Investigating the role of the inhibitor of apoptosis protein, Apollon, in the regulation of autophagy in breast cancer cells

by

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Macroautophagy (autophagy) is a lysosomal process for degrading cytoplasmic proteins and organelles for maintenance of homeostasis as well as for bioenergetic and biosynthetic needs. During nutrient deprivation and chemotherapy, both tumour-related stresses, autophagy is upregulated. The molecules and pathways involved in the regulation of autophagy in response to these stresses are still not well understood. Several recent studies have uncovered links between components of autophagy and apoptosis. As there is increasing evidence indicating that many anti-cancer therapeutics affect both autophagy and apoptosis, it is critical to identify the relationships between these pathways to develop more rational therapies.

A previous study identified several *Drosophila melanogaster* genes with autophagy-regulating functions. One of these genes was *dBruce*, a member of the inhibitor of apoptosis (IAP) gene family, which was found to negatively regulate autophagy in *Drosophila* cells *in vitro* and *in vivo*. The mammalian homologue of *dBruce*, Apollon, is overexpressed in several cancers, and Apollon knockdown has been shown to sensitize some cancer cells to chemotherapy. These findings led to the suggestion that Apollon may be a promising target for cancer therapy. As autophagy has been shown to play a role in cancer development and treatment, a link between Apollon and autophagy may have clinical implications.

My hypothesis in this study was that Apollon, the human homologue of *dBruce*, is a negative regulator of autophagy in human breast cancer cells. I tested this hypothesis using three different breast cancer cell lines, SKBR3, BT474 and MCF-7, to determine whether Apollon knockdown had an effect on autophagy under fed or starved conditions. After Apollon knockdown, MCF-7 and SKBR3 cells showed a significant increase in GFP-LC3 and/or MDC puncta under both fed and starved conditions. Further analysis in MCF-7 cells confirmed that Apollon knockdown led to an induction of the complete autophagy process as determined by two autophagy flux assays. These results show that reducing Apollon expression induces autophagy and thus Apollon is a negative regulator of autophagy in human breast cancer cell lines. Further studies will be required to determine whether Apollon knockdown-induced autophagy would be beneficial for cancer therapy.
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CHAPTER 1: INTRODUCTION

1.1 Autophagy

The term autophagy, literally self (auto) eating (phagy), was coined in the 1960’s by Christian de Duve to describe a self-degradative process in cells (De Duve, 1963). Autophagy is a process that occurs at basal levels in eukaryotic cells for the turnover of long-lived proteins and organelles to maintain homeostasis. Autophagy is also upregulated in response to cellular stresses such as starvation and chemical treatment. There are three forms of autophagy - macroautophagy, microautophagy, and chaperone-mediated autophagy – with a common theme of delivering cytoplasmic contents to lysosomes for degradation (Levine & Kroemer, 2008). The most commonly studied of these pathways is that of macroautophagy. Macroautophagy occurs when a double membrane of unknown origin, called a phagophore, forms and encompasses a portion of cytoplasm to create an autophagosome. The autophagosome can fuse with an endosome to form an amphisome. The outer membrane of the autophagosome or amphisome fuses with a lysosome to form an autolysosome. The hydrolytic enzymes from the lysosome degrade the inner membrane as well as the cytoplasmic components (Levine & Kroemer, 2008; Xie & Klionsky, 2007). This process is diagrammed in Figure 1.1. The term macroautophagy will hereafter be referred to as autophagy.
Figure 1.1 Steps in macroautophagy

Macroautophagy begins with the engulfment of cytoplasm by a phagophore. Closure of the membrane results in an autophagosome which is then able to fuse with either an endosome to first form an amphisome, or directly with a lysosome to form an autolysosome. Acid hydrolases in the lysosome break down the inner membrane and the cytoplasmic contents for release back into the cytosol for use by the cell. Figure from (Hannigan & Gorski, 2009) © 2009 Landes Bioscience.

1.1.1 Molecular mechanisms and machinery of autophagy

The process of autophagy is highly conserved among eukaryotes, from yeast to flies to mammals (reviewed in (Reggiori & Klionsky, 2002). The first genetic studies to reveal genes involved in autophagy were performed in the yeast *Saccharomyces cerevisiae* (Thumm et al., 1994; Tsukada & Ohsumi, 1993). Currently, over 30 autophagy genes have been identified in yeast (reviewed in (Klionsky, 2007) and orthologues of many of these genes have been found in various other species, including *C. elegans*, *Arabidopsis*, *Drosophila* and mammals (reviewed in (Diaz-Troya et al., 2008; Lum et al., 2005b)).

Genes that are involved in autophagy are termed Atg (autophagy-related) genes
Induction refers to the transmission of a signal, such as starvation, to the appropriate regulatory elements for autophagy to be induced. The main regulatory component is the target of rapamycin (TOR), an inhibitor of autophagy (Carrera, 2004). TOR inhibits autophagy by hyperphosphorylating Atg13 and preventing its interaction with Atg1. Signals such as starvation cause inhibition of TOR and subsequent
dephosphorylation of Atg13, which is then free to interact with Atg1 and 17 (Kamada et al., 2000) resulting in vesicle nucleation. It is still unclear how exactly the phagophore is formed, whether it is a *de novo* process or whether it forms from an existing membrane, but its formation requires several proteins. Central to phagophore formation is activation of Vps34, a class III phosphatidylinositol 3-kinase. Activation of Vps34 requires the formation of a complex consisting of beclin 1, UVRAG, and Vps15 (Zhong et al., 2009).

Elongation and completion of the autophagosome membrane requires activity of Atg9. Atg9, a highly conserved transmembrane protein, shuttles between the forming phagophore, the golgi, and endosomes (Young et al., 2006). It is unclear exactly what function Atg9 is performing during this shuttling, but it is necessary for autophagosome formation and is required for recruitment of the ubiquitin-like proteins (below) to the forming membrane.

Elongation also requires the activity of two ubiquitin-like conjugation systems. In one system, Atg12 is conjugated to Atg5 (Kametaka et al., 1996) via the E1 and E2-like enzymes Atg7 (Tanida et al., 1999) and Atg10 (Mizushima et al., 1998). The other system involves the conjugation of Atg8 (LC3) to phosphatidlyethanolamine (PE). This occurs with the help of Atg4, a protease which cleaves full-length LC3 near its C-terminus to reveal a glycine residue. This form of LC3 is referred to as LC3-I. A subsequent reaction requires the E1 and E2-like enzymes Atg7 and 3 which covalently conjugate LC3-I to PE. The resulting LC3 conjugated to PE, LC3-II, is recruited and inserted into the elongating autophagosome membrane (Ichimura et al., 2000; Kabeya et al., 2000; Tanida et al., 2004). Finally, Atg16 binds Atg12-Atg5 conjugates and facilitates the formation of a tetrameric complex of Atg12-Atg5-Atg16 conjugates (Mizushima et al., 1998).
Most of the Atg proteins involved in autophagosome formation are cytosolic and only transiently interact with the membrane, thus they can be retrieved and reused. LC3 on the outer membrane of the autophagosome is recycled via cleavage by Atg4, which cleaves off the C-terminal PE and releases LC3 back into the cytoplasm as LC3-I (Kirisako et al., 2000). Atg9, which is a transmembrane protein, also requires a retrieval system for recycling. The Atg9 retrieval system is still poorly understood but requires the activity of Atg1, Atg2 and Atg18 (Reggiori et al., 2004).

1.1.2 Methods for measuring autophagy

There are several methods for measuring autophagy. The most widely accepted method for detecting autophagy is by using a protein called MAP1LC3β (LC3 in mammals or Atg8 in yeast), which is a core autophagy protein that is inserted into the autophagosome membrane (Kabeya et al., 2000). Tagging LC3 with green fluorescent protein (GFP) or another fluorescent protein at its N-terminus provides a method for monitoring LC3 localization. In cells where autophagy has not been induced, LC3 mainly appears diffuse in the cytoplasm. However, in cells undergoing autophagy, LC3 is inserted into the membrane of forming autophagosomes and is visualized as punctate structures throughout the cytoplasm. Alternatively, autophagy can be measured by analyzing levels of LC3-I and LC3-II on a western blot. LC3-I appears at 19kDa while LC3-II appears at 17kDa. An increase in LC3-II represents an increase of autophagosomes.

Caution must be exercised when assessing both GFP-LC3 localization and LC3-I and II levels via western blot. Both assays are static measures of autophagosome numbers, but an increase in autophagosomes does not necessarily represent degradative
completion of autophagy. An increase in autophagosomes may be indicative of autophagy induction, however it is also possible that there is a block in autophagy at a later stage leading to an accumulation of this early structure.

To determine whether autophagy has been induced and gone to completion requires measuring flux through the pathway. This can be done using late stage autophagy inhibitors such as bafilomycin A1 or chloroquine in combination with measurements of LC3-II levels. If autophagy has been induced, the introduction of these inhibitors will lead to a further increase in autophagosomes while failure to cause further accumulation indicates that the observed increase in autophagosomes was due to a block in the pathway (Klionsky et al., 2008; Tanida et al., 2005).

Other methods for assessing autophagic flux are to measure p62 levels or generation of free GFP from GFP-LC3. p62 (also called sequestosome 1, or SQSTM1) contains an LC3-interacting region (Pankiv et al., 2007) which allows for its translocation to the autophagosome and subsequent degradation in the autolysosome. A decrease in p62 levels, as determined by western blot, indicates an increase in flux through the autophagy pathway, while an increase in p62 would be indicative of an inhibition of autophagy (Bjørkøy et al., 2009). Similarly, the generation of free GFP from GFP-LC3 also indicates an increase in autophagic flux as fusion of autophagosomes with lysosomes leads to the degradation of LC3 while GFP is more stable in acidic compartments and remains intact (Klionsky et al., 2008).

Monodansylcadaverine (MDC) is also a commonly used marker for autophagy (Biederbick et al., 1995). MDC stains acidic compartments, which include autolysosomes, but also other acidic compartments such as endosomes and lysosomes (Bampton et al., 2005). Under normal cellular conditions there are relatively few MDC stained puncta; however under autophagy-inducing conditions MDC staining reveals an
increase in these acidic compartments. Presumably, a large majority of these structures are autolysosomes, but ultimately MDC analysis includes all lysosomal activity and thus requires validation by a specific autophagy marker or analysis of autophagic flux.

1.2 Autophagy in cell survival and cell death

Autophagy is a catabolic process that occurs at a basal level in all cells but is upregulated in response to cell stresses such as starvation (Filkins, 1970; Mortimore & Schworer, 1977), radiation (Paglin et al., 2001) and an increase in reactive oxygen species (Scherz-Shouval et al., 2007). There is much controversy over the role of autophagy in cells under such stresses with evidence to support roles in both cell survival and cell death.

Several studies have shown autophagy to have a role in cell survival during stress. For example, Ravikumar et al. (2006) showed that autophagy inhibition sensitized a variety of cell lines to proapoptotic stimuli while autophagy induction prevented death (Ravikumar et al., 2006). Supporting this cytoprotective role is the finding that cells deficient for autophagy show decreased viability under starvation conditions (Boya et al., 2005; Kang et al., 2007). Similarly, growth factor (GF) dependent Bax/Bak-deficient mouse cells also require autophagy for survival during GF withdrawal. Autophagy knockdown during GF withdrawal led to cell death, confirming a survival role for autophagy. Lum and colleagues determined that survival of these GF dependent cells relied on the substrates generated from autophagy, such as amino acids and fatty acids, feeding into the tricarboxylic acid (TCA) cycle to generate ATP (Lum et al., 2005a).

In contrast, autophagy sometimes appears to have a role in cell death which led to the term “autophagic cell death”. This term was initially coined to refer to the observation
of autophagic vacuoles in dying cells (Schweichel & Merker, 1973). The current recommendation for using this term is to describe a specific morphology, namely cell death without chromatin condensation but with the presence of autophagic structures (Kroemer et al., 2005). It is important to realize that this does not necessarily imply that cell death is occurring via autophagy, or is dependent on autophagy, just that autophagy is observed in the dying cells. Functional studies are required to determine what role autophagy is playing in these scenarios.

One example of a study showing autophagy involvement in cell death was performed by Chen and colleagues who showed that cells exposed to oxidative stress undergo autophagy and subsequent cell death. Cells deficient for apoptosis still died in response to oxidative stress, while cells deficient for autophagy showed a decrease in cell death, suggesting that autophagy was contributing to the death process (Chen et al., 2008). Several other studies have also shown autophagy acting to promote cell death after exposure to various death stimuli (Berry & Baehrecke, 2007; Koike et al., 2008; Maiuri et al., 2007a; Puissant et al., 2010; Samara et al., 2008). The factors that decide whether autophagy promotes cell survival or cell death are still unknown, although it has been suggested that genetic background, timing, and the type of stimulus may play into the decision. For example, a paper by Wang and colleagues showed that inducing autophagy in mouse embryonic fibroblasts using various death stimuli could cause either cell survival or cell death depending on the stimuli (Wang et al., 2008).
1.3 Autophagy and cancer

Autophagy is an important catabolic process in all cells and thus it is not surprising that it has been implicated in many diseases such as neurodegeneration and cancer. Given the previous description of autophagy acting as both a survival and a cell death mechanism, it is possible that autophagy may be beneficial but also problematic with regards to cancer. A tremendous amount of research has been conducted to sort out these opposing roles of autophagy in cancer and so far the consensus appears to be that autophagy is protective prior to cancer development but tumour promoting in the early stages of tumour development and plays a varying role with regards to cancer treatment (reviewed in Hippert et al., 2006; Hoyer-Hansen & Jaattela, 2008; Levine & Kroemer, 2008; Mathew et al., 2007a; Ogier-Denis & Codogno, 2003).

Autophagy may help to prevent normal cells from becoming cancerous. Autophagy maintains the integrity of normal cells by removing damaged proteins and organelles that may otherwise lead to the production of reactive oxygen species. This role is supported by the finding that mice heterozygous for Beclin 1 exhibited a higher rate of spontaneous tumour development that correlated with a decrease in autophagy (Yue et al., 2003). In addition, several genes involved in autophagy are found in chromosomal regions that are frequently mutated in cancers (S. Yousefi & Simon, 2007). These observations suggest that autophagy may be protective, and loss of autophagy may promote tumorigenesis. There are three mechanistic suggestions to explain this protective role of autophagy. The first is that when cells first lose the ability to die by apoptosis in response to metabolic stresses, autophagy may allow these cells to survive. When autophagy is lost, these cells would be more likely to undergo necrosis which would increase local inflammation and may promote tumour growth (Degenhardt et al., 2006).
The second suggestion is that loss of autophagy may lead to genomic instability in cells under metabolic stress resulting in activation of oncogenes (Mathew et al., 2007b).

Thirdly, autophagy may have a more direct role in cell growth. For example, expression of Beclin1, a gene required for autophagy, leads to a decrease in some cell growth genes (Liang et al., 1999), while knocking down Beclin1, and thus inhibiting autophagy, causes an increase in proliferation and thus may contribute to the uncontrolled growth observed in tumours (Qu et al., 2003).

Conversely, autophagy appears to promote cancer progression (Degenhardt et al., 2006). In these instances autophagy may be acting as a survival mechanism for cancer cells allowing them to survive the harsh conditions of the tumour microenvironment, such as lack of oxygen and nutrients. There is also evidence of many anti-cancer therapies causing an accumulation of autophagosomes (Maiuri et al., 2007a). Several studies have shown that knocking down autophagy actually causes the therapies to be more effective, supporting a protective or survival role for autophagy (Abedin et al., 2007; Amaravadi et al., 2007; Bellodi et al., 2009; Boya et al., 2005; Li et al., 2009; Maiuri et al., 2007a; Qadir et al., 2008; Samaddar et al., 2008).

In other cases, when autophagy is acting to promote death, autophagy induction may be useful as a therapeutic strategy (Bergmann, 2007; Hoyer-Hansen & Jaattela, 2008; Rubinsztein et al., 2007). Already some studies have shown that inducers of autophagy can be used to treat cancer by causing cancer cells to undergo autophagy-dependent cell death. For example, Concavalin A, a lectin from Jack bean seeds (Chang et al., 2007), curcumin, the active ingredient in turmeric (Aoki et al., 2007), and resveratrol, a phytoalexin found in grapes (Puissant et al., 2010), have therapeutic potential and appear to be cytotoxic due to their ability to induce autophagic cell death.
As discussed previously, there are likely several factors determining whether autophagy promotes cell survival or cell death. This is important to consider with regards to cancer therapy as several chemotherapeutics have been shown to induce autophagy. In some instances, therapy may be enhanced by inhibiting autophagy, while others could benefit from inducing autophagy.

1.4 Apoptosis and cancer

Apoptosis, also referred to as type I programmed cell death, is a genetically regulated form of cell death that is involved in many physiological and pathological processes. Apoptotic cell death is morphologically distinct from other cell death processes and is characterized by membrane blebbing, cell shrinkage, chromatin condensation, DNA fragmentation (Kerr et al., 1972), and phosphatidylserine exposure (Fadok et al., 1992). The resulting apoptotic bodies are eventually cleared by neighboring cells or macrophages (reviewed in (Renehan et al., 2001)). There are two main apoptotic pathways: the intrinsic (mitochondrial) pathway that responds to death signals from within the cell, and the extrinsic pathway that involves signaling from death receptors on the surface of the cell. Both pathways result in the activation of a caspase cascade and ultimately the cleavage of various cytoplasmic and nuclear substrates (reviewed in (Cohen, 1997).

As uncontrolled apoptosis could have detrimental effects at both the cellular and organismal level, the apoptotic process is tightly regulated. For example, caspases are synthesized as inactive zymogens which require cleavage for activation. In addition, there are several upstream regulators of caspases, including the inhibitor of apoptosis proteins.
(IAPs) which are able to bind and inhibit caspases and some pro-apoptotic mitochondrial molecules such as Smac and HtrA2 (Hao et al., 2004; Sekine et al., 2005) (reviewed in (Degterev et al., 2003; LaCasse et al., 1998)).

Apoptosis is a normal response to cell stresses such as DNA damage, hypoxia, starvation, and oncogene activation (Evan & Littlewood, 1998). Such stresses are commonly encountered by cancer cells in the early stages of tumourigenesis, before the tumour has developed a vascular network. The ability of these early tumour cells to resist apoptosis, and thus continue to grow, is a hallmark of cancer (Hanahan & Weinberg, 2000). Evasion of apoptosis can be achieved through various means including loss-of-function mutations or decreased expression of pro-apoptotic molecules and gain-of-function mutations or increased expression of anti-apoptotic molecules (reviewed in (Degterev et al., 2003)). For example, BAX, a proapoptotic bcl-2 family member that promotes mitochondrial membrane permeabilization, is inactivated by a frameshift mutation in over half of colon and gastric cancers, a mutation which contributes to tumour progression (Ionov et al., 2000). Similarly, anti-apoptotic bcl-2 is overexpressed in some breast cancers and contributes to the metastatic potential of the disease ((Del Bufalo et al., 1997)). Discovery of the various mechanisms used by cancer cells to evade apoptosis provides potential targets for the design of novel anticancer therapeutics as well as the potential for use as molecular markers for predicting treatment response (Degterev et al., 2003; Ferreira et al., 2002).
1.5 The inhibitor of apoptosis protein family

The inhibitor of apoptosis (IAP) protein family is a highly conserved group of proteins (Duckett et al., 1996; Uren et al., 1996) that contain at least one baculoviral IAP repeat (BIR) domain. In mammals, eight IAP family members have been identified: Neuronal apoptosis inhibitory protein (NAIP), X-linked IAP (XIAP), cellular IAP1 and 2 (cIAP1 and 2), Testis-specific IAP (Ts-IAP), Apollon, Survivin, and Livin (reviewed in (Hunter et al., 2007; LaCasse et al., 1998).

Expression of IAPs is an effective way to inhibit apoptosis, the mechanism of which was initially discovered by Deveraux et al. (1997). Their initial studies showed that XIAP is able to bind caspase-3 and -7, and further investigation showed that XIAP was able to prevent processing of caspase-3 into its mature subunits (Deveraux et al., 1997). Subsequent in vitro studies showed that XIAP, cIAP1 and cIAP2 are able to inhibit processing of pro-caspase-3, -6, and -7 (Deveraux et al., 1998). Caspase-9 has also been shown to be a target of some IAPs, such as XIAP, cIAP1, cIAP2 (Deveraux et al., 1998), Livin (Vucic et al., 2000), Ts-IAP (Lagace et al., 2001), and Apollon (Hao et al., 2004; Qiu & Goldberg, 2005). Many of the IAPs contain E3 ligase activity which, in combination with an E2 enzyme, allows them to attach ubiquitin to substrates. This E3 ligase activity can be used for ubiquitination of substrates and their subsequent degradation by the proteasome (Hao et al., 2004; Suzuki et al., 2001; Yang et al., 2000).

As IAPs play a critical role in regulating apoptosis, it is necessary that they themselves are under tight regulation. A careful balance of IAPs is maintained by negative regulators which are able to negate the caspase-inhibitory functions of IAPs and thus allow cell death to occur. There are several proteins that play a role in this negative regulation such as XIAP-associated factor 1 (XAF1) (Liston et al., 2001), Smac/DIABLO
(Verhagen et al., 2000), Omi/HtrA2 (Hedge et al., 2002), and other IAP (Verhagen et al., 2007) binding motif (IBM) containing proteins. Figure 1.3 illustrates the role of IAPs and their antagonists in the apoptotic pathway.

Figure 1.3 The role of IAPs and their antagonists in the apoptotic pathway

As overexpression of IAPs, which is quite common in cancer cell lines and primary tumor biopsy samples (Ambrosini et al., 1999; Fong et al., 2000; Li et al., 2001; Tamm et al., 2000), could lead to increased resistance to apoptosis, it is not surprising that IAPs have become targets for anti-cancer therapies. To date, several IAP antagonistic
therapies have been investigated with promising results. One potential therapy is the use of anti-sense oligonucleotides to various IAPs (Ansell et al., 2004; Hu et al., 2003; Lima et al., 2004; McManus et al., 2004; Tu et al., 2003). Much research has also been done investigating the use of negative IAP regulators as therapeutics. For example, over-expression of XAF1 (Leaman et al., 2002; Qi et al., 2007), Smac-like peptides (Sun et al., 2006), other IAP small molecule antagonists and dominant-negative adenoviral expression mutants (reviewed in (LaCasse et al., 2007) have also shown possibility as cancer therapeutics.

1.6 Apollon

Apollon (BIRC6/BRUCE) is a 528 kDa member of the IAP family. Apollon is a membrane-bound protein that localizes primarily to the trans-golgi network and other vesicular structures (Hauser et al., 1998). Apollon contains an amino-terminal baculoviral IAP repeat (BIR) domain and a carboxy-terminal ubiquitin-conjugating enzyme (UBC) domain (Bartke et al., 2004) as shown in Figure 1.4.

![Figure 1.4 Domain structure of Apollon](image)

The total size of Apollon is 4,829 amino acids (528kDa), with two functionally validated domains: an N-terminal BIR domain and a C-terminal UBC domain.

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The BIR domain is responsible for Apollon’s interactions with pro-apoptotic caspase-9, Smac/DIABLO (Hao et al., 2004; Qiu & Goldberg, 2005) and HtrA2/Omi (Sekine et al., 2005). The UBC domain is a characteristic of ubiquitin-conjugating (E2) enzymes. This is a highly conserved domain of 150-200 amino acids that is used for binding activating enzymes (E1s), protein ligases (E3s) and ubiquitin or ubiquitin like proteins (Burroughs et al., 2008). Apollon is a unique member of this family as it does not require the presence of an E3 enzyme for ubiquitination of substrates. This has led to the categorization of Apollon as a chimeric E2/E3 ubiquitin ligase (Bartke et al., 2004). Both the BIR and UBC domains are required for Apollon’s anti-apoptotic activity. The BIR domain allows Apollon to bind caspase-9, Smac/DIABLO and HtrA2, while its UBC domain facilitates their ubiquitination and ultimately degradation by the proteasome (Hao et al., 2004; Qiu & Goldberg, 2005). Apollon itself is also regulated by ubiquitination-dependent degradation via the E2 UbcH5 and E3 Nrdp1 (Qiu et al., 2004). IAP regulators, such as Smac/DIABLO and Omi/HtrA2 also negatively regulate Apollon. These interactions are illustrated in Figure 1.3.

Apollon has been shown to play a role in a variety of cellular processes. Apollon was first discovered in mice, where it was found that deletion of the UBC containing C-terminal portion led to massive apoptosis and embryonic lethality (Ren et al., 2005). Apollon has also been shown to have some functions outside of apoptosis inhibition. For example, another study in mice showed Apollon to be required for placental development which appeared to be unrelated to cell death and rather connected to insufficient differentiation (Lotz et al., 2004). Apollon has also been shown to have a role in cytokinesis, the final stage of mitosis (Pohl & Jentsch, 2008). Pohl and Jentsch showed Apollon to be required for formation of the midbody ring, a structure contained in the intercellular bridge joining the two daughter cells just before separation. Apollon was
found to act as a type of platform for recruitment of membrane delivery proteins and mitotic regulators. Loss of Apollon led to defective formation of the midbody ring and disruption of ubiquitin localization to this area. Similarly, Apollon has been shown to be involved in the control of cell division in neural progenitor cells (Sippel et al., 2009).

Overexpression of Apollon has been observed in some drug resistant brain cancers (Chen et al., 1999) and has been associated with a poor prognosis in childhood acute myeloid leukemia (Sung et al., 2007). These findings led to research investigating the effect of knocking down Apollon expression in cancer cells. Chen and colleagues showed that Apollon specific antisense oligonucleotides were able to sensitize brain cancer cells to cisplatin and camptotheacin treatment (Chen et al., 1999). Similarly, Chu et al. assessed the effect of adenovirus-mediated shRNA against Apollon in tumour cells treated with 5-fluorouracil (5-FU). They showed that knocking down Apollon caused an inhibition of tumor growth and enhanced the effect of 5-FU as a chemotherapeutic agent both in vitro and in vivo (Chu et al., 2008). A third study by Lopergolo et al. showed that knocking down Apollon in breast cancer cells using siRNA induces apoptosis through stabilization of p53 and activation of caspase-3 (Lopergolo et al., 2009). These findings have led to the suggestion that Apollon may be a promising target for cancer therapy.

1.7 Autophagy and apoptosis

In many situations, autophagy and apoptosis are shown to occur either sequentially or simultaneously. Three different relationships between these two pathways have been identified (reviewed in (Eisenberg-Lerner et al., 2009)). Autophagy may 1) co-operate
with apoptosis to promote death, 2) antagonize apoptosis by promoting survival, or 3) enable apoptosis by participating in one or more stages of apoptosis.

Several studies have shown autophagy to be induced alongside apoptosis, and act to contribute to cell death. For example, agents such as etoposide (Feng et al., 2005), ceramide (Pattingre et al., 2009), and the activation of TRAIL receptor-2 (Park et al., 2007) have been shown to be able to induce both apoptosis and autophagy resulting in cell death. Cooperation of these pathways can require both pathways to be active for cell death to occur, or it may refer to the ability of autophagy to compensate for the loss of apoptosis and promote cell death and vice versa.

Conversely, many studies have shown autophagy to act in opposition to apoptosis to promote cell survival (Maiuri et al., 2007a; Thorburn, 2008). For example, after mitochondrial membrane permeabilization and cytochrome c release, activities that generally lead to an apoptotic response, autophagy can allow cells to recover and grow rather than die (Colell et al., 2007). Similarly, autophagy has been shown to protect cells from apoptosis during ER and metabolic stress (Karantza-Wadsworth et al., 2007; Ogata et al., 2006), nutrient deprivation (Boya et al., 2005), drug treatment (Amaravadi et al., 2007; Qadir et al., 2008), and radiation (Paglin et al., 2001). In this type of scenario, inhibition of autophagy promotes cell death.

Finally, autophagy may act to enhance apoptosis by contributing to various stages or features of the apoptotic pathway. For example, some features of apoptosis, such as membrane blebbing and phosphatidylserine exposure are ATP-dependent processes (Inbal et al., 2002; Qu et al., 2007). Autophagy may contribute to the execution of these energy-dependent events by providing ATP (Eisenberg-Lerner et al., 2009).

It is important to determine which type of relationship exists between apoptosis and autophagy, especially in the context of cancer treatment. For example, if both
pathways are being induced by a cancer therapy and yet are acting in opposition, it may be beneficial to inhibit autophagy in such a setting. However, if apoptosis and autophagy are both acting to promote death, then autophagy inhibition would not be a beneficial treatment strategy.

While the specific molecular areas of crosstalk between apoptosis and autophagy are still largely unknown, some common regulators and genes shared by both pathways have been identified. mTOR is a key inhibitor of autophagy, however several factors upstream of mTOR have been shown to have functions in other cell survival and death pathways (such as Akt and ERK) (reviewed in (Ballif & Blenis, 2001; McCubrey et al., 2007; Song et al., 2007). Atg5, which is required for autophagy, can be cleaved and function to induce apoptosis (Yousefi et al., 2006). Similarly, bcl-2 family proteins, which are inhibitors of apoptosis, and p53, an activator of apoptosis, also have autophagy regulating functions (Crighton et al., 2006; Pattingre et al., 2005; Tasdemir et al., 2008b). Other components of the apoptotic pathway have also been shown to have autophagy regulating functions, such as tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) (Mills et al., 2004), Fas-associated death domain (FADD) (Thorburn et al., 2005), death-associated protein kinase (DAPk) and DAPk-related protein kinase (DRP-1) (Inbal et al., 2002). This list will likely continue to grow as more research goes into deciphering the complex relationship between apoptosis and autophagy.

1.8 Breast cancer, autophagy, and Apollon

Breast cancer is the most common incident cancer and leading cause of cancer-related death in women worldwide (World Cancer Report, 2008). Breast cancers are subdivided
into five distinct subtypes: luminal A and B, basal-like, epidermal growth factor receptor 2 (or Her-2)-associated, and normal-like (reviewed in (Kapp et al., 2006)). Subtypes are identified based on various markers including the estrogen receptor (ER), epidermal growth factor receptors (EGFR and Her-2), and the breast cancer susceptibility gene 1 (BRCA1) (reviewed in (Giancotti, 2006)). Classification of breast cancers into one of these five subtypes is used to predict which therapy options are likely to be effective for a particular patient.

Over 40% of human breast cancers demonstrate deletions of at least one allele of Beclin 1 (Aita et al., 1999), a core autophagy gene, and human tumours show decreased expression of Beclin 1 relative to the adjacent normal breast tissue (Liang et al., 1999). It has been shown that Beclin 1 can suppress MCF-7-induced tumorigenesis in nude mice (Liang et al., 1999). Beclin 1 knockout mice showed an increased incidence of lymphomas, liver and lung cancers, and breast hyperplasia (Qu et al., 2003 and Yue et al., 2003). Autophagy also appears to play a role with regards to breast cancer treatment. For example, Tamoxifen, an anti-estrogen used to treat ER+ breast cancers, induces autophagy in MCF-7 breast cancer cells. Qadir et al. (2008) and Samaddar et al (2008) showed that knocking down core autophagy genes increases the efficacy of Tamoxifen treatment, while John et al. (2008) showed that overexpression of Beclin 1 increases Tamoxifen resistance in MCF-7 breast cancer cells. Together these studies suggest that autophagy plays an important role in the therapeutic response of breast cancer cells to tamoxifen. Additional anti-cancer agents used to treat breast cancer, such as gefitinib and lapatinib (Cheng et al., 2010), trastuzumab (Vazquez-Martin et al., 2009), camptothecin, EB1089, and vincristine (reviewed in Armstrong and Gorski, 2010) were also shown to alter autophagy levels in breast cancer cells, suggesting that autophagy may have widespread implications for the treatment of breast cancer.
Apollon was implicated recently in the survival of multiple breast cancer cell lines (Lopergolo et al., 2009 and Chu et al., 2008). Two independent studies showed that knocking down Apollon by RNA interference can induce apoptosis in MCF-7, MDA-MB-231 and ZR75.1 breast cancer cell lines (Lopergolo et al., 2009 and Chu et al., 2008) and also sensitizes MCF-7 cells to 5-fluorouracil treatment (Chu et al., 2008). These studies indicate that Apollon warrants further investigation as a potential target for breast cancer therapy.

1.9 Objectives and hypothesis

1.9.1 Rationale

Several common regulators and areas of cross-talk have been identified between apoptosis and autophagy (Matsui et al., 2007; Mills et al., 2004; Pattingre et al., 2005; Yousefi et al., 2006; Yousefi & Simon, 2007) although the extent of this relationship is still largely unclear. As both processes have been implicated in cancer development, progression and treatment, it is important to learn more about the common regulators and interplay between these two pathways in order to better understand cancer and develop more effective therapies.

An RNAi screen in \textit{Drosophila melanogaster} identified Bruce, the fly homologue of Apollon, as a negative regulator of autophagy (Hou et al., 2008). In humans, overexpression of Apollon has been observed in some drug resistant brain cancers (Chen et al., 1999) and has been associated with a poor prognosis in childhood acute myeloid leukemia (Sung et al., 2007). In addition, decreasing Apollon expression can sensitize
some cancer cells to apoptosis (Chen et al., 1999; Chu et al., 2008; Lopergolo et al., 2009). Taken together, these findings led to the suggestion that Apollon may be a promising target for cancer therapy. If such an approach is to be investigated clinically, it is important to determine whether Apollon is also a regulator of autophagy in humans. If it is a regulator of autophagy, it will be valuable to know whether autophagy is acting to promote death or survival in various cancer types and stages and determine whether autophagy should thus be inhibited or promoted during Apollon knockdown to more effectively induce cancer cell death.

1.9.2 Hypothesis and specific aim

**Hypothesis:** Given that Bruce is a negative regulator of autophagy in *Drosophila* (Hou et al., 2008), I hypothesize that the human homologue, Apollon, is a negative regulator of autophagy in human breast cancer cell lines.

**Specific Aim:** Determine whether knockdown of Apollon expression in human breast cancer cells affects their ability to undergo starvation-induced autophagy.

1) Apollon expression was knocked down using siRNA in three different breast cancer cell lines: MCF-7, SKBR3 and BT474. 2) The effect of Apollon knockdown on cell growth and viability was measured. 3) To facilitate autophagy analysis, a stable GFP-LC3 SKBR3 cell line was created. 4) The ability of cells to undergo starvation-induced autophagy was assessed using MDC staining, GFP-LC3 localization, generation of free GFP and/or p62 degradation.
CHAPTER 2: MATERIALS AND METHODS

2.1 Cell culture and autophagy induction

Three human breast carcinoma cell lines were used in experiments: SKBR3 (ER-, Her2+) and BT474 (ER+, Her2+) from Dr. M. Bally (BC Cancer Research Centre) (originally from ATCC), and MCF-7 (ER+,Her2-) cells stably transfected with GFP-LC3 were provided by Dr. M. Roberge (University of British Columbia). All cell lines were grown in DMEM (Gibco) supplemented with 10% FBS (Gibco), 20mM HEPES (Gibco), 1X non-essential amino acids (Gibco), and 10 μg/ml insulin (Sigma-Aldrich). MCF-7 GFP-LC3 growth media was also supplemented with 500 μg/ml G418 for maintaining selection of cells containing the GFP-LC3 plasmid. Cells were maintained at 37 degrees Celsius with 5% CO2 and 95% air humidity.

Autophagy was induced by starvation in Earle’s Balanced Salt Solution (EBSS) (Gibco-Invitrogen) for 2h. Fed controls were treated with EBSS supplemented with 10% FBS (Gibco), 1X NEAA (Gibco), and 2 mM L-glutamine (Gibco). Autophagy was also induced with rapamycin (Sigma) in MCF-7 GFP-LC3 cells. Cells were treated for 4h with 20nM rapamycin diluted in EBSS.
2.2 Construction and sequencing of hrGFP-LC3 plasmid

Human Mammalian Gene Collection (MGC) verified full-length MAP1LC3β cDNA (Open Biosystems, MHS1010-97228175) was PCR amplified using primers with added EcoRI and BamHI sequences for cloning:

Forward primer: 5’ –CCGTCGGAGAAGACCTTC- 3’

Reverse primer: 5’ –GGTTTTACACTGACAATTTCATCC- 3’

The PCR product was cloned into phrGFP II-N (Stratagene) between the EcoRI and BamHI sites of the multiple cloning site (MCS) to generate an N-terminal hrGFP fusion protein. The construct was verified by sequencing using the following primers:

Forward primer: 5’ – GAGTACCACTTCATCCAGCA – 3’

Reverse primer: 5’ – AAGGACAGTGAGAGTGGCAC – 3’

Cycle sequencing was carried out using the BigDye Terminator reagents (Applied Biosystems).

2.3 Creation of stable SKBR3 hrGFP-LC3 cell line

The day before transfection, SKBR3 cells were plated at 10,000 cells/well in 6-well plates. 5.6 µg of hrGFP-LC3 was mixed with Lipofectamine2000™ (Invitrogen) in a total of 700 µl OPTI-MEM (Invitrogen) for 20 minutes at room temperature. The mixture was then applied to cells at 100 µl per well. After 24h cells were passaged at 1000 cells/well into 96-well plates. The following day, G418 (Gibco) was added for a final concentration of 1mg/ml to begin selection. Cells were grown under selection and expanded to generate stable polyclonal cell lines. Stable lines were maintained in 500 µg/ml G418.
2.4 Short-interfering RNA (siRNA)

One day prior to transfection BT474, MCF-7 GFP-LC3 and SKBR3 cells were plated at 10,000, 4,000 and 8,000 cells/well respectively in 96-well plates. Correspondingly, 3pmol, 5pmol and 3pmol of the appropriate chemically modified Stealth™ siRNA oligonucleotides (see Table 2.1) (Invitrogen) were transfected into cells for 72 h using Lipofectamine RNAiMAX™ (Invitrogen) as per manufacturer’s recommendations. A medium GC content negative control (scramble) siRNA was used in all experiments (Invitrogen, 12935-112). Two non-overlapping siRNA duplexes were used for Apollon knockdown, referred to as Ap1 and Ap2. A Beclin 1 (BecD) siRNA was used as a positive control in some experiments. The sequences of the siRNA duplexes used for knockdown are listed in Table 2.1:

<table>
<thead>
<tr>
<th>Table 2.1 siRNA sequences used in this study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ap1</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Ap2</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>BecD</td>
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To ensure that the siRNA treatment was not causing an interferon response, mRNA expression of oligoadenylate synthetase (OAS) and dsRNA-activated serine/threonine protein kinase (PKR) were also assessed by qRT-PCR as detailed below.
2.5 RNA isolation, cDNA preparation, and quantitative real-time PCR

Three days after siRNA treatment, RNA was collected from cells and purified using the RNeasy Plus Kit (Qiagen) as per manufacturers instructions. cDNA was reverse-transcribed in the presence of oligo-dT using Superscript III Reverse Transcriptase (Invitrogen). 100 ng of total RNA was used for first strand cDNA synthesis. Real time quantitative PCR was performed using Platinum SYBR Green qPCR SuperMix UDG with ROX (Invitrogen) as per manufacturer’s directions. Triplicate 10 µl reactions were carried out using 1 µl of cDNA each. The PCR reaction was performed using the ABI 7900 HT-Fast Real-Time PCR system (Applied Biosystems). mRNA expression was assessed using the primer pairs listed in Table 2.2:

<table>
<thead>
<tr>
<th>Primer pairs</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apollon-F</td>
<td>5′-AACCCTTACATTGGAGGTCTG-3′</td>
</tr>
<tr>
<td>Apollon-R</td>
<td>5′-GTTCTGCTTGCTGTTCAATGC-3′</td>
</tr>
<tr>
<td>Becn1-F</td>
<td>5′-GGAGAGGGAGCCATTATTGAAA-3′</td>
</tr>
<tr>
<td>Becn1-R</td>
<td>5′-AGAGTGAAGCTGTTGGCACTTT-3′</td>
</tr>
<tr>
<td>ActB-F</td>
<td>5′-CTGGAACGGTGAAGGTGACA-3′</td>
</tr>
<tr>
<td>ActB-R</td>
<td>5′-AAGGGACTTCCTGTAACAAATGCA-3′</td>
</tr>
<tr>
<td>OAS-F</td>
<td>5′-GCCATTGACATCATCTGTGG-3′</td>
</tr>
<tr>
<td>OAS-R</td>
<td>5′-AGGAAGACAACCAGGTCAGC-3′</td>
</tr>
<tr>
<td>PKR-F</td>
<td>5′-TCCACACTTCCGTGATTATCTG-3′</td>
</tr>
<tr>
<td>PKR-R</td>
<td>5′-TACTCCCTGCTTCTGACGGTAT-3′</td>
</tr>
</tbody>
</table>

A fragment from β-actin was amplified as the standard for the reaction. qRT-PCR data was analyzed using Q-Gene, an Excel-based software package (Visual Basic for
Applications) (Muller et al., 2002). One advantage of using qGene is that it does not require that amplification efficiencies of each primer set be equal. Instead, standard curves are generated for each primer pair using serial dilutions of cDNA to determine PCR amplification efficiency and these values are considered in further equations. The standard curve is plotted on a graph with log(cDNA) on the x-axis and mean C_T value on the y-axis. The slope for the line of best fit of each amplification curve is used to calculate E_ref and E_target (PCR amplification efficiency of reference and target genes respectively) as follows:

$$E_{\text{ref}} \text{ or } E_{\text{target}} = 10^{(-1/\text{slope})}$$

The software then calculates the normalized gene expression using the following calculation:

$$E_{\text{target}}^{(C_T \text{target})}/ E_{\text{ref}}^{(C_T \text{ref})}$$

The normalized gene expression from each reaction is then used to calculate the mean normalized expression and standard error for each set of triplicate samples. The mean normalized expression was later used for calculating percent knockdown after siRNA treatment by comparing expression in individual siRNA treated samples to expression in the scramble siRNA treated controls.

2.6 Protein extraction and western blot analysis

Protein was extracted from treated cells using the RIPA Lysis Buffer Kit (Santa-Cruz). Protein concentrations were determined using the BCA protein assay (Pierce). For analysis of p62, free GFP, and actin, 40 µg of protein were loaded on 12% NuPAGE bis-tris gels (Invitrogen) and run for 1h at 200V in 1X MES buffer (Invitrogen). Protein was
transferred to a PVDF membrane (Invitrogen) using the iBlot dry-blotting system (Invitrogen) and processed according to standard protocol for the Odyssey system (LI-COR Biosciences). Anti-p62 (1:200; Santa-Cruz), anti-GFP (1:1000; Clontech), and anti-actin (1:10,000; Abcam) were incubated overnight at 4 degrees Celsius. Anti-mouse IR800 (Rockland Immunochemicals) and anti-rabbit IR700 (Rockland Immunochemicals) were incubated for 1h at room temperature. Membranes were then imaged using the Odyssey Infrared Imaging System (Li-COR Biosciences). Quantitation was done by first normalizing p62 or GFP to the actin loading control, then consolidating three individual experiments to calculate an average and standard error. Significance was determined using the Student’s t-test.

For analysis of Apollon, 40 µg of protein from MCF-7 GFP-LC3 and SKBR3 and 60 µg from BT474 were loaded on 3-8% NuPAGE tris-acetate gels (Invitrogen) and run for 2.5h at 80V in tris-acetate running buffer (Invitrogen). Protein was transferred to PVDF membrane (Millipore) by standard wet-transfer for 2h at 100V. After blocking in skim milk, membranes were probed with anti-Apollon (1:250; BD Biosciences) overnight at 4 degrees Celsius. Anti-mouse IgG conjugated HRP was incubated for 1h at room temperature. The membrane was treated as per manufacturer’s instructions for the Amersham Biosciences Enhanced Chemiluminescence (ECL) Detection System (GE Healthcare) and the signal was detected with CL-Xposure film (Thermo Scientific). Membranes were then reblocked using Odyssey blocking buffer and probed with anti-ActB (1:10,000; Abcam) as a loading control followed by incubation with anti-rabbit IR700 (Rockland Immunochemicals) secondary for 1h at room temperature. Membranes were then imaged using the Odyssey Infrared Imaging System (Li-COR Biosciences).
2.7 Cell growth assay

For comparison of SKBR3 parental and transfected cells, SKBR3 and SKBR3 GFP-LC3 cells were plated in multiple optical plates at 8000 cells per well. A single plate was stained each consecutive day for 4 days with 4 µM Hoechst 33342 (Invitrogen) and 0.5 µM ethidium homodimer (Invitrogen) and the number of cells was counted using high-throughput fluorescence microscopy (described in detail in the following section).

For analysis of the effect of Apollon siRNA on growth of SKBR3 and MCF-7 GFP-LC3, cells were plated in multiple optical plates at 8000 and 3500 cells per well respectively. The following day one plate was stained with 4 µM Hoechst 33342 and 0.5 µM ethidium homodimer and subsequently analyzed on the InCell Analyzer. The remaining plates were treated with scramble and Apollon siRNA (as described in section 2.4) and the following three days a single plate was again stained and viewed on the InCell Analyzer (described in detail in the following section).

2.8 Fluorescence microscopy

High-throughput fluorescence microscopy was performed on the InCell Analyzer 1000 (GE Healthcare). 30 minutes prior to imaging, cells were fluorescently stained. When MDC was used for analysis, cells were stained with final concentrations of 3 µM DRAQ5 nuclear stain (Biostatus), 0.1 mM MDC (Sigma-Aldrich), and 0.5 µg/ml ethidium homodimer (Invitrogen). When GFP-LC3 stably transfected cells were used for autophagy analysis, cells were stained with 4 µM Hoechst 33342 (Invitrogen) and 0.5 µM ethidium homodimer (EthD1).
Image analysis was performed using InCell Developer Toolbox (Ver. 1.6, GE Healthcare). Individual cells were first identified with segmentation of nuclei based on DRAQ5 or Hoechst 33342 staining. Cell borders were defined by defining a radius around the nucleus. Cells were classified as “dead” if EthD1 staining was found to have more than 50% overlap with the cell compartment. The number of MDC or GFP-LC3 puncta within each live cell was then counted and the number of live cells with at least one, more than 3, more than 5, and more than 8 puncta was quantified. Puncta were defined as MDC or GFP positive structures of at least 1 pixel with a form factor (estimate of circularity) of at least 0.4.

For viability assays, the percent of cells dead was calculated as follows:

\[
\frac{\text{(# EthD1 stained nuclei)}}{\text{(# Hoechst stained nuclei)} + \text{(# EthD1 stained nuclei)} - \text{(# overlapping EthD1 and Hoechst stained nuclei)}}
\]

For the cell growth assays, individual cells were identified and counted based on segmentation of Hoechst 33342 stained nuclei. The number of cells counted at the first time point (24h after plating for SKBR3 and SKBR3 GFP-LC3 growth curves and at the time of siRNA treatment for analysis of the effect of Apollon knockdown on growth of MCF-7 GFP-LC3 and SKBR3) was used as the baseline for each experiment. The fold increase in the number of cells counted at each subsequent time-point was calculated and these values were plotted against time to generate a growth curve.

2.9 Statistical analyses

For analyses involving the comparison of only two means, specifically analysis of starvation responses and comparison of SKBR3 and SKBR3 GFP-LC3 growth, a two-
tailed student’s t-test (equal variances) was used to compare means between the fed and starved samples. For all other analyses which involved the comparison of means of several treatments to the mean of the control treatment, one-way ANOVA with Dunnett’s post test was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). In both cases, differences between means were considered significant if the resulting p-value was less than 0.05.
CHAPTER 3: RESULTS

3.1 Autophagy is upregulated in MCF-7 GFP-LC3, SKBR3 and BT474 breast cancer cells during starvation

To determine a potential regulatory role for Apollon in autophagy, it was necessary to first identify cell lines of interest which undergo autophagy. As starvation is known to be a potent inducer of autophagy (Aubert et al., 1996; Codogno et al., 1997) I analyzed various cell lines for their ability to undergo starvation-induced autophagy. MCF-7 cells stably transfected with GFP-LC3 (MCF-7 GFP-LC3), SKBR3 and BT474 breast cancer cell lines were subjected to two hours of complete starvation (see Materials and Methods) after which autophagy was assessed using MDC staining or GFP-LC3 localization (Figure 3.1). Fed and starved samples were imaged using the InCell Analyzer after which images were analyzed and MDC or GFP-LC3 puncta per cell were quantified using the InCell Developer Toolbox.

As the level of basal autophagy and/or method of detection in each cell line varied, different thresholds were chosen for indicating autophagy positive cells in each line. MCF-7 GFP-LC3 cells showed the greatest difference between control and starved conditions when analyzed for more than five GFP puncta per cell, while SKBR3 and BT474 cells both showed a consistent difference between control and starved conditions when assessed for more than three MDC puncta per cell (Figure 3.1).
Figure 3.1 Two hour starvation causes an increase in MDC or GFP-LC3 puncta in MCF-7 GFP-LC3, SKBR3 and BT474 breast cancer cell lines

Cells were subjected to two hours of complete starvation after which autophagy was assessed using MDC staining or GFP-LC3 localization. MCF-7 GFP-LC3: Hoechst 33342 staining shown in blue, GFP-LC3 shown in green. SKBR3 and BT474: DRAQ5 staining shown in blue, MDC staining shown in green. Reported values are mean ± SE of triplicate samples from one representative experiment of at least three independent experiments. *P-value<0.02
In order to assess autophagy specifically via LC3 localization in more than one cell line, an N-terminal humanized GFP II (hrGFP) tagged human MAP1LC3β construct was created and sequenced (Figure 3.2).

**Figure 3.2 hrGFP-LC3 construct**
Vitality® phrGFP II-N Mammalian Expression Vector from Stratagene-Agilent Technologies with insert of human MAP1LC3β indicated.

SKBR3 cells were stably transfected with the hrGFP-LC3 construct and two stable polyclonal lines were generated. One line was used for further analysis and was assessed for changes in morphology, growth rate and starvation response (Figure 3.3). The stably transfected cell line maintained the morphology and growth rate of the parental SKBR3 cells (Figure 3.3a and b) as well as the ability to undergo starvation-induced autophagy (Figure 3.3c).
Fed Starved

Percent of live cells with >5 GFP-LC3 puncta

Fed Starved

DRAQ5 MDC GFP-LC3

Fold change in cell numbers

Time after plating (hrs)

Fold change in cell numbers

Fed Starved

Percent of live cells with >3 MDC puncta

Percent of live cells with >5 GFP-LC3 puncta

Fed Starved

35
Figure 3.3 Characterization of SKBR3 hrGFP-LC3

a) Bright field images showing the morphology of the parental SKBR3 cell line (left) and the SKBR3 GFP-LC3 cell line (right). b) Fold change in cell numbers of SKBR3 GFP-LC3 and SKBR3 cell lines measured at 24hr intervals after plating. Data points represent mean ± SE for three independent experiments. c) SKBR3 GFP-LC3 cells were subjected to two hours of complete starvation (bottom panels) after which MDC and GFP-LC3 puncta were quantified (below). Blue = Draq5, Red = MDC, Green = GFP-LC3. Graph shows mean ± SE for one representative experiment of three independent experiments. *p-value<0.05
3.2 siRNA suppresses Apollon expression in breast cancer cells

To knock down expression of Apollon, two siRNA sequences for Apollon (Ap1 and Ap2) were assessed for their ability to decrease Apollon expression in MCF-7 GFP-LC3, SKBR3 and BT474 cells. Expression of Apollon was assessed 72 hours after transfection with siRNA as described in (Lopergolo et al., 2009). mRNA levels were analyzed using qRT-PCR which showed knockdowns of 78 ± 6, 96 ± 1, and 78 ± 2% for Ap1 and 92 ± 3, 97 ± 1, and 88 ± 6% for Ap2 in MCF-7 GFP-LC3, SKBR3 and BT474 cells, respectively, as compared to a medium GC content scramble control. The Ap2 siRNA consistently showed a greater knockdown, based on mRNA levels, than Ap1 (Figure 3.4). Western blot analyses were also conducted to determine whether the reduced levels of Apollon mRNA reflected a reduction in Apollon protein expression (Figure 3.5). In all cell lines, both Apollon siRNA sequences reduced the expression of Apollon protein to an undetectable level.

![Figure 3.4 Apollon transcript levels after siRNA treatment](image)

mRNA levels of Apollon were assessed 72 hours after siRNA treatment. Data presented shows the mean ± standard error of Apollon transcripts from consolidation of three independent experiments. Ap1 and Ap2 = Apollon siRNA-1 and Apollon siRNA-2

*P-value < 0.001
Figure 3.5 Apollon siRNA decreases endogenous Apollon protein levels in breast cancer cell lines

Representative western blots for Apollon show a dramatic decrease in Apollon protein levels after siRNA treatment. β-Actin (ActB) was used as a loading control. This trend was reproduced in 3 additional blots with MCF-7 GFP-LC3 and 1 additional blot for both SKBR3 and BT474.

Ap1 & Ap2 = Apollon siRNA-1 and Apollon siRNA-2 treated
3.3 Apollon knockdown does not affect cell growth or viability of SKBR3 or MCF-7 GFP-LC3 cells

To determine whether Apollon knockdown affected cell growth or viability of SKBR3 or MCF-7 GFP-LC3 cells, cell numbers and viability were assessed using high-throughput fluorescence microscopy. Cell numbers were counted at 24 hour intervals after siRNA treatment and normalized to the scramble siRNA treated control (Figure 3.6). Apollon knockdown did not significantly affect the number of SKBR3 or MCF-7 GFP-LC3 cells up to 72 hours after siRNA treatment.

![Figure 3.6 Effect of Apollon siRNA on number of MCF-7 GFP-LC3 and SKBR3 cells](image)

The number of a) MCF-7 GFP-LC3 and b) SKBR3 cells was analyzed at 24hr intervals after siRNA treatment at 0hrs. The fold increase in cell numbers relative to 0hrs is plotted. Each data point represents the mean ± SE for three independent experiments. Sc = scramble siRNA, Ap1 & Ap2 = Apollon siRNA-1 and Apollon siRNA-2
Cell viability following 72 hour siRNA treatment and 2 hour fed or starved treatment was assessed using ethidium homodimer staining to identify dead cells. The percentage of dead cells was determined for three independent experiments (Figure 3.7).

Figure 3.7 Effect of Apollon siRNA treatment on viability of MCF-7 GFP-LC3 and SKBR3 cells

Cell death was assessed via ethidium homodimer (EthD1) staining after 72hr siRNA treatment in combination with 2hr fed or starved treatment. The percentage of EthD1 positive a) MCF-7 GFP-LC3 and b) SKBR3 cells is represented as the mean ± SE for three independent experiments. Sc = scramble siRNA, Ap1 & Ap2 = Apollon siRNA-1 and Apollon siRNA-2
Both SKBR3 and MCF-7 GFP-LC3 cells exhibited increased death upon starvation treatment. No significant change in cell death was detected with Apollon siRNA treatment as compared to the similarly treated (fed or starved) scramble siRNA controls. As the amount of cell death under fed conditions was relatively low, and there was no significant increase in cell death after Apollon siRNA treatment, presumably the increase in cell numbers up to 72 hours after siRNA treatment was indicative of cell growth. Therefore, I can conclude that there is no significant change in cell growth up to 72 hours after Apollon siRNA treatment in MCF-7 GFP-LC3 or SKBR3 cells.

3.4 Apollon knockdown increases the number of MDC and/or GFP-LC3 puncta in MCF-7 and SKBR3 cells

To determine whether Apollon has an autophagy regulating effect in any of the three breast cancer cell lines, Apollon was knocked down using the verified Ap1 and Ap2 siRNA sequences and autophagy was assessed under fed and starved conditions using MDC staining or GFP-LC3 localization. As a control, siRNA for Beclin 1 (abbreviated as BecD siRNA), a positive regulator of autophagy, was also assessed for its ability to reduce MDC or GFP-LC3 puncta under starved conditions. A decrease in puncta after Beclin 1 siRNA treatment and starvation was seen in MCF-7 and SKBR3 cells stably transfected with GFP-LC3 (P < 0.05) and in BT474 cells stained with MDC (although the decrease in BT474 cells was not significant) (Figure 3.8b and 3.9b), however the decrease was not observed consistently in SKBR3 cells using MDC staining.

Under fed conditions, Apollon knockdown led to a significant increase in GFP-LC3 puncta in MCF-7 and SKBR3 cells (p < 0.05). Apollon knockdown also showed a
slight increase in MDC staining in BT474 cells under fed conditions, although a
significant increase was observed only once (as shown in Figure 3.8a). These findings
suggest that Apollon knockdown alone is sufficient to increase the number of
autophagosomes in MCF-7 and SKBR3 cells.

Under starved conditions, Apollon knockdown led to a significant increase in
GFP-LC3 puncta in MCF-7 (Figure 3.8b) and SKBR3 (Figure 3.9b) cells and an increase
in MDC puncta in parental SKBR3 cells, but had no significant effect on MDC staining
in BT474 cells (Figure 3.8b). All experiments were performed a minimum of three times
and at least one of the two Apollon siRNAs resulted in a significant increase in
autophagic structures in each experiment in MCF-7 and SKBR3 cells as summarized in
Table 3. 1.
Figure 3.8 Autophagy knockdown increases the number of MDC or GFP-LC3 puncta in all three breast cancer lines

a) Apollon knockdown significantly increased the number of GFP-LC3 or MDC puncta in both MCF-7 GFP-LC3 and BT474 cells under fed conditions. b) Apollon knockdown significantly increased the number of GFP-LC3 or MDC puncta in both MCF-7 GFP-LC3 and SKBR3 cells under starved conditions. Reported values are mean ± SE of triplicate samples from one representative experiment of three independent experiments. BecD = Beclin 1 siRNA, Ap1 and Ap2 = Apollon siRNA. *P-value<0.05 and **P-value<0.01
Figure 3.9 Autophagy knockdown increases the number of GFP-LC3 puncta in SKBR3 hrGFP-LC3 cells under starved conditions

a) Apollon knockdown did not affect the number of GFP-LC3 puncta under fed conditions. b) Apollon knockdown significantly increased the number of GFP-LC3 puncta under starved conditions. Reported values are the mean ± SE of triplicate samples from one representative experiment out of three independent experiments. BecD = Beclin 1 siRNA, Ap1 and Ap2 = Apollon siRNA. *P-value<0.05 and ***P-value<0.001

Table 3.1 Summary of Apollon siRNA sequences resulting in a significant increase in GFP-LC3 or MDC puncta under fed and starved conditions

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>MCF-7 GFP-LC3</th>
<th>SKBR3</th>
<th>BT474</th>
<th>SKBR3 GFP-LC3</th>
</tr>
</thead>
</table>

Ap1 and Ap2 = two different Apollon siRNA sequences (see Materials and Methods); - , no significant result; *significance of each result is indicated as follows: *p-value < 0.05, **p-value < 0.01, ***p-value < 0.001
3.5 Apollon knockdown results in increased autophagic flux in MCF-7 GFP-LC3 breast cancer cells

An accumulation of GFP-LC3 puncta can mean an induction of autophagy or a blockage at a later stage of the process leading to an accumulation of autophagosomes. The generation of free GFP from GFP-LC3 degradation in the autolysosome is a commonly used method for measuring autophagic flux (Klionsky et al., 2008). As rapamycin is a known inducer of autophagy (Takeuchi et al., 2005), I used rapamycin treatment to confirm that autophagy induction would induce formation of free GFP in my system (Figure 3.10a). Rapamycin treatment showed a significant increase in free GFP as compared to the control sample. To test whether Apollon knockdown also led to an induction of autophagic flux in MCF-7 GFP-LC3 cells, the generation of free GFP was measured (Figure 3.10b). A single representative western blot is shown representing the mean ± SE for three independent experiments. Apollon siRNA treatment showed a significant increase in free GFP under fed conditions as compared to the scramble siRNA treated control sample.
Figure 3.10 Generation of free GFP in MCF-7 GFP-LC3 cells after Apollon knockdown or rapamycin treatment

a) Left; a representative western blot showing free GFP and ActB levels after 2hr Rapamycin treatment (20nM). b) Left; a representative western blot showing free GFP and β-tubulin levels after 72hr Apollon siRNA treatment. a and b) Right; the amount of free GFP was normalized to the loading control for each experiment (β-tubulin or β-actin) and protein expression was reported as a percent relative to the scramble fed sample.

Graph represents mean values ± SE from three independent experiments. Sc = Scramble siRNA, Ap1 and Ap2 = Apollon siRNA. **P-value<0.01, ***P-value<0.001 vs scramble fed control.

Analysis of free GFP after both siRNA treatment and starvation did not prove to be a useful assay for autophagic flux (further discussed in section 4.1.6), therefore p62 degradation was explored for use as an assay to assess flux through the pathway under both fed and starved conditions. I used rapamycin treatment to confirm that autophagy
induction would induce p62 degradation in my system (Figure 3.11a). As expected, rapamycin treatment led to a decrease in p62 levels relative to a scramble siRNA treated fed sample, indicating that p62 degradation is a valid method for measuring autophagic flux in MCF-7 GFP-LC3 cells. Analysis of p62 levels following siRNA treatment with the scramble control showed a decrease in starved conditions compared to fed conditions, as expected, indicating that this assay is also useful in the context of siRNA treatment. Apollon siRNA treatment under both fed and starved conditions showed a decrease in p62 as compared to the similarly treated (fed or starved) scramble control sample (Figure 3.11b) although the decrease was only significant (p < 0.05) with Ap2 siRNA in combination with starvation. This decrease in p62 is indicative of an increase in autophagic flux. Together, the analysis of both free GFP generation and p62 degradation indicates that Apollon knockdown leads to an increase in autophagic flux in MCF-7 GFP-LC3 cells.
Figure 3.11 Degradation of p62 in MCF-7 GFP-LC3 cells following Apollon knockdown
a) Western blot showing p62 and β-actin (ActB) levels in scramble fed negative control cells and rapamycin (20nM) treated positive control cells. Graph on the right shows quantitation of p62 from blot on right relative to ActB. b) A representative western blot showing p62 and β-actin (ActB) levels after Apollon siRNA treatment. The amount of p62 was normalized to β-actin and protein expression was reported as a percent relative to the scramble fed sample. Graph represents mean values ± SE from three independent experiments. Sc = Scramble siRNA, Ap1 and Ap2 = Apollon siRNA, F = fed, S = starved. *P-value<0.05 vs scramble fed control
CHAPTER 4: DISCUSSION

The relationship between apoptosis and autophagy is complex, with common regulators and areas of crosstalk continually being discovered. Research conducted in *Drosophila* identified Bruce, an IAP family member, as a negative regulator of autophagy (Hou et al., 2008). Apollon, the human homologue of Bruce, had not previously been shown to have any connection to autophagy, and therefore the aim of this project was to determine whether Apollon is also a negative regulator of autophagy in human cells. This question was addressed by examining three different breast cancer cell lines, MCF-7 GFP-LC3, SKBR3 and BT474, to determine whether Apollon knockdown had an effect on autophagy levels in cells under fed and starved conditions. Both MCF-7 and SKBR3 cells showed a significant increase in autophagic structures after Apollon knockdown under both fed and starved conditions, while BT474 cells showed a slight, although insignificant, increase under fed conditions only. Further analysis in MCF-7 cells confirmed that Apollon knockdown indeed led to an induction of the complete autophagy process in both fed and starved conditions as determined by an autophagy flux assay. These findings support my initial hypothesis that Apollon is a negative regulator of autophagy in human breast cancer cells and provide the first evidence for autophagy regulation by a mammalian IAP family member.

4.1 Interpretation of results

Three breast cancer cell lines were chosen for use in this study: MCF-7 GFP-LC3, SKBR3 and BT474. These cells were chosen for their ability to undergo starvation-
induced autophagy (Figure 3.1) and based on the fact that they express Apollon, although
to varying degrees (Figure 3.5). These cell lines also represent variable genetic
backgrounds with regards to some important classifiers of breast cancers, namely the
estrogen receptor (ER) and human epidermal growth factor receptor 2 (Her2), as follows:
MCF-7 (Luminal type A; ER+, Her2-), SKBR3 (Luminal Type B; ER-, Her2+), and
BT474 (Luminal type A; ER+, Her2 +) (Kao et al., 2009).

4.1.1 Analysis of starvation-induced autophagy

As shown in Figure 3.1, all three cell lines showed an increase in MDC or GFP-LC3
puncta after 2hrs of complete starvation. The levels of puncta under fed conditions and
the degree of starvation response varied considerably between cell lines. These
differences may be related to variability in the basal levels of autophagy in each cell line
as well as the difference in detection methods. The same threshold of three or more MDC
puncta was used to define autophagy-positive cells in both SKBR3 and BT474 cells,
which showed 4.2 ± 0.6 and 14.8 ± 1.7% autophagy-positive cells respectively, under fed
conditions. This suggests that the basal level of autophagy may be about three-fold higher
in BT474 cells as compared to SKBR3 cells. A threshold of five or more GFP-LC3
puncta was used to define autophagy-positive cells in the MCF-7 GFP-LC3 cell line,
which also indicated approximately 15% autophagy-positive cells under fed conditions.
This observation may again indicate a higher level of basal autophagy in MCF-7 cells as
compared to SKBR3 cells, or may be related to the method of detection used, GFP-LC3
localization vs. MDC staining, as discussed further in section 4.1.2.
4.1.2 Generation of a polyclonal SKBR3 cell line stably transfected with hrGFP-LC3

To facilitate specific analysis of autophagy in SKBR3 cells, a stable hrGFP-MAP1LC3β (GFP-LC3) polyclonal SKBR3 cell line was generated. Humanized *R. reniformis* GFP (hrGFP) was chosen as the fluorescent reporter as it has been shown to be brighter and less toxic than the commonly used eGFP (Sinclair, 2001). The transfected cell line maintained a similar morphology and growth rate to the parental SKBR3 cell line. This stable cell line was also able to undergo starvation-induced autophagy as indicated by both MDC staining and GFP-LC3 localization (Figure 3.3). To confirm that the increase in puncta observed after starvation was due to autophagy, Beclin 1 siRNA was used, which was able to reduce the observed response to starvation. As expected, this result confirms that the increase in GFP-LC3 puncta after starvation is autophagy related.

In the SKBR3 GFP-LC3 cell line, MDC staining under fed and starved conditions was approximately a third lower than in the parental cell line (Figure 3.3 and 3.1 respectively). One possible reason for this may be that a different filter set is used for analysis of GFP-LC3 cells that may not be as sensitive for detection of MDC. Conversely, the level of GFP-LC3 puncta was relatively high, and thus a threshold of five or more GFP-LC3 puncta was used to define autophagy-positive cells; the same threshold was used in the MCF-7 GFP-LC3 cell line. The apparently high background GFP-LC3 puncta could be due to a number of factors: 1) the cells may be under more stress from expression of the construct which may cause an increase in autophagy, 2) GFP-LC3 can associate with aggregates, especially when overexpressed, which may account for some of the puncta seen (reviewed in (Klionsky et al., 2008)), 3) or the variance may be related to the difference in detection methods and timing of analysis. GFP-LC3 labels autophagosomes, an early step in the autophagy pathway, while MDC stains
autolysosomes, a late step in the pathway. Perhaps the reason there appears to be such high GFP-LC3 puncta as compared to MDC puncta is because at the time-point assessed, namely two hours starvation, autophagy has been induced and autophagosomes are primarily being formed, but many have not yet fused with lysosomes to form autolysosomes, and thus are identified with GFP-LC3 but not with MDC.

4.1.3 Apollon knockdown

All three breast cancer cell lines showed a dramatic decrease in Apollon mRNA and protein levels 72 hours after siRNA treatment (Figures 3.4 and 3.5). The mRNA data showed variability in Apollon knockdown between cell lines and between the two Apollon siRNA sequences, however western blot analyses showed undetectable levels of Apollon in all lines after treatment with both siRNAs. The difference in transcript levels after knockdown with the two different Apollon siRNAs may indicate a reason for the variability in results achieved with each siRNA. For example, in MCF-7, Ap1 resulted in an average mRNA knockdown of 78% while Ap2 achieved an average of 92% knockdown. Alternatively, western blotting indicated comparable knockdown in protein levels with both siRNA sequences, but perhaps the sensitivity of detection is insufficient to detect a difference at the protein level. The variation in knockdown at the transcript level, and potentially at the protein level, between the two siRNA sequences may explain why Ap2 showed more consistent results in MCF-7, while Ap1 effects were variable.

Another possible reason for the observed variability in response to Apollon knockdown may be due to inconsistencies in factors such as transfection efficiency between experiments. Presumably the level of Apollon knockdown in the western blot
and fluorescence microscopy experiments was comparable, however siRNA treatment had to be scaled to deal with the different formats for these experiments, 6-well and 96-well plates respectively, which may have also introduced some variability with respect to knockdown. The potential for differences in Apollon knockdown between experiments, which is a concern in siRNA studies (Ovcharenko et al., 2005), may explain some of the variability observed in my results.

4.1.4 Effect of Apollon knockdown on cell growth and viability

There was no significant change in cell growth or viability in MCF-7 or SKBR3 cell lines 72 hours after Apollon knockdown (Figures 3.6 and 3.7). This data agrees with previously published findings regarding Apollon knockdown in MCF-7 cells. Both Lopergolo et al. (2009) and Chu et al. (2008) showed that MCF-7 cells did not demonstrate any change in growth or viability 72 hours after Apollon knockdown (Chu et al., 2008; Lopergolo et al., 2009). However, Chu and colleagues analyzed viability of MCF-7 cells up to 5 days after knockdown and on day 5 saw reduced viability. This suggests that timing, may be important with regards to the effect of Apollon knockdown on cell death. As Apollon is an inhibitor of apoptosis, one might expect that loss of Apollon would lead to an increase in cell death, especially with an added stimulus like starvation, however this was not observed in my study. One possible reason for this is that autophagy may be an early event induced by Apollon knockdown and may be acting to protect cells from apoptosis, and thus cell death is not seen to be increased by 72 hours. Perhaps after 5 or more days of Apollon knockdown autophagy is no longer able to protect cells from death and apoptosis takes over, which would explain the results seen
by Chu et al. (2008) (Chu et al., 2008). It would be interesting to further assess the levels of autophagy and apoptosis after Apollon knockdown to see if there is a shift from autophagy to apoptosis at a time point after 72hrs.

Cell type and/or breast cancer subtype may be additional factors influencing cell growth or viability following Apollon knockdown. For example, Lopergolo et al (2009), demonstrated significant effects on viability as early as 72 hours after Apollon knockdown in MDA-MB-231 breast cancer cells (ErbB2-dependent; ER-, low Her2 (Artemov et al., 2003)) and ZR75-1 breast cancer cells (Luminal type A; ER+, Her2- ) (Kao et al., 2009). Thus, it would also be valuable to investigate alternate breast cancer cell lines, such as these, for Apollon-related effects on autophagy levels. The cell viability effect of Apollon knockdown combined with Atg gene knockdown (or other forms of autophagy inhibition) would also be an interesting avenue of investigation.

4.1.5 Effect of Apollon knockdown on autophagic structures

In MCF-7 and SKBR3 GFP-LC3 cell lines treated with Apollon siRNA, a significant increase in GFP-LC3 puncta was evident even under fed conditions, suggesting that Apollon knockdown alone is sufficient to cause an increase in autophagosomes. A similar trend was also seen in BT474 cells, although a significant increase in MDC puncta was only observed once. Parental SKBR3 cells showed an increase in MDC puncta after Apollon knockdown only in combination with starvation. MCF-7 and SKBR3 GFP-LC3 cells also showed a further increase in GFP-LC3 puncta after Apollon knockdown in starvation conditions. BT474 cells did not demonstrate increased MDC puncta after Apollon siRNA treatment and starvation.
The lack of a significant difference in levels of puncta in BT474 cells may be due to the already high basal levels of MDC puncta in BT474 cells that may have masked any additional difference after siRNA treatment. BT474 cells also appear to be quite sensitive to the stress of siRNA treatment as MDC puncta were increased after scramble siRNA treatment even under fed conditions (see Figure 3.8 and 3.1). Another possible reason for the lack of a detectable response in BT474 cells may be due to the method used to detect autophagic structures, namely MDC staining. MDC does not specifically label autolysosomes, which may account for the high level of background, and does not produce as clear images as GFP-LC3. These factors may lead to reduced sensitivity (or greater variability) with respect to quantitation compared to GFP-LC3. Given the potential for issues in detecting autophagy using MDC staining, one may question whether the results obtained with MDC are valid. Comparing the results obtained in SKBR3 cells using both MDC and GFP-LC3 (Figure 3.8) indicates that the MDC results are indeed valid. While a significant response to Apollon knockdown was observed with MDC staining, confidence in this result was enhanced using GFP-LC3, a specific marker for autophagy. This comparison suggests that the results obtained with MDC are in fact valid, but that MDC may lack the sensitivity to pick up all positive results.

The difference in response to Apollon knockdown seen in parental SKBR3 cells as compared to SKBR3 GFP-LC3 cells may, however, also indicate that there is a real physiological difference between the parental SKBR3 cell line and the stable SKBR3 GFP-LC3 line. If this is the case, one reason for the observed difference in response to Apollon knockdown may be that the overexpression of GFP-LC3 in the stable cell line is on its own a stress on the cell and thus is akin to the stress put on the parental SKBR3 line by starvation. A possible reason for why some cell lines may display an increase in
puncta after Apollon knockdown under fed conditions while other lines may require starvation is discussed in section 4.2.

4.1.6 Effect of Apollon knockdown on autophagic flux

The MCF-7 GFP-LC3 cell line was utilized for analyses of autophagy flux. The first method used to assess flux after Apollon knockdown was an assay for measuring the generation of free GFP. Fusion of the autophagosome with the lysosome exposes the GFP-LC3 contained on the inner autophagosome membrane to the acid hydrolases from the lysosome. In this environment, LC3 is degraded while GFP is more stable in the acidic environment and remains intact for a longer period of time (reviewed in (Klionsky et al., 2008)). Comparison of fed samples after scramble siRNA treatment and Apollon siRNA treatment revealed a significant increase in free GFP after Apollon knockdown, suggesting autophagy was upregulated and the process was going to completion as observed for the rapamycin positive control (Figure 3.10a).

A comparison of starved samples with and without siRNA treatment indicated that analysis of free GFP after the combination of treatments was not appropriate for measuring flux. Analysis of samples without siRNA treatment at 15 and 30 minutes and then at 30 minute intervals over a two hour starvation period revealed a steady increase in free GFP, as expected. However, this trend was lost with the combination of scramble siRNA and starvation treatment. Rather, a slight increase in GFP was seen initially at 15 minutes of starvation, followed by a drastic decrease in free GFP up to two hours. One possible explanation is that the combination of siRNA treatment and starvation induces autophagic flux to such a rate that even the GFP is degraded by two hours.
A second assay was then employed to analyze autophagic flux after Apollon siRNA treatment in both fed and starved MCF-7 GFP-LC3 cells. Analysis of p62 degradation revealed a decrease in p62 levels, similar to the rapamycin control, after Apollon knockdown under both fed and starved conditions. This result confirmed that Apollon knockdown led to the induction of the complete autophagy process in MCF-7 cells.

Starvation using complete nutrient withdrawal was the only stress stimulus used in this study, but it would be interesting to see whether other stresses, such as hypoxia, radiation or chemical treatment which are more physiologically relevant, especially with regards to cancerous cells, show similar increases in autophagy in combination with Apollon knockdown. For example, investigations in our laboratory have shown that autophagy is induced in response to Tamoxifen treatment in all three cell lines used in this study (unpublished data), and thus it would be interesting to determine whether Apollon knockdown in combination with Tamoxifen also results in enhanced autophagy or affects viability in these cell lines.

4.2 Possible molecular mechanism of autophagy regulation by Apollon

Apollon is most commonly known for its role as a negative regulator of apoptosis, and was also found to have a critical role in cytokinesis (Pohl & Jentsch, 2008), the final step in mitosis. Both of these processes were shown to depend largely on the ability of Apollon to act as an E2/E3 ligase and ubiquitinate substrates (Hao et al., 2004; Sekine et al., 2005). In addition, Apollon was shown to be a regulator of p53 (Lopergolo et al.,
The ability of Apollon to regulate p53 protein levels and ubiquitinate substrates via its UBC domain suggests two possible mechanisms by which Apollon may regulate autophagy, illustrated in Figure 4.1.

Figure 4.1 Potential mechanisms for the negative regulation of autophagy by Apollon

Lopergolo et al. (2009) showed that Apollon knockdown via siRNA in breast cancer cells led to the stabilization of p53. This finding was also shown by Ren et al. (2005) in vivo in mice missing the C-terminal portion of Apollon and in vitro in mouse embryonic fibroblasts (MEFs). Ren and colleagues also showed that Apollon knockdown
resulted in the translocation of p53 to the nucleus (Ren et al., 2005). These findings lead me to hypothesize that Apollon may regulate autophagy indirectly via p53, as p53 has been shown to be a regulator of autophagy. Namely, cytosolic p53 was shown to be a negative regulator of autophagy (Tasdemir et al., 2008a), while nuclear p53 induced autophagy via transcriptional and non-transcriptional events leading to the suppression of mTOR (Tasdemir et al., 2008b). Apollon may negatively regulate autophagy by maintaining low levels of p53, particularly in the nucleus, leading to mTOR activation and autophagy suppression (Figure 4.1). Upon knockdown of Apollon, p53 stabilization and transport to the nucleus may be leading to mTOR inactivation and thus induction of autophagy. However, both SKBR3 and BT474 breast cancer lines contain mutant p53 (Wasielewski et al., 2006). If Apollon is regulating autophagy solely via p53, then p53 must be expressed at sufficient levels in MCF7 and SKBR3 cells, and the mutant form of p53 in SKBR3 must still be functional, at least in its capacity to regulate autophagy. BT474 cells also contain mutant p53. The possibility that Apollon exhibits autophagy regulation via p53 may suggest that mutant p53 in BT474 cells is not functional (with respect to autophagy regulation) and may explain the lack of response observed in BT474 cells after Apollon knockdown.

Another way Apollon may regulate autophagy is via its E2/E3 ubiquitin ligase activity. Apollon has been shown to ubiquitinate Smac/DIABLO (Hao et al., 2004), Omi/HtrA2 (Sekine et al., 2005) and caspase-9 (Hao et al., 2004), leading to their degradation by the proteasome. A recent paper by Li et al. (2010) showed that Omi/HtrA2 is a positive regulator of autophagy by promoting the digestion of Hax-1, a Bcl-2 family member that is able to bind and inhibit Beclin 1. Given that Apollon is known to negatively regulate Omi (Sekine et al., 2005), this may be the mechanism whereby Apollon regulates autophagy. Similarly, in the fruit fly, *Drosophila*
It was shown that Bruce (the fly homologue of Apollon) required Dcp-1, an effector caspase, to exhibit autophagy regulating activity (Hou and Gorski, unpublished data). Apollon may function in a similar fashion, via a downstream caspase. Caspase-9, as a known substrate for ubiquitination by Apollon, may be a potential candidate, although the most functionally similar mammalian caspase to Dcp-1 is caspase-3 (Song et al., 2000), and thus caspase-3 may also be a candidate. Alternatively, Apollon may have other downstream targets for ubiquitination that have not yet been identified. Perhaps Apollon leads to ubiquitination and proteasomal degradation of essential or activating autophagy proteins or positive regulators of autophagy. In this way, Apollon knockdown would lead to an increase in these proteins which could allow for autophagy to be increased. One possible candidate for this proposed mechanism of autophagy regulation by Apollon is LC3. Apollon knockdown led to an increase in GFP-LC3 (Figure 3.10) which presumably indicates stabilization of the protein rather than an increase in transcription and translation as the fusion protein is downstream of the CMV promoter. This may indicate that Apollon normally leads to the turnover of GFP-LC3 (and presumably endogenous LC3). As LC3 is necessary for autophagosome formation (Weidberg et al., 2010), this may be the mechanism whereby Apollon regulates autophagy.

The mechanisms proposed above suggest that Apollon is responsible for the turnover of proteins relevant to autophagy and that Apollon knockdown allows for stabilization and/or reduced turnover of these proteins that are already present within the cell. This leads to a potential reason for the differences observed in the cell lines in this study; namely, whether autophagy is increased upon Apollon knockdown alone, or whether a starvation signal is required to observe an increase in autophagy. In MCF-7 and SKBR3 GFP-LC3 cells, Apollon knockdown alone led to an increase in autophagic
structures, while parental SKBR3 required an additional starvation signal. If Apollon is acting to regulate autophagy at a post-translational level, then this observed difference may be due to different expression levels of the proteins targeted for ubiquitination by Apollon within the various cell lines. Perhaps in MCF-7 and SKBR3 GFP-LC3 cells, the proteins Apollon normally ubiquitinates are expressed at high enough levels that their stabilization alone is sufficient to cause an increase in autophagy. In parental SKBR3 cells, these proteins may be expressed at lower levels, such that their stabilization is not sufficient to cause an increase in autophagy. In parental SKBR3 cells, it may be that the starvation signal leads to upregulated expression of these proteins and/or increased transcription of their respective genes and only then can they cause an increase in autophagy.

4.3 Negative regulators of autophagy

The finding that Apollon negatively regulates autophagy is particularly interesting as few negative regulators of autophagy have been identified. mTOR is the main inhibitor of autophagy, while PI3K, Akt, and Rheb also negatively regulate autophagy (reviewed in (Baehrecke, 2005; Eisenberg-Lerner et al., 2009; Kondo et al., 2005; Lum et al., 2005a)). A more recently identified inhibitor of autophagy is bcl-2, commonly known for its apoptosis inhibiting function. Bcl-2 indirectly inhibits autophagy by interacting with Beclin-1, a core autophagy protein, and preventing its interaction with Vps34, an essential step in autophagosome formation (Pattingre et al., 2005).

Similar to Apollon, bcl-2 and other anti-apoptotic family members, such as bcl-XI, are overexpressed in many cancers and have been investigated as targets for cancer
therapy. There are currently several therapeutic modalities in various stages of development targeting anti-apoptotic bcl-2 family members (reviewed in (Kang & Reynolds, 2009; Marzo & Naval, 2008). Three of these bcl-2 family-targeting therapies have been shown to induce autophagy (Kessel & Reiners Jr., 2007; Lin et al., 2009; Maiuri et al., 2007a). The cytotoxic effects of Z36, a bcl-xl and bcl-2 inhibitor, were even shown to depend entirely on the induction of autophagy. Z36 competitively inhibits the interaction of bcl-Xl with Beclin 1 and causes cell death that is completely independent of apoptosis but dependent on autophagy, suggesting that the role of bcl-Xl in regulating autophagy is a critical function of this protein, at least in Hela cells (Lin et al., 2009). This demonstration provides support to the notion that proteins which negatively regulate autophagy may be effective therapeutic targets.

4.4 Apollon: A potential target for cancer therapy

Several IAP family members have already been investigated as potential cancer targets, with many therapeutic modalities currently in clinical trials (reviewed in (Hunter et al., 2007; LaCasse et al., 1998)). Recently, Apollon was suggested as a potential therapeutic target based on the finding that Apollon is overexpressed in some drug resistant cancers (Chen et al., 1999) and Apollon knockdown increases sensitivity of some cancer cells to chemotherapeutics (Chu et al., 2008). My results indicate that Apollon is a negative regulator of autophagy, a function that was previously unknown, and this finding leads to new considerations about Apollon as a potential cancer target.

My results suggest that targeting Apollon would lead to an increase in autophagy. With regards to cancer therapy, this may or may not be beneficial. In some cases,
autophagy acts to promote cell death (Bergmann, 2007; Chang et al., 2007; Hoyer-Hansen & Jaattela, 2008; Puissant et al., 2010; Rubinsztein et al., 2007). If this is the role that autophagy is playing after Apollon knockdown then this indeed may be a promising target for therapy. Alternatively, autophagy sometimes acts to promote cell survival (Abedin et al., 2007; Amaravadi et al., 2007; Bellodi et al., 2009; Boya et al., 2005; Li et al., 2009; Maiuri et al., 2007a; Qadir et al., 2008; Samaddar et al., 2008). If this is the role autophagy is playing after Apollon knockdown, then it may be necessary to inhibit autophagy alongside Apollon knockdown in order to effectively induce cell death. It will be necessary to determine the role that Apollon knockdown-induced autophagy is playing in cells and the effects of combined Apollon and autophagy knockdown before an Apollon targeted approach could be clinically applied for cancer treatment.

4.5 Summary of strengths and weaknesses

This study is the first to implicate a mammalian IAP family member as an inhibitor of autophagy, identifying a new common regulator between apoptosis and autophagy in human cells. While there are still several questions that remain regarding Apollon’s role as a regulator of autophagy, this finding provides a new direction for further investigating the complex relationship between apoptosis and autophagy.

The initial assessment for autophagy regulation by Apollon was conducted in three different breast cancer cell lines. Two of the cell lines used were stably transfected with GFP-LC3, a specific marker for autophagy, and both of these cell lines showed a significant increase in GFP-LC3 puncta upon Apollon knockdown. While the follow-up study examining autophagic flux was performed in only one cell line, two methods were
employed to measure flux, both of which showed consistent results in fed conditions. Another strength of this study was the use of two non-overlapping siRNA sequences for knocking down Apollon. While the Ap2 siRNA sequence gave the most consistent results, similar trends were seen with both siRNA sequences, providing confidence that the autophagy effects seen were in fact due specifically to the knockdown of Apollon. These results together provide convincing evidence for the novel finding that Apollon is a negative regulator of autophagy.

One weakness of this study was the fact that all three cell lines employed were breast cancer lines, and thus it is possible that the findings may not be universal but may instead be specific to breast cancer cells. Additionally, as the degree of the starvation response was variable between siRNA experiments in each cell line, it was not possible to normalize the data and consolidate experiments. As such, a single representative experiment had to be presented, although the findings shown were validated in multiple replicate experiments. As the results of this study may have clinical implications, a limitation was that siRNA was used for Apollon knockdown. This is not yet a clinically viable therapeutic strategy, although research into targeted delivery methods for RNAi therapeutics is rapidly progressing (Gondi & Rao, 2009).

4.6 Future directions

The finding that Apollon is a negative regulator of autophagy in breast cancer cell lines raises many new questions and provides several directions for future research in an effort to further understand this common regulator of apoptosis and autophagy.
1) What role does autophagy play in Apollon deficient cells?

This study showed that autophagy is induced in breast cancer cells when Apollon expression is knocked down. As Apollon has been a proposed target for cancer therapy, it is important to determine what cell fate autophagy is promoting in these cells and whether it may be different between cell types. If autophagy is acting to promote cell survival then it may be necessary to combine Apollon knockdown with autophagy inhibition to more efficiently induce cell death. It will be important to determine whether autophagy is acting as a cell survival or cell death mechanism before Apollon can be effectively investigated as a potential cancer target.

To determine what cell fate autophagy is promoting, one could simultaneously knock down Apollon and a core autophagy gene, such as Beclin 1 or Atg7, and assess the effect of this double knockdown on cell viability. If simultaneous Apollon knockdown and autophagy inhibition leads to an increase in cell death, then this would suggest that Apollon knockdown-induced autophagy is acting to promote cell survival. However, if autophagy inhibition causes a decrease in cell death, then this would suggest that autophagy is promoting cell death, a function that would be beneficial for cancer treatment.

2) What is the mechanism whereby Apollon negatively regulates autophagy?

As described in section 4.2, there are at least two possible mechanisms whereby Apollon may regulate autophagy (Figure 4.1). To determine whether Apollon regulates autophagy via p53, at least in some cell lines (e.g. MCF-7 GFP-LC3), one could knock down Apollon in combination with p53. If Apollon regulates autophagy through the
stabilization of p53, this double knockdown should result in decreased autophagy levels. Alternatively, to determine whether Apollon regulates autophagy via ubiquitination of proteins that are required for and/or positively regulate autophagy, one could first perform an immunoprecipitation (IP) mass-spectrometry study to determine what proteins Apollon interacts with and choose potential candidates from that list that may affect autophagy. To determine whether Apollon leads to ubiquitination of these proteins, a similar approach to that taken by Hao et al. (2004) could be taken. Briefly, cells could be transfected with ubiquitin, the protein of interest and Apollon. An IP could then be performed for the protein of interest and a subsequent western blot conducted for ubiquitin immunostaining. A protein smear, only with the presence of Apollon, would indicate that indeed the protein of interest was ubiquitinated by Apollon. To test whether the interaction and ubiquitination of this protein were responsible for autophagy regulation by Apollon, one could simultaneously knock down Apollon and the protein of interest and observe whether this double knockdown is in fact able to reduce the increased level of autophagy typically observed, indicating that this mechanism is important for Apollon’s autophagy regulating function.

3) Is overexpression of Apollon sufficient to suppress autophagy?

Apollon was shown to be overexpressed in some brain cancers and acute myeloid leukemias (Chen et al., 1999; Sung et al., 2007). This overexpression is likely playing a role in helping the cancer cells evade apoptosis, but since I have now shown that Apollon is a negative regulator of autophagy, it raises the question about whether overexpression of Apollon is also playing a role to inhibit autophagy in addition to inhibiting apoptosis. Perhaps Apollon upregulation is an early event in cancer development that allows for
cells to avoid apoptosis and inhibit autophagy, thus accumulating cell damaging organelles and proteins which may contribute to tumorigenesis.

One way to test whether Apollon overexpression is sufficient to suppress autophagy is by overexpressing full-length Apollon in cells that undergo starvation-induced autophagy (such as the cell lines used in this study) and determine whether Apollon overexpression reduces their autophagy response to starvation. If overexpression of the full-length Apollon is sufficient for suppression of autophagy, one could then overexpress various deletion constructs to determine which particular domains are involved in the autophagy regulating function of Apollon.

4) **Is autophagy regulation a universal role of Apollon? Or is this role specific to breast cancer cells?**

In mice, Apollon has been shown to be expressed in almost all tissues (Qiu & Goldberg, 2005). This project showed that in breast cancer cells Apollon is a negative regulator of autophagy, but this finding needs to be investigated in other cell types to determine whether this is a universal function of Apollon, or whether this finding is specific to breast cancer cells.

5) **Do other IAP family members have autophagy regulating functions?**

Apollon is one of eight mammalian IAP family members, which raises the question of whether autophagy regulation is specific to Apollon, or whether other family members may also exhibit autophagy regulating functions.
Overall, this study was the first to show that a mammalian IAP family member has a role in autophagy regulation. While there are still several questions that need to be addressed regarding the mechanism and role of Apollon regulated autophagy, this finding provides new insight into the common regulators of apoptosis and autophagy and may have clinical implications with regards to cancer therapy.
REFERENCES


