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Kirsten Anne Meyer White

Kirsten Anne Meyer White
Candidate

Internal Medicine
Department

This dissertation is approved, and it is acceptable in quality and form for publication:

Approved by the Dissertation Committee:

Marianne Berwick, Chairperson

Chien-An A. Hu, Co-chair

Todd Thompson

Helen Hathaway

ONCOGENE-DEPENDENT REGULATION OF AUTOPHAGIC FLUX IN MELANOMA

by

KIRSTEN ANNE MEYER WHITE

B.S., Biology, University of New Mexico, 1995
M.S., Biology, University of New Mexico, 1999

DISSERTATION

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DEDICATION

To Dr. Lynn Hertel who lead the way;

The Meyer/White Families

Those who inspired me to fight the good fight against cancer;

And the melanoma patients who gave everything for my research

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ONCOGENE-DEPENDENT REGULATION OF AUTOPHAGIC FLUX IN MELANOMA

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Kirsten Anne Meyer White

B.S., Biology, University of New Mexico, 1995

M.S., Biology, University of New Mexico, 1999

Doctor of Philosophy, Biomedical Sciences, University of New Mexico, 2015.

ABSTRACT

Skin cancer is the one of the most diagnosed cancers in the United States with increasing incidence of 6% every year. In 2015, it is estimated 73,870 new cases of melanoma will be identified and 9,480 individuals will die of their disease. While melanoma only accounts for approximately 2.4% of all cancer related deaths and is the 5th leading diagnosed cancer (US) it is the one of the most common cancers in young adults, age 25-29, particularly for young women. Of particular importance, the mean survival rate of patients diagnosed with metastatic melanoma is six months, with five-year survival rates of less than 5%.

One reason for the increasing incidence in young adults may be due to the use of indoor tanning. UV exposure causes DNA damage and can induce the activation of a metabolic pathway called autophagy. Autophagy is activated by stress, including DNA damage, and melanoma risk is associated with UV exposure.

Here I present three studies investigating oncogene differences in rates of autophagy as well as the relationship of UV exposure and genotypic variants to autophagy. In this project, I determine whether oncogene status in melanoma differentially regulates apoptosis by modifying autophagic flux. The central hypothesis of this project is that cutaneous malignant melanomas (CMM) with *BRAF* mutations may be autophagy-addicted while tumors with *NRAS* mutations may be less dependent on autophagy.

I found that *BRAF/NRAS* mutations differentially alter autophagic flux to suppress apoptosis in melanoma. Our results show that oncogene status in melanoma correlates with differential regulation of autophagic flux and that inhibition of autophagy in *BRAF* mutant melanoma cells results in apoptosis. These data suggest that *BRAF* mutant melanoma cells suppress apoptosis by modifying autophagic flux and that these cells may be autophagy addicted in order to promote survival.

I also determined that proxy autophagy markers LC3 and Beclin1 are associated with UV exposure and clinical stage when evaluating tissue sections from melanoma patients and controls. Surprisingly, the *NRAS* wide-type sections had elevated LC3 levels when compared to the *NRAS* mutant tissue sections suggesting that autophagy may be inhibited *NRAS* in melanoma tumors. These results indicate that autophagic flux varies by tumor stage and is associated with UVR exposure.

Finally, I also determined that several SNPs in autophagy related genes are melanoma prognostic indictors. Of note, one SNP that has previously been shown to be inversely associated with other diseases, with a functional variant which increases disease

susceptibility was inversely associated with Breslow thickness, the most important indicator of melanoma outcome.

The work from my study helps address the inconsistencies in the literature regarding autophagy's impact on melanoma progression. Furthermore, these studies provide a basis to investigate the role *ATG* gene SNPs, UV exposure and in autophagy and melanoma.

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CHAPTER ONE: INTRODUCTION

APOPTOSIS AND AUTOPHAGY: THE YIN-YANG OF HOMEOSTASIS IN CELL DEATH IN CANCER

1.1 ABSTRACT

Apoptosis and autophagy are physiologically necessary pathways which are vital for cell homeostasis. Apoptosis facilitates type I programmed cell death, while autophagic survival mechanism counteracts apoptosis. Dysregulation in the homeostatic balance between these two essential cellular pathways has been linked to various diseases. We review relevant Janus molecules and their interactomes, as well as lysosomes which play multiple roles in apoptosis and autophagy, and to discuss how targeted interventions can be used in cancer prevention and/or therapy.

1.2 INTRODUCTION

Apoptosis and autophagy are both normal, genetically and biochemically regulated, and physiologically necessary pathways which are vital for cell homeostasis. Like two sides of a coin, apoptosis facilitates purposeful suicide by tightly controlled reactions followed by phagocytosis, while autophagy usually counteracts apoptosis and affords a survival mechanism for stressed cells until exaggerated stress or nutrition depletion forces the cells to compromise. Interestingly, compromised cells may use autophagy as a type II programmed cell death mechanism to die. Dysregulation in the balance between these two essential cellular pathways has been reported to be significant in the onset and pathogenesis of almost all diseases. Work from my laboratory research and others has shown that inhibition of autophagy induces cell death in many cancer cell types and chemical and genetic inhibition of autophagy increases apoptotic cell death. Disrupted regulation of autophagy creates an environment that facilitates the initiation and progression of many diseases including cancer. In addition, this deregulation is associated with increased cancer risk, particularly for pancreatic, colorectal, and breast

cancer (Gukovsky et al., 2013). The goal of this article is twofold: (1) to review relevant Janus molecules and their interactomes, as well as lysosomes which play multiple roles in apoptosis and autophagy, and (2) to discuss how targeted interventions can be used in cancer prevention and/or therapy.

1.3 APOPTOSIS (THE YIN)

Apoptosis or type I programmed cell death is an ATP-dependent, multi-step process which occurs in response to both internal (intrinsic) and external (extrinsic) signals and as part of normal cell development and homeostasis (**Figure 1**) (Elmore, 2007). Hallmark characteristics of apoptosis in the conserved, late stage include the appearance of phosphatidylserine (PS) on the outer leaflet of the cell membrane that is recognized by neighboring cells/macrophages which then rapidly phagocytose cells displaying PS (Li et al.,2003). Other key features of late-stage apoptosis include an intact membrane with signs of blebbing/membrane protrusions, cellular condensation, chromatin condensation, and site-specific DNA fragmentation (Coleman et al., 2001). In so processing, apoptosis does not result in inflammation and/or tissue damage as is typical of necrosis which is acute, unregulated, and accidental cell death. The intrinsic apoptotic signaling pathway is dependent on the formation of apoptosome, an interactome of apoptotic protease activating factor 1 (Apaf-1), (pro-) caspase 9, cytochrome C and (d) ATP (Zou et al., 1997). While the extrinsic pathway utilizes transmembrane death receptors (for example FasR, tumor necrosis factor receptor 1 (TNFR1), DR3, and DR4/DR5) (Bazzoni and Beutler, 1996), and their corresponding ligands (for example, FasL, TNF-α, Apo3L, and Apo2L) (Elmore, 2007). Apoptosis takes place in four sequential stages: stimulus, signaling, regulation, and execution. Stimulus

occurs in response to ligand-receptor interaction, which is extrinsic, or intracellular stress, which is intrinsic; an example of which is genomic toxicity. Subsequently, regulatory proteins, such as p53, Bcl-2, **I**nhibitors of **AP**optosis (IAPs), and signaling interactomes, such as apoptosome, fine tune this dynamic process, and then a group of **C**ysteine-activated **ASP**artate-specific prote**ASES** (Caspases) will be activated and used for cleaving critical proteins in cell structure, survival and proliferation. The inability to eliminate cells with genomic instability or aberrant proliferation through apoptotic mechanisms can eventually lead to the development of cancer. This inability to eliminate cells is often associated with the loss of function of p53, a tumor suppressor protein which is important in the signaling stage of apoptosis (Yu and Zhang, 2005). When p53 gets activated, it prevents the cell from replicating by stalling the cell at the G1 and G2/M cell cycle checkpoints. Characteristic uncontrolled cellular proliferation, known as hyperplasia, and a lack of clearance of these cells leads to accumulation of genetic and/or epigenetic mutations, to transform proliferating cells into invasive, malignant cells.

1.4 AUTOPHAGY (THE YANG)

Autophagy is a catabolic process, which utilizes lysosomal hydrolases to recycle and degrade cytosolic components, proteins, and other macromolecules and organelles in response to nutrient depletion and other stresses (**Figures 2 and 3**) (Rikiishi, 2012). The word autophagy literally means "eating of the self"; this process involves the cell digesting its own intracellular components to reallocate nutrients as a means of survival during nutritional deprivation. Autophagy is important during normal development as well as in response to environmental stimuli and is vital to the maintenance of cellular homeostasis and, unlike apoptosis, can be reversible. Autophagy rids the body of aged

and damaged organelles and can help in the elimination of pathogens. While autophagy references typically focus on macroautophagy, other types of autophagy, including microautophagy and chaperone-mediated autophagy, share the same lysosomal degradation mechanism but differ in the way that material is delivered to the lysosome (Mizushima, 2007; Sahu et al., 2011).

Similar to apoptosis, autophagy has several characteristic features, including the formation of distinct cellular interactomes and structures (e.g., isolation membrane/phagophore, autophagic vesicles). It has three major phases: initiation, elongation and completion. Initiation involves the formation of a double membrane structure (from isolation membrane to autophagosome) which captures both the cytoplasm and organelles and then fuses with endosomes and lysosomes (becoming the amphisome and the autolysosome, respectively) which degrades the contents of the vesicle. The formation of this double membrane structure is a complex process involving many **A**u**T**opha**G**y-related proteins (Atgs) (Schmid and Münz, 2007). Currently, over 30 genes encoding Atgs have been identified, including the microtubule-associated protein light-chain 3 (LC3). LC3, a homologue of yeast Atg8, is required for the formation of autophagosomal membranes. LC3 is recruited to the isolation membrane, which will ultimately develop into the autophagosome where cellular targets are sequestered in preparation for degradation. The autophagosome will then fuse with endosomes/ lysosomes to create the autolysosome where cellular targets are degraded (Hippert, 2006). Under nutrient-deprived conditions, autophagy can be induced at the transcriptional and post-translational level. With regard to post-translational protein modifications, LC3, for example, exists in both a (normal) cytoplasmic and a

(autophagic) membrane-associated form (Kirisako et al., 1999). The process of incorporating LC3 to the membrane is accomplished through LC3 cleavage, lipidation and translocation. The LC3 designation is modified once cleavage has occurred as well as when the protein is localized to the membrane. Under this nomenclature, LC3-I refers to cytosolic localization following cleavage and LC3-II refers to a membrane bound LC3 (Kirisako et al., 1999). Atg4, a specific cysteine protease belonging to the caspase family, initiates LC3 processing by post-translationally cleaving LC3's C-terminal amino acid (arginine) (Ichimura et al., 2000). This cleavage generates LC3-I. The newly exposed C-terminal glycine (Gly 116) is bound by phosphatidylethanolamine (PE), a lipid constituent of plasma membranes, lipidation occurs next resulting in the formation of LC3-II. Atg4 can also act by delipidating LC3 at the lysosomal fusion step and during apoptosis Atg4 is cleaved by caspase-3 becoming highly toxic (Betin et al., 2009).

LC3-II associates with both the inner and outer membranes of the isolation membrane. Transient conjugation of LC3 to the autophagosomal membrane through an ubiquitin like system is essential for macroautophagy. The conversion of LC3-I to LC3-II has been used as an indicator of autophagic state in *in vitro* model systems (Mizushima et al., 2010). The biochemical reactions involved in LC3 cleavage, lipidation, and translocation to the isolation membrane are defining features of this type of autophagy. Autophagy uses two conjugation systems similar to the ubiquitin targeting system**.** For comparison, ubiquitin directs proteins targeted for degradation to the proteasome and is activated by an E1 enzyme. Following activation, E1 transfers the activated ubiquitin to E2 enzymes and then with E3 enzymes catalyzes the conjugation of ubiquitin to substrates. Important binding residues on LC3 can function in an ubiquitin-like fashion and LC3's crystal

structure demonstrated folds similar to ubiquitin, illustrating the resemblance of these two proteins in structure (Sugawara et al., 2004; Suzuki et al., 2005). Therefore, terms ubiquitin-like, E1-like, E2-like and E3-like are applied to these autophagic ubiquitin-like systems that produce the conjugated proteins (LC3-II-PE and Atg5-Atg12-Atg16L), which play a role in autophagosome formation, elongation and completion. In brief, the LC3 targeting system works with LC3 acting as the ubiquitin-like protein that is transferred to phosphatidylethanolamine (PE), while Atg7 functions in the same manner as an E1 enzyme; Atg3 like an E2 enzyme and the Atg12-Atg5-Atg16L complex like an E3 ligase for the LC3-II–PE complex (**Figure 4**). Residues Phe 77 and Phe 79 are located on one surface of Atg8, and residues Tyr 49 and Leu 50 are on the opposite surface. Researchers have shown that Phe 77 and Phe 79 recognize Atg4, which can also act as a de-ubiquitination enzyme. Residues Tyr 49 and Leu 50 act downstream of lipidation (Amar et al., 2006) (**Figure 5**). In addition, these multi-complex interactions potentially facilitate the loading of large proteins into a developing autophagosome (Bjorkoy et al., 2005). Upstream of the LC3-II-PE linkage, Atg12 is conjugated to Atg5 to form a stable Atg12-Atg5 conjunction. This process is mediated by Atg7, an E1-like activating enzyme, and Atg10, an E2-like conjugating enzyme. Atg16 then forms a complex with the Atg12–Atg5 conjugate (Mizushima et al., 2002).

The isolation membrane structure serves as both a signaling platform and a compartment for holding targeted proteins. Autophagy requires precise signaling and sorting of cargo in order to transport the appropriate materials to the autophagosome for degradation and recycling. The induction of autophagy causes the translocation of proteins like LC3 to the isolation membrane from the cytosol. During the elongation of the isolation membrane,

the Atg5-Atg12-Atg16L complex localizes to the membrane to form a cup-shaped structure. LC3-II then localizes to the isolation membrane, while the Atg5-Atg12-Atg16L complex dissociates. Autophagosome formation occurs and finally the autophagosome's outer membrane fuses with lysosomes to form the autolysosome which can then degrade targeted contents. LC3 is the only protein that remains associated with the completed autophagosome. Following autolysosomal formation, lysosomal hydrolases, including cathepsins, degrade the targeted proteins, while cathepsins degrade LC3-II on the inner autophagosomal surface. Following target degradation, Atg4 is involved in separating outer membrane LC3-II from the autophagosome, although LC3-II is still present in late autophagic vesicles (Mizushima et al., 2010).

Derailed autophagy has been associated with many diseases including cancer, neurodegenerative disease, and cardiovascular disease (Shintani and Klionsky, 2004). Important crosstalk between apoptosis and autophagy has been identified in colorectal cancer with possible therapeutic implications including the use of a new class of anticancer agents, histone deacetylase (HDAC) inhibitors, which induce autophagy. Autophagy has been shown to be induced in hormone-sensitive prostate cancer cells either by incubation in androgen deficient medium or by treatment with an androgen inhibitor (Kaini et al., 2012). These findings suggest that autophagy protects prostate cancer cells during androgen deprivation allowing the cells to survive nutrient depletion. Conversely, investigators were able to show that induction of autophagy has a pro-death role in pancreatic cancer cells (Mujumdar and Saluja, 2010). Pancreatic tumors have been shown to have elevated autophagy under basal conditions and studies involving autophagy inhibition using either selective inhibitors (chloroquine) or RNA interference

of essential autophagy genes appear to influence sensitivity to chemotherapy. In addition, autophagy appears to play multiple roles in pancreatic cancer as a higher LC3 expression in pancreatic cancer tissue is associated with enhanced expression of the hypoxia marker carbonic anhydrase IX in the peripheral area, correlating with poor outcome and shorter disease-free period (Kang et al., 2012). Moreover, melanoma cell lines have also been shown to exhibit high basal levels of both p62 and LC3II (Xie et al., 2013) and cutaneous malignant melanoma cells display high overall levels of autophagy (Lazova et al., 2010) suggesting that autophagy may provide an active metabolic state for invasive melanoma tumors.

1.5 P53 IN APOPTOSIS AND AUTOPHAGY

P53, a sequence-specific DNA binding protein, is a sensor of both DNA and cellular stress and is commonly called the guardian of the genome for its ability to regulate the cell cycle. The Janus or bi-functional role of p53 is illustrated by the ability of this protein to both trigger and inhibit apoptosis and autophagy based on its subcellular localization. Under normal low stress conditions, p53 is basally activated and promotes DNA repair and cell survival. However, under high stress conditions or in response to DNA damage, p53 is highly activated and upregulates cell cycle arrest and/or apoptosis. P53 directly affects the expression of two BH3-only proteins, Bad and (t)Bid, which are important regulators of both apoptosis as well as autophagy (Zhaorigetu et al., 2008).

P53 also induces apoptosis through transcription independent mechanisms including the generation of reactive oxygen species (ROS) and through its direct associations with mitochondria. P53 interacts with several apoptogenic proteins including Bax and Fas and p53-dependent apoptosis typically follows the intrinsic apoptosis pathway. However, p53

can also influence cell death through the death receptors. P53 has the ability to activate the transcription of pro-apoptotic genes, including Bax, Noxa, and PUMA, and can also trigger apoptosis through the inhibition of anti-apoptotic genes including survivin (Mirza et al., 2002), which allows the activation of caspases. Conversely, in addition to its inhibitory characteristics, p53 also has the ability to upregulate several processes including the activation of the death domain receptor (DR5) for TRAIL (TNF-related apoptosis-inducing ligand), which is activated in response to DNA damage (Wu et al., 1997) and induces apoptosis through caspase 8 activation. Once the TRAIL receptor binds its ligand, the receptor trimerizes facilitating the recruitment of pro-caspase 8 to the death domain as well as the Fas-associated death domain protein (FADD). These interactions cause the formation of a death inducing signaling complex or DISC which then assists in the cleavage of pro-caspase 8 into its active state (Sprick et al., 2000). An increase in p53 expression also enhances the levels of Fas on the cell surface by promoting its ability to translocate from the Golgi (Sionov and Haupt, 1999). In apoptosis p53 is important in the activation of the apoptosome. Upregulation of p53 is associated with an increase in Apaf-1 expression (Haupt et al., 2003), and can also trigger apoptosis through the upregulation of the pro-apoptotic Bcl-2 family members, such as Bax and PUMA (Fridman and Lowe, 2003). While p53 is a strong nuclear transcription factor it can also function in the cytoplasm where it increases mitochondrial permeability. In general, the basal level of cytosolic p53 is present and maintained through the ubiquitin proteasome pathway but at higher concentrations it may translocate into the mitochondria. Once in the mitochondria, p53 interacts with both Bcl-xL and Bcl-2, down regulating their ability to inhibit apoptosis (Tasdemir et al., 2008).

P53's role in autophagy is also multifaceted. In the cytosol it operates at the mitochondrial level to suppress autophagy through its binding to Bcl-2 and Bax (Tasdemir et al., 2008), as well as potentially through its interactions with mTOR (Tasdemir et al., 2008). Nuclear p53 upregulates apolipoprotein L1 (ApoL1), a novel BH3-only, (phospho)lipid binding protein with high affinity for phosphatidic acid (PA), that directly interacts with mTOR to increase autophagy. P53 post transcriptionally down-regulates LC3 resulting in a reduction of autophagic flux (**Figures 2 and 3**). In tumors where p53 function is lost or altered through mutations, the accumulation of excessive LC3 may ultimately result in apoptosis. P53 helps sustain the viability of cells by helping to maintain homeostasis and adjusting the rate of autophagy to nutrient stimulus (Scherz-Shouval et al., 2010). Not only does the direct activation of p53 in cell lines induce autophagy but it also upregulates the expression of the Damage Regulated Autophagy Modulator (DRAM) gene. DRAM, a p53 target, encodes lysosomal proteins which upregulate autophagy (Zhaorigetu et al., 2008) (**Figures 2 and 3**). Chemical inhibition of p53 in cell lines results in the characteristic signs of autophagy including the depletion of p62, the presence of GFP-LC3 in the cytoplasmic vacuoles (puncta), and the presence of autophagosomes and autolysosomes (Tasdemir et al., 2008). In apoptosis, p53 can activate the pro-apoptotic proteins in a transcription-independent manner and DNA-damage leads to mitochondrial translocation of p53 and intrinsic pathway upregulation by increasing PUMA. P53 can bind directly to the Bcl-2 family member Bcl-xL and influence cytochrome c release through direct activation of Bax to upregulate apoptosis. P53 can release both pro-apoptotic multi-domain proteins and BH3-only proteins that are sequestered by Bcl-xL. Finally, the cross-talk between apoptosis and

autophagy is shown through the ability of p53 to post transcriptional inhibition LC3 by p53, which reduces the overall rate of autophagy ultimately causing excessive LC3 accumulation resulting in apoptosis (Scherz-Shouval et al., 2010).

Targeting p53 in order to develop novel anti-cancer treatments has broad implications. The two major strategies include targeting of wildtype (wt.) p53 or mutant p53. With respect to wt. p53, drugs that activate endogenous p53 at protein or transcription levels through small molecules would be preferable. For example, **R**eactivation of p53 and **I**nduction of **T**umor cell **A**poptosis (RITA), which binds to and activates p53 thereby inducing apoptosis, has shown promise in suppressing tumor growth in culture (Roh et al., 2010). CP-31398 was the first small molecule developed that increases expression of wt. p53 through the reduction of p53 ubiquitination. In addition, small molecules which target p53 transcription may reduce the adverse effects of chemotherapy or radiation in normal proliferating cells. One such compound, Pifithrin, can reversibly inhibit p53 transcriptional activation, block p53 activation in normal cells, and thus reduce apoptosis. In targeting mutant p53, new compounds which re-activate wt. p53 activities through conformational changes of mutant p53 have been developed (Wang and Sun, 2010). For example, ellipticine, **P**53 **R**eactivation and **I**nduction of **M**assive **A**poptosis (PRIMA-1), and **M**utant p53 reactivation and **I**nduction of **R**apid **A**poptosis (MIRA-1) have been shown to induce a conformational change of mutant p53 that revert the protein to wild type activity. Interestingly, UCN01, a bifunctional modulator of cyclin-dependent kinases, can arrest cancer cells harboring mutant p53 at the G2 checkpoint leading to cell death (Facchinetti et al., 2004).

1.6 THE BCL-2 FAMILY MEMBERS IN APOPTOSIS AND AUTOPHAGY

Bcl-2 (B cell lymphoma/leukemia type 2) is a member of a family of proteins, with the same name, that convey either pro- or anti-death signaling. The interactions of the Bcl-2 proteins determine the fate of a cell tipping the homeostatic balance to favor apoptosis, or autophagic survival. The anti-death Bcl-2 members (e.g. Bcl-2 and Bcl-xL) suppress apoptosis and promote cell survival and mutations in the Bcl-2 gene have been shown to be associated with many cancers. The BH3-only proteins which contain a single Bcl-2 homology (BH) domain can interact with the anti-death Bcl-2 family members to induce apoptosis (Tuffy et al., 2010). These interactions also trigger downstream events which can ultimately permeabilize the mitochondrial membrane, resulting in the release of the cytochrome c and resulting in the activation of caspases (**Figure 1**) (Shintani and Klionsky, 2004). The BH3-only proteins can also propagate stress signals and indirectly activate Bax and Bak, the pro-death members, responsible for the induction of apoptosis and/or blockage of autophagy. For example, ApoL6, a newly identified members of the BH3-only proteins, when overexpressed, induces apoptosis and blocks Beclin 1-mediated autophagy simultaneously (Liu et al., 2005; Zhaorigetu et al., 2011) (**Figures 1 and 3**). Interestingly, Beclin 1 has also been shown to be a *bona fide* BH3-only protein, however, when overexpressed, it does not induce apoptosis but rather autophagy (Fan et al.,2013). On the other hand, Bcl-2 interferes with Bax or Bak function by forming heterodimers with them. When separated from Bcl-2, Bax or Bak forms homodimers or heterodimers which allow permeabilization of the mitochondrial membrane (Ruvolo et al., 2001). Interestingly, the interplay of Bcl-2 kinase and phosphatase allows for rapid and reversible regulation of Bcl-2's activity in response to stress signals or damage (Ruvolo et al., 2001). Bcl-2 interacts either directly or indirectly

with siblings including Bik, Bid, Bim and PUMA (see below) to affect cell death and survival.

Bcl-2 also plays a Janus role regulating both apoptosis and autophagy through its interactions with their common proteins linking these pathways, allowing for a more responsive cell survival/ cell death switching mechanism. Bcl-2 inhibits both autophagy and apoptosis through direct binding to Beclin 1 (Jegga et al., 2011). Beclin 1 is a core component in the enzymatic complex phosphoinositides-3-kinase class III (PI3KC3) which initiates the formation of autophagosomes (Moscat and Diaz-MecoSee 2009). Bcl-2 binding to Beclin 1 allows the survival of cells when they are deprived of their growth factors. Disruption of this interaction by pro-apoptotic proteins such as Bad and ApoL6 induces autophagy (Zhaorigetu et al., 2008, Li et al., 2007). Under starvation conditions, the stress activated signaling molecule, c-Jun N-terminal protein kinase 1 (JNK1) mediates the induction of multisite phosphorylation of cellular Bcl-2. This phosphorylation results in the dissociation of Bcl-2 from Beclin 1 and the subsequent activation of autophagy (Wei et al., 2008). Other research has indicated that cells containing mutations in Beclin 1 inhibit its ability to bind Bcl-2 and induce autophagy related cell death (Pattingre et al., 2005).

Other findings implicate autophagy as a mechanism of resistance to anti-angiogenic therapies in colon cancer cells supporting the importance of investigations into inhibitory approaches in the management of this disease (Selvakumaran et al., 2013). In addition, researchers have shown that autophagosomes are actively produced in colorectal cancer cells under nutrient starvation, and treatment with autolysosome inhibitors like 3-methyladenine, which inhibits autophagy by blocking autophagosome

formation through inhibition of PI3KC3, enhances apoptosis. Overall it appears that autophagy is activated in colorectal cancers both in vitro and in vivo, and may contribute to the cancer cell survival (Sato et al., 2007, Prabhudesai et al., 2007). Overexpression of the pro-survival Bcl-2 family members, for example, Bcl-2 and Bcl-xL, is associated with tumor progression, as well as resistance to chemotherapy. Bcl-2 small molecule inhibitors (**Figure 1**), including mimics of BH3-only proteins and Bcl-2 antagonists, induce activation of pro-apoptotic proteins and overcome chemoresistance in cancer cells. Inhibitors like ABT-263 (Navitoclax) and AT-101, both mimics of the BH3-only domain, have a broader range of targets binding to both Bcl-2 and Bcl-xL. On the other hand, inhibitors, like GX15-070 (Obatoclax), are antagonists against only Bcl-2 and lower the apoptotic threshold in tumor cells already damaged by chemotherapy. Functions of several Bcl-2 drugs are listed in **Table 1** and shown on **Figure 3**. Please refer to other outstanding reviews and articles for details (Azmi et al., 2011; Bodur and. Basaga 2012; Kamal et al., 2014; Bai and Wang 2014).

1.7 ATG PROTEINS IN AUTOPHAGY AND APOPTOSIS

Atgs are regulated in response to the availability of nutrients and growth factors including amino acids. These signals are generated through a serine/threonine kinase known as the mammalian/mechanistic target of rapamycin (mTOR) (Kamada et al., 2004). MTOR is an important negative regulator of autophagy that exists in two distinct complexes, mTORC1 and mTORC2. MTORC1 is typically active and serves as a nutrient deprivation sensor. MTORC2 is a regulator of AKT phosphorylation, thus inhibition of mTORC2 results in G1 cell cycle arrest as a consequence of the inhibition of AKT phosphorylation (Feldman et al., 2009). Under normal conditions mTOR signaling

results in the hyper-phosphorylation of several Atgs which leads to inhibition of the autophagic process. For example, mTOR phosphorylates Atg13 and disrupts its binding to two orthologs of Atg1, ULK1 and ULK2, which are required for autophagy (Grasso et al., 2012). ULK1/2 is essential for both the initial construction of the autophagosome as well as acting as a nutrient sensing center in a complex with Atg13 and Atg17. Atg13 in conjunction with a second protein, FIP200, is critical for correct localization of ULK1 to the isolation membrane and for the stability of ULK1 protein. Interestingly, ULK1 also has bi-functionality that can be unregulated in response to activated p53, resulting in cell death (Gao et al., 2011).

1.8 P62 IN APOPTOSIS AND AUTOPHAGY

Other proteins can facilitate the targeting of LC3. Initiation signals from autophagy can recruit p62, a multi-domain ubiquitin-binding, signaling adaptor protein also known as sequestosome 1 (47). P62 can assemble proteins that have been tagged by ubiquitin through its polymerization with other p62 molecules. This ability to aggregate permits p62 clusters to recognize, assemble, and deliver the targeted cargo into the autophagosomes via binding to LC3. P62 aggregates are often termed speckles due to their cytosolic visibility and these speckles act as organizing centers where p62 is able to interact with caspase 8 as well as other factors (Sanz et al., 2000). Inhibition of autophagy results in larger p62 speckles which appear experimentally to co-localize with poly ubiquitinated proteins that are normally degraded by autophagy (Komatsu et al., 2007). It is postulated that in addition to its autophagic role in aggregation p62 functions to compact toxic proteins (Moscat and Diaz-MecoSee 2009). P62 is also implicated in the activation of NF-κB, a transcription factor (Baldwin, 2012) that plays an important role in

the inhibition of apoptosis. These results indicate that p62 plays a role in the apoptosis pathway as well as in autophagy. Further experiments suggest that p62 is required for the extrinsic apoptosis pathway in particular for the proper functioning of the death receptors DR4 and DR5 also needs p62. These data imply that p62 speckles are signaling centers which determine either cell survival through autophagy, triggering the NF-κB pathway, or by aggregating caspase-8 to signal the cell to undergo apoptosis.

Research has identified two caspase cleavage sites in Beclin-1 that once cleaved result in fragments which are unable to induce autophagy. The cleavage site at the c-terminus of Beclin 1 sensitizes cells to apoptosis once cleaved was able to induce the release of proapoptotic factors. These findings point to a mechanism by which caspase -dependent generation of a cleaved Beclin-1 enhances apoptosis. In addition, growth factor withdrawal in a murine pro-B cell line (Ba/F3) results in caspase mediated cleavage of both Beclin-1 and PI3K (Moscat and Diaz-MecoSee 2009). Finally, research has shown that increased ApoL6 expression has been shown to induce the degradation of Beclin 1, which results in p62 accumulation, as well as reducing LC3-II formation and translocation, ultimately hindering autophagy (Zhaorigetu et al., 2008).

1.9 THE LYSOSOME IN APOPTOSIS AND AUTOPHAGY

The lysosome is one of the required organelles of autophagy, as well as a way to distinguish three different types of autophagy which are partially defined by the way the lysosome interact with cargo (Hotchkiss et al., 2009). In general, the term autophagy typically refers to macroautophagy, where the double membrane autophagosome envelops its cargo, fuses with lysosomes and forms the autolysosome. Microautophagy refers to the second mechanism where the lysosome appears to bring cargo in from the

cytosol. Lastly, chaperone-mediated autophagy utilizes heat shock proteins to deliver substrates to lysosomes for degradation (Hotchkiss et al., 2009).

The lysosome, a major depot of non-specific hydrolases within the cell, has a single lipid-bilayer membrane. Changes in lysosomal structure or damage to its membrane can lead to lysosomal destabilization. Factors including ROS, proteases, p53, and Bcl-2 family proteins which contribute to the permeabilization of the lysosomal membrane are similar to those which can affect apoptosis and autophagy. Lysosomal destabilization or lysosomal membrane permeabilization (LMP) can occur in response to cell death signals resulting in the leakage or release of the lysosomal cathepsins. The release of cathepsins regulates apoptosis signaling and LMP can be triggered by a wide range of apoptotic and autophagic signals including death receptor activation, ER stress, DNA damage, and growth factor starvation (Boya and Kroemer, 2008; Chwieralski et al.,2006; Guicciardi et al.,2004; Stoka et al.,2007). The induction of apoptosis can be dependent on this early release of cathepsins, but it can also occur late in the apoptotic process and amplify the death signal. The cysteine cathepsins and the aspartic protease cathepsin D are both very abundant in the lysosome and are involved in apoptosis signaling (66-69). Excess ROS may destabilize the lysosomal membrane resulting in the rapid release of cathepsins to the cytosol (Werneburg et al., 2007). Cell death by LMP has also been linked to the direct activation of caspases by lysosomal proteases (Ishisaka et al., 1998). In addition, pro-apoptotic Bax relocates to the lysosomes and co-localizes with Bim suggesting that this localization may result in Bax-mediated LMP and ultimately apoptosis. Bcl-2 family members may also regulate LMP through the mitochondria (Yuan et al., 2002). There is also evidence that LMP may occur in p53-

induced apoptosis where p53 actually localizes to the lysosomes and triggers LMP (Li et al., 2007). It may also upregulate LMP and Bax-mediated mitochondrial permeabilization without translocating to the lysosome but rather through the upregulation of the gene p53 up-regulated modulator of apoptosis (PUMA) and Noxa (Oda et al., 2000; Yu et al., 2001; Nakano and Vousden, 2001). Noxa encodes a Bcl-2 homology 3 (BH3)-only member of the Bcl-2 family of proteins. When ectopically expressed, Noxa localizes to the mitochondria where it interacts with and suppresses the anti-apoptotic Bcl-2 family members and also results in the activation of caspase-9.

Following the activation of apoptosis, another protein called lysosome associated apoptosis inducing protein (LAPF) associates with lysosomes and is essential for DNA damage induced LMP (Li et al., 2007, Chen et al., 2005). Downregulation of either p53 or LAPF prevents the induction of LMP (48). LMP may also occur during other types of cell death, including autophagic cell death (Kroemer et al., 2005). Transcriptional analysis has established common binding sites both in autophagy and on lysosomal genes showing an additional association (46). ApoL1 localizes in the cytosol and lysosomes suggesting that ApoL1 regulated autophagy, may involve the lysosomes (Zhaorigetu et al., 2008). The lysosome is an extremely important structure in both homeostasis and cell death.

Targeting the lysosome by chemical inhibitors is typically accomplished using lysosomotropic agents. It is well known that intermittent use of the lysosomotropic weak bases like chloroquine or hydroxychloroquine can prevent cancer in mouse models. This data suggests that chemicals which target the lysosome may be effective in cancer prevention. Chloroquine, a weak base, functions through its preferential accumulation in

the lysosome where it becomes protonated and cannot diffuse out (Maclean et al., 2008). This accumulation results in the inactivation of lysosomal hydrolases, including cathepsins, which are necessary for lysosomal degradation.

Other drugs which target the lysosome include the macrolide antibiotic bafilomycins, particularly bafilomycin A1 (Baf-A1). This antibiotic derived from *Streptomyces griseus* is a specific inhibitor of vacuolar-type H+ ATPases (V-ATPase) and prevents maturation of autophagic vacuoles by preventing the fusion between autophagosomes and lysosomes (Yamamoto et al., 1989). Cancer cells overexpress V-ATPase when compared to non-tumorigenic cells, and may be more sensitive to treatment with Baf-A1 or concanamycin A (ConA), another potent V-ATPase inhibitor (**Figure 2**; Morimura et al., 2008; Huss et al., 2002). Interestingly, am emerging drug, Concanavalin A (ConVA), induces lysosome- and mitochondria-mediated apoptosis. ConVA is a lectin or carbohydrate-binding protein which binds mannose displayed on cell surfaces. ConVA is then internalized preferentially to the mitochondria, although some endocytosed ConVA will accumulate in the lysosome and also changes the membrane permeability on the mitochondria and initiates apoptosis. Many tumors have increased expression of lectins on their plasma membrane, therefore ConVA preferentially binds to tumor cells (Alonso et al., 2006). Thus, through the identification of small molecules or chemical inhibitors that induce lysosomal permeabilization or increase lysosomal facilitated autophagy–associated cell death, targeting the lysosome may prove to be an effective strategy for treatment of cancer (Fehrenbacher and Jäättelä, 2005; **Figure 2**; **Table 1**).

1.10 PUMA IN APOPTOSIS AND AUTOPHAGY

PUMA, which is a BH3-only protein (Nakano and Vousden, 2001), has an expression pattern consistent with a causative role in p53-dependent apoptosis. PUMA is a pro-apoptotic protein and rapidly induces apoptosis through a Bax- and mitochondria-dependent pathway (Han et al., 2001). PUMA can be directly activated p53 through p53-responsive elements in its promoter region and can also be induced by the chemotherapeutic (e.g., Adriamycin/doxorubicin) in a p53-dependent fashion (Yu et al., 2003). Protein isoforms encoded by PUMA have also been shown to localize in the mitochondria where they interact with Bcl-2 and Bcl-xL via the BH3 domain (Yu et al., 2003). Structural analysis has shown that PUMA directly binds to these anti-apoptotic Bcl-2 family proteins (Day et al., 2008) and is essential for apoptosis induced by both exogenous and endogenous p53. In cancer cell lines PUMA has been shown to dissociate Bax and Bcl-xL to induce apoptosis (Ming et al., 2006). Following chemicals and/or genotoxic-induced DNA damage (Yu and Zhang, 2008), both p53 and PUMA are activated. Independent of p53, PUMA expression can also be induced by oncogenic stress (Fernandez et al., 2003; Maclean et al., 2003), growth factor withdrawal, inhibition of kinases including FOXO (Han et al., 2001, Maclean et al., 2003, You et al., 2006), ER stress and altered redox (Reimertz et al., 2003;, Ward et al., 2004), as well as infection (Castedo et al., 2004) all resulting in apoptosis upregulation. It have been shown that nuclear factor-kB (NF-kB) can assist in p53-dependent PUMA induction through recruitment to the PUMA promoter during response to DNA damage (Yu and Zhang, 2008). Additionally, many tumors possess p53 mutations (Vogelstein and Kinzler, 2004) which prevent PUMA induction during DNA damage response (Yu and Zhang, 2005). PUMA induces apoptosis through the generation of both superoxide and hydrogen

peroxide in a Bax-dependent manner. Mitochondrial translocation and multimerization of Bax is a critical event in mitochondrial-mediated apoptosis (Nakano and Vousden, 2001). Interestingly, hypoxia results in metabolic changes which induce p53 to transcriptionally activate genes including PUMA. After its activation, PUMA will interact with all the anti-apoptotic Bcl-2 family members, releasing Bax or Bak which induce apoptosis in the mitochondria (Yu and Zhang, 2008). The induction of PUMA causes Bax to form multimers resulting in mitochondrial dependent cell death. PUMA has recently been shown to induce autophagy which appears to lead to targeting of the mitochondria for removal by autophagy, or mitophagy (Yee et al., 2009), mediated through Bax and Bak. This is interesting since PUMA also induces apoptosis in a Bax-dependent manner (Yu et al., 2003). In addition, the inhibition of PUMA- or of Bax-induced autophagy results in a reduction in apoptosis suggesting that targeting mitophagy could enhance apoptosis. Because of its role in both apoptosis and autophagy, PUMA is extremely interesting and may provide a vital link in the communication between these two programmed cell death pathways.

1.11 INHIBITORS OF APOPTOSIS (IAPS)

Inhibitor of apoptosis proteins (IAPs) are a family of anti-apoptotic proteins that promote cancer cell survival. IAPs exert a range of biological activities that promote cell survival and proliferation. X chromosome-linked IAP (XIAP) is a direct inhibitor of caspases whereas cellular IAPs block the assembly of pro-apoptotic protein signaling complexes and mediate the expression of anti-apoptotic molecules. In general, expression and function of IAPs are deregulated in human cancer cells due to genetic aberrations, increase of their mRNA or protein expression levels, or loss of endogenous inhibitors,

such as second mitochondria-derived activator of caspase (SMAC). As IAPs are expressed at high levels in various types of cancer and have been linked to tumor progression, treatment failure and poor prognosis, IAPs are promising targets for cancer therapeutic intervention (Stern et al., 2012). Several therapeutic targeting strategies that have been designed to target IAPs thus far, such as small-molecule IAP antagonists and antisense oligonucleotides. Among them, the most common approach is based on mimicking the IAP-binding motif of SMAC, an endogenous IAP antagonist. These antagonists include AT-406, and YM155. It has been shown that AT-406 selectively inhibits the activity of XIAP, cellular IAPs 1 (c-IAP1) and 2 (c-IAP2), and melanoma inhibitor of apoptosis protein (ML-IAP). This inhibition results in the promotion of apoptosis (Brunckhorst et al., 2012). AEG35156, a XIAP antisense oligonucleotide, has been shown to sensitize malignant cells to chemotherapies both *in vitro* and murine in vivo models (Schimmer et al., 2009) (**Table 1**). Preclinical studies have indicated that the therapeutic potential of IAP antagonists might best be exploited in combination protocols, including conventional chemotherapeutic drugs, signal transduction modulators, death receptor agonists or radiation therapy (Fulda and Vucic, 2012).

In conclusion, the environment encountered by tumor cells requires adaption to low blood supply, low nutrients, and hypoxic conditions that contributes to the cell's avoidance of multiple cell death pathways. The loss of homeostasis between apoptosis and autophagy has been postulated as both a causative factor in tumorigenesis as well as an area for therapeutic intervention. In general, the autophagic survival mechanism blocks apoptosis in most cancer types (Stern et al., 2012; Rubinsztein et al. 2012; Yao et al., 2011; Harhaji-Trajkovic et al., 2012). In designing therapies to eliminate cancer cells,

specifically through apoptosis rather than necrosis, one might consider (a). targeting the activation of the cytosolic pro-apoptotic p53 interactome as well as inactivating the pro-autophagic p53 interactome; (b). inducing degradation of Beclin 1 and Atg5 for the generation of pro-apoptotic form of Beclin 1-C and Atg5N; (c). mobilization of Bcl-2 and Bcl-xL from Bax/Bak interactome to Beclin 1 interactome; and (d). activation of PUMA, ApoL6, or ApoL1 in a cancer cell specific manner. Thus, the crosstalk between apoptosis and autophagy remains a highly investigated and promising area for the identification of novel therapeutics and research into the interactions which maintain molecular homeostasis between these two death pathways will continue to further our understanding of the dynamics between cell death and cancer etiology.

1.12 ABBREVIATIONS:

ApoL1, Apolipoprotein L1; ApoL6, Apolipoprotein L6; AMPK, AMP-activated protein kinase; Apaf-1, apoptotic proteinase-activating factor-1; Atg, Autophagy-related gene; BAD, Bcl-2-associated death promoter; Bcl-2, B-cell lymphoma 2; Beclin 1, Bcl-2 interacting protein 1; BH3, Bcl-2-homology-3 domain; BH, Bcl-2 homology; BID, BH3 interacting-domain death agonist; DISC, death-inducing signaling complex; DR5, the death domain receptor; DRAM, damage-regulated modulator of autophagy; FADD, FAS-associated death domain protein; FLICE, FADD-like IL-1 β-converting enzyme; FIP200, focal adhesion kinase -interacting protein FIP 200 fragment; HDAC, histone deacetylase; IAPs, inhibitors of apoptosis proteins; JNK, c-Jun N-terminal kinase; LC3, microtubule-associated protein 1 light chain 3; MAPK, mitogen-activated protein kinase; MOMP, mitochondrial outer membrane permeabilization; mTOR, mammalian (mechanistic) target of rapamycin; PA, phosphatidic acid; PCD, programmed cell death; PE, phosphatidylethanolamine; PI3K, phosphoinositide 3-kinase; ROS, reactive oxygen species; SMAC, second mitochondrial-derived activator of caspase; TNF-α, tumor necrosis factor-α;

TRAIL, tumor necrosis factor-related apoptosis inducing ligand; TSC1/2; tuberous sclerosis complex1/2; ULK1, unc-51-like kinase 1; TNFR1 tumor necrosis factor receptor 1; Vps34, vacuolar protein sorting-34.

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1.14 REFERENCES:

Alonso, E., Gómez, L., Madrid, J.F., and Sáez, F.J. 2006. Identification of mannose moieties in N- and O-linked oligosaccharides of the primordial germ cells of Xenopus embryos. Microsc. Res. Tech. 69: 595–599.

Amar, N., Lustig, G., Ichimura, Y., Ohsumi, Y., and Elazar, Z. 2006. Two newly identified sites in the ubiquitin-like protein Atg8 are essential for autophagy. EMBO Reports 7: 635–642.

Azmi, A.S., Wang, Z., Philip, P.A., Mohammad, R.M. and Sarkar, F.H. 2011. Emerging Bcl-2 inhibitors for the treatment of cancer. Expert Opin. Emerg. Drugs 16:59-70.

Bai, L., and Wang, S., 2014. Targeting apoptosis pathways for new cancer therapeutics. Annu. Rev. Med. 65:139-155.

Baldwin, A.S. 2012. Regulation of cell death and autophagy by IKK and NF-κB: critical mechanisms in immune function and cancer. Immunol. Rev. 246:327-345.

Bazzoni, F., and Beutler, B., 1996. The tumor necrosis factor ligand and receptor families. New Engl. J. Med. 334:1717-1725.

Betin, V.M., and Lane, J.D., 2009. Atg4D at the interface between autophagy and apoptosis. Autophagy 5:1057-1059.

Bjorkoy, G., Lamark, T., Brech, A., Outzen, H., Perander, M., Overvatn, A., Stenmark, H., and Johansen,T. 2005. p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. J. Cell Biol. 171:603–614.

Bodur, C., and Basaga, H. 2012. Bcl-2 inhibitors: emerging drugs in cancer therapy. Curr. Med. Chem. 19:1804-1820.

Boya, P., and Kroemer, G. 2008. Lysosomal membrane permeabilization in cell death. Oncogene 27:6434–6451.

Brunckhorst, M.K., Lerner, D., Wang, S., and Yu, Q. 2012. AT-406, an orally active antagonist of multiple inhibitor of apoptosis proteins, inhibits progression of human ovarian cancer. Cancer Biol. Ther. 13:804-811.

Castedo, M., Perfettini, J.L., Piacentini, M., and Kroemer, G. 2005. p53-A pro-apoptotic signal transducer involved in AIDS. Biochem. Biophys. Res. Commun. 331: 701–706.

2005. The lysosome-associated apoptosis-inducing protein containing the pleckstrin homolog PH and FYVE domains LAPF, representative of a novel family of PH and FYVE domain-containing proteins, induces caspase-independent apoptosis via the lysosomal-mitochondrial pathway. J. Biol. Chem. 280: 40985–40995.

Chwieralski, C.E., Welte, T., and Bühling, F. 2006. Cathepsin-regulated apoptosis. Apoptosis 11:143–149.

Coleman, M.L., Sahai, E.A., Yeo, M., Bosch, M., Dewar, A., and Olson, M.F. 2001. Membrane blebbing during apoptosis results from caspase-mediated activation of ROCK I. Nat. Cell Biol. 3:339-345.

Day, C.L., Smits, C., Fan, F.C., Lee, E.F., Fairlie, W.D., and Hinds, M.G. 2008. Structure of the BH3 domains from the p53-inducible BH3-only proteins Noxa and Puma in complex with Mcl-1. J. Mol. Biol.380:958–971.

Elmore, S. 2007. Apoptosis: a review of programmed cell death. Toxicol. Pathol. 35: 495-516.

Facchinetti, M.M., De Siervi, A., Toskos, D., and Senderowicz, A.M. 2004. UCN-01-induced cell cycle arrest requires the transcriptional induction of p21waf1/cip1 by activation of mitogen-activated protein/extracellular signal-regulated kinase kinase/extracellular signal-regulated kinase pathway. Cancer Res. 64:3629-3637.

Fan, Y.J., and Zong, W.X. 2013. The cellular decision between apoptosis and autophagy. Chin. J. Cancer. 323: 121–129.

Feldman, M.E., Apsel, B., Uotila, A., Loewith, R., Knight, Z.A., Ruggero, D., and Shokat, K.M. 2009. Active-Site Inhibitors of mTOR Target Rapamycin-Resistant Outputs of mTORC1 and mTORC2. PLoS Biol. 7:e38.

Fehrenbacher, N., and Jäättelä, M. 2005. Lysosomes as Targets for Cancer Therapy. Cancer Res. 65: 2993-2995.

Fernandez, P.C., Frank, S.R., Wang, L., Schroeder, M., Liu, S., Greene, J., Cocito, A., and Amati, B. 2003. Genomic targets of the human c-Myc protein. Genes Dev. 17:1115–1129.

Fridman, J.S., and Lowe, S.W. 2003. Control of apoptosis by p53. Oncogene 22: 9030–9040.

Fulda, S., and Vucic, D. 2012. Targeting IAP proteins for therapeutic intervention in cancer. Nat.Rev. Drug Discov. 11:109-124.

Gao W., Shen, Z., Shang, L. and Wang, X. 2011. Upregulation of human autophagy initiation kinase ULK1 by tumor suppressor p53 contributes to DNA damage induced cell death. Cell Death Differ. 18:1598-1607.

Grasso, D., Garcia, MN., and Iovanna, J.L. 2012. Autophagy in Pancreatic Cancer. Int J Cell Biol. 760498, 7 pages.

Guicciardi, M.E., Leist, M., and Gores, G.J. 2004. Lysosomes in cell death. Oncogene 23:2881–2890.

Gukovsky, I., Li, N., Todoric, J., Gukovskaya, A., and Karin, M. 2013. Inflammation, autophagy, and obesity: common features in the pathogenesis of pancreatitis and pancreatic cancer. Gastroenterology 144:1199-1209.

Han, J., Flemington, C., Houghton, A.B., Gu, Z., Zambetti, G.P., Lutz, R.J., Zhu, L., and Chittenden, T. 2001. Expression of bbc3, a pro-apoptotic BH3-only gene, is regulated by diverse cell death and survival signals. Proc. Natl. Acad. Sci. USA 98: 11318–11323.

Harhaji-Trajkovic, L., Arsikin, K., Kravic-Stevovic, T., Petricevic, S., Tovilovic, G., Pantovic, A., Zogovic, N., Ristic, B., Janjetovic, K., Bumbasirevic, V., and Trajkovic, V.

2012. Chloroquine-mediated lysosomal dysfunction enhances the anticancer effect of nutrient deprivation. Pharm. Res. 29:2249-2263.

Haupt, S., Berger, M., Goldberg, Z., and Haupt, Y. 2003. Apoptosis – the p53 network. J. Cell Sci. 116:4077-4085.

Hippert, M.M., O'Toole, P.S., and Thorburn, A. 2006. Autophagy in Cancer: Good, Bad, or Both? Cancer Res. 66: 9349-9951.

Hotchkiss, R.S., Strasser, A., McDunn, J.E., and Swanson, P.E. 2009. Cell Death. N Engl. J. Med. 361:1570-1583.

Huss, M., Ingenhors, G., Konig, S., Gaßel, M., Drose, S., Zeeck, A., Altendorf, K., and Wieczorek, H. 2002. Concanamycin A, the Specific Inhibitor of V-ATPases, Binds to the Vo Subunit c. J. Biol. Chem. 277:40544–40548.

Ichimura, Y., Kirisako, T., Takao, T., Satomi, Y., Shimonishi, Y., Ishihara, N., Mizushima, N., Tanida, I., Kominami, E., Ohsumi, M., Noda, T., and Ohsumi, Y. 2000. A ubiquitin-like system mediates protein lipidation. Nature 408: 488–492.

Ishisaka, R., Utsumi, T., Yabuki, M., Kanno, T., Furuno, T., Inoue, M., and Utsumi, K. 1998. Activation of caspase-3-like protease by digitonin-treated lysosomes. FEBS. Letters 435:233–236.

Jegga, A.G., Schneider, L., Ouyang, X., and Zhang J. 2011. Systems biology of the autophagy-lysosomal pathway. Autophagy 5:477-89.

Kaini, R.R., Sillerud, L.O., Zhaorigetu, S., and Hu, C.A. 2012. Autophagy regulates lipolysis and cell survival through lipid droplet degradation in androgen-sensitive prostate cancer cells. Prostate 72:1412-1422.

Kamada, Y., Sekito, T., and Ohsumi, Y. 2004. Autophagy in yeast: a TOR-mediated response to nutrient starvation. Curr. Top. Microbiol. Immunol. 279: 73–84.

Kamal, A., Faazil, S., and Malik, M.S. 2014. Apoptosis-inducing agents: a patent review 2010 - 2013. Expert Opin. Ther. Pat. 24:339-354.

Kang, R., and Tang, D. 2012. Autophagy in pancreatic cancer pathogenesis and treatment. Am. J. Cancer Res. 2: 383–396.

Kirisako, T., Baba, M., Ishihara, N., Miyazawa, K., Ohsumi, M., Yoshimori, T., Noda T., and Ohsumi, Y. 1999. Formation Process of Autophagosome Is Traced with Apg8/Aut7p in Yeast. J. of Cell Biol. 147:435–446.

Komatsu, M., Waguri, S., Koike, M., Sou, Y.S., Ueno, T., Hara, T., Mizushima, N., Iwata, J., Ezaki, J., Murata, S., Hamazaki, J., Nishito, Y., Iemura, S., Natsume, T., Yanagawa, T., Uwayama, J., Warabi, E., Yoshida, H., Ishii, T., Kobayashi, A., Yamamoto, M., Yue, Z., Uchiyama, Y., Kominami, E., and Tanaka, K. 2007. Homeostatic levels of p62 control cytoplasmic inclusion body formation in autophagy-deficient mice. Cell 131:1149–1163.

Kroemer, G., and Jaattela, M. 2005. Lysosomes and autophagy in cell death control. Nat. Rev. Cancer. 5:886–897.

Lazova, R., Klump, V., and Pawelek, J. 2010. Autophagy in cutaneous malignant melanoma. J. Cutan. Pathol. 37: 256–268.

Li, M.O., Sarkisian, M.R., Mehal, W.Z., Rakic, P., and Flavell, R.A. 2003. Phosphatidylserine receptor is required for clearance of apoptotic cells. Science 302:1560–1563.

Li, N., Zheng, Y., Chen, W., Wang, C., Liu, X., He, W., Xu, H., and Cao, X. 2007. Adaptor protein LAPF recruits phosphorylated p53 to lysosomes and triggers lysosomal destabilization in apoptosis. Cancer Res. 67:11176–11185.

Liu, Z., Lu, H., Jiang, Z., Pastuszyn, A., and Hu, C.A. 2005. Apolipoprotein L6, a novel proapoptotic Bcl-2 homology 3-only protein, induces mitochondria-mediated apoptosis in cancer cells. Mol. Cancer Res. 3:21-31.

Maclean, K.H., Dorsey, F.C., Cleveland, J.L. and Kastan, M.B. 2008. Targeting lysosomal degradation induces p53-dependent cell death and prevents cancer in mouse models of lymphomagenesis. J. Clin. Invest. 118:79-88.

Maclean, K.H., Keller, U.B., Rodriguez-Galindo, C., Nilsson, J.A., and Cleveland, JL. 2003. c-Myc augments gamma irradiation-induced apoptosis by suppressing Bcl-XL. Mol. Cell Biol. 23:7256–7270.

Ming, L., Wang, P., Bank, A., Yu, J., and Zhang, L. 2006. PUMA Dissociates Bax and Bcl-XL to Induce Apoptosis in Colon Cancer Cells. The J. Biol. Chem. 281:16034–16042.

Mirza, A., McGuirk, M., Hockenberry, T.N., Wu, Q., Ashar, H., Black, S., Wen, S.F., Wang, L., Kirschmeier, P., Bishop, W.R., Nielsen, L.L., Pickett, C.B., and Liu, S. 2002. Human survivin is negatively regulated by wild-type p53 and participates in p53-dependent apoptotic pathway, Oncogene 21: 2613-2622.

Mizushima, N. 2007. Autophagy: process and function. Genes Dev. 21: 2861-2873

Mizushima, N., Ohsumi, Y., and Yoshimori, T. 2002. Autophagosome Formation in Mammalian Cells. Cell Struct. Funct. 27: 421-429.

Mizushima, N., Yoshimorim, T., and Levine, B. 2010. Methods in Mammalian Autophagy Research. Cell 140: 313–326.

Morimura, T., Fujita, K., Akita, M., Nagashima, M., and Satomi, A. 2008. The proton pump inhibitor inhibits cell growth and induces apoptosis in human hepatoblastoma. Pediatr. Surg. Int. 24:1087-1094.

Moscat, J., and Diaz-MecoSee, M.T. 2009. p62 at the Crossroads of Autophagy, Apoptosis, and Cancer. Cell 137: 1001-1004.

Mujumdar, N., and Saluja, AK. 2010. Autophagy in pancreatic cancer: An emerging mechanism of cell death. Autophagy 6:997-998.

Nakano, K., and Vousden, K.H. 2001. PUMA, a novel proapoptotic gene, is induced by p53. Mol Cell. 7: 683–694.

Oda, E., Ohki, R., Murasawa, H., Nemoto, J., Shibue, T., Yamashita, T., Tokino, T., Taniguchi, T., and Tanaka, N. 2000. Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. Science 288:1053–1058.

Pattingre, S., Tassa, A., Qu, X., Garuti, R., Liang, X.H., Mizushima, N., Packer, M., Schneider, M.D., Levine, B. 2005. Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. Cell 122:927-939.

Prabhudesai, S.G., Rekhraj, S., Roberts, G., Darzi, A.W., Ziprin, P. 2007. Apoptosis and chemo-resistance in colorectal cancer. J. Surg. Oncol. 96:77–88.

Reimertz, C., Kögel, D., Rami, A., Chittenden, T., and Prehn, J.H. 2003. Gene expression during ER stress-induced apoptosis in neurons: induction of the BH3-only protein Bbc3/PUMA and activation of the mitochondrial apoptosis pathway. J Cell Biol. 162:587–597.

Rikiishi, H. 2012. Novel Insights into the Interplay between Apoptosis and Autophagy. Int. J. Cell Biol. 2012:317645.

Roh, J.L., Ko, J.H., Moon, S.J., Ryu, C.H., Choi, J.Y., and Koch, W.M. 2010. The p53-reactivating small-molecule RITA enhances cisplatin-induced cytotoxicity and apoptosis in head and neck cancer. Cancer Lett. 325:35-41.

Rubinsztein, D.C., Codogno, P., and Levine, B. 2012. Autophagy modulation as a potential therapeutic target for diverse diseases. Nat. Rev. Drug Discov. 119: 709–730.

Ruvolo, P.P., Deng, X., and May, W.S. 2001. Phosphorylation of Bcl2 and regulation of apoptosis. Leukemia 15:515-522.

Sahu, R., Kaushik, S., Clement, C.C., Cannizzo, E.S., Scharf, B., Follenzi, A., Potolicchio, I., Nieves, E., Cuervo, A.M., and Santambrogio, L. 2011. Microautophagy of cytosolic proteins by late endosomes. Dev. Cell. 20:131-139.

Sanz, L., Diaz-MecoSee, M.T., Nakano, H., and Moscat, J. 2000. The atypical PKC-interacting protein p62 channels NF-κB activation by the IL-1–TRAF6 pathway. EMBO. Journal 19:1576–1586.

Sato, K., Tsuchihara, K., Fujii, S., Sugiyama, M., Goya, T., Atomi, Y., Ueno, T., Ochiai, A., and Esumi, H. 2007. Autophagy is activated in colorectal cancer cells and contributes to the tolerance to nutrient deprivation. Cancer Res. 67:9677-9684.

Scherz-Shouval, R., Weidberg, H., Gonen, C., Wilder, S., Elazar, Z., and Oren, M. 2010. p53-dependent regulation of autophagy protein LC3 supports cancer cell survival under prolonged starvation. Proc. Natl. Acad. Sci. USA. 107:18511-18516.

Schimmer, A.D., Estey, E.H., Borthakur, G., Carter, B.Z., Schiller, G.J., Tallman, M.S., Altman, J.K., Karp, J.E., Kassis, J., Hedley, D.W., Brandwein, J., Xu, W., Mak, D.H.,

LaCasse, E., Jacob, C., Morris, S.J., Jolivet, J., and Andreeff, M. 2009. Phase I/II trial of AEG35156 X-linked inhibitor of apoptosis protein antisense oligonucleotide combined with idarubicin and cytarabine in patients with relapsed or primary refractory acute myeloid leukemia. J. Clin. Oncol. 27:4741-4746.

Schmid, D., and Münz, C. 2007. Innate and adaptive immunity through autophagy. Immunity 27:11–21.

Selvakumaran, M., Amaravadi, R., Vasilevskaya, I.A., and O'Dwyer, P.J. 2013. Autophagy Inhibition Sensitizes Colon Cancer Cells to Anti-angiogenic and Cytotoxic Therapy. Clin. Cancer Res. 19:2995-3007.

Shintani, T., and Klionsky, D.J. 2004. Autophagy in health and disease: a double-edged sword. Science 306:990-995.

Sionov, R.V., and Haupt, Y. 1999. The cellular response to p53: the decision between life and death. Oncogene 18:6145-6157.

Sprick, M.R., Weigand, M.A., Rieser, E., Rauch, C.T., Juo, P., Blenis, J., Krammer, P.H., and Walczak, H. 2000. FADD/MORT1 and caspase-8 are recruited to TRAIL receptors 1 and 2 and are essential for apoptosis mediated by TRAIL receptor 2. Immunity 12:599–609.

Stern, S.T., Adiseshaiah, P.P., and Crist, R.M. 2012. Autophagy and lysosomal dysfunction as emerging mechanisms of nanomaterial toxicity. Part Fibre Toxicol. 9:20.

Stoka, V., Turk, V., and Turk, B. 2007. Lysosomal cysteine cathepsins: signaling pathways in apoptosis. Biol. Chem. 388:555–560.

Sugawara, K., Suzuki, N.N., Fujioka, Y., Mizushima, N., Ohsumi, Y., and Inagaki, F. 2004. The crystal structure of microtubule-associated protein light chain 3, a mammalian homologue of Saccharomyces cerevisiae Atg8. Genes Cells. 9:611–618.

Suzuki, N.N., Yoshimoto, K., Fujioka, Y., Ohsumi, Y., and Inagak,i F. 2005. The crystal structure of plant ATG12 and its biological implication in autophagy. Autophagy 1:119–126.

Tasdemir, E., Maiuri, M.C., Galluzzi, L., Vitale, I., Djavaheri-Mergny, M., D'Amelio, M., Criollo, A., Morselli, E., Zhu, C., Harper, F., Nannmark, U., Samara, C., Pinton, P., Vicencio, J.M., Carnuccio, R., Moll, U.M., Madeo, F., Paterlini-Brechot, P., Rizzuto, R., Szabadkai, G., Pierron, G., Blomgren, K., Tavernarakis, N., Codogno, P., Cecconi, F., and Kroemer, G. 2008. Regulation of autophagy by cytoplasmic p53. Nat. Cell Biol. 10:676-687.

Tasdemir, E., Maiuri, M.C., Morselli, E., Criollo, A., D'Amelio, M., Djavaheri-Mergny, M., Cecconi, F., Tavernarakis, N., and Kroemer, G. 2008. A dual role of p53 in the control of autophagy. Autophagy 4:810-814.

Tuffy, L.P., Concannon, C.G., D'Orsi, B., King, M.A., Woods, I., Huber, H.J., Ward, M.W., and Prehn, J.H.M. 2010. Characterization of Puma-Dependent and Puma-Independent Neuronal Cell Death Pathways following Prolonged Proteasomal Inhibition. Mol. Cell Biol. 30:5484-5501.

Vogelstein, B., and Kinzler, KW. 2004. Cancer genes and the pathways they control. Nat Med. 10:789–799.

Wang, Z., and Sun, Y. 2010. Targeting p53 for Novel Anticancer Therapy. Transl. Oncol. 3:1–12.

Ward, M.W., Kögel, D., and Prehn, J.H. 2004. Neuronal apoptosis: BH3-only proteins the real killers? J. Bioenerg. Biomembr. 36: 295–298.

Wei, Y., Pattingre, S., Sinha, S., Bassik, M., and Levine, B. 2008 JNK1-Mediated Phosphorylation of Bcl-2 Regulates Starvation-Induced Autophagy. Mol Cell. 30:678-688.

Werneburg, N.W., Guicciardi, M.E., Bronk, S.F., Kaufmann, S.H., and Gores, G.J. 2007. Tumor necrosis factor-related apoptosis-inducing ligand activates a lysosomal pathway of apoptosis that is regulated by Bcl-2 proteins. J. Biol. Chem. 282: 28960–28970.

Wu, GS., Burns, T.F., McDonald, E.R. 3rd, Jiang, W., Meng, R., Krantz, I.D., Kao, G., Gan, D.D., Zhou, J.Y., Muschel, R., Hamilton, S.R., Spinner, N.B., Markowitz, S., Wu, G., and el-Deiry, W.S. 1997. KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor gene. Nat. Genet. 17:141-143.

Xie, X., White, E.P., and Mehnert, J.M. 2013. Coordinate Autophagy and mTOR Pathway Inhibition Enhances Cell Death in Melanoma. PLoS ONE 8: e55096.

Yamamoto, A., Tagawa, Y., Yoshimori, T., Moriyama, Y., Masaki, R., and Tashiro, Y. 1998. Bafilomycin A1 prevents maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes in rat hepatoma cell line, H-4-II-E cells. Cell. Struct. Funct. 23:33-42.

Yao, J.C., Shah, M.H., Ito, T., Bohas, C.L., Wolin. E.M., Van Cutsem, E., Hobday, T.J., Okusaka, T., Capdevila, J., de Vries, E.G., Tomassetti, P., Pavel, M.E., Lincy, J., Lebwohl, D., and Öberg, K. 2011. Everolimus for advanced pancreatic neuroendocrine tumors. N. Engl. J. Med. 364: 514–523.

Yee, K.S., Wilkinson, S., James, J., Ryan, K.M., and Vousden, K.H. 2009. PUMA- and Bax-induced autophagy contributes to apoptosis. Cell Death Differ. 16:1135–1145.

You, H., Pellegrini, M., Tsuchihara, K., Yamamoto, K., Hacker, G., Erlacher, M., Villunger, A., and Mak, T.W. 2006. FOXO3a-dependent regulation of Puma in response to cytokine/growth factor withdrawal. J. Exp. Med. 203:1657–1663.

Yu, J., Wang, Z., Kinzler, K.W., Vogelstein, B., and Zhang, L. 2003. PUMA mediates the apoptotic response to p53 in colorectal cancer cells. Proc. Natl. Acad. Sci. USA. 100:1931–1936.

Yu, J., and Zhang, L. 2005. The transcriptional targets of p53 in apoptosis control. Biochem Biophys. Res. Commun. 331: 851–858.

Yu, J., and Zhang, L. 2008. PUMA, a potent killer with or without p53. Oncogene 27:S71-83.

Yu, J., Zhang, L., Hwang, P.M., Kinzler, KW., and Vogelstein, B. 2001. PUMA induces the rapid apoptosis of colorectal cancer cells. Mol. Cell. 7:673–682.

Yuan, XM., Li, W., Dalen, H., Lotem, J., Kama, R., Sachs, L., and Brunk, UT. 2002. Lysosomal destabilization in p53-induced apoptosis. Proc. Natl. Acad. Sci. USA. 99: 6286–6291.

Zhaorigetu, S., Wan, G., Kaini, R., Jiang, Z., and Hu, C.A.A. 2008. ApoL1, a BH3-only lipid-binding protein, induces autophagic cell death. Autophagy 4:1079–1082.

Zhaorigetu, S., Yang, Z., Toma, I., McCaffrey, T.A., and Hu, C.A.A. 2011. Apolipoprotein L6, Induced in Atherosclerotic Lesions, Promotes Apoptosis and Blocks Beclin 1-dependent Autophagy in Atherosclerotic Cells. J. Biol. Chem. 286: 27389–27398.

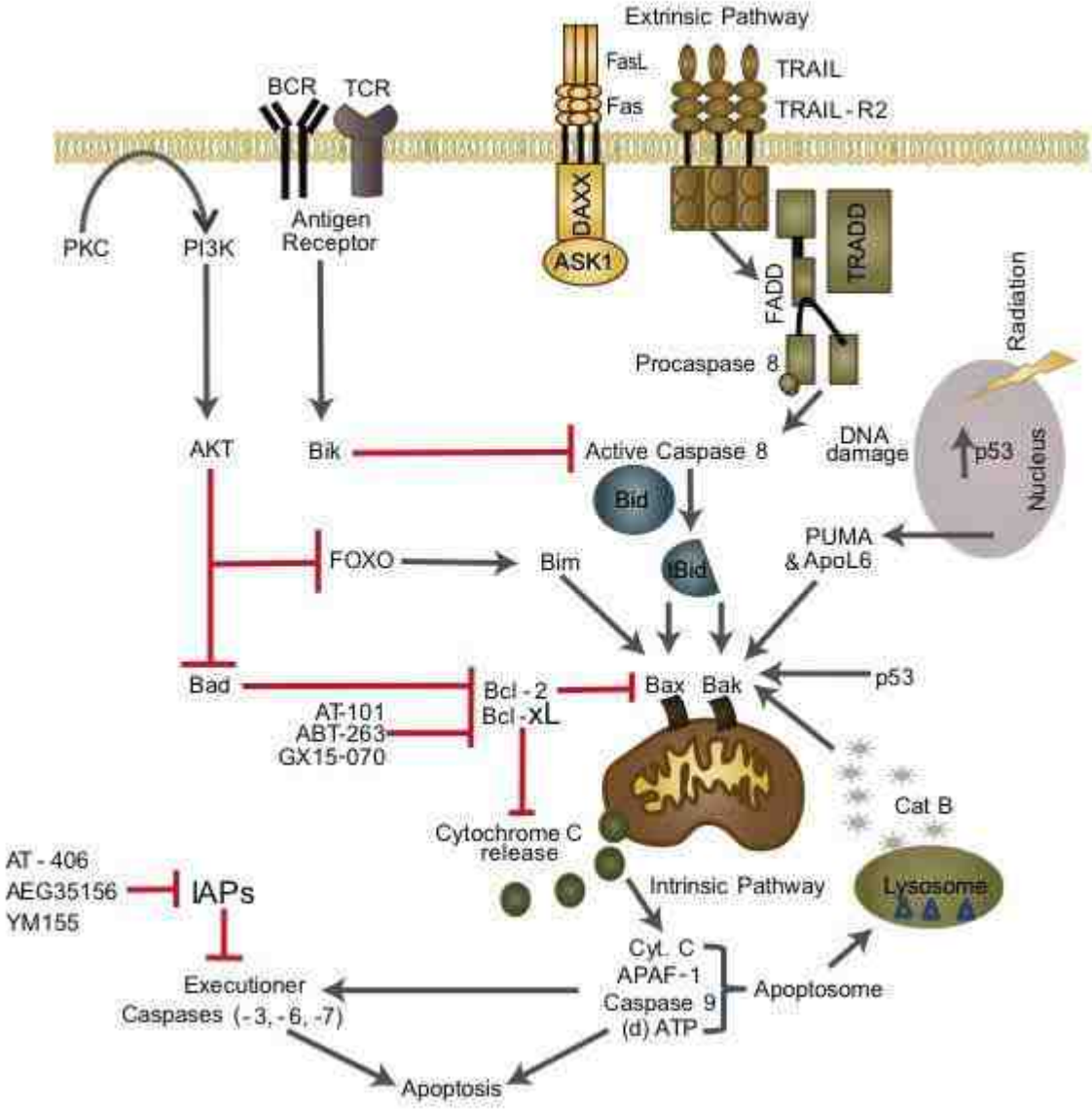
Zou, H., Henzel, W.J., Liu, X., Lutschg, A., and Wang, X. 1997. Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. Cell 90: 405–413.

1.15 Chapter Table and Figures.

1.15.1 Table 1: Inhibitors of Therapeutic Targets in Autophagy and Apoptosis

Inhibitors of autolysosome formation	
Compound:	**Mechanism of Action**
(Hydroxy) Chloroquine	Lysosomotropic drug
Bafilomycin A1	Specific inhibitor of vacuolar H+ ATPase (V-ATPase)
Concanavalin A	Apoptosis induction/immunomodulating activity
Concanamycin A	Specific inhibitor of V-ATPase
Inhibitors of caspase inhibitors	
AT- 406	A Smac mimetic and an antagonist of the inhibitor of apoptosis proteins (IAPs)
AEG35156	An antisense oligo of XIAP
YM155	Inhibits Survivin promoter activity
Inhibitors of Bcl-2	
Obatoclax (GX15-070)	Antagonist of Bcl-2
ABT-263 (Navitoclax)	Potent inhibitor of Bcl-xL and Bcl-2
AT101	Binds with Bcl-2, Bcl-xL

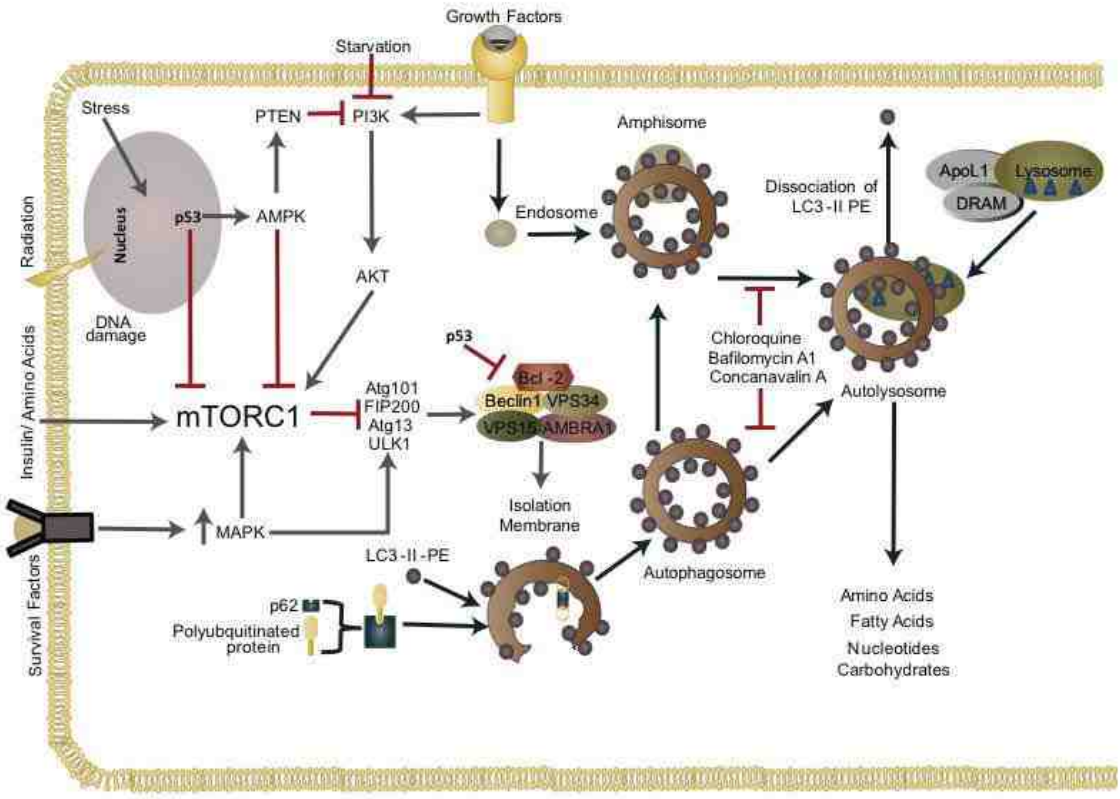
1.15.2 Figure 1. Apoptosis (the Yin)



Apoptosis is an ATP-dependent, multi-step process which occurs in response to both internal (intrinsic) and external (extrinsic) signals and as part of normal cell development and homeostasis. The intrinsic apoptotic signaling pathway is dependent on the formation of the apoptosome, an interactome of apoptotic protease activating factor 1 (Apaf-1), (pro-) caspase 9, cytochrome C and (d) ATP. The extrinsic pathway utilizes

transmembrane death receptors (FasR, TNFR1, DR3, and DR4/DR5) (6), and their corresponding ligands (FasL, TNF-α, Apo3L, and Apo2L). Apoptosis takes place in four sequential stages: stimulus, signaling, regulation, and execution. Stimulus occurs in response to ligand-receptor interaction (extrinsic) or intracellular stress (intrinsic). Subsequently, regulatory proteins (p53, Bcl-2, IAPs) and apoptosomes fine tune this dynamic process. A group of caspases are then activated and used for cleaving proteins critical to cell survival and proliferation.

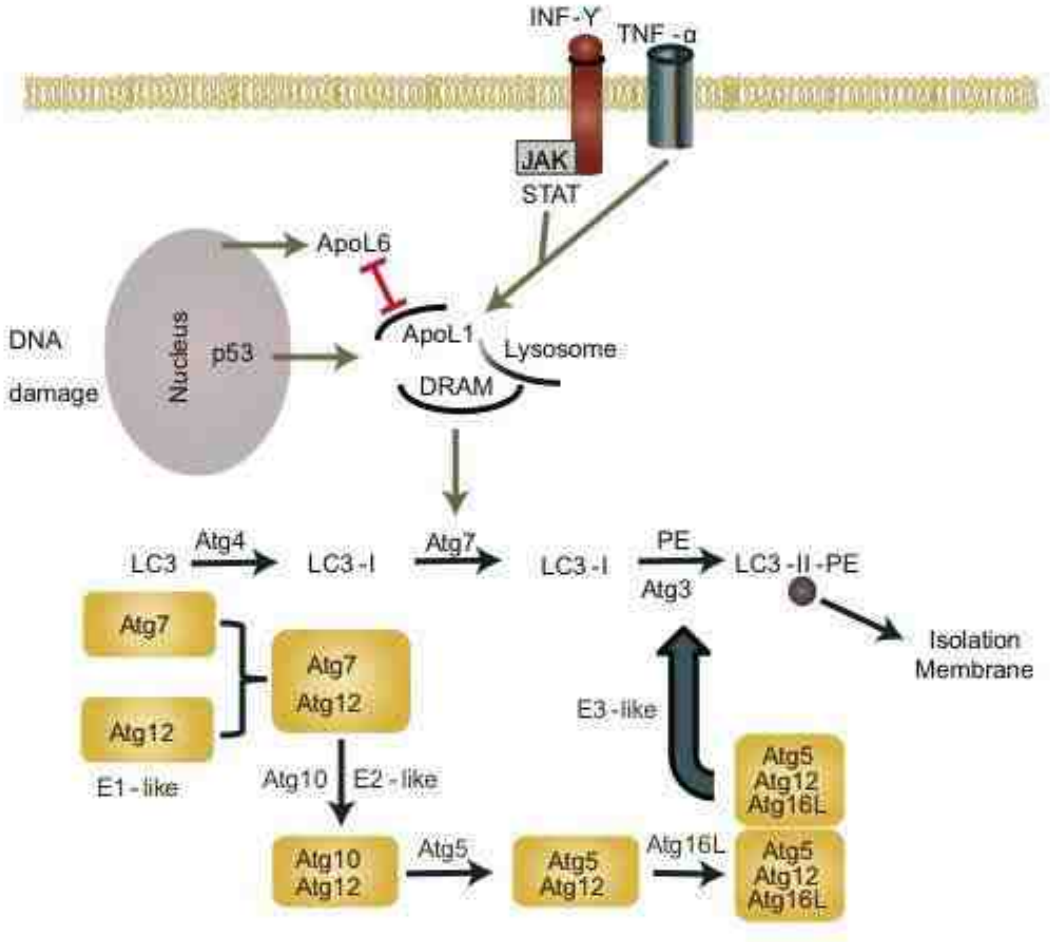
1.15.3 Figure 2. Autophagy (the Yang), Conjugation Pathways.



Autophagy uses two conjugation systems similar to the ubiquitin targeting system**.** The process of attaching LC3 to the membrane is accomplished through LC3 cleavage, lipidation and translocation. The LC3 designation is modified once cleavage has occurred

as well as when the protein is localized to the membrane. Under this nomenclature LC3-I refers to cytosolic localization following cleavage and LC3-II refers to a membrane bound LC3. During the elongation of the isolation membrane, the Atg5-Atg12-Atg16L complex localizes to the membrane to form a cup-shaped structure. LC3-II then localizes to the isolation membrane, while the Atg5-Atg12-Atg16L complex dissociates.

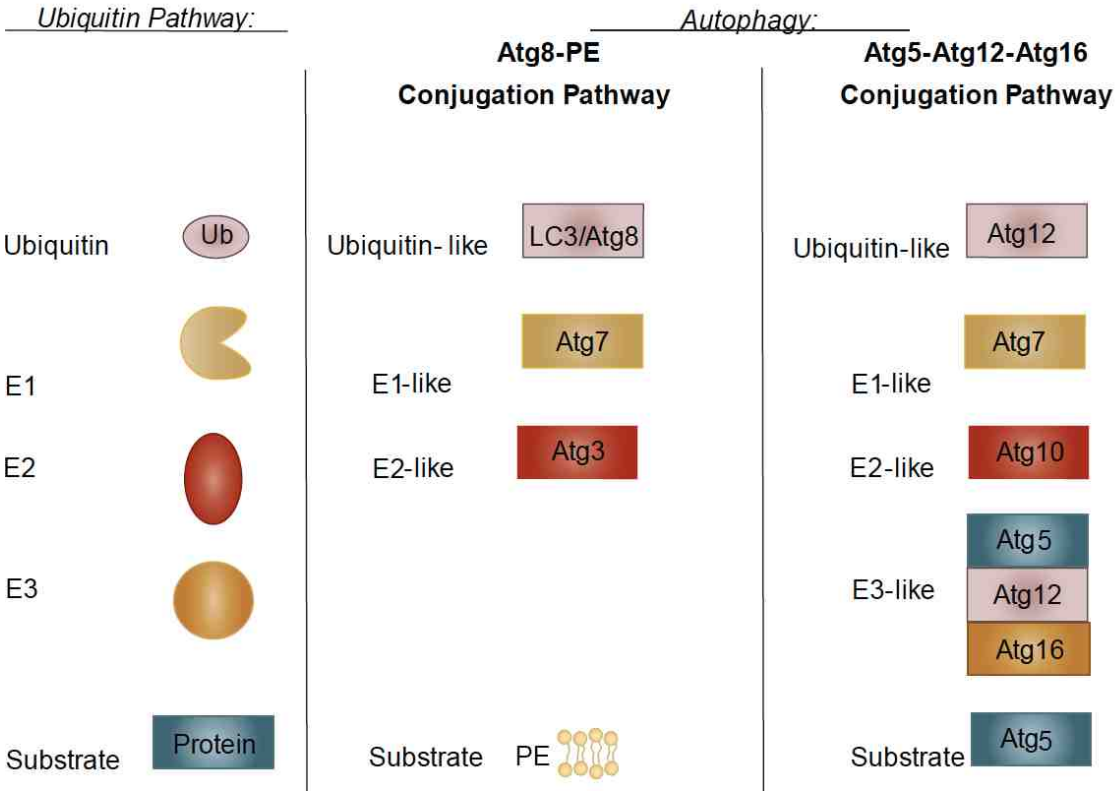
1.15.4 Figure 3. Autophagy (the Yang), Autophagic flux.



Autophagy has three major phases: initiation, elongation and completion. Initiation involves the formation of a double membrane structure (autophagosome). The formation of this double membrane structure is a complex process involving many Atg proteins. LC3 is required for the formation of autophagosomal membranes. LC3 is recruited to the

isolation membrane, which ultimately develops into the autophagosome where cellular targets are sequestered in preparation for degradation. LC3 is the only protein that remains associated with the completed autophagosome. The autophagosome then fuses with endosomes/lysosomes to create the autolysosome where cellular targets are degraded. Following autolysosomal formation, the lysosomal hydrolases, including cathepsins, degrade the targeted proteins, while the cathepsins degrade LC3-II on the inner autophagosomal surface. Following target degradation, Atg4 is involved in separating outer membrane LC3-II from the autophagosome, although LC3-II is still present in late autophagic vesicles. Crosstalk between the regulatory proteins, such as ApoL6, ApoL1, and DRAM, can dictate the outcome of autophagic flux.

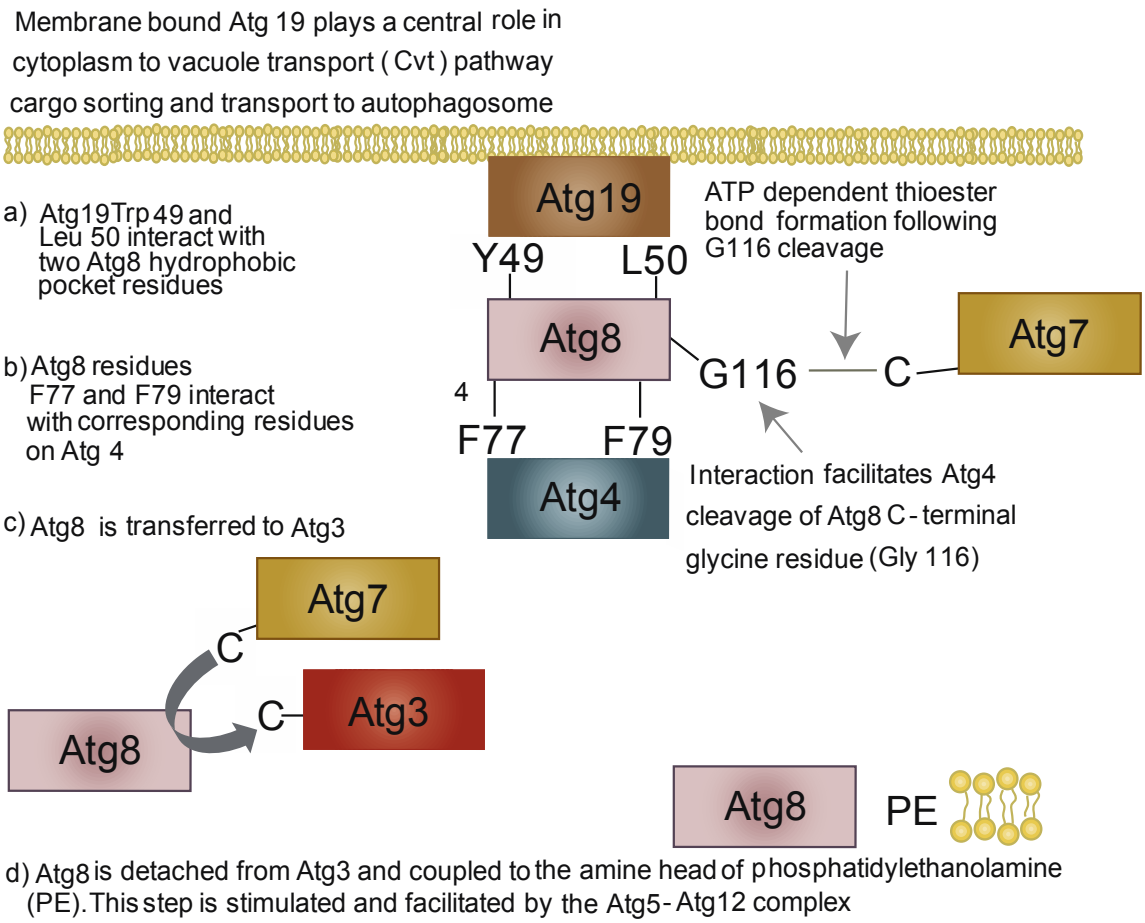
1.15.5 Figure 4. Similar Molecules Between Two Protein Degradation Pathways.



Transient conjugation of LC3 to the autophagosomal membrane through an ubiquitin like system is essential for macroautophagy. Ubiquitin directs proteins targeted for degradation to the proteasome and is activated by an E1 enzyme. Following activation, E1 transfers the activated ubiquitin to E2 enzymes and then with E3 enzymes catalyzes the conjugation of ubiquitin to substrates. LC3 binding residues can function in an ubiquitin-like fashion and terms ubiquitin-like, E1-like, E2-like and E3-like are applied to these autophagic ubiquitin-like systems producing the conjugated proteins (LC3-II-PE and Atg5-Atg12-Atg16L). LC3 targeting system works with LC3 acting as the ubiquitin-like protein that is transferred to phosphatidylethanolamine (PE), while Atg7 functions in the same manner as an E1 enzyme; Atg3 like an E2 enzyme and the Atg12-Atg5-Atg16L

complex like an E3 ligase for the LC3-II–PE complex. Upstream of the LC3-II-PE linkage, Atg12 is conjugated to Atg5 to form a stable Atg12-Atg5 conjunction. This process is mediated by Atg7, E1-like activating enzyme, and Atg10, an E2-like conjugating enzyme. Atg16 then forms a complex with the Atg12–Atg5 conjugate.

1.15.6 Figure 5. Biochemical reactions in Atg8 lipidation.



Atg8 in budding yeast *Saccharomyces cerevisiae* shares significant homology with mammalian LC3. In yeast Atg8, residues Phe 77 and Phe 79 are located on one surface, and residues Tyr 49 and Leu 50 are on the opposite surface. It has been shown that Phe 77 and Phe 79 recognize Atg4, an endopeptidase and a de-conjugation enzyme. Atg4 is a

specific cysteine protease belonging to the caspase family, initiates LC3 processing by post-translationally cleaving LC3's C-terminal amino acid (arginine). This cleavage generates LC3-I. The newly exposed C-terminal glycine (Gly 116) is bound by phosphatidylethanolamine (PE), a lipidation conjugation reaction catalyzed by Atg12-5-16L complex, which results in the formation of LC3-II.

1.16 Dissertation Summary: Background

The American Cancer Society estimates that in 2015, 73,870 new melanomas will be diagnosed and 9,940 people will die from their disease. [1]The 5-year survival rate for stage IV melanoma also remains unchanged at only 15-20% but the incidence continues to rise.[1] Once melanoma has metastasized the available treatment options all have significant challenges. Immunotherapy with IL-2 and IFN-α offers some patients complete and long-lasting remission, but has a very low percentage of responders. [2,3]Chemotherapy, including alkylating agents (dacarbazine, temozolomide), platinum analogs, and microtubular toxins [4] have a larger percentage of responders. [2,5] However, these agents used alone or in combination still have less durability.[6] Advances in the field of melanoma have successfully identified targeted therapies including selective oncogene inhibitors. Activating mutations in the *BRAF* and *NRAS* proto-oncogenes are found in approximately 58% of primary melanomas (43% *BRAF* ^{V600E}; 15% *NRAS* ^{Q61R}) and 63% of melanoma metastases (48% *BRAF* ^{V600E}; 15% *NRAS* ^{Q61R}) .[7] Long term effective treatments for invasive melanoma typically use targeted immunotherapy combined with therapeutics that target the critical MAPK pathway through the inhibition of *BRAF*. [2,8] However, inhibition of these targets has not significantly increased patient survival, as tumors eventually develop resistance. [1,9] Recently, immunotherapy has shown

encouraging advances in metastatic melanoma treatment through the recognition of neoantigens and through deactivation of immune checkpoint blockades, such as CTLA-4 antagonist-antibodies, and PD-L1. These have shown an increase in ten month survival in phase I and II clinical trials. [10,11] While activating mutations in *BRAF/NRAS* are critical mediators of melanomagenesis [12–14] and the most common somatic alterations in melanoma, the mechanisms by which these mutations promote tumorigenesis are still under investigation. One potential pathway that may provide a mechanism to support tumorigenesis is the metabolic pathway of autophagy. Autophagy, or self-eating, is a catabolic process that assists the removal of unnecessary or dysfunctional components, damaged proteins and organelles through lysosomal degradation.[15,16] A tightly regulated process autophagy, plays a role in a wide variety of normal physiological processes including energy metabolism, stress responses, growth regulation, and aging[17,18] and can be induced in response to nutrient deprivation.[19] Macroautophagy, the most widely discussed type of autophagy (referred to hereafter as autophagy) involves the initial formation of a double membrane structure (isolation membrane) which encapsulates organelles and targeted elements in the cell, separating these targets from the rest of the cytoplasm and fusing them into a circular autophagosome. The autophagosome then fuses with the lysosomes (autophagolysosome), which degrades or recycles the contents of the vesicles. [20] Autophagy has been shown to play a Janus role in cancer,[21] suppressing the early stages of tumor formation [22,23] yet enhancing tumorigenesis in the later stages of development.[24–28] Research has shown that while autophagy plays similar roles in tumor cells as in normal cells, tumor cells' energetic needs are greater and they have a more hypoxic environment, so dependence on autophagy may be greater. [29–31] Conversely,

deficiencies in autophagy in normal cells can promote cancer through the accumulation of damaged mitochondria and other components that autophagy normally degrades or recycles. [32,33] Finally autophagy has been reported to control other processes relevant to the etiology of cancer, including oxidative stress [32] inflammation [18,34,35] and both innate and acquired immunity.[21,35–38] Tumor cells have increased levels of DNA and protein synthesis and require a constant nutrient source; autophagy appears to promote tumor survival by providing energy to the hypoxic tumor environment or in response to chemotherapy. [30,39–41] Autophagy also localizes to hypoxic tumor areas that occur far from blood vessels and supports tumor cell survival.[30] Importantly, the mechanism by which BRAF and NRAS influence autophagy remains to be elucidated. Recent evidence for metabolic resistance to BRAF inhibitors and the speculation that some cancers are "autophagy addicted" suggests that activating mutations in BRAF may generate tumors with this phenotype.[42] There is thus a need to investigate the mechanism and extent to which mutations in oncogenes affect the rate of autophagic flux and ultimately sensitivity to inhibition of this process.

1.17 Single Nucleotide Polymorphisms (SNPs):

1.17.1 SNP Definition

A single nucleotide polymorphism is a DNA (germline) sequence variation that occurs commonly within a population; in which a single nucleotide (adenine, thymine, cytosine, or guanine) is altered. There are many types of SNPs and the most commons are those that occur in between genes in introns (intronic). SNPs, in a majority of cases have no clear effect on human health or development but some act as biological marker that allow for the identification of disease associated genes. Even intronic SNPs may have on

impact if they are located in enhancers, super enhancers, promoters or introduce splice variants. [43–47]Some SNPs occurring near or within genes potentially play a more direct role, effecting the functioning of an associated gene or protein stability and impacting risk, progression or disease specific survival. [48]

There is evidence that autophagy related genes have SNPs which impact the function of both promoters as well as protein stability.[49,50] The role of autophagy-related SNPs in melanoma progression and survival has not been investigated and may provide insight into the interactions between this disease and the autophagy pathway.

1.18 Purpose and Hypotheses

Invasive melanoma is a complex disease with an increasing incidence of 6% each year and few clinical treatment options. The American Cancer Society estimates that in 2015 the 5-year survival rate for stage IV melanoma will remain unchanged at only 15%, even with new therapies. One emerging hallmark of cancer is the reprogramming of energy metabolism [51] which includes the role of autophagy.

Autophagy is a cellular recycling pathway involved in normal physiological processes including nutrient and/or stress responses, antigen presentation, and aging. In cancer, autophagy appears to be an important energy source for nutrient depleted tumors.[29] Studies of solid tumors have shown autophagy's role is biphasic as it initially suppresses the early stages of tumor formation [22] and inhibits metastasis by promoting anti-tumor inflammatory responses but later promotes metastasis by enhancing the tumor's ability to respond to environmental stress. [15,52] However, the mechanisms supporting the Janus nature of autophagy remain to be elucidated.

Studies have indicated that evaluated tumors differ in their sensitivity to chloroquine

(CQ), a potent inhibitor of autophagy.[26,53] Currently, multiple clinical trials are underway using CQ and its derivatives to evaluate the effectiveness of these inhibitors in cancer therapies.[54,55] In melanoma, basal levels of autophagy appear to vary by tumor stage, and higher levels of autophagic flux are associated with increased hypoxia and poor clinical prognosis.[56] In 2010, Lazova and colleagues reported that melanoma cells contain high levels of autophagosomes, a cellular structure associated with autophagy, as measured by the proxy marker LC3, an autophagy related (ATG) protein.[57] Ma et al. (2014) reported increased autophagy in BRAF inhibitor resistant cells.[58] These findings suggest autophagy provides metabolic support for tumors.

Other environmental and genetic factors also have an influence on autophagy. Ultraviolet-radiation (UVR) exposure increases autophagic flux[59,60] and UVR is an established risk factor for melanoma. Furthermore, in oncogenic *BRAF*-mutated thyroid cancer a single nucleotide polymorphism (SNP) in a crucial *ATG* gene, *ATG5*, has been shown to have a positive association with cancer susceptibility and poor outcome.[50] To date there are no studies examining *ATG* gene SNPs in melanoma outcomes, so the role of these variants is unclear, particularly in relation to the presence of *BRAF/NRAS* oncogenes. Variants identified in these patients could affect *ATG* gene expression and ultimately, influence the rate of autophagy.

The objective of the present study is to determine whether in melanoma oncogene status and UV exposure are independently associated with levels of autophagy and if autophagy markers associate with increased melanoma survival. Our long term goal is to reduce mortality from melanoma by identifying novel targets for treatment using the autophagy pathway.

1.19 Rationale, Hypothesis, and Specific Aims:

1.19.1 Rationale

The rationale of the proposed research is that oncogene status in melanoma differentially suppresses apoptosis by modifying autophagic flux.

1.19.2 Hypothesis

Our hypothesis is that cutaneous malignant melanomas (CMM) with BRAF mutations may be autophagy-addicted while tumors with NRAS mutations may be less dependent on autophagy.

I interrogated this hypothesis through the following specific and testable aims:

1.19.3 Specific Aim 1

Determine if BRAF/NRAS mutations alter autophagic flux at the cellular level in order to suppress apoptosis in melanoma.

1.19.4 Specific Aim 2

Determine if autophagic flux is associated with individual UV exposure and/or clinical stage and modified by oncogenic BRAF/NRAS in melanoma tumor.

1.19.5 Specific Aim 3

Determine whether SNPs in ATG genes are associated with BRAF/NRAS mutations in melanoma patients.

1.20 Overall Approach.

In order to determine if *BRAF/NRAS* mutations alter autophagic flux to suppress apoptosis in melanoma, I used melanoma cell lines to quantify the complete process of autophagy (autophagic flux), beginning with the formation of an isolation membrane, autophagosome and eventually an autolysosome where unnecessary or dysfunctional cellular components are degraded. [61,62] The goal was to examine the model that metabolic stress caused by mutations in oncogenes leads to alterations in gene expression, as shown in tissue samples, increasing the rate of autophagy, and ultimately leading to melanoma progression. In addition, variants in autophagy genes may modify autophagic flux leading to melanoma progression.

Our long term goal is to reduce mortality from melanoma by identifying novel targets for treatment using the underexplored autophagy pathway. In six melanoma cell lines (e.g. SK-Mel 2, 19, 29, 94,103, and 147) containing *BRAF* or *NRAS* mutations, the sensitivity and cell viability associated with chloroquine (CQ) treatment were characterized by autophagic flux, to determine decreases in cellular viability caused by autophagy inhibition. Using quantification of LC3-II puncta and western blots, I evaluated the differential rate of autophagy by oncogene status. These two common CMM activating mutations, *BRAF* and *NRAS*, appear to have differential effects on the autophagy pathway. Our data indicate that the targeting of autophagy using CQ in the *BRAF* oncogenic cell lines increases apoptosis and leads to a significant decrease in viability

(Figure 1; Chapter 2). In addition, *BRAF* cell lines appear to have higher basal protein expression of LC3-II (Figure 4; Chapter 2) and a higher number of basal LC3-II puncta when compared to *NRAS* cell lines (Figure 4; Chapter 2) indicating dependence on the autophagy pathway for survival. Finally, the induction of autophagy through serum starvation results in differential LC3 expression as measured by number of puncta in the *BRAF* and *NRAS* cell lines (Figure 4; Chapter 2). These results indicate that *BRAF* mutant melanoma is more dependent on autophagy for survival than *NRAS* mutant melanoma and that oncogene status affects the sensitivity of melanoma cells to CQ by altering autophagic flux, subsequently increasing apoptosis. Ongoing experiments include the evaluation of autophagy in patient samples to quantify expression of known autophagy markers as well an evaluation of LC3-II expression correlated with oncogene status and autophagy SNPs. These findings will be immediately translatable for patients who would benefit from CQ co-therapy, which is approved for clinical use.

1.21 Significance of the Dissertation Study

Long term survival remains a challenge for metastatic melanoma patients as there are few durable treatment options available. Melanoma treatment can be divided into four major types: surgery, chemotherapy, radiation therapy and biological therapy.[63] Treatment of stage IV and recurrent melanoma may include the following: targeted therapy with ipilimumab or *BRAF* inhibitors (e.g. vemurafenib); biological therapy with interleukin-2 (IL-2); chemotherapy including alkylating agents; or palliative therapy to relieve symptoms and improve the quality of life. [63,64] Unfortunately, in most cases, treatment fails to halt the advance of melanoma and patients do progress, typically within a few

months.[9] Exciting immunotherapy treatment options including IL-2, [2,3,8] drugs that block Cytotoxic T lymphocytes (CTLs) inhibitory mechanism (CTLA-4) or Programmed cell death 1 (PD-1) boost the immune response against melanoma cells. These combinations which circumvent the tumor's ability to evade immune destruction are resulting in tumor regression and increased survival, although there remains heterogeneity in responses to treatment.[65–67] The Phase Ib study of MEDI4736 for a combinations of CTLA-4 and Programmed cell death ligand 1 (PD-L1) antagonist-antibodies have shown promising results while illuminating the relationship between melanoma progression and immune system.[68] These results indicate that combining immunotherapy with oncogene (*BRAF, NRAS*)-targeted drugs may provide durable responses in melanoma for a larger number of patients.

In addition to evading immune destruction, another hallmark of cancer is the reprogramming of pathways involved in energy metabolism to support continuous growth, development and progression of cancer using mechanisms including the autophagy pathway.[51] Autophagy is often dysregulated in the hypoxic, low nutrient regions of tumors and can support cancer survival through its ability to provide tumors an energy source.[15,18,29,34,69,70] Autophagy upregulation has been documented in lung, prostate and other cancer types but the mechanisms supporting its role in melanoma tumorigenesis have not been well characterized. In addition, the role of autophagy in modifying cancer cell death or survival remains controversial.[71] Interestingly, it appears that oncogene status, an important contributor to cellular deregulation of proliferation and apoptotic pathways in melanoma also influences the rate of autophagic flux. *BRAF* ^{V600E} mutations have been reported to promote lung tumorigenesis by utilizing autophagy's

ability to preserve mitochondrial function,[72] and inhibition of autophagy in vemurafenib-resistant melanoma decreases cell survival. The concept that oncogenes can directly influence autophagic rates has important implications for cancer development as well as the management of treatment options for this difficult disease. Furthermore, in non-medullary thyroid cancers with activating *BRAF* mutations, a SNP in *ATG5*, a crucial *ATG* gene, has been shown to be associated with disease susceptibility and outcome. [73] To date there are no studies examining *ATG* gene SNPs in melanoma progression or survival, so the role of these variants is unclear. Variants identified in these melanoma patients could affect *ATG* gene expression and ultimately influence the rate of autophagic flux.

The significance of this proposal is that autophagy may represent a novel area for identifying biomarkers and potential targets for treatment in melanoma. Given that oncogenes influence autophagy and $BRAF^{V600E}$ melanoma cells appear to use late-stage autophagy to maintain an active metabolic state,[57,74] the current study was designed to determine whether oncogenic mutations drive melanomas to become autophagy-addicted for survival and if inhibition of this pathway increases apoptosis in these cells. If this is so, we would anticipate finding that autophagy or oncogenically driven autophagy as an important factor in progression to metastasis and death. Our study design is innovative as it combines both molecular epidemiology and molecular biology techniques allowing the proposed research to determine if oncogene status is an important modifier of autophagy in melanoma moving from bench to bedside. Autophagy has been shown to promote tumor survival [21,58] and support survival of radiation and chemotherapy resistant tumor cells [54,57] thus highlighting the importance of investigating this process in melanoma.

Upon completion of the proposed aims, this research will clarify the relationships between autophagy, melanoma and oncogene status and will elucidate some of the molecular mechanisms/pathways contributing to this disease. This project has the potential to make a significant contribution because (i) it will characterize the cell death response to autophagy inhibition in the context of *BRAF/NRAS* oncogenic status; (ii) it will characterize the relationship at between autophagy and UV exposure in tissue sections and (iii) it is an important first step in identifying autophagy related SNPs that may influence autophagic flux and melanoma tumorigenesis.

1.22 Limitations of Dissertation Study

There are several limitations to this study.

1. While immortalized cells lines can provide some information about how metastasis in melanoma is facilitated, there are limitations to using them as a resource. Some of the strengths of cell lines are they often show similar allelic populations compared to the actual tumors and they provide a rich source of RNA which under other circumstances would be difficult to obtain. They also provide good model systems for evaluating invasion and the potential for the tumors to metastasize to distant sites. Weaknesses include an increase in chromosomal instability in many cancer cell lines as well as chromosomal gains and losses as the number of passages increase, which could affect experimental outcomes.

2. Problems with reliable labeling of autophagosomes have created controversies around using immunofluorescence.[62,75] To address this potential issue, I used a proven colocalization experiment to identify the autolysosomes indicating true target degradation and autophagy completion. Staining protocols have been developed for quantitative

analysis using antibodies to LC3 (ATG8). In addition, quantitative fluorescent microscopy will be used to assess relative quantities of LC3 estimating the accumulation of autophagic vacuoles. Although my aims have little interdependence, this aim was limited by the reality that many cells use other mechanisms, such as methylation or acetylation, to regulate protein expression so LC3 may stay consistently expressed under variable conditions. Flow cytometry experiments have resulted in higher levels of cell death (Figure 2a-d Chapter 2), so I used an alternative method to examine cell death, Caspase activity, in order to obtain more definitive results. Furthermore, the use of the IHC could result in variable or incomplete staining and subjective quantification of protein expression. For this reason I developed a novel quantification method which removes the subjective component of IHC quantification. An alternative approach would be to use standard immunofluorescent techniques using anti-LC3 antibodies to quantify expression of tumor cells from tumor-associated melanophages. The immunofluorescent nature of melanoma samples makes this method a less desirable but still viable alternative.

1.23 References:

1. American Cancer Society Facts and Statistics 2015 | Research | American Cancer Society. at <http://www.cancer.org/research/cancerfactsstatistics/cancerfactsfigures2015/>

2. Flaherty, K. T. & McArthur, G. BRAF, a target in melanoma: implications for solid tumor drug development. *Cancer* **116,** 4902–13 (2010).

3. Buzaid, A. C. Management of metastatic cutaneous melanoma. *Oncology (Williston Park).* **18,** 1443–50; discussion 1457–9 (2004).

4. Bhatia, S., Tykodi, S. S. & Thompson, J. A. Treatment of metastatic melanoma: an overview. *Oncology (Williston Park).* **23,** 488–96 (2009).

5. O'Day, S. J., Kim, C. J. & Reintgen, D. S. Metastatic melanoma: chemotherapy to biochemotherapy. *Cancer Control* **9,** 31–8

6. Bajetta, E. *et al.* Metastatic melanoma: chemotherapy. *Semin. Oncol.* **29,** 427–45 (2002).

7. Colombino, M. *et al.* Heterogeneous distribution of BRAF/NRAS mutations among Italian patients with advanced melanoma. *J. Transl. Med.* **11,** 202 (2013).

8. Chapman, P. B. *et al.* Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N. Engl. J. Med.* **364,** 2507–16 (2011).

9. Jarkowski, A. & Khushalani, N. I. BRAF and beyond: Tailoring strategies for the individual melanoma patient. *J. Carcinog.* **13,** 1 (2014).

10. Tarhini, A., Lo, E. & Minor, D. R. Releasing the brake on the immune system: ipilimumab in melanoma and other tumors. *Cancer Biother. Radiopharm.* **25,** 601–13 (2010).

11. Johnson, D. B., Peng, C. & Sosman, J. A. Nivolumab in melanoma: latest evidence and clinical potential. *Ther. Adv. Med. Oncol.* **7,** 97–106 (2015).

12. Haq, R. *et al.* Oncogenic BRAF regulates oxidative metabolism via PGC1α and MITF. *Cancer Cell* **23,** 302–15 (2013).

13. Davies, H. *et al.* Mutations of the BRAF gene in human cancer. *Nature* **417,** 949–54 (2002).

14. Hawryluk, E. B. & Tsao, H. Melanoma: clinical features and genomic insights. *Cold Spring Harb. Perspect. Med.* **4,** a015388 (2014).

15. White, E., Karp, C., Strohecker, A. M., Guo, Y. & Mathew, R. Role of autophagy in suppression of inflammation and cancer. *Curr. Opin. Cell Biol.* **22,** 212–7 (2010).

16. Mizushima, N. Autophagy: process and function. *Genes Dev.* **21,** 2861–73 (2007).

17. Shintani, T. & Klionsky, D. J. Autophagy in health and disease: a double-edged sword. *Science* **306,** 990–5 (2004).

18. Choi, A. M. K., Ryter, S. W. & Levine, B. Autophagy in human health and disease. *N. Engl. J. Med.* **368,** 651–62 (2013).

19. Russell, R. C., Yuan, H.-X. & Guan, K.-L. Autophagy regulation by nutrient signaling. *Cell Res.* **24,** 42–57 (2014).

20. Patel, A. S. *et al.* Autophagy in idiopathic pulmonary fibrosis. *PLoS One* **7,** e41394 (2012).

21. Rosenfeldt, M. T. & Ryan, K. M. The multiple roles of autophagy in cancer. *Carcinogenesis* **32,** 955–63 (2011).

22. Sun, K. *et al.* Paradoxical roles of autophagy in different stages of tumorigenesis: protector for normal or cancer cells. *Cell Biosci.* **3,** 35 (2013).

23. Panda, P. K. *et al.* Mechanism of autophagic regulation in carcinogenesis and cancer therapeutics. *Semin. Cell Dev. Biol.* **39,** 43–55 (2015).

24. Zhang, J. *et al.* Statins, autophagy and cancer metastasis. *Int. J. Biochem. Cell Biol.* **45,** 745–52 (2013).

25. Qu, X. *et al.* Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. *J. Clin. Invest.* **112,** 1809–20 (2003).

26. Yang, Z. J., Chee, C. E., Huang, S. & Sinicrope, F. A. The role of autophagy in cancer: therapeutic implications. *Mol. Cancer Ther.* **10,** 1533–41 (2011).

27. Kenific, C. M., Thorburn, A. & Debnath, J. Autophagy and metastasis: another double-edged sword. *Curr. Opin. Cell Biol.* **22,** 241–5 (2010).

28. Mathew, R., Karantza-wadsworth, V. & White, E. Role of autophagy in cancer. **7,** 961–967 (2007).

29. Sato, K. *et al.* Autophagy is activated in colorectal cancer cells and contributes to the tolerance to nutrient deprivation. *Cancer Res.* **67,** 9677–84 (2007).

30. Chatterjee, S. J. & Pandey, S. Chemo-resistant melanoma sensitized by tamoxifen to low dose curcumin treatment through induction of apoptosis and autophagy. *Cancer Biol. Ther.* **11,** 216–28 (2011).

31. Strohecker, A. M. *et al.* Autophagy sustains mitochondrial glutamine metabolism and growth of BrafV600E-driven lung tumors. *Cancer Discov.* **3,** 1272–85 (2013).

32. Lee, J., Giordano, S. & Zhang, J. Autophagy, mitochondria and oxidative stress: cross-talk and redox signalling. *Biochem. J.* **441,** 523–40 (2012).

33. Boland, M. L., Chourasia, A. H. & Macleod, K. F. Mitochondrial dysfunction in cancer. *Front. Oncol.* **3,** 292 (2013).

34. Levine, B. & Kroemer, G. Autophagy in the pathogenesis of disease. *Cell* **132,** 27–42 (2008).

35. Levine, B., Mizushima, N. & Virgin, H. W. Autophagy in immunity and inflammation. *Nature* **469,** 323–35 (2011).

36. Zhou, X.-J. & Zhang, H. Autophagy in immunity: implications in etiology of autoimmune/autoinflammatory diseases. *Autophagy* **8,** 1286–99 (2012).

37. Sumpter, R. & Levine, B. Autophagy and innate immunity: triggering, targeting and tuning. *Semin. Cell Dev. Biol.* **21,** 699–711 (2010).

38. Oh, J. E. & Lee, H. K. Autophagy in innate recognition of pathogens and adaptive immunity. *Yonsei Med. J.* **53,** 241–7 (2012).

39. Marino, M. L. *et al.* Autophagy is a protective mechanism for human melanoma cells under acidic stress. *J. Biol. Chem.* **287,** 30664–76 (2012).

40. Martinez-Outschoorn, U. E. *et al.* The autophagic tumor stroma model of cancer or "battery-operated tumor growth." *Cell Cycle* **9,** 4297–4306 (2014).

41. Ávalos, Y. *et al.* Tumor suppression and promotion by autophagy. *Biomed Res. Int.* **2014,** 603980 (2014).

42. Altman, B. J. & Rathmell, J. C. Metabolic stress in autophagy and cell death pathways. *Cold Spring Harb. Perspect. Biol.* **4,** a008763 (2012).

43. Hnisz, D. *et al.* Super-enhancers in the control of cell identity and disease. *Cell* **155,** 934–47 (2013).

44. Martini, S. *et al.* From single nucleotide polymorphism to transcriptional mechanism: a model for FRMD3 in diabetic nephropathy. *Diabetes* **62,** 2605–12 (2013).

45. De Gobbi, M. *et al.* A regulatory SNP causes a human genetic disease by creating a new transcriptional promoter. *Science* **312,** 1215–7 (2006).

46. Martin, L. J. *et al.* Functional variant in the autophagy-related 5 gene promotor is associated with childhood asthma. *PLoS One* **7,** e33454 (2012).

47. Erichsen, H. C. & Chanock, S. J. SNPs in cancer research and treatment. *Br. J. Cancer* **90,** 747–51 (2004).

48. NCI Dictionary of Genetics Terms - National Cancer Institute. at <http://www.cancer.gov/publications/dictionaries/genetics-dictionary?expand=S>

49. Huijbers, A. *et al.* The effect of the ATG16L1 Thr300Ala polymorphism on susceptibility and outcome of patients with epithelial cell-derived thyroid carcinoma. *Endocr. Relat. Cancer* **19,** L15–8 (2012).

50. Plantinga, T. S. *et al.* Role of genetic variants of autophagy genes in susceptibility for non-medullary thyroid cancer and patients outcome. *PLoS One* **9,** e94086 (2014).

51. Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: the next generation. *Cell* **144,** 646–74 (2011).

52. Xu, Y., Xia, X. & Pan, H. Active autophagy in the tumor microenvironment: A novel mechanism for cancer metastasis. *Oncol. Lett.* **5,** 411–416 (2013).

53. Yang, S. *et al.* Pancreatic cancers require autophagy for tumor growth. *Genes Dev.* **25,** 717–29 (2011).

54. Sui, X. *et al.* Autophagy and chemotherapy resistance: a promising therapeutic target for cancer treatment. *Cell Death Dis.* **4,** e838 (2013).

55. The BAMM Trial: BRAF, Autophagy and MEK Inhibition in Metastatic Melanoma: A Phase I/2 Trial of Dabrafenib, Trametinib and Hydroxychloroquine in Patients With Advanced BRAF Mutant Melanoma - No Study Results Posted - ClinicalTrials.gov. at <https://clinicaltrials.gov/ct2/show/NCT02257424>

56. Schaaf, M. B. E. *et al.* The autophagy associated gene, ULK1, promotes tolerance to chronic and acute hypoxia. *Radiother. Oncol.* **108,** 529–34 (2013).

57. Lazova, R., Klump, V. & Pawelek, J. Autophagy in cutaneous malignant melanoma. *J. Cutan. Pathol.* **37,** 256–68 (2010).

58. Ma, X. *et al.* Targeting ER stress – induced autophagy overcomes BRAF inhibitor resistance in melanoma. **124,** (2014).

59. Chen, L.-H. *et al.* Targeting protective autophagy exacerbates UV-triggered apoptotic cell death. *Int. J. Mol. Sci.* **13,** 1209–24 (2012).

60. Zhao, Y. *et al.* Autophagy is induced by UVA and promotes removal of oxidized phospholipids and protein aggregates in epidermal keratinocytes. *J. Invest. Dermatol.* **133,** 1629–37 (2013).

61. Hansen, T. E. & Johansen, T. Following autophagy step by step. *BMC Biol.* **9,** 39 (2011).

62. Klionsky, D. J. *et al.* Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. *Autophagy* **4,** 151–75 (2008).

63. Coit, D. G. *et al.* Melanoma, version 4.2014. *J. Natl. Compr. Canc. Netw.* **12,** 621–9 (2014).

64. Krammer, R. & Heinzerling, L. Therapy preferences in melanoma treatment--willingness to pay and preference of quality versus length of life of patients, physicians and healthy controls. *PLoS One* **9,** e111237 (2014).

65. Snyder, A. *et al.* Genetic Basis for Clinical Response to CTLA-4 Blockade in Melanoma. *N. Engl. J. Med.* **371,** 141119140020009 (2014).

66. Dolan, D. E. & Gupta, S. PD-1 pathway inhibitors: changing the landscape of cancer immunotherapy. *Cancer Control* **21,** 231–7 (2014).

67. Madore, J. *et al.* PD-L1 expression in melanoma shows marked heterogeneity within and between patients: implications for anti-PD-1/PD-L1 clinical trials. *Pigment Cell Melanoma Res.* **28,** 245–53 (2015).

68. Mahoney, K. M., Freeman, G. J. & McDermott, D. F. The Next Immune-Checkpoint Inhibitors: PD-1/PD-L1 Blockade in Melanoma. *Clin. Ther.* **37,** 764–82 (2015).

69. Selvakumaran, M., Amaravadi, R. K., Vasilevskaya, I. A. & O'Dwyer, P. J. Autophagy inhibition sensitizes colon cancer cells to antiangiogenic and cytotoxic therapy. *Clin. Cancer Res.* **19,** 2995–3007 (2013).

70. Wu, W. K. K. *et al.* The autophagic paradox in cancer therapy. *Oncogene* **31,** 939–53 (2012).

71. Xie, X., White, E. P. & Mehnert, J. M. Coordinate autophagy and mTOR pathway inhibition enhances cell death in melanoma. *PLoS One* **8,** e55096 (2013).

72. Strohecker, A. M. & White, E. Autophagy promotes BrafV600E-driven lung tumorigenesis by preserving mitochondrial metabolism. *Autophagy* **10,** 384–5 (2014).

73. Plantinga, T. S. *et al.* Role of genetic variants of autophagy genes in susceptibility for non-medullary thyroid cancer and patients outcome. *PLoS One* **9,** e94086 (2014).

74. Maddodi, N. *et al.* Induction of autophagy and inhibition of melanoma growth in vitro and in vivo by hyperactivation of oncogenic BRAF. *J. Invest. Dermatol.* **130,** 1657–67 (2010).

75. Mizushima, N., Yoshimori, T. & Levine, B. Methods in mammalian autophagy research. *Cell* **140,** 313–26 (2010).

CHAPTER TWO:

Melanoma Cells Carrying *BRAF* ^{V600E} or *NRAS* ^{Q61R} Differ in Autophagy Status and Sensitivity

2.1 Abstract: Metastatic melanoma has no cure. Although mutations of *BRAF* or *NRAS* (*BRAF** or *NRAS**) are identified in 63 percent of melanomas, single inhibition of these targets has not increased survival. Recently, autophagy has been examined as a potential target for overcoming *BRAF* inhibitor resistance and there is evidence that survival of melanoma cells may be autophagy-dependent. Our study investigated whether *BRAF or NRAS* mutations alter autophagic flux to suppress apoptosis in melanoma, whether basal levels of autophagic flux differ based on oncogene status and whether chemical inhibitor of autophagy would differentially alter autophagy in *BRAF** vs. *NRAS** melanoma cells. Our study has shown that autophagy is upregulated in *BRAF** melanoma cells compared to *NRAS**. Interestingly, *BRAF** melanoma cells demonstrated a four-fold decrease in cell viability following treatment with 20 μM chloroquine compared < 20 percent decreased in *NRAS** cells. In addition, knockdown of *BRAF* gene expression corresponded with a decrease in *LC3* gene expression not seen in *NRAS* knockdown. Our research supports that *BRAF** melanoma cell lines have a higher basal level of autophagic flux, are more sensitive to autophagy inhibition, and were less able to respond to autophagy induction than *NRAS** melanoma cells. Findings from this study indicate that oncogene status influences autophagic flux and that *BRAF* mutant melanoma may be addicted to autophagy for survival while *NRAS* mutant melanoma may not be as dependent on this pathway.

2.2 Keywords: Autophagy; *NRAS; BRAF; LC3*; cell death; melanoma

2.3 Introduction:

One of the emerging hallmarks of cancer is the reprogramming of pathways involved in energy metabolism to support continuous growth using mechanisms like autophagy.[1] Autophagy, or self-eating, is a catabolic process that assists the removal of unnecessary or dysfunctional components, damaged proteins and organelles through lysosomal degradation.[2] It is a tightly regulated process that plays a role in normal cell growth and development [3]and can be induced in response to nutrient deprivation.[4] Macroautophagy, the most widely discussed type of autophagy (hereafter referred to as autophagy), involves the initial formation of a double membrane structure (isolation membrane) which encapsulates cytoplasm, organelles and targeted elements in the cell, separating these targets from the rest of the cytoplasm and fusing them into a circular autophagosome. The autophagosome is characterized by the microtubule-associated protein 1A/1B-light chain 3 (LC3) protein, which is proteolytically cleaved during autophagy and then the conjugated/ lipidated form (LC3-II) localizes to the autophagosome membrane. Subsequently, LC3-II can be used as a proxy marker for the rate of autophagy as Immunofluorescent punctate structures are visible using microscopy.[5] The autophagosome then fuses with the lysosomes (autophagolysosome), which degrades or recycles the contents of the vesicles. [6] The dysregulation of autophagy has implications for many diseases including cancer. [3,7–11]

Autophagy has been shown to play a dual role in cancer[11], suppressing the early stages of tumor formation[12] yet having a pro-tumorigenic influence in the later stages of development. [13–16] Autophagic flux refers to the complete process of autophagy, beginning with the formation of an isolation membrane, autophagosome and eventually

an autolysosome where unnecessary or dysfunctional cellular components are degraded.[17] Autophagy is often upregulated in the hypoxic, low nutrient regions of tumors and can support cancer survival through its ability to provide tumors an energy source.[18,19] This upregulation has been documented in lung, prostate and other cancer types but autophagy's role in melanoma has not been well characterized.

In addition, the rate of autophagic flux appears to vary by tumor type, and it has been reported that the effect of autophagy on cancer cells may be dependent upon the stage of the cancer.[20] For example, in early primary melanomas, autophagy has been reported to play a role in suppression of melanoma tumorigenesis through the induction of senescence.[21]

Interestingly, it appears that oncogene status, an important contributor to cellular deregulation of proliferation and apoptotic pathways in melanoma also influences the rate of autophagic flux. *BRAF* ^{V600E} mutations have been reported to promote lung tumorigenesis by utilizing autophagy's ability to preserve mitochondrial function.[22] The concept that oncogenes can directly influence autophagic rates has important implications for cancer development as well as the management of treatment options for this difficult disease.

Invasive melanoma is a complex disease with an increasing incidence and the cause of the majority of skin cancer deaths. In 2015, the American Cancer Society estimates that 73,870 new melanomas will be diagnosed and 9,940 people will die from their disease. [23] The 5-year survival rate for stage IV melanoma also remains unchanged at only 15-20% but the incidence continues to rise, as it has for the past 30 years, at a rate of 6% every year. [24] Activating mutations in the *BRAF and NRAS* proto-oncogenes are

found in approximately 58% of primary melanomas (43% *BRAF* ^{V600E}; 15% *NRAS* ^{Q61R}) and 63% of melanoma metastases (48% *BRAF* ^{V600E}; 15% *NRAS* ^{Q61R}).[25] Advances in the field of melanoma have successfully identified targeted therapies including selective oncogene inhibitors (*BRAF/NRAS*); however, single inhibition of these targets has not significantly increased patient survival, as tumors eventually develop resistance.[26,27] While activating mutations in *BRAF* are critical mediators of melanomagenesis[28] and the most common genetic alterations in melanoma, the mechanisms by which these mutations promote tumorigenesis are still under investigation. Importantly, many of the mechanism by which proto-oncogenes like *BRAF* influence autophagy are under investigation. [29,30] Recent evidence for metabolic resistance to *BRAF* inhibitors and the speculation that some cancers are "autophagy addicted" suggests that activating mutations in *BRAF* may generate tumors with this phenotype.[31] These findings demonstrate the need to investigate the mechanism and extent to which mutations in proto-oncogenes affect the rate of autophagic flux and ultimately sensitivity to inhibition of this process.

Given that oncogenes influence the rate of autophagy and *BRAF* ^{V600E} -mutant melanoma cells, in particular, appear to use late-stage autophagy to maintain an active metabolic state,[29,32,33] the current study was designed to determine whether proto-oncogene mutations differentially drive metastatic melanomas to become autophagy-addicted for survival.

2.4 Materials and Methods:

2.4.1 Cell Lines.

Six melanoma cell lines, authenticated using short tandem repeat loci profiling by ATCC (Table 1), were established from human metastatic melanomas at Memorial Sloan Kettering Cancer Center and were a generous gift from Dr. Paul Chapman (SK-Mel 2, SK-Mel 19, SK-Mel 29, SK-Mel 94, SK-Mel 103, and SK-Mel 147) (Table1). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (250 units/ml), and streptomycin (250 µg/ml) (Pen-Strep) and referred to as normal media (NM) or DMEM without FBS referred to as serum free media (SFM) at 37°C and 5% CO_2.

2.4.2 In Vitro Cytotoxicity Assay: Cell lines were seeded at a density $1.20X10^6$ in 25 cm^2 flasks. Twenty-four hours after plating, cells were treated with 20µM chloroquine (CQ) and incubated at 37˚C and 5% CO_2 for a period of 24 hours Synthetic CQ was purchased from Sigma-Aldrich (C6628) and prepared in H_2O (vehicle). Control cells were treated with equivalent volume of vehicle. Following treatment, floating cells were collected; adherent cells were washed with PBS, trypsinized and mixed with the corresponding floating cells before staining. Cells were stained with 0.4% trypan blue and counted to estimate the number of live and dead cells. Cell viability is expressed as the percentage of live cells compared to the total number of cells counted. Comparisons of percent viability in vehicle vs. treated cells were determined using one way ANOVA with a cut-off of $P<0.01$.

2.4.3 Annexin 5/PI Staining – Flow Cytometry: *BRAF* ^{V600E} mutant melanoma cell lines and *NRAS* ^{Q61R} mutant melanoma cell lines were trypsinized and incubated with FITC-conjugated Annexin V (BD Biosciences) and propidium iodide. Ten thousand events were analyzed by flow cytometry with CellQuest Pro software. Data is presented

as the average percentage of viable cells +/- SD that was quantified 24 hours post treatment with either vehicle or 20 μM CQ. Statistical significance was set at 1%.

2.4.4 Caspase Activity: Caspase 3/7 activity was quantified using the Caspase-Glo assay kit (Promega, Madison USA) following the manufactures instructions. 24 hrs. following vehicle or CQ treatment, the plates containing cells were removed from the incubator and allowed to equilibrate to room temperature. 100 μl of Caspase-Glo reagent was added to each well; the plate was gently mixed and then incubated at room temperature for 1 hour. The luminescence of each sample was measured in a plate-reading luminometer (Infinite® Tecan). The experiments were performed in triplicate on two separately-initiated cultures.

2.4.5 Immunofluorescent Staining of Lysotracker Red: Autophagic flux was assessed using immunofluorescence imaging of LC3 as an indicator of the steady state level of autophagosomes and co-staining with LysoTracker (red-Cy3 dye) to examine the co-localization of lysosomal vesicles and autophagosomes in the cell. Cells were grown in normal media (NM) or serum free media (SFM), fixed, and examined by microscopy for the presence of autophagosomes and autolysosomes visualized by autophagosome membrane fluorescent LC3 or LysoTracker. LysoTracker Red was used to visualize lysosomes in vehicle and CQ treated cells. Cells were grown on coverslips as described below and once 70% confluence was reached, pre-warmed (37°C) media containing LysoTracker Red DND-99 at 50nM concentration was added. Cells were incubated for 30 minutes under normal growth conditions (37°C, 5% CO2), and then the media was replaced with normal media prior to imaging to ensure sufficient lysosomal staining prior to fixation and co-staining.

2.4.6 Immunofluorescent Staining of Microtubule-associated Protein 1 Light Chain 3 (LC3): Monolayers of cells were grown on coverslips to 70%confluence prior to experimental treatments. Cells were grown in NM or SFM media containing DMEM only. Cells were then treated with vehicle, 20 μM CQ, 20nmol/L Bafilomycin A1 (BAF) (Wako) or 1 μM Rapamycin (RAP) (Sigma, St. Louis, MO). Following treatment conditions, cells were washed twice in cold 1X PBS then fixed with 4% paraformaldehyde for 10 min, washed with 1X PBS and incubated in blocking buffer at room temperature for 30minutes. Cells were incubated with a selective antibody against LC3 (Microtubule-associated protein1A/1B-light chain 3; MBL, Woburn, MA) to evaluate autophagic flux following treatment with 20 μm CQ, 20nmol/L BAF or 1 μM RAP or vehicle for 24 hours. Cells were labeled with LC3 primary antibody in blocking solution for 1 hour. After 40 minutes, media containing DAPI was added to the coverslips for the final 20 minute incubation of LC3. After washing with 1X PBS, coverslips were incubated in anti-goat FITC-conjugated secondary antibody in blocking buffer (1:1000) in the dark for 1 hour at room temperature and then mounted on microscope slides using prolong gold (Life Technologies, NY). Autophagic flux was determined using the percentage of cells displaying LC3 punctate (rather than diffuse) fluorescence out of a 100 total cells counted in three biological replicates for each cell line and treatment type grown in cell line in either NM or a SFM for 2 hours. Formation of acidic vesicular organelles as an indication of autophagic flux was monitored by LysoTracker DND-99 (Cy3 –red) and 4',6-diamidino-2-phenylindole (DAPI) staining was used to visualize nuclei. BAF treatment was used to inhibit lysosomal function, resulting in a decrease in autophagic flux. Conversely autophagic rate was increased by treating with RAP and the

change in flux was measured. Cells were examined by Zeiss Axioskop 2MOT microscope, magnification × 620 oil immersion objective lens, and representative images of cells were taken. Co-localization of punctate Anti-LC3 FITC, LysoTracker (Cy3) and DAPI staining is shown by the pink and yellow color in a composite panel. One-way repeated measures ANOVA were used to compare means among the experimental groups and vehicle control. Pair wise comparisons between treatment groups and vehicle control were performed after adjusting for multiple tests to evaluate the effect of each treatment. Statistical significance was set at 1%

2.4.7 Quantification of Co-localized Puncta: SlideBook version 5.0 software, which analyzes the entire three-channel images by measuring the intensity of each label, was used to quantify the number of cells displaying autophagosomes. The quantification of cells with higher levels of LC3 puncta per cell is represented by area in microns compared to background. Mask segments of the low- and high- FITC/Cy3 fluorescents were created in SlideBook using in both channel images and the background was corrected using the same threshold values for all images to be analyzed. The merged images were then statistically analyzed using cross-mask and cross-channel functions. The ratio of fluorescence was then calculated allowing for quantification of the number of fluorescently-labeled co-localized puncta in fixed cells.

2.4.8 Cell Transfection and RNA Interference: For RNA interference experiments, cells were grown in 12-well plates at a density of 1×10^5 cells/well for 24. The cells were then transfections with siRNA using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA) following the manufacturer's recommendations using a final concentration of 10 nM siRNA against BRAF (Origene SR300470) or NRAS (Origene,

SR303236). siRNA transfected cells were collected 24, 48 and 72 hours following transfections. Efficiency of knockdown of the target mRNA was evaluated by real-time PCR.

2.4.9 RNA Isolation and Real-Time PCR with SYBR Green Assay: Total RNA (1 µg) was isolated with RNeasy Mini Kit from Qiagen (Valencia, CA) and reverse transcribed in 50 µl at 48 °C for 2 h using High Capacity cDNA Archive Kit from Applied Biosystems (Foster City, CA, USA). Amplification of cDNA was carried out using the following primers and SYBR green PCR master mix in an Applied Biosystems 7900 Real-Time PCR System. The primers used for amplification are: MAP1 LC3B forward primer (AGCAGCATCCAACCAAAATC) and reverse primer (CTGTGTCCGTTCACCAACAG). For both *BRAF* and *NRAS* gene expression pre-designed KiCqStart™ SYBR green primers were purchased (Sigma St. Louis, MO). The threshold cycle (Ct) values, as defined by the default setting, were measured by an ABI Prism 7900 Sequence Detection System. B-actin was used as an internal control. Triplicate samples were measured and averaged.

2.4.10 Western Blot Analysis of LC3 Protein Expression: Cells were washed twice with PBS and lysed for 30 min at 4°C in RIPA buffer (Sigma-Aldrich, France) prior to harvesting by scraping. After an ultra-centrifugation at 16,000 ×g for 10 min at 4°C, protein concentration was determined using the BCA Protein Assay Kit (Pierce, IL). Proteins were electrophoresed on a4-20% SDS–polyacrylamide gel for 45 minutes at 200 V. Proteins were then transferred onto nitrocellulose membranes (30 min, 100 mA) (BioRad Laboratories, France). Membranes were blocked with 3% bovine serum albumin (BSA), 0.1% Tween 20 in 10 mM Tris–HCl pH 7.5, 0.1 M NaCl for 1 hour at room

temperature. Incubation was overnight at 4°C with primary polyclonal rabbit anti-human LC3 at 1:500 (Cell Signaling Technology, MA) and monoclonal anti-actin at 1:1,000 (Sigma-Aldrich, France) (loading control). Subsequently the membranes were washed and incubated with 0.02 µg/ml Horseradish peroxidase–conjugated secondary antibody (HRP)-conjugated IgG (Cell Signaling, MA) for 1 hour and visualized using Super Signal West Fempto Chemiluminescent Substrate System (Pierce Biotechnology, IL). The intensity of bands was analyzed by FluorChem™ R System and AlphaView Analysis Software (Protein Simple, California). Three biologic replicates were generated for each endpoint. LC3-I/LC3-II ratio has been previously used as a surrogate of autophagic flux. However, documented differences in across cell lines and tissue dependent expression levels of LC3-I and LC3-II as well as differences in antibody affinities for LC3-I and LC3-II have indicated that this type of analysis has produced false-positive or false-negative results. Additionally, as LC3-II itself is subject to autophagic degradation at the lysosome, the current study is in agreement with the consensus to utilize overall levels of LC3-II normalized to loading control as a measure of autophagic flux (30). One-way repeated measures ANOVA were used to compare means among the experimental groups and vehicle control. Pair wise comparisons between treatment groups and vehicle control were performed after adjusting for multiple tests to evaluate the effect of each treatment. Statistical significance was set at 1%.

2.5 Results:

2.5.1 In vitro Viability Assessments: Melanoma cell lines treated for 24 hours with CQ demonstrated a decrease in percent cell viability as measured by trypan blue exclusion (Figure 1). Further, differential sensitivity to this chemical inhibition of

autophagy appeared to be influenced by *BRAF/NRAS* status in these cell lines. When cultured in NM and treated with CQ, *BRAF* ^{V600E} cell lines (SK-MEL 2, 19 and 29) had significantly decreased viability when compared to cells treated with vehicle (SK-MEL 19, *P*=0.00014; SK-MEL 29, *P*=0.0013; SK-MEL 94, *P*= 0.0008) (Figure 1A). *NRAS* ^{Q61R} cell lines (SK-MEL 2, SK-MEL 103 and SK-MEL 147) treated with the same dose of CQ had a significantly less pronounced response with <20% decrease in viability compared to vehicle (SK-MEL 2, *P*= 0.003; SK-MEL 103, *P*= 0.937; SK-MEL147, *P*=0.005 (Figure 1B). This data suggests that oncogenic status may influence the level of autophagic flux utilized by these cells.

2.5.2 Characterization of Apoptotic Cell Death: We used Annexin V/PI staining and a capase3/7 activity assay in order to characterize the type of cell death associated with the observed decrease in cell viability after treatment with CQ (Figure 2a-e; Figure 3). Overall, there was a high rate of cell death (15-20%) in the control samples potentially due to the adherent cell removal. Despite this baseline, following CQ treatment of *BRAF* cell lines, a significantly larger subpopulation of apoptotic cells was present when compared to vehicle treated cells (21.2% vs. 48.6%). Conversely, the *NRAS* cell population was predominantly composed of viable cells subsequent to CQ treatment. Further, when compared to *NRAS* treated cells, *BRAF* treated cells had a greater percentage of cells undergoing apoptosis demonstrating that the decrease in cell viability seen following CQ treatment was a consequence of inhibiting autophagy rather than overt toxicity. In addition, the activity of caspase 3/7 was over 5X greater in the cells with 20 μM CQ compared to untreated *BRAF** cells, while there was no significant difference between the *NRAS** treated or untreated cells (Figure 3)

2.5.3 Effect of Serum Starvation on Oncogenic BRAF and NRAS Melanoma Cells.

Under nutrient-deprived conditions, autolysosomes become more acidic as demonstrated by an increase in Cy3-red lysosomal staining of the cells. Following incubation for 0.5 hour in SFM, both *NRAS* and *BRAF* showed a marked increase in LC3-puncta co-localizing with lysosomal staining, indicating an increase in the number of autolysosomes (Figure 4). Quantification by SlideBook software of LC3 positive puncta (~ 100 cells/ triplicate experiment) from either *BRAF** and *NRAS** melanoma cells grown in NM or SFM indicated that serum deprivation induces LC3 puncta to form large structures at 0.5 hour in *BRAF** cells (Figure 4a and c). Serum deprivation also induces LC3 puncta formation at 0.5 and at 2 hours in *NRAS* cells (Figure 4b and c). Note that although serum starvation induces both autophagosomes (green) and autolysosomes (yellow) in *BRAF** melanoma cells, indicating autophagic induction, the quantity of autolysosomes are inhibited at 2 hours of serum starvation. In addition, in *BRAF** cells extended serum deprivation (2 hours) shows a decrease in LC3 size and number with continued starvation (Figure 4a). As shown in the quantification of LC3 immunofluorescence puncta, as a ratio of the percentage of cells displaying punctate fluorescence out of 100 cells, compared to control cells in an average of three independent experiments the attenuated response in *BRAF** cells lines shortly after serum starvation suggesting an exhaustion of the autophagy pathway (Figure 4).

2.5.4 Effect of Autophagy Inhibition and Induction on Oncogenic BRAF and NRAS Melanoma Cells. Following treatment with autophagy inhibitors (BAF or CQ) or an inducer of autophagy (RAP) *BRAF** and *NRAS** cells displayed differential autophagic responses to these chemical treatments (Figure5a-e). Following CQ or BAF

treatment *BRAF* cell lines reveal an up-regulation of LC3-II protein expression, indicating a higher rate of autophagy in these melanoma cell lines as indicated by the numerous, large LC3 puncta. These large LC3 puncta are indicative of impaired autophagic flux and demonstrate decreases co-localization with lysosomes (red) which represent a higher basal autophagic flux in *BRAF** cells. Conversely, in the *NRAS** cells autophagic inhibition by CQ or BAF addition induces very few LC3 puncta to form large structures. For autophay induction, RAP addition results in an increase in lysosome/LC3 co-localization as well as LC3 puncta indicating autophagy induction in both types of cell lines. Figure 5a-e). *BRAF** cells display a much higher basal level of autophagic flux as indicated by large number of autophagosomes (green) and autolysosomes (yellow) while RAP addition induced a substantial increase in lysosome/LC3 co-localization. The large statistical increase in *NRAS* autolysosomes following RAP induction confirms the capacity of these cells to utilize autophagy (Figure 5c) but indicates low levels of basal autophagy.

2.5.5 Differential Rates of Autophagic Flux: Differential rates of autophagic flux were validated using LC3 protein quantification and the LC3-II turnover assay by western blot (Figure 6 a-e). *BRAF* LC3-I and LC3-II protein levels were higher than the expression levels in represented in the *NRAS* cell lines. *BRAF* cell lines also demonstrated significantly increased level of LC3-II expression after CQ treatment, suggesting a break in autophagy flux. A subsequent accumulation of LC3-II that would have otherwise been degraded following conjugation into the inner autophagosomal membrane during the completion of autophagic flux may explain the eventual increase in LC3-II levels. Conversely, *NRAS* cell lines demonstrated a negligible change in LC3-II expression

following inhibition of autophagy, suggesting that these cells are not as dependent on this process.

2.5.6 Association of *LC3* Gene Expression with *NRAS/BRAF* Expression: Gene expression experiments examining the relationship between *NRAS*, *BRAF* and *LC3* using RT-PCR indicate a trend between *BRAF* expression and *LC3* expression in our cell lines. *LC3* gene expression decreased at 24, 48 and 72 hours following *BRAF* knockdown. While LC3 gene expression did decrease slightly at 24 hours following *NRAS* knockdown there was no continued trend with *NRAS* expression.

2.6 Discussion.

2.6.1 *BRAF*^{V600E} or *NRAS*^{Q61R} Genotype Determines Melanoma Sensitivity to CQ Treatment.

In this study, our data demonstrated CQ attenuated cell viability in *BRAF*^{V600E} cell lines when compared to *NRAS* ^{Q61R} cell lines treated with an equivalent dose. This data implies a variation in the basal levels of autophagic flux of these two oncogenic cell lines that are subsequently differentially affected by chemical inhibition.

Upregulation of *autophagy related (ATG*) genes results in the conjugation of cytosolic LC3 (LC3-I) to form LC3-II-PE which is recruited to the autophagosomal membranes.[6] When the intra-autophagosomal components are degraded by lysosomal hydrolases, LC3-II-PE is also degraded. Lysosomal turnover of LC3-II-PE reflects autophagic flux, and the detection of LC3 by immunofluorescence is a reliable method for monitoring autophagy.[34] CQ disruption of lysosomal function causes a dysregulation in autophagosome turnover resulting in an increase in the number of autophagosomes

present in the cell.[5,34] Similarly, a defect in autophagy turnover results when autophagy has been induced but there is limited autophagic flux. Subsequently, this leads to a reduction in the overall rate of autophagic flux and an increase in type II cell death. Our data support that *BRAF** cells are dependent on late-stage autophagy for maintenance of an active metabolic state and undergo apoptosis when treated with autophagy inhibiting agents such as CQ (Figures 1-5). [35,36] Some cancer cell types have been shown to rely on autophagy for survival as evidenced by an increase in autophagosome production and autophagic flux while under nutrient depleted conditions (Figure 4). [18,37,35] Further, this dependence on autophagy for survival has been described as "autophagy-addiction".[31] To that end, recent reports in the literature have revealed that tumor cells harboring *BRAF* ^{V600E} mutations develop an addiction to autophagy as a means to preserve mitochondrial function, as well as for glutamine metabolism, suggesting that oncogene mutation status may dictate basal levels of autophagy. [38] These findings suggest that inhibiting autophagy may be a powerful strategy for *BRAF* ^{V600E} -driven malignancies that develop resistance to selective inhibition.

2.6.2 Oncogene status dictates melanoma CQ sensitivity, altering autophagic flux and increasing apoptosis. Through *in vitro* assays we showed a significant decrease in cell viability following inhibition of autophagy in *BRAF* ^{V600E} melanoma cell lines which we then characterized as apoptotic cell death. Supporting the idea that oncogene status alters autophagic flux, we observed a diminished effect on cell viability in *NRAS*^{Q61R} after CQ treatment. This phenomenon was evident as an 8-fold difference in the IC_{50} for CQ in BRAF^{V600E} melanoma cell lines compared to NRAS^{Q61R} was observed (Table 1; Figure 1). We verified that the reduction in cell viability following CQ

exposure resulted in a significant increase in apoptotic cell death in *BRAF** cell lines following CQ inhibition compared to the *NRAS** cell lines (Figure 2 and 3). These data indicate that *BRAF* ^{V600E} melanoma cell lines are more sensitive to CQ induced inhibition of autophagy and that inhibition of autophagy in these cells as shown by our data (Figures 2 and 3) results in apoptotic cell death. While CQ is currently being investigated in cancer trials, without a clear understanding of the biological mechanisms underlying the mode of action for this treatment, we may be unable to appropriately target those patients who can benefit from this combination therapy. Our data indicate that only patients with *BRAF** melanomas may be responsive to autophagy inhibition with CQ as a co-therapy (Figure 1-3; Figure 5).

2.6.3 *BRAF* cells have more basal LC3 puncta and, following autophagy inhibition or induction, oncogenic cell lines display different rates of autophagic flux. In this study dysregulation is represented by higher basal expression of LC3-II puncta in the *BRAF* ^{V600E} cell lines compared to *NRAS* ^{Q61R} cell lines (Figure 4 and 5). Thus these data are consistent with the hypothesis that cell lines with *BRAF* ^{V600E} mutations may be more dependent upon autophagy for survival than *NRAS* ^{Q61R} cell lines. In addition, the inability of *BRAF** cells to upregulate autophagy over 2 hours following serum starvation (Figure 4) reinforcing the elevated basal level maintained in these cells. The failure of these cells to have the capacity to increase autophagic degradation rates in response to serum deprivation suggests these cells have upregulated basal autophagy to threshold. [39] The data that *NRAS**cells can increase autophagy following serum starvation as well as following RAP induction indicate that these cells can use the autophagy pathway to survive nutrient depletion but they are not dependent upon this pathway under

basal conditions. In addition, we demonstrated an increase size in LC3 puncta in CQ-treated *BRAF* ^{V600E} cell lines (Figure 4), suggesting autophagosome accumulation as a consequence of lysosomal inhibition by CQ. By contrast, we did not observe the same level of LC3-II aggregation and autophagosome accumulation in the *NRAS* ^{Q61R} cell lines treated with the same dosage of CQ. Further, using the western assay, we showed a decrease in overall LC3-I and an increase in LC3-II protein expression following CQ treatment (Figure 6) in *BRAF** cells but not a corresponding decrease in *NRAS** cells indicating that autophagy is induced at higher basal levels in *BRAF** than *NRAS** melanoma cells.

To determine if BRAF^{V600E} was associated with autophagy activation, we knocked down either *BRAF* or *NRAS* in melanoma cells and evaluated the corresponding change in LC3 gene expression (Figure 7). These results indicate showed knock-down of *BRAF* significantly decreased *LC3* expression. These results are consist with the association of the *BRAF** and increased rates autophagy. We did not see a corresponding consistent trend with *NRAS* knockdown providing evidence that, while *BRAF** correlates with *LC3* expression, *NRAS** expression does not. Previously, a positive correlation between LC3 protein and levels of *BRAF* has been also reported lending further support to our conclusions.[32]

Currently, *BRAF* inhibition is accomplished clinically by treatment with vemurafenib, a specific *BRAF* ^{V600E} ATP-competitive, small-molecule inhibitor. Studies have reported the development of *NRAS* mutation in patients following vemurafenib treatment for *BRAF* melanoma.[40] This important finding reiterates the significance of understanding the biological mechanisms underlying the induction of the autophagy

pathway, as *BRAF* inhibition resistant tumors might also lose their sensitivity to chloroquine following acquisition of *NRAS* mutations. *BRAF* is an amino acid sensor and has been shown to positively regulate autophagy in colon cancer cells. Under nutrient deprivation conditions or with constitutive activation of *BRAF,* MAPK is induced and results in the upregulation of autophagy. As part of the MAPK pathway, Extracellular Signal-regulated Kinase (ERK) regulates the maturation of autophagic vacuoles and constitutive activation of the MAPK.[41] MAPK activation results in the inhibition of mTORC1/2, as well an increase in the expression of Beclin 1 (Atg6), a protein with a central role in autophagy initiation. Through these interactions we speculate that *BRAF* may be facilitating an increase in basal autophagic flux (Figure 8). This speculation is supported by recent data reporting that the use of combination *BRAF* and autophagy inhibitors promoted tumor regression in *BRAF* inhibition -resistant xenografts.[42]

Limitations of our study include the absence of the characterization of the rate of autophagic flux in a *BRAF*/*NRAS* wildtype cell line. However, as melanoma is a highly mutagenic cancer, even a cell line that that expressed wildtype *NRAS* and *BRAF* would have other mutations which might impact autophagy independently diminishing the relevance of this comparison.

In the current study we determined that autophagy was up-regulated in $BRAF^{V600E}$ melanoma cells when compared with $NRAS^{Q61R}$ melanoma cells as measured by immunofluorescence and western blot analyses and that inhibition of autophagy resulted in apoptotic cell death specifically in the *BRAF** cells. Finally we identified a trend in the gene expression of a proxy marker of *LC3* and *BRAF* expression but no corresponding trend with *NRAS* expression.

Despite the recent success of *BRAF*^{V600E} specific inhibitors, chemo-resistance quickly occurs which suggests that other signaling mechanisms, like autophagy, may contribute to progression in this subtype of melanoma. Our findings implicate a positive association between the expression of *BRAF* ^{V600E} mutations in melanoma cells lines and the rate of autophagic flux. While both *BRAF* and *NRAS* mutations activate ERK/MAPK signaling,[43] our research reveals that melanoma cells harboring *BRAF* ^{V600E} mutations utilize autophagy as a metabolic resource to a greater extent than *NRAS** cells. In addition, the *BRAF** and *NRAS** pathways, may have opposing effects on the rate of autophagic flux in melanoma. Our results indicate that *BRAF** melanoma cells are addicted to autophagy and support *BRAF* activation of autophagy, potentially through Beclin1, [44,45] thereby promoting tumor survival (Figure 8). [30,43]

In conclusion, *BRAF** melanoma cancer cells are more susceptible to autophagic inhibition, while *NRAS** melanoma cells appear less dependent on this pathway which implicates this process as a viable and attractive target for future therapies. The identification of an association of autophagy dependence and *BRAF* ^{V600E} melanoma suggests that drugs like CQ, that suppress this process, may also have therapeutic potential for aggressive tumor types and that there is differential activation of the autophagy pathway in *BRAF** vs. *NRAS** melanomas.

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2.8 References:

1. Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: the next generation. *Cell* **144,** 646–74 (2011).

2. Jain, M. V *et al.* Interconnections between apoptotic, autophagic and necrotic pathways: implications for cancer therapy development. *J. Cell. Mol. Med.* **17,** 12–29 (2013).

3. Choi, A. M. K., Ryter, S. W. & Levine, B. Autophagy in human health and disease. *N. Engl. J. Med.* **368,** 651–62 (2013).

4. Lum, J. J., DeBerardinis, R. J. & Thompson, C. B. Autophagy in metazoans: cell survival in the land of plenty. *Nat. Rev. Mol. Cell Biol.* **6,** 439–48 (2005).

5. Klionsky, D. J. *et al.* Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. *Autophagy* **4,** 151–75 (2008).

6. Mizushima, N. Autophagy: process and function. *Genes Dev.* **21,** 2861–73 (2007).

7. White, E., Karp, C., Strohecker, A. M., Guo, Y. & Mathew, R. Role of autophagy in suppression of inflammation and cancer. *Curr. Opin. Cell Biol.* **22,** 212–7 (2010).

8. Wu, W. K. K. *et al.* The autophagic paradox in cancer therapy. *Oncogene* **31,** 939–53 (2012).

9. Rubinsztein, D. C., Bento, C. F. & Deretic, V. Therapeutic targeting of autophagy in neurodegenerative and infectious diseases. *J. Exp. Med.* **212,** 979–90 (2015).

10. Deretic, V. Autophagy in Tuberculosis. *Cold Spring Harb. Perspect. Med.* **4,** a018481–a018481 (2014).

11. Rosenfeldt, M. T. & Ryan, K. M. The multiple roles of autophagy in cancer. *Carcinogenesis* **32,** 955–63 (2011).

12. Brech, A., Ahlquist, T., Lothe, R. A. & Stenmark, H. Autophagy in tumour suppression and promotion. *Mol. Oncol.* **3,** 366–75 (2009).

13. Mathew, R. *et al.* Autophagy suppresses tumorigenesis through elimination of p62. *Cell* **137,** 1062–75 (2009).

14. Yang, S. *et al.* Pancreatic cancers require autophagy for tumor growth. *Genes Dev.* **25,** 717–29 (2011).

15. Yang, S. *et al.* Autophagy regulation by the nuclear factor κB signal axis in acute pancreatitis. *Pancreas* **41,** 367–73 (2012).

16. Patil, S., Rao, R. S. & Raj, A. T. Dual Role of Autophagy in Oral Cancer. *J. Int. oral Heal. JIOH* **7,** i–ii (2015).

17. Hansen, T. E. & Johansen, T. Following autophagy step by step. *BMC Biol.* **9,** 39 (2011).

18. Selvakumaran, M., Amaravadi, R. K., Vasilevskaya, I. A. & O'Dwyer, P. J. Autophagy inhibition sensitizes colon cancer cells to antiangiogenic and cytotoxic therapy. *Clin. Cancer Res.* **19,** 2995–3007 (2013).

19. Sato, K. *et al.* Autophagy is activated in colorectal cancer cells and contributes to the tolerance to nutrient deprivation. *Cancer Res.* **67,** 9677–84 (2007).

20. Kimura, T., Takabatake, Y., Takahashi, A. & Isaka, Y. Chloroquine in Cancer Therapy: A Double-Edged Sword of Autophagy. *Cancer Res.* **73,** 3–7 (2013).

21. Liu, H., He, Z. & Simon, H.-U. Targeting autophagy as a potential therapeutic approach for melanoma therapy. *Semin. Cancer Biol.* **23,** 352–60 (2013).

22. Strohecker, A. M. & White, E. Autophagy promotes BrafV600E-driven lung tumorigenesis by preserving mitochondrial metabolism. *Autophagy* **10,** 384–5 (2014).

23. American Cancer Society Facts and Statistics 2015 | Research | American Cancer Society. at <http://www.cancer.org/research/cancerfactsstatistics/cancerfactsfigures2015/>

24. What are the key statistics about melanoma skin cancer? at <http://www.cancer.org/cancer/skincancer-melanoma/detailedguide/melanoma-skin-cancer-key-statistics>

25. Colombino, M. *et al.* Heterogeneous distribution of BRAF/NRAS mutations among Italian patients with advanced melanoma. *J. Transl. Med.* **11,** 202 (2013).

26. Das Thakur, M. *et al.* Modelling vemurafenib resistance in melanoma reveals a strategy to forestall drug resistance. *Nature* **494,** 251–5 (2013).

27. Chapman, P. B. *et al.* Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N. Engl. J. Med.* **364,** 2507–16 (2011).

28. Haq, R. *et al.* Oncogenic BRAF regulates oxidative metabolism via PGC1α and MITF. *Cancer Cell* **23,** 302–15 (2013).

29. Xie, X., Koh, J. Y., Price, S., White, E. & Mehnert, J. M. Atg7 Overcomes Senescence and Promotes Growth of BrafV600E-Driven Melanoma. *Cancer Discov.* **5,** 410–23 (2015).

30. Ma, X. *et al.* Targeting ER stress – induced autophagy overcomes BRAF inhibitor resistance in melanoma. **124,** (2014).

31. Altman, B. J. & Rathmell, J. C. Metabolic stress in autophagy and cell death pathways. *Cold Spring Harb. Perspect. Biol.* **4,** a008763 (2012).

32. Maddodi, N. *et al.* Induction of autophagy and inhibition of melanoma growth in vitro and in vivo by hyperactivation of oncogenic BRAF. *J. Invest. Dermatol.* **130,** 1657–67 (2010).

33. Lazova, R., Klump, V. & Pawelek, J. Autophagy in cutaneous malignant melanoma. *J. Cutan. Pathol.* **37,** 256–68 (2010).

34. Mizushima, N., Yoshimori, T. & Levine, B. Methods in mammalian autophagy research. *Cell* **140,** 313–26 (2010).

35. Yang, Z. J., Chee, C. E., Huang, S. & Sinicrope, F. A. The role of autophagy in cancer: therapeutic implications. *Mol. Cancer Ther.* **10,** 1533–41 (2011).

36. Chatterjee, S. J. & Pandey, S. Chemo-resistant melanoma sensitized by tamoxifen to low dose curcumin treatment through induction of apoptosis and autophagy. *Cancer Biol. Ther.* **11,** 216–28 (2011).

37. Sui, X. *et al.* Autophagy and chemotherapy resistance: a promising therapeutic target for cancer treatment. *Cell Death Dis.* **4,** e838 (2013).

38. Strohecker, A. M. *et al.* Autophagy sustains mitochondrial glutamine metabolism and growth of BrafV600E-driven lung tumors. *Cancer Discov.* **3,** 1272–85 (2013).

39. Knecht, E., Hernández-Yago, J. & Grisolía, S. Regulation of lysosomal autophagy in transformed and non-transformed mouse fibroblasts under several growth conditions. *Exp. Cell Res.* **154,** 224–32 (1984).

40. Callahan, M. K. *et al.* Progression of RAS-mutant leukemia during RAF inhibitor treatment. *N. Engl. J. Med.* **367,** 2316–21 (2012).

41. Corcelle, E. *et al.* Control of the autophagy maturation step by the MAPK ERK and p38: lessons from environmental carcinogens. *Autophagy* **3,** 57–9

42. Ma, X.-H. *et al.* Targeting ER stress–induced autophagy overcomes BRAF inhibitor resistance in melanoma. *J. Clin. Invest.* **124,** 1406–1417 (2014).

43. Xie, X., White, E. P. & Mehnert, J. M. Coordinate autophagy and mTOR pathway inhibition enhances cell death in melanoma. *PLoS One* **8,** e55096 (2013).

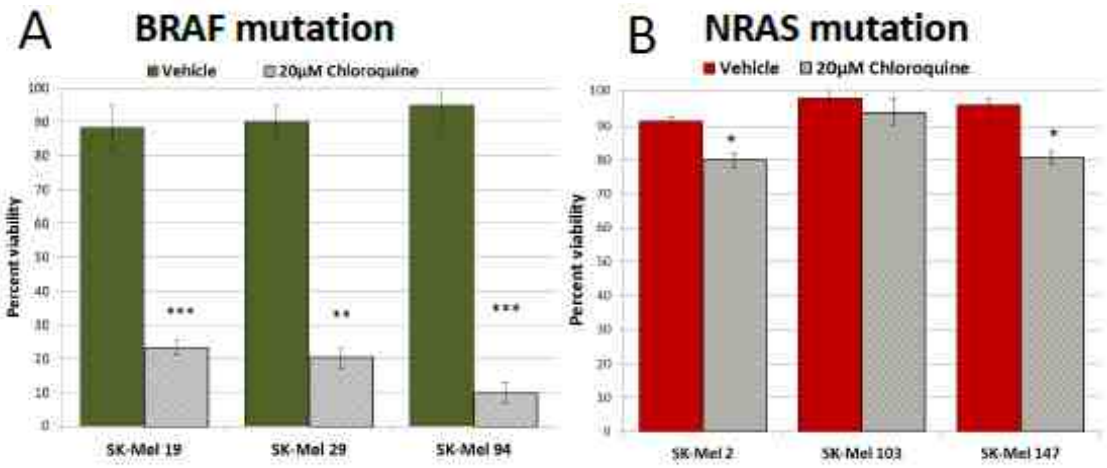
44. Lovat, M. C. and P. E. Harnessing Autophagy for Melanoma Benefit. *Cell Biol. Res. Ther.* at <http://www.scitechnol.com/harnessing-autophagy-for-melanoma-benefit-xYl7.php&&article_id=1019>

45. Deng, Q. *et al.* Lower mRNA and protein expression levels of LC3 and Beclin1, markers of autophagy, were correlated with progression of renal clear cell carcinoma. *Jpn. J. Clin. Oncol.* **43,** 1261–8 (2013).

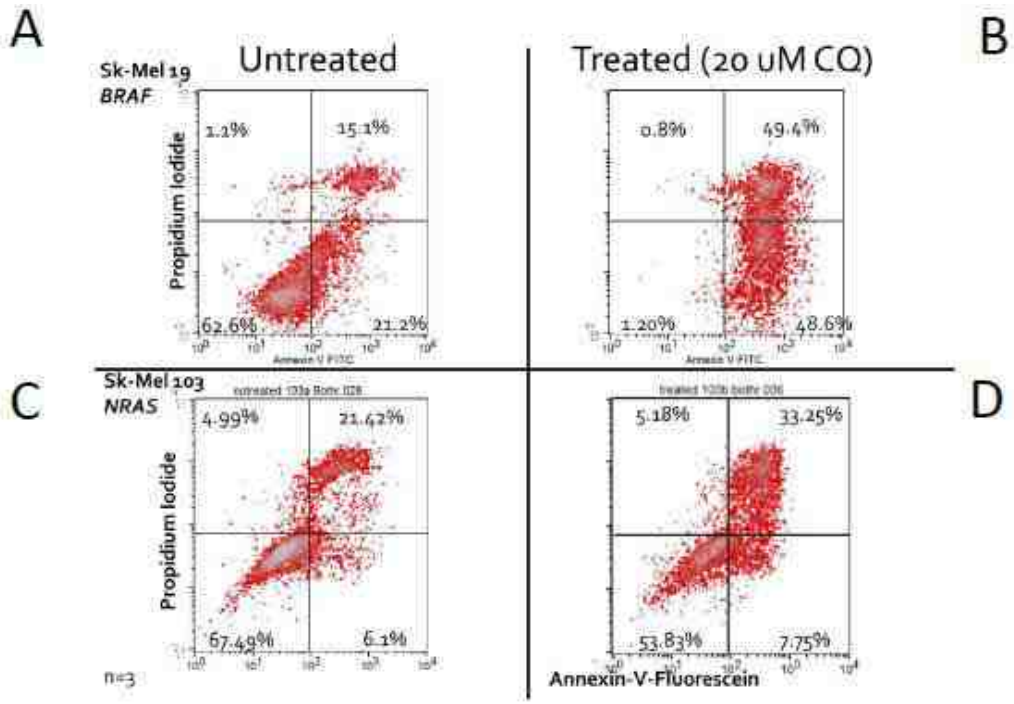
2.9 Tables and Figures

2.9.1 Table 1: Oncogene status and gender summary for Memorial Sloan Kettering melanoma cell lines (SK-Mel).

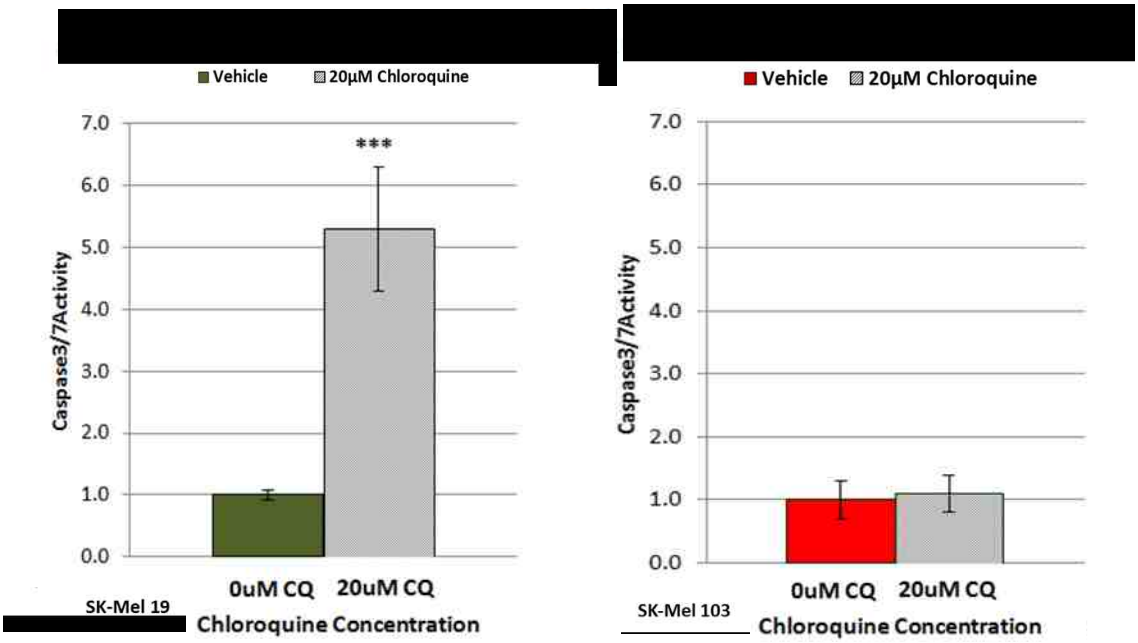
Table 1: Memorial Sloan Kettering Melanoma Cell				
Cell Line	Gender	p53	B-RAF	N-Ras
19	Female	WT	V600E	WT
29	Male	WT	V600E	WT
94	Male	WT	V600E	WT
2	Male	WT	WT	Mut/Q61R
103	Male	WT	WT	Mut/Q61R
147	Male	WT	WT	Mut/Q61R



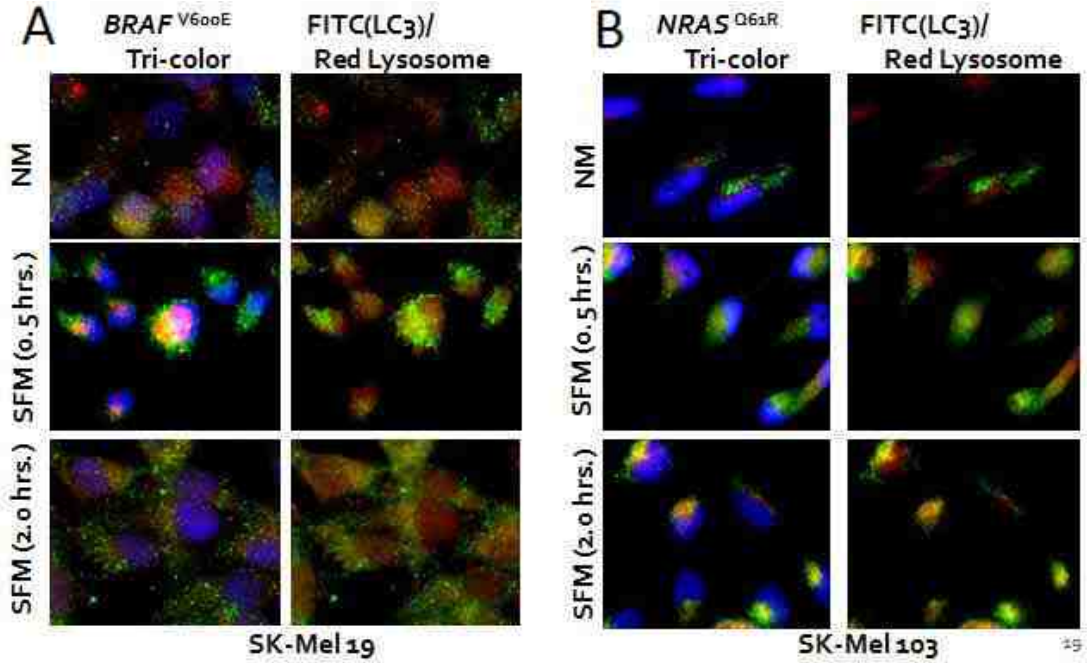
2.9.2 Figure 1: Cell Treatment with 20 μM CQ. Average percentage of viable cells (n=3) following treatment with vehicle or 20 μM CQ (A) *BRAF* ^{V600E} mutant melanoma cell lines (SK-Mel 2, 19, 29) (B) *NRAS* ^{Q61R} mutant melanoma cell lines (SK-Mel 94, 103, 147) (mean ± SD *, P <0.01; **, P <0.001; ***, P <0.0001).

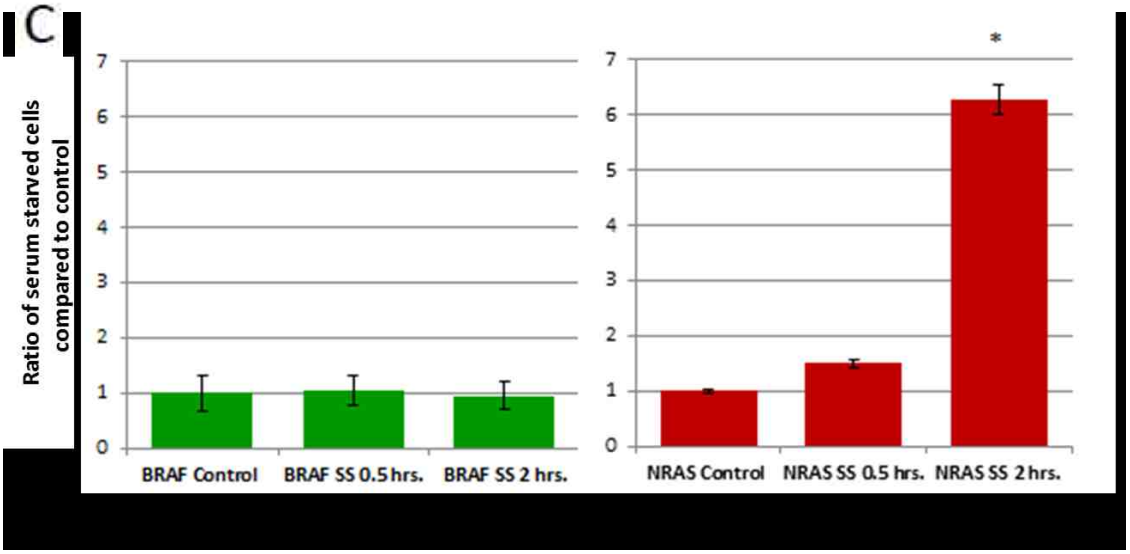


2.9.3 Figure 2a-d: Annexin V/PI staining by flow cytometry. Flow cytometric analysis of melanoma cell apoptosis in culture. Untreated and CQ treated cells collected after 24 h of culture were stained with PI and FITC-labelled Annexin V. Two melanoma cell lines, one *BRAF* and one *NRAS* were treated either with vehicle (Figure 2a and c) or c 20 μM CQ (Figure 2b and d) for 24 hours prior to staining with Annexin V and propidium iodide (PI). Viable cells with intact membranes were represented by the unstained portion (Annexin V and PI negative) in the lower left, while and Annexin V stained cells, indicating early apoptotic cells, are represented in the lower right (Annexin V positive/ PI negative). The upper left portion represents necrotic cells (Annexin V negative/PI positive) while cells with disrupted membranes are undergoing late apoptosis or are already dead are represented in the upper right (Annexin V positive/ PI positive). Insets represent the percentage of cells in each quadrant.



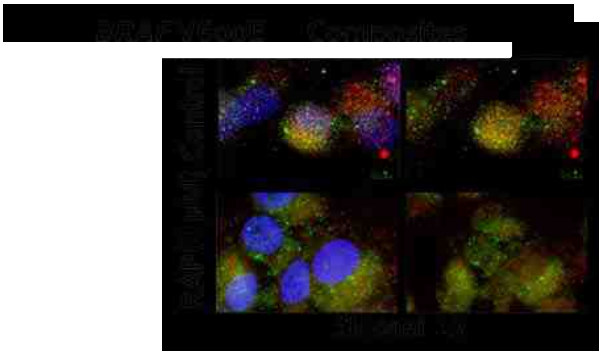
2.9.4 Figure 3a-b: Caspase activity luminescence assay. *BRAF* (SK-MEL19) or *NRAS* (SK-MEL 103) mutant cells were treated with either vehicle or 20μM chloroquine for 24 h. Following treatment the caspase 3/7 activity of each triplicate group of cells was quantified by a luminescence assay. Data are mean ± SD of two sets of different triplicate experiments. * $P < 0.005$.





2.9.5 Figure 4a-c: Immunofluorescent staining of melanoma cell lines before and after autophagy induction by serum starvation. (A-B) Autophagy was induced by serum starvation through culture in serum-free medium (SFM) for indicated time points. Control cells were cultured in normal media (NM) containing FBS and vehicle. Live cells were treated with lysotracker and then cells were fixed prior to additional staining with Anti-LC3 FITC and DAPI. Composite images of LC3 (green) with lysosomal (red) and nuclear stain (blue) or co-localization shown by yellow dual staining of LC3 (green) with lysosomal (red). Cells were examined by Zeiss Axioskop 2MOT microscope × 620. *$P < 0.05$. A) *BRAF* mutant melanoma cells in NM or SFM. B) *NRAS* mutant melanoma cells in NM or in SFM. C) Histogram of quantification of LC3 immunofluorescence puncta as a ratio of the percentage of cells displaying punctate fluorescence out of 100 cells compared to control cells in an average of three independent experiments. *$P < 0.05$.

A



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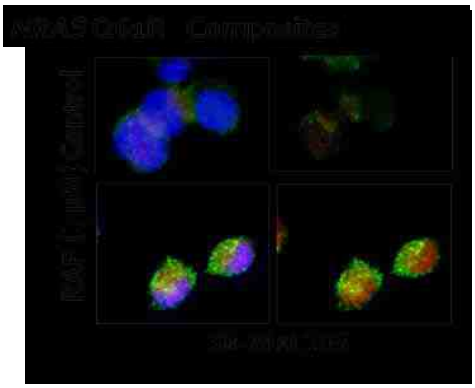

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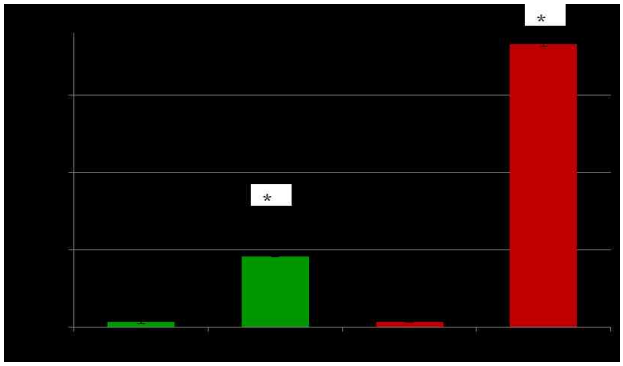
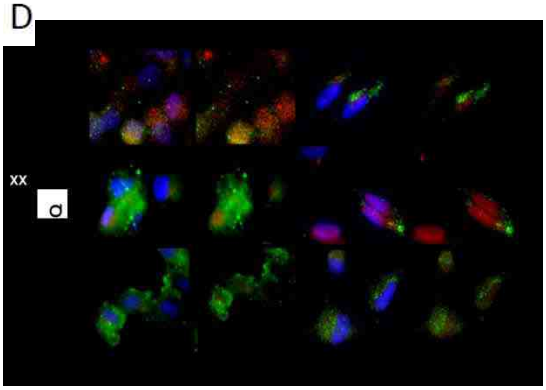
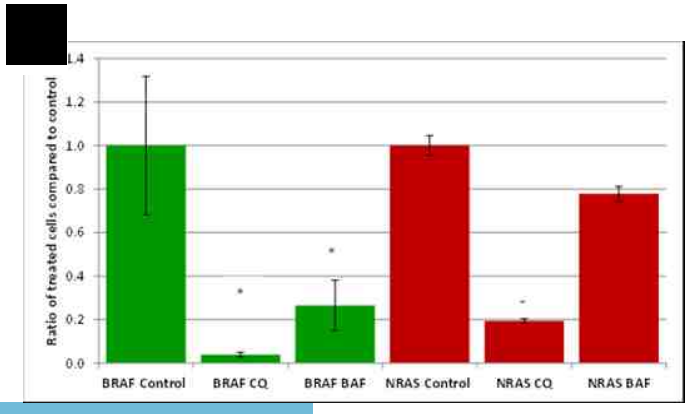


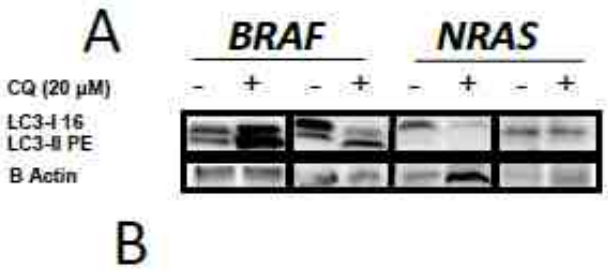
D

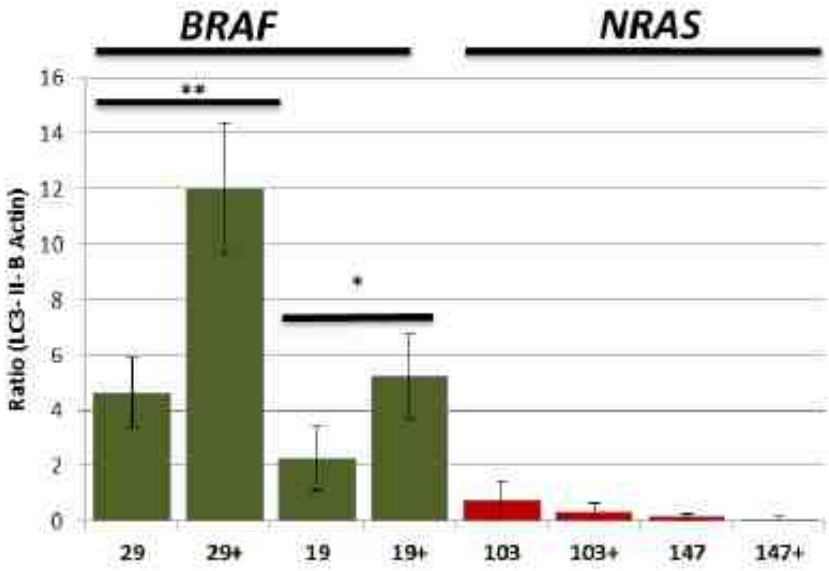




2.9.6 Figure 5a-e: The effect of autophagic chemical inhibition by CQ or BAF treatment, or RAP induced autophagy, in melanoma cells.

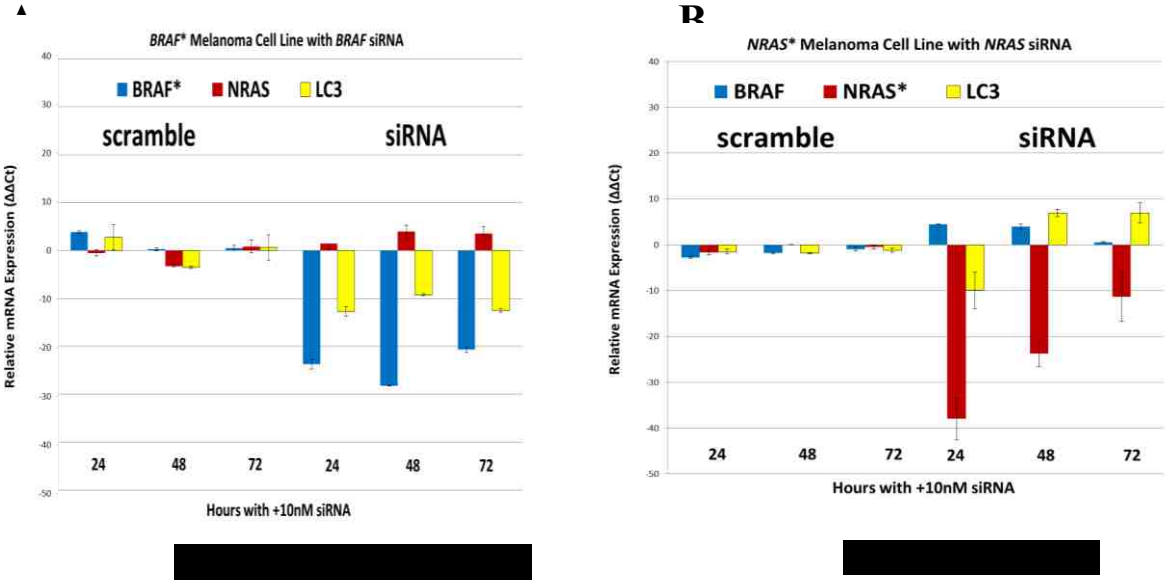
Oncogenic melanoma cells were treated with vehicle, (A) rapamycin (1 μM)(RAP), (D) cloroquine (CQ)(20 μM) or (D) bafilomycin A (BAF)(20nmol/L) for three hours. Quantification of autophagy induction (C) or inhibition (E). Control cells were cultured in normal media containing FBS and vehicle. Live cells were treated with lysotracker and then cells were fixed prior to additional staining with Anti-LC3 FITC (green) and DAPI nuclear stain (blue). Composite images of LC3 (green) with lysosomal (red) and nuclear stain (blue) or co-localization shown by yellow dual staining of LC3 (green) with lysosomal (red). Cells were examined by Zeiss Axioskop 2MOT microscope. × 620.





2.9.7 Figure 6a-b: Western blot analysis of LC-3B.

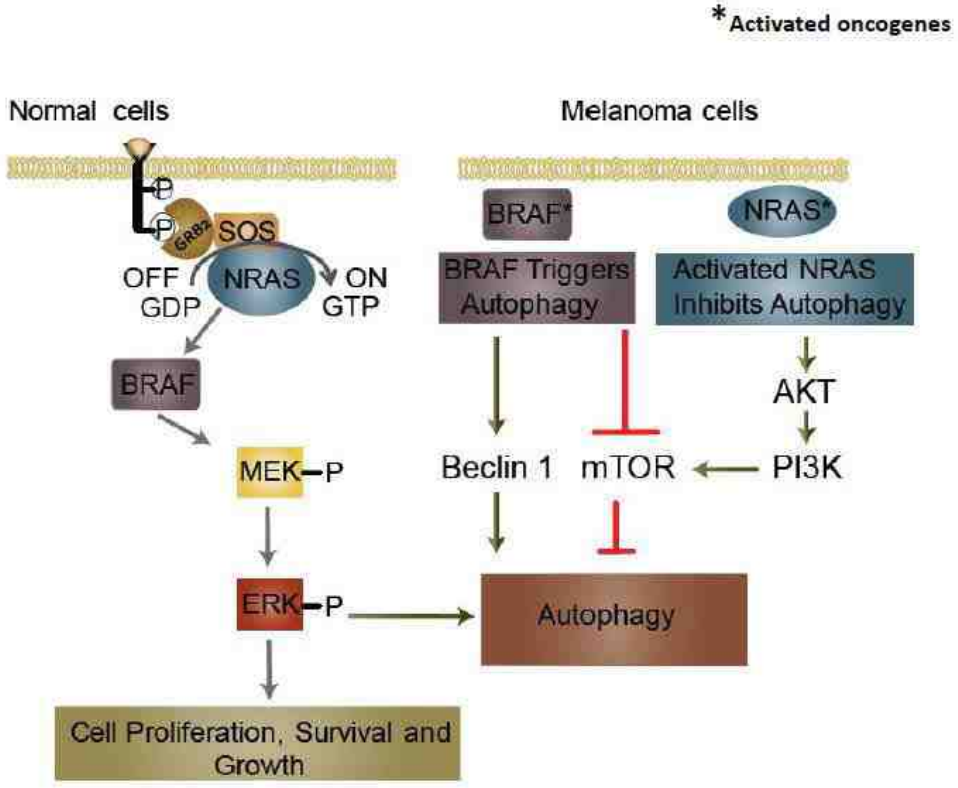
(A) Western blot and (B) Quantitative Analysis of (A). Relative LC3 protein expression in *BRAF* and *NRAS* mutant melanoma cell lines when treated with vehicle (-) or 20 μM CQ (+) for 24 hrs. (B) Protein quantification from Western blot using densitometry of LC-3B-II in *BRAF* or *NRAS* mutant melanoma following treatment with vehicle or 20 μM CQ as indicated. Results are shown as arbitrary units (AU) normalized to β-Actin (mean ± SD; **, P < 0.01; ***, P < 0.001; n =3).



2.9.8 Figure 7: Suppression of BRAF or NRAS mRNA expression by siRNA.

SK-Mel cells were transiently transfected with si-*BRAF*; si-*NRAS* or scrambled siRNA (Scramble). Twenty-four, forty-eight or seventy-two hours after siRNA or scramble treatment, total RNA was extracted from transfected cells and quantified using real-time PCR. Expression levels of BRAF and LC3 mRNA were significantly suppressed by si-

BRAF (A) and expression levels of NRAS mRNA were significantly suppressed by si-*NRAS* (B) but had an insignificant influence on LC3 mRNA. Results are normalized to the expression level of B-actin mRNA and are expressed as the ratio of *BRAF*, *NRAS* or *LC3* expression to scramble. Data are presented as mean ± SD of three replicate experiments.



2.9.9 Figure 8: Melanoma cells with activating BRAF* or NRAS* have differential dependence on autophagy

CHAPTER THREE:

Quantitative Analysis of Immunohistochemistry in Melanoma Tumors and LC3 and Beclin 1 Expression in Melanoma Lesions: Association with UV Exposure and Prognostic Indicators.

Supplemental note: Due to the current subjective quantification of protein expression, in order to rigorously address whether autophagic flux is associated with UV exposure and/or clinical stage and modified by oncogenic BRAF/NRAS in melanoma tumors, I developed a novel quantification method which removes this aspect from the evaluation of proteins by IHC in melanoma.

3.1 Abstract:

Aims: Interpretation of protein expression, by immunohistochemistry (IHC), in melanoma tissue sections is difficult and subjective. Inter/intra-observer variability even among experienced dermatopathologists and inherent pigmentation, make diagnostic reproducibility challenging.

Methods: We sought to identify a quantitative method for measuring IHC protein expression in melanoma tissues. In the current study, we used IHC HRP-DAB with an Azure counterstain, to develop a quantitative measurement of protein expression using spectral imaging technology.

Results: We examined the distribution of mean intensities from DAB-labeled protein in different participants using different Azure reference spectra to remove (unmix) melanin stain. We identified no significant differences in mean DAB intensities ($p=0.73$; Kruskal-Wallis).

Conclusions: To our knowledge, this is the first study to use spectral imaging to quantify IHC protein expression in melanoma lesions. Using this methodology, the absorbance spectra of the reference is not affected by overall label intensity, allowing IHC interpretation to be independent of high visual contrast chromogens. This has important

implications particularly for pigmented tissue sections. This quantification method reduces the subjectivity of protein expression analysis and provides a valuable tool for accurate evaluation.

Keywords: Immunohistochemistry; melanoma; spectral imaging

3.2 Introduction:

Immunohistochemistry (IHC) is an important technique to both researchers and clinicians[1], and is used to identify the presence and location of protein[2]. Most cancer research employing IHC utilizes formalin-fixed paraffin embedded (FFPE) tissues that have been sectioned and mounted onto slides. For clinicians, IHC is particularly important in the diagnosis of cancers, including melanoma, as it allows for identification of overexpressed proteins. These protein biomarkers, for example S100 and HMB-45 in melanoma, can predict disease progression and identify potential therapeutic targets.

Standard IHC is a multi-step technique that has two major stages—antigen retrieval/staining and analysis. The first step in the staining stage is deparaffinization and rehydration of the tissue section. The tissue section is incubated in a buffer under high heat to remove cross-links formed during the fixation process and "expose" proteins of interest. Antigen retrieval unmasks antigens within the tissue section for binding to antibodies, followed by a series of steps to block antibody binding to non-specific proteins and the removal of endogenous peroxidases. Next, the tissue is incubated with a high affinity primary antibody specific to the protein of interest. Visualization of the protein is accomplished using a chromogen substrate and an enzyme, such as horseradish

peroxidase (HRP), is conjugated to either the primary antibody or a secondary antibody against the primary antibody.

The addition of the chromogen bound to the substrate-conjugated antibody results in substrate cleavage, producing a colored stain at that location, and thereby indicating the presence of the target protein. The most commonly utilized substrate-chromogen combination is HRP (substrate) 3,3'-Diaminobenzidine (DAB) (chromogen), which results in a brown stain and there are a limited number of substrate-chromogen combinations available for IHC [3].

Once the tissues are developed, they are dehydrated and mounted with a stabilizing mounting medium and coverslip for visualization. The stains are visualized using microscopy and quantified through a variety of methods. Current IHC analysis is semi-quantitative; typically several readers use a subjective scoring system. The scores are subsequently compared in order to assess inter-reader variability[4].

The limitations of this semi-quantitative method have been particularly troublesome in melanoma, because the melanin pigment is brown, creating challenges for both researchers and clinicians to accurately differentiate between DAB staining and melanin, potentially impacting accurate diagnoses. Given the importance of this tool, there is a need for a non-subjective quantitation method that can be applied to IHC in melanoma tissue sections.

In 1991, Kamino and Tam identified that Azure B, hereafter referred to as Azure, acts as an appropriate counterstain in melanoma sections[5]. They reported that Azure stains melanin green-blue, allowing for its identification in contrast to the surrounding tissue. However, this method was still quantified using the scoring system, and therefore relies on subjective interpretation.

Azure, a cationic dye, is one of the major metabolites of methylene blue and is used in chromosomal tissue staining[6]. While the selective binding is not well characterized in the literature, we propose, as a provisionary mechanism, that the heterocyclic nitrogen in Azure functions as a base[7] to deprotonate carboxylic acids found in melanin. This would allow for ionic interactions between melanin's anion and the cations in Azure. Acidic interplay, in conjunction with hydrogen bonding, likely results in the preferential staining of the melanin (Supplemental Figure 1).

With advances in imaging and associated software, we sought to identify a quantitative method for measuring protein expression in melanoma tissues. In the current study, we used IHC HRP-DAB staining in melanoma tissues, with Azure as a counterstain, to develop a quantitative measurement of protein expression using spectral imaging technology.

3.3 Methods:

3.3.1 Tissue samples:

Melanoma tissue sections were obtained from two different sources: University of California Surgical Pathology Laboratory and the University of New Mexico Hospital (UNMH) Surgical Pathology Laboratory. California Surgical tissues were selected from a

residual bio-repository of de-identified FFPE tumor blocks from patients diagnosed with malignant melanoma between 1990 and 1999 in Los Angeles County. New Mexico tissues were selected from the University of New Mexico Ultraviolet Light Exposure and Immunosuppression in Melanoma bio-repository (INST 0815 HRRC 08-433).

Non-pigmented control tissue was obtained from breast reduction mammoplasty surgery between November 2007 and January 2011 as previously described[8]. This sample was collected with IRB approval and was de-identified.

3.3.2 Immunohistochemistry (IHC):

All IHC protocols were performed according to the antibody manufacturer's instructions. Briefly tissue sections were stained with antibody produced in rabbit and generated against a C-terminus peptide in G protein-coupled estrogen receptor 1 (GPER) (clone number 8073) at a dilution of 1:200 for one hour and fifteen minutes. The GPER antibody was a generous donation from the laboratory of Dr. Eric Prossnitz. Tissue sections were then incubated with 1:100 dilution of secondary goat anti rabbit-HRP antibody for one hour (Sigma, St. Louis, MO).

HRP activity was visualized using the Liquid DAB Plus Substrate Kit (Life Technologies, Carlsbad, CA) according to manufacturer's instructions. Following incubation with DAB, sections were stained with Azure for 10 minutes[5]. Tissue sections were then dehydrated and mounted with a coverslip using permount.

3.3.3 Image Acquisition and Analysis:

Brightfield, spectral images of IHC labeled sections were generated at the UNM Cancer Center Fluorescence Microscopy and Cell Imaging Shared Resource using a Nikon

TE2000 microscope (Melville, NY) in transmitted light mode, which had been adjusted for Koehler illumination. Images were obtained using a 60x oil objective, at 1.5x intermediate magnification, and a Nuance Spectral Imaging Camera and software (Perkin Elmer). The Nuance camera uses a liquid crystal tunable filter (LCTF), set to collect transmitted light in 20 nm bandwidths, at 10 nm step intervals, from 420 to 720 nm. The resulting spectral image cube consists of 16 separate images each acquired at a different wavelength range. Each pixel in the resulting image cube has an absorbance spectrum that depends on the absorbing materials (labels) that are present at that pixel location. Nuance camera software controls both the LCTF and spectral image acquisition. Prior to imaging of the tissues, a 100% transmission reference image cube was acquired from a region of the slide with no tissue or other debris and was used to convert all images to optical density (OD) images. Spectral image cubes collected from melanoma sections labeled only with Azure or anti-GPER-DAB were used to generate pure absorbance spectra for each of these labels. These pure spectra were then used by the Nuance software to unmix (using a linear unmixing algorithm) image cubes acquired from slides labeled with both Azure and DAB, generating single component images of each label. Absorbance spectrum imaging and unmixing of IHC labeled sections has been previously described[9].

Three reference spectra (pure absorbance spectra) of Azure were generated from 3 separate patient tumors, each labeled only with Azure. A single reference spectrum of GPER-DAB was generated from a GPER-positive, non-pigmented tissue section labeled only with anti-GPER DAB. To quantify the staining, we generated three spectral libraries, each consisting of two spectra: 1) GPER-DAB reference spectrum and Azure

spectrum from patient tumor section A; 2) GPER-DAB reference spectrum and Azure spectrum from patient tumor section B; 3) GPER-DAB reference spectrum and Azure spectrum from patient tumor section C.

Additional tissue sections from the same three patient tumors were doubly labeled with GPER-DAB and Azure and spectral image cubes were acquired with the Nuance camera. These spectral image cubes were unmixed with each of the three spectral libraries described above. The Azure component images show location of the counterstain and tissue structure; GPER-DAB component images show GPER-expressing cells in the section.

GPER-DAB component images were exported from the Nuance software as TIF images, imported into Slidebook software 6.0 (3i, Intelligent Imaging Innovations, Denver) and the GPER-DAB staining was quantified. Component GPER-DAB images were inverted from absorbance to pseudofluorescence for analysis. In the inverted images, higher pseudofluorescence intensity corresponds to higher absorbance (DAB concentration). A segment mask was created by setting a threshold to eliminate background. Intensity values above the threshold represent DAB labeling. A single threshold level was determined by examination of multiple images and was used for quantifying all of the DAB component images. Segmentation masks were created on each doubly labeled patient section. Utilizing the mask statistics function in Slidebook, we exported the mean intensity value of each masked region in the GPER-DAB component images for statistical analysis.

3.3.4 Statistics:

Mean intensity of anti-GPER-DAB in the three sections stained with GPER-DAB and Azure was compared following unmixing with the three reference spectral libraries described above, generating three mean intensities of GPER-DAB staining for each of the participants. Since the distribution of mean intensities of GPER-DAB staining (distribution) might not meet normality assumptions, we used the Kruskal–Wallis one-way analysis of variance (ANOVA) to compare the distributions. The null hypothesis was that the distributions for each of the participants would be equal. The alternative hypothesis is that the distribution for at least one of the participants is different from the distribution for other participants.

3.4 Results:

3.4.1 Azure reference spectra do not vary between tissue sections:

Because the absorbance spectrum of melanin may vary from person to person, the spectrum of Azure bound to melanin may also vary from person to person, as the melanin pigment masked by Azure staining will be included in the absorbance spectrum. Additionally, we were concerned that the affinity of Azure to bind to melanin may also vary from person to person. To address these concerns, we compared Azure spectra across melanoma tissues samples from three different participants.

Figure 1 shows the three Azure reference spectra (blue, green, and red lines) generated from melanoma tissues sections labeled with Azure only from three different participants. A GPER-DAB reference spectrum (brown line) was generated in a non-pigmented tissue section stained only with GPER-DAB. Importantly, the three Azure spectra overlap and are visually comparable (Figure 1).

3.4.2 Quantification of GPER-DAB staining in tissue sections with Azure counterstain:

As described previously, from the spectra represented in Figure 1, we generated three spectral libraries which included one Azure spectra derived from the three different participants and the GPER-DAB spectra derived from the non-pigmented tissue section. Each of these spectral libraries is named A, B, or C (Figure 2 and Table 1) consistent with the participant (A, B, or C) from which the Azure spectra was derived.

Melanoma sections stained with both GPER-DAB and Azure for each participant (A, B, and C) were imaged with the Nuance spectral camera, generating spectral image cubes, referred to in Figure 2 as Original Image. The Nuance software creates a single image representation of the spectral cube, using a lookup table that maps different patterns of absorbance to different colors which we refer to as the *original image*. Although the original image appears to show good localization of label, accurate location information is obtained only after unmixing the original image, using a spectral library with reference spectra for each of the labels on the section. The original image from participant A is shown in Figure 2.

Using spectral library A, comprised of the Azure spectra from participant A and the spectra from the non-pigmented GPER-DAB tissue, we unmixed the original image from participant A. We also unmixed the original image from participant A using the spectral libraries that included Azure spectra generated from participants B and C. As shown in Figure 2, these GPER-DAB component images appear nearly identical.

Similarly, we unmixed the original images from participants B and C with all three spectral libraries, resulting in three GPER-DAB component images per participant, and nine GPER-DAB component images total for participants A, B, and C. The component images for GPER-DAB are shown for participant A in Figure 2, and the mean intensities are reported for each of the nine GPER-DAB component images in Table 1.

To compare the distribution of mean GPER-DAB intensities of participants compared to themselves but unmixed with different Azure reference spectra, we performed a Kruskal-Wallis test and identified no significant differences (Table 1). These results indicate that the reported mean intensities vary slightly depending on the Azure reference spectra. However, these differences are not significant for any of the patient samples ($p=0.73$ $p>0.05$; One-way ANOVA with Kruskal-Wallis). Importantly, this suggests that one Azure reference spectrum is sufficient to unmix dual-stained images from multiple participants, eliminating the need for a separately stained section and Azure reference spectrum for every individual.

3.5 Discussion:

We have shown that using Azure as a counterstain in pigmented tissue sections, such as melanoma tumors, allows for quantification of HRP-DAB staining using spectral imaging techniques. Acquisition of spectral images of IHC stained melanoma tissue samples and subsequent unmixing using reference spectra, allows DAB-labeled protein to be quantified without interference from highly pigmented melanin. Although not demonstrated here, this method also allows for the observation of co-localization and quantitative measurement of multiple staining components. Unmixing is based on the absorbance spectra of the labels, is not affected by overall label intensity and is non-

subjective, meaning that IHC is no longer dependent on the chromogens showing a high visual contrast with pigmentation or other chromogens.

Importantly, this method allows for elimination of inter-reader variability by using spectral imaging to evaluate highly pigmented tissues. This finding improves the current semi-quantitative method of IHC, and increases the utility of IHC for both researchers and clinicians.

This study has many strengths. First, we have identified a non-subjective method to quantify protein expression in pigmented melanoma tissues. Furthermore, it is likely that this method would also provide a non-subjective method for quantification of protein expression in non-pigmented tissues, along with tissues using multiple chromogens. Additionally, we used archival tissues to validate that this technique can be applied to stored samples. Stored samples serve as a valuable resource for biomarker validation studies, but are often limited by the quality of the specimen and the loss of antigenicity over time.

The discovery that one Azure reference spectrum can be used for every participant has important implications. First, it will lessen the amount of time needed to image tissue sections by eliminating the need to image an Azure reference for every participant. This will also hasten the unmixing process, as each participant's dual-stained image will be unmixed using the same spectral library, allowing for batch unmixing. Finally, this will reduce the number of tissue sections needed from each participant, thereby allowing precious samples to be used for additional biomarker testing.

In summary, to our knowledge, this is the first study to use spectral imaging to quantify protein expression in pigmented tissue sections. This quantification method reduces the subjectivity and hastens analysis of protein expression detected by IHC. For clinicians, non-subjective quantification of protein expression in melanomas may impact the current diagnosis and staging standards. For researchers, quantification of protein expression may further inform melanoma etiology, progression and identification of therapeutic targets. Therefore, we expect that these findings will advance the field of melanoma.

3.6 Acknowledgements: The authors gratefully acknowledge the support of the UNM Clinical Translational Science Center staff, particularly Susan Tigert and Amy Overby. Images in this study were generated in UNM Cancer Center Fluorescence Microscopy Shared Resource (supported as detailed on http://hsc.unm.edu/crtc/microscopy/Facility.html). We appreciate the generous donation of the GPER antibody from Dr. Eric Prossnitz's laboratory, particularly assistance received from Dr. Natalie Fredette. We also appreciate the generous donation of melanoma tissue sections from USC, as well as the donation of the mammoplasty tissue from the laboratory of Dr. Helen Hathaway with assistance from Tamara Howard.

Dr. Jenna Lilyquist and Kirsten White, as co-authors, created the research's conception, designed all experiments, stained tissue sections, acquired data, conducted the data analysis and interpretation. Dr. Salina Torres, corresponding author, has provided expertise and guidance throughout the project from conception, supplied most if the tissue samples and provided expertise in melanin immunohistochemistry. Dr. Rebecca Lee and Genevieve Phillips provided experimental design expertise, data acquisition, interpretation and guidance in optimization of spectral imaging. Christopher Hughes

contributed to analysis and interpretation of the data, as well as the azure melanin interaction content and created Supplemental Figure 1. All the authors have been involved in the drafting of the article and its revisions for intellectual content.

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The authors and contributors of this work report no conflict of interest.

3.7 References

1. Matos LL de, Trufelli DC, de Matos MGL, da Silva Pinhal MA. Immunohistochemistry as an important tool in biomarkers detection and clinical practice. *Biomark Insights*. 2010;5:9-20.

2. Brandtzaeg P. The increasing power of immunohistochemistry and immunocytochemistry. *J Immunol Methods*. 1998;216(1-2):49-67. http://www.ncbi.nlm.nih.gov/pubmed/9760215. Accessed July 28, 2015.

3. Chromogens in Multiple Immunohistochemical Staining Used for Visual Assessment and Spectral Imaging: The Colorful Future . http://www.nsh.org/sites/default/files/March2010Article.pdf. Accessed July 28, 2015.

4. Taylor CR, Levenson RM. Quantification of immunohistochemistry--issues concerning methods, utility and semiquantitative assessment II. *Histopathology*. 2006;49(4):411-424. doi:10.1111/j.1365-2559.2006.02513.x.

5. Kamino H, Tam ST. Immunoperoxidase technique modified by counterstain with azure B as a diagnostic aid in evaluating heavily pigmented melanocytic neoplasms. *J Cutan Pathol*. 1991;18(6):436-439.

6. Horobin RW. How Romanowsky stains work and why they remain valuable - including a proposed universal Romanowsky staining mechanism and a rational troubleshooting scheme. *Biotech Histochem*. 2011;86(1):36-51. doi:10.3109/10520295.2010.515491.

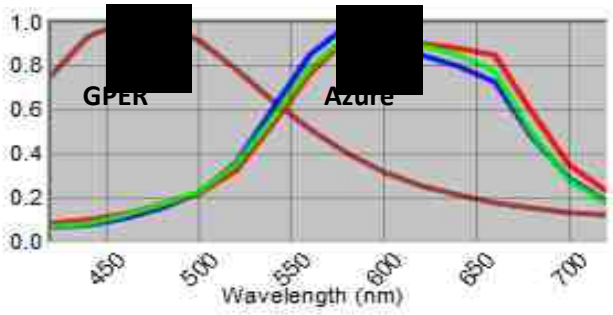
7. Sipponen MH, Pihlajaniemi V, Littunen K, Pastinen O, Laakso S. Determination of surface-accessible acidic hydroxyls and surface area of lignin by cationic dye adsorption. *Bioresour Technol*. 2014;169:80-87. doi:10.1016/j.biortech.2014.06.073.

8. Scaling AL, Prossnitz ER, Hathaway HJ. GPER mediates estrogen-induced signaling and proliferation in human breast epithelial cells and normal and malignant breast. *Horm Cancer*. 2014;5(3):146-160. doi:10.1007/s12672-014-0174-1.

9. Levenson RM, Mansfield JR. Multispectral imaging in biology and medicine: slices of life. *Cytometry A*. 2006;69(8):748-758. doi:10.1002/cyto.a.20319.

3.8. Tables and Figures

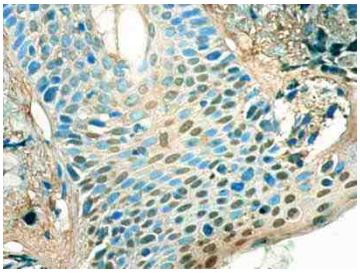
3.8.1 Figure 1: Azure Spectra do not vary between melanoma tissue sections



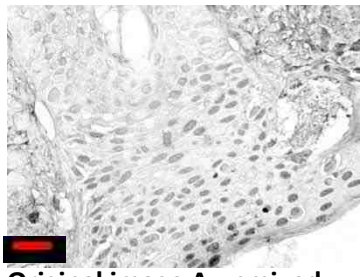
Absorbance spectra of GPER ( ) and Azure acquired from 3 separate melanoma tissues ( ).

3.8.2 Figure 2: Component images unmixed with different spectral libraries

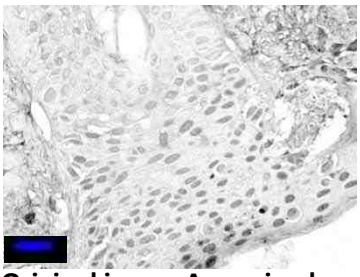
A:



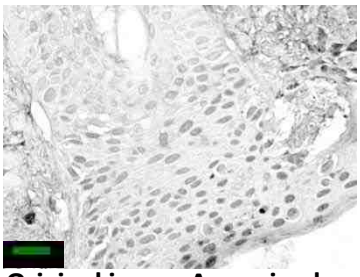
B.



Original image A unmixed with Spectral Library A



Original image A unmixed with Spectral Library B

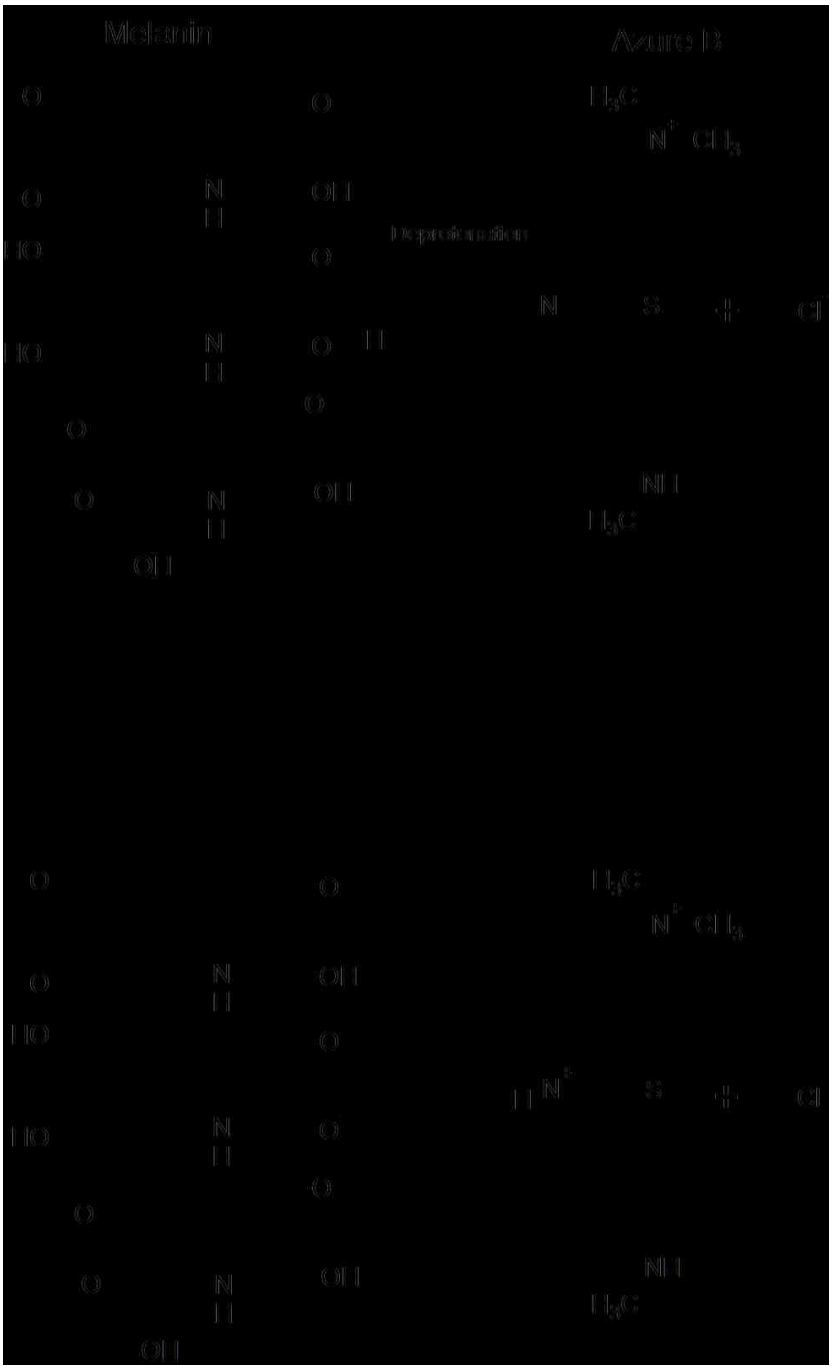


Original image A unmixed with Spectral Library C

A: Original image: GPER-DAB and Azure in Melanoma (Participant A). This is a single image representation of a spectral image cube (containing 16 individual images).

B. Component images representing GPER-DAB in melanoma: Original image (Participant A) unmixed with three spectral libraries.

3.8.3 Supplemental Figure 1: Proposed mechanism for Azure binding to melanin



3.9 Abstract

LC3 and Beclin 1 Expression in Melanoma Lesions: Association with UV Exposure and Prognostic Indicators.

Background: The activation of the autophagy pathway in BRAF melanomas is one proposed mechanism involved in progression. Autophagy, a catabolic process, has been shown to both inhibit and promote tumorigenicity. Autophagy inhibition is under investigation in melanoma treatment. However, the melanoma risk factor, UV exposure, activates autophagy. Here we investigated UV exposure and autophagy by *BRAF/NRAS* status to test for differential contributions to melanoma prognostic factors and survival.

Methods: Sections from n melanoma tumors and n benign nevi were analyzed for Beclin1 and LC3 expression, using immunohistochemistry. *BRAF* /*NRAS* status was determined by sequencing. Beclin1 and LC3 expression levels measured by IHC were correlated with UV exposure data. The effect of *NRAS* and *BRAF* pathways on autophagy proteins was evaluated.

Results: We found that the autophagy pathway is activated with UV exposure in melanoma tumors compared to benign nevi. We observed critical proteins in the autophagy pathway associated with decreased Breslow depth (Beclin1) as well as decreased mortality (LC3).

Conclusions: The expression of Beclin1 and LC3 was not directly linked *BRAF* or *NRAS* mutations in melanoma; rather, autophagy was activated in response to UV exposure and associated with prognostic indicators and survival. These findings may have implications concerning the biology of melanoma and for ongoing autophagy inhibition clinical trials.

3.10 Introduction:

Invasive melanoma has an increasing incidence of 6% each year but with new clinical treatment options for advanced melanoma. The American Cancer Society estimates that in 2015, 73,870 new melanomas will be diagnosed and 9,940 people will die from their disease. Despite the introduction of new therapies, the 5-year survival rate for stage IV melanoma will remain unchanged at 15% to 20%.[1]

Autophagy is a cellular recycling pathway involved in normal physiological processes including nutrient and/or stress responses, antigen presentation, and aging.[3–8] In cancer, autophagy appears to be an important energy source for nutrient depleted tumors.[9–11] In melanoma, basal levels of autophagy appear to vary by tumor stage, and higher levels of autophagic flux are associated with increased hypoxia and poor clinical prognosis.[11–15] Mutations in *BRAF* and *NRAS* differentially regulate autophagy.[16–22] In 2010, Lazova and colleagues reported that melanoma cells contain high levels of autophagosomes, a cellular structure associated with autophagic flux, as measured by the proxy marker microtubule-associated protein 1 light chain 3 (LC3), an autophagy related (ATG) protein.[11] LC3 is integrated into the membrane of a critical structure in autophagy, called the autophagosome, in order to facilitate the recycling of products targeted for degradation. This integration creates a punctate LC3-staining pattern, which is used as a proxy marker for the rate of autophagy.[11] The role of LC3 and another critical protein in autophagy initiation, Beclin 1 (Becn1), may have a biphasic role in cancer.[3] LC3 levels have been shown to be elevated in the cytoplasm of cancer cells including gastrointestinal[23] and pancreatic cancer.[24] and have been reported to be associated with stress response, metastasis and poor clinical prognosis in melanoma.[15,19,25,26] Becn1 has

been reported to be associated with increased stage[27], lower relapse-free survival [28] and decreased 5-year survival in several cancers.[29] Elevated levels of LC3 and Becn1 as well as other autophagic proteins correlated with poor prognosis or cancer progression have prompted Phase I/II clinical trials to investigate autophagy inhibition as a co-therapy, particularly in melanoma.[30–32]

Conversely, it also appears that there is an inverse relationship between expression of autophagy markers and malignant potential, so in some cancers lower levels of autophagy are associated with worse prognosis. This implies that defects in cellular autophagy may contribute to the development of cancer[33] and that cancer cells may have lower basal autophagic activity. [34–37] Low levels of both Becn1 and LC3 have also both been reported to associate with poor clinical pathological markers and decreased survival [38–40] in melanoma, among other tumors.[41,42] Becn1 in particular has been shown to exhibit anti-oncogenic functions, has been found to be monoallelically deleted in breast, ovarian and prostate tumors.[43–46] Finally, low levels of Becn1 have also been reported in nodular melanomas to be associated with increased risk of early death.[47] The dual role of autophagy in the regulation and progression of melanoma require further investigation.

The role of these two autophagy proteins (LC3 and Becn1) in melanoma may, in part, be influenced by their interaction with other factors including ultra-violet (UV) exposure. UV's impact on progression and survival in melanoma is also complex and unclear. [48,49] As autophagy appears to have a role in the resolution of double-stranded DNA breaks[50,51], it may be that UV induces autophagy and improves melanoma-specific survival at the same time that it is the major risk factor for melanoma. The impact of UV exposure on autophagic processes in skin has not been well characterized and there is a

need for in-depth evaluation of role of autophagy in a set of well-defined melanoma samples.[52]

In the current study, we seek to determine whether autophagy differentially regulates prognostic characteristics and survival in melanoma depending on the timing of UV exposure. We evaluated autophagy in 51 melanoma and 17 benign nevus tissue sections, for LC3 and Becn1 protein expression, using immunohistochemistry (IHC) to determine if autophagic flux is associated with clinical stage in melanoma. For the same samples, using extensive questionnaire data, we evaluated the impact of estimated lifetime sun exposure on survival and correlated these data with prognostic factors as well as available information on autophagy levels. These results assessed whether autophagic flux is modified by UV exposure. We hypothesized that UV exposure is associated with levels of autophagy and autophagy markers are prognostic for clinical characteristics of melanoma survival.

3.11 Material and Methods:

3.11.1 Patient Characteristics:

The New Mexico Tumor Registry and the University of New Mexico Cancer Center identified study participants in which a skin biopsy was taken to rule out melanoma. Study participants with melanoma or a benign skin biopsy (n=98) were interviewed using a validated questionnaire for UV exposure.[48,53] Institutional Review Boards of all participating institutions approved the protocol and informed consent was obtained from each participant. 46 melanoma and 17 patients who had a benign nevi excised, were consented, enrolled and interviewed and selected based on availability of a tissue block

(see patient characteristics below, Table 1-2). Survival was determined using the National Death Index and provided by the Tumor Registry.

3.11.2 IHC Staining of LC3 and Becn1:

Formalin-fixed paraffin embedded (FFPE) control and melanoma sections were made using archival tissue blocks from patient biopsies. Expression of LC3 and Becn1 in these sections were measured as a proxy for levels of autophagy. Standard immunoperoxidase techniques for immunohistochemistry using anti-LC3 and anti-beclin1 antibodies (MBL, Woburn, MA; Cell Signaling Technology, Danvers, MA; 1:200; with citric acid/ antigen retrieval) was used to quantify expression in melanoma cells (brown) compared to tumor-associated melanophages (azure). Azure B stains melanin green blue and is easily distinguished from the brown diaminobenzidine chromogen[54]. Original images were obtained using a Nikon Scope from the UNM Fluorescence Microscopy Shared Resource, equipped with a spectral imaging camera. Becn1 and LC3 staining intensity were quantified by Spectral imaging software.[55]

3.11.3 BRAF, NRAS status:

Genomic DNA was isolated from FFPE tumor sections using Qiagen QIAamp DNA FFPE kit (Qiagen, Inc., Valencia, CA). Patients' *BRAF/NRAS* mutation status was evaluated using standard PCR technique to amplify the coding region of interest. All amplicons were directly sequenced on an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA) using BigDye Terminators (Applied Biosystems) according to the manufacturer's specifications for sequencing. A 224 bp *BRAF* PCR product

(chr7:140453033-140453256) was amplified using the primers 5′-TCATAATGCTTGCTCTGATAGGA-3′ and 5′-GGCCAAAAATTTAATCAGTGGA-3′ and a 272 bp *NRAS* PCR product (chr1:115256298-115256569) was amplified using the primers 5′- GGTGAAACCTGTTTGTTGGA-3′ and 5′-AACCTAAAACCAACTCTTCCCA-3′ in a 50 ml reaction containing the 10 µL (40ng/µl) DNA template, 5 µM of each primer, 2× Premix F (Epicentre, Madison, Wisconsin) and 0.5 U of Taq polymerase in a PCR reaction as described above. DNA template was denatured at 94°C for 4 min and cycled 26 times through steps of denaturing at 94°C for 1 min, annealing 63-0.5c and denaturation. An additional 10 cycles of denaturing at 94°C for 1 min, annealing 50c and extension at 72°C; final DNA extension was at 72°C for 10:00 minutes. PCR products were purified using ExoSap (Affymetrix, Santa Clara, CA) according to manufacturer's instructions.

Sequencing chromatograms were read with the aid of FinchTV software version 1.4.0 (Geospiza, Inc., http://www.geospiza.com/Products/finchtv.shtml)

3.11.4 Clinical Stage:

Histopathology was characterized by a board-certified pathologist using Hematoxylin & Eosin (H&E) stained slides and clinical stage was determined including Breslow thickness, mitotic index and ulceration. Mitoses were defined as present or absent. [56] All cases were characterized using these variables. Patient vital status was obtained through linkage with the NM Tumor Registry, using an honest broker system with IRB approval (HRRC 08-433).

3.11.5 UV Exposure:

Using extensive questionnaire data, participants were retrospectively evaluated for UV ambient exposure in the first decade of life and near the time of diagnosis. Questionnaire data, which has been previously described,[53] includes the following variables: daily sun exposure on weekdays and weekends at each decade; sun exposure at the site of the melanoma at each decade; and waterside recreational sun exposure at each decade.

3.11.6 Data Analysis:

Frequency tables were used to summarize the clinical distributions and UV exposures for controls and melanoma cases (Table 1-2). Analysis of UV data with participant characteristics was correlated with oncogene status and levels of LC3 and Becn1 protein in melanoma tissue sections. Wilcoxon rank-sum tests and Pearson chi-squared tests were performed for pair-wise associations. ORs and 95% CIs were calculated from non-parametric logistic regression models using data from cases and benign nevi controls to assess the association with LC3 and Becn1 expression. The Pearson's coefficient was used to assess linear relationships. The relationship between categorical variables was analyzed by Chi-square test or Fisher's exact test. Univariate and multivariate analyses of prognostic factors were performed by the Cox proportional hazards regression model. ORs and 95% confidence intervals estimated from coefficients were used to summarize the associations. For all tests, a two-sided P value <0.05 was considered statistically significant.

3.12 Results:

In the overall population we identified 24 *BRAF* T1799A (V600E) point mutations in 16 cases and 8 controls. Additionally, we found 6 cases with *NRAS* A182G (Q61R) mutations. No controls had an NRAS mutation. There were 24 melanoma cases and 9 controls without a mutation in BRAF or NRAS. Protein levels were evaluated in 48 melanoma biopsies and 17 control tissues using the novel IHC quantification method we developed and is described previously in chapter 3 (Table 2; Table 4).

After adjustments for age and sex, high Becn1 expression was associated with decreased Breslow thickness (p=0.05), whereas high levels of LC3 were correlated with decreased mortality among patients (p=0.02). Furthermore, females were more likely to have tumors with high Becn1 expression (p=0.02) (Table 3). Melanoma participants who reported ever having a blistering sunburn were more likely to have high Becn1 expression (p=0.01) (Table 4). Becn1 expression was not associated with *NRAS** or *BRAF** status. Superficial spreading histology (Table 3) and report of blistering sunburns in early life had borderline associations with high levels of Becn1 and LC3 (P = 0.09, P = 0.08, respectively) (Table 4).

High LC3 expression was also positively associated with the expression of punctate LC3 (p=0.0007) (Table 3). High cytosolic LC3 expression correlated with *NRAS* WT genotype (p=0.01) when compared to *NRAS* mutant Table 3). A lack of painful sunburns for two or more days (p=0.03) was associated with high LC3 expression (Table 4). High LC3 expression had a borderline significant associations with unprotected prolonged sun exposure (p=0.06), boating activities and any outdoor activity sun exposure (p=0.08) (Table 4).

3.13 Discussion:

This study was designed to assess the association of oncogene status and UV exposure with protein levels of Becn1 and LC3 in cutaneous melanoma. UV is associated with both autophagy activation and melanoma incidence, and as both UV and autophagy are complex.

Previously, it has been shown that Becn1 and LC3 protein levels are altered in several human cancer types including melanoma and our data supports the fact that protein levels vary between melanoma and control patient samples. [57] We also found that expression of autophagy markers varied by Clark's level and survival status.

High levels of Becn1 and LC3 are consistently inversely associated with poor prognostic markers and/or survival. High Becn1 expression was associated with decreased Breslow depth (the strongest prognostic indicator for melanoma) ($p=0.05$) (Table 3), whereas high levels of punctate LC3 indicating autophagosome formation, had an association with decreased mortality among patients ($p=0.04$) (Table 5). Early autophagy, as represented by Becn1, was associated with lower stage at diagnosis, while late stage autophagy, as represented by lower punctate LC3 expression, and was associated with decreased mortality from melanoma (Table 5). Autophagy genes have previously been implicated both in tumor suppression and tumor development.[44] Becn1 has multiple functions. In addition to its role in autophagy initiation, it is implicated in both the differentiation and apoptosis of cancer cells.[58,59] Lower Becn1 expression has been reported in both breast cancer,[60] and melanoma[42] and the loss of Becn1 has been correlated with poor prognosis in colon[61] and liver[50] cancer, lymphomas[62] and squamous cell carcinoma.[27] Overexpression of Becn1 is also correlated with progression of gastric and colorectal

cancer[63] and with poor prognosis in endometrial cancer. [29]In our study the association of high Becn1 with decreased Breslow thickness might indicate that it is acting as a tumor suppressor in melanoma patients.

In our study, the expression of the Becn1 protein and also of the autophagosome protein LC3 was found to be lower in melanomas with more aggressive prognostic indicators, similar to findings in ovarian cancer. [38]The expression of Becn1 and LC3 could also be associated with UV in melanoma as both of these proteins were associated with UV exposure

The relationship between UV and autophagy may have important implications for data describing the autophagy pathway in melanoma progression.[17,26] While melanoma only accounts for approximately 2.4% of all cancer related deaths, it is the one of the most common cancers in young adults, age 25-29, particularly in young women. This is of particular importance as the mean survival rate of patients diagnosed with metastatic melanoma is six months, with five year survival rates of less than 5%. UV exposure causes DNA damage and can induce the activation of autophagy.

Extensive studies of the role of UV in survival with melanoma have been based on careful measurement of UV and melanoma risk,[53] and sun exposure and survival with melanoma.[48,64] We evaluated sun exposure at three times in life: early life up to the age of 10, in the 10 years prior to diagnosis, and averaged over the lifetime. Studies have shown that an individual's lifetime sun exposure is well represented by the sum of their exposures at each decade (e.g., 10, 20) of age. [65] We also evaluated sunburns and water-related activities. While not significant our study identified an association between water activities and boating activities and increased Becn1 expression (Table 3).

Previously it has been reported that the only significant UV predictors of melanoma survival are UVB dose up to the age of 10 and sunburns in the decade prior to diagnosis. [48] Strangely, UVB dose up to the age of 10 *increased* the risk of dying from melanoma, yet any sunburns in the decade prior to diagnosis *decreased* the risk of dying from melanoma, even after adjusting for clinical characteristics. As autophagy acts as a cytoprotective mechanism against ultraviolet (UV)-induced apoptosis, Becn1 and LC3 expression may be upregulated following DNA damage from sun exposure.[66]

UV-radiation is an established risk factor for melanoma and increases autophagic flux. [67] UV exposure triggers defense mechanisms, including an increase in DNA repair capacity,[49,68] and autophagy has an established role in DNA repair by removing products of DNA damage. [67] Some researchers have found evidence that high levels of sun exposure prior to diagnosis were associated with better melanoma survival.[64] However, contradictory evidence indicates high levels of UV exposure in childhood are associated with worse survival and that high levels of UV exposure prior to diagnosis are associated with better overall survival but not with better melanoma-specific survival. [48] In our study we identified that UV exposure is independently associated with levels of autophagy as shown by the high LC3 protein expression in individuals with decreased sunburns or no participation in water sports (Table 4).

Our study is limited by the possibility that cells use other mechanisms, including post translational modifications, to regulate protein expression so LC3/Beclin1 may stay consistently expressed under variable conditions. In addition, our small sample size may not really be representative of a larger population-based sample.

In our study, high level of Beclin 1 and LC3 expression in tumors, correlated with less aggressive histopathological markers including Breslow thickness, as well as better overall survival of the patients. The present data support the idea that while autophagy is associated with melanoma tumors, low level of autophagy favors cancer progression and that autophagy may support more indolent melanoma phenotype. Understanding the relationships between autophagy status, UV exposure and melanoma progression will help to elucidate the molecular mechanisms that contribute to this disease.

Conflict of interest: The authors have declared that no conflict of interest exists.

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3.15 REFERENCES

1. American Cancer Society Facts and Statistics 2015 | Research | American Cancer Society. http://www.cancer.org/research/cancerfactsstatistics/cancerfactsfigures2015/. Accessed June 8, 2015.

2. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144(5):646-674. doi:10.1016/j.cell.2011.02.013.

3. Shintani T, Klionsky DJ. Autophagy in health and disease: a double-edged sword. *Science*. 2004;306(5698):990-995. doi:10.1126/science.1099993.

4. Choi AMK, Ryter SW, Levine B. Autophagy in human health and disease. *N Engl J Med*. 2013;368(7):651-662. doi:10.1056/NEJMra1205406.

5. Jones RG, Thompson CB. Tumor suppressors and cell metabolism: a recipe for cancer growth. *Genes Dev*. 2009;23(5):537-548. doi:10.1101/gad.1756509.

6. Yang Z, Goronzy JJ, Weyand CM. Autophagy in autoimmune disease. *J Mol Med (Berl)*. 2015;93(7):707-717. doi:10.1007/s00109-015-1297-8.

7. Yang ZJ, Chee CE, Huang S, Sinicrope FA. The role of autophagy in cancer: therapeutic implications. *Mol Cancer Ther*. 2011;10(9):1533-1541. doi:10.1158/1535-7163.MCT-11-0047.

8. Espert L, Beaumelle B, Vergne I. Autophagy in Mycobacterium tuberculosis and HIV infections. *Front Cell Infect Microbiol*. 2015;5:49. doi:10.3389/fcimb.2015.00049.

9. Sato K, Tsuchihara K, Fujii S, et al. Autophagy is activated in colorectal cancer cells and contributes to the tolerance to nutrient deprivation. *Cancer Res*. 2007;67(20):9677-9684. doi:10.1158/0008-5472.CAN-07-1462.

10. Townsend KN, Hughson LRK, Schlie K, Poon VI, Westerback A, Lum JJ. Autophagy inhibition in cancer therapy: metabolic considerations for antitumor immunity. *Immunol Rev*. 2012;249(1):176-194. doi:10.1111/j.1600-065X.2012.01141.x.

11. Lazova R, Klump V, Pawelek J. Autophagy in cutaneous malignant melanoma. *J Cutan Pathol*. 2010;37(2):256-268. doi:10.1111/j.1600-0560.2009.01359.x.

12. Lazova R, Pawelek JM. Why do melanomas get so dark? *Exp Dermatol*. 2009;18(11):934-938. doi:10.1111/j.1600-0625.2009.00933.x.

13. Schaaf MBE, Cojocari D, Keulers TG, et al. The autophagy associated gene, ULK1, promotes tolerance to chronic and acute hypoxia. *Radiother Oncol*. 2013;108(3):529-534. doi:10.1016/j.radonc.2013.06.015.

14. Lazova R, Camp RL, Klump V, Siddiqui SF, Amaravadi RK, Pawelek JM. Punctate LC3B expression is a common feature of solid tumors and associated

with proliferation, metastasis, and poor outcome. *Clin Cancer Res*. 2012;18(2):370-379. doi:10.1158/1078-0432.CCR-11-1282.

15. Marino ML, Pellegrini P, Di Lernia G, et al. Autophagy is a protective mechanism for human melanoma cells under acidic stress. *J Biol Chem*. 2012;287(36):30664-30676. doi:10.1074/jbc.M112.339127.

16. Xie X, Koh JY, Price S, White E, Mehnert JM. Atg7 Overcomes Senescence and Promotes Growth of BrafV600E-Driven Melanoma. *Cancer Discov*. 2015;5(4):410-423. doi:10.1158/2159-8290.CD-14-1473.

17. Strohecker AM, Guo JY, Karsli-Uzunbas G, et al. Autophagy sustains mitochondrial glutamine metabolism and growth of BrafV600E-driven lung tumors. *Cancer Discov*. 2013;3(11):1272-1285. doi:10.1158/2159-8290.CD-13-0397.

18. Guo JY, Chen H-Y, Mathew R, et al. Activated Ras requires autophagy to maintain oxidative metabolism and tumorigenesis. *Genes Dev*. 2011;25(5):460-470. doi:10.1101/gad.2016311.

19. Mito- WEAS. Autophagy promotes. 2014;10(2):384-385.

20. Armstrong JL, Corazzari M, Martin S, et al. Oncogenic B-RAF signaling in melanoma impairs the therapeutic advantage of autophagy inhibition. *Clin Cancer Res*. 2011;17(8):2216-2226. doi:10.1158/1078-0432.CCR-10-3003.

21. Corazzari M, Lovat PE. Cell Biology : Research & Therapy Harnessing Autophagy for Melanoma Benefit. 2013:2-4.

22. Corazzari M, Fimia GM, Lovat P, Piacentini M. Why is autophagy important for melanoma? Molecular mechanisms and therapeutic implications. *Semin Cancer Biol*. 2013;23(5):337-343. doi:10.1016/j.semcancer.2013.07.001.

23. Yoshioka A, Miyata H, Doki Y, et al. LC3, an autophagosome marker, is highly expressed in gastrointestinal cancers. *Int J Oncol*. 2008;33(3):461-468. http://www.ncbi.nlm.nih.gov/pubmed/18695874. Accessed September 1, 2015.

24. Fujii S, Mitsunaga S, Yamazaki M, et al. Autophagy is activated in pancreatic cancer cells and correlates with poor patient outcome. *Cancer Sci*. 2008;99(9):1813-1819. doi:10.1111/j.1349-7006.2008.00893.x.

25. Han C, Sun B, Wang W, et al. Overexpression of microtubule-associated protein-1 light chain 3 is associated with melanoma metastasis and vasculogenic mimicry. *Tohoku J Exp Med*. 2011;223(4):243-251. http://www.ncbi.nlm.nih.gov/pubmed/21415575. Accessed August 26, 2015.

26. Ma X, Piao S, Dey S, et al. Targeting ER stress – induced autophagy overcomes BRAF inhibitor resistance in melanoma. 2014;124(3). doi:10.1172/JCI70454DS1.

27. Wang J, Pan X-L, Ding L-J, Liu D-Y, Da-Peng Lei, Jin T. Aberrant expression of Beclin-1 and LC3 correlates with poor prognosis of human hypopharyngeal

squamous cell carcinoma. *PLoS One*. 2013;8(7):e69038. doi:10.1371/journal.pone.0069038.

28. Zhao Y, Chen S, Gou W, Xiao L, Takano Y, Zheng H. Aberrant Beclin 1 expression is closely linked to carcinogenesis, differentiation, progression, and prognosis of ovarian epithelial carcinoma. *Tumour Biol*. 2014;35(3):1955-1964. doi:10.1007/s13277-013-1261-6.

29. Giatromanolaki A, Koukourakis MI, Koutsopoulos A, Chloropoulou P, Liberis V, Sivridis E. High Beclin 1 expression defines a poor prognosis in endometrial adenocarcinomas. *Gynecol Oncol*. 2011;123(1):147-151. doi:10.1016/j.ygyno.2011.06.023.

30. The BAMM Trial: BRAF, Autophagy and MEK Inhibition in Metastatic Melanoma: A Phase I/2 Trial of Dabrafenib, Trametinib and Hydroxychloroquine in Patients With Advanced BRAF Mutant Melanoma - No Study Results Posted - ClinicalTrials.gov. https://clinicaltrials.gov/ct2/show/NCT02257424. Accessed August 23, 2015.

31. Vogl DT, Stadtmauer EA, Tan K-S, et al. Combined autophagy and proteasome inhibition: a phase 1 trial of hydroxychloroquine and bortezomib in patients with relapsed/refractory myeloma. *Autophagy*. 2014;10(8):1380-1390. doi:10.4161/auto.29264.

32. Rangwala R, Chang YC, Hu J, et al. Combined MTOR and autophagy inhibition: phase I trial of hydroxychloroquine and temsirolimus in patients with advanced

solid tumors and melanoma. *Autophagy*. 2014;10(8):1391-1402. doi:10.4161/auto.29119.

33. Mathew R, Karantza-wadsworth V, White E. Role of autophagy in cancer. 2007;7(december):961-967.

34. Kopitz J, Kisen GO, Gordon PB, Bohley P, Seglen PO. Nonselective autophagy of cytosolic enzymes by isolated rat hepatocytes. *J Cell Biol*. 1990;111(3):941-953. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2116292&tool=pmcentrez&rendertype=abstract. Accessed July 20, 2015.

35. Kisen GO, Tessitore L, Costelli P, et al. Reduced autophagic activity in primary rat hepatocellular carcinoma and ascites hepatoma cells. *Carcinogenesis*. 1993;14(12):2501-2505. http://www.ncbi.nlm.nih.gov/pubmed/8269618. Accessed August 26, 2015.

36. Otsuka H, Moskowitz M. Differences in the rates of protein degradation in untrasformed and transformed cell lines. *Exp Cell Res*. 1978;112(1):127-135. http://www.ncbi.nlm.nih.gov/pubmed/631208. Accessed August 26, 2015.

37. Knecht E, Hernández-Yago J, Grisolía S. Regulation of lysosomal autophagy in transformed and non-transformed mouse fibroblasts under several growth conditions. *Exp Cell Res*. 1984;154(1):224-232. http://www.ncbi.nlm.nih.gov/pubmed/6088263. Accessed August 26, 2015.

38. Valente G, Morani F, Nicotra G, et al. Expression and clinical significance of the autophagy proteins BECLIN 1 and LC3 in ovarian cancer. *Biomed Res Int*. 2014;2014:462658. doi:10.1155/2014/462658.

39. Sakurai T, Okumura H, Matsumoto M, et al. The expression of LC-3 is related to tumor suppression through angiogenesis in esophageal cancer. *Med Oncol*. 2013;30(4):701. doi:10.1007/s12032-013-0701-x.

40. Deng Q, Wang Z, Wang L, et al. Lower mRNA and protein expression levels of LC3 and Beclin1, markers of autophagy, were correlated with progression of renal clear cell carcinoma. *Jpn J Clin Oncol*. 2013;43(12):1261-1268. doi:10.1093/jjco/hyt160.

41. Lovat MC and PE. Harnessing Autophagy for Melanoma Benefit. *Cell Biol Res Ther*. http://www.scitechnol.com/harnessing-autophagy-for-melanoma-benefit-xYl7.php&&article_id=1019. Accessed August 26, 2015.

42. Miracco C, Cevenini G, Franchi A, et al. Beclin 1 and LC3 autophagic gene expression in cutaneous melanocytic lesions. *Hum Pathol*. 2010;41(4):503-512. doi:10.1016/j.humpath.2009.09.004.

43. Aita VM, Liang XH, Murty V V, et al. Cloning and genomic organization of beclin 1, a candidate tumor suppressor gene on chromosome 17q21. *Genomics*. 1999;59(1):59-65. doi:10.1006/geno.1999.5851.

44. Qu X, Yu J, Bhagat G, et al. Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. *J Clin Invest*. 2003;112(12):1809-1820. doi:10.1172/JCI20039.

45. Russell SE, Hickey GI, Lowry WS, White P, Atkinson RJ. Allele loss from chromosome 17 in ovarian cancer. *Oncogene*. 1990;5(10):1581-1583. http://www.ncbi.nlm.nih.gov/pubmed/2250914. Accessed August 26, 2015.

46. Eccles DM, Russell SE, Haites NE, et al. Early loss of heterozygosity on 17q in ovarian cancer. The Abe Ovarian Cancer Genetics Group. *Oncogene*. 1992;7(10):2069-2072. http://www.ncbi.nlm.nih.gov/pubmed/1408149. Accessed August 26, 2015.

47. Sivridis E, Koukourakis MI, Mendrinos SE, et al. Beclin-1 and LC3A expression in cutaneous malignant melanomas: a biphasic survival pattern for beclin-1. *Melanoma Res*. 2011;21(3):188-195. doi:10.1097/CMR.0b013e328346612c.

48. Berwick M, Reiner AS, Paine S, et al. Sun exposure and melanoma survival: a GEM study. *Cancer Epidemiol Biomarkers Prev*. 2014;23(10):2145-2152. doi:10.1158/1055-9965.EPI-14-0431.

49. Gilchrest BA, Eller MS, Geller AC, Yaar M. The pathogenesis of melanoma induced by ultraviolet radiation. *N Engl J Med*. 1999;340(17):1341-1348. doi:10.1056/NEJM199904293401707.

50. Lin W, Yuan N, Wang Z, et al. Autophagy confers DNA damage repair pathways to protect the hematopoietic system from nuclear radiation injury. *Sci Rep*. 2015;5:12362. doi:10.1038/srep12362.

51. Liu EY, Xu N, O'Prey J, et al. Loss of autophagy causes a synthetic lethal deficiency in DNA repair. *Proc Natl Acad Sci U S A*. 2015;112(3):773-778. doi:10.1073/pnas.1409563112.

52. Liu H, He Z, Simon H-U. Targeting autophagy as a potential therapeutic approach for melanoma therapy. *Semin Cancer Biol*. 2013;23(5):352-360. doi:10.1016/j.semcancer.2013.06.008.

53. Kricker A, Armstrong BK, Goumas C, et al. Ambient UV, personal sun exposure and risk of multiple primary melanomas. *Cancer Causes Control*. 2007;18(3):295-304. doi:10.1007/s10552-006-0091-x.

54. Kamino H, Tam ST. Immunoperoxidase technique modified by counterstain with azure B as a diagnostic aid in evaluating heavily pigmented melanocytic neoplasms. *J Cutan Pathol*. 1991;18(6):436-439. http://www.ncbi.nlm.nih.gov/pubmed/1723081. Accessed May 25, 2015.

55. White KAM, Lilyquist J, Lee RJ, Philips GK, Hughes CR TS. Quantitative Analysis of Immunohistochemistry in Melanoma Tumors. *Appl Immunohistochem*. 2015;In submiss.

56. Thomas NE, Kricker A, Waxweiler WT, et al. Comparison of clinicopathologic features and survival of histopathologically amelanotic and pigmented melanomas: a population-based study. *JAMA dermatology*. 2014;150(12):1306-1314. doi:10.1001/jamadermatol.2014.1348.

57. Eisenberg-Lerner A, Kimchi A. The paradox of autophagy and its implication in cancer etiology and therapy. *Apoptosis*. 2009;14(4):376-391. doi:10.1007/s10495-008-0307-5.

58. Kang R, Zeh HJ, Lotze MT, Tang D. The Beclin 1 network regulates autophagy and apoptosis. *Cell Death Differ*. 2011;18(4):571-580. doi:10.1038/cdd.2010.191.

59. Wirawan E, Vande Walle L, Kersse K, et al. Caspase-mediated cleavage of Beclin-1 inactivates Beclin-1-induced autophagy and enhances apoptosis by promoting the release of proapoptotic factors from mitochondria. *Cell Death Dis*. 2010;1:e18. doi:10.1038/cddis.2009.16.

60. Liang XH, Jackson S, Seaman M, et al. Induction of autophagy and inhibition of tumorigenesis by beclin 1. *Nature*. 1999;402(6762):672-676. doi:10.1038/45257.

61. Li B-X, Li C-Y, Peng R-Q, et al. The expression of beclin 1 is associated with favorable prognosis in stage IIIB colon cancers. *Autophagy*. 2014;5(3):303-306. doi:10.4161/auto.5.3.7491.

62. Huang J-J, Li H-R, Huang Y, et al. Beclin 1 expression: a predictor of prognosis in patients with extranodal natural killer T-cell lymphoma, nasal type. *Autophagy*.

2010;6(6):777-783. http://www.ncbi.nlm.nih.gov/pubmed/20639699. Accessed August 30, 2015.

63. Ahn CH, Jeong EG, Lee JW, et al. Expression of beclin-1, an autophagy-related protein, in gastric and colorectal cancers. *APMIS*. 2007;115(12):1344-1349. doi:10.1111/j.1600-0463.2007.00858.x.

64. Berwick M, Armstrong BK, Ben-Porat L, et al. Sun exposure and mortality from melanoma. *J Natl Cancer Inst*. 2005;97(3):195-199. doi:10.1093/jnci/dji019.

65. Yu C-L, Li Y, Freedman DM, et al. Assessment of lifetime cumulative sun exposure using a self-administered questionnaire: reliability of two approaches. *Cancer Epidemiol Biomarkers Prev*. 2009;18(2):464-471. doi:10.1158/1055-9965.EPI-08-0894.

66. Sui X, Chen R, Wang Z, et al. Autophagy and chemotherapy resistance: a promising therapeutic target for cancer treatment. *Cell Death Dis*. 2013;4:e838. doi:10.1038/cddis.2013.350.

67. Zhao Y, Zhang C-F, Rossiter H, et al. Autophagy is induced by UVA and promotes removal of oxidized phospholipids and protein aggregates in epidermal keratinocytes. *J Invest Dermatol*. 2013;133(6):1629-1637. doi:10.1038/jid.2013.26.

68. Eller MS, Gilchrest BA. Tanning as part of the eukaryotic SOS response. *Pigment Cell Res*. 2000;13 Suppl 8:94-97. http://www.ncbi.nlm.nih.gov/pubmed/11041364. Accessed September 5, 2015.

3.16 Tables:

3.16.2 Table 1. Demographic and pathological summary of participants.

N=68	**Overall**		**Control**		**Case**	
Age at diagnosis/Presentation No.	59.5		54.9		60.7	
	No.	**%**	**No.**	**%**	**No.**	**%**
Gender						
Male	48	51.1	11	61.1	37	48.7
Female	44	46.8	6	33.3	38	50
Oncogene Status						
*NRAS**	7	7.4	.	.	7	9.2
*BRAF**	25	26.6	1	5.6	24	31.6
Wild-type	45	47.9	9	50	36	47.4
Breslow thickness (mm)						
0.01-1.00	30	31.9	.	.	30	39.5
> 1.00	32	34	.	.	32	42.1
LC3 Punctate						
Absent	30	31.9	6	33.3	24	31.6
Present	39	41.5	11	61.1	28	36.8
Anatomic site						
Trunk/pelvis	15	16	.	.	15	19.7
Scalp/neck	6	6.4	.	.	6	7.9
Face/ears/other	13	13.8	.	.	13	17.1
Upper extremities	20	21.3	.	.	20	26.3
Lower extremities	17	18.1	.	.	17	22.4
Histological subtype						
SSM	38	40.4	.	.	38	50
NM	19	20.2	.	.	19	25
LMM	9	9.6	.	.	9	11.8
Other	2	2.1	.	.	2	2.6
Mitosis						
Absent	21	30.8	.	.	21	41.2
Present	29	42.7	.	.	29	56.9
Regression						
Absent	28	29.8	.	.	28	36.8
Present	26	27.7	.	.	26	34.2
Ulceration						
Absent	13	13.8	.	.	13	17.1
Present	44	46.8	.	.	44	57.9

Clark's Level						
Level 2	21	22.3	.	.	21	27.6
Level 3	8	8.5	.	.	8	10.5
Level 4	23	24.5	.	.	23	30.3
Level 5	7	7.4	.	.	7	9.2
Vital Statistics						
Death from melanoma*	68	72.3	6	33.3	62	81.6
Alive or death from other causes	12	12.8	.	.	12	15.8

3.16.3 Table 2. Summary of participants UV exposure.

	Overall		Control		Case	
	No.	**%**	**No.**	**%**	**No.**	**%**
UV exposure						
Sunlamp Use						
Never	64	68.1	13	72.2	51	67.1
Ever	24	25.5	5	27.8	19	25
Missing	6	6.4	.	.	6	7.9
Sun exposure without any protection						
Get a severe sunburn with blistering	13	13.8	3	16.7	10	13.2
Have a painful sunburn for a few days with peeling	34	36.2	5	27.8	29	38.2
Get mildly burnt followed by some tanning	27	28.7	7	38.9	20	26.3
Go brown without any sunburn	11	11.7	3	16.7	8	10.5
Don't Know	1	1.1	.	.	1	1.3
Missing	8	8.5	.	.	8	10.5
Repeatedly exposed to bright sunlight						
Go very brown and deeply tanned	11	11.7	2	11.1	9	11.8
Get moderately tanned	39	41.5	10	55.6	29	38.2
Get mildly or occasionally tanned	24	25.5	4	22.2	20	26.3
Get no suntan at all or only get freckled	11	11.7	2	11.1	9	11.8
Don't Know	2	2.1	.	.	2	2.6
Missing	7	7.4	.	.	7	9.2
Ever Sunburn						
Never	16	17	3	16.7	13	17.1
Ever	72	76.6	15	83.3	57	75
Missing	6	6.4	.	.	6	7.9
Sunburn at age 10y						
No	26	27.7	6	33.3	20	26.3
Yes	62	66	12	66.7	50	65.8

	Overall		Control		Case	
	No.	**%**	**No.**	**%**	**No.**	**%**
Missing	6	6.4	.	.	6	7.9
Ever Blister						
No	47	50	13	72.2	34	44.7
Yes	41	43.6	5	27.8	36	47.4
Missing	6	6.4	.	.	6	7.9
Blister at age 10y						
No	59	62.8	14	77.8	45	59.2
Yes	29	30.9	4	22.2	25	32.9
Any occupational sun exposure						
No	35	37.2	8	44.4	27	35.5
Yes	53	56.4	10	55.6	43	56.6
Occupational sun exposure						
None	35	37.2	8	44.4	27	35.5
<P50	29	30.9	6	33.3	23	30.3
>=P50	24	25.5	4	22.2	20	26.3
Any beach or waterside activities						
Never	55	58.5	10	55.6	45	59.2
Yes	33	35.1	8	44.4	25	32.9
Any outdoor poolside activities						
Never	37	39.4	6	33.3	31	40.8
Yes	51	54.3	12	66.7	39	51.3
Other sunbathing						
Never	23	24.5	4	22.2	19	25
Yes	64	68.1	14	77.8	50	65.8
Boating Activities						
Never	31	33	3	16.7	28	36.8
Yes	57	60.6	15	83.3	42	55.3
Any Water Activity						
Never	7	7.4	.	.	7	9.2
Yes	81	86.2	18	100	63	82.9
Missing	6	6.4	.	.	6	7.9

* Death from melanoma recorded during 2 years of follow-up.
Abbreviations: SSM, Superficial spreading melanoma; NM, Nodular melanoma; LMM, Lentigo maligna melanoma; UV, ultraviolet radiation

3.16.4 Table 3: Characteristics of Control and Melanoma Participants Analyzed for Demographic, Clinical Characteristics by LC3 and Beclin1

	LC3 Protein Expression							Beclin 1 Protein Expression						
	All		Control		Case			All		Control		Case		
	No.	Mean	No.	Mean	No.	Mean	P-value	No.	Mean	No.	Mean	No.	Mean	P-value
All	68	1543	17	1587	51	1528	0.67	59	2524	14	326	45	3207	
Age at Presentation							0.07							**0.02**
Gender	21	1552	17	1587	4	1403		18	930	14	326	4	3045	**0.02**
Male	23	1580	-	-	23	1580		18	3040	-	-	18	3040	
Female	24	1499	-	-	24	1499		23	3366	-	-	23	3366	
Clinical Characteristics:														
Death from Melanoma	44	1610	5	1908	39	1572	**0.03**	12	670	10	191	2	3065	
Alive or death from other causes	10	1383	-	-	10	1383		37	2991	4	663	33	3273	
Breslow Thickness							0.17							**0.05**
< 1	20	1497	-	-	20	1497		17	2986	-	-	17	2986	
≥ 1	27	1538	17	1587	10	1455		23	1442	14	326	9	3179	
BRAF* and *NRAS							**0.01**							0.56
WT	43	1567	9	1697	34	1532		37	2703	6	504	31	3128	
NRAS+	6	1317	-	-	6	1317		6	2868	-	-	6	2868	
BRAF+	24	1512	8	1465	16	1536		21	2188	8	192	13	3416	
Punctate LC3							**>0.01**							0.84
Absent	38	1641	11	1763	27	1592		34	2366	10	391	24	3189	
Present	26	1550	17	1587	9	1478		22	1374	14	326	8	3209	

Table 4. Characteristics of Control and Melanoma Participants Analyzed for UV Exposure, LC3 and Beclin1

	LC3 Protein Expression							Beclin 1 Protein Expression						
	All		Control		Case			All		Control		Case		
UV Exposure:	**No.**	**Mean**	**No.**	**Mean**	**No.**	**Mean**	**P-value**	**No.**	**Mean**	**No.**	**Mean**	**No.**	**Mean**	**P-value**
Repeated Sun Exposure Without Protection							0.06							0.46
Go Very Brown/Deeply Tan	28	1597	10	1613	18	1588		24	2270	8	389	16	3210	
Moderately Tan	18	1539	3	1786	15	1489		16	2990	2	301	14	3374	
Get Mildly Tan	6	1279	2	934	4	1451		5	1932	2	208	3	3082	
No Suntan/Only Freckled	1	1556	-	-	1	1556		1	3203	-	-	1	3203	
Don't Know	4	1608	-	-	4	1608		3	2831	-	-	3	2831	
Missing	10	1519	2	1816	8	1445		9	2437	2	217	7	3072	
Painful Sunburn (2 or more days)							**0.03**							0.5
No	52	1494	14	1518	38	1485		45	2544	11	346	34	3256	
Yes	4	1608	-	-	4	1608		3	2831	-	-	3	2831	
Missing	12	1734	3	1914	9	1674		11	2354	3	253	8	3143	
Ever Blistering Sunburn							0.32							**0.02**
No	28	1464	4	1378	24	1479		24	2976	3	194	21	3374	
Yes	4	1608	-	-	4	1608		3	2831	-	-	3	2831	
Any Beach/ Waterside Activities							0.69							0.93
Never	26	1554	7	1471	19	1585		23	2529	6	502	17	3245	
Ever	4	1608	-	-	4	1608		3	2831	-	-	3	2831	

Subjects consisted of 68 participants from a New Mexico tissue collection. OR values are adjusted for age as a continuous variable. Abbreviations: CI = confidence limit; NM = nodular melanoma; HR = Hazard ratio; SSM = superficial spreading melanoma; SD = standard deviation; Death from melanoma recorded during 2.5 years of follow-up. *Statistical significance was set at 5%. **Unless noted otherwise odds ratios (OR) are for a one unit increase.

3.14.6 Table 5. Univariate Logistic Regression for Melanoma Specific Mortality Correlated with Histopathological Characteristics, UV Exposure and LC3/ Beclin 1 Expression

	OR (95% CI)**	p-value*
Age at Diagnosis	1.00 (0.95-1.05)	0.95
Sex (Female)	1.00 (0.29-3434)	0.99
Clinical Characteristics:		
Breslow thickness (continuous)	1.5 (1.11-2.01)	**0.01**
Breslow Thickness ≥ 1	5.33 (1.03-27.8)	**0.05**
Clark (Per Stage Increase)	1.87 (0.90-3.92)	0.1
Mitoses	1.31 (1.03-1.67)	0.03
Moles	0.94 (0.84-1.05)	0.24
Ulceration (Absent vs. Present)	13.67 (2.48-75.27)	**0.003**
Regression (Absent vs. Present)	0.25 (0.05-1.4)	0.12
Oncogene Status:		
NRAS (heterozygous vs wt.)	35,0 (3.21-381.59)	**0.004**
BRAF (heterozygous vs wt.)	0.63 (0.12-3.44)	0.6
Autophagy Markers:		
LC3 Expression (log-transformed)	0.004 (0.001-0.77)	0.04
LC3 Punctate Expression	0.25 (0.06-1.08)	0.06
Beclin1 Expression (log-transformed)	1.35 (0.38-4.72)	0.64
Histological Sub-type:		
SSM	0.15 (0.04-0.64)	0.01
NM	2.60 (0.69-9.8)	0.16
LMM	8.39 (1.81-38.98)	0.01
Melanoma in situ	1.03 (0.23-4.61)	0.97
UV Exposure:		
Weekly Sun Hours Age 10 (per 8 hour increase)	0.94 (0.45-1.92)	0.86
Weekly Sun Hours at Diagnosis (per 8 hour increase)	1.08 (0.68-1.73)	0.73
Sunlamp Use	1.92 (0.48-7.67)	0.36
Painful Sunburn (2 or more days)	0.77 (0.14-4.17)	0.76
Early Sunburn (by age 10)	0.88 (0.20-3.81)	0.87
Decades with Sunburn (per 1 decade increase)	0.76 (0.39-1.48)	0.42
Ever Had a Blistering Sunburn	0.88 (0.23-3.34)	0.85
Early Blistering Sunburn (by age 10)	1.21 (0.31-4.76)	0.78
Decades with Blistering Sunburn (per 1 decade increase)	0.62 (0.26-1.49)	0.28
Any Occupational Exposure to Sunlight	0.95 (0.24-3.71)	0.94

Total Weekly Occupational Exposure to Sunlight (per 1 hour increase)	1.01 (0.98-1.05)	0.47
Total Occupational Exposure to Sunlight	1.22 (0.54-2.77)	0.64
Activities:		
Any Beach/Waterside Activity	2.93 (0.74-11.53)	0.12
Swimming Pool Activities	2.05 (0.485-8.67)	0.33
Sunbathing Other than Beach or Pool	0.76 (0.17-3.32)	0.72
Boating Activities	1.57 (0.37-6.69)	0.54
Any Outdoor Activity Sun Exposure	0.96 (0.10-8.97)	0.97
Number of Outdoor Activities (per 1 activity increase)	1.42 (0.7-2.88)	0.34

Subjects consisted of 68 participants from a New Mexico tissue collection. OR values are adjusted for age as a continuous variable. Abbreviations: CI = confidence limit; NM = nodular melanoma; OR = odds ratio; SSM = superficial spreading melanoma; SD = standard deviation; Death from melanoma recorded during 2.5 years of follow-up. *Statistical significance was set at 5%. **Unless noted otherwise odds ratios (OR) are for a one unit increase

CHAPTER 4

Variants in Autophagy Related Genes and Clinical Characteristics in Melanoma:

A Population-Based Study

4.1 Abstract

Autophagy has been linked with melanoma, but polymorphisms in autophagy related (*ATG*) genes have not been investigated for association with survival or histopathological features known to be important in melanoma survival. We examined 5 *ATG* gene single nucleotide polymorphisms (SNPs) in a multicenter population-based case-control study of melanoma. DNA from 911 melanoma patients was genotyped for SNPs with suspected impact on autophagic flux. While no association was identified with survival, several associations with prognostic features were noted. A decreased Breslow thickness (p = 0.03) and earlier stage at diagnosis (OR 0.47, 95% CI 0.27-0.81, p = 0.02) was identified with the minor allele for an *ATG16L* polymorphism (rs2241880) and the heterozygous genotype was associated with younger diagnosis age (p = 0.02). In addition, two SNPs in *ATG5* (rs2245214 and rs510432) were associated with increased stage of melanoma (OR 1.84 95% CI 1.12-3.02, p=0.05; OR 1.47 95% CI 1.11-1.94, p=0.03). Finally, although not significant at the global p-value, we identified an inverse association between the minor alleles of ATG5 (rs2245214) and scalp or neck melanomas (OR 0.20, 95% CI 0.05-0.86, p= 0.03); ATG10 rs1864182 (OR 0.42, 95% CI 0.21-0.88, p= 0.02) and brisk TILs, and non-brisk TILs and ATG5 rs510432 (OR 0.55 95% CI 0.34-0.87, p= 0.01). In summary, our data suggest *ATG* SNPs might be associated with prognostic characteristics although not with survival and that the direction of the ATG SNPs may be differential due to low. It may be informative to evaluate these and other SNPs in the larger population as these associations may be helpful to understand the role of autophagy in melanoma.

4.2 Introduction:

The American Cancer Society estimates that in 2015, 73,870 new melanomas will be diagnosed and 9,940 people will die from their disease.[1] The long term prognosis for melanoma patients has not improved at the same rate as other cancers.[2] One mechanism of tumorigenesis that is under intensive investigation is autophagy. Autophagy is a catabolic process that assists the removal of unnecessary or dysfunctional cellular components, including damaged proteins and organelles through lysosomal degradation.[3] Macroautophagy (hereafter referred to as autophagy) is tightly regulated and plays a role in a wide variety of normal physiological processes including energy metabolism, stress responses, growth regulation, and aging[4,5] and can be induced in response to nutrient deprivation.[6] Accumulating evidence indicates that autophagy is involved in cancer progression.[5] In addition, the idea of melanoma addiction to autophagy[7–11] has important implications for cancer development as well as treatment options.[12] There are clinical trials ongoing at the National Institutes of Health to target inhibition of the autophagic pathway in multiple cancer types including melanoma.[13] However, the extent to which the autophagy impacts melanoma progression and/or survival remains to be elucidated.

Single nucleotide polymorphisms (SNPs) have been implicated in the pathogenesis of many types of cancers[14] including melanoma.[15–17] Relevant data from the current literature suggests that the frequency of variants in autophagy-related (*ATG*) genes may be altered in cancer patients.[18] Autophagy gene variants have been associated with risk prognosis and/or survival in autoimmune diseases, including Crohn's disease, tuberculosis[19] and gastric, breast and thyroid cancers[20–24]. To our knowledge, there are no

studies examining the relationship between *ATG* gene SNPs and histopathological markers or survival in melanoma.

In this study, we analyzed germline DNA for variants (i.e. SNPs) in *ATG* genes from a large population-based cohort of melanoma patients from Australia and the US. The SNPs investigated were chosen having been identified as significantly associated with disease outcomes and≥10% minor allele frequency in Caucasians. Our goal was to determine whether SNPs in *ATG* genes were associated with factors associated with tumor characteristics and survival in melanoma patients. We hypothesize that *ATG* gene variants are associated with histopathological markers of melanoma progression.

4.3 Materials and Methods

4.3.1 Patient Characteristics:

Over four years, 3,579 individuals from 9 study sites including eight population-based cancer registries in the United States (New Jersey, North Carolina, California), Australia (New South Wales, Tasmania), Canada (Ontario, British Columbia), and Italy (Turin), and one hospital center in Michigan were enrolled in the Genes, Environment and Melanoma (GEM), a large international population-based case control study. GEM recruitment procedures and data collection have been previously described.[25] The Institutional Review Boards of all participating institutions approved the protocol and informed consent was obtained from each participant. GEM is a population-based international consortium studying risk for melanoma development and progression and survival. From this study of a population of 1,206 individuals with multiple primary melanoma and 2,373 with single primary melanoma, 911 genomic DNA samples were

chosen based on tumor availability (previously reported; Thomas et. al 2015)(see patient characteristics below, Table 1)[26] and genotyped to compared *ATG* SNP frequencies with histopathological data.

4.3.2 Clinical Stage:

Histopathology slides were reviewed as previously described[27] and clinical stage was determined based on pathology reports and includes Breslow thickness, mitotic index and ulceration. Mitoses were defined as present or absent. TIL grade was scored as absent, nonbrisk, or brisk using a previously defined grading system.[27] All cases were characterized using the T classification which describes the state of the primary tumor in the AJCC TNM (tumor, regional nodes, and distant metastasis) melanoma staging system.

4.3.3 Selection of SNPs and Genotyping:

5 SNPs in three critical *ATG* genes were selected from those identified with functional SNPs in the literature or that were associated with cancer or disease outcomes (see Figure 1). [18,19,21,28,29] Taqman Real-Time PCR Assays (ThermoFisher Scientific, Grand Island, NY) were used to identify SNPs in *ATG* genes performed with a 7900HT Fast Real-Time PCR System (ThermoFisher Scientific, Grand Island, NY) following manufacture recommendations. DNA was isolated from buccal cells as previously described.[25,30] A Nanodrop 2000 spectrophotometer (ThermoFisher Scientific, Grand Island, NY) was used for quantification of DNA. The ratio of fluorescence in amplification during the logarithmic phase was quantified to identify specific alleles in genes of interest using a commercially available Taqman primer assay on a 7900HT Applied Biosystems qPCR

machine. Genomic DNA samples were chosen from those samples with sufficient DNA and tumor availability to allow for somatic analysis in future studies. [26] The genotyping call rate for the 911 chosen samples ranged from 96% to 99% and biological replicates were generated for 10% of the samples with 100% concordance.

4.3.4 Data Analysis:

Frequency tables were used to summarize the genotype distributions for each *ATG* SNP. To assess the genotyping quality, we calculated the genotype call rates and tested the departure from Hardy Weinberg Equilibrium for each SNP in all subjects. The association between SNPs and histopathological features were tested under the general genotypic inheritance (co-dominant) model. No assumptions were made on the model of inheritance, and the genotypes for each SNP were treated as a three-level nominal variable. Using a genotypic model to simultaneously compare heterozygous genotype versus wildtype, and homozygous minor genotype versus wildtype, we report a global p - value representing the overall significance of the two comparisons for our analysis. Linear regression analyses were performed to assess the association between *ATG* gene SNPs and log transformed Breslow thickness, which was non-normally distributed. To evaluate the association between *ATG* SNPs and histopathological features, we conducted logistic regression analyses for binary outcomes (mitosis, ulceration), ordinal logistic regression analysis for ordinal outcome (stage), and polytomous logistic regression analyses for nominal outcomes (histology and tumor subtype). The exponentiated regression coefficient modeled Breslow thickness representing Breslow thickness increases per mm. ORs and 95% confidence intervals estimated from the regression

models were used to summarize the associations. Wald tests were used to assess the significance of the association and statistical significance was two-sided at 5%.

4.4 Results:

Five SNPs within *ATG* genes that have been previously reported associated with disease outcomes were genotyped and similar allele frequencies were identified in males and females (data not shown). GEM minor allele frequencies are presented in Table 2 and genotypes do not deviate from Hardy Weinberg Equilibrium (HWE) (data not shown).

After adjustment for age, sex, status (single or multiple primaries) and study center, three *ATG* SNPs (rs2241880, rs510432, rs2245214) were significantly associated with melanoma prognostic indicators. An analysis of all 5 *ATG* gene SNPs with Breslow thickness, the most important prognostic marker in melanoma, revealed a significant association between the minor allele (A) of rs2241880 (*ATG16L)* (Table 3a) and a decrease in Breslow thickness (p= 0.02). A significant association was also identified between the rs2241880 (*ATG16L*) minor allele and an earlier stage at diagnosis (OR 0.47 95% CI 0.27-0.81, p = 0.02) when defined as a binary variable of Stage T1a/T1b/T2a versus T2b and higher (Table 4). Finally, presence of heterozygous (AG) of rs2241880 was significantly associated with a younger age of melanoma diagnosis (p = 0.02) (Table 3b).

In addition, two *ATG5* SNPs (rs2245214 and rs510432) were found significantly associated with increased stage of melanoma (OR 1.47, 95% CI 1.11-1.94, p=0.03; OR 1.84, 95% CI 1.12-3.02, p=0.05) (Table 4). The minor allele (CC) of rs510432 had a borderline association with increased stage. Interestingly, only the heterozygous (CG) of

rs2245214 was associated with this increase in stage when it was defined as a continuous variable (Supplemental Table S1), while the other two SNPs (rs2241880, rs510432) did not retain significance in this analysis

No associations between the 5 autophagy SNPs and mitosis, ulceration, histological subtype or melanoma specific survival (Table 5; Supplemental Table S1) were identified.

Of interest, while they were not significant at the level of the global p-value, a decrease in OR (OR 0.42 95% CI 0.21-0.88, p=0.02) was identified between the presence of brisk TILs and the minor allele of SNP rs1864182, and the presence of non-brisk TILs and the minor allele of rs510432 (OR 0.55 95% CI 0.34-0.87, p=0.01). Finally, while not significant at the global p-value, an inverse association between the minor allele of rs2245214 and scalp/neck melanomas was also identified (OR 0.20 95% CI 0.05-0.86, p=0.03).

4.5 Discussion:

There are clear associations between autophagy and cancer and the role of germline SNPs in melanoma progression and survival has remained unexplored. Autophagy in cancer is context dependent, acting as both a tumor suppressor and as a tumor promoter depending on the stage of development of the tumor. While a recent GWAS study reported no association between melanoma susceptibility[31] and *ATG* gene SNPs, we know of no other study addressing the associations between common genetic variants in *ATG* genes and melanoma development and survival.

In the current study, five SNPs were selected for analysis based on their association with other diseases. The SNPs investigated in this study are located in genes that are critical

to the early stage of the autophagy pathway (Figure 1) and necessary for the formation of the autophagosome.[3] As shown in Figure 1 ATG10 is essential for ATG12 conjugation to ATG5 and ultimately to ATG16L.

Previously, variants in *ATG* genes have been associated with risk, prognosis or survival in cancer[18,28,29] and autoimmune conditions.[32–35] In the current analysis, we examined one SNP (rs2241880) in *ATG16L* which increases risk and is associated with poorer prognosis in disease and might contribute to progression in melanoma. A nonsynonymous polymorphism in *ATG16L*, rs2241880 (T300A), has been extensively studied because its variant allele has been linked with increased risk of Crohn's disease.[23] This *ATG16L* SNP (AA) creates a caspase 3- and caspase 7- cleavage site and reduces the stability of the protein resulting in decreased autophagy; clinically, presence of this variant is associated with decreased survival and increased risk for ileal Crohn's disease in adults. [23] While this SNP is associated with increased susceptibility, it is also associated with childhood (early) onset of this disease.[36]As illustrated in Figure 1, *ATG16L* is essential for the formation of the autophagosome. Through its noncovalent interaction with *ATG12–ATG5,* it facilitates the conjugation of other critical ATG proteins. Other studies have identified a genetic association for rs2241880 and another SNP in *ATG5* (rs2245214) with nearly two-fold susceptibility to non-medullary thyroid cancers [18] as well as an increase in disease severity associated with rs2241880.[28] This SNP (rs2241880) has also been shown to be associated with almost double the risk of developing colorectal cancer.[37]

ATG5 is part of an ubiquitin-like conjugation pathway which links ATG5 with ATG16L (ATG5-ATG16L). Specifically, ATG5 membrane binding is activated through its

conjugation with ATG16L. Membrane binding by the ATG12–ATG5-ATG16 exerts an E3 enzyme-like function and this binding is critical for the correct formation of the autophagosome (Figure 1). Importantly, both rs1864182 and rs1051423, located in *ATG10*, have been reported associated with a decreased risk of breast cancer.[29]

In the current study, three SNPs were associated with melanoma prognostic indicators. One SNP, in a critical autophagy gene (*ATG16L*), had multiple associations in our analysis. A significant association was identified between the minor allele for rs2241880 and a decrease in Breslow thickness (p = 0.03) and an earlier stage at diagnosis (OR 0.47 95% CI 0.27-0.81, p = 0.02). This SNP (rs2241880) also showed an association between the heterozygous variant and a younger age at melanoma diagnosis (p = 0.02). This SNP is also associated in the literature with decreased autophagy and may mediate melanoma progression through the accumulation of protein aggregates and damaged organelles in patients.[38,39]There is evidence in the literature that decreased autophagy may inhibit melanoma tumorgeneis.[7,8] Further, this *ATG16L* SNP has been reported in the literature to be associated with increased IL-1β production in primary cells.[23] Metastatic melanoma cells spontaneously secrete active IL-1β[40] and the association between melanoma and this *ATG* variant warrants further investigation.

In *ATG5,* we identified a positive association with increased stage between two SNPs, rs510432 minor allele (CC) (defined as a Stage T1a/T1b/T2a vs. T2b and higher OR 1.26 95% CI 0.81-1.95, p = 0.05) and rs2245214 heterozygous genotype (CG) (defined as categorical stage OR 1.47 95% CI 1.11-1.94 p = 0.03), and increased stage in melanoma. SNP rs510432 had a borderline association when it was. Interestingly, rs510432 is in the 5′ untranslated region (UTR) upstream of its first exon in the promotor region. In

addition, this SNP (rs510432) (C) has a reported positive association with asthma (p= 0.003) [21] and is reported to confer increased promoter activity of this gene. As we also identified a positive association with increased stage and rs510432 (C) in our population, further studies exploring the functional role of this SNP in the rate of autophagy and melanoma progression may elucidate *ATG5* promoter activity in these participants, leading to more advanced melanoma stage.

In addition, *ATG5* has functions independent of autophagy, including critical roles in apoptosis, mitotic catastrophe and regulation of the β-Catenin signaling pathway[41,42]. As *ATG5* is often down-regulated in primary melanomas,[43] the association of two SNPs in this critical *ATG* gene with increased melanoma stage is significant as they have the potential to become new markers of melanoma risk, progression and/or therapeutic targets.

Our study identified no association between the 5 *ATG* gene SNPs and melanoma specific survival. There were also no significant associations identified between the SNPs and ulceration, mitosis, or histological subtype.

While they were not significant at the global p-value, a decrease in OR (0.42 95% CI 0.21-0.88, p = 0.02) was identified between the presence of brisk TILs and the minor allele of SNP rs1864182, and non-brisk TILs and the minor allele of rs510432 (OR 0.55 95% CI 0.34-0.87 p = 0.01). The association of TILs with autophagy variants is significant as increased number of TILs, particularly brisk TILs, in primary melanomas are associated with improved melanoma-specific survival.[44] TIL absence in melanoma is associated with higher stage then either the presence of non-brisk or brisk TIL. The presence of non-brisk TILs is also associated with a 30% decrease in melanoma death

(HR 0.7; 95% CI 0.5 to 1.0) while there is a 50% decrease associated with brisk TILs (HR 0.5 95% CI 0.3 to 0.9).[44]

Finally, while not significant at the global p-value, an inverse association between the minor allele of rs2245214 and scalp/neck melanomas was also identified (OR 0.20 95% CI 0.05-0.86 p=0.03). As it has been previously documented that individuals with scalp/neck melanomas have poorer outcomes than patients with melanomas on other sites, [45] this inverse relationship warrants further studies to determine if there is a functional significance for *ATG5* and this anatomic site.

Autophagy has had a role in cancer; however the relationship between genetic variants in autophagy genes and cancer development, progression and survival remains under explored. In the present study, we assessed the impact of variants in *ATG* genes necessary for autophagic flux in relationship to melanoma prognostic indicators and survival. Drugs targeting the autophagy pathway are being investigated as effective therapy for many cancers including melanoma. SNPs that alter autophagic rates may affect the effectiveness of current treatment strategies and have clinical significance.[9,46–49] *In silico* analysis of results from multiple studies, and/or coordination of large studies, will have to assess the reproducibility of these *ATG* gene interactions in melanoma.

This study is limited by the knowledge that alteration of autophagy might not be due to variants in *ATG* genes but possibly due to other signaling pathways that regulate autophagy or post-translational modifications. In addition, there are other probably other functional genetic variants not included in the current study, as there are approximately 38 *ATG* genes specifically required for autophagy in *Saccharomyces cerevisiae*.[50] We found no direct association between any of the 5 *ATG* gene SNPs and survival although

this may be due to insufficient sample size. Enlarging the number of samples and incorporating functional studies may help to establish if an association with survival exists. Our analyses did not control for multiplicative significance and false discovery rate but employed a more stringent global p-value to reduce type 1 errors. These limitations must be addressed in future experiments by screening for SNPs in other relevant genes potentially using alternative technologies including deep sequencing.

In conclusion, while we found no association with survival, we have identified three *ATG* gene SNPs as a genetic factors affecting melanoma progression, which, in melanoma patients, may cause changes in ATG protein levels and alter autophagy regulation, affecting melanomagenesis. These findings emphasize the significance of the autophagy pathway in melanoma. As the role of autophagy in melanoma is complex and context dependent, the reported associations may provide important insight into how SNPs in critical autophagy genes impact melanoma progression.

4.6 Acknowledgments:

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4.7 References:

1. American Cancer Society Facts and Statistics 2015 | Research | American Cancer Society. http://www.cancer.org/research/cancerfactsstatistics/cancerfactsfigures2015/. Accessed June 8, 2015.

2. What are the key statistics about melanoma skin cancer? http://www.cancer.org/cancer/skincancer-melanoma/detailedguide/melanoma-skin-cancer-key-statistics. Accessed June 8, 2015.

3. Mizushima N. Autophagy: process and function. *Genes Dev*. 2007;21(22):2861-2873. doi:10.1101/gad.1599207.

4. Shintani T, Klionsky DJ. Autophagy in health and disease: a double-edged sword. *Science*. 2004;306(5698):990-995. doi:10.1126/science.1099993.

5. Choi AMK, Ryter SW, Levine B. Autophagy in human health and disease. *N Engl J Med*. 2013;368(7):651-662. doi:10.1056/NEJMra1205406.

6. Jones RG, Thompson CB. Tumor suppressors and cell metabolism: a recipe for cancer growth. *Genes Dev*. 2009;23(5):537-548. doi:10.1101/gad.1756509.

7. Xie X, Koh JY, Price S, White E, Mehnert JM. Atg7 Overcomes Senescence and Promotes Growth of BrafV600E-Driven Melanoma. *Cancer Discov*. 2015;5(4):410-423. doi:10.1158/2159-8290.CD-14-1473.

8. Strohecker AM, Guo JY, Karsli-Uzunbas G, et al. Autophagy sustains mitochondrial glutamine metabolism and growth of BrafV600E-driven lung tumors. *Cancer Discov*. 2013;3(11):1272-1285. doi:10.1158/2159-8290.CD-13-0397.

9. Ma X, Piao S, Dey S, et al. Targeting ER stress – induced autophagy overcomes BRAF inhibitor resistance in melanoma. 2014;124(3). doi:10.1172/JCI70454DS1.

10. Zhang J, Yang Z, Xie L, Xu L, Xu D, Liu X. Statins, autophagy and cancer metastasis. *Int J Biochem Cell Biol*. 2013;45(3):745-752. doi:10.1016/j.biocel.2012.11.001.

11. Xie X, White EP, Mehnert JM. Coordinate autophagy and mTOR pathway inhibition enhances cell death in melanoma. *PLoS One*. 2013;8(1):e55096. doi:10.1371/journal.pone.0055096.

12. Corazzari M, Fimia GM, Lovat P, Piacentini M. Why is autophagy important for melanoma? Molecular mechanisms and therapeutic implications. *Semin Cancer Biol*. 2013;23(5):337-343. doi:10.1016/j.semcancer.2013.07.001.

13. The BAMM Trial: BRAF, Autophagy and MEK Inhibition in Metastatic Melanoma: A Phase I/2 Trial of Dabrafenib, Trametinib and Hydroxychloroquine in Patients With Advanced BRAF Mutant Melanoma - No Study Results Posted - ClinicalTrials.gov. https://clinicaltrials.gov/ct2/show/NCT02257424. Accessed August 23, 2015.

14. Li W-Q, Pfeiffer RM, Hyland PL, et al. Genetic polymorphisms in the 9p21 region associated with risk of multiple cancers. *Carcinogenesis*. 2014;35(12):2698-2705. doi:10.1093/carcin/bgu203.

15. Gibbs DC, Orlow I, Kanetsky PA, et al. Inherited genetic variants associated with occurrence of multiple primary melanoma. *Cancer Epidemiol Biomarkers Prev*. April 2015. doi:10.1158/1055-9965.EPI-14-1426.

16. Fang S, Wang Y, Chun YS, et al. Association of Common Genetic Polymorphisms with Melanoma Patient IL-12p40 Blood Levels, Risk, and Outcomes. *J Invest Dermatol*. April 2015. doi:10.1038/jid.2015.138.

17. Barrett JH, Taylor JC, Bright C, et al. Fine mapping of genetic susceptibility loci for melanoma reveals a mixture of single variant and multiple variant regions. *Int J Cancer*. 2015;136(6):1351-1360. doi:10.1002/ijc.29099.

18. Plantinga TS, van de Vosse E, Huijbers A, et al. Role of genetic variants of autophagy genes in susceptibility for non-medullary thyroid cancer and patients outcome. *PLoS One*. 2014;9(4):e94086. doi:10.1371/journal.pone.0094086.

19. Songane M, Kleinnijenhuis J, Alisjahbana B, et al. Polymorphisms in autophagy genes and susceptibility to tuberculosis. *PLoS One*. 2012;7(8):e41618. doi:10.1371/journal.pone.0041618.

20. Plantinga TS, van de Vosse E, Huijbers A, et al. Role of genetic variants of autophagy genes in susceptibility for non-medullary thyroid cancer and patients outcome. *PLoS One*. 2014;9(4):e94086. doi:10.1371/journal.pone.0094086.

21. Martin LJ, Gupta J, Jyothula SSSK, et al. Functional variant in the autophagy-related 5 gene promotor is associated with childhood asthma. *PLoS One*. 2012;7(4):e33454. doi:10.1371/journal.pone.0033454.

22. Chen D, Zhu C, Wang X, et al. Neuroscience Letters A novel and functional variant within the ATG5 gene promoter in sporadic Parkinson ' s disease. *Neurosci Lett*. 2013;538:49-53. doi:10.1016/j.neulet.2013.01.044.

23. Lassen KG, Xavier RJ. An alteration in ATG16L1 stability in Crohn disease. *Autophagy*. 2014;10(10):1858-1860. doi:10.4161/auto.29963.

24. Grimm WA, Messer JS, Murphy SF, et al. The Thr300Ala variant in ATG16L1 is associated with improved survival in human colorectal cancer and enhanced production of type I interferon. *Gut*. February 2015:gutjnl - 2014-308735 - . doi:10.1136/gutjnl-2014-308735.

25. Millikan RC, Hummer A, Begg C, et al. Polymorphisms in nucleotide excision repair genes and risk of multiple primary melanoma: the Genes Environment and Melanoma Study. *Carcinogenesis*. 2006;27(3):610-618. doi:10.1093/carcin/bgi252.

26. Thomas NE, Edmiston SN, Alexander A, et al. Association Between NRAS and BRAF Mutational Status and Melanoma-Specific Survival Among Patients With Higher-Risk Primary Melanoma. *JAMA Oncol*. April 2015. doi:10.1001/jamaoncol.2015.0493.

27. Thomas NE, Kricker A, Waxweiler WT, et al. Comparison of clinicopathologic features and survival of histopathologically amelanotic and pigmented melanomas: a population-based study. *JAMA dermatology*. 2014;150(12):1306-1314. doi:10.1001/jamadermatol.2014.1348.

28. Huijbers A, Plantinga TS, Joosten LAB, et al. The effect of the ATG16L1 Thr300Ala polymorphism on susceptibility and outcome of patients with epithelial cell-derived thyroid carcinoma. *Endocr Relat Cancer*. 2012;19(3):L15-L18. doi:10.1530/ERC-11-0302.

29. Qin Z, Xue J, He Y, et al. Potentially functional polymorphisms in ATG10 are associated with risk of breast cancer in a Chinese population. *Gene*. 2013;527(2):491-495. doi:10.1016/j.gene.2013.06.067.

30. Begg CB, Orlow I, Hummer AJ, et al. Lifetime risk of melanoma in CDKN2A mutation carriers in a population-based sample. *J Natl Cancer Inst*. 2005;97(20):1507-1515. doi:10.1093/jnci/dji312.

31. Law MH, Bishop DT, Lee JE, et al. Genome-wide meta-analysis identifies five new susceptibility loci for cutaneous malignant melanoma. *Nat Genet*. August 2015. doi:10.1038/ng.3373.

32. Salem M, Ammitzboell M, Nys K, Seidelin JB, Nielsen OH. ATG16L1: A multifunctional susceptibility factor in Crohn disease. *Autophagy*. 2015;11(4):585-594. doi:10.1080/15548627.2015.1017187.

33. Naser SA, Arce M, Khaja A, et al. Role of ATG16L, NOD2 and IL23R in Crohn's disease pathogenesis. *World J Gastroenterol*. 2012;18(5):412-424. doi:10.3748/wjg.v18.i5.412.

34. López P, Alonso-Pérez E, Rodríguez-Carrio J, Suárez A. Influence of Atg5 mutation in SLE depends on functional IL-10 genotype. *PLoS One*. 2013;8(10):e78756. doi:10.1371/journal.pone.0078756.

35. Murthy A, Li Y, Peng I, et al. A Crohn's disease variant in Atg16l1 enhances its degradation by caspase 3. *Nature*. 2014;506(7489):456-462. doi:10.1038/nature13044.

36. Fisher SA, Tremelling M, Anderson CA, et al. Genetic determinants of ulcerative colitis include the ECM1 locus and five loci implicated in Crohn's disease. *Nat Genet*. 2008;40(6):710-712. doi:10.1038/ng.145.

37. Nicoli ER, Dumitrescu T, Uscatu CD, et al. Determination of autophagy gene ATG16L1 polymorphism in human colorectal cancer. *Rom J Morphol Embryol*. 2014;55(1):57-62. http://www.ncbi.nlm.nih.gov/pubmed/24715166. Accessed May 30, 2015.

38. Lerena C, Calligaris SD, Colombo MI. Autophagy: for better or for worse, in good times or in bad times. *Curr Mol Med*. 2008;8(2):92-101. http://www.ncbi.nlm.nih.gov/pubmed/18336290. Accessed August 22, 2015.

39. Panda PK, Mukhopadhyay S, Das DN, Sinha N, Naik PP, Bhutia SK. Mechanism of autophagic regulation in carcinogenesis and cancer therapeutics. *Semin Cell Dev Biol*. 2015;39:43-55. doi:10.1016/j.semcdb.2015.02.013.

40. Okamoto M, Liu W, Luo Y, et al. Constitutively active inflammasome in human melanoma cells mediating autoinflammation via caspase-1 processing and secretion of interleukin-1beta. *J Biol Chem*. 2010;285(9):6477-6488. doi:10.1074/jbc.M109.064907.

41. Yousefi S, Perozzo R, Schmid I, et al. Calpain-mediated cleavage of Atg5 switches autophagy to apoptosis. *Nat Cell Biol*. 2006;8(10):1124-1132. doi:10.1038/ncb1482.

42. Maskey D, Yousefi S, Schmid I, et al. ATG5 is induced by DNA-damaging agents and promotes mitotic catastrophe independent of autophagy. *Nat Commun*. 2013;4:2130. doi:10.1038/ncomms3130.

43. Liu H, He Z, von Rütte T, Yousefi S, Hunger RE, Simon H-U. Down-regulation of autophagy-related protein 5 (ATG5) contributes to the pathogenesis of early-stage cutaneous melanoma. *Sci Transl Med*. 2013;5(202):202ra123. doi:10.1126/scitranslmed.3005864.

44. Thomas NE, Busam KJ, From L, et al. Tumor-infiltrating lymphocyte grade in primary melanomas is independently associated with melanoma-specific survival in the population-based genes, environment and melanoma study. *J Clin Oncol*. 2013;31(33):4252-4259. doi:10.1200/JCO.2013.51.3002.

45. Tseng WH, Martinez SR. Tumor location predicts survival in cutaneous head and neck melanoma. *J Surg Res*. 2011;167(2):192-198. doi:10.1016/j.jss.2010.10.008.

46. Kondo Y, Kanzawa T, Sawaya R, Kondo S. The role of autophagy in cancer development and response to therapy. *Nat Rev Cancer*. 2005;5(9):726-734. doi:10.1038/nrc1692.

47. Kim J, Yip MLR, Shen X, et al. Identification of anti-malarial compounds as novel antagonists to chemokine receptor CXCR4 in pancreatic cancer cells. *PLoS One*. 2012;7(2):e31004. doi:10.1371/journal.pone.0031004.

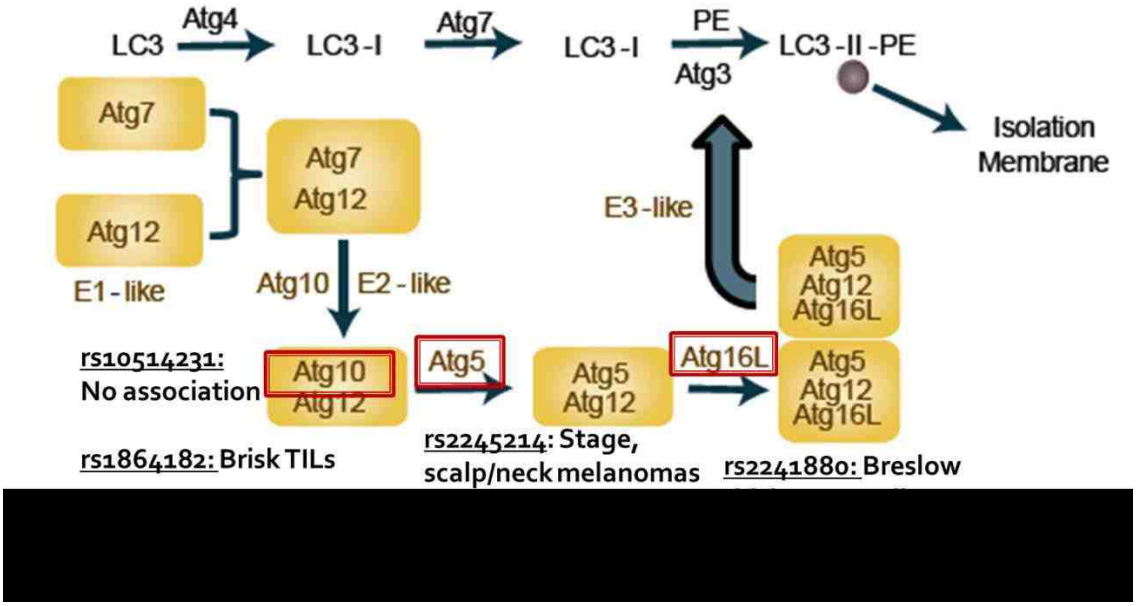
48. Amaravadi RK. PUMA: a puzzle piece in chloroquine's antimelanoma activity. *J Invest Dermatol*. 2013;133(9):2133-2135. doi:10.1038/jid.2013.135.

49. Vogl DT, Stadtmauer EA, Tan K-S, et al. Combined autophagy and proteasome inhibition: a phase 1 trial of hydroxychloroquine and bortezomib in patients with relapsed/refractory myeloma. *Autophagy*. 2014;10(8):1380-1390. doi:10.4161/auto.29264.

50. Klionsky DJ. Citing recent declines in the discovery of new ATG genes, some scientists now suggest that the end of autophagy research may be within sight. *Autophagy*. 2014;10(5):715-716. doi:10.4161/auto.28813.

4.7 Figure and Tables:

4.7.1 Figure 1: Overview of the Autophagy related (ATG) conjugation pathway focusing on the gene variants investigated in this study, including those in the *ATG5, ATG 10* and *ATG 16L* genes.



* Five variants from three autophagy related genes were selected for either an association with or functional impact on risk or progression of disease. Two SNPs were chosen from *ATG10*, two from *ATG5* and one from *ATG16L*. From our analysis a significant association was identified between the *ATG16L* polymorphism (rs2241880) and a decrease in Breslow thickness (p = 0.03), younger age (p = 0.02) and earlier stage at diagnosis (OR 0.47, 95% CI 0.27-0.81, p = 0.02). In addition, two SNPs in *ATG5* (rs2245214 and rs510432) were associated with increased stage of melanoma (OR 1.84 95% CI 1.12-3.02, p=0.05; OR 1.47 95% CI 1.11-1.94, p=0.03). We identified an inverse association between the *ATG5* SNP rs2245214 and melanomas on the scalp or neck (OR 0.20, 95% CI 0.05-0.86, p= 0.03); rs1864182 (*ATG10*) (OR 0.42, 95% CI 0.21-0.88, p= 0.02), and with brisk TILs. Finally, we identified an association between non-brisk TILs and the *ATG5* SNP rs510432 (OR 0.55 95% CI 0.34-0.87, p= 0.01) although not significant at the global p-value.

4.7.2 Table 1. Clinicopathologic characteristics among 911 melanoma cases*

Characteristics		No.	%
Age at diagnosis	60		
Breslow thickness, median , mm	0.8		
Gender	Male	534	59
	Female	377	41
Breslow thickness (mm)	0.01-1.00	547	60
	1.01-2.00	212	23
	2.01-4.00	108	12
	> 4.00	44	5
Status	SPM	603	66
	MPM	308	34
Anatomic site	Trunk/pelvis	394	43
	Scalp/neck	56	6
	Face/ears/other	116	13
	Upper extremities	172	19
	Lower extremities	173	19
Histological subtype	SSM	610	67
	NM	92	10
	LMM	116	13
	Other	93	10
	Missing	0	0
Ulceration	Absent	794	92
	Present	73	8
Mitosis	Absent	454	52
	Present	415	48
AJCC stage	T1a	397	46
	T1b	124	14
	T2a	183	21
	T2b	16	2
	T3a	73	8
	T3b	32	4
	T4a	21	2
	T4b	21	2
AJCC stage (T1a/T1b/T2a vs. T2b+)	T1a/T1b/T2a	704	81
	T2b+	163	19
TIL grade	Absent	194	22
	Non-Brisk	563	65
	Brisk	111	13
Growth phase	Absent	255	29

	Present	614	71
Death from melanoma*		76	8
Alive or death from other causes		835	92

*Subjects comprised 911 participants from the GEM study from Australia and the United States. OR values are adjusted for age as a continuous variable. Abbreviations: AJCC= American Joint Committee on Cancer; CI = confidence limit; Mpm= Multiple primary melanoma; NM = nodular melanoma; OR = odds ratio; SSM = superficial spreading melanoma; SD = standard deviation; TIL= Tumor infiltrating lymphocytes; Spm=Single primary melanoma. Death from melanoma recorded during 7.5 years of follow-up.

4.7.3 Table 2. Allele frequencies of ATG genes in melanoma patients

ATG SNP	Genotype	Number of patients (%)
ATG5		
rs510432	CC	190 (0.22)
	CT	425 (0.48)
	TT	266 (0.30)
ATG5		
rs2245214	CC	331 (0.38)
	CG	427 (0.49)
	GG	110 (0.13)
ATG16L		
rs2241880	AA	198 (0.23)
	AG	418 (0.490
	GG	245 (0.29)
ATG10		
rs10514231	CC	116 (0.13)
	CT	403 (0.47)
	TT	345 (0.40)
ATG10		
rs1864182	AA	238 (0.28)
	AC	424 (0.49)
	CC	200 (0.23)

4.7.4 Table 3. Relationship between ATG genotype, Breslow thickness and age of diagnosis in melanoma

4.7.4a Table 3a. Relationship between ATG genotype and Breslow thickness in melanoma

Breslow thickness (Continuous)

ATG gene SNP	Genotype	Coeff (95% CI)	p-value*	Global p-value*
rs10514231	TT	1.00		0.18
	CT	1.09 (0.98-1.22)	0.09	
	CC	0.99 (0.84-1.16)	0.91	
rs1864182	CC	1.00		0.72
	AC	1.05 (0.92-1.19)	0.46	
	AA	1.05 (0.9-0.82)	0.50	
rs2241880	GG	1.00		**0.03**
	AG	1.04 (0.92-0.85)	0.55	
	AA	0.87 (0.97-0.99)	0.06	
rs22445214	TT	1.00		0.28
	CG	1.09 (0.98-1.22)	0.11	
	CC	1.06 (0.9-1.26)	0.49	
rs510432	TT	1.00		0.30
	CT	1.05 (0.94-1.19)	0.37	
	CC	1.12 (0.97-1.3)	0.12	

Genotypic model adjusted for age (continuous) sex, study center and status. Abbreviations: CI, confidence interval; Coeff, coefficient

4.7.4b Table 3b. Age at diagnosis by genotype status among melanoma cases

Age at diagnosis, y

ATG gene SNP	Genotype	n	Coeff (95% CI)	p-value*	Global p-value*
rs10514231	TT	864	1.00		
	CT		0.22 (-1.95-2.39)	0.84	0.71
	CC		1.33 (-1.84-4.50)	0.41	
rs1864182	CC	862	1.00		
	AC		0.72 (-1.67-3.11)	0.55	0.70
	AA		-0.26 (-3.09-2.57)	0.86	
rs2241880	GG	861	1.00		**0.02**

	AG		-3.25 (-5.60--0.91)	**0.01**	
	AA		-2.04 (-4.83-0.74)	0.15	
rs2245214	TT	868	1.00		
	CG		-0.59 (-2.77-1.58)	0.59	0.75
	CC		-1.17 (-4.43-2.09)	0.48	
rs510432	TT	881	1.00		
	CT		0.43 (-1.86-2.73)	0.71	0.75
	CC		-0.55 (-3.33-2.23)	0.70	

Genotypic model adjusted for sex, study center and status.

4.7.5 Table 4. Relationship between ATG genotype and AJCC stage in melanoma

		Melanoma Stage		
ATG gene SNP	Genotype	≥ Stage T2b vs. Stage T1a/T1b/T2a	p-value*	Global p-value*
rs10514231	TT	1.00		
	CT	1.18 (0.80-1.76)	0.41	0.46
	CC	0.84 (0.46-1.54)	0.57	
rs1864182	CC	1.00		
	AC	1.22 (0.79-1.89)	0.38	0.52
	AA	0.98 (0.57-1.66)	0.93	
rs2241880	GG	1.00		
	AG	0.88 (0.59-1.33)	0.55	**0.02**
	AA	0.47 (0.27-0.81)	**0.01**	
rs2245214	TT	1.00		
	CG	1.46 (0.98-2.17)	0.06	0.14
	CC	1.05 (0.57-1.92)	0.88	
rs510432	TT	1.00		
	CT	1.26 (0.81-1.95)	0.30	**0.05**
	CC	1.84 (1.12-3.02)	**0.02**	

Genotypic model adjusted for age (continuous) sex, study center and status. Abbreviations: AJCC, American Joint Committee on Cancer

4.7.6 Table 5. Relationship between ATG Genotype and melanoma-specific survival

ATG gene SNP	Genotype	HR (95% CI)	p-value	Wald p-value
rs10514231	TT	1 [Reference]		0.72
	CT	1.24 (0.74-2.07)	0.42	

	CC	1.12 (0.54-2.35)	0.76	
rs1864182	CC	1 [Reference]		
	AC	1.83 (0.93-3.59)	0.08	0.18
	AA	1.89 (0.90-3.98)	0.10	
rs2241880	GG	1 [Reference]		
	AG	1.04 (0.60-1.80)	0.89	0.85
	AA	0.86 (0.43-1.72)	0.67	
rs22445214	TT	1 [Reference]		
	CG	0.86 (0.52-1.43)	0.56	0.80
	CC	0.82 (0.37-1.81)	0.63	
rs510432	TT	1 [Reference]		
	CT	0.68 (0.40-1.17)	0.16	0.13
	CC	1.19 (0.67-2.12)	0.55	

Survival analysis adjusted for age, sex, study center, status (Spm vs. Mpm) and time dependent crossover in a genotypic model. Abbreviations: HR, hazard ratio; CI, confidence interval

4.7.7 Supplemental Table S1a. Clinicopathologic characteristics by genotype status among melanoma cases*

		Anatomic Site								
ATG gene SNP	Genotype	Scalp/neck vs. Trunk/pelvis	p-value*	Face/ears/ other vs. Trunk/pelvis	p-value*	Upper extremities vs. Trunk/pelvis	p-value*	Lower extremities vs. Trunk/pelvis	p-value*	Wald p-value
rs10514231	TT	1.00		1.00		1.00		1.00		
	CT	0.74 (0.39-1.39)	0.34	1.06 (0.65-1.74)	0.80	0.8 (0.53-1.2)	0.28	0.97 (0.63-1.47)	0.87	0.73
	CC	0.46 (0.15-1.4)	0.17	1.36 (0.71-2.62)	0.35	0.76 (0.41-1.42)	0.39	0.97 (0.52-1.82)	0.92	
rs1864182	CC	1.00		1.00		1.00		1.00		
	AC	1.56 (0.77-3.14)	0.22	1 (0.58-1.71)	0.99	0.79 (0.5-1.24)	0.31	0.68 (0.25-1.81)	0.44	0.22
	AA	0.83 (0.52-1.33)	0.44	1.33 (0.72-2.47)	0.37	0.94 (0.55-1.62)	0.83	1.26 (0.73-2.17)	0.41	
rs2241880	GG	1.00		1.00		1.00		1.00		
	AG	1.68 (0.83-3.4)	0.15	0.9 (0.53-1.52)	0.68	0.96 (0.62-1.5)	0.87	0.99 (0.62-1.59)	0.97	0.70
	AA	0.88 (0.34-2.27)	0.79	1.18 (0.65-2.16)	0.59	0.96 (0.57-1.63)	0.88	1.09 (0.63-1.89)	0.75	
rs2245214	TT	1.00		1.00		1.00		1.00		
	CG	0.73 (0.4-1.33)	0.31	0.9 (0.56-1.45)	0.66	0.73 (0.49-1.1)	0.14	0.84 (0.55-1.28)	0.41	0.51
	CC	0.20 (0.05-0.86)	**0.03**	0.88 (0.44-1.77)	0.72	0.68 (0.37-1.26)	0.22	0.72 (0.38-1.37)	0.32	
rs510432	TT	1.00		1.00		1.00		1.00		
	CT	0.65 (0.35-1.23)	0.18	0.67 (0.41-1.11)	0.12	1.27 (0.82-1.98)	0.28	1.23 (0.78-1.95)	0.38	0.20
	CC	0.45 (0.19-1.07)	0.07	0.68 (0.37-1.24)	0.21	0.88 (0.51-1.52)	0.65	0.98 (0.56-1.71)	0.94	

		Histological sub-type						
ATG gene SNP	Genotype	NM vs. SSM	p-value	LMM vs. SSM	p-value	Other vs. SSM	p-value	Wald p-value
rs10514231	TT	1.00		1.00		1.00		
	CT	1.21 (0.70-2.07)	0.50	1.08 (0.68-1.73)	0.74	0.95 (0.59-1.54)	0.83	0.36
	CC	1.78 (0.90-3.52)	0.10	1.10 (0.57-2.13)	0.77	0.46 (0.19-1.14)	0.09	
rs1864182	CC	1.00		1.00		1.00		
	AC	1.58 (0.85-2.94)	0.15	1.52 (0.90-2.57)	0.12	1.00 (0.59-1.71)	0.99	0.46
	AA	1.63 (0.81-3.27)	0.17	1.12 (0.59-2.11)	0.73	0.80 (0.41-1.56)	0.52	
rs2241880	GG	1.00		1.00		1.00		
	AG	1.63 (0.92-2.88)	0.09	1.31 (0.78-2.19)	0.30	0.89 (0.53-1.51)	0.66	0.54
	AA	1.18 (0.60-2.36)	0.63	1.18 (0.65-2.15)	0.59	0.74 (0.39-1.42)	0.36	
rs2245214	TT	1.00		1.00		1.00		
	CG	1.59 (0.95-2.67)	0.08	0.88 (0.56-1.39)	0.59	1.41 (0.84-2.37)	0.20	0.26
	CC	0.76 (0.31-1.85)	0.54	0.80 (0.40-1.60)	0.53	1.40 (0.68-2.90)	0.36	
rs510432	TT	1.00		1.00		1.00		
	CT	1.03 (0.60-1.78)	0.91	0.88 (0.54-1.43)	0.60	0.89 (0.53-1.49)	0.65	0.96
	CC	1.33 (0.71-2.49)	0.38	0.98 (0.54-1.78)	0.94	0.93 (0.49-1.76)	0.83	

All genotypic analyses were adjusted for age (continuous), sex, study center and status (Spm/ Mpm/ crossover)
*Bold significance set p= 0.05

Ulceration

ATG gene SNP	Genotype	Ulceration present vs. absent	p-value	Wald p-value
rs10514231	TT	1.00		
	CT	1.04 (0.60-1.81)	0.90	0.82
	CC	0.80 (0.35-1.86)	0.61	
rs1864182	CC	1.00		
	AC	1.23 (0.66-2.29)	0.52	0.61
	AA	0.90 (0.41-1.95)	0.78	
rs2241880	GG	1.00		
	AG	0.94 (0.53-1.68)	0.84	0.41
	AA	0.61 (0.28-1.31)	0.20	
rs2245214	TT	1.00		
	CG	1.33 (0.77-2.28)	0.31	0.29
	CC	0.70 (0.27-1.78)	0.45	
rs510432	TT	1.00		
	CT	1.27 (0.68-2.37)	0.45	0.36
	CC	1.68 (0.83-3.43)	0.15	

Mitosis

ATG gene SNP	Genotype	Mitosis present vs. absent	p-value	Wald p-value
rs10514231	TT	1.00		
	CT	1.09 (0.81-1.48)	0.56	0.84
	CC	1.02 (0.65-1.58)	0.95	
rs1864182	CC	1.00		
	AC	0.95 (0.68-1.33)	0.77	0.50
	AA	1.17 (0.79-1.74)	0.43	
rs2241880	GG	1.00		
	AG	1.06 (0.76-1.48)	0.73	0.49
	AA	0.86 (0.58-1.27)	0.44	
rs2245214	TT	1.00		
	CG	1.33 (0.98-1.79)	0.07	0.17
	CC	1.30 (0.83-2.03)	0.25	
rs510432	TT	1.00		
	CT	1.18 (0.86-1.63)	0.31	0.34
	CC	1.33 (0.90-1.96)	0.15	

TIL Grade

ATG gene SNP	Genotype	Non Brisk vs. Absent	p-value	Brisk vs Absent	p-value	Wald p-value
rs10514231	TT	1.00		1.00		
	CT	0.98 (0.68-1.41)	0.91	0.92 (0.54-1.59)	0.78	0.88
	CC	1.15 (0.67-1.98)	0.62	0.80 (0.34-1.86)	0.6	
rs1864182	CC	1.00		1.00		
	AC	1.12 (0.74-1.68)	0.60	0.87 (0.49-1.58)	0.66	0.14
	AA	0.81 (0.51-1.29)	0.37	0.42 (0.21-0.88)	**0.02**	

rs2241880	GG	1.00		1.00		
	AG	0.91 (0.61-1.35)	0.63	1.02 (0.55-1.90)	0.96	0.42
	AA	0.89 (0.55-1.43)	0.62	1.53 (0.76-3.08)	0.23	
rs2245214	TT	1.00		1.00		
	CG	0.93 (0.65-1.34)	0.70	0.86 (0.50-1.49)	0.59	0.97
	CC	0.91 (0.53-1.57)	0.74	1.01 (0.46-2.22)	0.98	
rs510432	TT	1.00		1.00		
	CT	0.74 (0.49-1.11)	0.15	0.99 (0.54-1.79)	0.96	0.12
	CC	0.55 (0.34-0.87)	**0.01**	0.72 (0.36-1.45)	0.36	

Melanoma Stage (continuous)

ATG gene SNP	Genotype	OR (95% CI)	p-value	Wald p-value
rs10514231	TT	1.00		0.32
	CT	1.18 (0.90-1.56)	0.23	
	CC	0.92 (0.61-1.38)	0.69	
rs1864182	CC	1.00		
	AC	1.07 (0.79-1.46)	0.65	0.90
	AA	1.06 (0.74-1.53)	0.74	
rs2241880	GG	1.00		
	AG	1.01 (0.75-1.37)	0.93	0.14
	AA	0.74 (0.52-1.06)	0.10	
rs2245214	TT	1.00		
	CG	1.47 (1.11-1.94)	**0.01**	**0.03**
	CC	1.23 (0.82-1.85)	0.32	
rs510432	TT	1.00		
	CT	1.30 (0.97-1.75)	0.08	0.14
	CC	1.37 (0.96-1.95)	0.09	

4.8 Supplemental research to chapter 4 manuscript:

4.9 Introduction:

The importance of the autophagy pathway, particularly in cancer, has been previously described in this chapter. In addition, it has been reported that knockout of autophagy related genes results in multiple disease states including neurodegenerative disease, autoimmunity, and cancer. Cells dependent on this catabolic pathway with decreased autophagic capacity to recycle nutrients will undergo programmed cell death which can result in tissue damage and inflammation. [1] Inflammation has an important and emerging role in cancer and has particular significance for melanoma. Melanoma inflammatory cells, including tumor-infiltrating macrophages, have prognostic implications.[2] The presence of TILs in vertical growth phase of cutaneous melanomas has been reported to be associated with a better prognosis [3,4] Conversely, high levels of an inflammatory marker, C-reactive protein is a poor prognostic indicator.[5] Cytokines, chemical messengers of the immune system, have a dynamic range of expression patterns in melanoma that changes with cancer progression. A variant in one SNP previously described in this chapter, autophagy related 16-like 1 (T300A) (Chapter 4.4), enhances the degradation of the ATG16L protein by caspase 3.[1] Under certain conditions ATG16L is critical for negative regulation of inflammatory cytokines[9] and the T300A variant impacts IL-1β and IL-6 production. [10]

Characterizing the correlation of this SNP, which can directly impact the rate of autophagy in cells, with the two major oncogenes in melanoma BRAF and NRAS, should allow us to elucidate the role of autophagy in these important subtypes melanoma. For

this analysis, I used only single primary samples which had been genotyped for *BRAF/NRAS* status from GEM participants. In order to determine whether SNPs in ATG genes are associated with *BRAF/NRAS* mutations in melanoma patients, I used the procedures and premises listed in the previous part of this chapter to investigate 5 SNPs in 3 autophagy related genes in melanoma.

4.10 Materials and Methods:

4.10.1 Patient Characteristics:

From the study of a population of 2,373 participants with single primary melanomas, we chose 602 genomic DNA samples were based on tumor availability (previously described in 4.3.1 and previously reported) [3] and compared *ATG* SNP frequencies with oncogene status.

4.10.2 Clinical Stage:

Previous described in 4.3.2 and summary characteristics were previous reported [3]

4.10.3 Selection of SNPs and Genotyping:

5 SNPs in three critical *ATG* genes were selected from those identified with functional SNPs in the literature or that were associated with *BRAF* and *NRAS* status as previously described. DNA was isolated from buccal cells as previously described in 4.3.3.[4,5] SNPs in *ATG* genes were characterized using Taqman Real-Time PCR Assays (ThermoFisher Scientific, Grand Island, NY) on a 7900HT Fast Real-Time PCR System (ThermoFisher Scientific, Grand Island, NY) following manufacture recommendations as previously

described in 4.3.3. The identification of ATG gene alleles were identified using the ratio of amplification fluorescence during the logarithmic phase generated from a commercially available Taqman primer assay on a 7900HT Applied Biosystems qPCR machine as previously described 4.3.3. Genomic DNA samples were chosen from those samples for single primary melanoma with sufficient DNA and tumor availability to allow for somatic analysis in future studies. [3] The genotyping call rate for the 602 chosen samples ranged was 98 to 100% and biological replicates were generated for 10% of the samples with 100% concordance.

4.10.4 Data Analysis:

Frequency tables were used to summarize the genotype distributions for each *ATG* SNP (see Table 2). The association between SNPs and oncogene status were tested under the general genotypic inheritance (co-dominant) model allowing for simultaneous comparison of the heterozygous genotype versus wildtype, and homozygous minor genotype versus wildtype. No assumptions were made on the model of inheritance, and the genotypes for each SNP were treated as a three-level nominal variable. From this analysis we report a global p -value which represents the overall significance for our analysis as described previously. To evaluate the association between *ATG* SNPs and BRAF or NRAS status, we conducted logistic regression analyses. ORs and 95% confidence intervals estimated from the regression models were used to summarize the associations. Wald tests were used to assess the global significance of the association and statistical significance was two-sided and set at 5%.

4.11 Results:

Five SNPs within *ATG* genes that have been previously reported associated with disease outcomes were genotyped and the result correlated with oncogene status. After adjustment for age, sex and study center, none of the 5 *ATG* gene SNPs were significantly associated with *BRAF* or *NRAS* status in melanoma participants. However the "A" allele of one SNP in *ATG10* (rs1864182) showed a positive correlation with *BRAF* status although it did not retain significance at the global p-value (AC) (OR 1.79 95%CI 1.12-2.87, p= 0.02) (AA) (OR 1.82 95%CI 1.06-3.12, p= 0.03). This may be due to insufficient sample size as our power calculation was originally based on 795 samples. I was unable to obtain DNA for all 795 participants and information on *BRAF NRAS* status was unavailable for some tumors, resulting in decreased sample size.

4.12 Discussion:

In this study a SNP in *ATG10* (rs1864182), while not significant at the global level, correlated with *BRAF* status in our melanoma participants. Of interest, the significance of both allelic specific models, one which examines the relationship between the heterozygous genotype versus wildtype, and homozygous minor genotype versus wildtype were both below .05 (p=0.02; p=0.03 respectively). Elevated expression of ATG10 in colon cancer has been shown to be associated with increased lymph node metastasis[6] and previously this SNP has showed to be associated with increased IL-8 production (p = 0.04).[7]

Melanoma cells express a variety of cytokines, and their level of expression changes as melanoma progresses. [6] Early stage melanoma with lesions less than 1 mm Breslow

thickness exhibit low levels of cytokines including IL-8. [7] More advanced stage is associated with increased expression of pro-inflammatory cytokines IL-1α, β, IL-6, and IL-8. [7] The expression of IL-6 in particular has been associated with melanoma malignancy and its production has been associated with defective autophagy.[8] Of interest, this particular SNP in *ATG10* (rs1864182) was also previously reported to be associated with decreased breast cancer risk.[8] Induced autophagy in breast cancer cells has been shown to result in autophagic regulated cell death.[9] The impact of this SNP in the function of ATG10 remains to be elucidated. Analysis of the rate of autophagy associated with this SNP would provide insights into its association both with BRAF oncogene status as well as correlations with melanoma progression and survival.

4.13 Additional References:

1. Naser, S. A. *et al.* Role of ATG16L, NOD2 and IL23R in Crohn's disease pathogenesis. *World J. Gastroenterol.* **18,** 412–24 (2012).

2. Jensen, T. O. *et al.* Macrophage markers in serum and tumor have prognostic impact in American Joint Committee on Cancer stage I/II melanoma. *J. Clin. Oncol.* **27,** 3330–7 (2009).

3. Thomas, N. E. *et al.* Association Between NRAS and BRAF Mutational Status and Melanoma-Specific Survival Among Patients With Higher-Risk Primary Melanoma. *JAMA Oncol.* (2015). doi:10.1001/jamaoncol.2015.0493

4. Begg, C. B. *et al.* Lifetime risk of melanoma in CDKN2A mutation carriers in a population-based sample. *J. Natl. Cancer Inst.* **97,** 1507–15 (2005).

5. Millikan, R. C. *et al.* Polymorphisms in nucleotide excision repair genes and risk of multiple primary melanoma: the Genes Environment and Melanoma Study. *Carcinogenesis* **27,** 610–8 (2006).

6. Jo, Y. K. *et al.* Increased expression of ATG10 in colorectal cancer is associated with lymphovascular invasion and lymph node metastasis. *PLoS One* **7,** e52705 (2012).

7. Songane, M. *et al.* Polymorphisms in autophagy genes and susceptibility to tuberculosis. *PLoS One* **7,** e41618 (2012).

8. Qin, Z. *et al.* Potentially functional polymorphisms in ATG10 are associated with risk of breast cancer in a Chinese population. *Gene* **527,** 491–5 (2013).

9. Yuk, J.-M. *et al.* Vitamin D3 induces autophagy in human monocytes/macrophages via cathelicidin. *Cell Host Microbe* **6,** 231–43 (2009).

10. Viros, A. *et al.* Ultraviolet radiation accelerates BRAF-driven melanomagenesis by targeting TP53. *Nature* **511,** 478–82 (2014).

4.14 Tables:

4.14.1 Table 1: BRAF/ NRAS status by SNP genotype among melanoma cases*

ATG SNP	Genotype	*BRAF/ NRAS* status	OR (95% CI)	p-value	Wald p-value
rs10514231	TT	wt.		1 [Reference]	
rs10514231	CT	*BRAF**	1.36 (0.90-2.06)	0.15	
rs10514231	CC	*BRAF**	1.39 (0.77-2.50)	0.26	0.36
rs10514231	CT	*NRAS**	1.37 (0.79-2.37)	0.27	
rs10514231	CC	*NRAS**	0.81 (0.34-1.92)	0.62	
rs1864182	CC	wt.		1 [Reference]	
rs1864182	AC	*BRAF**	1.79 (1.12-2.87)	**0.02**	
rs1864182	AA	*BRAF**	1.82 (1.06-3.12)	**0.03**	0.09
rs1864182	AC	*NRAS**	1.09 (0.61-1.95)	0.77	
rs1864182	AA	*NRAS**	0.78 (0.38-1.62)	0.51	
rs2241880	GG	wt.		1 [Reference]	
rs2241880	AG	*BRAF**	0.97 (0.62-1.53)	0.90	
rs2241880	AA	*BRAF**	0.90 (0.53-1.53)	0.61	0.94
rs2241880	AG	*NRAS**	1.17 (0.64-2.12)	0.70	
rs2241880	AA	*NRAS**	0.91 (0.45-1.86)	0.80	
rs2245214	TT	wt.		1 [Reference]	
rs2245214	CG	*BRAF**	1.12 (0.75-1.68)	0.57	
rs2245214	CC	*BRAF**	0.89 (0.47-1.69)	0.73	0.57
rs2245214	CG	*NRAS**	0.71 (0.41-1.21)	0.20	
rs2245214	CC	*NRAS**	0.93 (0.43-2.02)	0.86	
rs510432	TT	wt.		1 [Reference]	
rs510432	CT	*BRAF**	1.12 (0.72-1.73)	0.63	
rs510432	CC	*BRAF**	1.03 (0.59-1.78)	0.93	0.38
rs510432	CT	*NRAS**	0.86 (0.47-1.55)	0.61	
rs510432	CC	*NRAS**	1.51 (0.78-2.93)	0.22	

Genotypic model adjusted for age (continuous) sex, and study center. *Bold significance set p= 0.05 Abbreviations: OR, odds ratio; CI, confidence interval

4.14.2 Table 2: Power calculation for detecting oncogene status in 795 melanoma patients

		Minimum detectable per-allele OR	
SNP	Minor allele	*NRAS* vs wt.	*BRAF* vs wt.
rs510432	0.447	1.75	1.53
rs2241880	0.471	1.75	1.53
rs10514231	0.359	1.76	1.55
rs2245214	0.367	1.76	1.55

* sample size of 795 , 80% power and 5% significance level

CHAPTER FIVE:

5.1 Conclusions:

My research offers insight into the interactions between *BRAF*/*NRAS* status, autophagy and melanoma. Despite new treatment options and therapies the long term survival rate for melanoma is stilllow.[1] A common hypothesis has been that the autophagy pathway is upregulated early in tumorigenesis, suppressing cancer progression, but then the pathway is subverted and in later stages promotes tumorigencity.[2,3] However, I have demonstrated at the cellular level that, particularly in late stage melanoma, oncogene status influences autophagy regulation.

My research involves a broad approach. Starting with cell lines from metastatic melanoma patients, moving to melanoma and benign nevus tissues of patients in a pilot study, and finally to a larger study of melanoma patients at the population level, I have examined the complex relationship between melanoma, autophagy and the oncogenes that characterize a majority of melanoma tumors. Using this broad approach, I have identified dependence in metastatic *BRAF** mutant cell lines on autophagy. In addition, in *NRAS** cell lines I identified that these cells have the ability to utilize the autophagy pathway in response to serum starvation or chemical induction but they are not upregulating this process for survival under basal conditions. In addition, the data supporting the concept that *BRAF** melanoma cancer cells are more susceptible to autophagic inhibition suggest that constitutively activated *BRAF** selectively upregulated autophagy. The way in which autophagy is upregulated beyond the activation of the MAPK pathway remains to be elucidated, but this research suggests there is an important connection between the tumorigenicity of *BRAF** melanoma and autophagy. My research also identifies the important but subtle finding that *NRAS** melanoma cells appear less

dependent autophagy for survival at later in later stage. This differential activation of the autophagy pathway suggests that these two oncogenes, which mutually signal through the MAPK pathway, are interacting with other proteins to regulate autophagy. This finding is not counter intuitive as the uncoupling of mutant *NRAS* signaling from *BRAF*, and subsequent preferential signaling through *CRAF* has been previously reported.[4] Still the role of autophagy in *NRAS* mutant melanoma etiology presents an area for additional research.

The relationship between autophagy regulation and melanoma was also investigated in melanoma and control tissue sections showing that high Beclin1 expression was associated with decreased Breslow thickness potentially implying a tumor suppressive effect. LC3 levels correlated with decreased melanoma specific mortality. Of note my research also identified a gender specific association in the melanoma patients. Females in our sample population were more likely to have high Beclin1 expression in their tumor tissue sections. This is a particularly stimulating observation as female melanoma patients exhibit longer survival over male melanoma patients with similar stage.[5] If Beclin1 is acting as a tumor suppressor in melanoma, the higher levels of this protein may also have prognostic implications.

Further, evaluation of UV exposure in melanoma tissue sections identified that certain outdoor activities, indicated consistent UV exposure, and were associated with upregulated autophagy. Melanoma participants reporting blistering sunburn were more likely to have elevated Beclin1 expression while high LC3 was associated with a lack of blistering sunburns. At first this finding may seem counter intuitive for a potential role for Beclin1 as a tumor suppressor. However, previous studies have shown an inverse

correlation between sunburn and death from melanoma.[6] If Beclin1 is acting to suppress tumorigenesis following UV exposure this interaction may help to clarify the complex between relationships between sun exposure and melanoma progression.

Interestingly, Beclin1 expression was not associated with *NRAS* mutant or *BRAF* mutant status in our analysis. While there may be no direct relationship between Beclin1 expression and these oncogenes, another possible reason I did not identify a relationship between Beclin1 expression and *BRAF* or *NRAS* status might be due to low statistical power. We did see a borderline significance with the melanoma histological subtype, superficial spreading melanoma, that is more commonly associated with *BRAF* mutant melanoma but additional investigations will be necessary to clarify this relationship.

LC3 expression is generally accepted as a proxy marker for the rate of autophagy and, interestingly, in our sample population high LC3 was correlated with decreased melanoma specific mortality. When I examined the interactions between mortality and LC3 compared to the interaction of mortality with either Beclin1 or Beclin1 combined with LC3, it was clear that LC3 protein expression alone in this model is associated with decreased mortality. As punctate LC3 in cases was significantly associated with LC3 expression in cases, this potentially indicates an increase in the number of autophagosomes present in these patients. To add to the complexity, while *BRAF* and *NRAS* status were not significantly associated with LC3 expression, high cytosolic LC3 expression correlated with wildtype status in our population genotype. The presence of high levels cytosolic of expression, coupled with the lack of punctate LC in these participants potentially indicates deacetylation of LC3, which has been reported to increase LC3s expression in the cytoplasm.[7] This would indicate that LC3 has been

primed for autophagy induction in the tissue of our non-oncogenic samples but that autophagosome formation may be impaired or inhibited as indicated by the absence of punctate LC3. The reasons for this association remain unclear and require additional investigation. In addition, while not significant, high LC3 expression was also associated with prolonged sun exposure on unprotected skin, boating activities and any outdoor activity sun exposure. This indicates a role for UV exposure with autophagy activation as evidenced by the high LC3 expression. Ultimately my results indicated that autophagy proteins vary by tumor stage and their expression is associated with UV exposure. My research also indicate that the expression of Beclin1 and LC3 could be beneficial as prognostic indicators and that elevated level of these two proteins may support the maintenance of a more indolent melanoma phenotype.

In the final study of this project, I identified SNPs associated with markers of melanoma progression. To date, there are no other studies investigating the impact of *ATG* gene SNPs in melanoma progression and survival. While the association between oncogene status and these SNPs was not significant at a global p level, the linear correlation between *BRAF* status and a SNP in *ATG10* was significant for the allelic model. This analysis could benefit from additional studies using increased sample size to ensure sufficient power in order to adequately evaluate this correlation. In addition, I identified three *ATG* SNPs (rs2241880, rs510432, rs2245214) that were associated with prognostic indictors in melanoma. The SNP in ATG16L (rs2241880) creates a cleavage site that results in the degradation of ATG16L and an overall decrease in the rate of autophagy. This variant was associated with better prognostic indicators including Breslow thickness, decreased stage and a younger age of melanoma diagnosis. This may

indicate that reduced levels of ATG16L are associated with less aggressive melanoma. In addition, two other SNPs in another autophagy related gene *ATG5* (rs2245214 and rs510432) were associated with increased stage of melanoma. Of particular interest, the rs510432 SNP located in the ATG5 promoter has been associated with an increased rate of autophagy.[8] This data would appear to suggest that increased levels of ATG, potentially resulting in increased rates of autophagy, is associated with more aggressive stage in melanoma. The apparent contrary nature of these two findings implies that, beyond the impact of these genetic factors that could be modulating disease severity, other factors are regulating autophagy potentially including UV exposure. Throughout this study, several important interactions have been elucidated implicating the interactions between oncogenes and the autophagy pathway as impacting melanoma progression and survival. Overall, my results support the literature indicating the dual role of autophagy, acting tumor suppressive in earlier stages and tumor promoting, particularly in mutant *BRAF* melanomas as the tumor progresses. Autophagy, as represented by the proxy marker LC3, is upregulated in mutant BRAF melanoma cells compared to *NRAS*. This is also consistent with high levels of autophagy being associated with late stage *BRAF* tumor cells.

As shown at the individual level in melanoma tissue sections, and supported by the literature, high levels of autophagy are associated with earlier prognostic indictors suggesting that autophagy may be tumor suppressive in earlier stages of melanoma development. This is particularly true as Beclin1, which was associated with many positive prognostic indicators in my study, has tumor suppressive roles in many cancers.

Finally at the population level my results indicated that decreased lifelong autophagy, resulting from the degradation of an important autophagy related protein (Atg16L), was associated with better prognosis, while increased autophagy, resulting from upregulation of the promoter of an important ATG gene (ATG5), was associated with increased melanoma stage.

Overall my results indicate that dysregulation of autophagy can impact melanoma progression.

5.2 Future studies:

My research has broad implications for melanoma and cancer research as a whole. With a demonstrated but context specific impact of UV exposure and oncogene status on autophagic regulation, coupled with interactions with SNP in ATG genes potentially modifying melanoma progression and survival, this data builds knowledge applicable to the many autophagy associated diseases. This knowledge suggests several future directions.

The differential activation of the autophagy pathway suggests that these two oncogenes, *BRAF* and *NRAS*, which mutually signal through the MAPK pathway, are interacting with other proteins to regulate autophagy. This finding is not counter intuitive as the uncoupling of mutant *NRAS* signaling from *BRAF*, and subsequent preferential signaling through *CRAF* has been previously reported.[4] *NRAS* mutant cell lines have the ability to use the autophagy pathway in response to nutrient deprivation but mutant *NRAS* signaling of MAPK preferentially through *CRAF* appears to occur without the support of the autophagy pathway at least under basal condition. The cyclic AMP pathway (cAMP), which is inhibited in *NRAS*- mutant melanomas compared to *BRAF* oncogenic melanomas, has been reported to have a role in autophagy.[4] *BRAF* signaling through cAMP has been reported to upregulate the autophagy initiating protein Beclin 1. Further characterization of this interaction, including the impact of oncogenic *BRAF* on Beclin1 as well as the role of cAMP in autophagy and how these interaction impacts melanoma progression would help to clarify the biological regulation of autophagy. As I have stated the importance of Beclin1 warrants further investigation in melanoma. It has already been reported that the generation of Vitamin D3, following UV exposure, enhances the expression of Beclin-1 resulting in increased autophagy. [9] This interaction between UV exposure and a potential tumor suppressive role for Beclin1 could be characterized using clinical markers for sun exposure including serum Vitamin D levels and histopathological data from patients whose *BRAF NRAS* status has been established.

In addition, there is data implicating high rates of autophagy as a mechanism of acquired resistance to *BRAF* mutant inhibitors including vemurafenib.[10] This data supports the need for clinical studies in which autophagy inhibition is combined with

oncogene specific therapies in order to overcome resistance. However the effect of these combinations on different stages of melanoma is not clear. As our data have shown there remains a complex balance between the tumor suppressive effects of autophagy, particularly by Beclin 1, and the tumorigenic metabolic support provide by this pathway. Functional studies are needed in order to further characterize the anticipated outcome of these combination therapies. The use of cell lines to characterize these interactions further would allow for some clarification of these relationships. Ultimately, the use of animal models would be beneficial for determining how the modulations of these proteins, as well as the impact of UV exposure, has on autophagy and melanoma progression and would allow for refinement in our understanding of these complex systems.

In addition, in order to further investigate the correlations we identified in this study, autophagy SNPs should be functionally evaluated and it should be established what, if any linkage disequilibrium is present between these and other SNPs. At the present time, many novel and exciting therapies are in clinical trials and some, including CTLA-4 and PD-1/PD-L1 blockade, are showing promise for durable impact on survival and ATG16L and ATG5 have both been shown to impact cytokine production in disease.[8,11–13] It is also important to note that focused my investigation on 5 SNPs in 3 genes. There are currently 38 *ATG* genes identified and the impact of variants in any of the other critical autophagy genes has not been established for melanoma progression or survival. Research characterizing deep sequencing and epigenetic changes including methylation patterns, could help to characterize the impact of the SNPs identified in this research. Finally, functional studies designed to evaluate the impact of the SNPs investigated in

this study as a well other *ATG* gene SNPs on cytokines production and autophagy regulation will provide insights into the relationship between autophagy and melanoma progression.

5.3 Overall conclusions and perspectives:

This dissertation has presented data suggesting that the relationships between autophagy, melanoma and oncogene status are complex and that careful examination of these interactions will be pivotal in the successful application of autophagy inhibitors for melanoma co-therapy. In particular, the dependence of *BRAF* mutant cell lines on autophagy to suppress apoptosis as well as the relationship between SNPs and melanoma clinical characteristics has important implications for understanding the etiology and progression of this disease. Factors impacting the rate of autophagy including SNPs and UV exposure interact with oncogenic differences to impact the melanoma progression and survival.

These findings have important repercussions in beyond melanoma. First, there are other diseases where the autophagy pathway plays a critical role including in cardiovascular, neurodegenerative, infectious, and metabolic diseases and the data present in this research may inform biological mechanisms in those diseases as well.

In addition, epidemiological studies can be used to identify unique interactions between independent and complex variables, including autophagy and UV exposure, in order to provide insights into melanoma. The use of large population studies when

properly modelled, allows for the statistical power to examine interactions between multiple SNPs, exposure variables and clinical characteristics.

Importantly, these results have implications for the interaction of the autophagy pathway in disease, particularly, where oncogenes have a critical developmental role. Overall, this data indicates that while *BRAF* cells may be dependent on autophagy for survival, and that the expression of Beclin1 and LC3 may support a more indolent melanoma phenotype. This suggests that the role of autophagy, as well as its interactions with other mitigating factors, should be carefully considered before inhibition of autophagy is used universally in melanoma treatment.

5.4 References

1. American Cancer Society Facts and Statistics 2015 | Research | American Cancer Society. at <http://www.cancer.org/research/cancerfactsstatistics/cancerfactsfigures2015/>

2. Eskelinen, E.-L. The dual role of autophagy in cancer. *Curr. Opin. Pharmacol.* **11,** 294–300 (2011).

3. Yang, Z. J., Chee, C. E., Huang, S. & Sinicrope, F. A. The role of autophagy in cancer: therapeutic implications. *Mol. Cancer Ther.* **10,** 1533–41 (2011).

4. Fedorenko, I. V, Gibney, G. T. & Smalley, K. S. M. NRAS mutant melanoma: biological behavior and future strategies for therapeutic management. *Oncogene* **32,** 3009–18 (2013).

5. Joosse, A. *et al.* Gender differences in melanoma survival: female patients have a decreased risk of metastasis. *J. Invest. Dermatol.* **131,** 719–26 (2011).

6. Berwick, M. *et al.* Sun exposure and mortality from melanoma. *J. Natl. Cancer Inst.* **97,** 195–9 (2005).

7. Huang, R. *et al.* Deacetylation of Nuclear LC3 Drives Autophagy Initiation under Starvation. *Mol. Cell* **57,** 456–66 (2015).

8. Martin, L. J. *et al.* Functional variant in the autophagy-related 5 gene promotor is associated with childhood asthma. *PLoS One* **7,** e33454 (2012).

9. Yuk, J.-M. *et al.* Vitamin D3 induces autophagy in human monocytes/macrophages via cathelicidin. *Cell Host Microbe* **6,** 231–43 (2009).

10. Ahn, J.-H., Han, B.-I. & Lee, M. Induction of Resistance to BRAF Inhibitor Is Associated with the Inability of Spry2 to Inhibit BRAF-V600E Activity in BRAF Mutant Cells. *Biomol. Ther. (Seoul).* **23,** 320–6 (2015).

11. Ott, P. A., Hodi, F. S. & Robert, C. CTLA-4 and PD-1/PD-L1 blockade: new immunotherapeutic modalities with durable clinical benefit in melanoma patients. *Clin. Cancer Res.* **19,** 5300–9 (2013).

12. López, P., Alonso-Pérez, E., Rodríguez-Carrio, J. & Suárez, A. Influence of Atg5 mutation in SLE depends on functional IL-10 genotype. *PLoS One* **8,** e78756 (2013).

13. Sorbara, M. T. *et al.* The protein ATG16L1 suppresses inflammatory cytokines induced by the intracellular sensors Nod1 and Nod2 in an autophagy-independent manner. *Immunity* **39,** 858–73 (2013).