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Chapter II

Breast Cancer and Autophagy

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Abstract

Stress on a cellular level can have detrimental effects on an organism and has been implicated in many different diseases, including cancer. Breast cancer, one of the most prevalent forms of the disease, is often initiated by abnormal cellular mechanisms and damage to DNA brought about by different types of cellular stress. One method of minimizing damage caused by stress is a process that occurs within the cell known as autophagy. Autophagy is a catabolic process in which old and damaged components of the cell are degraded into their elemental forms and recycled in order to synthesize additional proteins and energy. Recently a gene involved in the regulation of autophagy was found to be altered in breast cancer cells, leading researchers to hypothesize that autophagy may play a pivotal role in the disease; however the definite role that autophagy plays in breast cancer is still unclear. Some evidence suggests

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that autophagy acts as a tumour suppressor and should be stimulated to decrease the incidence of cancer in breast cells. Other evidence indicates that autophagy's survival effects may actually help breast cancer cells persist and contribute to chemotherapy resistance in the body. With increasing experimental evidence supporting each claim, it may be that the role of autophagy varies depending on the particular type of tumour and the stage of disease progression. Establishing an accurate link between autophagy and breast cancer is therefore a very important objective that could eventually lead to significant therapeutic implications.

Cell Stress and Disease

Traffic jams, project deadlines, final exams; these events all share a common link – stress. Just as we can undergo stress on a daily basis, so can the cellular components that make us up. Cell stress can come in many different forms from both the environment, as well as within the cell. Environmental cues can include starvation, high temperatures, low oxygen, and hormonal stimulation, while intracellular stress may involve damaged organelles, accumulation of mutant proteins, or microbial invasion (Valko et al, 2005; Levine and Kroemer, 2008). Cellular stress can directly result in improper protein folding, protein breakdown, and DNA damage within the confines of the cell (Rockwell et al, 2001). These types of insults to the cells of the body and accumulation of damaged components have been implicated in a number of diseases including liver, muscle and heart disease, many types of cancer, inflammatory bowel disease (Hampe et al, 2007; Cadwell et al, 2008) and certain neurodegenerative disorders such as Parkinson's (Alam et al, 1997) and Huntington's disease (Anne et al, 2007; Levine and Kroemer, 2008).

Normally in instances of severe cellular stress, an organism can avoid these potentially life-threatening consequences by initiating death in damaged cells via the apoptotic pathway. Apoptosis, a type of programmed cell death, is a normal occurrence in the lifespan of an organism, especially in early development, and functions to remove both non-essential and damaged cells. In the case of those irreversibly damaged, there are genes and proteins within the cell, such as the p53 gene, which are activated upon receiving information on the status of the injured cell (Levine, 1997). This activation can lead to the induction of apoptotic protein activity which results in cellular events such as nuclear chromatin condensation, cytoplasmic shrinking, dilated endoplasmic

reticulum, and membrane blebbing that eventually lead to cell death (Kerr et al, 1972). By eliminating the cells that are beyond repair the organism can avoid more negative repercussions. However, in terms of cell viability, apoptosis is an extreme resolution to cell stress. There are other processes within the cell that work to avoid an accumulation of damaged components; one of these important mechanisms is known as autophagy, and its emerging roles in breast cancer cells will be the focus of this chapter.

Autophagy

Autophagy, a term coined from Greek roots literally meaning "self-eating", is a catabolic (breakdown) process of self-digestion that, under most conditions, promotes cell survival by degrading and recycling internal components. Three forms of autophagy have been described - macroautophagy, microautophagy and chaperone-mediated autophagy – that differ in terms of their physiological functions and mode of cargo delivery (Reggiori and Klionsky, 2002; Wang and Klionsky, 2003). This chapter will focus on the most studied form, macroautophagy, which will be referred to simply as autophagy for the remainder of this article. Autophagy is an evolutionarily conserved process that involves the formation of a double-membrane within the cell that elongates and engulfs a bulk portion of the cytoplasm. Although the process is thought to be nonspecific, the engulfed cytoplasm often contains old or damaged cellular organelles and aberrant proteins (Levine and Klionsky, 2004). Following cytoplasmic engulfment, the double membrane fuses to form a vesicle, termed an autophagasome, which in turn fuses with the lysosome, an acidic organelle, to form an autolysosome. The acid hydrolases within the lysosomes degrade the components of the autophagosome into their elemental forms (free nucleotides, amino acids and fatty acids), which can then be recycled back into the cytoplasm of the cell, to make new proteins as well as provide building blocks for ATP generation (Figure 1) (Noda and Ohsumi, 1998; Levine and Klionsky, 2004).

Autophagy occurs at low levels in virtually all cells to perform homeostatic functions such as the turnover of misfolded proteins and damaged organelles; however, in situations of high cellular stress and damage-inducing conditions when the cell needs to generate additional intracellular nutrients and energy, autophagy is up-regulated and performs at a higher capacity (Levine and Klionsky, 2004). It is therefore seen as an important tool in the everyday housekeeping duties as well as a continued survival mechanism of

the cell. Additionally in extreme cases of stress or starvation, often in the absence of properly functioning apoptosis, this type of degradation within the cell presumably can't keep up to its energy demands and actually results in what is known as autophagic cell death, as the cell literally eats itself beyond sustainability (Clark, 1990; Bursch et al, 2000).

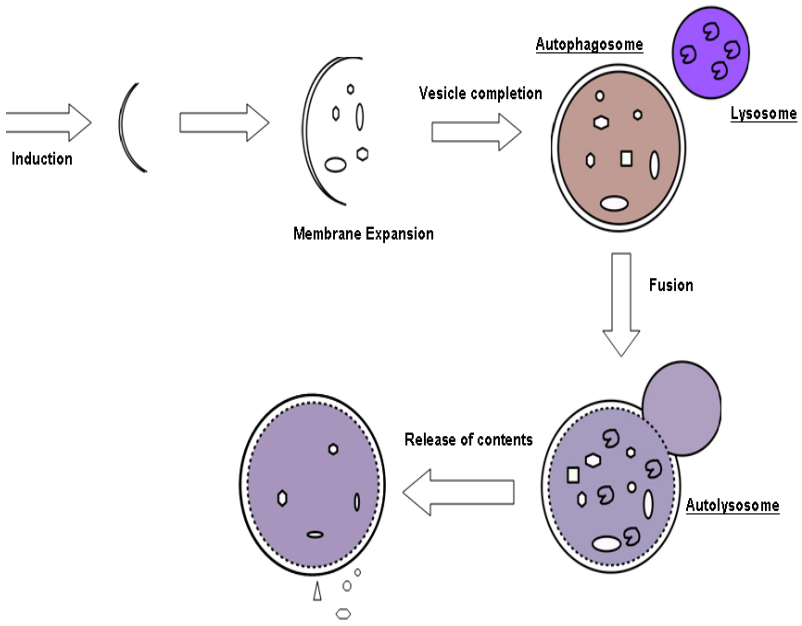


Figure 1. *The cellular events during autophagy* – Autophagy begins with the formation of an isolation membrane, which elongates and takes up cytosolic components such as proteins and organelles. Once the double membrane has fused to form an autophagosome, the vesicle fuses with a lysosome to form an autolysosome. Inside the autolysosome, the cytosolic components are broken down into their elemental forms and released back into the cytosol to be re-used in processes such as protein synthesis or energy production.

Autophagic Regulation and Core Machinery

Autophagy involves a complex pathway of cellular events, containing many different proteins. The process can be divided into several distinct steps: induction and signaling, autophagosome nucleation, membrane expansion and vesicle completion, autophagosome targeting, docking and fusion with the lysosome, and degradation and export of materials to the cell's cytoplasm

(Figure 1) (Melendez and Neufeld, 2008; Levine and Yuan, 2005). One of the key proteins known to regulate autophagy is TOR kinase, which inhibits the autophagic process in the presence of growth factors and abundant nutrients - when the cell doesn't need to break down internal components to synthesize energy (Noda and Ohsumi, 1998). Also involved in autophagy inhibition are the class I PI3K/Akt signaling molecules that link receptor tyrosine kinases to TOR activation (Figure 2) (Takeuchi et al, 2005; Debnath et al, 2003). Some activators of autophagy include the proteins AMPK (Meley et al, 2006), which responds to low ATP levels to represses TOR kinase, and eIF2a (eukaryotic Initiation Factor 2 alpha), which responds to nutrient starvation (Levine and Kroemer, 2008). The cellular checkpoint protein involved in apoptosis, p53, is also involved in autophagy regulation. The p53 protein can positively control autophagy, at least in some cases through a stress-induced regulator termed DRAM (Crighton et al, 2006). The protein DRAM, or damage-regulated autophagy monitor, is a downstream transcriptional target of p53 that when activated has been shown to induce autophagy and is critical in p53-induced cell death (Crighton et al, 2006). More recently it was shown that the cytoplasmic form of p53 might instead act as a negative regulator of autophagy, suggesting a more complex model that may be dictated by the specific nature of the stress signal (Tasdemir et al, 2008).

Downstream of TOR kinase there are more than 30 genes (in yeast), termed autophagy-related (*atg*) genes that encode proteins that are essential to the execution of autophagy (Klionsky et al, 2003). One of the major breakthroughs in studying the autophagic process came from the initial identification of these genes in yeast (Tsukada and Ohsumi, 1993; Thumm et al, 1994; Harding et al, 1995). Homologues of these genes have been found in humans and include *atg8* (known as *MAP1LC3B* or *LC3*) and *atg6* (known as *beclin 1*), both of which are needed for proper autophagosome formation (refer to Table 1). A uniform nomenclature was agreed upon for homologues of *atg* genes in multiple species; essentially a species identifier precedes "*atg*". Table 1 lists *atg* genes conserved between yeast and human, and briefly describes their role(s) in autophagy that are further detailed in other reviews (e.g. Klionsky et al, 2003; Meijer et al, 2007).

Table 1. Core autophagy genes found in yeast and humans*

Yeast Name	Human Homologue	*Role in autophagy	References
ATG1	*Ulk1, Ulk2*	Atg1 is a serine/threonine protein kinase; involved in the regulation of autophagy induction; may regulate subcellular re-distribution of mammalian Atg9 that takes place following nutrient starvation	Matsuura et al, 1997; Kametaka et al, 1998; Kamada et al, 2000; Young et al, 2006
ATG3	*atg3*	Functions as an ubiquitin-conjugating-like enzyme that covalently attaches Atg8 to phosphatidylethanolamine	Ichimura et al, 2000; Tanida et al, 2002
ATG4	*atg4 (4A,4B,4C, 4D)*	Atg4 is a cysteine protease that cleaves the C-terminus of Atg8 to expose a glycine residue for subsequent conjugation	Kirisako et al 2000; Hemelaar et al, 2003; Tanida et al, 2004a
ATG5	*atg5*	Atg5 is covalently attached to Atg12 to facilitate autophagosome formation (Atg12 conjugation system)	Kametaka et al, 1996; Mizushimi et al, 1998; Kuma et al, 2004; Shao et al, 2007
ATG6	*beclin 1*	Atg6 is a component of the class III phosphotidylinositol-3-kinase complex that is required for autophagosome formation (forms a complex with Atg14)	Kametaka et al, 1998; Liang et al, 1999; Liang et al, 2000; Kihara et al, 2001
ATG7	*atg7/GSA7*	Atg7 functions as an ubiquitin-activating-like enzyme; it activates both Atg8 and Atg12 before conjugation	Kim et al, 1999; Tanida et al, 2006; Shao et al, 2007
ATG8	*MAP1LC3B, MAP1LC3A, MAP1LC3C, GABARAP, GATE16*	Atg8 is involved in autophagosome formation and is used as a marker for autophagosomes; lipidation of LC3 paralogues is involved in the closure of autophagosomes.	Kirisako et al, 1999; Kirisako et al 2000; He et al, 2003; Fujita et al, 2008
ATG9	*atg9 (9A and 9B)*	Atg9 is a transmembrane protein that may be involved in delivering membrane to the forming autophagosome	Noda et al, 2000; Lang et al, 2000
ATG 10	*atg10*	Atg10 functions as an ubiquitin-conjugating-like enzyme that covalently attaches Atg12 to Atg5 to	Mizushima et al, 1998; Boya et al, 2005;

Table 1. (Continued)

Yeast Name	Human Homologue	*Role in autophagy	References
		aid in autophagosome formation (Atg12 conjugation system)	Shao et al, 2007
ATG 12	*atg12*	Atg12 is conjugated to an internal lysine of Atg5 through its C-terminal glycine to facilitate autophagosome formation (Atg12 conjugation system)	Mizushimi et al, 1998; Shao et al, 2007; Boya et al, 2005
ATG 13	*atg13*	Induction (modulates Atg1 response)	Funakoshi et al, 1997; Kamada et al, 2000; Chan et al, 2009
ATG 14	*atg14/barkor*	Autophagosome formation (forms a complex with Atg6)	Itakura et al, 2008; Sun et al, 2008
ATG 16	*atg16 (16L1 and 16L2)*	Atg16 binds Atg5 and homo-oligomerizes to form a tetrameric complex for autophagosome formation (Atg12 conjugation system)	Mizushima et al, 1999; Kuma et al, 2002; Mizushima et al, 2003

* Klionsky et al, 2003; Meijer et al, 2007. Human homologues are also designated as *HsATG* or *Atg*.

Quantifying Autophagy *in vitro* Using LC3

LC3 (Atg8) is an especially important autophagy protein as it can be used in the laboratory to monitor autophagy in a variety of ways. This protein exists in two forms within the cell: LC3-I and LC3-II. LC3-I, the form most commonly found within the cytoplasm, is predominant under normal conditions. When autophagy is induced, LC3-I is converted to LC3-II by Atg4-mediated C-terminus cleavage (Tanida et al, 2004a; Tanida et al, 2004b) and lipidated with phosphatidylethanolamine (PE), which allows it to be inserted into the autophagosome membrane (Kabeya et al, 2000). The LC3-II form is the only Atg protein in higher eukaryotes that is known to be associated with the fully formed autophagosome and can be found on both the outer and inner surfaces of the autophagosome membrane (Mizushima and Yoshimori, 2007). LC3 conversion can be visualized via Western blot analysis, and comparing the levels of LC3-II to appropriate controls can indicate the relative number of autophagosomes between samples (Kabeya et al, 2000; Kirisako et al, 1999). Another method used to visualize LC3 in the cell is by tagging its N-terminus with a fluorescent marker, such as green fluorescent protein (GFP) (Kabeya et al, 2000; Mizushima and Yoshimori, 2007). Under normal conditions LC3 (LC3-I) appears diffuse in the cell, but under autophagy-inducing conditions, LC3 appears as localized structures

(puncta) which represent its incorporation into autophagosomes (LC3-II) (Kabeya et al, 2000). One issue in monitoring GFP tagged LC3 is that when the autophagosome fuses with the lysosome and the contents are degraded, the GFP loses its fluorescent signal, making quantifying levels of autophagy more difficult (Klionsky et al, 2008). However, a recent solution to this problem has become available; Kimura et al (2007) developed an alternate marker whereby the LC3 protein is tagged with an additional fluorescent protein, monomeric red-fluorescence protein or mRFP, which is relatively stable and whose signal is maintained in the acidic environment of the lysosome (Kimura et al, 2007). Therefore tagging LC3 with both mRFP and GFP allows researchers to visualize both the red and green LC3-II puncta (i.e. yellow overlay) in the autophagosomes, and just the red labeled LC3-II after autolysosomal degradation (Kimura et al, 2007; Iwai-Kanai et al, 2008).

Autophagy is a dynamic, multi-step process that can be monitored at various steps (seen in figure 1). It is important to distinguish between measurements that monitor the numbers of autophagosomes (steady-state) versus those that measure flux through the autophagic pathways. Autophagic flux refers to the complete process of autophagy, including the delivery of cargo to the lysosomes and subsequent breakdown of its components (Klionsky et al, 2008). Some steady-state autophagy tests may indicate that autophagy has occurred due to the fact that there is an increase in autophagsosomes, however a defect in the degradation stage may actually prevent the completion of the autophagic process (Kavacs et al, 1982). Therefore these types of circumstances have to be differentiated from fully functional autophagy (flux) that includes delivery to, and degradation within lysosomes. In a steady-state experiment, the above methods (LC3 conversion or GFP-LC3 puncta quantitation) can be used to only monitor the formation of autophagosomes (Mizushima and Klionsky, 2007), however, when used in combination with autophagy inhibitors and various other assays, they can also be used to measure autophagic flux (Tanida et al, 2005). Recently, Klionsky and 231 other researchers published a comprehensive set of autophagy-monitoring guidelines to assist in the interpretation of various assays (Klionsky et al, 2008). Most researchers agree that the best way to measure autophagy in an *in vitro* or *in vivo* system is to use a combination of these techniques; however applying such dynamic assays to *in vivo* samples is often technically difficult (Tsuchihara et al, 2008). New assays and tools are required to enable more facile *in vivo* validation of results that are gathered using proven *in vitro* methods.

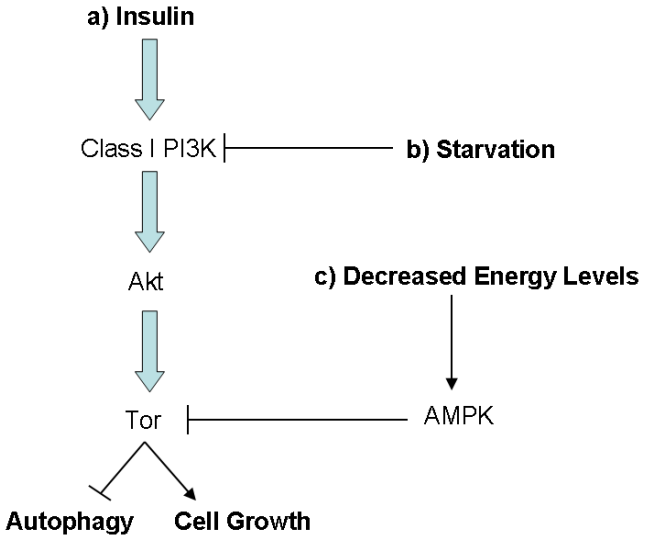


Figure 2**.** *Cellular responses to different environmental conditions.* The insulin signaling pathway in a cell with adequate nutrition leads to cell growth as depicted in a), while an autophagic response to amino acid starvation and reduced ATP levels is illustrated in b) and c) respectively.

Autophagy and Breast Cancer

What effect does altering the autophagy process have on cancer cells? There has been a dramatic increase in research dedicated to answering this question during the last decade. A cancerous cell is one whose normal machinery malfunctions, causing the cell to grow and divide much faster than normal, often resulting in the development and proliferation of a tumour. Breast cancer is one of the most prevalent forms of the disease in women and affects millions of people worldwide (American Cancer Society, 2007). Today, breast cancer, like many other forms of cancer, is considered to be an outcome of both environmental and genetic factors leading to development of cancer cell traits, including self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis, impairment of DNA repair mechanisms, and metabolic transformation (Hanahan and Weinburg, 2000; Rockwell et al, 2001; Tennant et al, 2009). The precise mechanisms responsible for these cancer cell traits are still poorly understood but some of the molecules involved are beginning to be elucidated. In 1999, the first direct

association between a core autophagy gene and human cancer was discovered by Liang et al, who found that the autophagy gene *beclin 1* was monoallelically deleted in 40-75% of sporadic human breast and ovarian cancers (Liang et al, 1999), suggesting a possible tumour suppressor function for this autophagy protein. Since then, there have been an increasing number of scientific papers published on the relationships between autophagy and cancer, examining not only *beclin 1*, but several of the other *atg* genes required for autophagy (refer to Table 1). While studies to elucidate the role of autophagy in tumorigenesis have been carried out in various cancer types, predominantly in cancer cell lines, here we will mainly describe studies conducted specifically in breast cancer.

Establishing exactly what the link is between breast cancer cells and autophagy is an important research area that has increased in recognition over the past few years. The solution to this quandary is more complicated than it might seem at first glance. There is experimental evidence that supports the theory that autophagy acts as a tumour suppressor and should therefore be stimulated to reduce the occurrence of cancer in breast cells (Liang et al, 1999; Qu et al, 2003; Karantza-Wadsworth et al, 2007; Mathew et al, 2007). However there is other evidence supporting the idea that the survival effects of autophagy may actually help breast cancer cells endure and subsequently proliferate, particularly in adverse environmental conditions, and thus autophagy should be inhibited to help reduce breast cancer (Paglin et al, 2001; Abedin et al, 2007; Qadir et al 2008; Samaddar et al, 2008; Apel 2008). This leads to the question of whether the process of autophagy acts to help or hinder cancerous breast cells. The answer to this could help determine whether we should attempt to therapeutically activate or inhibit autophagy and if so, at what stage of tumour development. Below we describe the supporting evidence for the roles autophagy may play in cancer and discuss how these opposing roles can be reconciled.

The Tumour-Suppressor Role of Autophagy in Breast Cancer

Over the past 10 years several genetic links have emerged between autophagy defects and cancer, providing increasing support for the concept that autophagy functions as a tumour-suppressor pathway (Liang et al, 1999; Mathew et al, 2007; Marino et al, 2007; Karantza-Wadsworth et al, 2007). In addition, an overlap between the regulation of autophagy and signaling pathways that regulate tumour formation has been established. Several

autophagy-inducing genes involved in the upstream inhibition of TOR signaling pathways including PTEN, TSC1, and TSC2 are known tumour suppressors that act to decrease the incidence of cancer (Arico et al, 2000; Inoki et al, 2003; Levine and Kroemer, 2008). TOR-activating oncogene products on the other hand, such as class I PI3K and Akt, inhibit autophagy, and when over-expressed increase the likelihood of developing cancer (Blommaart et al, 1997) (Figure 2). The PI3K pathway is particularly relevant to breast tumorigenesis as a recent large-scale sequence analysis of genes mutated in human breast cancer identified mutations in multiple PI3K pathway components (Wood et al. 2007). Lastly, the apoptosis checkpoint protein p53 is the most commonly mutated tumour suppressor gene in human breast cancers, and is known to be involved in regulating autophagy in DNA-damaged cells (Bursch et al, 2000; Crighton et al, 2006; Tasdemir et al, 2008).

The first core *atg* gene discovered that is linked to human breast cancer, as mentioned previously, is *beclin 1*, located on chromosome 17q21. *Beclin 1* is required for autophagosome formation in a complex with class III PI3K and was found to be monoallelically deleted in human ovarian, breast, and prostate cancers (Liang et al 1999; Aita et al, 1999). Experiments have shown that many breast carcinoma cell lines, although polyploid for chromosome 17, exhibit deletions of one *beclin 1* allele, and human breast tumours show decreased Beclin 1 protein levels compared to normal adjacent tissue (Liang et al, 1999). In mouse knock-out studies, it was shown that *beclin 1* heterozygous mice have hyperproliferative, pre-neoplastic changes in mammary cells (Qu et al, 2003) and are more prone to the development of spontanteous lung and liver tumours, as well as lymphoma (Qu et al, 2003, Yue et al, 2003). These findings suggest that the mono-allelic deletions of *beclin 1* in human breast cancers are likely mechanistically important in tumorigenesis. Karantza-Wadsworth et al (2007) showed that allelic loss of beclin 1 resulted in attenuated and delayed autophagy induction compared to wild-type, and also sensitized mammary epithelial cells (iMMECs) to metabolic stress (*in vitro* ischemia) as well as accelerated lumen formation in mammary acini. Autophagy defects activated the DNA damage response and promoted gene amplification and drug resistance *in vitro*. In allograft mouse mammary tumours *in vivo*, allelic loss of *beclin 1* activated the DNA damage response and, when combined with defective apoptosis, promoted mammary tumorigenesis (Karantza-Wadsworth et al, 2007).

A study by Mathew et al in 2007 used similar experimental methods to confirm that monoallelic deletion of *beclin 1* does decrease the likelihood of cell survival *in vitro*. The Mathew (2007) study also analyzed cells harboring

a homozygous deletion of another essential autophagy gene, *atg5*, and its effects on cell survival. They found that the *atg5* deficiency in iBMK (immortalized baby mouse kidney epithelial cells) cell-lines impaired survival of the cells and promoted DNA damage under metabolic stress *in vitro,* but increased tumourigenicity in allograft mouse models *in vivo* (Mathew et al, 2007). Marino et al (2007) generated mutant mice deficient in *atg4C*/*autophagin-3*, whose gene product is a member of the mammalian Atg4 family of cysteine proteases. These mutant mice appeared both viable and fertile, with no apparent abnormalities; however upon further analysis these mice were found to have decreased autophagy in the diaphragm when starved and an increased susceptibility to developing fibrosarcomas when exposed to chemical carcinogens (Marino et al, 2007). Together, these findings suggest that defective autophagy increases the susceptibility to DNA damage and genomic instability.

In addition to autophagy's cell autonomous mechanism as a suppressor of genomic damage, a non-cell-autonomous tumor suppressor function for autophagy has been proposed. In apoptosis-defective iBMK cells, autophagy inhibition by AKT activation or *beclin 1* knockdown resulted in necrotic cell death *in vitro* and *in vivo* (Degenhardt et al, 2006). This cell death by necrosis was associated with inflammation, providing a potential non-cell-autonomous mechanism by which autophagy defects resulted in the observed acceleration in tumour growth (Degenhardt et al, 2006). Inflammatory infiltration and cytokine production are found in necrotic tumours and are thought to foster the growth of such tumours (Balkwill, 2004). Therefore, promoting autophagy may also act to restrict necrosis and inflammation, and such tumor suppressor effects would ultimately discourage breast cancer progression.

Autophagy, Tumour Suppression and Ageing

The notion that decreased levels of autophagy can lead to a higher probability of tumour formation is consistent with the anti-ageing theory of autophagy, as it has been shown that both the formation of autophagosomes and their removal by lysosomal fusion decrease with age (Terman, 1995, Cuervo et al, 2005), negatively correlating with the incidence of most types of cancer initiation, including breast cancer. The first genetic evidence linking autophagy and longevity was found by Melendez et al using *C. elegans* models where specific mutations in genes related to the insulin-like signaling pathway doubled the lifespan of the worms, however when autophagy was

blocked in these mutated worms, their lifespan returns to normal values (Melendez et al, 2003). This suggests that the activation of autophagy is one of the downstream effectors that lead to a prolonged existence (Melendez et al, 2003, Cuervo et al, 2004). A study by Bergamini et al in 2003 also described an association between ageing and autophagy. They proposed that caloric restriction and decreased levels of IGF-1, both known factors in life longevity, may act by stimulating an increase in the basal levels of autophagy (Bergamini et al, 2003). Decreased basal levels of autophagy would allow for the accumulation of damaged or non-functioning components of the cell. Normally in humans, these events would result in DNA instability and often lead to the activation of the apoptotic pathway and eventually cell death (Moll and Zaika, 2001; Yen and Klionsky, 2008). However, it has been shown that a large percentage of breast cancer cells have defective or inactive apoptotic proteins (such as p53), leading to an inhibition of this programmed cell death. The accumulation of increased sources of oxidative stress because of decreased levels of both autophagy and apoptosis may result in damaged DNA or other known initiators of cancer (Karantza-Wadsworth et al, 2007). Although this evidence so far comes from different *in vitro* models, if applied to the human population, it may help explain why cancer is more prevalent in the elderly than in younger populations who have higher levels of autophagy.

With this information, one might instinctively think that the link between autophagy and breast cancer is fairly obvious; autophagy must play a tumour suppressor role and when not functioning properly it may lead to chromosomal instability (Karantza-Wadsworth et al, 2007; Mathew et al, 2007) and eventually cancer cell development. Therefore therapeutically targeting pre-cancerous cells to upregulate components of autophagy might be a method of decreasing the initiation of the disease in breast tissue. However, there needs to be an important distinction made between preventing the initiation of a cancerous tumour and treating a tumour that has already been established. Most successful cytotoxic chemotherapy agents utilized by oncologists in treating cancers (including breast cancer) actually promote significant DNA damage and chromosomal instability, leading to activation of the apoptosis pathway in cancerous cells. In fact, there are a number of drugs either already in clinical use or in the development stage for the treatment of already-established breast tumors, that promote DNA damage in tumour cells, and also have an effect on autophagy (Table 2). There is increasing evidence to suggest that up-regulating autophagy in already-established cancerous cells would aid in tumour survival and perhaps resistance to chemotherapy treatment by degrading damaged organelles, preventing apoptosis, and/or by

providing additional energy the tumour cells would otherwise lack. This leads to the second role - that increasing autophagy in established cancerous cells promotes tumour viability. In this context, therapeutic strategies employing autophagy inhibition may be warranted, with the resulting cytotoxic effects on the tumour outweighing any possible tumor-promoting side effects secondary to DNA damage.

The Tumour Survival Role of Autophagy in Breast Cancer

As described above, autophagy is an important process for maintaining cell viability under conditions of stress within the cell. The rapid proliferation of breast tumour cells puts them under tremendous stress and, without an adequate blood supply, the ability to properly sustain nutrition and energy decreases exponentially. If sufficient nutrition or energy cannot be supplied to the proliferating cells, then it would seem the appropriate response would be for the cancerous cells to stop dividing and die off at a certain threshold. However, this is often not the case in breast tumours and it has therefore been suggested that perhaps the increase in autophagy levels in breast tumour cells leads to enhanced survival properties (Abedin et al, 2007). This mechanism seems very plausible from a functional standpoint as the autophagic pathway can be used to break down proteins and organelles to provide the basic elements needed to assemble new proteins crucial to the growth of the cell, as well as energy to perform the necessary functions. During the initial phase of tumour formation, new blood vessels have not yet been created and the nutritional demands of tumour cells are likely to surpass the supply from normal vasculature (Harris, 2002). Applying the autophagic survival mechanism to these rapidly growing cancer cells, which have outgrown their vascular supply and face oxygen shortage or metabolic stress, would allow them to continue to grow and divide with little to no outside resources (Maiuri et al, 2007). In support of this general concept, Debnath et al. used a three-dimensional mammary epithelial cell (MCF10A) culture model to show that during detachment induced apoptosis (termed *anoikis* [Frisch and Francis, 1994]) the incidence of autophagy increased dramatically (Debnath et al, 2002; Fung et al, 2008). It was demonstrated further that knockdown of core autophagy genes using siRNA techniques resulted in an increased ability of the detached cells to undergo apoptosis (Fung et al, 2008; Debnath, 2008). These findings led the researchers to speculate that autophagy may contribute to the survival of tumour cells lacking matrix contact either early on in

carcinoma development or later during dissemination and metastasis (Fung et al, 2008; Debnath, 2008).

A study by Abedin et al, showed in human breast adenocarcinoma MCF7 cells that upon DNA damage, autophagy significantly delayed the apoptotic response by the cell resulting in extended cell viability. This delay occurred even in the presence of camptothecin (CPT), a drug that binds topoisomerase I, resulting in DNA damage and the upregulation of p53 expression, normally leading to apoptosis of the cell. When the researchers down-regulated autophagy-related proteins (Beclin 1 and Atg7) they unmasked a caspase-dependant apoptotic response to DNA damage, which eventually led to cell death (Abedin et al, 2007). Therefore it was concluded that in the presence of DNA damage induced by CPT, the subsequent increase in autophagy led to an increased cell survival and delayed cell-death (Abedin et al, 2007).

Autophagy also appears to have a pro-survival role in the cellular response to the endocrine therapy agent, tamoxifen, possibly implicating the pathway in the high incidence of tamoxifen resistance in breast cancer patients. Approximately 30-50% of women treated with anti-estrogen therapy (a type of endocrine therapy for estrogen-positive breast cancers) do not initially respond or their breast cancer cells ultimately acquire resistance during treatment (Clark et al, 2001); therefore it is crucial that we understand how resistance occurs in breast cancer patients. In 1996, Bursch et al showed that high-dose tamoxifen (10^{-6} M) treatment, along with its cyto-toxic properties, resulted in an increase in autophagy, which they proposed aided MCF-7 cell death. A more recent functional study by Qadir et al. (2008) employed small interfering RNAs (siRNAs) to knock down three different autophagy-related proteins, Atg5, Atg7, and Beclin 1 in three different estrogen receptor positive (ER+) breast cancer cell-lines (MCF-7, T47D, and MCF7-HER2) in the presence of tamoxifen (2.5-5.0 uM) treatment. This study showed that autophagy knockdown with Atg7 or Beclin 1-siRNAs resulted in enhanced mitochondrial depolarization and reduced cell viability even in breast cancer cells with reduced sensitivity (T47D) or resistance (MCF7-HER2) to tamoxifen. Thus, tumour sensitization to tamoxifen can be enhanced when autophagy gene function is knocked down, perhaps due to increased cytotoxic effects of tamoxifen that lead to increased apoptosis and significantly reduced cell viability. Similar findings were made by Samaddar et al. (2008) who demonstrated that 4-hydroxytamoxifen (1-5 uM) induces autophagy in ER+ breast cancer cells that do not die and facilitates the development of anti-estrogen resistance, allowing the cells to survive in culture despite the presence of toxic drug concentrations (Samaddar et al,

2008; Schoenlein et al, 2009). Reduction of autophagy by 3-MA treatment or Beclin 1-RNAi in combination with 4-hydroxytamoxifen resulted in increased cell death, indicated by increased cleavage of caspase-9 and the caspase-6 substrate Lamin A (Samaddar et al, 2008). While further studies are required to uncover the molecular pathways involved, these results suggest that autophagy may represent a general mechanism responsible for avoiding or delaying tamoxifen-induced apoptosis and that autophagy knockdown may be useful in a combination therapy setting to sensitize breast cancer cells to high dose tamoxifen therapy that is used in the treatment of late stage or recurrent breast cancer.

The induction of autophagy by irradiation, a common treatment modality for breast cancer, has also been demonstrated. An early report by Paglin et al. (2001) described the accumulation of acidic vesicular organelles following radiation treatment in MCF7 cells and associated these with a protective autophagy response. In a more recent study, Apel et al. (2008) showed that breast cancer cells (MDA-MB-231) can be re-sensitized to radiation by autophagic inhibition. They hypothesized that autophagy protects the cells against radiation damage by providing catabolites required for repair processes, and possibly by physically containing the toxic molecules, thereby preventing cytoplasmic acidification (Apel et al, 2008). Their proposed model was that in situations where autophagy was inhibited, the increased needs for catabolite supplies for enhanced DNA repair in radio-resistant cells could not be fulfilled, resulting in induction of the apoptotic pathway and eventually cell death (Apel et al, 2008). Their results supported this model, in that cancer cell-lines that had previously been resistant to radiation treatment became re-sensitized following inhibition of the autophagic pathway (Apel et al, 2008). The increased number of recent findings designating the autophagic pathway as a mechanism that can delay activation of apoptosis in breast cancer cells (Abedin et al, 2007; Apel et al, 2008, Qadir et al, 2008, Samaddar et al, 2008) have strengthened the notion that autophagy has a primary tumour survival role in established breast cancer cells.

Resistance to therapeutic drugs is a negative outcome in chemo-therapy and a major issue in the clinical treatment of breast cancers. There are several therapeutic drugs that have been shown to alter the levels of autophagy in breast cancer cells – at least in cell-lines (Table 2). A more thorough list of clinically relevant drugs demonstrated to modulate autophagy in a variety of other cancer cell types can be found in a recent review by Høyer-Hansen and

Table 2. Treatments shown to have autophagy-modulating effects in breast cancer cells

Agent	Target	Step of autophagy affected	Effect on autophagosomes & autolysosomes (& methods)	Effect on Autophagic Flux	References
Tamoxifen	Estrogen Receptor; Other	Induction	Increase in autophagosomes (punctate GFP-LC3) and corresponding increase in MDC fluorescence	Induce*	Bursch et al, 1996; Qadir et al, 2008; Samaddar et al, 2008
Radiation	DNA	Induction	Increase in autophagosome and autolysosome formation (EM, GFP-LC3, acridine orange and LAMP-1)	ND	Paglin et al, 2001; Apel et al, 2008
Campto-thecin	Topoisomerase I; leads to DNA damage	Induction	Increase in autophagosomes (punctate GFP-LC3) & increase in association of mitochondria with autophagic vesicles	ND	Abedin et al, 2007
mTOR inhibitors (eg. Rapamycin)	mTOR	Induction	Increase in autophagosome and autolysosome formation (EM, punctate DsRed1-LC3 & GFP-LC3, MDC, Lamp-1)	Induce	Noda et al, 1998; Hoyer-Hansen et al, 2005; Kim et al, 2006, Abedin et al, 2007
EB1089	Ca2+ mobilization; leads to AMPK activation and mTor inhibition	Induction	Increase in autophagosome and autolysosome formation (EM, punctate DsRed1-LC3, GFP-LC3, MDC)	Induce	Høyer-Hansen et al, 2005; Demasters et al, 2006

Table 2. (Continued)

Agent	Target	Step of autophagy affected	Effect on autophagosomes & autolysosomes (& methods)	Effect on Autophagic Flux	References
Chloroquine	Lysosomal pH	Fusion/ Degrada-tion	Increase in number of autophagosomes (EM and punctate GFP-LC3)	Inhibit*	Amaravadi et al, 2007 (lymphoma model)
Microtubule-targeting agents e.g. vincristine	Tubulin	Fusion	Increase in number of autophagosomes (punctate GFP-LC3); Decrease in fusion of autophagosomes with lysosomes (lack of GFP-LC3 and Lamp2 colocalization)	Inhibit	Groth-Pedersen et al, 2007

***** Gorski laboratory; effect demonstrated in breast cancer cell lines using western blot-based LC3 flux assay (unpublished). ND = Not Demonstrated

Jäättelä, 2008, and includes treatments such as HDAC inhibitors, angiogenesis inhibitors, Imatinib, HIV protease inhibitors, and Resveratrol among others. Since these drugs affect multiple cellular processes (eg. autophagy, cell division), the extent that autophagy modulation plays a role in their therapeutic effects remains to be determined. Many of the drugs listed in Table 2 and in Høyer-Hansen and Jäättelä (2008), like tamoxifen and rapamycin, were found to promote autophagy. Thus, recent data implicating autophagy in chemoresistance warrants further investigation and consideration with respect to chemotherapy drugs that induce autophagy in cancerous cells. This is true especially since much of the incriminating data comes from either breast cancer cell-lines, or limited work with mouse models, both of which have their own shortcomings and are difficult to apply directly to human patients (Wagner, 2004; Jessani et al, 2005). If these results are in fact applicable to humans, then inhibiting the autophagic pathways in breast cancer cells in combination with autophagy-inducing treatments may actually increase the cytotoxicity of these therapies, thereby increasing cellular apoptosis and reducing the emergence of therapy resistance. However, as described above, in apoptosis-defective iBMK cells, autophagy inhibition by AKT activation or Beclin 1 knockdown resulted in cell death by necrosis that was associated with inflammation and accelerated tumor growth (Degenhardt et al, 2006). Thus, the apoptotic competence of a cell and likely other factors may have an important impact on potential autophagy-related therapeutic strategies.

A Role for Autophagy in Breast Cancer Cell Death?

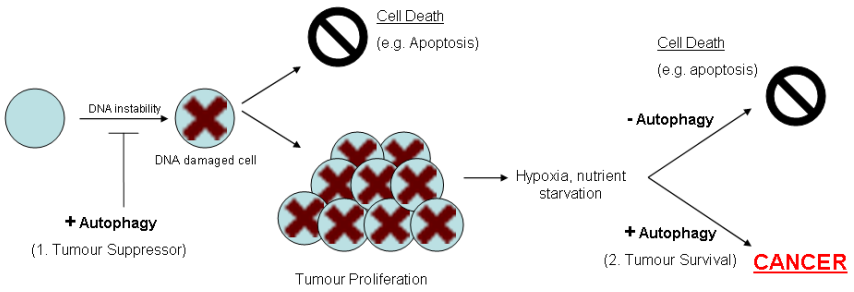
Another proposed role of autophagy in breast cancer cells is one that leads to cell death. It is possible that increasing autophagy to a high enough level may be unsustainable to the cell and result in cellular death. A study in 2005 in the MCF-7 cancer cell-line showed that EB1089, a chemotherapeutic vitamin D analogue and Ca^{2+} mobilizing agent, kills tumor cells via a caspase-independent pathway that results in chromatin condensation and DNA fragmentation, similar to apoptosis. Using electron microscopy and LC3 fluorescent tags, the researchers concluded that it was likely a dramatic increase in autophagy that resulted in cell death (Høyer-Hansen et al, 2005). In support of this conclusion, they showed that upon inhibition of autophagy (via Beclin 1 knockdown), the incidence of chromatin condensation and cell death decreased significantly (Høyer-Hansen et al, 2005). Despite these findings, the

exact cause of this proposed autophagic cell death is still unknown; however in a more recent paper, Høyer-Hansen et al (2007) demonstrated that the anti-apoptotic protein Bcl-2 regulates autophagy by calcium homeostasis and signalling, with increased levels of calcium leading to repression of mTOR. Using L929 murine fibrosarcoma cells, the Lenardo research group showed that autophagic cell death caused by caspase inhibition is achieved through the selective autophagic degradation of the reactive oxygen species (ROS) scavenger catalase, which in turn leads to elevated levels of ROS that kill the cell (Yu et al, 2006). Though the experiment was not done on breast cancer cells themselves, applying these findings to a clinical setting, it might be logical to try and increase autophagy in cancerous breast tissue instead of the treatment options explored above. It is still undetermined in many cases whether or not autophagy has a direct role in the death of breast cancer cells or whether the increase in autophagy when treating breast cancer with certain chemotherapy agents is instead an associated attempt at cell protection. However, when considering the application of either of these concepts to cancer patient treatment, there are certainly many more important questions that need to be answered, and further research into using the autophagic pathway components as therapeutic targets to treat breast cancer cells is needed.

Reconciling the Dual Roles of Autophagy

The compelling experimental data supporting autophagy as both a tumour suppressor and tumour survival mechanism give rise to the idea that the role of autophagy changes depending on the tumour type, and more importantly, the stage of development (Figure 3). Therefore under normal conditions, autophagy would act as a tumour suppressor, and through its homeostatic housekeeping role, prevent an accumulation of potentially harmful agents. If this process were not working properly or efficiently, such as in the monoallelic deletion of *beclin 1*, these deficient components would not be properly broken down and could potentially damage the cell or cause genetic instability, leading to development of cancer (Mathew et al, 2007; Karantza-Wadsworth et al, 2007; Liang et al, 1999). Therapeutically up-regulating missing/faulty components in the pathway of autophagy defective cells would therefore likely decrease the incidence of tumour formation. However, most gene/protein abnormalities associated with autophagy are discovered after a tumour has been initiated. According to this dual-function model, now that the

A. Dual roles of autophagy in cancer development



B. Dual roles of autophagy in response to chemotherapy

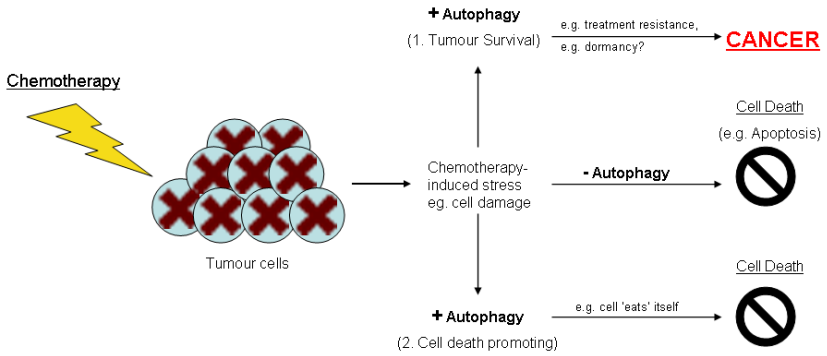


Figure 3. *Possible roles of autophagy in breast cancer cells. A. Dual roles of autophagy in cancer development:* 1. Tumour Suppressor: Housekeeping and stress-induced autophagy functions to limit DNA damage and maintain genome integrity. 2. Tumour Survival: Under environmental stress conditions, the autophagic process may be upregulated in breast tumours, providing an internal source for protein synthesis and energy production to facilitate survival. *B. Dual roles of autophagy in the response to chemotherapy:* 1. Tumour Survival: Autophagy acts as a cytoprotective mechanism in response to the cellular damage resulting from chemotherapy. If autophagy is reduced or inhibited in these cells, cell death (e.g. apoptotis in apoptosis-competent cells) occurs. 2. Cell Death Promoting: In some contexts, autophagy is increased to high levels that the cell cannot sustain and thus cell death ensues.

cell is known to be cancerous, autophagy may instead play a role in tumor promotion or tumor survival. At this point, cancerous cells can amplify the autophagic pathway, taking advantage of its cell-survival properties and preventing apoptosis. By breaking down proteins and organelles in the cytoplasm to produce additional energy, the cell is able to flourish in its surroundings despite little to no additional nutrients or blood supply (Noda and Ohsumi, 1998). At this stage, therapeutically targeting components in the autophagic pathway might act to decrease the ability of the cancer cells to survive. As described above, combining this type of autophagy inhibition with cytotoxic autophagy-inducing therapeutics such as tamoxifen may help increase the sensitivity of the cells to the toxic treatment and decrease the likelihood of resistance. In both contexts, tumor suppressor vs. tumor survivorship, autophagy is conferring a cyto-protective function: at pre-cancerous stages, this helps to prevent cancer initiation, but in already-established cancer cells or tumours, this helps to promote cancer survival. However, there still remains the third possibility – that autophagy may also act to kill breast cancer cells in a therapeutic context in established cancers. Again it should be noted that most findings to date resulted from *in vitro* cell culture studies and applying these conclusions to mouse models and then to human patients is currently speculative at best.

Conclusion and Future Directions

Breast cancer arises from the uncontrolled proliferation and the inappropriate survival of damaged cells, which increases the likelihood of tumour formation. Cells have developed several defense mechanisms to ensure that cell division, differentiation and death occur properly and in a coordinated fashion, both during development and in the adult body (Esquela-Kerscher and Slack, 2006). Much of the cell survival research over the past decade has given us good reasons to believe that the manipulation of autophagy may provide a useful way to prevent cancer development, limit tumor cell proliferation, and increase the effectiveness of cancer treatments. With the increased amount of interest and research put into this developing field, we are rapidly gaining a better understanding of how the autophagy process works. We already have drugs on the market that both induce autophagy such as rapamycin or tamoxifen (Noda and Ohsumi, 1998; Bursch et al, 1996), as well as inhibit the process, such as chloroquine (Amaravadi et al, 2007) (Table 2).

However, the answer to the question of whether we should try to switch autophagy on or off is not straightforward and researchers are still trying to resolve the overall picture. Consequently, unlike some other common aspects of cancer cell biology, such as cell growth, apoptosis, or angiogenesis, where we always know how we would like to manipulate the process in the tumor (reduce, increase, and reduce respectively), our goals for manipulation of autophagy will likely depend both on the tumour type, genetic background and, most importantly its stage of development (Hippert et al, 2006). This has important implications when discussing therapeutic options; for those individuals at risk of cancer, perhaps we should provide drugs that increase autophagy, which might decrease the likelihood of cellular DNA damage and tumour formation. There are, however, alternatives to drug treatment to alter autophagy levels in the body. For example, dietary components can also increase cellular autophagy levels (Hannigan and Gorski, 2009) and perhaps this would be the more appropriate cancer-prevention strategy in relation to maintaining healthy cell viability. On the other hand, if a cancerous tumour has already been established, using drugs that inhibit autophagy, such as chloroquine, in combination with chemotherapy might prevent continued cell survival. Since neither of these drugs is a specific inhibitor of autophagy and both have known side effects (Yam and Kwok, 2006), there is also substantial interest in developing more specific autophagy inhibitors. Some of the core autophagy machinery components, such as Ulk-1 (Atg1) or Atg4, may make more suitable molecular targets. An important question that also needs to be addressed concerns the role, if any, autophagy might play in breast cancer metastasis and whether we want more or less autophagy to prevent metastatic progression (Hippert et al, 2006). Additional unexplored areas include the possible role of autophagy in breast tumor dormancy (Lu et al, 2008) and in breast tumor-initiating or stem-like cells.

Much of the current research has been done on cell lines in the laboratory, and further *in vivo* research on animals and, pending further support from the animal studies, ultimately information from human trials is needed to put the process in a more relatable context. Because all current anti-cancer chemotherapy agents that have an effect on autophagy also target other pathways in the cell, it is difficult to decipher the complete role of autophagy stimulation in their therapeutic action (Levine and Kroemer, 2008). New drugs that specifically target the autophagic process in breast cancer cells have to be developed and tested to determine whether the positive effects of blocking a tumour cell survival pathway outweigh the potential negative effects of blocking a tumour suppressor pathway (Levine and Kroemer, 2008). There is

also an outstanding need to develop predictive markers for autophagy, particularly if therapeutic efficacy of autophagy modulation is validated in animal models. We will need to know which cancer patients will respond best to autophagy-modulating therapies. Antibodies to autophagy proteins such as Atg5 or LC3 will likely not be sufficient since the expression levels or even subcellular distribution of these proteins is not necessarily indicative of autophagy flux. We will likely need a combination of markers, including ones for autophagic substrates such as p62 (Pursiheimo et al, 2009; Bjorkoy et al, 2009), to accurately predict a functional autophagy response in a variety of human breast and other cancers. Only after more fully understanding the autophagic process in mammalian systems will we begin to comprehend how to translate progression in our knowledge of the autophagy pathway into therapeutic approaches to treating cancer.

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