activated AMPK through the activation of the BECN1/PI3K complex. (Adapted from Kim et al. 2011)

B.1.2.2. Regulation of autophagy nucleation by the PI3K/BECN1 complex

Phophatitylinositol-3-phophat (PI3P) is required for the very first step of the initiation and nucleation of the first recognizable autophagosomal structure, the isolation membrane, called phagophore, in its early and open state (Devereaux et al., 2013; Klionsky and Codogno, 2013). In contrast to yeast (*S. cerevisiae*) where the autophagosomes are generated at the phagophore assembly site (PAS) (Itakura and Mizushima, 2010) the origin of which mammalian autophagosomes arise is still under controversial debate that may expect multiple sources of the membrane (Axe et al., 2008; Carlsson and Simonsen, 2015; Hailey et al., 2010; Hayashi-Nishino et al., 2009; Karanasios and Ktistakis, 2015; Ravikumar et al., 2010a). For example the endoplasmatic reticulum, forming so called omegasome, which are positive for the transmembrane protein ATG9 are highly discussed. Building and transport of membrane vesicles is controlled by a variety of proteins, including Ras-related in brain (Rab)-Proteine, whereby our working group could show that Rab3GAP1/2 regulates autophagosome builiding (Spang et al., 2014). The recruitment of PI3P to the initiation sites of autophagosomes depends on a macromolecular PI3K-complex. This complex countains a class III PI3PK (also known as vacuolar protein sorting: Vps34) producing PI3P (Kim et al., 2013). Furthermore, the complex consists of BECN1 (ATG6), which directly enhances the kinase activity, by acting as a scaffold protein for PI3PK and its co-factors. Moreover, the autophagy/BECN1 regulator 1 (AMBRA1) and ATG14/barkor as well as Vps15/p150 are building up the core complex (He and Levine,2010). If BECN1 is in its inactive form it is bound to two apoptosis regulators BCL2 (B cell CLL/ lymphoma 2) and BCL-XL (BCL2-like protein). In that state BECN1 could not provide further activation of the profound PI3K activity leading to the repression of autophagy induction. If the situation in the cell change and autophagosomal structure is needed, BECN1 gets displaced from the inhibitory interaction partners and drives autophagosome biosynthesis (Pattingre et al., 2005; Sica et al., 2015). Different regulators starting this event are known, including MAPK8 (mitogen-activated protein kinase 8), also called JNK1, DAPK1 (death associated protein 1) (Ravikumar, 2010) as

well as the “BH3-only” proteins (Maiuri et al., 2007). In addition, recent studies discovered further posttranslational modifications regulating BECN1 (Sun et al., 2015; Wang et al., 2012). Commonly used autophagy inhibitors, 3-methyladenine (3MA) and wortmannin are targeting the BECN1 complex, as they are direct inhibitors of PI3K (Ravikumar et al., 2010b).

The detection of PI3P at the nascent phagophore is followed by its binding to DFCP1 (Double FYVE-containing protein 1) and WIPI (WD repeat domain phosphoinositide interacting 1-3) (Axe et al., 2008). Not until the detection of these early autophagosomal markers, the microtubule-associated protein 1 light chain 3 (MAP1-LC3), a marker for matured autophagosomes is recruited at the omegasome, which is afterwards released into the cytoplasm forming the autophagosome (Karanasios and Ktistakis, 2015).

B.1.2.3. Two ubiquitin-like conjugation systems directing autophagosome maturation

Membrane expansion is carried out by two ubiquitin-like conjugating systems. The first one is the ATG12-ATG5-Atg16 conjugation system, which is formed with the help of ATG7 (E1 ubiquitin activating enzyme-like) and ATG10 (E2 ubiquitin conjugating enzyme-like) and is essential for phagophore expansion The complex dissociates upon full maturation of the autophagosome. The second conjugating system, assisting in elongation and maturation of the phagophore, includes LC3 (ATG8) a small ubiquitin-like modifier (UBL). The autophagosomal membrane protein is a known Atg8 family member that binds to autophagy receptors (e.g. p62/SQSTM: sequestosome1) tagged with substrates, and directly links cargo to autophagosomal degradation. Therefore LC3 has a main function in selective sequestration of proteins in autophagosomal degradation. Precursor LC3 is cleaved by ATG4B at its COOH terminus to form cytosolic LC3-I. Next, ATG7 and ATG3 accelerate the conjugation of phosphatidylethanolamine (PE) to LC3I to form the membrane associated LC3-II (Kabeya et al., 2000; Tanida et al., 2004), a process also facilitated by the ATG12–ATG5 and ATG16L1 complex (Saitoh et al., 2008). LC3-II on the outer membrane is most likely delipidated and recycled by ATG4 whereas LC3-II at the inner membrane gets degraded after fusion. Hence, LC3II serves as a useful marker for autophagosomes, as they contain LC3II until they are finally degraded upon fusion with lysosomes (Jiang and Mizushima, 2015; Klionsky et al., 2012;

Mizushima et al., 1998; Mizushima et al., 2003; Rubinsztein et al., 2015). All the before mentioned hierarchical regulators of autophagy are needed to induce the so-called "canonical autophagic pathway" and are is summarized below (Figure 3).

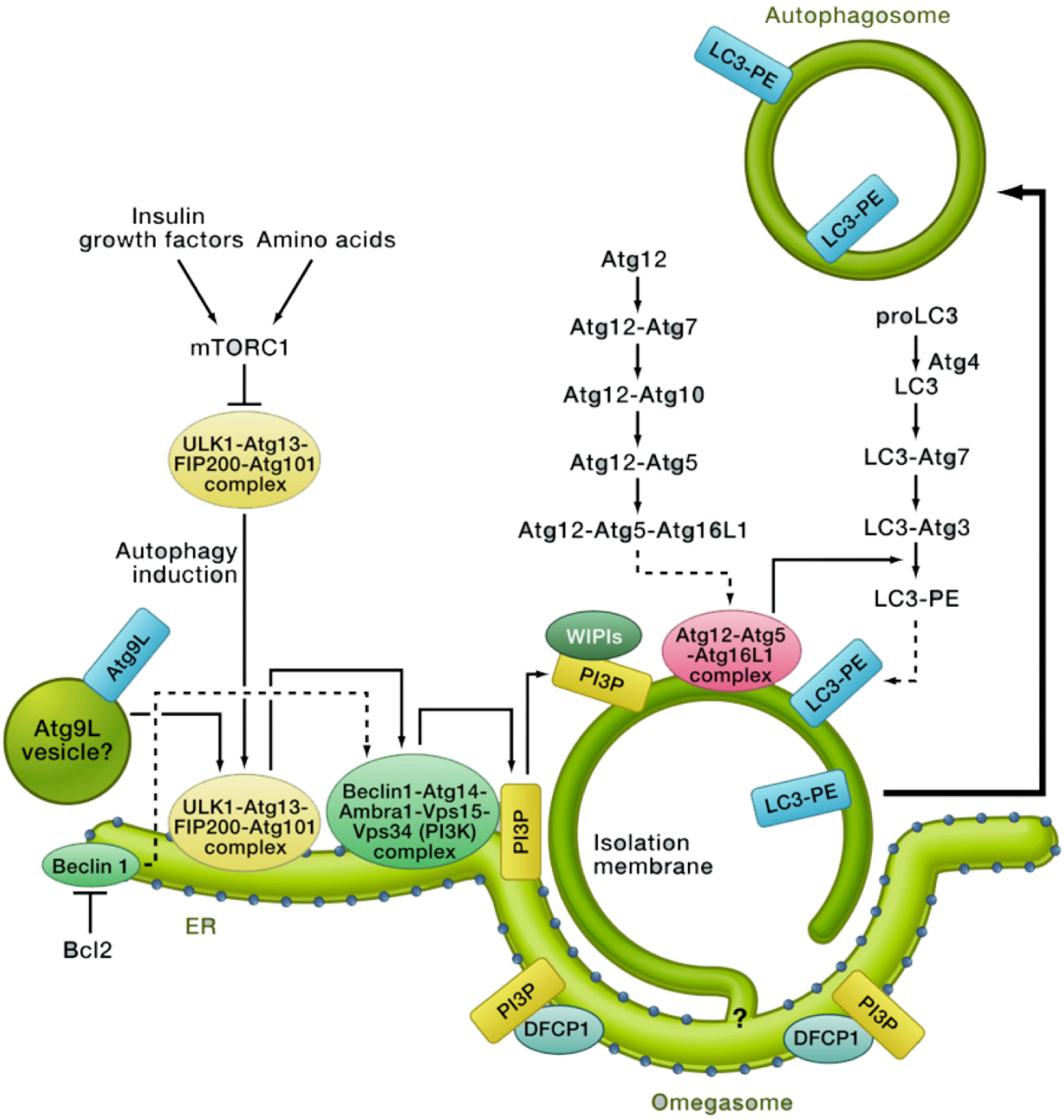

Figure 3: Overview on canonical autophagy: Autophagosomal vesicle biogenesis is regulated tightly by interconnected protein complexes. For the initiation step Atg9-vesicles, the ULK complex, the BECN1/PI3K complex WIPIs as well as DFCP1 are of particular importance. Elongation and maturation of the autophagosomal membrane is furthermore regulated by two ubiquitin-like conjugation systems Atg12-Atg5-Atg16L-complex and LC3-PE. (Adapted from Mizushima and Komatsu,2011)

After the full maturation, autophagosomes can fuse subsequently with lysosomes to form autolysosomes. The trafficking of autophagosomes is bidirectional along microtubule- dynein-dynactin motor complexes with a tendency towards a perinuclear region enriched with lysosomes, the microtubule-organizing center (MTOC) (Ravikumar et al., 2005; Ravikumar et al., 2010b) (Kimura S, 2008). At the MTOC autophagosomes may fuse conclusively with lysosomes whereupon the degradation of the delivered cargo by digestion enzymes starts. After the breakdown of the contents, the processed products are released through permeases back to the cytosol (Feng et al., 2015).

Non-canonical autophagy pathways have recently been discovered that differ from canonical signaling, as they do not necessarily require the hierarchical action of the above-mentioned ATG proteins and protein complexes (Codogno et al., 2012a). For example, BECN1-independent or mTORC1- and ULK1 complex-bypassing non-canonical autophagy routes have been described (Hiebel et al., 2014; Scarlatti et al., 2008a; Scarlatti et al., 2008c). Although these discrete pathways differ in their upstream signaling cascades they also finally lead to the fusion of autophagosomes with lysosomes and to the degradation of material in this acidic compartments.

B.1.2.4. Selective Autophagy

Many studies suggest that in situations of nutrient deprivation the autophagic response occurs unselectively, providing metabolic substrates for survival from degradation of variable cell components and proteins. On the other hand autophagy could occur in a very selective manner targeting specific substrates, for example in response to perturbations of homeostasis, such as oxidative stress, where specific intracellular damaged components have to get cleared precisely via special autophagy adaptors and receptors (Okamoto, 2014; Sica et al., 2015)(Sika&Galluzzi 2015.Okamoto 2014).

At least more than 20 autophagy receptors have been described until now, linking the cargo to the autophagosomal machinery (Figure 4). Thereby, the receptors recognize molecular determinants, unfolded regions of a protein as well as posttranslational modifications like ubiquitination, arginylation and acetylation (Cha-Molstad et al., 2015; Jeong et al., 2009).

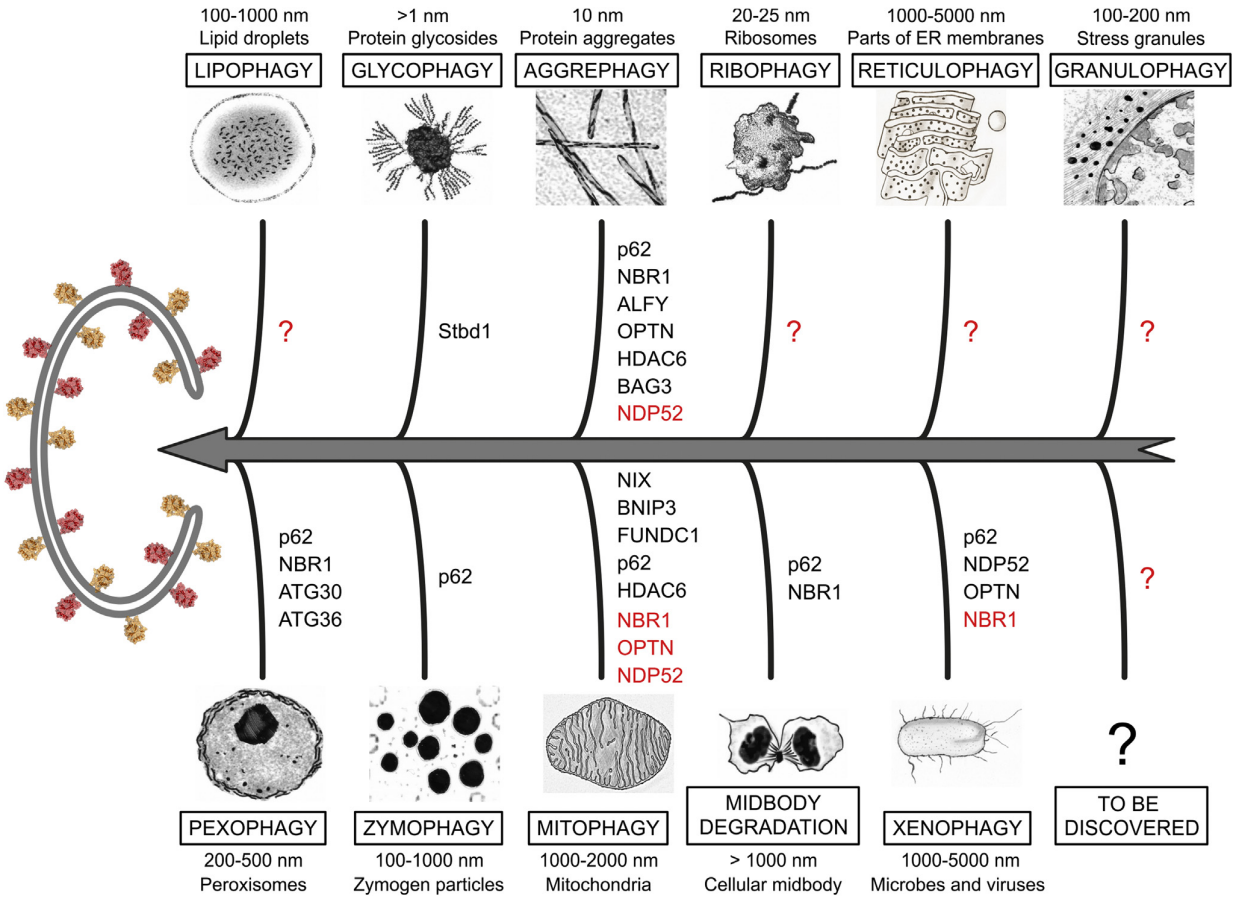

Figure 4: Selective autophagy in mammals. Different forms of selective autophagy have been detected in mammalian cells. Several types of cargo could be recognized, giving the name for the autophagy form (E.g. mitophagy; aggrephagy) and degraded with the help of specialized autophagy receptors and adaptors (established=black; putative=red). (Adapted from Rogov et al.,2014)

Ubiquitination is the best-described signal tethering degradation material. Thereby K63-linked-polyubiquitinated proteins are preferred to be degraded via autophagosomal pathway and are preferentially found to be conductive in aggresome formation (Tan et al., 2008; Thrower et al., 2000). Thus far ubiquitin dependent but also -independent receptor-mediated degradation pathways have been described. Some selective autophagy receptors, like p62/SQSTM1, recognize and bind to K63-linked Ubiquitin at the surface of cargo. Furthermore, autophagy receptors selfoligomerize and initiate autophagosomal building to promote the degradation process (Johansen and Lamark, 2011; Kirkin and Dikic, 2007; Kirkin et al., 2009). Besides that, autophagy receptors are able to bind to Atg8 protein family members, MAPLC3 and the GABARAPs/GATE16, on the surface of the autophagosomes, linking the cargo to the autophagosomal machinery (Slobodkin and Elazar, 2013). The conjunction of the receptors to the autophagosomal

structure is possible by defined protein structures, the LC3 interaction region, called LIR motif.

So far several autophagy-receptors besides p62/SQSTM have been described targeting ubiquitin-dependent and independent degradation signals. Among these, NBR1 (neighbor of BRCA1 gene 1) was found to act as a cargo receptor in cooperation with p62/SQSTM in autophagic clearance of ubiquitinated proteins. Besides autophagy receptors, which themselves get degraded in the same process, autophagy adaptors, including BAG3 (BCL2-associated athanogene 3), and auxiliary proteins are found as accessory factors but are not degraded by autophagy (Khaminets et al., 2016). Molecular chaperones and co-chaperones are critical actors in this process, which thereby promote protein homeostasis and are therefore discussed in the next section in detail.

B.1.3. HSC/HSP70 chaperones and their co-chaperones of the BAG protein family

Initially chaperones were found to counteract aggregation of non-native proteins under stress and denaturizing conditions such as nutrient deprivation, pathogenic infection as well as heat stress and therefore they were termed “heat shock proteins” (HSP) (Petersen and Lindquist, 1988; Scheuner et al., 2001). Molecular chaperones assist during *de novo* protein folding, drive protein transport across membranes and modulate protein-protein interaction by controlling conformational changes. Besides this, they are responsible for detecting unfolded or misfolded proteins, by recognizing, unstructured, hydrophobic regions of non-native proteins and transfer them to degradation systems (Hartl and Hayer-Hartl, 2002; Morimoto, 2008).

One of the best-studied chaperone families is called HSP70. One family member is HSC70, which is constitutively expressed in the mammalian cytosol. Otherwise, HSP70 is inducible and is kept at low expression levels under physiological conditions (Sherman and Gabai, 2015) and are hereafter named HSC/HSP70 complex. The cycle between an ATP and ADP bound state of HSC/HSP70 regulates substrate binding and release and many co-regulators of the HSC/HSP70 complex are known conducting the action of the chaperone (Figure 5). Mainly, these co-regulators are J-proteins (also termed HSPs40) and NEFs (nucleotide exchange factors) (Hennessy et al., 2005; Sondermann et al., 2001; Szabo et al., 1994).

BAG proteins (BCL2-associated athanogenes) BAG1-BAG6, initially found as anti-apoptotic proteins are found to act as NEFs and bind to the ATPase domain of HSC/HSP70. Hence, BAG proteins act as co-chaperones, that induce conformational changes and regulate nucleotide-, and subsequently, substrate-binding and release (Hartl et al., 2011; Kampinga and Craig, 2010; Lee, 1999). In the next section the focus is on the BAG protein family, since they build a link to different downstream cellular processes including autophagy.

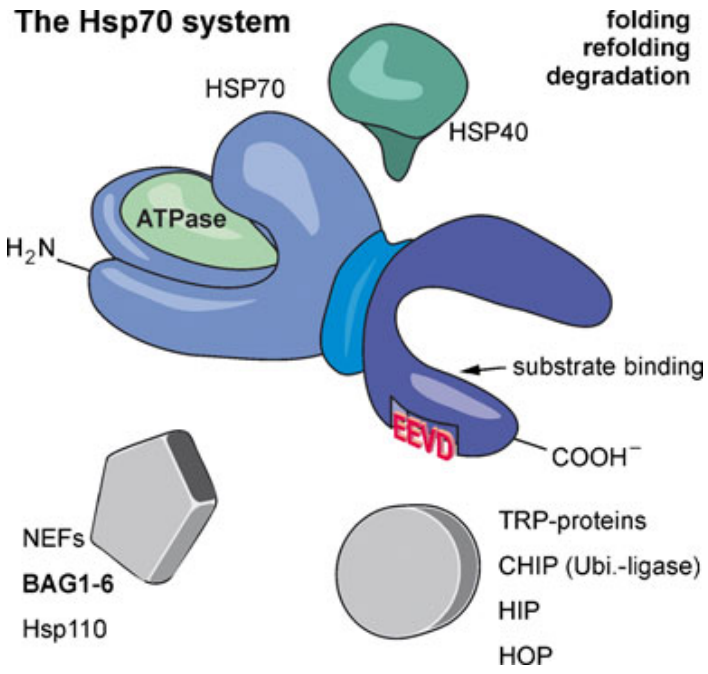

Figure 5: The molecular chaperone HSP70 and interaction partners. With the help of co-chaperones and co-regulators HSP70 chaperones are regulating protein folding, misfolding as well as protein degradation. The cycle between an ATP and ADP bound state regulates HSP70 substrate binding and release. The ATP consuming cycle is regulated by co-chaperones, which thereby controls the delivering of a given protein to different degradation pathways (Gamerdinger, Behl, Carra; 2009).

Genes of the BAG family are evolutionary conserved and present in yeasts (e.g. Saccharromyces cerevisiae), invertebrates (*Caenorhabditis elegans*, *Drosophila melanogaster*), plants (e.g. *Arabidopsis thaliana*) as well as in mammals (e.g. humans and mice) suggesting a crucial biological role (Takayama and Reed, 2001). Originally they were found as pro-survival and anti-apoptotic factors by the interaction with BCL2, one of the best known inhibitor of apoptosis under stress conditions. But BAG proteins function also in a variety of cell processes (e.g. cell signaling, transcription factors

regulation), which are important for cellular stress response as well as apoptosis, proliferation, migration, hormone action and autophagy (Doong et al., 2003; Doong et al., 2002; Rosati et al., 2011b).

BAG1-BAG6 are characterized by their evolutionary conserved BAG domain, which is located at the C-terminus. The BAG domain has approximately 110-124 amino acids and includes three antiparallel helices that enable the proteins to interact with and to bind to HSC/HSP70 chaperones (Briknarova et al., 2001). The fate of the substrate, which is bound to the HSC/HSP70 complex, depends on the involved co-chaperone, as BAG proteins are suggested to act competitively.

Through its BAG domain BAG1 binds to the ATPase domain of HSC/HSP70. Simultaneously it is also able to bind directly to the proteasome with its ubiquitin-like domain (UBL) coupling HSC/HSP70 substrate degradation to the proteasome (Gamerdinger et al., 2009a; Luders et al., 2000). Thereby it is likely that BAG1 as a nucleotide exchange factor induces substrate transfer from HSC/HSP70 to the proteasome. Additionally BAG1 is also found in chaperone-mediated autophagy and link HSC/HSP70 machinery to lysosomal degradation via the KFERQ sequence (Arias and Cuervo, 2011). On the other hand BAG3, also known as BIS/CAIR-1, stimulates macroautophagy through the interaction with two HSP/HSC70. These findings have been made when the reciprocal regulation from BAG1-dependent proteasomal degradation to BAG3-mediated macroautophagy during conditions of aging and stress was discovered and interpreteted as an adaptive physiological response of the cell (Gamerdinger et al.,2009). Within these studies the autophagy-adaptor protein BAG3 was found acting as an initiator of selective autophagy, whereupon a new pathway the "*BAG3-mediated macroautophagy"* was discovered (Figure 6) (Gamerdinger et al., 2009a; Gamerdinger et al., 2011a).

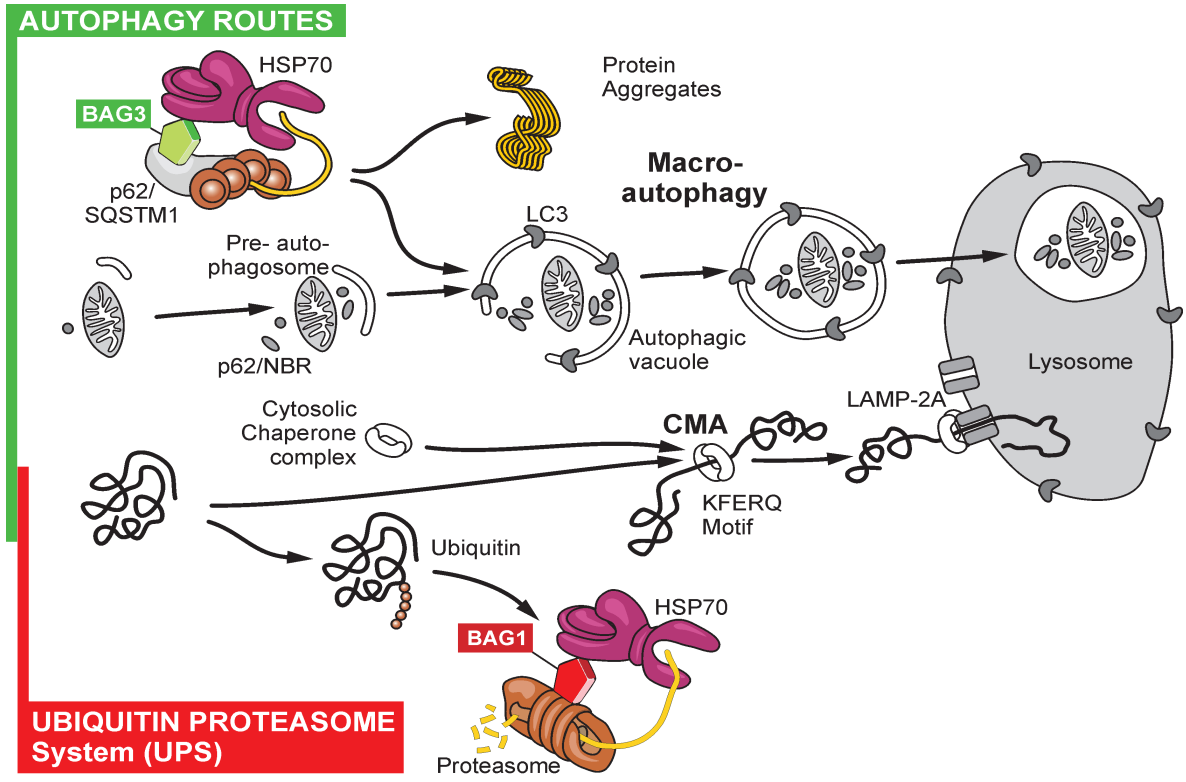

Figure 6: BAG1 and BAG3 regulating protein degradation pathways. The BAG proteins are involved in protein degradation regulation. For instance, BAG1-HSP70 complex delivers cargo to the proteasome, whereas BAG3-HSP70 complex favours auophagosomal degradation of a given HSP70 substrat. (Morawe et al., 2012)

In several studies it has been shown that this hypothecical BAG3-HSP70- small heat shock protein 8 (HSBP8) complex is responsible for the selective degradation of misfolded proteins via autophagy (Figure 7). HSBP8 was also found to clear misfolded proteins via autophagy (Crippa et al., 2010). In detail, BAG3 was described to recruit HSC/HSP70 to the autophagy receptor p62/SQSTM (Arndt et al., 2010; Gamerdinger et al., 2009a). This action is propagated by the direct physical binding of BAG3 to p62/SQSTM assuming a role for BAG3 in substrate delivery to the autophagosomal machinery. The interaction of p62/SQSTM and BAG3 furthermore enhances autophagosomal activity. BAG3-positive sequestration structures have been found to be p62/SQSTM positive, but as well positive for ubiquitinated substrates (Gamerdinger et al., 2011a). The autophagy receptor p62/SQSTM is able to bind ubiquitinated substrates as well as the autophagosomal membrane protein LC3 (Pankiv et al., 2007). Through the direct interaction of p62/SQSTM with LC3, as well as the binding of BAG3, the BAG3-chaperone-substrate complex could directly be linked to the autophagosomal machinery and furthermore gets acces to the lysosomal degradation system.

Disease-related mutated proteins, like poly-Q-expanded huntingtin and the amyotrophic lateral sclerose (ALS) associated mutant Superoxid dismutase 1 (SOD1), where also found to get cleared by the BAG3-mediated autophagic pathway Interestingly, in the case of mutant SOD the BAG3-mediated degradation of the aggregates was not depending on ubiquitination (Gamerdinger et al., 2011a). Overexpressed mutant huntingtin-derived aggregates get also cleared by BAG3, while this characteristic of BAG3 was independent of the BAG domain but dependent on the PxxP region (Carra et al., 2009; Carra et al., 2008).

If misfolded proteins and protein aggregates are excessively formed, they will be able to build inclusion bodies. A special form of inclusion body, the aggresome, is formed through retrograde dynein-dependent transport of aggregates and degradation substrates to a region near the MTOC (Kopito, 2000). Aggresomes are places of high autophagosomal activity, where degradation substrates are linked spatially to lysosomes. Studies suggest that this reaction is triggered by the histone deacetylase 6 (HDAC6) coupled with dynein and microtubules (Iwata et al., 2005; Kawaguchi et al., 2003; Wang et al., 2015) and include the function of molecular chaperones and co-chaperones including BAG3 (Gamerdinger et al., 2009a; Gamerdinger et al., 2011a). BAG3 thereby binds directly to dynein via its PxxP domain and allows the active transport of substrates along microtubules via the dynein motor complex, directing them to the aggresome (**Figure 7**). Additionally the formation of aggresomes leads to significant reduction in toxicity as they are concentrated at one point and do not disturb cellular function at multiple locations (Rodriguez-Gonzalez et al., 2008).

In addition, BAG3 seems to promote muscle functions as its highest expression levels in mouse tissue are found in striated muscles and heart (Homma et al.,2006), where it co-localizes with z-discs. Mice with homozygous BAG3 disruption are growth retarded and die by week four after birth. They showed severe muscle defects resulting in a myopathy and displayed non-inflammatory myofibrillar degeneration with apoptotic features (Homma et al., 2006; Youn et al., 2008).

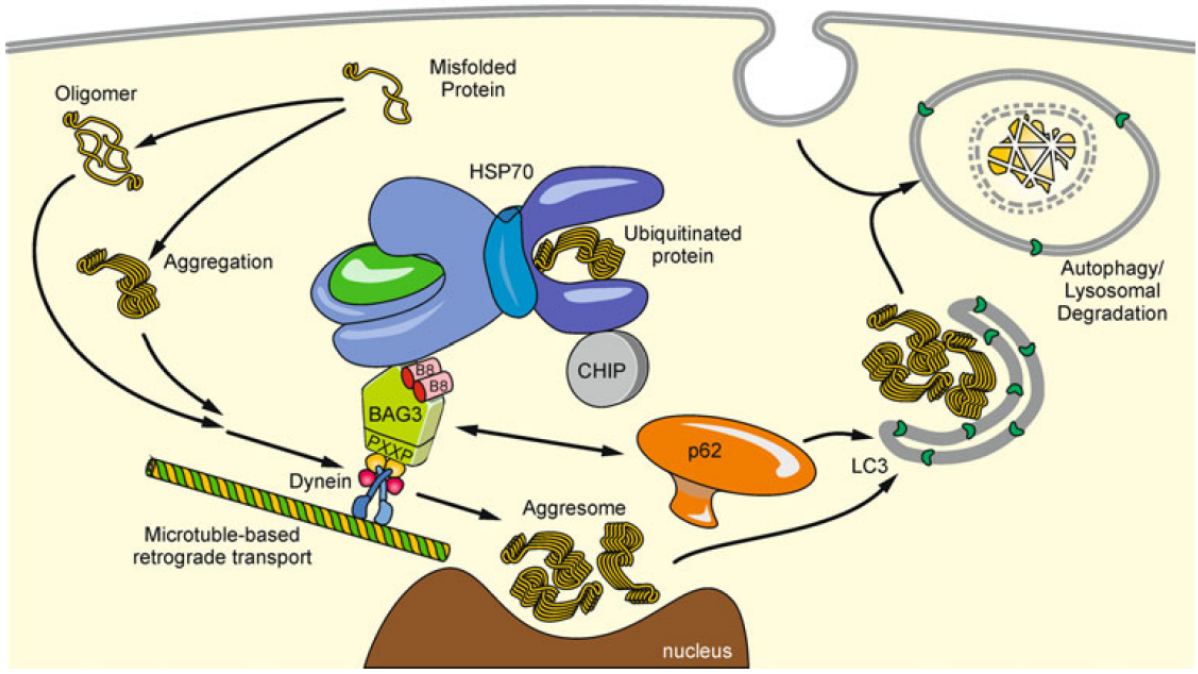

Figure 7: The putative BAG3-HSPB8-HSP70 complex. Molecular chaperones and co-chaperones are able to target misfolded and aggregated proteins and deliver them eather to the proteasome (not shown) or to the macroautophagic machinery for degradation. (Gamerdinger,Carra,Behl,2011)

Initially BAG proteins have been as found to interact with the anti-apoptotic protein BCL2, while *bag1* (BCL2-associated athanogene 1) was the first gene identified as an enhancer of the BCL2 anti-apoptotic activity(Takayama et al., 1995). Later on B-CLLs (B-cell chronic lymphatic leukemia) survival was found to be mediated via BAG3 (Romano et al., 2003). In various following studies focusing on tumor cells, BAG3 silencing was shown to be related to enhance drug induced apoptosis (Liu et al., 2009; Rosati et al., 2007). It was also described, that caspases are able to cleave BAG3 thereby supporting the apoptotic procedure (Wang et al., 2010). Further studies suggests that the interaction of BAG3 and HSP/HSC70 is also regulating cell survival by either modulating the degradation of apoptosis regulating-proteins or by competing with BAG1 and depressing protein delivery to the proteasome (Du et al., 2008; Rosati et al., 2007). Additionally, BAG3 is, with its proline-rich region (PxxP) as well as its WW domain, able to interact with several other proteins, which could facilitate the anti-apoptotic function (McCollum et al., 2010).

B.1.4. Autophagy function in pathophysiology

In line with the complexity of the so far described autophagy pathways are the numerous implications of autophagy in pathophysiological processes ranging from neurodegeneration to cancer (**Figure 8**). In almost all of the phenomena's, autophagy is described as a double-edged sword. It could have repressive function in the development and onset of different disease, which could switch into a supportive function for disease progression after disease onset (Mizushima et al., 2008). Some of these contrary functions in disease are summarized in the following illustration. In the next section the focus is on two pathophysiology conditions, relevant for the present work, where autophagy is ment to play a crucial role and is discussed also in therapy treatment: 1. breast cancer and 2. neurodegeneration.

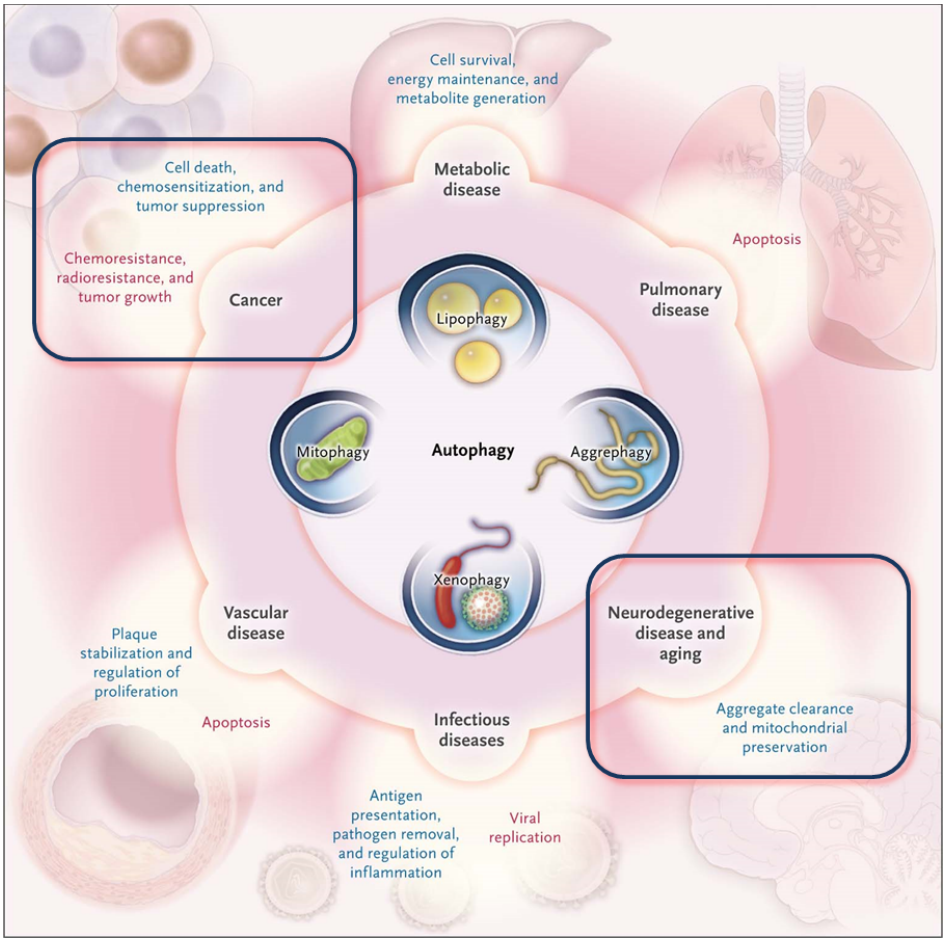

Figure 8: Autophagy is related to different human diseases. Autophagy can influence disease progression by the ability to either act as a beneficial mechanism thereby inhibiting disease progression (shown in blue) or by acting as a detrimental process and promoting disease progression (shown in red). Mainly autophagy plays a role in the clearance of dysfunctional protein aggreagtes and disease could evolve when this mechanism failed. Moreover in cardiac and lung disease, autophagy is related to cell death and proliferation whereas in cancers autophagy is a tumorsuppressor, but could support established tumors in cell survival and supports resistance to anticancer-therapy. Furthermore autophagy

is regulating the immune response and the clearance of pathogens (xenophagy) as well as it might contribute to metabolic disease as it was found to be involved in the lipid metabolism. (adapted from Choi AMK et al., 2013)

B.1.4.1. Autophagy in breast cancer

Early tumorigenesis and tumor maintenance as well as the effectiveness of therapeutic intervention were shown to be affected by autophagy (Apel et al., 2008; Berardi et al., 2011; Cheong et al., 2012; Degenhardt et al., 2006). Altered ATG protein expression and altered autophagic activity has been shown in a variety of cancer tissues ranging from glioblastoma stem cells to breast cancer cells. Breast cancer is one of the most leading causes of cancer-related death among women and a lot of effort has been made to develop new strategies of treating various forms of this cancer type and is in the focus of the present study.

Breast cancer is traditionally subdivided into three classes: (I) hormone receptor (estrogen and/or progesterone receptors) positive breast cancers that represent approximately 70-80% of all cases, (II) human epidermal growth factor receptor 2 (HER2) overexpressing cancers in approximately 10-15% of all cases and (III) the remaining 10-15% of breast cancers that are defined by hormone receptor and HER2 negativity (triple negative cancers) (Brendel et al., 2013; Cleator et al., 2007; Konecny et al., 2003).

Estrogen receptor (ER)-positive breast cancers could express the two structurally related receptors ERα and ERβ. Of these, ERα is the only ER that is detected by immunohistochemistry in breast cancer biopsies and is the predominant subtype expressed in breast tumor tissue as it primarily stimulates the growth of cancer cells (Dawson et al., 2009). Both receptors bind estrogen (17β-estradiol, E2) as a ligand and patients with ER-positive tumors are currently treated with anti-hormone therapy to inhibit ER signaling. In the first line, therapeutic intervention either uses anti-estrogens (AE, e.g. tamoxifen) interfering with ER signaling directly or blocking E2 synthesis with aromatase inhibitors. However, almost 40% of ER-positive breast cancers fail to respond to tamoxifen and tumor cells often develop resistance to anti-hormone therapy (Bieche et al., 2001). Although, there is much progress in understanding this disease the number of patients dying from breast cancer is not decreasing substantially. This emphasizes the need for alternative strategies or add-on concepts in treating breast cancer. Recently,

BAG3 was shown to diminish proteotoxicity via selective autophagy (Galavotti et al., 2013; Jo et al., 2012; Lorin et al., 2013; Rapino et al., 2014) and has been been shown to direct a selective form of autophagy. Furthermore, autophagy inhibitors (e.g. histone deacetylase 6 inhibitor ST80 or the lysosomal inhibitors chloroquine or hydroxychloroquine) sensitize cancer cells to anti-cancer therapy and therefore currently represent attractive therapeutic tools (Ma et al., 2004). Nevertheless, as breast cancer is a heterogeneous disease and the acquired resistance to anti-hormone therapy and the efficacy of autophagy inhibitors can vary from patient to patient personalized medicine and individual treatment concepts with a focus on detailed description of the autophagy pathway is needed.

B.1.4.2. Autophagy in neurons and neurodegenerative disease

The induction of autophagy is described to be organ and cell specific. Recent studies showed that autophagy could be induced by fasting not in every brain region, but at least in cortical neurons, Purkinje cells and hypothalamic neurons (Alirezaei et al., 2010; Kaushik et al., 2011). So there might be a very complicated role of autophagy for its metabolic need in the brain, whereas the role in PQC is rather obvious, because neurons are not dividing anymore as they are postmitotic and differentiated cells and therefore not able to clear accumulating components by cell division. Severe neurological deficits have been detected in mice lacking autophagy in the central nervous system as lack of coordination as well as loss of large pyramidal neurons in the cerebral cortex (Hara et al., 2006; Komatsu et al., 2006). All regions of autophagy deficient brains showed accumulation of p62/SQSTM positive inclusion bodies enriched with polyubiquitinated proteins. Purkinje cells with disrupted autophagy exhibit progressive axonal dystrophy and degeneration of axon terminals that is followed by cell death and behavioral deficits. These findings indicate a special role of autophagy for axonal homeostasis (Nishiyama et al., 2007).

In stress conditions, misfolding of proteins occurs more frequently and the successive overload of the compensatory mechanism of protein homeostasis could lead to protein aggregation. Accordingly, the appearance of protein aggregates is a pathological hallmark of neurodegenerative disease like ALS, Parkinson's disease (PD), Huntington's disease (HD) and Alzheimer's disease (AD) (Gundersen, 2010; Kopito, 2000; Morimoto,

2008; Selkoe, 2001; Shaw and Valentine, 2007). A central factor for the risk of developing neurodegenerative disease is thought to be oxidative stress. Especially the central nervous system is shown to be highly sensitive to oxidative stress that might be due to its high oxygen turnover and possibly due to the quantity of unsaturated fatty acids (Behl et al., 1997; Coyle and Puttfarcken, 1993; Moosmann and Behl, 2002; Uttara et al., 2009). Some of the ROS species are causing modifications of proteins by oxidation of amino acid residues side chains, resulting in protein-protein cross linking and oxidation of the protein backbone which leading to protein fragmentation, misfolding and aggregation.

Under physiological conditions oxidative stress is held in balance but chronic pathophysiological conditions as well as acute insults as stroke or traumatic brain injury cause oxidative damage. Indeed oxidative stress has also been causally linked to the appearance of neurodegeneration in chronic disease as AD, PD as well as it is a main trigger of cell death also following acute insults as traumatic brain injury (TBI) (Behl, 1997; Behl et al., 1994; Butterfield and Kanski, 2001; Kontos and Povlishock, 1986; Moosmann and Behl, 2002; Tyurin et al., 2000). Accordingly, TBI is discussed as a risk factor for developing chronic CNS (central nervous sytem) disease and is discussed as associated with increased risk of late-onset Alzheimer disease (Gandy and Dekosky, 2012; Moretti et al., 2012). After single severe TBI an amyloid pathology similar to that detected in AD was described (Ciallella et al., 2002; Moretti et al., 2012). An explanation causing this correlation might be due to the high oxidative stress levels after the insult in combination with the aging process where PQC is overwhelmed, that leads to a secondary chronic disease (Djordjevic et al., 2016). The secondary brain damage, occurring after the mechanical impact, is mediated by a variety of mechanisms where hypo- and hyperperfusion and the oxygenation status in the damaged brain and the surrounding brain areas are changing that result in crucial rearrangements in cellular processes and the oxidative stress response that ends up in cell death, whereas some cells occur to be stress resistant. These alteratons in many physiological processes come along with the accumulation of protein aggregates detected in cells and brain parenchyma (DeKosky et al., 2010; Moretti et al., 2012). The advantage of the autophagosomal pathway compared to the UPS, in which just soluble and unfolded proteins could be degraded, is the ability to abolish insoluble components like aggregates (Ding and Yin, 2008). Substrates need to be unfolded for degradation by the

proteasome but aggregated proteins are shown to be resistant to the unfolding process. Some aggregate species as polyQ Huntingtin, responsible for HD, could furthermore cause malfunction to the proteasome (Bence et al., 2001). The clearance of misfolded proteins in these conditions is highly depending on autophagy. Therefore autophagy is an important mechanism for the quality control system under protein stress and might be challenged in disease status. Indeed, an accumulation of autophagosomes was described in AD in humans which likely arise from an impaired clearance by lysosomes (Nixon et al., 2008).

Different toxins including H_2O_2 or glutamate are found to induce oxidative stress triggered neuronal death. Oxidative glutamate toxicity is glutamate-receptor independent and causes intracellular cysteine depletion and subsequent glutathione depletion, which results in increasing oxidative stress in the cell (Schubert, 2001). Functioning against hypoxia, heat shock and oxidative stress, HSC/HSP70 could provide stress resistance. Furthermore, the interaction with the ubiquitin ligase CHIP connects the HSC/HSP70 complex to different degradation pathways, including the turnover of tau, which is related to Alzheimer's disease (Petrucelli et al., 2004). BAG3 as a co-chaperone of HSC/HSP70 is also found to be upregulated under oxidative stress conditions in vitro and was found to stimulate macroautophagy, hypothesizing a role for autophagy as an adaptive response mechanism against oxidative damage (Gamerdinger et al., 2009a). Indeed increased ROS levels could be measured in the paradigm of starvation-induced autophagy, which induces autophagy, and which could be enhanced via antioxidants. ROS may regulate autophagy by inactivating ATG4 through oxidation, resulting in the blockage of the delipidation of LC3-II from cytosolic membranes (Scherz-Shouval et al., 2007). ROS in form of H_2O_2 are also found to induce autophagy at least in part by BECN1 expression in cancer cell lines (Chen et al., 2008). However the interplay between ROS, autophagy and neurodegeneration is not exactly understood and requires further investigations and is therefore a main content of this work.

B.2. **Aim of the project:**

Autophagy is a key mechanism to control PQC and to keep protein homeostasis. Hence, it was reported that onset as well as progression of different diseases are regulated by autophagy. For instance autophagy facilitates stress resistance in cancer cells and was found to be activated after oxidative stress. Recently, a new pathway of autophagy has been described: *BAG3-mediated selective macroautophagy*, which was also found to be induced in paradigms of oxidative stress.

In order to analyze a potential role of autophagy on cytoprotection, and subsequenty, to get further insights about BAG3 in these conditions, autophagy was investigated in two different models for stress resistance: breast cancer cells and tissue and neuronal cells and tissue.

It is well known, that breast cancer cells can develop endocrine resistance to anti-hormone therapy and this can be facilitated via the autophagy pathway, but so far the description of a detailed autophagy expression profile of ER-positive cancer cells is missing. Therefore, the main work of this thesis was to investigate the BAG3-mediated autophagy in breast cancer and neuroblastoma cells characterized by their oxidative stress resistance.

The second part of this work concentrates on neuronal autophagy in the context of oxidative stress resistance. Neurons are highly vulnerable to disturbed proteostasis in particular as they are post-mitotic cells. Therefore, autophagy might come into play to keep PQC in stress conditions. The focus in this section was an *in vitro* model of hippocampal cells adapted to oxidative stress and was transferred to an *in vivo* model for traumatic brain injury, where oxidative stress is causatively linked to the pathophysiologic consequences.

The relevance of autophagy in the resistance to oxidative stress was analyzed in detail for both of the mentioned systems, and the results are presented the following sections.

C. Results

C.1. Impact of autophagy in estrogen receptor-dependent stress resistance and breast cancer

C.1.1. Regulation of caspase 3 and BAG proteins in stress-resistant cancer cells

The co-chaperone BAG3 plays a crucial role in two main processes in the cell: autophagy and apoptosis. Both processes are known to interact with each other and are thought to have an impact on cytoprotection and the genesis of stress resistance.

ERs, the physiological target of the steroid molecule E2, are transcription factors that can regulate gene transcription by interaction with estrogen response elements (ERE) or via interaction and activation of other transcription factors and activation of signaling cascades in the cytoplasm. As ERα and ERβ are usually co-expressed and both receptors can be activated by the same ligand a differential analysis of the function of each receptor is experimentally challenging. The ERα, but not ERβ, was proposed to be involved in cytoprotection against different toxins including Ionomycin, which induces Ca^{2+} toxicity (Gamerdinger et al., 2006; Manthey and Behl, 2006). Similar effects have been also described in our lab after induction of apoptosis by Staurosporine, which was 17β-estradiol-independent (Brendel et al., 2013).

In order to analyze a potential role of autophagy on cytoprotection, and subsequent to get further insights about a role for BAG3 and the ERs in this process, a well characterized neuroblastoma cell line (SK-N-MC) lacking expression of ERs was employed, which was stably transfected with mock-plasmid (empty vector) (SK-01), ERα (SK-ERα) or ERβ (SK-ERβ) (Zschocke et al., 2002b). These cell lines are used in our laboratory for characterization of ER-action for more than a decade now and enable us to study the differential function of ERs in tumor cells under controlled and well described conditions. In addition, throughout the whole analysis, the patient derived MCF-7 breast cancer cell line was employed as an ERα-positive cellular breast cancer model and as a positive control for pathophysiological ERα function as these cells endogenously express ERα and are ERβ-negative.

All cell lines were characterized for ER expression by PCR analysis (Figure 9, A). Further, the cell lines were tested for the inducibility of their transcription factor activity on ERE binding sites by the ligand E2. By using an ERE-luciferase reporter plasmid the monitoring of transcription factor activity from the receptors was possible. The cell lines expressing one of the receptors showed classical ER signaling as we could induce ERE-mediated transcription factor activity via E2 and also reduce the ERE-Luciferase signal after ICI treatment inhibiting both of the receptor types (Figure 9, B).

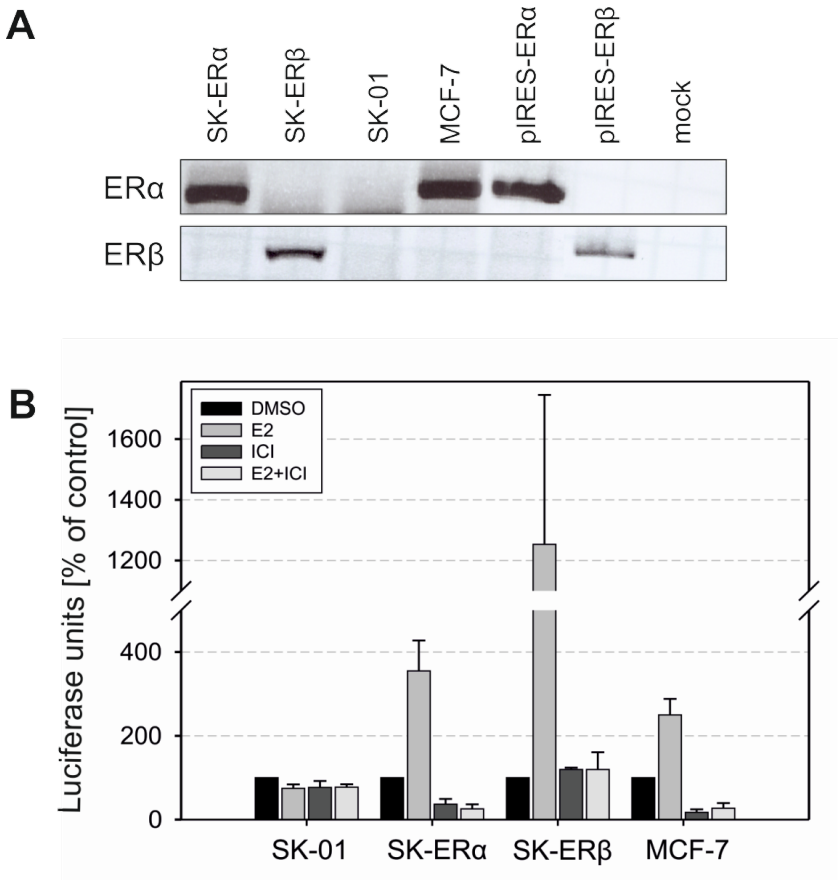

Figure 9 Estrogen receptor reporter assay. SK-01, SK-ERα, SK-ERβ and MCF-7 cells were treated with vehicle control, E2 (10 nM), ICI (1 µM) or a co-treatment with E2 and ICI for 24 h. Activation of the ER response element (ERE) was plotted as Luciferase units in % of control cells (SK-01). Values of 3 independent experiments are expressed as mean ± s.e.m..

C.1.1.1. Increased survival of ERα expressing cells

The resistance of ER expressing cells against hydrogen peroxide (H_2O_2)-induced acute oxidative stress was analyzed by Propidium Iodid (PI) exclusion assay to investigate

possible beneficial effects of ER signaling on cell survival. Indeed, also an enhanced survival of ERα expressing SK-N-MC cells could be detected after 18 h of H_2O_2 treatment compared to cells that do not express ERs (SK-01) (Figure 10). Equal findings were observed in MCF-7 cells employed as control cell line endogenously expressing ERα.

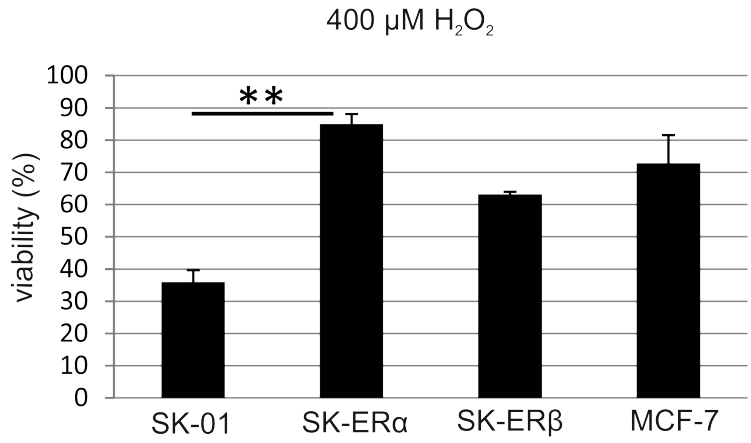

Figure 10: Increased survival of ERα expressing clonal cells. Survival after exposure to oxidative stress induced by hydrogen peroxide for 18 h is strongly enhanced in ERα expressing cells, SK-ERα and MCF7, as shown by Propidium iodid exclusion assay.SK-ERβ cells showed also a slightly higher increased survival as compared to SK-0 cells but are not as resistant as ERα expressing cells.(Brendel & Felzen et al.,2013)

In paradigms of apoptosis, caspases (cysteine-aspartic acid proteases) are crucial mediators of programmed cell death. Interestingly, SK-ERα cells also showed an increased survival in the presence of the kinase inhibitor staurosporine (Brendel et al., 2013) which is known to induce apoptosis via the downstream activation of caspase 3 (Chae et al., 2000) or caspase-independent cell death via decrease of the mitochondrial membrane potential (Deas et al., 1998).

Since caspase 3 is the key downstream executioner caspase of apoptotic cell death, caspase 3 at the protein and activity level was investigated. In fact, in Western blot analysis, Procaspase 3, the procapase form which gets activated by cleavage, was not detectable in ERα expressing cells in contrast to SK-01 control cells (Figure 11, A;B). These results could be strengthened by immunocytochemistry: compared to SK-01 cells and SKERβ, ERα expressing cells, showed just weak signals in the caspase 3 staining, consistent with the Western blotting data (Figure 11, C).

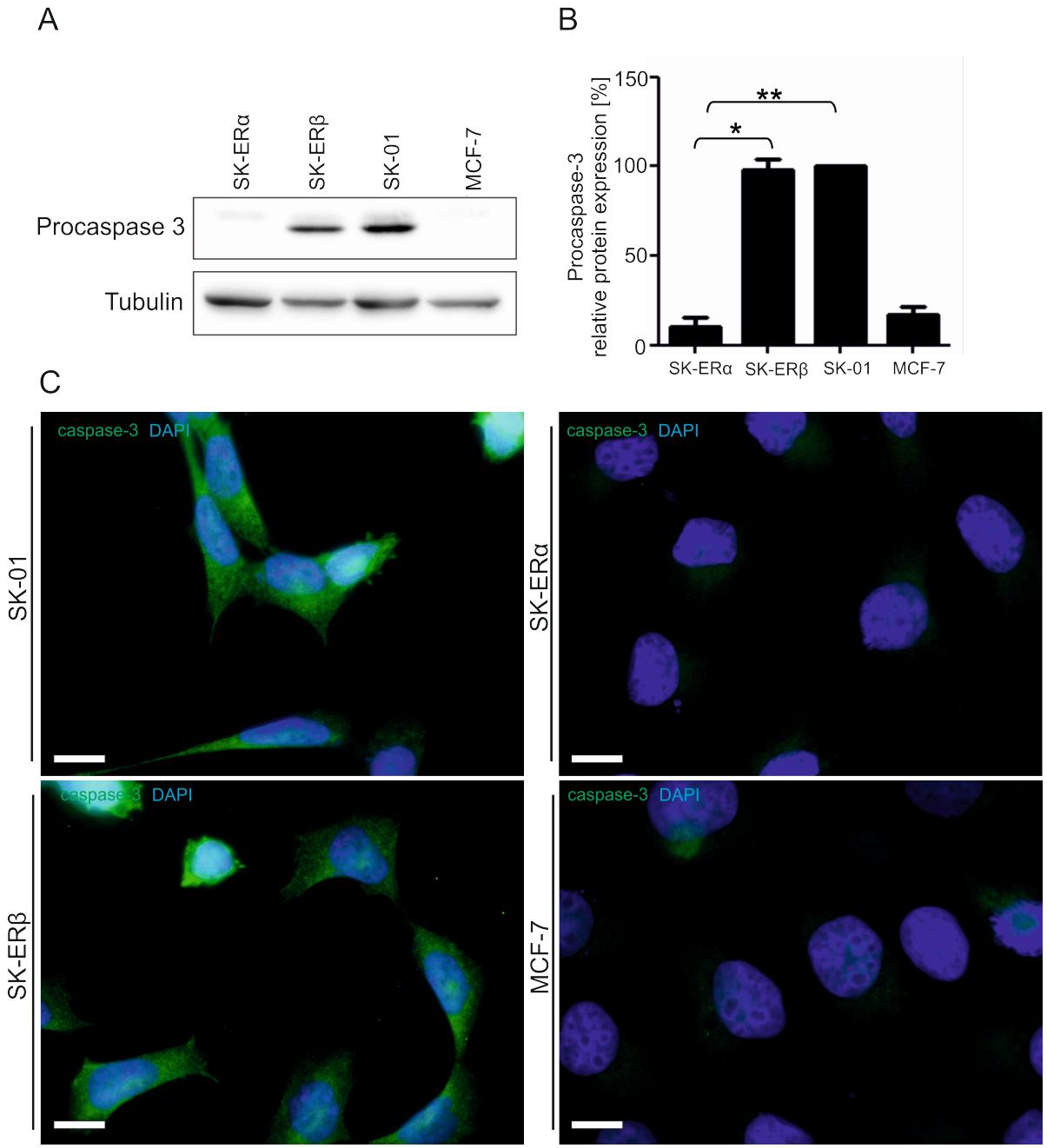

Figure 11: Decreased caspase 3 levels in ERα expressing cells. (A) Western blot analysis of caspase 3 levels indicates that caspase 3 is almost not detectable in SK-ERα and MCF-7 cells, whereas SK-01 cells, or SK-cells expressing exclusively ERβ clearly show caspase 3 expression. (B) Densitometric analysis of Western blots represented in (A); for densitometric quantification, results were normalised to tubulin. (* $p < 0,05$; ** $p < 0,01$, $n = 3$). Furthermore, cells were analysed by immunocytochemistry for caspase 3 (green channel) and nuclei were counterstained with DAPI (blue channel) (C). Compared to SK-01 cells, ERα expressing cells, SK-ERα and MCF-7, showed only very weak caspase 3 staining, supporting the Western blotting data. Scale bars: 10 µm. (Brendel and Felzen et al., 2013)

C.1.1.2. Increased expression of BAG1 and BAG3 and reduction of Caspase 3-specific BAG3-cleavage products in the presence of ERα

Besides the down regulation of caspase 3, in ERα expressing cells, previous gene array analysis in our lab showed that also other apoptosis genes are regulated in ERα expressing cells compared to control cells (Brendel et al. 2012). Interestingly, the expression of *Bcl2* and *Bcl2l11*, another member of the *Bcl2* gene family, is increased in the presence of ERα. While BCL2l11 functions as a pro-apoptotic molecule, BCL2 is a known inhibitor of apoptosis. BCL2 is also described to interact with BCL2-associated athanogenes (Kabbage and Dickman, 2008). BAG3, one member of the BAG protein family, is known to protect non-small cell lung cancer cells from apoptosis, by regulating BCL2 and BCL-XL expression (Wang et al., 2010).

The expression profile of the BCL2 regulators BAG1 and BAG3 were analyzed, and indeed, the immunoblot analysis of BAG1 revealed a strong up-regulation of all BAG1 isoforms in ERα expressing cells (Figure 12, A). BAG1 isoforms where formed as a result of an alternative translation initiation from one mRNA. Further the expression of BAG3 was detected as highly up regulated just in ERα expressing cells, SK-ERα and MCF-7. On the other hand ERβ expressing cells showed no difference in the expression pattern compared to control cells, revealing the significance of the finding is restricted to the stress resistant ERα expressing cells.

The functional relevance of the results of the expression studies in the context of apoptosis were investigated by, performing immunoblot analysis of BAG3 cleavage products. BAG3 is known to be cleaved by caspase 3 and, therefore, BAG3 cleavage products are useful indicators for caspase 3 activity (Wang et al., 2010).

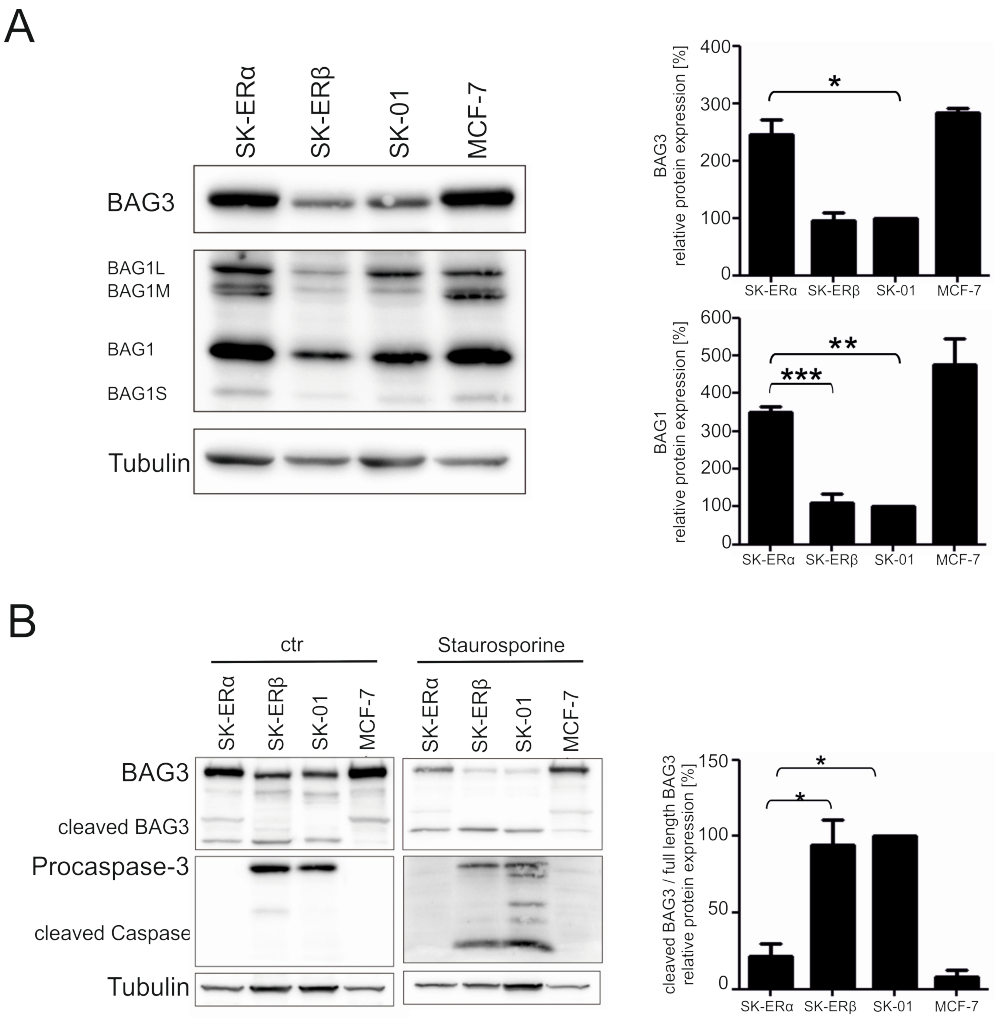

Figure 12: (A) Increased BAG1 and BAG3 protein levels in ERα expressing cells. (A) Immunoblot analysis, performed on the same blot as caspase detection, revealed a strong up-regulation of BAG1 and BAG3 in SK-ERα cells compared to mock transfected SK-01 cells. MCF-7 cells, which endogenously express ERα also showed stronger BAG1 and BAG3 immunoreactivity. Densitometric analysis of Western blots represented in. For densitometric quantification, results were normalised to tubulin (* $p < 0,05$; ** $p < 0,01$; *** $p < 0,001$ $n = 3$). **(B) Decreased caspase 3-specific BAG3-cleavage products in ERα expressing cells**. Western blot analysis of caspase 3-specific BAG3-cleavage products revealed decreased BAG3-cleavage products in ERα expressing cells compared to SK-01 (and SK-cells expressing ERβ). Full length BAG3 is clearly increased in ERα expressing cells despite the treatment with the apoptosis inducer staurosporine. In contrast, cells lacking ERα expression showed cleaved caspase 3 bands indicating a staurosporine induced acvtivation of caspase 3 and consequently also an enhanced BAG3 cleavage. (B) Densitometric analysis of full length BAG3 and caspase-specific BAG3-cleavage products within the staurosporine treatment group (right panel of Western blot in A). The graph shows the ratio of caspase-specific BAG3-cleavage products to the corresponding full length BAG3 band. As control for equal protein loading, blots were re-probed with an antibody directed against tubulin (* $p < 0,05$; $n = 3$). (Brendel and Felzen et al., 2013)

Hence, cells were incubated with staurosporine to induce caspase 3 activity. Indeed, the amount of BAG3 cleavage products (relative to full length BAG3) following staurosporine treatment was reduced in SK-ERα cells, while as the amount of BAG3-cleavage products in SK-01 cells was highly increased upon staurosporine treatment

(Figure 12, B). Again, MCF-7 cells showed a result similar to the SK-ERα expressing cells, while ERβ expressing cells displayed a BAG3-cleavage pattern comparable to control-transfected SK-01 cells. As revealed by densitometric analysis the BAG3-cleavage products were reduced by approx. 80 - 90% in both cell lines expressing either ERα ectopically or endogenously (Figure 12,B).

Taken together, these results suggest that the observed enhanced survival of ERα expressing cells under oxidative stress and apoptosis inducing conditions may be due (at least in part) to a significant downregulation of the main executioner caspase 3 and through the upregulation of BCL2 regulating co-chaperones BAG1 and BAG3.

In addition to the role of BAG1 as enhancer of BCL2 activity, BAG1 is known to be involved in protein degradation via the ubiquitin-proteasome system. In addition, besides the role of BAG3 in apoptosis, the molecule was linked to protein clearance via macroautophagy in aged and acutely stressed cells (Gamerdinger et al., 2009a). The function of BAG proteins in PQC is enabled by their binding affinity to the molecular chaperone HSC/HSP70. The complex around HSC/HSP70 is involved in protein homeostasis by conducting protein folding as well as degradation. In cooperation with the BAG co-chaperones the HSC/HSP70 complex directs a degradation-prone substrate either to the proteasomal- or the macroautophagic degradation pathway. HSP70 protein expression level were found highly up regulated in ERα expressing cells (Figure 13), suggesting a role for degradation pathways in the maintenance of protein homeostasis in resistant ERα expressing cells.

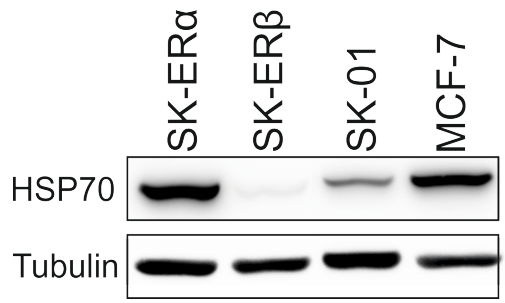

Figure 13: ERα expressing cells display increased HSP70 protein level. Protein expression levels of HSP70, one of the main chaperones in the eukaryotic cell, have been detected by Western blotting in all of the cell lines. ERα expressing cell lines, SK-ERα and MCF-7, show highly increased HSP70 protein expression in comparisons to the non ER-expressing cells SK-01 as well as SK-ERβ. (Brendel and Felzen et al., 2013)

Despite the role in apoptosis, BAG proteins are involved in PQC acting as co-chaperones of HSP70, which itself was found to be highly expressed in the resistant cells. Regarding these findings, the role of protein homeostasis in ERα dependent signaling and the developing of resistance was the fundament of the further studies within this work. Therefore in the next section a detailed analysis of the autophagic degradation pathway in the ER cells was analyzed.

C.1.2. The regulation of autophagy in stress-resistant cancer cell

In the last years autophagy was critically investigated in different diseases and disease models, including neurodegeneration as well as cancer and recent studies suggest, that autophagy and BAG3, may play a critical role in stress resistance. Therefore the role of autophagy in the context of estrogen-dependend cytoprotection was investigated in detail within the next sections of this thesis.

C.1.2.1. Estrogen receptor expression differentially regulates transcription of autophagy-related genes.

As described previously, ER expression as well as enhanced autophagic activity contributes to metastatic potential and resistance to therapy of different cancer types (Galavotti et al., 2013; Gattelli et al., 2013). First a gene expression profiling of the different cell types employing a human autophagy PCR array containing 88 genes involved in autophagy was performed. These genes included ATGs, genes out of the BCL2/PI3KC3 complex, the mTOR complex as well as autophagy adaptors, lysosomes associated and additionally some other known upstream autophagy inducers and regulators. Utilizing SK-01 cells as control cells that do not express ERs, gene expression of cells ectopically or endogenously expressing ERα or ERβ were calculated. Subsequent groups of genes that display the different regulatory complexes (ULK1-, PI3K- and mTOR-complex), various other ATGs that are associated with lysosomal function and candidates that are not part of the core autophagy machinery were analyzed. The results of the PCR analysis showed reduced expression of multiple genes and key autophagy components in ERα expressing cells (29 genes in SK-ERα cells and 32 genes in MCF-7 cells) although a higher autophagic activity could be detected (Figure 16).

	vehicle treated					
	SK-01 vs. SK-ERα		SK-01 vs. SK-ERβ		SK-01 vs. MCF-7	
	p-value	fold change	p-value	fold change	p-value	fold change
ATG1/ULK complex						
ULK1	0.008	-2.62	0.557	1.14	0.020	-1.95
ULK2	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
FIP200	0.000	-4.32	0.034	-1.25	0.000	-4.24
other ATGs						
ATG2A	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
ATG2B	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
ATG3	0.032	-1.41	0.221	1.20	0.009	-1.54
ATG4A	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
ATG4B	0.015	-2.32	0.622	-1.05	0.010	-2.65
ATG4C	0.004	-2.45	0.794	1.09	0.002	-2.96
ATG4D	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
ATG5	0.017	-1.57	0.085	-1.32	0.006	-1.80
ATG6/BECLIN1	0.029	-1.59	0.167	1.22	0.008	-1.54
ATG7	0.014	2.34	0.030	1.90	0.021	1.94
ATG8/GABARAP	0.080	-1.17	0.000	1.61	0.000	-1.32
ATG8/GABARAPL1	0.000	-57.74	0.000	2.83	0.000	-85.97
ATG8/GABARAPL2	0.000	2.25	0.002	1.65	0.000	2.16
ATG8/MAP1LC3A	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
ATG8/MAP1LC3B	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
ATG8/MAP1LC3B2	0.000	2.10	0.051	1.25	0.000	1.96
ATG8/MAP1LC3C	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
ATG9A	0.020	-1.61	0.095	-1.32	0.012	-1.90
ATG10	0.000	-4.03	0.294	-1.12	0.000	-3.85
ATG12	0.000	-2.41	0.115	-1.13	0.000	-2.12
ATG14/BAKOR	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
ATG16L1	0.000	-4.15	0.007	-1.53	0.000	-3.91
ATG16L2	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
ATG18/WIPI1	0.000	18.49	0.584	1.36	0.000	15.72
ATG18/WIPI2	0.000	-5.78	0.338	-1.15	0.000	-4.94
ATG18/WIPI3	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
ATG24A/SNX30	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
BCL2/PI3K C3 complex						
BAX	0.010	1.38	0.002	1.63	0.234	1.13
BCL2	0.036	1.57	0.795	1.05	0.002	2.05
BCL2L1	0.002	1.52	0.008	1.32	0.007	1.41
BECN1L1	0.099	-2.45	0.509	-1.32	0.050	-2.58
UVRAG	0.003	-2.55	0.044	-1.58	0.007	-2.18
MCL1	0.000	-2.10	0.173	-1.16	0.000	-1.80
PIK3C3	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
PIK3R4	0.002	-2.10	0.184	1.22	0.001	-2.34
autophagy adaptor						
SQSMT1	0.031	1.98	0.008	1.87	0.015	1.71
mTOR complex						
mTOR	0.049	-2.62	0.903	1.00	0.011	-2.80
AKT1S1	0.033	1.22	0.003	1.70	0.272	1.14
RAPTOR	0.109	-1.83	0.634	-1.05	0.035	-2.51
RICTOR	0.001	-2.59	0.052	1.44	0.001	-2.54
Lysosome associated						
LAMP1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
LAMP2	0.711	1.09	0.020	-1.67	0.081	1.41
LAMP3	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
DRAM	0.000	18.75	0.025	1.54	0.000	13.68
other						
TP73	0.014	-2.93	0.028	1.48	0.001	-4.07
EPS15L1	0.000	6.36	0.995	1.10	0.000	4.84
BIRC5	0.000	-2.41	0.033	1.29	0.000	-2.47
ATF4	0.003	-1.68	0.004	1.56	0.005	-1.68
DDIT3	0.004	-1.86	0.065	1.48	0.000	-2.65
ULK3	0.000	-2.59	0.042	-1.20	0.000	-1.77
ULK4	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
EIF4EBP1	0.000	-5.47	0.794	1.03	0.000	-6.35
EIF4EBP2	0.012	-1.83	0.006	1.56	0.061	-1.46
GPSM1	0.000	-5.78	0.055	-1.76	0.000	-5.52
GPSM3	0.000	-6.92	0.425	1.12	0.000	-6.00
HIF1A	0.002	-1.57	0.004	1.38	0.001	-1.77
SEC16A	0.803	1.02	0.042	-1.86	0.643	-1.17
SEC23A	0.377	-1.61	0.982	1.06	0.018	-2.18
SEC23B	0.060	1.40	0.160	1.32	0.038	1.51
SEC24D	0.157	-1.17	0.006	1.40	0.003	-1.54
SH3GLB1	0.003	-1.75	0.726	1.04	0.000	-2.04
TPR	0.001	-3.66	0.461	1.17	0.001	-3.64

Figure 14: Estrogen receptors differentially regulate autophagy pathway- associated gene expression. (A) Total RNA from SK-01, SK-ERα, SK-ERβ and MCF-7 cells was characterized by using the Human Autophagy Primer Library 1 (HATPL-1) and com-paring ER expressing cells to mock-plasmid transfected controls (SK-01). Red numbers indicate an up-regulation greater than 1.5 fold, blue numbers indicate an down-regulation greater than 1.5 fold, dark red numbers denote a p-value<0.05, black numbers depict no significant change and n.s. no significant change in gene expression within the whole group. Results represent the mean values of five independent experiments.(Felzen et al.,2015)

A significant upregulation was shown for 9 genes in SK-ERα and MCF-7 cells. In contrast, SK-ERβ cells showed a negative regulation for 4 genes and an upregulation for 10 genes that was not accompanied by higher autophagic activity and these genes were mainly different to those regulated in ERα expressing cells.

An "autophagy-footprint" of cells expressing ERα or ERβ was established from this data and showed fundamental differences in autophagy-related gene expression in cells expressing ERα (SK-ERα) compared to SK-ERβ and cells lacking ER (Figure **15***). Additionally, it was clearly shown that the SK-ERα cell line has almost an identical "autophagy-footprint" as MCF-7 breast cancer cells derived from a breast cancer patient. Taken together, these data demonstrate that ER expression differentially regulates autophagy-related gene transcription in tumor cells.*

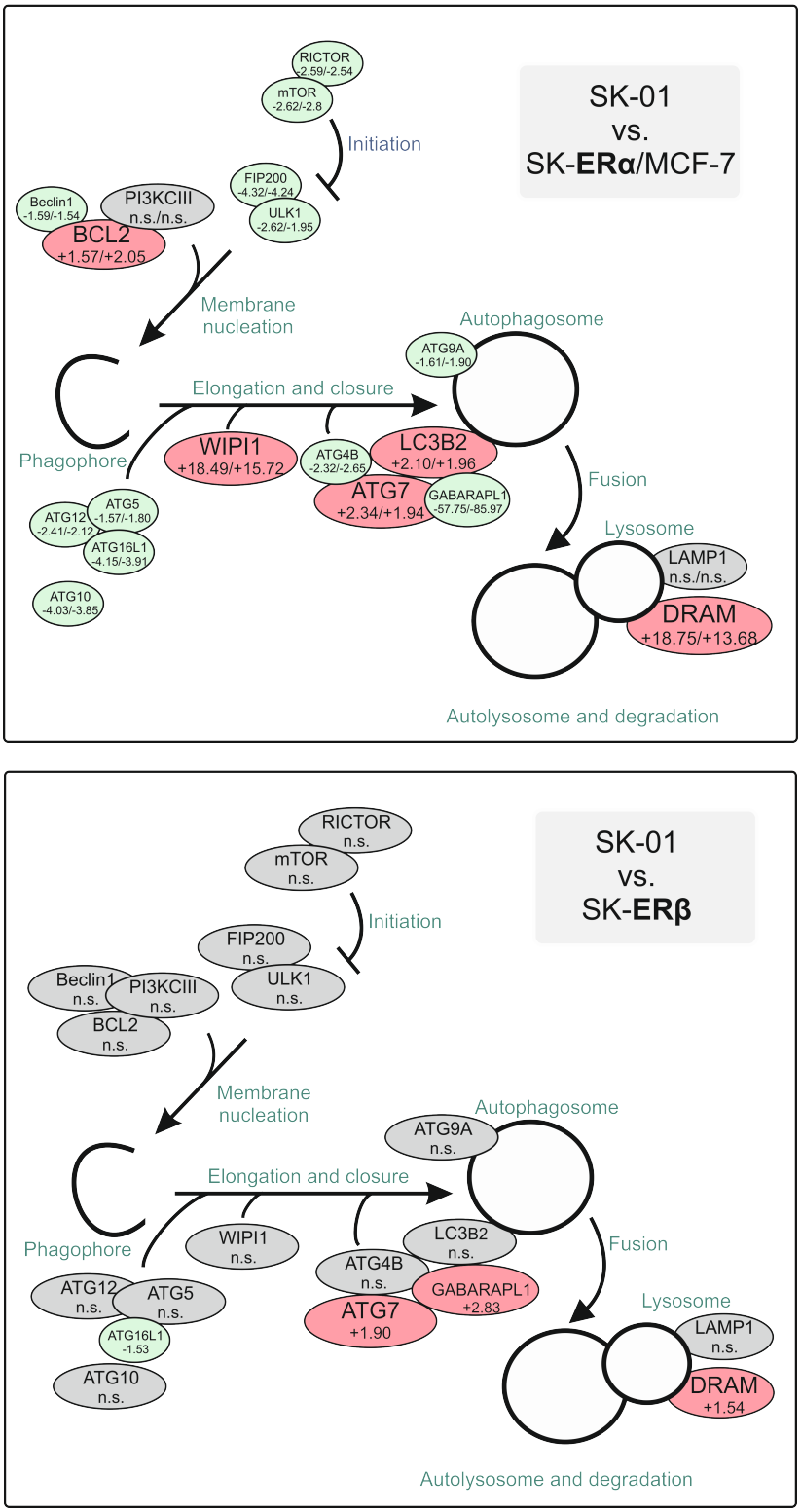

Figure 15: Key autophagic genes regulated in ERα or ERβ expressing cells show crucial differences. Cartoons display a selection of key autophagic genes of the corresponding PCR array analysis in and show the "autophagy-footprint" of ERα (SK-ERα and MCF-7) and ERβ expressing cells. Genes highlighted in blue were downregulated, in grey not altered in expression whereas genes highlighted in red were upregulated. First numbers in (A) show the regulation in SK-ERα cells and following numbers the regulation in MCF-7 cells. In (B) numbers display the regulation of genes in SK-ERβ cells compares to SK-01. n.s. indicates that there is no significant change in gene expression comparing SK-ERα, SK-ERβ and MCF-7 cells to mock transfected controls. (Felzen et al., 2015)

C.1.2.2. Estrogen receptor α induces non-canonical autophagic activity

Observing differential regulation of autophagy-related genes in ERα expressing cells vs. SK-ERβ or SK-01 cells further were investigated. Therefore, several experiments to determine whether SK-ERα and MCF-7 cells show an altered autophagic activity were performed. We first detected LC3B (hereafter referred to as LC3), a widely accepted marker for autophagosomes, by Western blotting. The autophagic flux can be measured by monitoring LC3-II accumulation after inhibiting lysosomal activity via Bafilomycin A_1 ($BafA_1$) -treatment. In ERα expressing cells the accumulation of LC3-II was significantly higher than in mock-transfected SK-01 or ERβ expressing cells (Fig. 2a). There was no statistical difference between SK-01 and SK-ERβ cells. In line with LC3-II immunoblotting, NBR1 a LC3 binding protein and additional well established marker for autophagic degradation (Johansen and Lamark, 2011) also shows an enhanced accumulation in ERα cells (Figure 16).

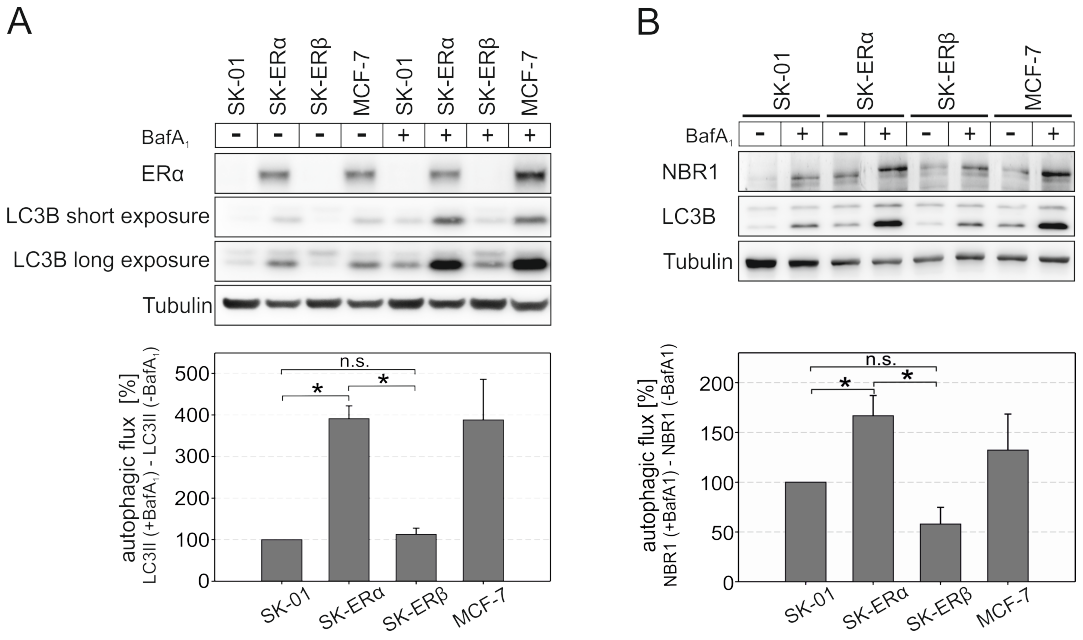

Figure 16: ERα expression enhances autophagic flux. (A and B) Protein extracts from vehicle or BafA1 treated SK-01, SK-ERα, SK-ERβ and MCF-7 cells were subjected to Western blot analysis and calculation of autophagic flux using anti-LC3 and NBR1 antibodies. Tubulin was used as loading control. (A) ERα expression of the different cell lines was shown via Western blot analysis and autophagic flux was determined by the accumulation of LC3-II in a 6 h treatment period with 500 nM BafA1. Therefore, normalized LC3-II levels in the absence of the lysosomal inhibitor were subtracted from corresponding levels obtained in the presence of BafA1. (B) Cells were treated as in (a) and autophagic flux was calculated by NBR1 expression determined by western blot. Values of 3 independent experiments in each panel are expressed as mean ± s.e.m. and control SK-01 cells were set to 100%. (*) on bars represent statistical significance of $p<0.05$ comparing 2 groups and n.s. displays no statistical significant difference. (Felzen et al., 2015)

Additionally, transient overexpression of ERα in the ER-lacking native SK-N-MC cell line that was the basis for the generation of SK-01, SK-ERα and SK-ERβ cells leads to an enhanced autophagic flux and elevated BAG3 protein level (Figure 17).

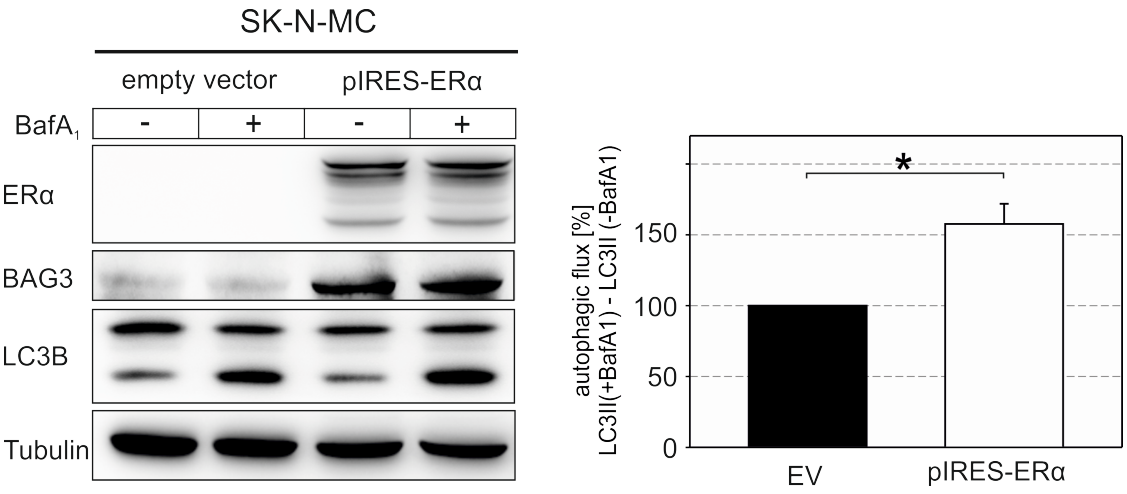

Figure 17: ERα overexpression leads to an enhanced autophagic flux and elevated BAG3 protein level in SK-N-MC cells. Overexpression of ERα in SK-N-MC cells was performed by transient transfection of pIRES-ERα plasmid. Transfection efficiency and calculation of autophagic flux was determined by using anti-ERα and anti-LC3 antibody, respectively. Tubulin was used as loading control. Autophagic flux was determined by the accumulation of LC3-II in a 6 h treatment period with 500 nM BafA1. Therefore, normalized LC3-II levels in the absence of the lysosomal inhibitor were subtracted from corresponding levels obtained in the presence of BafA1. Values of 3 independent experiments in each panel are expressed as mean ± s.e.m. (*) on bars represent statistical significance of $p<0.05$ comparing 2 groups and n.s. displays no statistical significant difference. (Felzen et al., 2015)

Next, the effect of ERs on the expression of the major protein complexes that are involved in the initiation of autophagy, the nucleation of the phagophore, elongation and closure of the autophagosomal membrane were determined. In most eukaryotic cellular systems mTORC1 has been demonstrated to negatively regulate the induction of autophagy and activated mTOR is phosphorylated at serine 2448 (Chiang and Abraham, 2005; Meijer and Codogno, 2004). Although showing that ERα-positive cells display an enhanced autophagic flux no significant changes in the ratio of mTOR to phosphor-mTOR at serine 2448 in any of the tested cell lines were observed Figure 18, A). The phosphorylation state of p70S6K a downstream target of mTOR kinase was also examined. In line with the mTOR data an altered ratio of p70S6K to phospho p70S6K

was not detected, although it did not escape our notice that total p70S6K protein level differ between cell lines (Felzen et al., 2015).

The PI3KCIII, which generates PI3P, in complex with Beclin1 and BCL2 represents an evolutionarily conserved positive regulatory circuit which controls autophagosome formation. Surprisingly, we could not detect an upregulation of PI3KCIII in ERα expressing cells. Instead, we identified a significant upregulation in SK-ERβ cells compared to SK-01 and SK-ERα cells (Figure 18, B).

PI3KCIII activity is regulated by Beclin1 which itself is bound to, and inhibited by BCL2. As autophagic activity is indirectly negatively regulated via BCL2 (Kang et al., 2011) whether BCL2 levels change upon ER expression was tested. Western Blot analysis showed that SK-ERα and MCF-7 cells expressed BCL2 at a higher level than SK-01 control cells or the SK-ERβ clone, according to PCR array data (Figure 18, C).

Additionally, the activity of the PI3K complex was inhibited with the specific PI3K inhibitor Wortmannin (WN) known to reduce canonical autophagic flux. Again, we could not detect a decreased autophagic flux in SK-ERα and MCF-7 compared to SK-01 cells, determined by LC3-II and SQSTM1 immunoblotting after WN- and $BafA_1$-treatment (Figure 18, D).

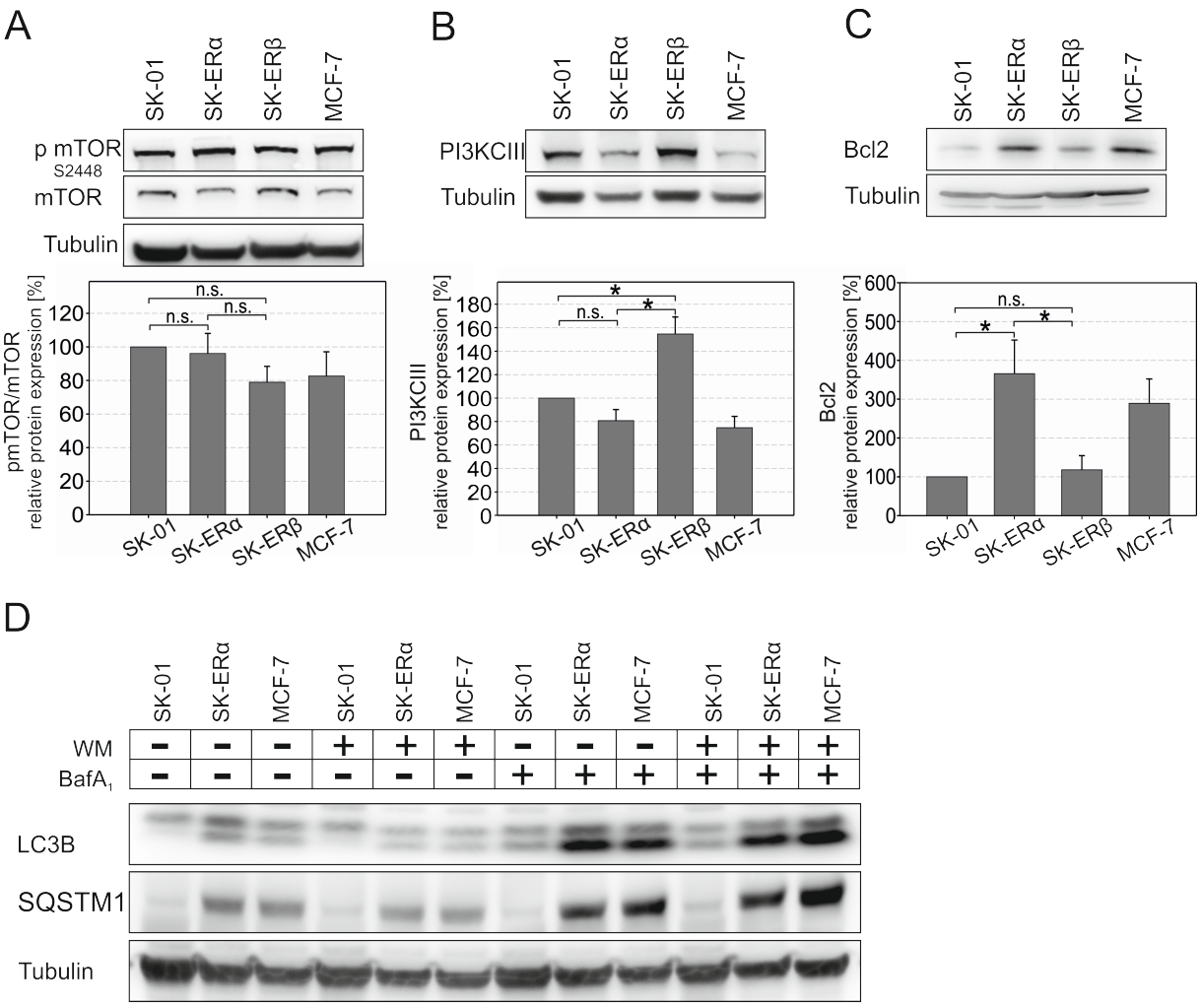

Figure 18: ERα expression differentially modulates key autophagy pathway proteins independent of mTOR phosphorylation and PI3KCIII protein expression and activity. Western blot analysis of protein extracts from untreated cells were performed for detection of indicated proteins. In the diagrams (lower panel each) levels of proteins are depicted after normalization to corresponding Tubulin levels.(D) SK-01, SK-ERα and MCF-7 cells were treated with vehicle control, Wortmannin (1 µM) and BafA1 (100 nM) for 20 h and were subjected to Western blot analysis. Autophagic flux was determined by the accumulation of LC3-II and p62/SQSTM1. Tubulin was used as loading control. Values of 3 independent experiments in each panel are expressed as mean ± s.e.m. and control SK-01 cells were set to 100%. (*) on bars represent statistical significance of $p<0.05$ comparing 2 groups and n.s. displays no statistical significant difference. (Felzen et al., 2015)

The influence of siRNA-mediated knockdown of Beclin1, a key factor for canonical autophagy (Galluzzi et al., 2015), on the autophagic activity in the respective cell lines was investigated. The autophagic flux in the ERα expressing cells was not reversible with knockdown of Beclin1, suggesting that a non-canonical Beclin1-independent autophagic pathway exists in the analyzed cell model (Figure 19). Interestingly, a higher autophagic activity after Beclin1 knockdown was detected, even though this effect was

present in all cell lines investigated (SK-ERα, MCF-7 and SK-01 control cells) pointing to an estrogen receptor-independent effect.

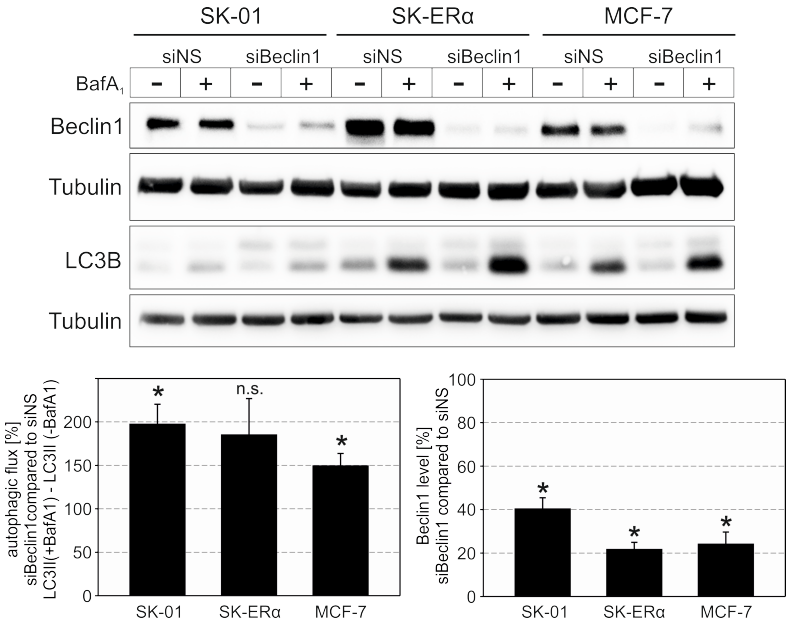

Figure 19: Beclin1 knockdown enhances the autophagic flux independent of ERα expression. SK-01, SK-ERα and MCF-7 cells were transfected with nonsense siRNA (siNS) and Beclin1 siRNA (siBeclin1) for 48 h, as indicated. Protein extracts from vehicle or BafA1 treated cells were subjected to Western blot analysis and calculation of autophagic flux using anti-LC3 antibody and knockdown efficiency was determined by using anti-Beclin1 antibody. Tubulin was used as loading control. Autophagic flux was determined by the accumulation of LC3-II in a 6 h treatment period with 500 nM BafA1. Therefore, normalized LC3-II levels in the absence of the lysosomal inhibitor were subtracted from corresponding levels obtained in the presence of BafA1. Values of 3 independent experiments in each panel are expressed as mean ± s.e.m. (*) on bars represent statistical significance of $p<0.05$ comparing 2 groups and n.s. displays no statistical significant difference.(Felzen et al., 2015)

Consecutively, investigating the protein level of effectors downstream of the major autophagy regulating protein complexes a strong upregulation of WIPI1 in ERα cells compared to SK-01 and SK-ERβ cells was detected (Figure 20, A), which has been identified earlier to function downstream of PI3P mediating events during the initiation of autophagosome formation (Gaugel et al., 2012). ATG7 another crucial component of the ATG8/LC3 system is also significantly upregulated in SK-ERα and MCF-7 cells (Figure 20, B).

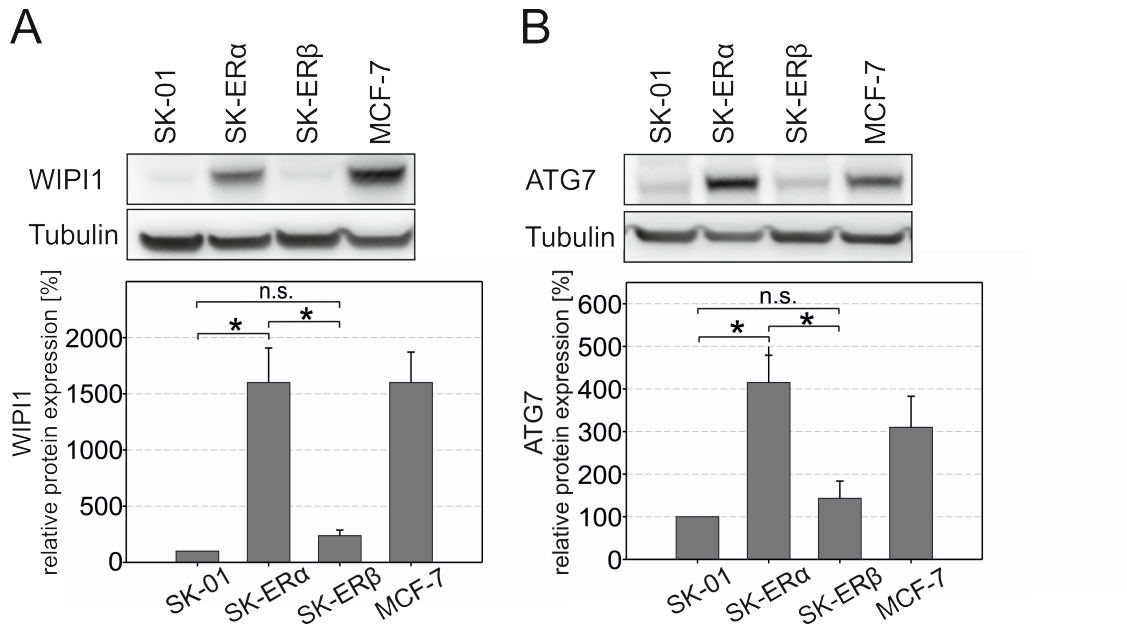

Figure 20: ERα expression enhances the expression of WIPI1 and ATG7 protein expression. Protein extracts from untreated cells, SK-01, SK-ERα, SKERβ and MCF-7, were used to perform Western blot analysis for detection of indicated proteins. The diagrams display the protein levels after normalization to corresponding Tubulin. Values of 3 independent experiments in each panel are expressed as mean ± s.e.m. and control SK-01 cells were set to 100%. (*) on bars represent statistical significance of $p<0.05$ comparing 2 groups and n.s. displays no statistical significant difference. (Felzen et al., 2015)

It has been shown that components of the mitogen-activated protein kinase (MAPK) pathway together with the autophagy-related proteins DRAM1 and SQSTM1 lead to enhanced autophagic activity and this revealed a novel function of DRAM1 and SQSTM1 in control of migration/invasion in cancer stem cells (Galavotti et al., 2013). Showing that DRAM1 and SQSTM1 mRNA are increased we asked if blocking the MAPK pathway activity in ERα-positive cells with the specific ERK1/2 inhibitor U0126 would result in lowered autophagic flux. The data shows no reduced autophagic flux after U0126 and subsequent $BafA_1$-treatment (Figure 21).

In this context it was investigated if reducing the level of DRAM1, one the most up-regulated genes in ERα expressing cells shown in the PCR array (Figure 9), results in a modulation of the autophagic flux. Interestingly, DRAM1 as part of the autophagic machinery partially contributes to this specific ERα-driven autophagic pathway, a reduced autophagic flux in ERα-expressing cells (significant reduction in MCF-7 cells and a clear but not significant tendency in SK-ERα cells) after knockdown of DRAM1 was detected (Figure 22).

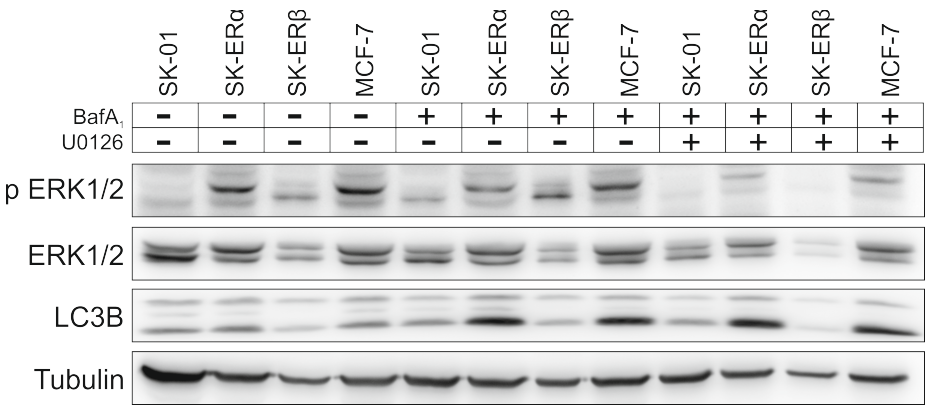

Figure 21: Inhibition of MAPK pathway by U0126 is not inhibiting ERα induced autophagy. Western blot analysis of protein extracts from untreated cells was performed for detection of indicated proteins. SK-01, SK-ERα and MCF-7 cells were treated with vehicle control, U0126 (50 µM) for 48h and BafA1 (100 nM) for 6 h and were subjected to Western blot analysis. Autophagic flux was determined by the accumulation of LC3-II. Tubulin was used as loading control. Inhibiting pERK levels displayd no difference in autophagic flux compared to controls. (Felzen et al., 2015)

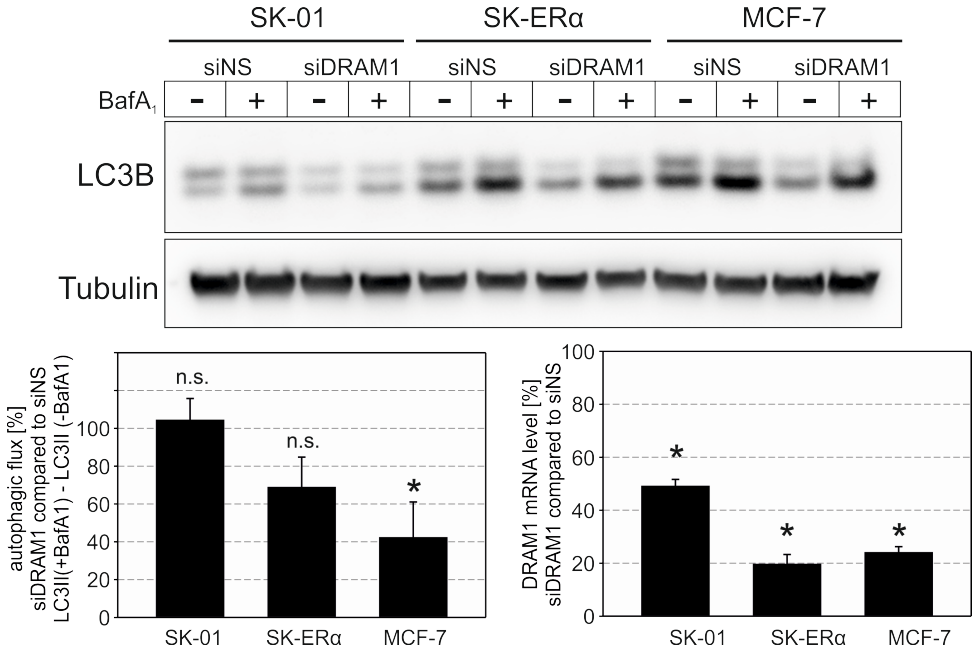

Figure 22: DRAM1 knockdown reduces autophagic activity in ERα-expressing cells. (a) SK-01, SK-ERα and MCF-7 cells were transfected with nonsense siRNA (siNS) and DRAM1 siRNA (siDRAM1) for 48 h, as indicated. Protein extracts from vehicle or BafA1 treated cells were subjected to Western blot analysis and calculation of autophagic flux using anti-LC3 antibody and knockdown efficiency was determined by qPCR analysis. For Western blot analysis Tubulin was used as loading control. Autophagic flux was determined by the accumulation of LC3-II in a 6 h treatment period with 500 nM BafA1. Therefore, normalized LC3-II levels in the absence of the lysosomal inhibitor were subtracted from corresponding levels obtained in the presence of BafA1. Values of 3 independent experiments in each panel are expressed as mean ± s.e.m. (*) on bars represent statistical significance of $p<0.05$ comparing 2 groups and n.s. displays no statistical significant difference. (Felzen et al., 2015)

Immunofluorescence stainings of endogenous LC3 and SQSTM1 clearly emphasize an enhanced accumulation after $BafA_1$-treatment in SK-ERα and MCF-7 cells (Figure 23, A).

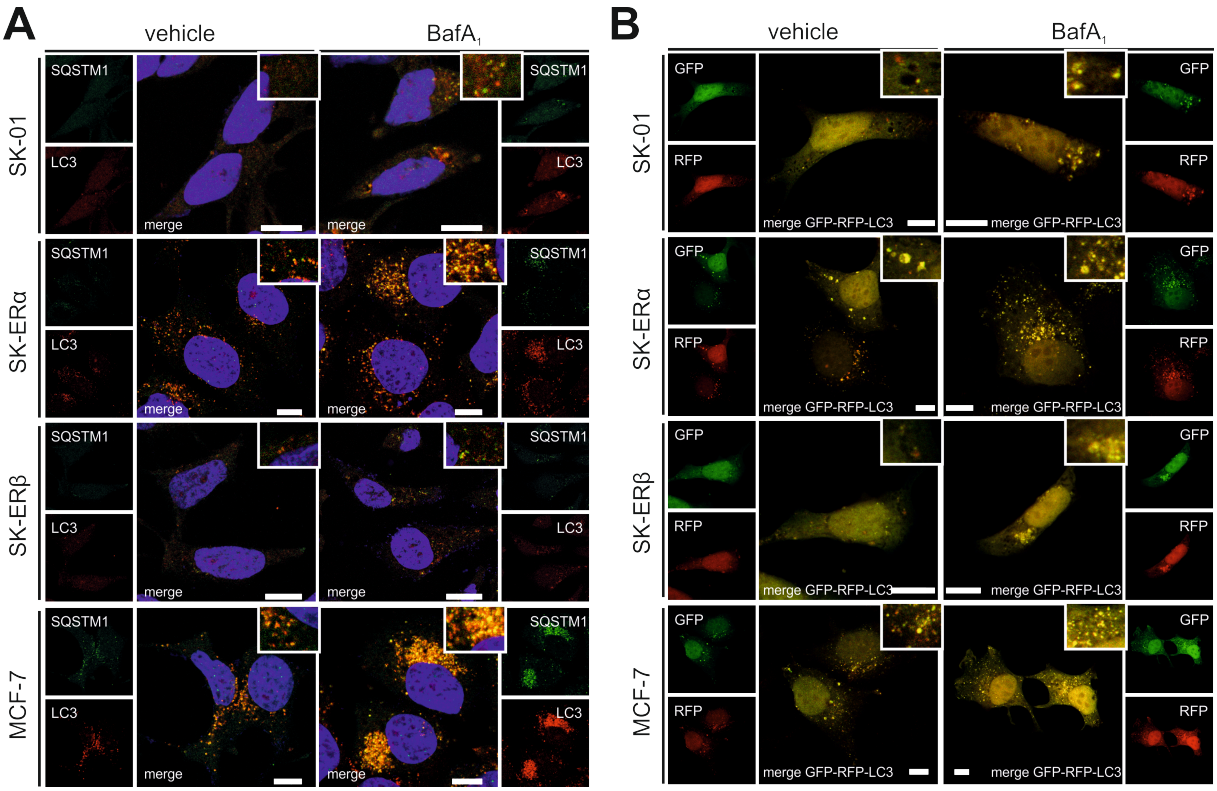

Figure 23: ERα expression promotes an increase of autophagosomes and autophagic flux. (A) Representative pictures of indirect immunofluorescence staining of endogenous LC3 (red) and SQSTM1 (green) in SK-01, SK-ERα, SK-ERβ and MCF-7 cells, treated with vehicle control and 500 nM of BafA1 for 6 h, are shown. DAPI (blue) was used to stain DNA. Scale bars: 10 µm. (B) SK-01, SK-ERα, SK-ERβ and MCF-7 cells were transiently transfected with a GFP-RFP-LC3 fusion protein and treated with vehicle control and 500 nM of BafA1 for 6 h. In autophagosomes, both RFP and GFP signals are apparent whereas under acidic conditions when autophagosomes fuse with lysosomes just the RFP signal persists. In contrast, GFP signal diminishes due to a labile enhanced GFP protein under acidification. Representative images acquired by confocal microscopy are shown. Scale bars: 10 µm. (C) GFP-RFPLC3-punctae were counted in all cell lines transfected with the ptfLC3 expression plasmid and treated with 500 nM of BafA1 for 6 h prior to fixation. At least six different positions of the corresponding stainings per experiment were documented utilizing confocal laser scanning microscopy and counted numbers of GFP-RFP-LC3-punctae were averaged. 59 SK-0 cells, 56 SK-ERα cells, 59 SK-ERβ cells and 58 MCF-7 cells were analyzed. 3 independent experiments in each panel are expressed as mean ± s.e.m. (*) on bars represent statistical significance of $p<0.05$ comparing 2 groups and n.s. displays no statistical significant difference. (Felzen et al., 2015)

Using an expression vector (ptfLC3) that encodes LC3 fused to red fluorescent protein (RFP) and green fluorescent protein (GFP) in tandem (GFP-RFP-LC3) (Kimura et al., 2007) was used to corroborate that these findings of an increased expression of autophagic markers in ERα cells reflect an enhanced autophagosome biogenesis rather than a decreased autophagosome clearance. All cell types were transiently transfected with the ptfLC3 vector and treated with vehicle control and $BafA_1$. All cell lines display exclusively red fluorescent punctae with no corresponding signal in the green channel under control conditions indicating a functional fusion of autophagosomes with lysosomes resulting in the quenching of the pH sensitive GFP in these acidic compartments. Consistent with the Western blot data, an enhanced accumulation of autophagosomes after $BafA_1$-treatment in ERα-positive cells was observed (Figure 23, B). The quantification of GFP-RFP-LC3 punctae in every cell line further underlines our findings and demonstrates a significantly higher amount of autophagolysosome formation in ERα-expressing cells (Figure 23, C).

Identifying the presence of autophagic vesicles by transmission electron microscopy is considered a gold standard of verifying autophagosome formation (Klionsky et al., 2012). Indeed, an abundance of autophagosomes and autophagolysosomes was detected in ERα expressing cells compared to SK-01 and SK-ERβ cells by electron microscopy (Figure 24).

These results suggest that ERα expression leads to an enhanced autophagic flux, which is not controlled by the mTOR-, PI3KCIII- and MAPK-pathway but key autophagy proteins such as BAG3, ATG7 and WIPI1 are strongly upregulated. Taken together, this newly described non-canonical autophagy pathway is driven by ERα.

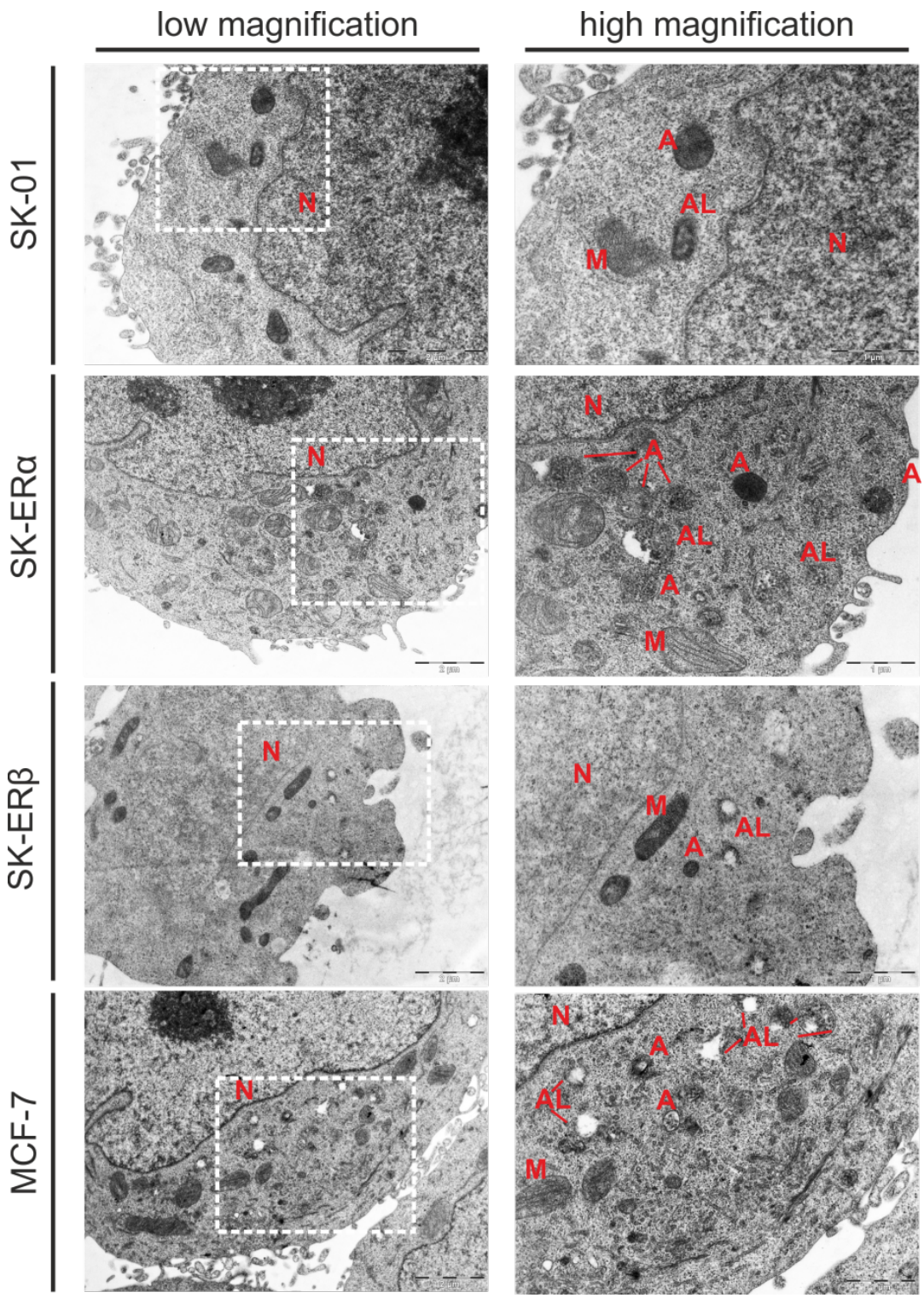

Figure 24: Transmission electron microscopy revealed the accumulation of autophagic vesicles in ERα expressing cells. Typical ultrastructure of SK-01, SK-ERα, SK-ERβ and MCF-7 cells visualized by using transmission electron microscopy. Magnifications of marked areas are shown in the lower panel. ERα expressing cells showed increased autophagic vesicles of different stages of maturation. Autophagosomes are indicated by A, autolysosomes by AL, nucleus by N and mitochondria by M. (Felzen et al., 2015)

C.1.2.3. Increased autophagic flux is independent of ERE-mediated transcriptional activity of estrogen receptor α

ER-mediated transcription factor activity can be induced via its ligand E2 or inhibited via the synthetic antagonist ICI 182780 (ICI). SK-01, SK-ERβ, SK-ERα and MCF-7 cells were treated with E2 and ICI and measured expression of autophagy-related genes according to (Figure 18). The cell lines tested showed classical ER signaling as ERE-mediated transcription factor activity via E2 was inducible and also reduce the ERE-Luciferase signal after ICI treatment (Figure 9). Interestingly, no major changes in autophagy-related gene expression were observed after compound treatment in any cell line (see Appendix (Figure 41)).

Next, whether autophagic activity in our cellular systems is altered after respective drug application was investigated. Again, no changes in autophagic activity in any of the analyzed cells following E2 or ICI administration determined by LC3 immunoblotting was observed. Vehicle or drug treatment resulted in a comparable autophagic activity as shown for untreated cells (

Figure 25). The key autophagy proteins studied also showed no significant changes in total protein expression and the ratio between phosphorylated and total mTOR was not altered.

The data provided here indicate that ERα-mediated enhanced autophagic activity is independent of its ERE-mediated transcription factor activity and the observed ER-driven non-canonical autophagy pathway is fundamentally different to classical canonical autophagy.

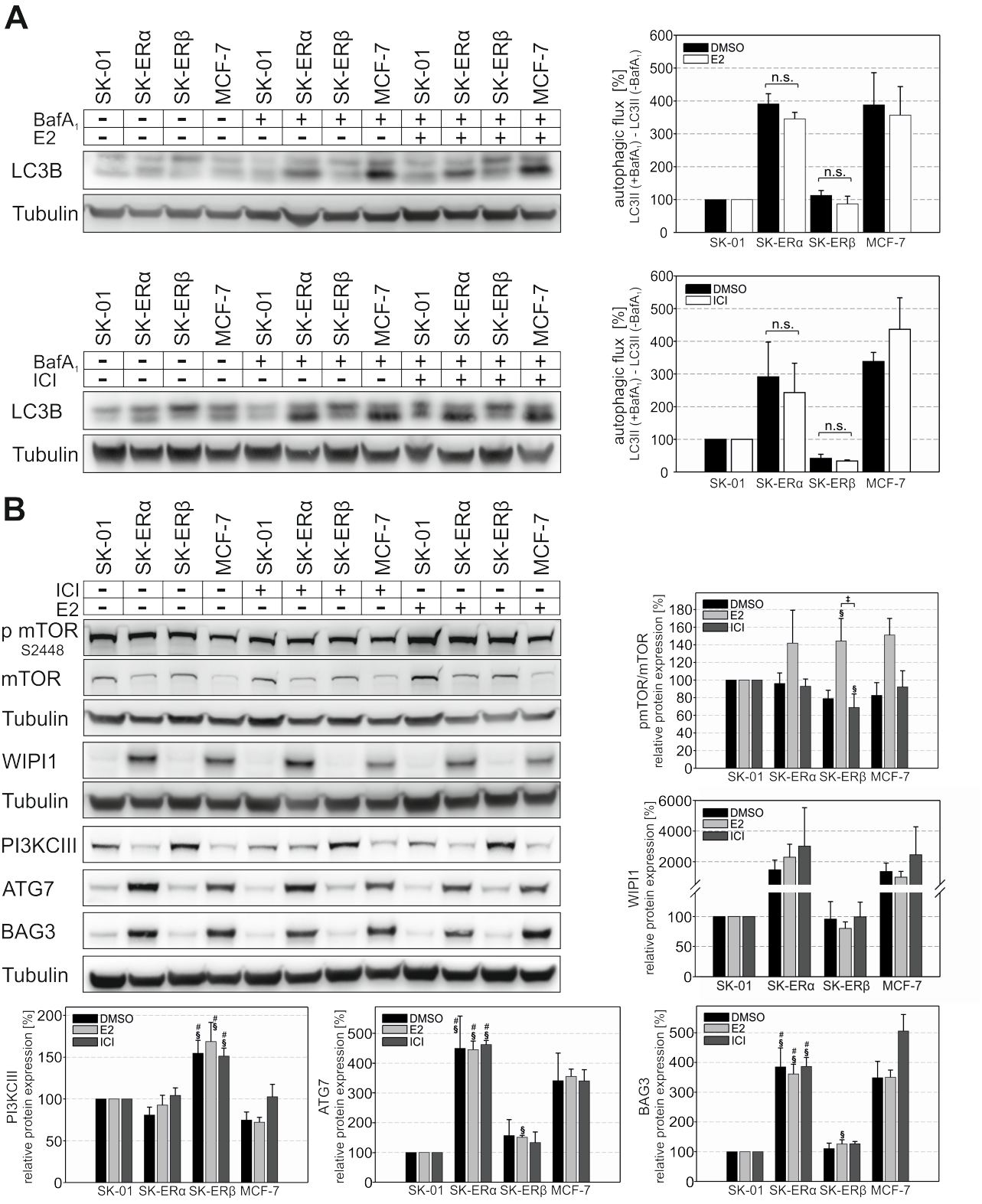

Figure 25: E2 or ICI treatment does not alter autophagic flux or expression of key autophagy pathway-related proteins. (A) SK-01, SK-ERα, SK-ERβ and MCF-7 cells were incubated with 10 nM E2 and 1 µM ICI or vehicle control for 24 h. 6 h prior to cell lysis indicated cells were additionally treated with 500 nM $BafA_1$ and subjected to Western blot analysis. Autophagic flux was determined by the accumulation of LC3-II. Therefore, normalized LC3-II levels in the absence of the lysosomal inhibitor were subtracted from corresponding levels obtained in the presence of $BafA_1$. Tubulin was used as loading control. E2 or ICI treatment does not affect autophagic flux in each of the analyzed cell types. (B) Cells were treated with E2 or ICI for 24 h and Western blot analysis of indicated proteins was performed.

Tubulin served as loading control. Except of the pmTOR/mTOR ratio of E2 and ICI treatment of SK-ERβ there was no effect on protein expression of pmTOR/mTOR, WIPI1, PI3KCIII, ATG7 and BAG3 after drug treatment. (A and B) Values of 3 independent experiments in each panel are expressed as mean ± s.e.m. and control SK-01 cells were set to 100%. (§) on bars represent statistical significance of $p<0.05$ comparing SK-ERα or SK-ERβ cells to SK-01 cells with the same treatment. (#) on bars represent statistical significance of $p<0.05$ comparing E2 or ICI treated SK-ERα cells to E2 or ICI treated SK-ERβ cells. (‡) on bars display a statistical significance of $p<0.05$ of E2 treated SK-ERβ cells compared to ICI treated SK-ERβ cells. All other combinations show no statistical significant difference. (Felzen et al., 2015)

C.1.2.4. Inhibition of autophagy reduces resistance of estrogen receptor α expressing cells to oxidative stress.

Oxidative stress can lead to the preferential killing of cancer cells and various pharmacological compounds, such as cytostatic drugs, with direct or indirect effects act via induction of reactive oxygen species and are in use for effective cancer therapies (Gorrini et al., 2013). Cells react on increased oxidative stress via the induction of the autophagy pathway, which in turn reduces oxidatively modified proteins, and organelles that would negatively modify the protein homeostasis of the cell. Additionally, it is also discussed that autophagy is a key mechanism of cell survival during anti-estrogen treatment and progression of drug resistance in breast cancer cells (Gonzalez et al., 2014; Schoenlein et al., 2009). ERα expression mediates increased resistance of neuroblastoma cells to oxidative stress, as shown in the first part of the results in this thesis. Whether this enhanced survival is mediated via the increased autophagic activity in these cell lines was tested. Consistent with our previously published results the data shows SK-01 and SK-ERβ cells are more susceptible to oxidative stress induced by H_2O_2-treatment in comparison to SK-ERα and MCF-7 cells measured by cell survival determination via FACS analysis after propidium iodid staining (Figure 26). Co-treatment of the cell lines with $BafA_1$ was performed to inhibit the late phase of autophagy to further investigate if ERα-induced autophagy contributes to the increased cell viability after stress induction. The results showed that inhibition of autophagy by $BafA_1$-treatment in ERα expressing cells significantly reduced cell survival after H_2O_2-treatment (Figure 26, B, D) that was not observed in ERα negative cells (Figure 26, A, C). These results suggest that enhanced autophagic activity mediated by ERα contributes to the resistance of cells to oxidative stress and drug treatment.

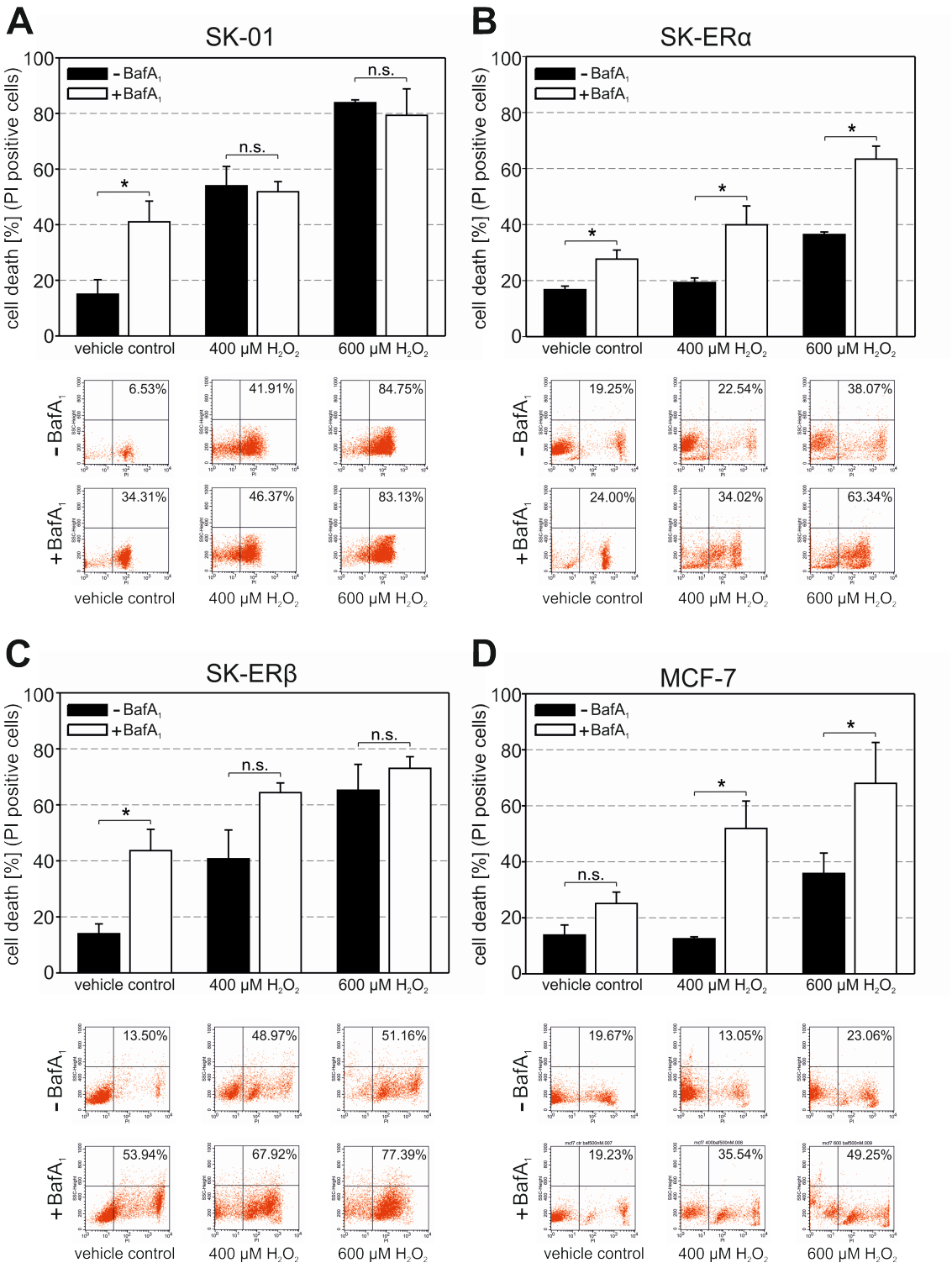

Figure 26: Inhibiting the late phase of autophagy via $BafA_1$ results in enhanced cell death in ERα expressing cells upon exposure to oxidative stress induced by H_2O_2. SK-01, SK-ERα, SK-ERβ and MCF-7 cells were treated with vehicle control or 400 µM and 600 µM H_2O_2 with or without $BafA_1$ (500 nM) for 24 h and cell death was quantified by flow cytometry after propidium iodid staining. Values of 3 independent experiments in each panel are expressed as mean ± s.e.m. and representative data from the experiments is shown in FACS dot plot profiles. (*) on bars represent statistical significance of $p<0.05$ comparing 2 groups and n.s. displays no statistical significant difference. (Felzen et al., 2015)

C.1.2.5. Knockdown of BAG3 reduces autophagic flux and contributes to a partial reversal of stress resistance in ERα-expressing cells.

BAG3 has previously been shown to modulate selective autophagy, proteotoxicity, resistance to stress and cancer-related signaling networks and data show that BAG3 expression is upregulated in SK-ERα cells (Brendel et al., 2013; Colvin et al., 2014b; Gamerdinger et al., 2009b; Rapino et al., 2013). Therefore, downregulation of BAG3 may result in reduced autophagic activity in concert with a higher vulnerability of cells to oxidative stress. To answer this question, knockdown of BAG3 via siRNA in SK-01, SK-ERα and MCF-7 cells was induced and additionally the cells were treated for the flux experiments with $BafA_1$, as indicated. Knock down of BAG3 was accompanied by reduced levels of LC3-II after lysosomal inhibition, demonstrating that BAG3 downregulation significantly impairs autophagic flux in SK-ERα cells as well as in the endogenous ERα expressing MCF-7 cell line and to a lesser extend in SK-01 cells (**Figure 27**, A-C). Next, SK-01, SK-ERα and MCF-7 cells were transfected with non-sense and BAG3 siRNA followed by treatment with vehicle control or H_2O_2 according to experiments performed; cell viability was measured via FACS analysis after propidium iodid staining. The data showed that knockdown of BAG3 does not modulate cell death induced by H_2O_2 compared to non-sense treated controls in SK-01 cells and that cell death was in the same range as with or without $BafA_1$- and H_2O_2-treatment. Interestingly, upon siRNA-mediated BAG3 knockdown and H_2O_2-treatment a significant increase in sensitivity in SK-ERα and MCF-7 cells was detected (Figure 27 D-F), which was in total even higher than after inhibiting just the late phase of autophagy via $BafA_1$-treatment. Note, that higher, although not significant, cell death in SK-ERα and MCF-7 cells already after BAG3 knockdown and vehicle control treatment was detected that might argue for a more general role of BAG3 and basal autophagy in maintaining cancer cell viability in ERα-positive cells.

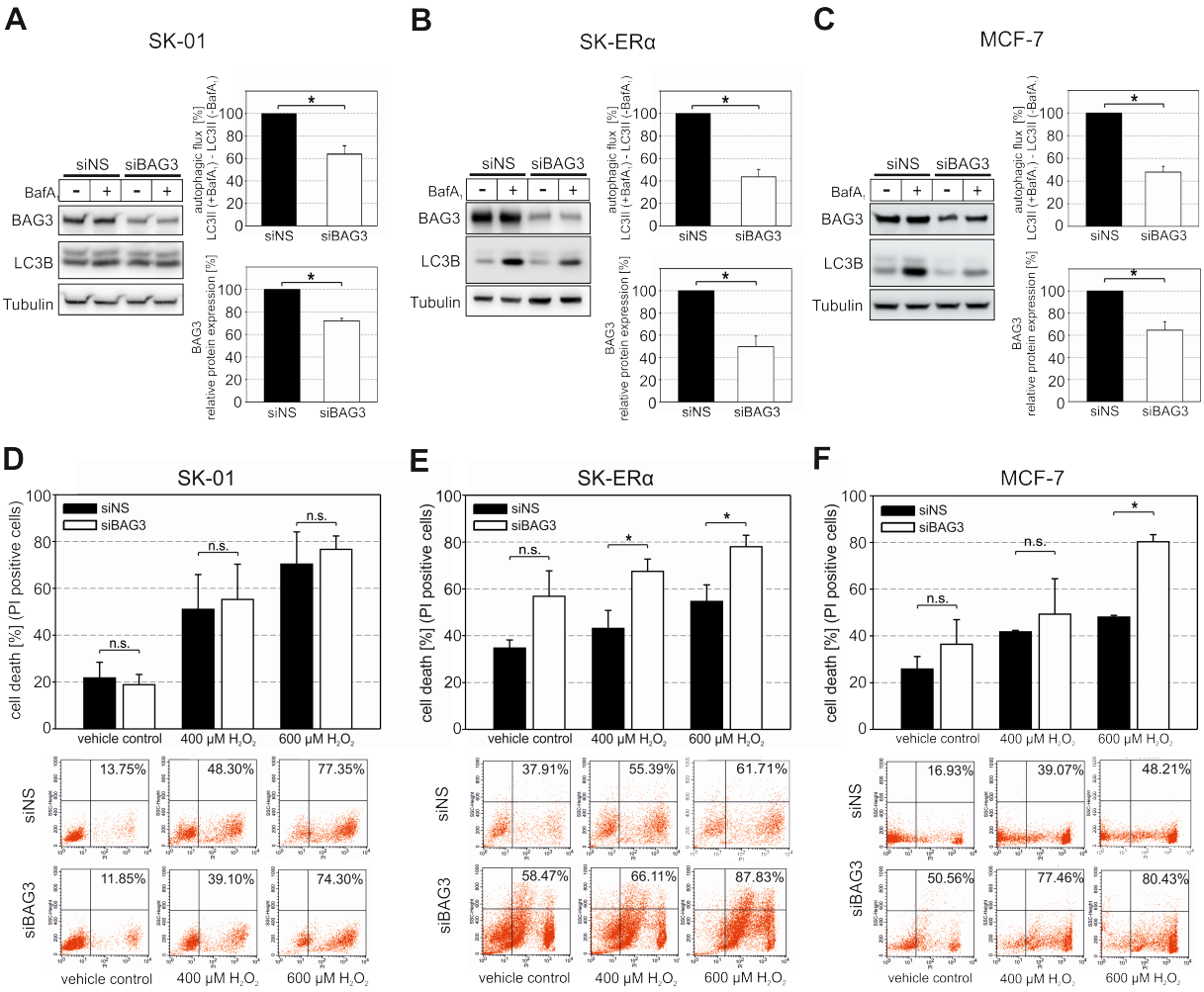

Figure 27: BAG3 is required for enhanced autophagic flux and resistance to oxidative stress in SK-ERα and MCF-7 cells. (A,B,C) SK-01, SK-ERα and MCF-7 cells were transfected with non-sense siRNA (siNS) and BAG3 siRNA (siBAG3) for 48 h, as indicated. Protein extracts from vehicle or $BafA_1$ treated cells were subjected to Western blot analysis and calculation of autophagic flux using anti-LC3 antibodies. Tubulin was used as loading control. Autophagic flux was determined by the accumulation of LC3-II in a 6 h treatment period with 500 nM $BafA_1$. Therefore, normalized LC3-II levels in the absence of the lysosomal inhibitor were subtracted from corresponding levels obtained in the presence of $BafA_1$. (D,E,F) SK-01, SK-ERα and MCF-7 cells were transfected with non-sense siRNA (siNS) and BAG3 siRNA (siBAG3) for 48 h and treated with vehicle control or 400 µM and 600 µM H_2O_2 for 24 h, as indicated. Cell death was quantified by flow cytometry after propidium iodid staining. Values of 3 (SK-01), 4 (SK-ERα) and 3 (MCF-7), independent experiments in each panel are expressed as mean ± s.e.m. and representative data from the experiments is shown in FACS dot plot profiles. (*) on bars represent statistical significance of $p<0.05$ comparing 2 groups and n.s. displays no statistical significant difference. (Felzen et al., 2015)

Tissue from breast cancer patients that were characterized to be ERα-positive or ERα-negative was analyzed to translate our results obtained in our cellular model system to clinically relevant conditions. Samples of seven ERα-positive and seven ERα-negative patients were randomly picked and immunohistochemistry with antibodies against ERα, BAG3, LC3 and p62/SQSTM1 was performed.

The data showed overall the staining intensity of autophagy markers LC3 and p62/SQSMT1 is higher in ER-positive compared to ER-negative breast cancer tissue (Figure 28). BAG3 expression generally was increased in some ER-positive patients but interestingly in one case investigated (patient 17889) BAG3 protein levels were reduced in regions with high ERα expression.

Together, this set of experiments shows that interfering with the autophagy pathway reduces the resistance of ERα expressing cells and sensitizes them to cell death inducing stress. Additionally, in ER-positive breast cancer patients autophagy markers are strongly increased although individual changes in BAG3 expression occur.

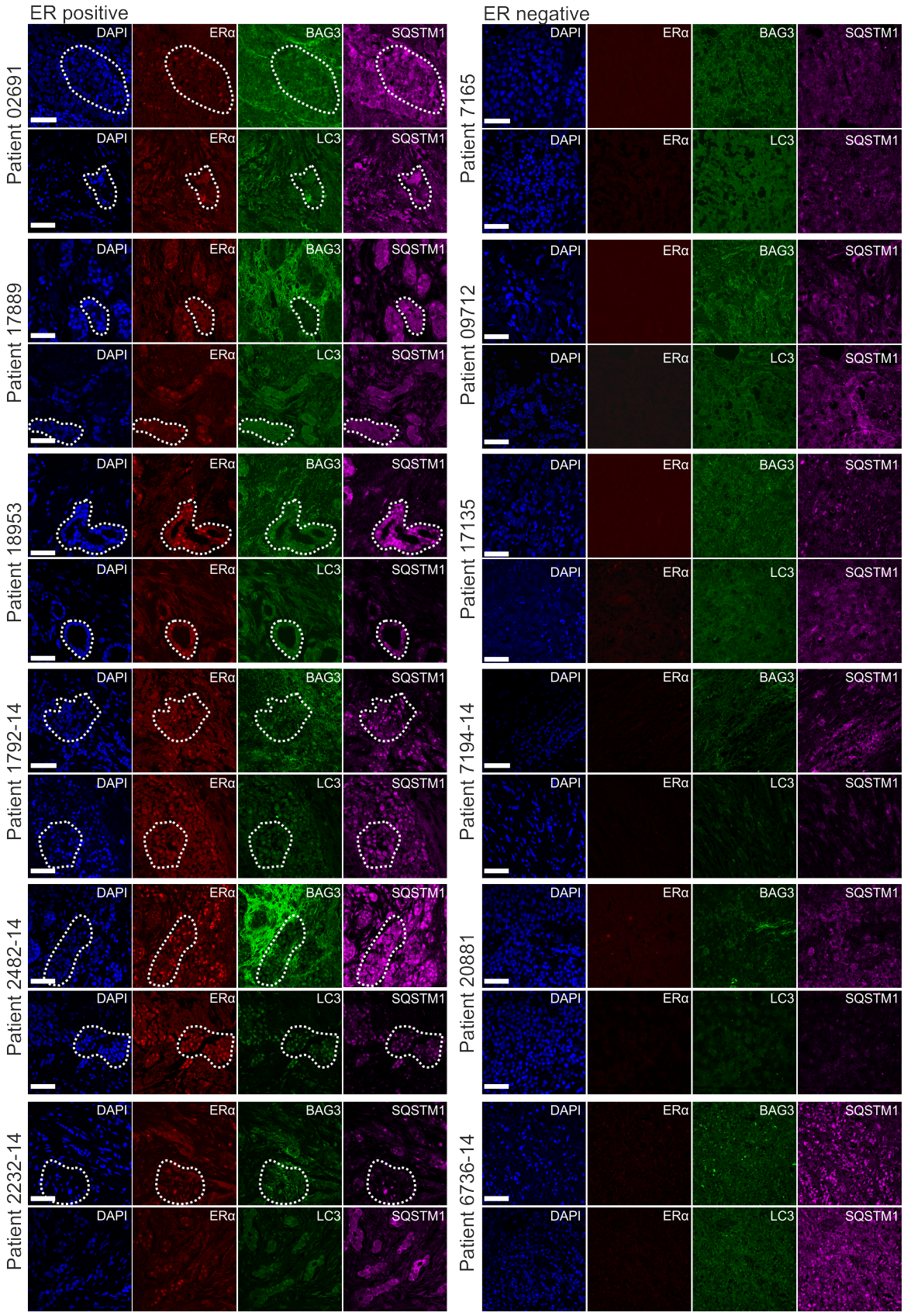

Figure 28: Enhanced expression of autophagy makers in ERα-positive breast cancer patients. Formalin-fixed paraffin-embedded samples from ER-positive and ER-negative breast cancer patients stained for ERα, BAG3, LC3, SQSTM1 and DAPI, were used for immunocytochemistry, as indicated. Images acquired by confocal microscopy are shown. Insets (white dotted line) show regions of interest with high ERα expression. Scale bars: 50 µm. (Felzen et al., 2015)

C.2. The role of the autophagic protein degradation system in neuronal stress adaptation

C.2.1. Autophagic flux and autophagy is increased in hippocampal cells adapted to oxidative stress

Autophagy in situations of oxidative stress was analyzed in the HT22 murine hippocampal cell line. Out of the HT22 mother cell line 2 cell clones, adapted to chronic oxidative stress, were generated. On the one hand H_2O_2 (cell clone $HT22_{H202}$) and on the other hand glutamate (cell clone $HT22_{Glu}$) have been used to cause sub lethal oxidative stress levels in the cell and to induce adaptation processes. Importantly in our cell model, glutamate is not inducing cell death by excitotoxicity and in a glutamate-receptor-independent way, since HT22 cells don't express functional glutamate receptors. Glutamate is rather inducing oxidative cell death by inhibition of a cysteine-glutamate antiporter resulting in the depletion of intracellular cysteine and furthermore of GSH (glutathione: γ-L-Glutamyl-L-cysteinylglycin) one of the main antioxidant molecule within the cell (Bannai and Kitamura, 1980; Murphy et al., 1989; Sato et al., 1999)(Bannai and Kitamura 1980; Murphyet al. 1989; Sato et al. 1999). The used cell model is a well-established tool for the research of resistance and cytoprotection in neurons (Schäfer et al 2004, Clement&Gamerdinger 2009).

As it was shown by Clement et al. 2009 that HT22 cells resistant to oxidative stress show differences in the lysosomal compartment compared to the sensitive mother cell line we asked whether there are also changes upstream of the degradation pathways which ends up in the lysosomes. When stopping the lysosomal/autophagosomal fusion with the v-ATPase inhibitor bafilomycin (BafA1) the accumulation of LC3II and p62/SQSTM was measured, in all of the cell lines by Western blot. Indeed, the resistant cells show higher autophagic flux in comparison with HT22 cells when measuring the accumulation of LC3II as well as p62/SQSTM (Figure 29).

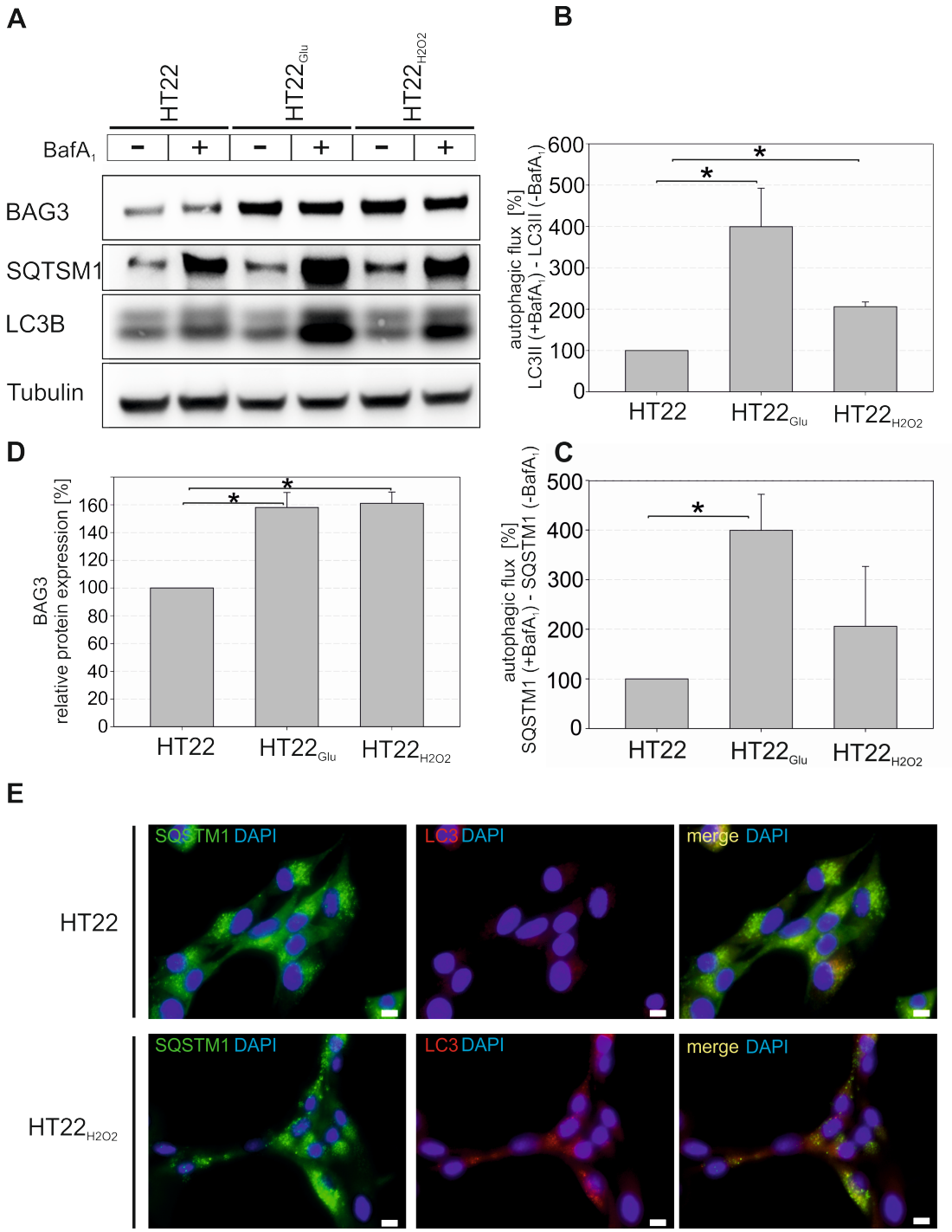

Figure 29 Autophagic markers are increased in hippocampal cells adapted to chronic oxidative stress. (A) Protein extracts from HT22, HT22Glu and HT22H2O2 have been taken after 6h of BafA1 treatment to stop the autophagosome/lysosomes fusion and expression of the indicated proteins was detected by western blotting. (B,C) The autophagic flux was measured after densitometric analysis. Therefore, normalized LC3-II levels in the absence of the lysosomal inhibitor were subtracted from corresponding levels obtained in the presence of BafA1. (D) Densitometric analysis of the BAG3 signal of untreated cells after normalization to tubulin is shown. (E) HT22 and HT22H2O2 cells were immunhistochemically stained against the indicated proteins. Scale bars:10µM. Pictures were taken by confocal microscopy. Values of 3 independent experiments in each panel are expressed as mean ± s.e.m. and control HT22 cells were set to 100%. (*) on bars represent statistical significance of $p<0.05$ comparing 2 groups.

C.2.1.1. BAG3 expression is increased in hippocampal cells exposed to chronic oxidative stress

The increased autophagic flux in the cells adapted to oxidative stress directed to further investigations to analyze the degradation pathway in more detail. It is known that under oxidative stress conditions, the BAG3-mediated selective autophagy could be activated (Gamerdinger, 2009). When detecting BAG3 by immunoblotting, it was found to be highly upregulated in the adapted cells compared to the control cell line (Figure 29). These findings suggest that the BAG3-mediated selective autophagy may be involved in the adaptation process of the cell, since BAG3 is known to clear aggregated prone cargo via autophagy by the interaction with HSP70 and p62/SQSTM (Gamerdinger 2009,2011). In the first part of this study BAG3 was already shown to be highly expressed in cells adapted to stress and HSP70 was also found to be highly upregulated in $HT22_{Glu}$ and $HT22_{H2O2}$ cells in fomer studies (Clement et al.2009). The chaperone HSP70 is cooperating with different co-chaperones including the BAG proteins to transfer substrates to suitable degradation machineries. In cellular models of aging, where oxidative stress levels rise, a switch from the BAG1-mediated proteasomal degradation to a BAG3-mediated selective macroautophagy was found (BAG1/BAG3 switch). That's why in a next step the BAG1 levels in the cell lines were investigated.

C.2.1.2. The co-chaperone BAG1L is downregulated in resistant cells

The co-chaperone BAG1 is binding to HSP70 but is rather directing substrates to the proteasomal degradation system/UPS than to the autophagosomes. Since a BAG1 to BAG3 switch was found in aged cells as well as in HEK cells exposed to acute oxidative stress, it was also of interest to know if this phenomenom is also true for neuronal cells and is a more general mechanism also in adaptation processes. Therefore the expression pattern of BAG1 in the oxidative stress adapted cells was investigated. BAG1 is expressed in 4 isoforms (BAG1, BAG1L, BAG1M and BAG1S), with the highest expression levels of BAG1L and S in mouse cells. All of the BAG1 isoforms were detected by immunoblotting against the BAG domain. One of the BAG isoforms, BAG1L, was found significantly downregulated in $HT22_{H2O2}$ and $HT22_{Glu}$ cells compared to the HT22 cell line (Figure 30), which is exactly the same isoform besides BAG1 that was also found to be downregulated in aged cells. Taken together BAG3 and BAG1L are reciprocally regulated in cells exposed to chronic oxidative stress and display an expression pattern equivalent to aged cells (Gamerdinger, 2009).

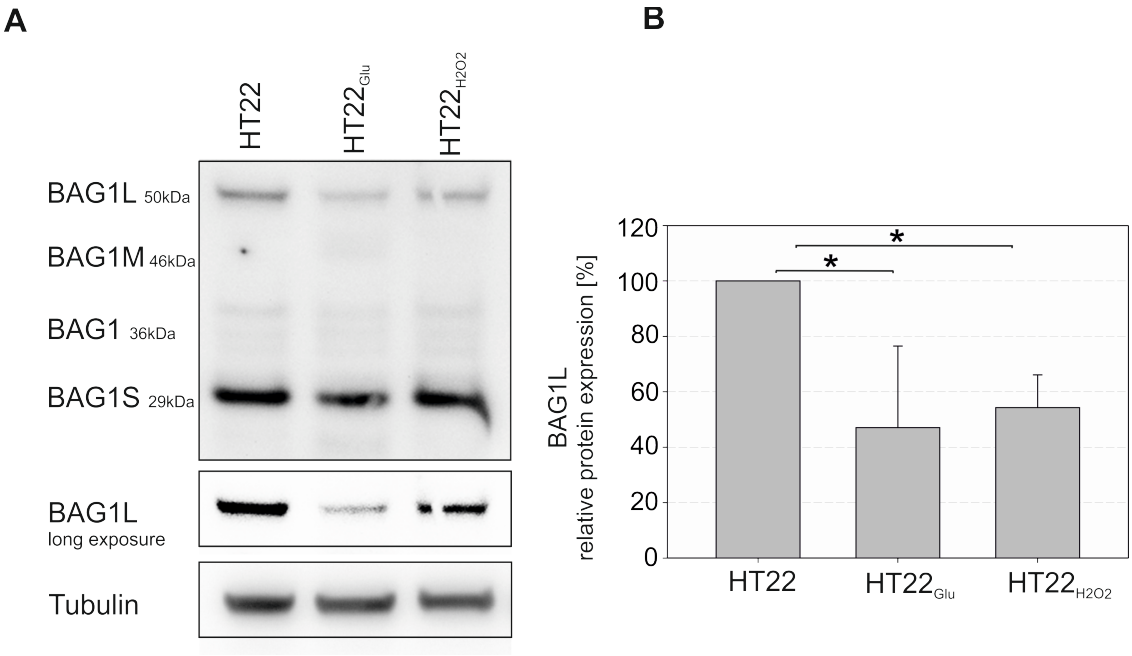

Figure 30: Adaptation to oxidative stress reduces BAG1L expression. (A)Protein extracts from untreated HT22, HT22Glu and HT22H2O2 cells were subjected to Western blot analysis for the indicated proteins. Tubulin was used as loading control. (B) Densitometric analysis of the expression profile of BAG1L are shown. Values of 3 independent experiments in each panel are expressed as mean ± s.e.m. and control HT22 cells were set to 100%. (*) on bars represent statistical significance of $p<0.05$ comparing 2 groups and n.s. displays no statistical significant difference.

C.2.1.3. Autopagy induction is independent of canonical mTOR regulation, Atg7 and Atg4 protein expression but displays a BECN1 protein upregulation

BAG3 was identified as autophagy adapter and inducer of selective autophagy (Gamerdinger et al.,2009/2011), and as in this study BAG3 was found highly upregulated in cells exposed to chronic oxidative stress, it was of interest to further focus on the macroautophagic pathway. A main regulator of autophagy is the mammalian target of rapamycin (mTOR). mTORC1, existing of mTOR and Raptor, represents a repressor of autophagy, as if it is hyperactivated inhibits the ULK-complex and represses downstream induction of autophagosome generation. In nutrient depletion mTOR is inactivated by Raptor and furthermore autophagy is initiated. One possibility to check for mTOR activity is the detection of the phosphorylation site S2448. Downregulated phosphorylation correlates with decreased mTORC1 activity (M.Rosner et al,2010) and hence autophagy activation takes place. When detecting mTOR and the phosphorylated protein there were no changes in phosphorylation status and ratio

detectable in the resistant cells $HT22_{Glu}$ and $HT22_{H2O2}$ compared to HT22 cells (Figure 31 A,D).

Atg7 accelerates the conjugation of phosphatidylethanolamine (PE) to LC3I to form the membrane associated LC3-II (Kabeya,2000) and is of main relevance for autophagy function. But again, there was no detection of a regulation of Atg7 in the adapted cell $HT22_{Glu}$ and $HT22_{H2O2}$ compared to HT22 (Figure 31 B,E).

A known autophagy-related protein, which is regulated directly by oxidative stress is ATG4. ATG4 cleaves the COOH-terminal of LC3 and also delipidates LC3II on the cytosolic membrane (Ravikumar 20010). The delipidation process is dependent on the oxidative state of Atg4. Under oxidative conditions Atg4 itself gets oxidized and is inactivated (Scherz-Shouval R. 2007). When investigating Atg4 via immunoblotting we could not detect any differences in the adapted cells in comparison to HT22 cells (Figure 31 B,E). It has to be mentioned that in this study the expression levels of the protein, but not the oxidation status has been controlled, which would give further hinds about the regulation status of Atg4 in more detail.

Taken together autophagy induction in the case of adaptation processes to oxidative cell stress is independent of mTOR phosphorylation as well as Atg7 and Atg4 protein expression.

Another main regulator of autophagy displays the PI3K/BECN1 complex, which is required for the recruitment of PI3P to the phagophore. Therefore, it was of interest whether BECN1 is responsible for the activation of the atutophagic pathway in the adapted cell lines while mTOR was shown to be unregulated. Indeed, higher BECN1 protein expression levels in $HT22_{Glu}$ and $HT22_{H2O2}$ cells were detected when compared to the HT22 mother cell line (Figure 31 C,G).

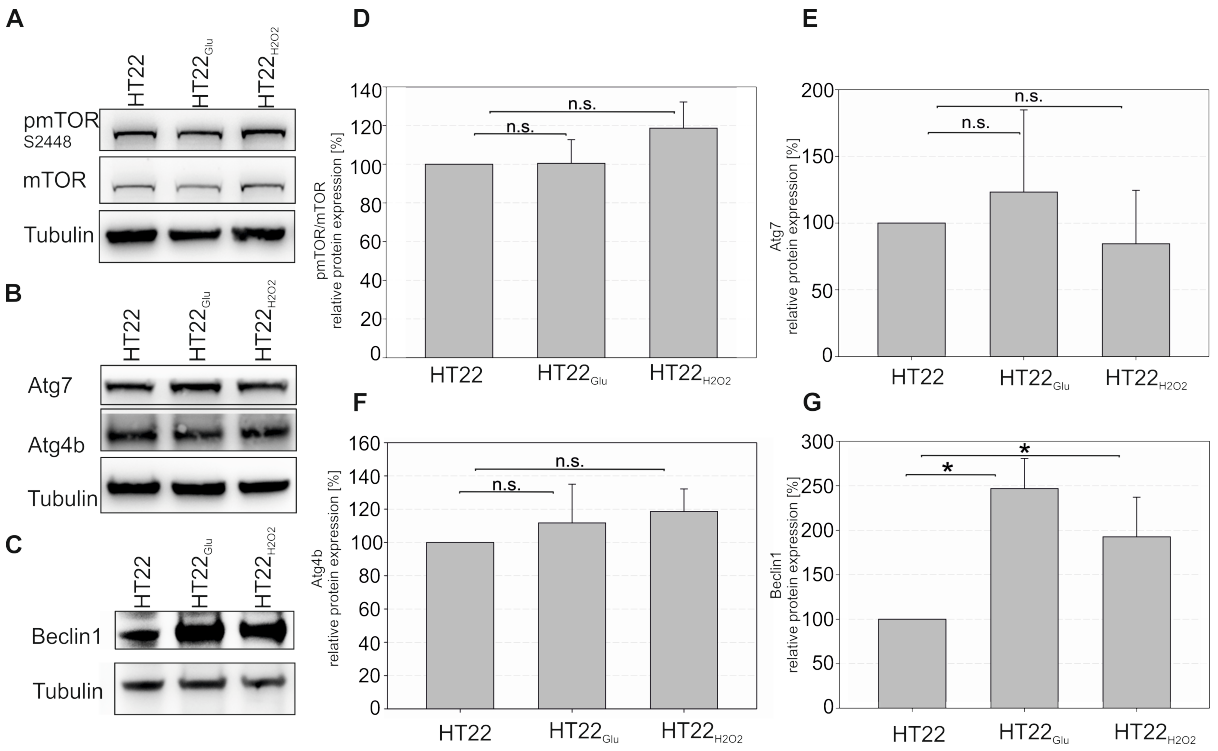

Figure 31 Adaptation to oxidative stress enhances the expression of Beclin1 protein expression but excludes regulation of main canonical autophagy regulators mTOR, Atg7 and Atg4. Protein extracts from untreated cells, HT22, HT22Glu and HT22H2O2 were used to perform Western blot analysis for detection of indicated proteins. The diagrams display the protein levels after normalization to corresponding Tubulin. Values of 3 independent experiments in each panel are expressed as mean ± s.e.m. and control SK-01 cells were set to 100%. (*) on bars represent statistical significance of $p<0.05$ comparing 2 groups and n.s. displays no statistical significant difference.

Taken together a mTOR independent autophagic pathway without changes in the expression pattern of main autophagy regulators Atg7 and Atg4 has been detected in cells which are resistant to chronic oxidative stress. Instead BECN1, a main inducer of autophagy is highly upregulated within the adaptation process.

The lysosomal compartment is an important component of the autophagy degradation pathway. In former studies the adaptation process of the HT22 cells have been shown to be associated with changes in the lysosomal compartment (Clement et al 2009) and have therefore been analyzed further in the next section of the current studies.

C.2.1.4. Lysosomal rearrangement in hippocampal cells adapted to oxidative stress is induced by BAG3 overexpression

Autophagosomes engulf cargo in double membranes and end up in fusion with lysosomes to form so called autolysosomes. It has already been shown in former studies of our lab that in oxidative stress-resistant cells the lysosomes, detected by lysosomal associated membrane protein 2 (LAMP2) stainings, are differentially distributed compared to the HT22 wild type cells. Furthermore lysotracker staining, visualizing the acidic compartments in the cell, indicated different amount and localization of lysosomes in oxidative stress-resistant cells. Western blot analysis revealed statistically significant higher LAMP1 protein expression levels in the resistant cells $HT22_{Glu}$ and a tendency of a higher expression in $HT22_{H2O2}$ cells in comparison to the non-resistant cells HT22 (Figure 32 A,B).

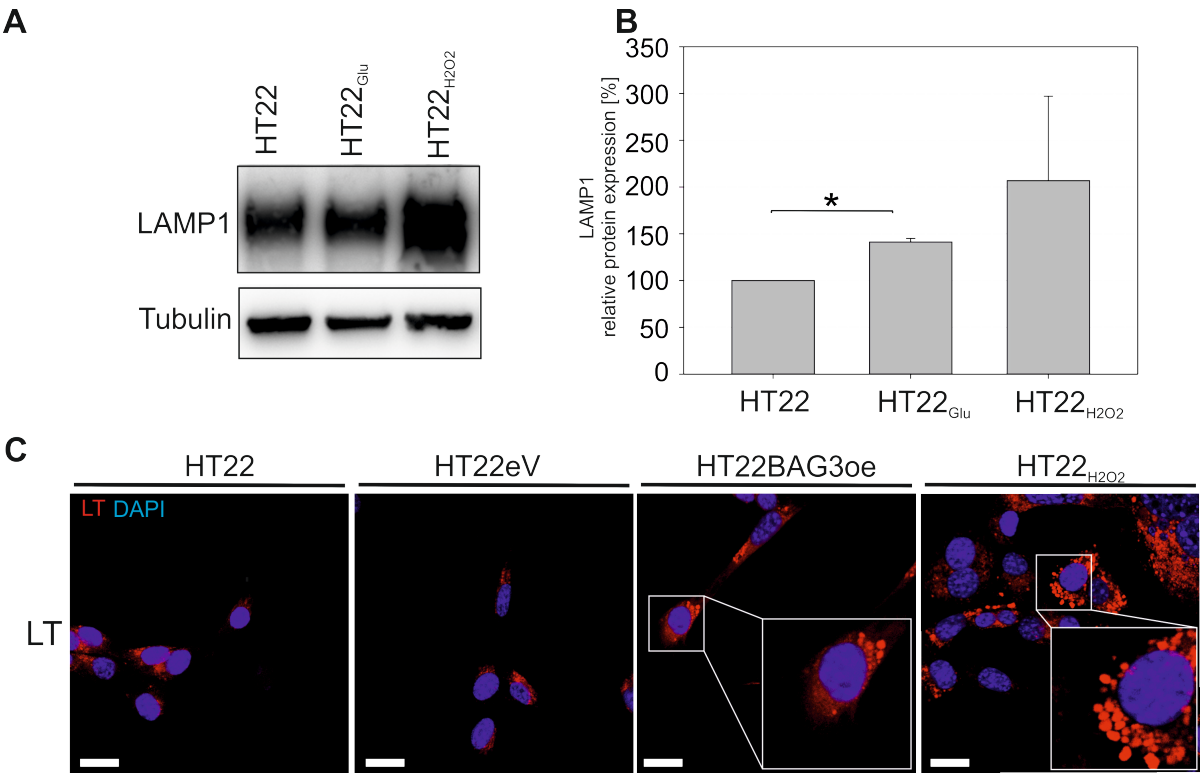

Figure 32: BAG3 expression promotes the rearrangement of the lysosomal compartment comparible as in cells exposed to chronic oxidative stress. (A,B) Protein extracts from untreated cells were analyzed via western blotting and quantification of LAMP1 expression was calculated by densitometric analysis after normalization to tubulin. (C) Representative pictures of lysotracker red staining (LT) in untreated HT22, HT22 empty vector transfected (HT22eV), HT22 overexpressing BAG3 and HT22 adapted to H2O2 (HT22H2O2) cells, are shown. DAPI (blue) was used to stain DNA. Scale bars: 10 µm. Representative images acquired by confocal microscopy are shown. 3 independent experiments in each panel are expressed as mean ± s.e.m. (*) on bars represent statistical significance of $p<0.05$ comparing 2 groups and n.s. displays no statistical significant difference.

Lysotracker stainings have been prepared in conditions of overexpressing of BAG3 to analyze the lysosomal compartment with respect to the BAG3-mediated selective autophagic pathway. Comparing untreated cells (HT22), cells transfected with empty vector (HT22eV), cells overexpressing BAG3 (HT22BAG3oe) and the resistant cells ($HT22_{H2O2}$) small dotted perinuclear accumulated lysosomes in HT22 and HTeV cells were found. Oxidative stress resistant cells ($HT22_{H2O2}$) showed elevated number of lysosomes with increased size in comparison to non-resistant cells, and the same results could also be detected in $HT22_{BAG3oe}$. After overexpression of BAG3 the distribution of lysosomes is similar to the resistant cell line (Figure 33 C).

The results indicate that the distribution of lysosomes as well as the size of the acidic compartments is changed in cells resistant to oxidative stress and that it is very likely influenced by BAG3 protein level.

In the previous work of this thesis protein homeostasis under oxidative stress conditions *in vitro* in hippocampal cells was studied. For this reason, it was of interest if the results are translatable into *in vivo* situations in the brain.

C.2.1.5. Proteinhomeostasis is altered after traumatic brain injury (TBI) *in vivo*

In traumatic brain injuries (TBI) oxidative stress is thought to play a crucial role in inducing cell death and served as a particularly suitable pathophysiological model (Kontos and Povlishock, 1986; Tyurin et al., 2000). A well-established model for traumatic brain injury in mice is the “controlled cortical impact” (CCI) (Xiong et al., 2013). CCI is a method to perform traumatic brain injuries in mice through the setting of a rigid impactor. Following a craniotomy mechanical energy is delivered to an intact dura whereby the impact is made under precise parameters to achieve a pre-determined deformation depth an reproducibility.

Characterizing changes in the BAG3-mediated selective macroautophagic pathway after TBI the BAG3 protein expression pattern in mice brain 12h, 24h and 48h after CCI was analyzed and compared to control animals. Western blot analysis of BAG3 protein expression revealed higher expression of BAG3 12 h and 24 h after CCI as compared to control animals. Strikingly after 48 h after CCI the BAG3 protein expression was significantly upregulated (Figure 34 A). Furthermore the CCI exposed site (ipsilateral)

was compared with the non-CCI exposed site of the brain (contralateral) for autophagy markers by immunohistochemistry. The Data revealed higher LC3, p62/SQSTM as well as BAG3 levels in the ipsilateral site of the lesion 6h after CCI compared to the contralateral site (Figure 34 B). Furthermore LC3_GFP mice showed the same distribution pattern as we found LC3 enriched in ipsilateral areas after CCI compared to contralateral sites (Figure 34 C).

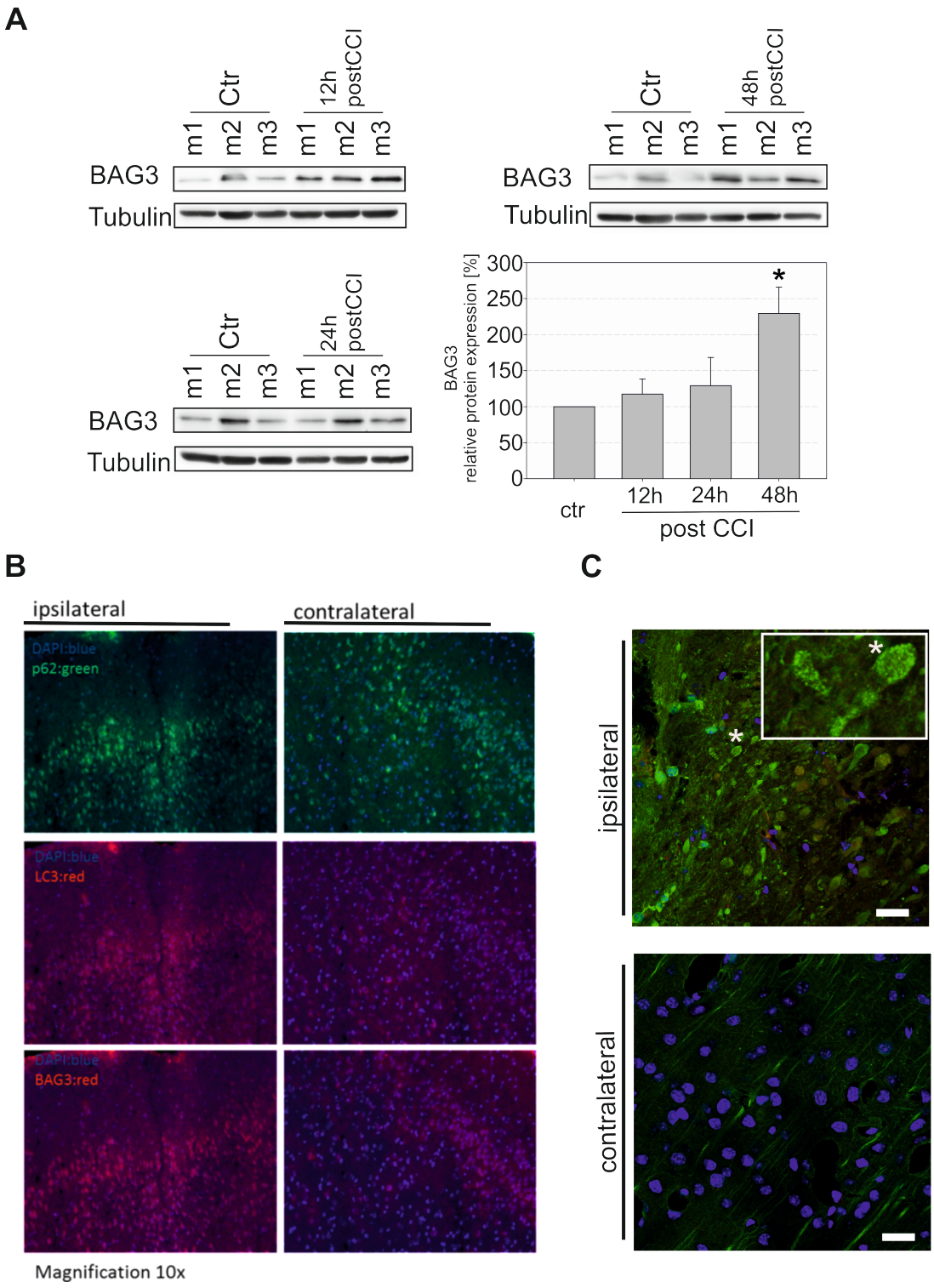

Figure 34: Autophagic markers increase in ipsilateral brain areas after CCI. (Studies were carried out in Cooperation with Christina Gölz; Univ.-Prof. Dr. med. Kristin Engelhard; clinics for anesthesiology University medical center of the Johannes Gutenberg-University; and LC3_GFP mice were kindly provided from Prof. Dr. Irmgard Tegeder, Frankfurt). (A) Protein extracts from brain lysates of untreated mice (Ctr) as well as ipsilateral brain sites 12 h, 24 h and 48 h post CCI were analysis by Western blotting for indicated proteins followed by densitometric analysis. (B) Brain slices 6h post CCI were used for immunocytochemistry to detect the contribution of indicated autophagosomal markers. Ipsi- and contralateral brain regions of equal hemisphere areas are shown. (C) LC3_GFP mice have been used to improve the quantification of LC3 signal after CCI. Again pericontusional ipsilateral sites have been

compared to equal areas in the contralateral hemisphere. Green dots show accumulation of LC3 signals that indicate autophagosomal structures. 3 independent experiments in each panel are expressed as mean ± s.e.m. (*) on bars represent statistical significance of p<0.05 comparing 2 groups and n.s. displays no statistical significant difference.

Answering the question whether the accumulation of LC3 and p62/SQSTM after CCI is due to an induction rather than to a disturbance of autophagy, the autophagic flux after CCI in acute brain slices was quantified. Western blot analysis of LC3II accumulation with and without chloroquine treatment of sham and CCI animals detected a significantly higher autophagic flux in the ipsilateral site of CCI exposed animals. (Figure 35).

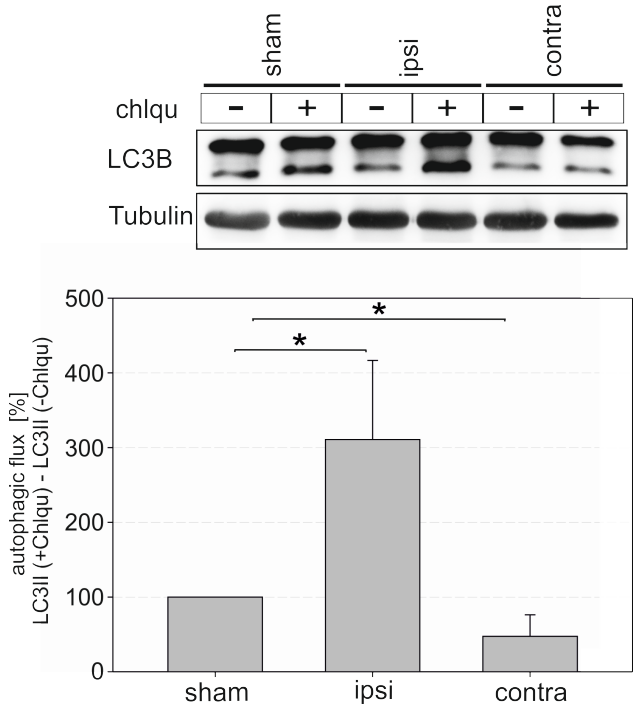

Figure 35: Ipsilateral brain areas are characterized by a higher autophagic flux compared to sham animals. (Studies were carried out in Cooperation with Florie Le Prieult; Prof Dr Thomas Mittmann; Institute of physiology; Universitymedicine of the Johannes Gutenberg-University). Acute brain slices have been prepared 48h post CCI as well as sham operated mice and incubated 2h with Chloroquine (200µM) in oxygenated incubation chambers (All done by Florie Le Prieult). Protein lysates have been analyzed by western blotting against indicated proteins and quantified by densitometric analysis (carried out by Vanessa Felzen). 3 independent experiments in each panel are expressed as mean ± s.e.m. (*) on bars represent statistical significance of p<0.05 comparing 2 groups and n.s. displays no statistical significant difference.

Taken together increased levels of BAG3 protein levels have been detected after CCI in mice in vivo, that might be due to the induction of BAG3-mediated selective autophagy. Strikingly, we detected higher autophagic flux in ipsilateral brain areas in acute brain slices of mice after CCI. The data found in vivo furthermore correlate with the findings of the in vitro studies and highlight a role for autophagy, with a special focus on BAG3, and stress adaptation processes.

D. Discussion

Recently *BAG3-mediated selective macroautophagy*, a new pathway of autophagy, has been discovered by one group of the Behl lab, but still the exact role of BAG3 in autophagy and pathophysiology has to be clarified in detail. In this work, macroautophagy with a focus on BAG3, was therefore the main field of interest. Detailed analysis has been performed to describe ER-dependent autophagy in cell lines ectopically and endogenously expressing ER as well as in breast cancer tissue. The study identified a non-canonical autophagic pathway induced by ERα, which is partially regulated by BAG3 and induced cell resistance to oxidative stress. In the second part of this work, research on autophagy and cytoprotection in neuronal cell systems was translated into an *in vivo* insult model. Hippocampal cells exposed to oxidative stress revealed, that a non-canonical autophagy pathway independent of mTOR, but including BECN1 and BAG3 expression, is strongly induced, that leads to a rearrangement of the lysosomal compartment and may act as an adaptation process to chronic and acute oxidative stress, which might maintain survival in neuronal cells.

In both mentioned *in vivo* and *in vitro* systems the relevance of autophagy in stress resistance was analyzed in detail and its implications in breast cancer and neurodegeneration are discussed in the following sections.

D.1. **ERα regulates apoptosis and autophagy providing cytoprotection**

This study employed a well-characterized model of transformed cells, which ectopically express ERα or ERβ. Moreover a patient-derived ERα-positive breast cancer cell line was used and revealed a down regulation of the expression of mediators of apoptosis, as well as a strong resistance of ERα expressing cells to oxidative stress. Furthermore, BAG3, was found to be highly up-regulated in ERα expressing cells. This suggests that autophagy may be involved in the induction of resistance against oxidative stress via ERα. Hence, a detailed analysis of the autophagic pathway was performed.

D.1.1. ERα expression leads to the repression of caspase 3 and high levels of BAG1 and BAG3 on protein level

The results link the expression of ERα in neuroblastoma and breast cancer cells to the down-regulation of main players modulating apoptosis including the key executioner caspase, *caspase* 3, as well as BAG proteins which correlates with the increased

resistance against oxidative stress. In addition to the observed down-regulation of *caspase* 3, an increased *bcl*2/*bax*-ratio in ERα expressing cells described by Brendel & Felzen et al.2013 might furthermore support the ERα-dependent suppression of apoptosis. Interestingly, the ERα-mediated protection against apoptosis occurs already in the absence of 17β-estradiol. In fact, we have previously observed effects of ERα without further addition of 17β-estradiol in the context of silencing *caveolin 1* and *caveolin 2* gene expression in SK-ERα cells (Zschocke et al., 2002). Down-regulation of caspase 3 expression may be induced via non-classical, cytosolic effects of ER or upstream cross-talk of ER signaling. ERs present in the cytoplasm have been reported to interact with various signaling pathways, including the PI3K pathway (Behl, 2002a; Simoncini et al., 2000).

Interestingly the expression of the anti-apoptotic BAG proteins BAG3 and BAG1 were highly regulated in ERα cells. BAG1 and its family member BAG3 maintain the expression of the anti-apoptotic proteins BCL2, BCL-XL and MCL1 (Aveic et al., 2011; Zhang et al., 2011). Furthermore BAG3 is cleaved by caspase 3 (Wang et al., 2010) and our results show strong stabilization of the full-length BAG3 protein, which supports the finding of an almost complete silencing of *caspase*3 activity. Residual BAG3 cleavage products might be caused via processing by caspase 1 or 8, which are also capable of shedding BAG3 into fragments (but to a lower extent) (Wang et al., 2010). The multifunctional protein BAG1 has also been shown to be involved in various cell processes involved in cell survival (Townsend et al., 2005) and was up-regulated in cells expressing ERα. Co-silencing of BAG1 and BAG3 caused an enhanced predisposition to cell death in cell lines and primary neurons (Aveic et al., 2011) that underlines our findings that ERα expressing cells with increased levels of BAG1 and BAG3 display enhanced survival capacity under oxidative stress conditions.

D.1.2. Impact of autophagy in estrogen receptor-dependent stress resistance and breast cancer

Beside the apoptosis-related impact of BAG1 and BAG3, they also represent key players to control cellular protein homeostasis. BAG1 controls proteasome dependent protein

degradation, whereas BAG3 is involved in the clearance of aggregated proteins in fibroblasts as well as in post-mitotic aged neurons (Gamerdinger et al., 2009a).

BAG3 regulates the degradation of target proteins via direct interaction with the chaperone complex HSP70/HSC70 controlling PQC, which is called BAG3-mediated selective macroautophagy. We found highly elevated HSP70 protein levels in ERα expressing cells, suggesting that this interaction might contribute to stabilize protein homeostasis, thereby leading to cytoprotection. A secured PQC is known to stabilize cell survival and in particular autophagy has been implicated in cytoprotection, ranging from cancer cell resistance to neuroprotection (Apel et al., 2008; Berardi et al., 2011; Mizushima et al., 2008).

Hence, the focus of our further studies was especially on the detailed analysis of the autophagic pathway.

Breast cancer is a heterogeneous disease, which is clinically classified as steroid hormone receptor-positive, HER2-positive and triple negative tumors. Approximately 70% of newly diagnosed breast cancers are ER-positive. Out of the two forms of ERs, ERα and ERβ, ERα levels are specifically analyzed in clinical specimens as binding of estrogen to ERα has been found to stimulate proliferation of breast cancer cells. It is well known that breast cancer cells may develop endocrine resistance and resistance to anti-hormone therapy which might be facilitated by up-regulation of the autophagic machinery (Cook et al., 2011). However up to now the description of a detailed autophagy expression profile of ER-positive cancer cells has not been done.

We could show that the expression of ERα, but not ERβ, is followed by a strong induction of the autophagic machinery. While the elevated autophagic activity triggered via ERα signaling was clearly demonstrated, analysis of autophagy arrays revealed a not clear-cut pattern. The expression of ERα, leads to a defined “Autophagy footprint” of related genes. One has to emphasize that both mentioned cell lines expressing ERα showed in most cases the same expression pattern of autophagy related genes. These results strongly suggest that ERα expression has an outstanding function in regulating specific genes related to autophagy, which in consequence may potentially stimulate autophagy.

Furthermore, our analysis showed that treatment of ERα- or ERβ-positive cells with E2 or ICI, inducing and inhibiting transcription factor activity of the receptors respectively,

did not alter the identified genetic "*autophagy-footprint*" of these cells suggesting an ER-modulated mechanism distinct to their function acting as bona fide transcription factors (**Figure 41**). It has been reported that ERs also function in a non-classical mode by interacting with various intracellular signaling pathways, thereby affecting indirectly the transcription of target genes independent of the classical genomic action (**Figure 37**)(Behl, 2002b; Heldring et al., 2007; Nilsson et al., 2011; Simoncini et al., 2000). This may explain the observed ERE-independent effects of ERα on the autophagic pathway.

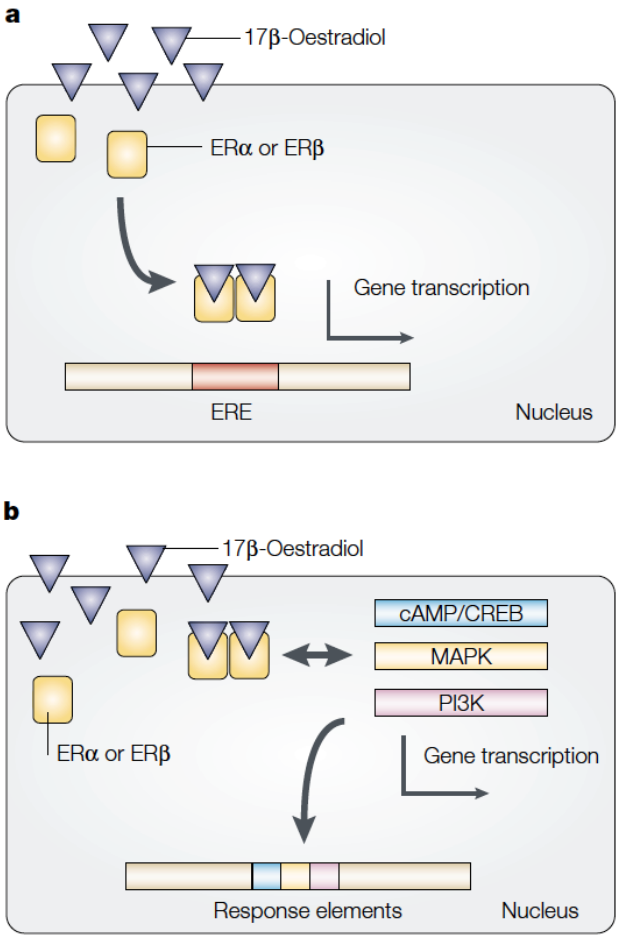

Figure 36: Classical and non-classical mode of ER signaling. Classical ER signaling (A): ERs in the cytoplasm bind their cognate ligand, followed by conformational changes inducing dimerization and translocation to the nucleus. The central DNA binding domain (DBD) recognizes and interacts with specific ERE binding sites thereby regulating transcription of target genes (canonical activity). Furthermore ERs are able to bind other transcription factors AP1 or SP1, resulting in indirect regulation of transcription. Further studies revealed new ER signaling pathways (B): non-genomic effects of ERs are known. In this case signaling cascades in the cytoplasm are activated (PI3K, MAPK) by second messangers by events, which remain to be elucidated. Furthermore ligand independent (for example growth factor dependent) activities of ERs have already been described. However they are not as well understood compared to classical modes of action. The findings of our studies support the hypothesis that ERα-induced autophagy is ERE independent and signals are transduced independently of ligand binding. (C. Behl et al., 2002)

ERα and ERβ genes are localized on different chromosomes, but share some characteristics in activity and in structure. They differ in three functional domains: the DNA binding domain (DBD), the COOH-terminal ligand-binding domain (LBD) and the NH2-terminal domain (NTD). Whereas the DBD is highly conserved between the two receptors and guarantees the transcription factor activity by binding to EREs, the AF1 located at the NTD show just 16% homology between the two ERs and is characterized by its ligand-independent function (Nilsson et al., 2011). Therefore, the E2 independency of the described autophagic activity that is just found for ERα but not ERβ might be due to its AF1 function. Furthermore, the AF1 domain triggers the interaction with different co-regulators of ERs, which might lead to the cytoplasmic activity of ERα rather than to the classical transcription factor activity. In contrast to the DNA binding domains of the two receptors, which are highly homologue, the interaction with co-regulators differs by 50% between the two ERs (Nassa et al., 2011). The characterized ERα regulated autophagy is likely due to its interaction with ERα specific co-regulators. For example, since 1995 it is known that the Ras-MAPK cascade of the growth factor signaling pathway modulates the phosphorylation of the AF1 domain thereby regulating ERα activity (Kato et al., 1995). This could be addressed in future studies by ERα-AF1 mutant experiments, which may lead to the interruption of the induced autophagic pathway. Another explanation for transcriptional changes of autophagy genes by ERα expression is the targeting of genes lacking the ERE structure.

Both forms of the estrogen receptors have already been described to display different characteristics that may also counteract each other. For instance, studies revealed that ERβ may inhibit ERα-mediated regulation of gene expression (Williams et al., 2008). For this reason, further studies should also investigate the impact on autophagy upon co-expression of both receptors.

D.1.2.1. ERα induces a non-canonical autophagy pathway…

When dissecting the autophagy-signaling pathway, canonical key protein complexes such as the mTOR- and PI3KCIII-complex were not specifically activated or elevated in ERα-mediated autophagy. ULK1 and FIP200, members of the upstream ULK complex, were rather found to be down-regulated in ERα positive cells. Furthermore, other key

autophagy-related genes out of the two ubiquitin-like conjugating systems (ATG12-ATG5 and the ATG8/LC3 system) were even markedly reduced in their expression in these cells. Canonical autophagy involves hierarchical activity of previously mentioned signaling complexes and ATG proteins. However, there is evidence that the formation of functional autophagosomes may bypass some of these steps (Codogno et al., 2012b; Mauthe et al., 2011; Scarlatti et al., 2008b) (Figure 37).

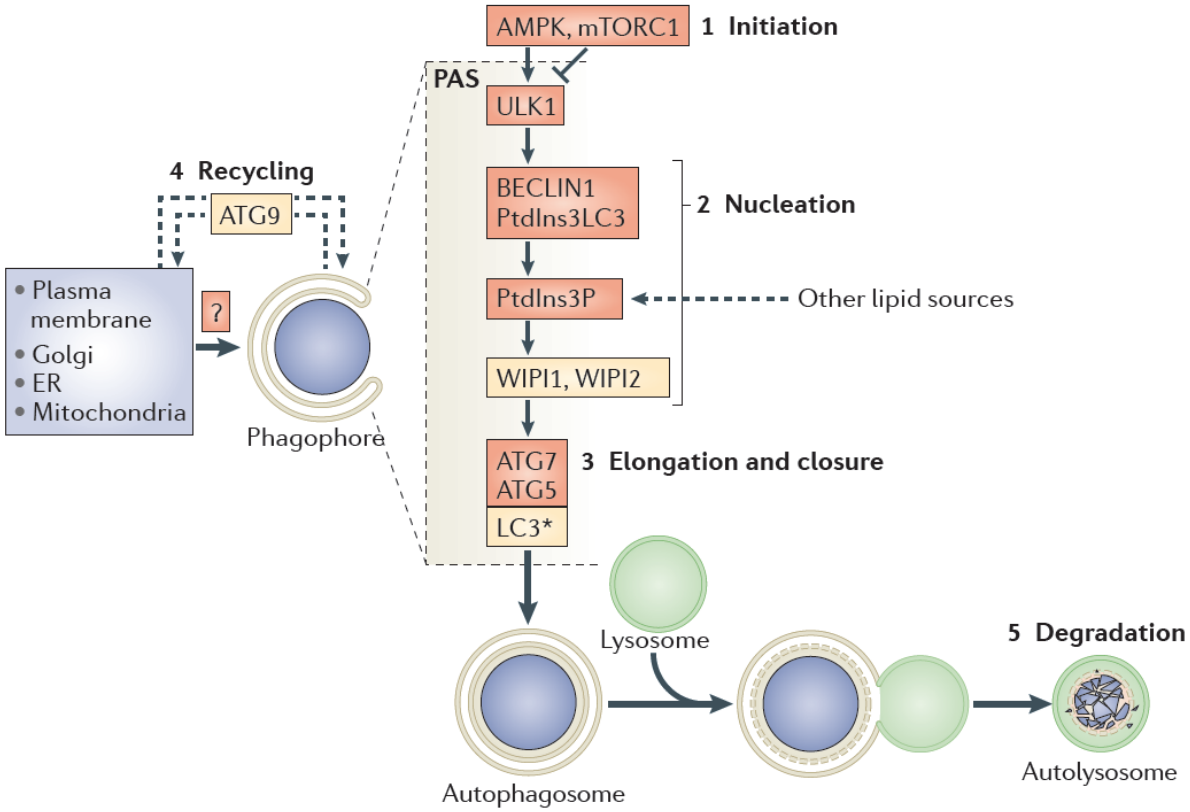

Figure 37: Canonical and non-canonical autophagy: Non-canonical autophagy routes emerged in the last years that could bypass some of the canonical steps (highlighted in red) as well as skipping some membrane sources (question mark). Key proteins in mammalian autophagy are shown. The building of PI3P as well as the proteins WIPI 1 and 2 are central of canonical autophagy. In ATG7–ATG5-independent autophagy LC3 lipidation is absent (indicated by Asterisk). Both canonical and non-canonical autophagy routes are described to end up in the production of canonical autophagosomal structures, namely autophagosomes and autolysosomes, as end result (Adapted from Codogno et al,2011).

D.1.2.2. ...independent of AMPK signaling and mTOR

A main regulator of autophagy the mTORC1 complex did not show any changes in its phosphorylation status in the highly autophagic active ERα positive cells. It has been shown that components of the MAPK pathway together with the autophagy-related proteins DRAM1 and p62/SQSTM1, also highly expressed in ERα, lead to enhanced

autophagic activity (Galavotti et al., 2013). During canonical autophagy, AMPK acts as mTOR inhibitor, thus triggering autophagy. Autophagy induced via ERα was not altered after inhibition of the AMPK pathway, supporting the hypothesis that mTOR is not involved in ERα-controlled autophagy. It was demonstrated that mTOR-dependent autophagy is mainly induced by starvation. The AMPK/PI3KCI/mTOR signaling pathway acts as a metabolic sensor, which might not be altered and is therefore not activated through ERα. Considering that ERα directly interacts with the PI3KCI this finding was quiet surprising as it showed that ERα-induced autophagy is not driven by direct interaction. Interestingly, recent studies have focused on the treatment of ERα-positive breast cancers by inhibition of PI3K (Yang et al,2016; Bosch et al, 2015). However, our results clearly show that this target would have no effect on ERα-induced autophagy, which is one mechanism of cancer cell resistance. In addition, various ongoing studies focus on overcoming endocrine resistance of breast cancer cells by the treatment with rapamycin thereby targeting mTOR-induced autophagy (Janku et al., 2014). It has to be carefully decided which type of breast cancer should be treated with rapamycin as inducing overwhelming autophagy might result in exacerbated cancer resistance.

1.2.1.3 ... independent of BECN1/PI3K complex

In the present study we identified the ERα-mediated autophagic pathway as independent from BECN1, one of the main canonical regulators of autophagy.

We found BECN1 mRNA levels even down-regulated in ERα positive cells although a higher autophagic activity was measured. But BCL2, a direct inhibitor of BECN1, was significantly up-regulated in these cells. BCL2 was already described as being up-regulated in ERα expressing cells in studies analyzing the regulation of apoptosis by ERα (Brendel, 2013). BCL2 is an anti-apoptotic molecule but also inhibits autophagy by forming a complex with BECN1. This process prevents the formation of the BECN1/PI3K complex, suppressing recruitment of ATGs to the phagophore. By over-expressing BCL2 through ERα signaling these interactions between BECN1 and BCL2 may be stabilized and inhibit BECN1-induced autophagy. BECN1 is also a haploinsufficient tumor suppressor (Liang et al., 1999; Qu et al., 2003; Yue et al., 2003), that is localized on the breast cancer susceptibility chromosomal locus 17q21 (Aita et al., 1999). In about 40% of breast cancers this monoallelic loss of BECN1 has been detected (Li et al., 2010) and

indeed mice with heterogeneous deletion of BECN1 show increased spontaneous carcinomas including breast carcinomas (Cicchini et al., 2014). Hence, BECN1/BCL2 binding inhibitors for therapy approaches of breast cancers are under debate at least for ER negative breast cancers to provoke BECN1 restorage and induction of autophagy (Tang et al., 2015). However, in these studies they did not verify if autophagy is really depressed in cancers with low BECN1 mRNA expression, or if its induced via non-canonical autophagy as we monitored at least in ERα expressing breast cancers cells. Under these circumstances provoking BECN1 activity would furthermore push the already highly active autophagy supporting therapy resistance and aggressiveness and would counteract a positive therapy outcome. Carefully decided therapy forms have to be of great interest when targeting BECN1 and autophagy in breast cancers.

There are already different other regulators of BECN1 identified, which could furthermore regulate expression levels and activity of BECN1 in tumors. For example Elgendy et al. (2014) detected MCL1, a bcl2 family member inhibiting apoptosis, which co-regulates and co-destabilizes BECN1 contributing to the degradation of both proteins by the proteasome during melanoma cancer progression independent of autophagy (Elgendy et al., 2014). Indeed we also found MCL1 mRNA levels slightly down regulated in ERα expressing cells that might be an explanation for the destabilization of BECN1 in the same way in our cell model leading to an by an autophagy induction independent of BECN1.

While BECN1 was thought to be an indispensable regulator of the autophagic machinery, recent studies have already pointed to BECN1-independent autophagy pathways (**Figure 37**). The neurotoxin 1-methyl-4-phenylpyridinium (MPP+) was found to be associated with neuronal cell death and induced BECN1 independent noncanonical autophagy (Zhu et al., 2007). Furthermore pro-apoptotic compounds such as staurosporine, induced BECN1-independent autophagy (Grishchuk,Y;2011) in primary neuronal cells. A study from Hiebel et al. described induction of BECN1-independent autophagy upon inhibition of the Cannabinoireceptor1 (CB1) in HEK cells and primary astrocytes (Hiebel et al., 2014). Also Resveratrol was found to induce BECN1-independent autophagy in MCF-7 breast cancer cells, which are identical to the cells used in our studies. While in most of the reports BECN1-independent autophagy was described to occur during cell death, we found BECN1-indepent autophagy pathways to be induced in cells highly resistant to

apoptosis. That highlights a new pathway of non-canonical autophagy crucial for cell survival rather than cell death.

D.1.2.3. Non canonical autophagy depends on BAG3, is regulated by DRAM1 and occurs with high levels of WIPI1, a known interactor of ERs

This study found DRAM1 and WIPI1 genes to show strongest up-regulation in ERα expressing cells. Strikingly the proteins have already been described to be connected to non-canonical autophagy (Galavotti et al., 2013). DRAM1 is a target gene of p53 and is supposed to be a lysosomal protein inducing autophagy (Crighton et al., 2007), but detailed studies about its exact function and localization are missing. One of the studies including 500 primary, high-grade glioblastoma samples revealed high mRNA levels of DRAM1, p62/SQSTM and WIPI1 and high DRAM1 levels correlate with shorter overall survival (Galavotti et al., 2013). Consistent with our findings, autophagy induced by DRAM1 was shown to be independent of the mTOR pathway, illustrating a non-canonical mode of action in autophagy. Functionally p62/SQSTM and DRAM1 were found to regulate invasion and cell motility of glioblastoma cells. In summary our findings persists with prior observations that directly link autophagy to the aggressiveness of cancer cells.

Despite the down-regulation of other key autophagy genes in our cellular system, BAG3 showed high abundance and diminishes the autophagic flux when downregulated by siRNA. The protein is a key factor in regulating and executing selective autophagy and has been implicated in non-canonical pathways of autophagy before (Gamerdinger et al., 2009b; Gamerdinger et al., 2011b; Liu et al., 2013; Mauthe et al., 2011). Supporting our data Liu et al (2013) described a non-canonical autophagic pathway, which was dependent on BAG3 but independent of BECN1. A recent study from Merabova and collegues showed an overexpression of the BAG3 protein in glioma cell lines is related to an enhanced BCL2/BECN1 binding, thereby inducing autophagy which is independent on the BECN1 complex (Merabova et al., 2015).

ERα-induced autophagy is independent on mTOR as well as BECN1 and may directly modulate downstream proteins of autophagy. Our data suggests that WIPI1 might be the first target of ERα as it was found to interact directly with ERα (Proikas-Cezanne et al., 2004). This interaction was, as in our case, found to be independent of E2. To confirm

this hypothesis, knock-down experiments should be considered which may confirm reciprocal regulation of ERα and WIPI1. WIPI1 is crucial for the binding of PI3P, which is localized in proximity to early phagophores and co-localizes with LC3 positive autophagosomes. Furthermore, BAG3 depletion was shown to reduce the amount of WIPI1 positive autophagosomes (Gamerdinger et al., 2009a), suggesting interplay between the two proteins. Pull-down experiments of BAG3 may further clarify the physical interaction of the three proteins and together with knock-down experiments may reveal the hierarchical action and would therefore help to gain a better understanding. An ERα-WIPI1-BAG3 interaction / pathway may induce and recruit autophagosomes and furthermore induce DRAM1 at the late stage of lysosomal degradation (Figure 38).

D.1.2.4. BAG3 is a novel mediator of ERα-induced autophagy and triggers oxidative stress resistance

We could show that the presence of ERα, but not ERβ, leads to increased autophagic activity. This process facilitates stress resistance and survival after oxidative stress.

We provide evidence that this novel ERα-mediated non-canonical autophagy pathway described in this study is, at least partially, mediated by the function of BAG3. The reversal of stress resistance of ERα-expressing cells is seen after inhibiting the late phase of autophagy via $BafA_1$-treatment as well as after knockdown of BAG3.

The monitored effect of BAG3 on cell survival could be explained by its role in autophagy but might also be due to its anti-apoptotic functions. The latter are thought to be regulated by interactions of BAG3 with the anti-apoptotic protein BCL2, resulting in a protection of cells from apoptotic cell death (Knezevic et al., 2015).

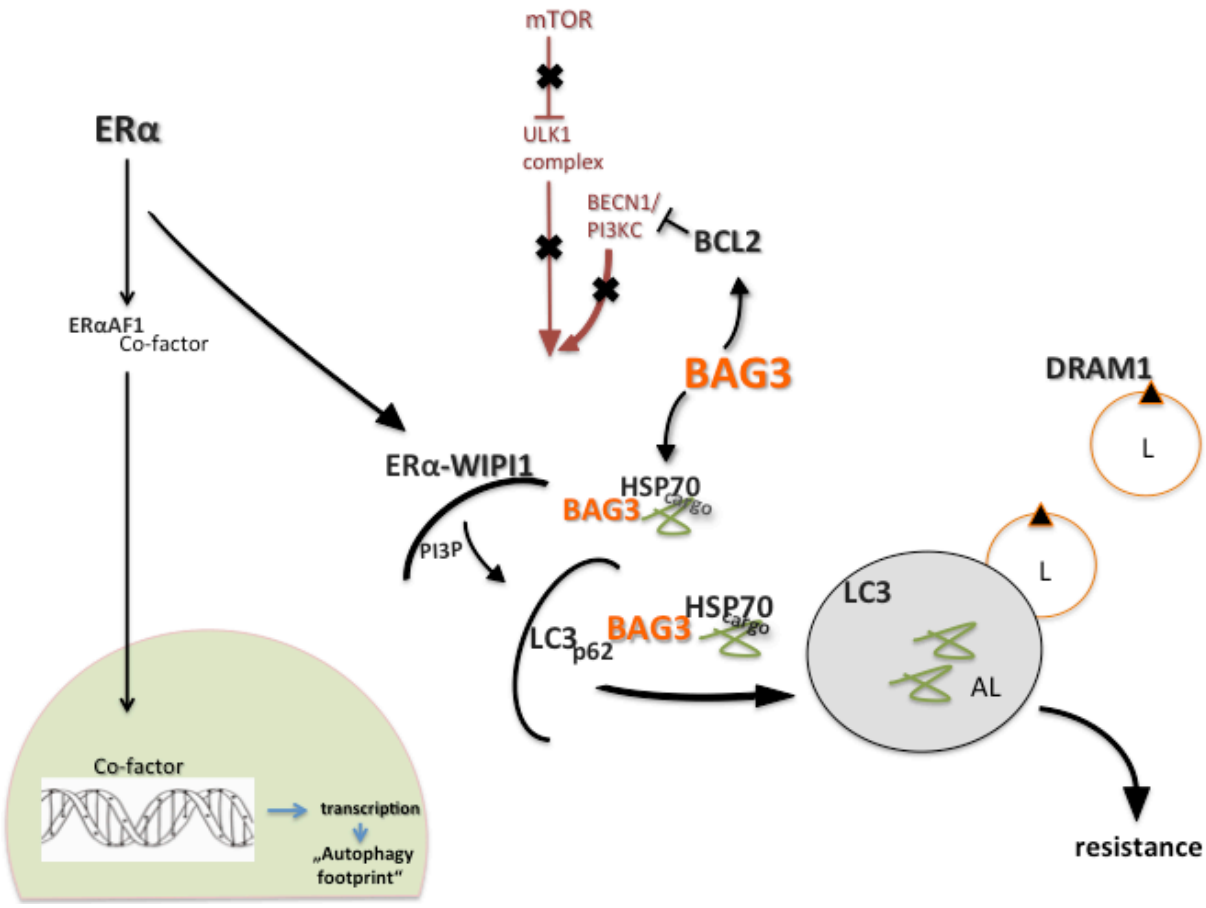

Figure 38: Hypothetical Model of BAG3-involved non-canonical autophagy induced by ERα. BAG3 stimulates autophagy upstream of LC3, independent of mTOR and downstream of the ULK- and the PI3KC, that contributes to autophagy and cancer cell resistance. ERα induced autophagy underlies a mechanism distinct to its function acting as bona fide transcription factors on EREs in the nucleus. Rather the indirect regulation of genes leading to the ERα induced "autophagy footprint" might expect the interplay with cytoplasmic Co-factors. Moreover, ERα could directly bind to WIPI1, a protein found on early PI3P positive autophagosomal membranes. Subsequently, BAG3 could be integrated, acting together with HSP70 in recruiting cargo to the autophagosomes, bind to p62 a well-known LC3 binding protein, and deliver the cargo for degradation. The autophagosome matures and fuses with DRAM1 enriched lysosomes. In addition, BAG3 is interacting with BCL2 inhibiting apoptosis and could thereby indirectly repress the BECN1/PI3K complex. **Red arrows/ font** : bypassed canonical steps of autophagy; L=lysosome; AL= autophagolysosome

BAG3 was furthermore found in mitigating proteotoxicity with the consequence of an inducible resistance of tumor cells (Rapino et al., 2015; Rapino et al., 2014). Additionally, tumor cell apoptosis was reported to be induced by silencing of BAG3 and improved drug induced apoptosis of neoplastic cells (Cotugno et al., 2014; Rosati et al., 2011a). BAG3 was also described to affect the HSP70-regulated pathways intensively which are related to cancer cell survival and also apoptosis (Colvin et al., 2014a). Knock-down of HSC/HSP family members is known to overcome therapeutic resistance. However,

whether BAG3 induces autophagy and cytoprotection due to its interaction with HSP/HSC70 remains to be clarified in more detail. This could be done by e.g. blockade of BAG3-HSP70 binding by designing a mutant form of BAG3 lacking the BAG-domain. Changes in autophagy induction and survival after oxidative stress should be monitored. Studies by Li et al 2015 showed that blocking BAG3-HSP70 interactions using the allosteric inhibitor JG98 led to anti-proliferative effects in cell lines and xenografts of MCF7 cells, which were also used in our studies (Li et al., 2015). If these observations are due to changes in autophagy was not analyzed. However the blockage of this interaction represents a novel therapeutic target for cancer therapy.

Lately huge efforts have been made to understand the role of BAG3 in different cancer types. Among many studies, Rosati et al. could show that pancreatic cancer cells secrete BAG3, through exosomes, which bind and activate macrophages, which then itself secrete cancer-supporting factors. Interestingly anti-BAG3 antibody treatment of a pancreatic cancer mouse model reduced tumor growth and metastasis, whereby this special BAG3 activity does not dependent on HSP70 interaction (Rosati et al., 2015). In line with our recent findings the results obtained by proteome analysis, revealed a role for BAG3 and Major Vault Protein as potent pro-survival factors that contribute to chemotherapy resistance in breast cancer cells (Pasillas,2014). For instance recent studies revealed crucial changes in microRNA expression after BAG3 knock-down which was related to anticancer drug resistance in ovarian cancer (Sugio et al 2014). Another study reports that down-regulation of BAG3 enhanced DDP-(Cisplatin) induced apoptosis in esophageal cancer through regulation of microRNA let-7g and let-7 expression (Wu et al., 2016) assuming a role for BAG3 in translational activity.

However BAG3 related resistance through elevated protein levels is obviously not a general phenomenon, as Bartsch et al.2015 described that autophagy markers BAG3 and p62/SQSTM were not found to be regulated in testicular cancers (Bartsch et al., 2016).

Induction of oxidative stress is also a substantial strategy in cancer therapy including radiation. This means that ERα-positive breast cancers characterized by enhanced autophagy and high BAG3 expression are likely to be resistant to oxidative stress. This cancer type would display resistance against therapy interventions relying on oxidative stress. In this case, BAG3-mediated selective autophagy is directly related to breast cancer cell survival.

D.1.2.5. Non-canonical autophagy markers, including BAG3 are novel targets for therapy of ER-postive breast cancer

As suggested by our study, immunofluorescence analysis of tissue from breast cancer patients also showed that markers for autophagy were highly expressed in ERα-positive samples. Clinical trials with substances inhibiting autophagy in cancer cells are already ongoing. Especially for treatment of breast cancers, hydroxychloroquine as well as Ixabepilone are already in clinical trial phases I/II. However, these substances inhibit the whole lysosomal process and are not very selective, assuming several side effects to be expected. Therefore, identification of specific biomarkers would be beneficial to target autophagy in cancer therapy.

BAG3 was recently shown to be involved in cancer-signaling networks mediating a selective macroautophagy pathway upon protein aggregation and to attenuate proteotoxicity in cancer cells (Colvin et al., 2014b; Gamerdinger et al., 2009b; Rapino et al., 2013). Colvin et al. reported, that BAG3 interacts with cellular sarcoma tyrosinkinase (Src), a well-known product of the protooncogene *c-src*. Thereby BAG3 mediates the effects of HSP70 on Src signaling by modulating the activity of the transcription factors NF-kB, FoxM1, Hif1a, the translation regulator HuR, and the cell-cycle regulators p21 and survivin. They identified a small-molecule inhibitor, YM-1, that disrupts the Hsp70–Bag3 interaction whereby in vivo administration of this drug was sufficient to suppress tumor growth in mice. Therefore, BAG3 also represents an interesting target for therapy of breast cancers that might have already acquired resistance to anti-hormone therapy. Interestingly, we found in our immunofluorescence analysis that one sample (out of ten) of ERα-positive tissue showed a reduced signal of BAG3. However as mentioned above there is great variability in breast cancer and its subtypes. Therefore, the different biological behavior of these variable cancer types calls for an individual and personalized medication and treatment concepts for breast cancer patients in respect to their individual disease status. For example some clinical approaches aiming to decrease autophagic activity in cancer cells target the mTOR- and PI3K-complexes. Several mTOR- and PI3K-inhibitors or mTOR/PI3K dual inhibitors are in early phase clinical trials or have been approved for therapy (Davis et al., 2014; Vicier et al., 2014). However in several breast cancer patients, who show an ERα-mediated "*autophagy-footprint*", comparable to our results, drugs targeting the canonical autophagy pathway would not

fulfill their autophagy reducing action. This novel ERα-mediated non-canonical autophagy pathway, which we describe here, was found to be mTOR- and PI3K-independent as the PI3K inhibitor Wortmannin did not reduce the autophagic flux in ERα-expressing cells and phosphorylation of mTOR was not altered. An unmet need exists for effective treatment strategies for anti-hormone resistant ERα breast cancer and BAG3 might represent an interesting alternative therapeutic target.

There is only limited data on biomarkers and target proteins that predict the efficacy of autophagy inhibitors in breast cancer in general (Gonzalez-Angulo and Blumenschein, 2013). Therefore, a detailed dissection of the autophagy pathway is needed. Analyzing autophagy networks and individual "*autophagy-footprints*" for patients could potentially improve effectiveness of treatment that targets the autophagy pathway and specialized medication for mTOR/PI3K-dependent canonical vs. non-canonical autophagy could be applied. This presents a major challenge for personalized medicine and biomarker development but also a novel therapeutic chance for cancer patients.

D.2. **Neuronal autophagy in oxidative stress conditions**

Keeping homeostasis is a major challenge for every neuronal cell, as they are differentiated and not able to divide anymore, wherefore PQC has to be very precise to keep homeostasis also during oxidative stress conditions. Autophagy, as a lysosomal degradation pathway, is therefore an important component of PQC to clear and recycle cellular components, damaged organelles and protein aggregates and was in focus of the second part of this thesis.

D.2.1. Oxdative stress induced autophagy with high levels of BAG3 is continually mTOR independent: a general mechanism in BAG3-mediated autophagy?!

In the present study, high levels of autophagic activity in oxidative stress resistant cells is independent of mTOR but is characterized by high BAG3 protein levels. In conditions of normal nutrient supply the essential aminoacid L-leucin, which is bidirectional transported through the efflux of L-glutamine (Nicklin et al,2009), is keeping the mTOR kinase in an active state, thereby facilitating protein translation, cell division as well as inhibiting autophagy. In amino-acid deprivation mTOR gets inactive (Pattingre,2008) resulting in induction of the autophagic machinery. One might explain our results that in oxidative stress conditions misfolded and aggregated proteins have to get cleared, which is independent of the starvation induced autophagy pathway that is regulated through mTOR. In the adaptation process mTOR-independent autophagy might be induced by stimulation of oxidized and misfolded proteins that result in the activation of the BAG3-mediated selective pathway. Supporting the above mentioned thesis, mTOR-independent autophagy has already been described when clearing aggregation prone autophagy substrates as α-synuclein or mutant Huntingtin (Sarkar,2006), whereby mutated Huntingtin (HTT43Q) is accumulating after knock down of BAG3 (Carra et al,2009), hypothesizing a role for BAG3-mediated clearance of the given protein. mTOR independent autophagy might be a general mechanism for BAG3 induced autophagy, as shown in the first part of the present thesis, the BAG3-mediated selective autophagy driven by ERα was also mTOR-independent. Studies published by Carra et al. 2009 could show that the BAG3-HspB8 protein transcription blockage through the phosphorylation of translation initiation factor eif2 resulting in autophagy induction is also not a down-stream effector of mTOR (Carra et al., 2009). In these studies the main action of HspB8

and BAG3 are described as PQC sensors inducing autophagy for diminishing denatured proteins rather than for nutrient supply. Besides these findings Gamerdinger et al. (2009) could show a decrease in BAG3 to BAG1 ratio upon amino-acid depletion that might occur through proteasomal degradation of BAG3, which underlines the function for BAG3-depended autophagy in PQC and might exclude its function for nutrient supply.

One might ask if the observed autophagy upregulation in the hippocampal oxidative stress-resistant cells is also triggered by the ERα, but until now there are contradictory publications showing ER expression in HT22 cell line. In our lab the expression of ER mRNA was detected but never the active ER protein.

D.2.2. Beclin1 is strongly regulated in hippocampal cells and might contribute to cytoprotective autophagy

We found high expression levels of BECN1 in the resistant cell lines, pointing to an autophagy route that depends on BECN1/PI3K complex. If this is the case should be evaluated with knock down BECN1 experiments and could be furthermore examined by inhibiting PI3K with Wortmannin thereby detecting the impact on the autophagic flux. In the first part of our studies we found out that ERα-induced autophagy was independent of BECN1 but also inducing autophagy via the BAG3-mediated selective pathway. In the ER expressing cells the BCL2 expressing level was markedly increased, which blocks the BECN1/PI3K complex. In contrast the HT22 oxidative stress resistant cell lines show decreased BCL2 protein levels as previously shown by Schäfer et al (2002). Hence, the BECN1/PI3K complex could be formed leading to autophagy induction. Furthermore it is already shown that the stress resistance in HT22 cells correlates with a higher phosphorylation status of the MAPK (Schäfer; 2003). The MAPK is a well-known positive regulator of autophagy. AMPK could induce autophagy via inactivating mTOR, which is not detected in our cell model. But furthermore MAPK is found to activate autophagy more downstream by the phosphorylation of BECN1. We detected mTOR not to regulated, but high expression levels of BECN1, which might be due to the MAPK pathway.

Furthermore we argument that especially in breast cancer cells BECN1 is often found to be haploinsufficient that results in low mRNA levels, which we also detected in the ERα

positive cells. This does not have to be true for hippocampal cells as the investigated HT22 cells show high levels of BECN1.

It is likely that under oxidative stress when misfolding of proteins occurs in exceptionally high rates the induction of autophagy takes place via BECN1 to induce directly autophagosome building rather than to the starvation dependent mTOR signaling.

D.2.3. BAG3 induces a rearrangement of the lysosomal compartment

Having a look to the lysosomal compartment we detected changes in the distribution as well as in the size of lysosomal acidic compartments in the resistant cells and we also detected this phenotype when overexpressing BAG3 in the sensitive cells. Previous studies in our lab demonstrate altered cholesterol and sphingolipid metabolism which was associated with changes in the lysosomal function of the resistant cells (Clement,2009). BAG3 might have influence on the lysosomal compartment by inducing autophagy, which is related to the activation and recruitment of lysosomes. BAG3 was already found to target ubiquitinated and nonubiquitinated aggregates for the retrograde transport via dynein motor to the aggresomes. Aggresomes are intracellular hot spots of high autophagic activity that involves autophagosomes as well as lysosomes. After induction of autophagy lysosomes have to be activated and recruited for the fusion with autophagosomes and for the active degradation of the substrates. To answer if and where the BAG3 activated lysosomes are fusing with the autophagosomes, one has to make double staining of markers of both the systems, including Vimentin detecting aggresomes and LAMP1 for lysosomal structures. Furthermore cathepsin B activity, one of the main proteases in the lysosomes, should be measured to see if these strongly accumulating lysosomes are effective.

D.2.4. A switch from BAG1-proteasomal degradation to BAG3-mediated selective autophagy: the way to oxidative stress resistance in neurons in vitro…and in vivo?

BAG1L downregulation correlates with a higher autophagic flux in the oxidative stress resistant cells. BAG1L acts as a co-chaperone of HSP70/HSC70 and links substrates to the UPS. Indeed, Schäfer (2003) reported the downregulation of the 26s subunit of the proteasome on mRNA level in the oxidative stress-resistant cells, suggesting a decreased proteasomal activity in these cells. Employing a proteasomal reporter assay would

clarify if the proteasomal activity slows down under oxidative conditions in the resistant cells and would underline our data. Studies of our lab showed already such a switch in aged fibroblasts, oxidative stressed cells and aged mouse neurons (Gamerdinger et al 2009) supporting our findings. The switch from BAG1 to BAG3 could be due to the overload of protein stress under oxidative conditions. This assumption might be true also for our studies of prolonged oxidative stress conditions in hippocampal cells, where the proteasome might be blocked or unprofitable for aggregate clearing but rather the autophagic pathway is needed to compete the oxidative stress and balance PQC, thereby facilitating oxidative stress resistance. The proteasome itself could be blocked by the enrichment of aggregates by blocking substrate-entry sites (Bence et al., 2001). Hence, the autophagic pathway is needed to clear those aggregate prone and defect organelles via the lysosomal pathway because protein aggregates get degraded by autophagy (Rubinsztein,2006), rather than by the proteasome. Reflecting our findings, knock down experiments in former studies of our lab showed that siRNA knock down of BAG1 increased autophagic flux (Gamerdinger, 2009). Lately HDAC6 (Histone deacetylase6), a sensor for proteasomal stress was found to be responsible for the retrograde transport of polyubiquitinated proteins to the aggresome and the clearance by autophagy. A switch from UPS to HDAC6 induced autophagy is also detected when blocking the proteasome in oligodendrocytes (Leyk et al,2013), illustrating the interconnection between the two pathways.

BAG3-mediated selective autophagy is already known to degrade misfolded or accumulating /aggregating prone proteins, which definitely will occur under oxidative conditions. Recently Ulbricht et al. (2015) reports highly upregulated autophagy related proteins p62/SQSTM, HSBP8 and as well BAG3 after 4 weeks of strenuous resistance exercise strength training in the skeleton muscle in humans (Ulbricht et al,2015). Muscle gaining high intensive action are exposed to mechanical, heat and as well as a huge amounts of oxidative stress, which all induce autophagy and the effect is furthermore interpreted as a resistance mechanism over time. Continuously the upregulation of the BAG3-mediated autophagy machinery could act as an adaptation mechanism that responds to high oxidative stress levels confirming our observations.

Besides that, BAG3 was found to inhibit proteasomal degradation by binding HSP70 abrogating proteasomal degradation of polyubiquitinted proteins (Doong et al., 2003), that might show the feedbackloop of BAG3-mediated autophagy which is needed when

aggregates have to be cleared and maybe sensors to the proteasome as BAG3 is degraded in some cases by the proteasome. For instance in nutrient starvation, when a quick proteasomal response is needed for catabolite supply, excluding a slow autophagosomal way, BAG3 is degraded by the proteasom. The link between BAG3 and induction of autophagy is an often-described phenomenon, but detailed analysis of the mechanism is missing. Lately one study could show the direct interaction of BAG3 with LC3B. LC3B a main protein of autophagosomal membranes is thereby regulated by BAG3 on the translational level (Rodriguez et al, 2015). In the first part of this thesis, BAG3 was also found to be highly upregulated in oxidative stress resistant breast and neuroblastoma cancer cells, giving the hind that BAG3 may play a more general and fundamental role in cytoprotective autophagy.

BAG1-proteasomal degradation is more likely slowing assuming by the downregulation of BAG1L, rather the BAG3-mediated selective macroautophagy may be responsible to keep homeostasis in conditions of chronic oxidative stress and a hypothetical mode of action is summarized in the next figure (**Figure 39**).

When analyzing brain areas after CCI we found BAG3 and other autophagy markers upregulated in the ipsilateral site of the lesion and higher autophagic flux could be measured pointing to the induction of autophagy, especially the BAG3-mediated selective autophagy. In contrast proteasomal activity slows down in brains after CCI compared to sham treated animals, which was determined in cooperation studies with T. Mittmann and F. Le Prieult (data not shown). The *in vivo* data thereby supports our *in vitro* findings where we already detected a switch in BAG3 and BAG1 expression and highly induced autophagy after long-term treatment of hippocampal cells with oxidative stress. Therefore BAG3 mediated autophagy might be a general mechanism due to the response to high oxidative levels and accumulating protein stress. Supporting this thesis, recent studies from Ulbricht et al. (2015) observed high levels of BAG3 after strenuous resistance exercise strength training in the skeleton muscle in human, which is also interpreted as an adaptation process to long term exposure to oxidative stress (Ulbricht et al, 2015).

Accumulation of reactive oxygen species causing oxidative stress, as well as chronic oxidative stress, is causally linked to neurodegenerative disease(Hensley et al., 1995). Oxidative modified molecules were found to accumulate within aging, and are thought to

be involved in neuronal cell death in chronic pathological disorders as well as in acute pathophysiologic events as brain trauma (Brunk and Terman, 2002; Dargusch and Schubert, 2002) and antioxidants are found to rescue neurons from oxidative cell death (Behl et al., 1997). Compared to the UPS, in which just soluble and unfolded proteins could be degraded, the autophagosomal pathway is able to abolish insoluble components like aggregates (Ding and Yin, 2008) that might occur after CCI/TBI. Misfolding and aggregation of proteins and organelles is challenging PQC and BAG3-mediated clearance via autophagy may thereby play a crucial role for neuronal adaptation and survival after CCI.

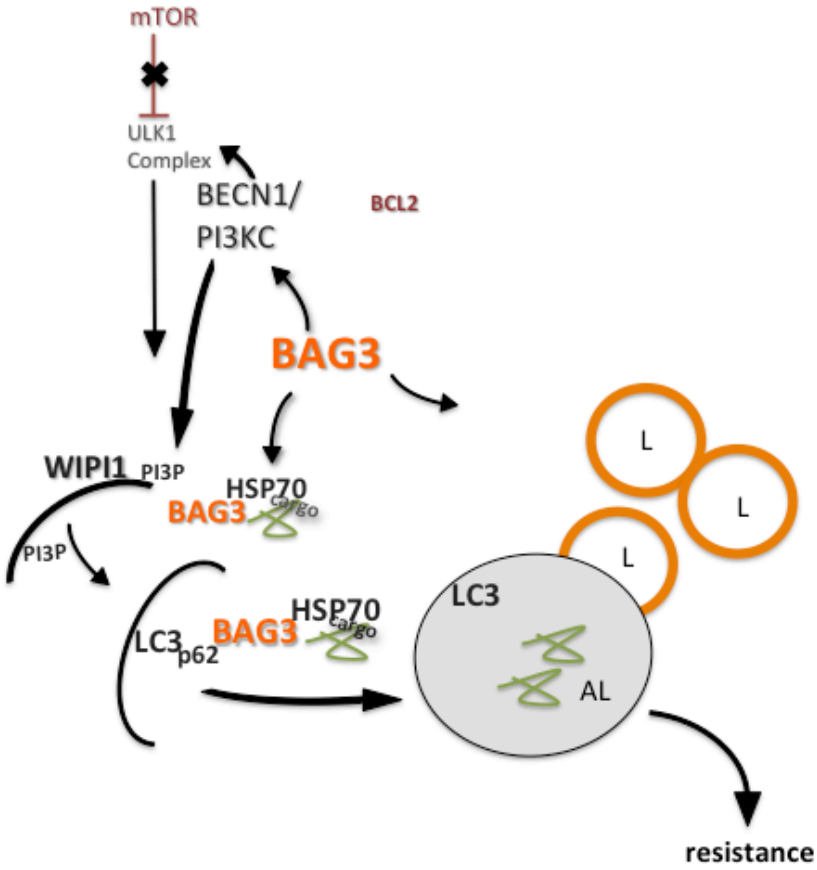

Figure 39: **Hypothetical model of autophagy in neuronal cells adapted to oxidative stress.** Autophagy is highly induced in hippocampal cells adapted to oxidative stress and bypass the canonical regulation of mTOR. The newly described pathway results in the upregulation BAG3 and BECN1. BECN1/PI3KC is not inhibited by BCL2 and could therefore induce autophagy by recruitment of PI3P to he early autophagosome, whereas BAG3 together with HSP70 recruits cargo to the autophagosomes for degradation. Furthermore the lysosomal compartment is rearranged in oxidative stress resistant cells, which could be due to the induction of the BAG3-mediated selective macroautophagy and acts for the optimum of the degradation capacity. **Red arrows/font**: bypassed canonical steps of autophagy; L=lysosome; AL= autophagolysosome

Altogether the findings of the study indicate that BAG3-mediated non-canonical autophagy may act as a general adaptation and cytoprotective mechanism that may point to a major role of this process in pathophysiological conditions ranging from cancer cells resistance to neuroprotection.

Thinking of new treatment approaches BAG3 could be a promising target to inhibit autophagy in cancer cells that show non-canonical characteristics and to combat cancer cell resistance to therapy. On the other hand inducing the BAG3-mediated autophagic pathway would be the goal in neuronal cells to diminish proteostatic stress and to support cells coping with neurodegenerative disorders.

E. Materials and methods

E.1. **Cell culture:**

Human SK-N-MC cells (ATCC HTB-10) and MCF-7 cells were obtained from the American Type Cell Collection and were cultured in phenol red-free Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 1 mM sodium pyruvate (Invitrogen), 10 % charcoal-dextran-treated fetal calf serum, 100 U/mL penicillin and 100 U/mL streptomycin (Invitrogen) at 37°C under a humidified atmosphere containing 5 % CO2. The medium for the SK-N-MC cell clones (SK-01, SK-ERα and SK-ERβ) was supplemented with 2 µg/mL G418 (Calbiochem). All reagents of the treatments were dissolved in dimethylsulfoxid (DMSO). 17β-estradiol (Sigma-Aldrich) was used in a concentration of 10 nM for 24 hours. The endconcentration of ICI 182780 (Tocris Biosciences) in the medium of stimulated cells was 1 µM. Bafilomycin A1 (Enzo Life Science) was used in a concentration of 500 nM. Exclusively in the case of the 20 hours 1 µM Wortmannin (Sigma-Aldrich) treatment Bafilomycin A1 was cotreated in a concentration of 100 nM. U0126 (Promega) was used in a concentration of 50 µM for 48 hours. 4-Hydroxytamoxifen (Sigma-Aldrich) was added for a period of 24 hours in a concentration of 1 µM. In the case of a double treatment, a 1-hour pretreatment with the inhibiting reagent was performed.

Mouse hippocampal cell line, HT-22, immortalized from primary mouse hippocampal neuronal culture, were obtained from salk institute as described previously (Schafer et al,2004). Glutamate resistant mixed cell clones were generated when plating HT-22 cells at low density and treating the cells with gradually rising concentations of glutamate up to 40mM within 4 month. H_2O_2 resistant cells were made with 1,2 µM H_2O_2 (Sigma-Aldrich) on low density HT-22 cells within 3 weeks, followed by the isolation of single cell clones. Later on cells were cultured in 450µM H_2O_2.

E.2. **Transfections:**

Cells were plated in 6-Well plates 24 h prior calcium phosphate transfection. All reagents were set at room temperature (RT) and 10 µg plasmid DNA or 20 µg siRNA was mixed with 105 µl H_2O and 15 µl $CaCl_2$ and incubated for 5 min. 120 µl 2x HEPES buffer saline were added and incubated for 30 min. The suspension was directly transferred to the medium and after 24 h fresh medium was added. For visualizing autophagic activity

GFP-RFP-LC3 plasmid (ptfLC3, Addgene) was used. After additional 24 h cells were harvested or subjected to immunocytochemistry. Generation of pIRES-ERα and pIRES-ERβ was described previously (Manthey et al., 2001; Zschocke et al., 2002a) and pIRES plasmid was purchased from Clonetech Laboratories.
For overexpression and siRNA mediated knockdown cells were transfected using the calcium phosphate method. Details are shown in the supplementary information. Non-sense siRNA (5` AUUCUCCGAACGUGUCACG 3`) was purchased as siMAX from MWG. siRNA against BAG3 was supplied from Sigma-Aldrich (SASI_Hs02_00337266). siRNA mix against DRAM1 was supplied by MWG (No1: 5´ACACCUCCAGAGAGUGGUA 3´; No 2: 5´GGAUUAUGUAUAUCACGUA 3´).

HT22 cells were transfected with Amaxa (Lonza). Electroporation was performed with 50-200 µg of DNA or RNA and 600000 mio cells in 1 reaction. Afterwards cells were plated on 2x 6cm^2 dishes / approach or 96 Well dishes.

E.3. **Western blot analysis:**

Adherent cells were washed two times with ice cold 1xPBS and afterwards lysed on ice with protein lysis buffer (50 mM Tris-HCl pH 7,5; 150 mM NaCl; 0,5 % NP40) plus protease inhibitor cocktail (P8340, Sigma-Aldrich) plus phosphatase Inhibitor cocktail (Roche) and were briefly sonicated and boiled for 5 min at 99C° shaking. Total protein concentration was measured by using bicinchoninic acid (BCA) protein assay reagent (23225, Pierce) and bovine serum albumin as standard. Lysates were resolved by SDS-PAGE and transferred to nitrocellulose membrane by electroblotting. Prestained protein ladder (#SM0671, Fermentas) was used as reference of molecular weights. Membranes were blocked for 1 hour at room temperature (RT) in blocking buffer (5 % non-fat dry milk in PBS supplemented with 0,05 % Tween 20). Membranes were washed three times for 15 min with PBS-T (PBS supplemented with 0,05% Tween 20) and incubated with the primary antibody at varying concentrations suggested by the manufacturer overnight at 4°C followed by three washing steps for 15 min with PBS-T at RT. Membranes were incubated with horseradish-peroxidase-linked secondary antibodies (1:5000) for 1 hour at RT and detected using the Super Signal procedure (Millipore) and visualized via the Fusion-SL 3500 WL system (Peqlab). Densitometric quantification was

done after normalizing the results to tubulin. Analysis was performed with Aida Image Analyzer v.4v26 software (raytest). For statistical analysis one-sample t-test was used.

Western blot analysis was carried out as described previously (Gamerdinger et al., 2009b). Analysis was performed with the Fusion-SL 3500 WL system (Peqlab) and Aida Image Analyzer v.4v26 software (raytest).

E.4. **Immunocytochemistry:**

Cells were plated on cover slips in 6-well or 24-well plates and fixed with 4 % paraformaldehyde for 20 min at RT. Subsequently, cells were permeabilized and blocked in blocking buffer (PBS, 0,5 % Triton-X100, 3 % bovine serum albumin) for 1 hour at RT. Incubation of primary antibodies with concentrations suggested by the manufacturer was done overnight at 4°C. After three washing steps for 15 min at RT with PBS cells were incubated with respective secondary antibodies (1:200) for 2 h at RT and counterstained with 4,6-diamidino-2-phenylindole (DAPI, 1 µg/ml) and again washed three times for 10 min at RT. Cells were mounted in Mowiol (Roth) containing 1,4-Diaza-bicyclo(2,2,2)octan (DABCO, Roth).

The paraffin embedded tumor samples were de-paraffinized and afterwards rehydrated with in Xylol, followed by a decreasing alcohol series (100% - 70% alcohol). The rehydration was completed by incubation with bidest H2O and slices were stored in water until antigen detection. Afterwards immunostaining was performed as described before. Cells and tissue were analyzed by microscopy using an inverted Leica TCS SP5 and Zeiss LSM710 meta confocal microscope and images were processed with Adobe Photoshop CS5 and Leica LAS AF lite software. GFP-RFP-LC3 dot formation (visualized using the GFP-RFP-LC3 expression plasmid mentioned above) was quantified by counting dots in confocal laser scanning microscopic pictures of corresponding transfected cell lines and after Bafilomycin A1 treatment (500 nM, 6 h). At least 56 cells per cell line were analyzed.

E.5. **Antibodies:**

The antibodies used for immunocytochemistry as well as for Western Blot analysis in were as follows: ATG7 (8558, Cell Signaling Technologies); BAG3 (ab47124, Abcam); BCL2 (sc-492, Santa Cruz); ERα (RM9101S0, Thermo Fischer Scientific); ERK1/2 (9102, Cell Signaling Technologies); p-ERK1/2 (9106, Cell Signaling Technologies); LC3B (L7543, Sigma-Aldrich), LC3B for immunostainings and western blot of tissue (XY); mTOR (OP97, Millipore); p-mTOR S2448 (ab51044, Abcam); NBR1 (00004077-M01, Abnova); SQSTM1 (GP62-C, Progen); PI3K Class III (4263, Cell Signaling Technologies); Tubulin (T9026, Sigma-Aldrich); WIPI1 (HPA007493, Sigma-Aldrich) Beclin1 (ab51031;abcam); p70S6K (9202, cell signaling); p70S6K Thr389 (9206, cell signaling). Secondary antibodies were anti-mouse/rabbit/guinea pig antibodies conjugated to DyLight 488, 649 and Cy3 (immunofluorescence, Jackson ImmunoResearch) or to HRP (immunoblotting, Jackson ImmunoResearch). The cBAG antibody, a kind gift from Prof. Dr. F. Ulrich Hartl, was raised in rabbits against the human BAG domain in BAG1M (aa 151–263).

E.6. **Transmission electron microscopy/ Glutaraldehyd-Osmium-fixation:**

The Transmission electron microscopy (TEM) analysis was carried out in co-operation with Prof. Dr U. Wolfrum. Cells were prefixed in 2,5 % glutaraldehyde, 0,1 M sucrose, 0,1 M cacodylate buffer (pH 7,4) for 1 hour at room temperature. After three washes for 10 min in 0,1 M cacodylate/sucrose buffer, cells were post-fixed for 1 hour in 1 ml 2 % OsO4, 0,1 M sucrose and 0,1 M cacodylate buffer, dehydrated and embedded in araldite resin. Ultrathin sections were cut with a Leica Ultracut S microtome and were counterstained with 2 % aqueous uranyl acetate. Sections were analysed in a FEI Tecnai 12 BioTwin transmission electron microscope and imaged with an SCCD SIS MegaView III camera.

E.7. **Human Breast cancer tissue:**

Human breast cancer tumor samples were acquired by surgery from cancer patients. Details about cancer patients can be found in Supplementary Table S1. Informed consent was obtained from all subjects and studies were approved by the Institutional Review

Boards and Ethical Committee of the collaborating Centrum für Tumorerkrankungen (UCT) of the University of Frankfurt.

E.8. **PCR, Reverse Transcription PCR and quantitative real-time PCR:**

RNA from cultured cells was extracted with the NucleoSpin RNA II Kit (Macherey-Nagel) according to the manufactures protocol. 1000ng of RNA was used for the Reverse transcription reaction in a final volume of 20 µl containing 2 µl reverse transcriptase buffer (Quiagen), 2µl dNTPs (5mM;Quiagen), 2µl oligo(dT)23 primer (10µM;Sigma), 10 U RNasin (Promega) and 4 U Omniscript Reverse Transcriptase (Quiagen).

Quantitative real-time PCR analysis was performed in a 20 µl reaction volume containing 1 µl cDNA, 2 µl of a 0,1 µM Primer mix (HATPL-1, Biomol), 10 µl 2x SensiMix SYBR & Fluorescein Mastermix (Bioline) and 7 µl H_20 using iCycler real-time thermocycler (Bio-Rad). Following 15 min denaturation at 95°C 35 cycles of amplification were performed. PCR cycle conditions were 95°C for 10 sec (denaturation), 58°C for 45 sec (annealing) and 72°C for 30 sec (extension). The specificity of the PCR products was confirmed by analyzing the melting curve.

Fold changes and p-values of target genes in SK-ERα, SK-ERβ and MCF-7 cells compared with SK-01 cells were calculated using RT^2 Profiler PCR Array Data Analysis Template v4.0 from Qiagen. All eight housekeeping genes spotted on the array plate were used as internal controls. Only genes with a higher fold change than +1.5 or -1.5 and a p-value $p \leq 0.05$ were treated as regulated genes.

For the detection of DRAM1 siRNA knock down we used primer supplied by Quiagen (Hs_DRAM1_1_SG). The housekeeping gene RPL19 was detected with the following primer sequences: forward 5' GAAATCGCCAATGCCAACTC 3` and reverse 5 TTCCTTGGTCTTAGACCTGCG 3`.

Primer sequences used for characterizing ERα and ERβ positive and negative cell lines are as follows: ERα-forward 5´ GTGCCAGGCTTTGTGGATTTG 3´; ERα-reverse 5´ GTTACTCATGTGCCTGATGTG 3´; ERβ-forward 5´ GAGGCCTCCATGATGATGTC 3´; ERβ-reverse 5´ TCTCCAGCAGCAGGTCAT 3´ and pIRES-ERα and pIRES-ERβ were used as positive controls. Samples were analyzed on a 1% agarose (Biozym) gel. Mass Ruler DNA ladder (#SM0403, Ferments) was used as reference.

E.9. **ERE-Luciferase-Assay:**

SK-01, SK-ERα, SK-ERβ and MCF-7 cells were transfected with the ERE-Luc reporter plasmid containing estrogen response elements (ERE) fused to the firefly luciferase gene (D-MTV-ERE-LUC; (Behl et al., 1995)). Transfection was carried out as described above. After 24 h cells were stimulated with 17β-estradiol (10 nM) and/or ICI (1 µM) for 24 h. Subsequently, cells were harvested by using the Luciferase Assay System (Promega, Cat. No. E4030). The protein concentrations were determined by BCA as described above and the luminescence readings were assigned in an automatic counter (Wallac Victor2, Wallac Inc.). Transfection experiments were performed in triplicates, repeated three times and normalized for identical protein contend.

E.10. **Survival experiments by Fluorescence Activated Cell Sorting (FACS):**

24 h prior to stimulation SK-01, SK-ERα, SK-ERβ and MCF-7 cells were seeded in 24-well plates. Following 1 h pre-incubation with Bafilomycin A_1 (500 nM) or DMSO, or 24h after transfection with siBAG3, cells were treated with vehicle control, 400 µM or 600 µM H_2O_2 (Sigma-Aldrich) for 24 h. After treatment the supernatant from the cells was collected and cells were dissolved from the plates with Trypsin digestion and again incubated with the prior collected supernatant. Following 5 min of centrifugation (800 g) the cells were resolved in 100 µl PBS. All steps were carried out on ice. Cell viability was measured by staining cells with propidium iodid (PI) (1 µg/ml). PI fluorescence was determined with a FACScan Flow Cytometer (Becton Dickinson). Cell populations were pre-gated by forward scatter (FSC-Height) and side scatter (SSC-Height). Afterwards, results were analysed with the BD CellQuest™ Pro Analysis software.

E.11. **Animal experiments**

Animal experiments have been carried out by the group of Anesthesiology Prof. Dr. Engelhardt Universitiy clinics Mainz. Animal testing license reference number 23 177-07/G 12-1-010 was approved by the Ethical Care Committee approval of the Federal Republic of Rhineland-Palatinate. Experimental TBI, Controlled Cortical impacts (CCI), have been done by Christina Gölz (Universitiy clinics Mainz, group of Anesthesiology Prof. Dr. Engelhardt) and were approved from TSchG §8 version 2006. C57Bl6-N mice were anesthetized with isoflurane via face mask and placed into a stereotactic frame. Trauma was induced by controlled cortical impact as described previously (Sebastiani

and Gölz et al 2015). After craniotomy on the right rostrocaudal plane, a mechanical lesion was induced on the right parietotemporal cortex by a custom fabricated impactor (L. Kopacz, Germany) with the following parameters: tip diameter of 3 mm, 1.5 mm brain penetration, impact duration of 150 msec, and impact velocity of 8 M/sec. The craniotomy was rapidly sealed with histoacrylic glue (B Braun Melsungen AG, Melsungen, Germany). The wounds were sutured closed. At the end of each experiment mice were anaesthetize with Isofluran and the head is kept via cervical dislocation.

Preparation of acute organotypic brain slices after CCI have been done in cooperation with Florie Le Prieult, group of Prof. Dr. Thomas Mittmann, Institute of Physiology, Mainz University Medical Center. After CCI or sham operation, 300µM slices have been prepared with the Vibratom. After incubation of the slices in control or Chloroquine (200µM; Sigma) conditions solved in artificial cerebral spinal fluid (ACSF) for 2h, the sections were gathered in lysis buffer (contents as in section western blotting), shock frozen in liquid nitrogen and analyzed by western blotting (carried out by Vanessa Felzen).

E.11.1. LC3EGFP mice

LC3EGFP mice (C57BL6/J-Tg(CAG-LC3-EGFP)53Nmz/Rbrc) were kindly provided from Prof. Dr. Irmgard Tegeder, Frankfurt (Developed by Noboru Mizushima, National Institute for Basic Biology). The GFP-LC3 mouse line was developed by random integration of GFP-LC3 fragments with the CAG promoter; this is a strong ubiquitous promoter containing the cytomegalovirus immediate-early enhancer and the chicken b-actin promoter. The GFP-LC3 transgene was inserted 106 bp upstream of a putative gene named “similar to transcriptional adaptor 3-like (LOC665264)”. This gene sequence was derived by automated computational analysis using the gene prediction method (GNOMON) and is predicted to be a pseudogene. Indeed, no transcript corresponding to this gene has been reported.

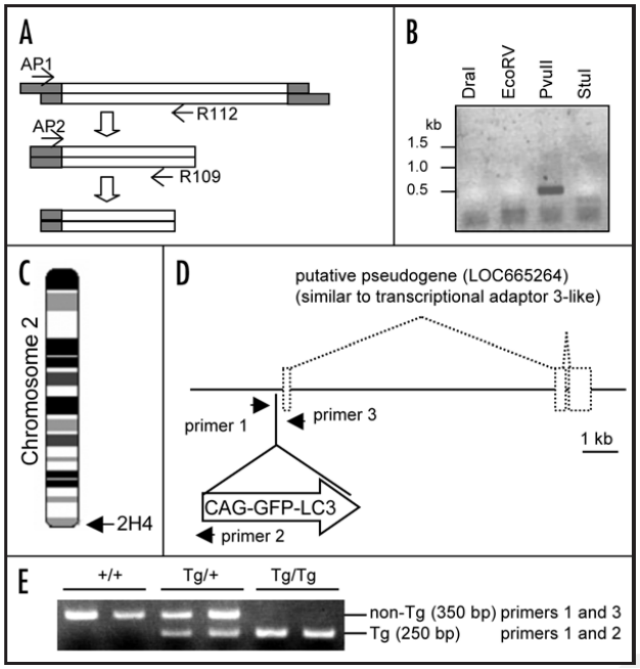

Figure 40: Chromosomal mapping of the GFP-LC3 transgene and zygosity determination in GFP-LC3 transgenic mice. (A) Scheme of the chromosome mapping of the GFP-LC3 transgene. The grey segments indicate the ligated adaptors. Primer sequences used were: AP1 5'-GTAATACGACTCACTATAGGGC-3'), AP2 (5'-ACTATAGGG CACGCGTGGT-3'), R112 (5'-CCAGGCGGGCCATTTACCGTAAGTTAT-3') and R109 5'-GGCGGGCCATTTACCGTAAGTTATGT-3'). (B) Result of the primary PCR amplification of the genomic DNA digested with the indicated restriction enzymes. (C, D) Integration site of the GFP-LC3 transgene. (E) Genomic DNA was extracted and analyzed by PCR with primers 1, 2 and 3 shown in panel D. Positions of the non-transgenic (wild-type) allele and transgenic allele are indicated. Primer sequences used were: Primer 1 5'-ATAACTTGCTGGCCTT TCCACT-3'), Primer 2 (5'-CGGGCCATTTACCGTAAGTTAT-3') and Primer 3 (5'-GCAGCTCATTGCTGTTCCTCAA -3'). The PCR program was: 94˚C for 4 min, followed by 25 cycles of 94˚C for 30 sec, 60˚Cfor 30 sec and 72˚C for 1 min, and an additional 7 min at 72˚C after the final cycle.

E.11.2. Statistical analysis:

Quantitative data are expressed as the means ± s.e.m.. Statistical comparisons between experimental groups were made using Student's *t*-test. Probability values of $p \leq 0.05$ were considered significant.

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G. Appendix

G.1. Publication output

G.1.1. Publications in journals

Felzen, V., C. Hiebel, I. Koziollek-Drechsler, S. Reissig, U. Wolfrum, D. Kogel, C. Brandts, C. Behl, and T. Morawe. 2015. Estrogen receptor alpha regulates non-canonical autophagy that provides stress resistance to neuroblastoma and breast cancer cells and involves BAG3 function. *Cell Death Dis*. 6:e1812.

Brendel, A. & **Felzen V.** et al., 2013. Differential regulation of apoptosis-associated genes by estrogen receptor alpha in human neuroblastoma cells. *Restor Neurol Neurosci*. 31:199-211.

G.1.2. Distribution to Meetings

Felzen,V.; Morawe,T.; Brendel,A.; Behl,C. Differential effect of estrogen receptors on apoptosis and autophagy; *2rd biennial rmn2 meeting,2012, Oberwesel*

Felzen Vanessa; Florie Le Prieult, Christina Gölz, Christian Behl, Thomas Mittmann, Kristin Engelhard, Serge Thal: Functional Role of Autophagy in Neuronal Homeostasis Following Traumatic Brain Injury; *3rd biennial rmn2 meeting,2014, Oberwesel*

Vanessa Felzen, Uwe Wolfrum, Christian Behl and Tobias Morawe: Estrogen receptors modulate non-canonical autophagy and contribute to resistance against oxidative stress in neuroblastoma and breast cancer cells. – *Keystone symposia , Autophagy Fundamentals to Disease, 2014, Austin (Texas)*

Kristin Engelhard, Thomas Mittmann, Christian Behl, **Vanessa Felzen**, Christina Gölz Florie Le Prieult: Functional Role of the Proteasome and Autophagic Protein Degradation System in Neuronal Homeostasis Following Traumatic Brain Injury ; *Collaborative Research Center 1080 International symposium Molecular and Cellular Mechanisms of Neural Homoeostasis 2015, Frankfurt Main*

G.2. **curriculum vitae**

Persönliche Daten

Wissenschaftlische Ausbildung

G.3. **Abbreviations**

AD	Alzheimer`s disease
ALS	Amyotrophic lateral sclerosis
ATG	Autophagy-related genes
ATP	Adenosisne tri-phosphate
BafA1	Bafilomycin A1
BAG	BCL2-associated athanogene
BECN1	Beclin1
CAIR1	carboxyamido-triazole-stressed-1
CCI	Controlled cortical impact
CMA	chaperone-mediated autophagy
DAPI	4',6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxid
DFCP1	Double FYVE-containing protein 1
EGFP	Enhanced green flourescent protein
ER	Estrogen receptor
ERE	Estrogen response elements
HSC70	70-kilodalton heat shock cognate protein
HSP70	70-kilodalton heat shock protein
kDa	Kilodalton
LAMP1	Lysosome associate membrane protein type 1
LC3	Microtubule-associated light chain 3
LIR	LC3-interacting region
mTOR	mammalian target of rapamycin
MTOC	microtubule-organizing center
NBR1	Neighbor of BRCA1 gene 1
NEF	Nucleotide exchange factor
p62/SQSTM	Sequestosome-1
PCR	Polymerase chain reaction

PD	Parkinsons Disease
PE	Phosphatidylethanolamine
PI3K	Phosphatidylinositol 3 Kinase
polyQ	Polyglutamine
PQC	Protein Quality Control
PxxP	Proline rich domain
rb	Rabbit
siNS	Nonsense RNA
siRNA	Small interfering RNA
SEM	Standard error of the mean
SH3	Src Homology 3
SOD1	Superoxid dismutase 1
TBI	Traumatic brain injury
TEM	Transmission electron microscopy
UBA	Ubiquitin-associated
UBL	Ubiquitin-like
ULK	UNC-51-like kinase
WIPI1	WD40 repeat protein interacting with phosphoinositides 1

G.4. **Results of the autophagy gene array**

10 nm E2 24h						1 µm ICI 24h					
SK-01 vs. SK-ERα		SK-01 vs. SK-ERβ		SK-01 vs. MCF-7		SK-01 vs. SK-ERα		SK-01 vs. SK-ERβ		SK-01 vs. MCF-7	
p-value	fold change	p-value	fold change	p-value	fold change	p-value	fold change	p-value	fold change	p-value	fold change
0.636	1.72	0.015	3.06	0.216	2.37	0.867	1.02	0.000	2.04	0.484	-1.09
n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
0.050	-2.90	0.501	-1.12	0.003	-2.90	0.000	-2.69	0.817	1.02	0.000	-3.17
n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.028	-1.46	0.465	1.09	0.001	-1.87
n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.006	-1.90	0.384	-1.14	0.000	-2.83
0.017	-2.39	0.033	-1.31	0.001	-2.78	0.000	-2.88	0.740	-1.07	0.000	-3.30
n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.005	1.57	0.095	1.19	0.024	1.32
0.021	-1.88	0.009	-1.31	0.025	-1.76	0.001	-1.72	0.207	-1.14	0.000	-1.87
0.024	-1.53	0.882	-1.01	0.192	-1.58	0.644	1.05	0.000	1.66	0.322	-1.09
0.040	1.82	0.025	1.51	0.082	2.00	0.001	3.02	0.055	1.73	0.004	2.08
0.250	-1.05	0.000	1.80	0.754	-1.01	0.009	1.51	0.000	1.73	0.040	1.25
0.004	-37.64	0.000	2.02	0.000	-80.85	0.000	-37.93	0.000	2.51	0.000	-44.10
0.005	1.48	0.000	1.43	0.124	1.77	0.000	3.19	0.000	1.73	0.000	2.82
n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
0.044	1.31	0.006	1.41	0.200	1.67	0.002	1.94	0.006	1.42	0.000	1.60
0.002	2.01	0.005	1.51	0.072	2.72	0.000	2.82	0.009	1.33	0.000	2.32
n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.380	-1.34	0.426	-1.22	0.032	-2.18
0.002	-3.88	0.000	-1.78	0.000	-3.20	0.000	-2.88	0.031	-1.19	0.000	-2.76
0.002	-2.14	0.344	-1.06	0.214	-1.62	0.000	-1.95	0.722	1.02	0.000	-1.92
n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.381	1.43	0.045	2.34	0.803	1.18
0.013	-3.52	0.116	-1.31	0.000	-4.85	0.000	-2.69	0.020	-1.45	0.000	-3.12
n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
0.004	8.26	0.174	1.31	0.004	13.39	0.000	16.16	0.555	1.09	0.000	12.97
0.050	-2.98	0.977	1.02	0.002	-3.24	0.000	-2.40	0.671	1.03	0.000	-2.64
n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
0.005	1.52	0.045	1.21	0.051	1.92	0.006	1.81	0.002	1.36	0.000	1.60
n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.049	1.39	0.004	1.75	0.461	1.12
0.003	2.61	0.099	1.22	0.021	4.06	0.167	1.26	0.092	1.17	0.000	1.64
n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.000	2.20	0.002	1.31	0.000	2.11
n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
0.013	-2.45	0.090	-1.40	0.003	-2.42	0.001	-2.09	0.251	-1.21	0.003	-2.00
0.043	-1.88	0.067	-1.38	0.039	-2.17	0.001	-1.90	0.022	-1.33	0.000	-1.79
n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.010	-1.40	0.002	1.40	0.000	-2.03
0.035	1.52	0.018	1.37	0.197	1.61	0.003	1.99	0.009	1.59	0.006	1.62
0.037	-1.91	0.162	-1.16	0.114	-2.05	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
0.147	1.46	0.002	2.22	0.187	1.58	0.033	1.49	0.000	1.78	0.059	1.37
n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.256	-1.32	0.152	1.42	0.025	-2.15
0.046	-2.05	0.054	1.55	0.001	-2.82	0.005	-1.80	0.026	1.68	0.002	-2.00
0.421	1.38	0.002	2.73	0.237	1.65	0.032	1.51	0.003	2.25	0.383	1.16
0.034	1.95	0.841	1.13	0.117	2.18	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.003	2.20	0.030	1.57	0.040	1.64
0.013	7.19	0.205	1.49	0.035	9.08	0.000	25.54	0.284	1.20	0.000	18.09
0.031	-2.42	0.024	1.31	0.001	-2.78	0.131	-1.72	0.028	2.10	0.001	-2.91
0.007	3.40	0.709	1.24	0.045	4.01	0.000	10.66	0.224	1.27	0.000	8.21
0.006	-2.49	0.243	1.10	0.000	-2.82	0.000	-2.88	0.160	1.06	0.000	-3.64
0.005	-1.81	0.992	1.01	0.532	-1.32	0.000	-2.65	0.782	1.04	0.000	-3.00
0.048	-1.83	0.122	-1.46	0.906	-1.76	0.009	-3.04	0.207	-1.37	0.003	-5.67
0.017	-1.60	0.793	1.02	0.607	-1.37	0.025	-1.52	0.853	1.03	0.043	-1.36
n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.198	-1.48	0.612	1.05	0.030	-2.24
0.002	-4.64	0.020	-1.42	0.000	-4.78	0.000	-7.39	0.056	-1.19	0.000	-11.03
0.010	-1.73	0.153	1.21	0.125	-1.32	0.006	-1.65	0.019	1.46	0.036	-1.38
0.126	-2.42	0.281	-1.20	0.040	-2.63	0.003	-3.13	0.539	-1.19	0.000	-4.01
0.033	-2.86	0.012	1.51	0.001	-3.33	0.000	-3.13	0.019	1.20	0.000	-3.39
n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.046	-1.31	0.002	1.57	0.001	-1.58
n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.005	-1.87	0.550	1.09	0.018	-1.70
0.077	2.84	0.309	2.10	0.027	3.26	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
0.039	-1.26	0.422	1.08	0.129	-1.51	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
0.011	-1.60	0.002	-1.70	0.166	-1.64	0.240	-1.15	0.237	-1.16	0.000	-1.50
n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.001	-2.44	0.084	1.52	0.000	-2.72

Figure 41: Estrogen receptors regulated autophagy pathway-associated gene expression is not altered after ICI or E2 treatments. (A) Total RNA from SK-01, SK-ERα, SK-ERβ and MCF-7 cells was characterized by using the Human Autophagy Primer Library 1 (HATPL-1) and comparing ER expressing cells to mock-plasmid transfected controls (SK-01). Red numbers indicate an upregulation greater than 1.5 fold, blue numbers indicate an downregulation greater than 1.5 fold, dark red numbers denote a p-

value<0.05, black numbers depict no significant change and n.s. no significant change in gene expression within the whole group. Results represent the mean values of five independent experiments.