Modulating autophagy for the therapeutic benefit of cutaneous melanoma.

Christopher Sean M^{c}Kee

Thesis submitted in partial fulfilment of the requirements of the regulations for the degree of

Doctor of Philosophy

Newcastle University

Faculty of Medical Sciences

Institute of Cellular Medicine

October 2013

Abstract: Modulating autophagy for the therapeutic benefit of cutaneous melanoma.

Abstract

Cutaneous metastatic melanoma remains largely untreatable, likely due to resistance to apoptosis and/or the activation of pro-survival signalling. Incidence continues to increase highlighting the urgent need for more effective treatment strategies. Many chemotherapeutic drugs induce autophagy, the principle lysosomal-mediated mechanism for the degradation/recycling of cellular products, as a compensatory mechanism to counteract apoptotic signals leading to the development of autophagy inhibition as a treatment for metastatic cancer. However, how to modulate autophagy for therapeutic benefit remains debatable since recent approaches demonstrate autophagy induction may also result in cell death in some tumour types. In line with the increasing evidence linking autophagy and apoptosis, including the discovery of interactions between anti-apoptotic Bcl-2 proteins and the autophagy regulatory protein Beclin-1, the principle aim of this study was thus to determine how autophagy modulation in metastatic melanoma may be harnessed for clinical benefit. To this aim drugs which activate pro-survival (bortezomib) or cytotoxic (Tetrahydrocannabinol, THC) autophagy were combined with a number of approaches to dissect the role of autophagy during cell death in response to these agents. Results demonstrated that while the anti-apoptotic protein Mcl-1 was able to limit autophagy, its over-riding function was the direct regulation of apoptosis. Furthermore, results confirmed autophagy was required for THC-induced death of melanoma cells, but that neither Beclin-1 nor Ambra 1 were required for autophagy, suggesting non-canonical autophagy activation in response to THC. Finally, THC was shown to promote lysosome membrane permeabilization (LMP) which was required for cell death.

In conclusion, these data suggest THC-induced apoptosis of melanoma cells requires induction of autophagy which, coupled with the activation of LMP that is also dependent on autophagic signalling, suggests a novel link between the processes of autophagy and apoptosis, ultimately translating into novel strategies through which to harness autophagy for the therapeutic benefit of melanoma.

This work is dedicated to

Matthew Preston M^{c}Kee

Aireoimid go mór uainn thú agus ní dhéanfar dearmad go deo ort, slán leat agus go dtuga Dia suaimhneas síoraí duit.

Acknowledgements

This work was funded by a British Skin Foundation.

I would like to thank my main supervisor; Dr Penny Lovat for her continued support, advice and guidance throughout the PhD and without whom I would not have had the opportunity to take part in such research.

I would also like to thank Dr Jane Armstrong for her continued advice and support even after moving to Sunderland University.

Dr Chris Redfern for crucial statistical advice.

Dr Robert Ellis for his support, endless (although often random) facts and general banter.

Mr Tom Ness for his support in providing patient tissues for our entire group throughout the project.

Members of Dermatology for their expertise and help on many occasions.

Dr Guillermo Velasco, my THC supplier and cannabinoid expert.

My family who have supported me throughout my life and without whom I could not have made it this far.

Finally, I would like to acknowledge someone very dear to me who is there for me through thick and thin and shows me how it feels to truly love someone. Emma, I love you and you mean the world to me.

Declaraction

This thesis is submitted for the degree of Doctor of Medicine at Newcastle University. The research was performed in the Deparyment of Dermatological Sciences under the supervision of Dr Penny Lovat as well as Dr Jane Armstrong (University of Sunderland). This thesis is my own work unless otherwise stated within the text. I certify that none of the material offered in this thesis has been previously submitted by me for a degree or any other qualification at this, or any other University.

Table of Contents

List of Tables and Figures

List of Tables

List of Figures

Abbreviations

AJCC - American Joint Committee On Cancer

µl - microlitre

µm - micrometer

µM - micromolar

Akt – Ak Thymoma

Ambra 1 - Activating Molecule In Beclin-1 Regulated Autophagy Protein 1

AMO - Antisense Morpholino Oligonucleotides

ANOVA - Analysis Of Variance

ARAF - V-Raf Murine Sarcoma Viral Oncogene Homolog A1

ASPP - Apoptosis-Stimulating Protein of p53

ATF4 - activating transcription factor 4

Atg - Autophagy Related Protein

β-Gal - β-Galactosidase

BAD - Bcl-2-Associated Death Promoter

Baf - Bafilomycin

Bak - Bcl-2 Homologous Antagonist Killer

Bax - Bcl-2-Associated X protein

BCC - Basal Cell Carcinoma

Bcl-xL - B-Cell Lymphoma Extra-Large

Bcl-2 - B-Cell Lymphoma 2

BECN-1 - Beclin-1

BH3 - Bcl-2 Homology Domain 3

BID - BH3 Interacting-Domain Death Agonist

BIM – Bcl-2 Interacting Mediator of Cell Death

BioGRID - Biological General Repository for Interaction Datasets

Bort - Botrezomib

BP - Base pair

BRAF - v-Raf Murine Sarcoma Viral Oncogene Homolog B1

BSA - Bovine Standard Albumin

CathB - Cathepsin B

CathBi - Cathepsin B Inhibitor

CB1/2 - Cannabinoid Receptor 1/2

CBD - Cannabidiol

CHO - Chinese Hamster Ovary

CHOP - C/EBP homologous protein

CMA - Chaperone Mediated Autophagy

CML - Chronic Myeloid Leukaemia

CNR1 - Cannabinoid Receptor Gene 1

CO_2 - Carbon dioxide

cSCC - Cutaneous Squamous Cell Carcinoma

CRAF - v-Raf Murine Sarcoma Viral Oncogene Homolog C1

Ctrl - Control

CQ - Chloroquine

CytC - Cytochrome C

DAPI - 4’,6-diamidino-2-phenylindole

Deptor - DEP Domain Containing MTOR-Interacting Protein.

DMEM - Dulbecco’s Modified Eagle’s Medium

DMSO - Dimethyl Supfoxide

DNA - Deoxyribonucleic Acid

EDTA - Ethylenediaminetetraacetic Acid

EGFP - Enhanced Green Fluorescent Protein

ELF2 - E74-Like Factor 2

ER - Endoplasmic Reticulum

ERK - Extracellular Signal-Regulated Kinase

FCS - Fetal Calf Serum

FFPE - Formalin-Fixed Paraffin-Embedded

FIP200 - Focal Adhesion Kinase Family Interacting Protein of 200 kD

GβL - G-Protein Beta-Subunit-Like Protein

GeneDB – Gene Database

GFP - Green Fluorescent Protein

GRB - Growth Factor Receptor-Bound Protein

GSPT1 - G1 to S Phase Transition 1

GTP - Guanosine Triphosphate

HCl - Hydrochloric Acid

HCQ - Hydroxychloroquine

Hr - Hour

HRAS - v-Ha-ras Harvey Rat Sarcoma Viral Oncogene Homolog.

IB - Immunoblotting

IHC – Immunohistochemistry

IF - Immunofluorescence

INF- α - Interferon-Alpha

IL2 - Interleukin 2

IGF - Insulin Growth Factor

ISP-1 - Thermozymocidin

ITCH - Itchy E3 Ubiquitin Protein Ligase

JNK- c-Jun N-terminal kinase

kDa - Kilodalton

KIAA0226 - Rubicon

KRAS - V-Ki-ras2 Kirsten Rat Sarcoma Viral Oncogene Homolog

LAMP - Lysosomal Associated Membrane Protein

LC3 - Microtubule-Associated Protein Light Chain 3

LMP - Lysosome Membrane Permeabilization

MAPK - Mitogen-Activated Protein Kinase

mAtg - Mammalian Autophagy Gene

MC1R - Melanocortin 1 Receptor Mcl-1 - Myeloid Cell Leukemia 1

MEK - Mitogen Activated Protein Kinase Kinase

Mitf - Microphthalmia-Associated Transcription Factor

ml - Millilitre

mM - Millimolar

1^0Mel - Primary Melanocyte

mRNA - Messenger Ribonucleic Acid

mTORC1 - Mammalian Target Of Rapamycin 1

MTS - (3-(4,5-Dimethylthiazol-2-yl)-5-(3-Carboxymethoxyphenyl)-2-(4-Sulfophenyl)-2H-Tetrazolium)

MTT - (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide, a Yellow Tetrazole)

NaCl - Sodium Chloride

NaF - Sodium Fluoride

NF-κB - Nuclear Factor Kappa-Light-Chain-Enhancer Of Activated B Cells

NOXA - Phorbol-12-Myristate-13-Acetate-Induced Protein 1

NRAS - Neuroblastoma Rat Sarcoma Viral Oncogene Homolog

p53 - Protein 53

PAS - Pre-Autophagosome Structure

PBS - Phosphate Buffered Saline

PBS/T - Phosphate Buffered Saline and Tween

PCR - Polymerase Chain Reaction

PE - Phosphatidylethanolamine

PEST - Proline, Glutamic Acid, Serine and Threonine (T) Domain

PI3K - Phosphatidylinositol 3-Kinase

PIKK - Phosphatidylinositol 3-Kinase-Related Kinase

PIP3 - Phosphatidylinositol 3-Phosphate

PRAS40 - Proline-Rich Akt/PKB Substrate 40 kDa

PRR5 – Psudo-Response-Regulator 5

PTEN - Phosphatase And Tensin Homolog

PTOV1 - Prostate Tumor Overexpressed 1

PUMA - p53 Upregulated Modulator of Apoptosis

RAF - V-Raf Murine Sarcoma Viral Oncogene Homolog

Raptor - Regulatory-Associated Protein of mTOR

RAS - Rat Sarcoma

Rap - Rapamycin

RFP - Red Fluorecent Protein

RGP – Radial Growth Phase

Rictor - Rapamycin-Insensitive Companion of mTOR

RNAi – Ribonucleic Acid Interfering

ROS - Reactive Oxygen Species

RTK – Receptor Tyrosine Kinase

RT-PCR – Reverse Transcription PCR

SD - Standard Deviation

SDS - Sodium Dodecyl Sulfate

SEH1L - Nup107-160 Cubcomplex Subunit SEH1 Like

SEM - Standard Error Of The Mean

Shc - Src Homology 2 Domain Containing

shRNA - Short-Hairpin Ribonucleic Acid

Sin1 - Short Integument 1

siRNA - Short Interfering Ribonucleic Acid

SMC3 - Structural Maintenance of Chromosomes

SOS - Son of Sevenless

TGF-β - Transforming Growth Factor B

THC - Tetrahydrocannabinol

TNFAIP3 – Tumour Necrosis Factor Alpha Induced Proteins

TP53P2 - Tumor Protein 53-Induced Nuclear Protein 2

TRB3 - Tribbles 3

TRP - Tyrosinase Telated Protein

ULK1/2 - Unc-51-Like Kinase 1/2

UV - Ultraviolet

UV-R - Ultraviolet Radiation

UVRAG - Ultraviolet Radiation Resistance-Associated Gene Protein

Vps34 - Vacuolar Protein Sorting 34

VGP - Vertical Growth Phase

WT - Wild-Type

XTT - (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide)

ZVAD- Benzyloxycarbonyl-V-A-D(OMe)-Fluoromethylketone)

ZWINT - ZW10 Interacting Kinetochore Protein

Chapter 1: Introduction

Chapter 1: Introduction

Table of Contents

1.1: Melanogenesis

In day to day activities our skin is exposed to high levels of ultraviolet radiation (UV-R), a major cause of DNA damage and a proven factor in skin cancer development (Thompson, Scolyer *et al*. 2005). In order to reduce the damaging effects of UV-R exposure a process known as melanogenesis is required in which photoprotective melanins are produced within melanocytes. These cells are pigment producing cells specialised for this purpose which are located in the basal layer of the epidermis known as the stratum basale (Fig. 1.1) (Costin and Hearing 2007). These cells function primarily to provide photoprotection for the skin against exposure to ultraviolet radiation.

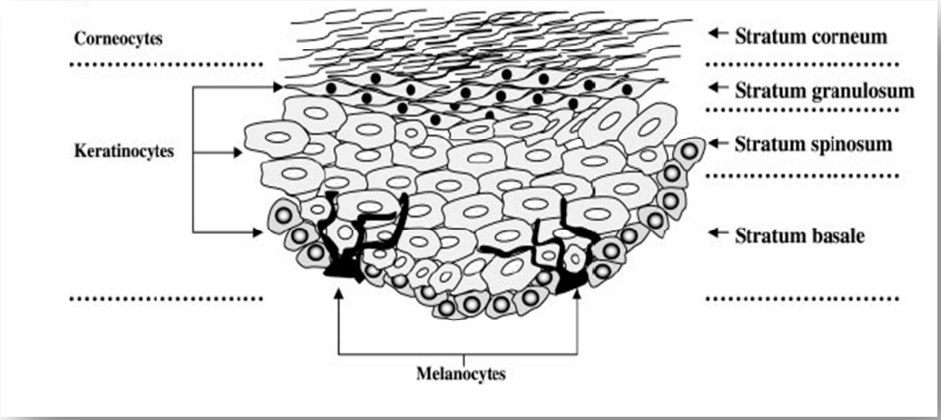

Figure 1.1: The location of melanocytes within the epidermis (Costin and Hearing 2007). Melanocytes are located within the stratum basale of the epidermis where they function to produce melanin and transport melanin containing melanosomes to adjacent keratinocytes via dendritic extensions.

Melanocytes are derived from highly migratory embryonic cells of the neural crest arising during gastrulation, an early stage of embryogenesis where the embryo is reorganised to form three germ layers; the ectoderm, mesoderm and endoderm (Erickson and Reedy 1998). Neural crest cells are pluripotent cells derived between the surface ectoderm and the neural plate, which give rise to many cell types including neurons, glial cells, adrenal medulla, cardiac cells and craniofacial tissue (Lin and Fisher 2007). It is these neural crest cells that specifically give rise to melanoblasts (the melanocyte precursor) which subsequently migrate, proliferate and differentiate en route to their final destination in both the basal epidermis and hair follicles (Lin and Fisher 2007).

Differentiation of melanoblasts into melanocytes requires the receptor tyrosine kinase c-Kit and the basic helix loop helix leucine zipper Microphthlamia-associated transcription factor (Mitf). C-Kit is activated by its associated ligand, Kit-ligand, which leads to activation of RAS/RAF signalling as well as post-translational modification of Mitf (Hemesath, Price *et al*. 1998). Both *c-Kit* and *Mitf* have been shown to be essential for melanocyte development and survival (Steingrimsson, Copeland *et al*. 2004; Vance and Goding 2004), and loss of *Mitf* results in almost complete loss of melanocytes in zebrafish and mice models (Lister, Robertson *et al*. 1999), likely explained by previous studies demonstrating that melanoblast survival involves transcriptional upregulation of anti-apoptotic members of the *Bcl-2* protein family by Mitf (McGill, Horstmann *et al*. 2002). At this point melanoblasts are committed to form either fully differentiated melanocytes or melanocyte stem cells which are found in the bulge region of hair follicles (Fig. 1.2) (Nishimura, Jordan *et al*. 2002). Fully differentiated melanocytes function to produce melanin (of which there are two types, the red/yellow pheomelanin and the brown/black eumelanin (Fig. 1.3)) which is then transferred to keratinocytes.

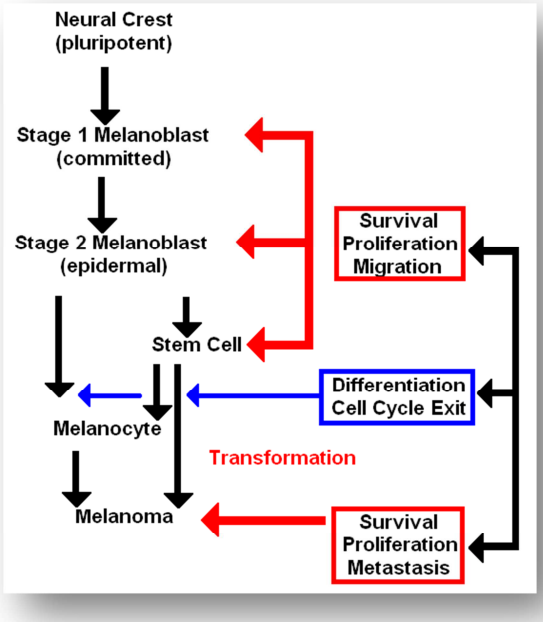

Figure 1.2: Development pathway of melanocytes and melanoma (Goding 2007). Pluripotent neural crest cells develop to form the precursor cells of melanocytes known as melanoblasts. These melanoblasts become committed to form both melanocytes and melanocyte stem cells and are thus lineage restricted. When fully differentiated, melanocytes function to produce melanin which is transferred to keratinocytes to protect against UV-induced DNA damage.

Melanin is derived from the same amino acid precursor, tyrosine, which undergoes hydroxylation to form dopaquinone (Land and Riley 2000). It is from dopaquinone that the eumelanin and pheomelanin pathways diverge, with the G-protein-coupled melanocortin 1 receptor (MC1R) being implicated in the switch between eumlanin and pheomelanin production. Eumelanogenesis is the process for producing eumelanin from dopaquinone and involves tyrosinase related proteins 1 and 2 (TRP 1 and TRP2). Pheomelanin is derived from the conjugation (by the thiol-containing cysteine) of glutathione rendering melanin much more photolabile, and often resulting in the production of hydrogen peroxide, superoxide, and hydroxyl radicals, all of which cause oxidative stress and possible DNA damage (Land and Riley 2000). These melanins are then packaged into melanosomes (a lysosome-like structure) and exported into adjacent keratinocytes via dendritic extensions (Fig. 1.1), with the keratinocytes using a 7 transmembrane receptor known as protease-activated receptor-1 (PAR2) for this process (Boissy 2003). This exportation of melanosomes to keratinocytes thus forms the basis for skin and hair pigmentation. In humans, pigmentation acts primarily to protect the skin from UV-R induced DNA damage and highly pigmented skin has been shown to be protected from carcinogenesis by a factor of 500-1000 times compared to that of skin with little pigmentation (Kollias, Sayre *et al*. 1991). Furthermore, melanocytes are able to respond to UV-R by increasing the amount of pigment manufactured in melanosomes as well as the number of melanosomes exported to adjacent keratinocytes and by the activation of many keratinocyte-to-melanocyte signalling pathways. Once inside keratinocytes, melanosomes are distributed and positioned over the 'sun exposed' side of the nucleus in response to UV-R exposure (Boissy 2003). However, rather than acting as a UV block, melanin likely acts as a sink for reactive oxygen species (ROS), which would otherwise lead to DNA damage (Rozanowska, Sarna *et al*. 1999).

Despite extensive preventative measures being in place to stop mutations occurring within melanocytes, occasionally atypical melanocytes develop. Atypical melanocytes are hyper-proliferative, often due to mutations in RAS/RAF signalling, and are able to escape from their usual location within the epidermis by overcoming their regulation by surrounding keratinocytes. The clustering of these cells within the same location leads to the formation of a benign naevus, more commonly known as a mole.

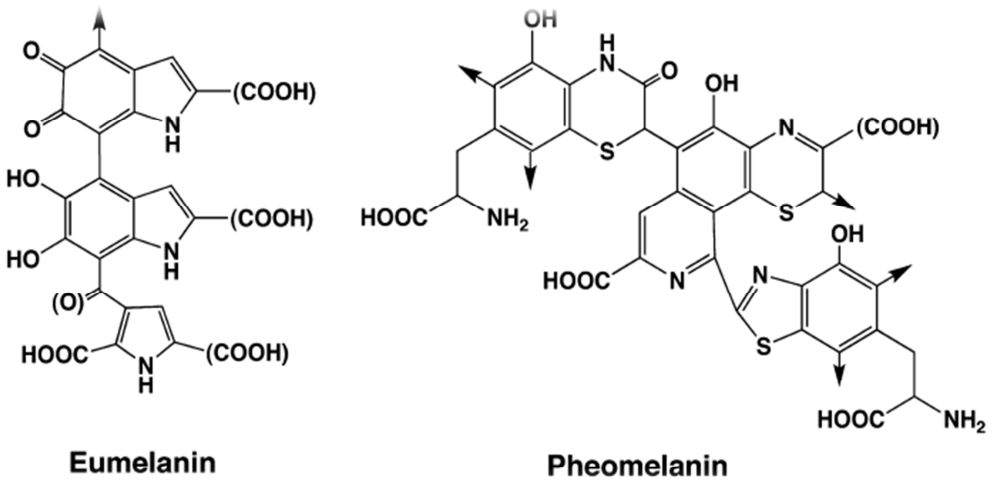

Figure 1.3: Structures of Eumelanin and Pheomelanin (Arrows represent sites for attachment to other units) (Ito and Wakamatsu 2008).

1.2: Cutaneous Melanoma

Cutaneous melanoma arises from the malignant transformation of melanocytes derived from either melanocytic naevi (Figure 1.4) or from previously normal looking skin. A stepwise model for melanoma development has been suggested in which a tumour arises from a cutaneous melanocyte to become a naevus and then a dysplastic naevus, which later develops into melanoma and eventually invasive disease. However, development of melanoma from a naevus only accounts for a quarter of cases, as roughly three quarters of melanomas develop in normal skin (Bevona, Goggins *et al*. 2003), suggesting melanoma may arise from melanocyte stem cells within the skin, (Grichnik 2008). Nevertheless, to date there is no direct evidence to support this theory; a specific stem cell type involved in transformation to melanoma has yet to be defined.

Melanoma is classified as a neuroectodermal tumour which is based on the lineage of melanocytes from neural crest cells. Although one of the rarer types of skin cancer, second to Merkel cell carcinoma only (Miller and Rabkin 1999), melanoma is the most aggressive and remains largely untreatable. With melanoma cases increasing by 3-8% per year since the mid 1960s for people of European decent (Thompson, Scolyer *et al*. 2005), melanoma is hence a major public health problem. Increased incidence of melanoma has been linked to environmental exposure to UV-R, where the highest age-standardised incidence and mortality rates are in countries with a higher UV index and a large Caucasian population (e.g. Australia and the USA) (Thompson, Scolyer *et al*. 2005). Additionally, genetic susceptibility of individuals, as well as the presence of many atypical or benign naevi, has been strongly linked with increased risk of melanoma (Thompson, Scolyer *et al*. 2005).

In the traditionally proposed model, it is suggested that melanoma development is classified in different stages arising from atypical melanocytes forming benign naevi and resulting in pre-malignant melanoma (Fig. 1.4). A radial growth phase then occurs due to further mutations within these naevi resulting in a radial growth pattern, primarily confined to the epidermis (Chin, Merlino *et al*. 1998). Malignant melanoma (Fig. 1.4 and Fig. 1.5) however, undergoes a vertical growth pattern resulting in the invasion of the tumour deep into the dermis and the subcutaneous fat layers of the skin (Fig. 1.4) which in many cases leads to further invasion into systemic metastasis (frequently involving the liver, bone, brain, lung and small intestine (Elder 1987)).

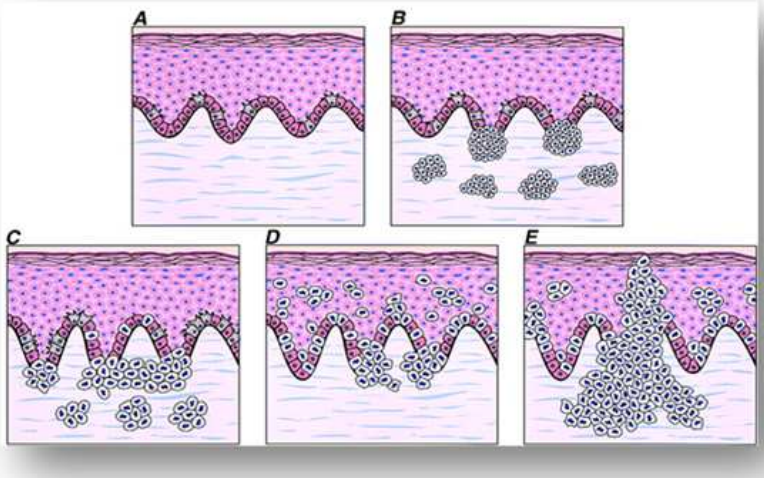

Figure 1.4: The progression of melanocyte transformation to metastatic malignant melanoma (Chin, Merlino et al. 1998). A: Normal melanocytes within the epidermis that are evenly distributed throughout the stratum basale. B: The benign proliferation of melanocytes due to mutations where they cluster together, forming benign naevi. C: Melanocyte dysplasia with many large atypical melanocytes. D: Radial growth phase (RGP) melanoma occurring due to further mutations. E: Metastatic malignant melanoma known as vertical growth phase (VGP).

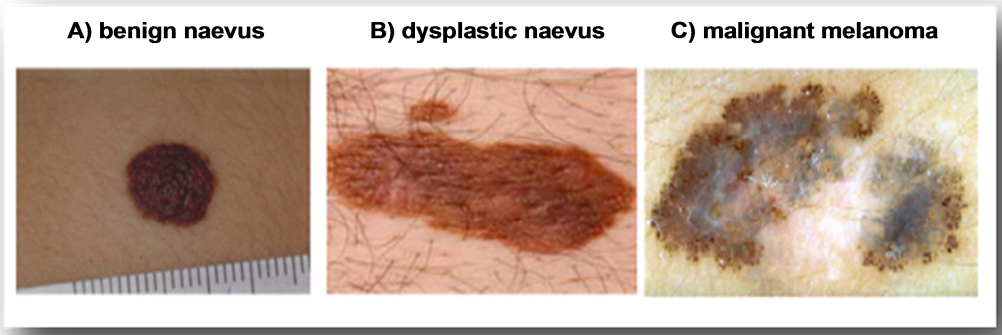

Figure 1.5: Progression of melanoma development from a benign naevus to malignant melanoma. A) A common benign naevus or mole (from Sydney Melanoma Diagnostic Centre, http://www.melanoma.net.au/Melanoma/melanoma.htm). B) A dysplastic naevus. (Cancer Research UK, http://www.cancerresearchuk.org) C) A malignant melanoma lesion with distinctive change in size and colour. (Melanoma Diagnostic Centre).

Prior to 2002 mainly two methods of diagnosis (one known as the Clark's classification system and the other described by Breslow) were used to classify the pathology of melanoma. Breslow's method measures tumour thickness from the granular cell layer to the peak depth of tumour invasion (Fig. 1.6) and is still widely used in diagnostic procedures while Clark's method, more recently abolished, considered the anatomical level of tumour invasiveness (Chin, Merlino *et al*. 1998) (Fig. 1.6). A recently updated staging by the American Joint Committee on cancer (AJCC) incorporating Breslow depth together with mitotic rate, micro-metastases and ulceration as well as metastatic spread now forms the basis of current melanoma diagnosis and staging. (Balch, Gershenwald *et al*. 2009).

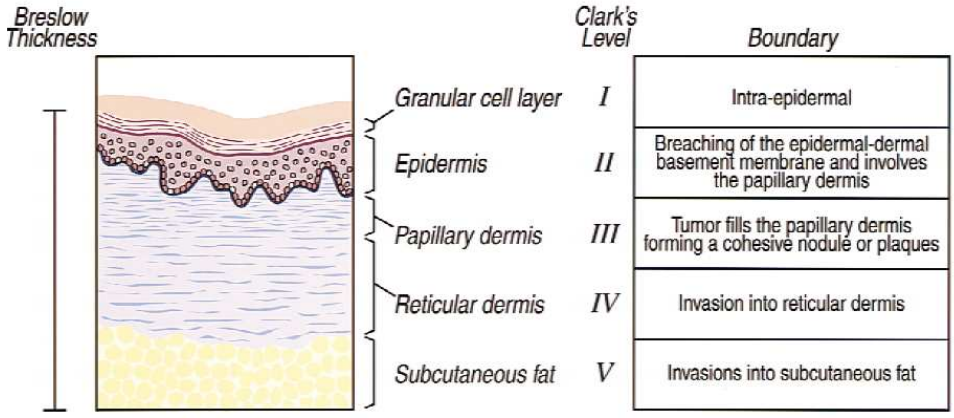

Figure 1.6: Pathological classification of melanoma (Chin, Merlino et al. 1998). The Clark's classification system (where anatomical invasion of melanoma in the skin is used rated from levels I-V) along with Breslow thickness (which measures the absolute thickness of melanoma invasion) are commonly used in the prognosis of melanoma.

While the prognosis for melanoma patients with early stage disease (Stage I and II) is highly favourable, the prognosis for patients with late stage disease (where either metastases to regional lymph nodes or systemic metastases have occurred) is extremely poor with associated 2 year survival rates of 18-40% (Balch, Gershenwald *et al*. 2009). Typically, systemic therapeutic treatment has been the most widely utilized approach for treatment of metastatic melanoma (Kasper, D'Hondt *et al*. 2007) where single-agent-therapy with dacarbazine, temozolomide or fotomustine are the most common therapeutic approaches for disseminated melanoma (Atallah and Flaherty 2005). However, despite

combination with chemokines such as interferon-α (INF- α) and interleukin 2 (IL2) (Kasper, D'Hondt *et al*. 2007), monoclonal antibodies, cancer-specific vaccines or immune adjuvants, response rates to any treatment combination remain poor with a median survival rate of 7.5 months. Poor response to current treatment regimes likely reflects the resistance of metastatic melanoma tumours to apoptosis, underpinning the serious need for novel and more effective systemic therapies. Recent breakthroughs have lead to the development of novel inhibitors specifically designed to exploit key signalling pathways known to be deregulated in melanoma. More specifically the RAS/RAF/MEK/ERK signalling pathway has been targeted by using a combination of BRAF and MEK inhibitors (Flaherty, Infante *et al*. 2012). These therapies have been designed to target specific patients bearing $BRAF^{V600E}$ mutant tumours providing the greatest therapeutic breakthrough in melanoma for more than 20 years. However, despite promising results within the first few months of treatment ultimately tumour resistance emerges with the long term survival rate for patients hence, ultimately unchanged (Flaherty, Infante et al. 2012; Nazarian, Shi et al. 2010; Poulikakos, Persaud et al. 2011; Sullivan and Flaherty 2013; Wagle, Emery et al. 2011). Additionally, the specificity of BRAF inhibitors to $BRAF^{V600E}$ mutant tumours limits their usage with still no effective therapies for BRAF wild-type or NRAS mutant tumours and highlighting the serious need for alternative and more effective systemic therapies.

1.3: Oncogenic BRAF signalling in melanoma

Progress in understanding the underlying molecular basis for melanoma pathogenesis has led to the development of numerous molecular based strategies for metastatic melanoma (Klinac, Gray *et al*. 2013). Increased knowledge of the underlying molecular biology of melanoma has shown that its development is strongly associated with the subversion of the pathways involved in development and proliferation of normal melanocytes. The two main pathways regulated by receptor tyrosine kinases are the PI3K/Akt/mTOR pathway and the RAS/RAF/MEK/ERK pathway. The RAS/RAF/MEK/ERK signalling pathway is a major stimulator of melanocyte proliferation which is often required to react to changes in conditions of the extracellular environment (Fig. 1.7) (Hocker, Singh *et al*. 2008). Under normal conditions this process is highly regulated and controlled, however (as is the case in many cancer types) activating mutations occur in key members of this cascade resulting in the loss of proliferative control, including those frequently mutated in melanoma (Garnett and Marais 2004). Of particular note are the RAF family of serine/threonine protein kinases, of which there are three members; ARAF, BRAF and CRAF. Additionally, the RAS subfamily of GTPases consisting of HRAS, KRAS and NRAS, has been implicated in many human cancers (Bos 1989). Activating mutations in BRAF, as well as NRAS which lies upstream of this pathway, have been found in 50-70% and 20% of melanoma cases respectively, and both *BRAF* and *NRAS* are oncogenes implicated in melanoma development (Davies, Bignell *et al*. 2002; Dong, Phelps *et al*. 2003; Uong and Zon 2009). The presence of both *NRAS* and *BRAF* mutation in the same tumour is rare and it is thought that cellular transformation occurs through a common mechanism involving the RAS/RAF/MEK/ERK signalling pathway (Sensi, Nicolini *et al*. 2006). The most common mutation in BRAF is the mutagenesis of a valine to a glutamic acid at the 600th residue (BRAF^{V600E}) resulting in a 700-fold hyper-activation of BRAF kinase activity allowing for the enhanced survival and division properties of melanoma (Davies, Bignell *et al*. 2002). Other mutations in BRAF have also been identified in melanoma including BRAF^{V600K} and BRAF^{V600D} present in 16.3% and 3.4% of melanoma cases bearing a BRAF mutation, respectively (Davies, Bignell *et al*. 2002; Rubinstein, Sznol *et al*. 2010). Although mutated BRAF has been shown to protect melanoma cells from apoptosis and is very likely required for development of melanoma, mutated BRAF is present in the majority of benign naevi and has been shown

to induce senescence after an 'initial burst' of proliferation when expressed in normal melanocytes (Pollock, Harper et al. 2003; Michaloglou, Vredeveld et al. 2005), suggesting BRAF is not the only factor required. The most convincing evidence for melanoma development being driven by mutant BRAF was described by Dhomen *et al* (Dhomen, Reis-Filho *et al*. 2009) in which an inducible *BRAF*^{V600E} transgenic mouse model was used to model melanoma development. Melanocyte-specific expression of this oncogene resulted in several melanocytic lesions such as benign neavi and areas of skin hyperpigmentation, as well as melanoma (Dhomen, Reis-Filho *et al*. 2009). However, the authors suggested that melanoma development only occurs following the acquisition of additional mutations. This has been validated experimentally using a zebrafish model to show that while BRAF activation leads to development of benign naevi, further progression to melanoma required inactivation of p53 (Patton, Widlund *et al*. 2005). As such, other molecular factors have been implicated in melanoma development in animal models including the inhibition of p53 and regulation of Mitf expression by activating transcription factor 2 (ATF2) (Patton, Widlund *et al*. 2005; Shah, Bhoumik *et al*. 2010). Based on RAS signalling, it has been suggested that a multistep process occurs in the development of malignancies with three distinctive events; low level oncogene activation to stimulate proliferation, the spontaneous upregulation of activated RAS levels to induce senescence, followed by evasion of senescence by other molecular events resulting in tumour progression (Sarkisian, Keister *et al*. 2007). It is therefore very likely that a similar multistep process is involved in melanoma with activating mutations in BRAF or NRAS. In support of this hypothesis it was reported that progression from benign melanocytic hyperplasia to metastatic melanoma could only occur in the presence of both *BRAF*^{V600E} and silenced *PTEN*, whereas BRAF^{V600E} expression alone was insufficient to promote melanoma development (Dankort, Curley *et al*. 2009).

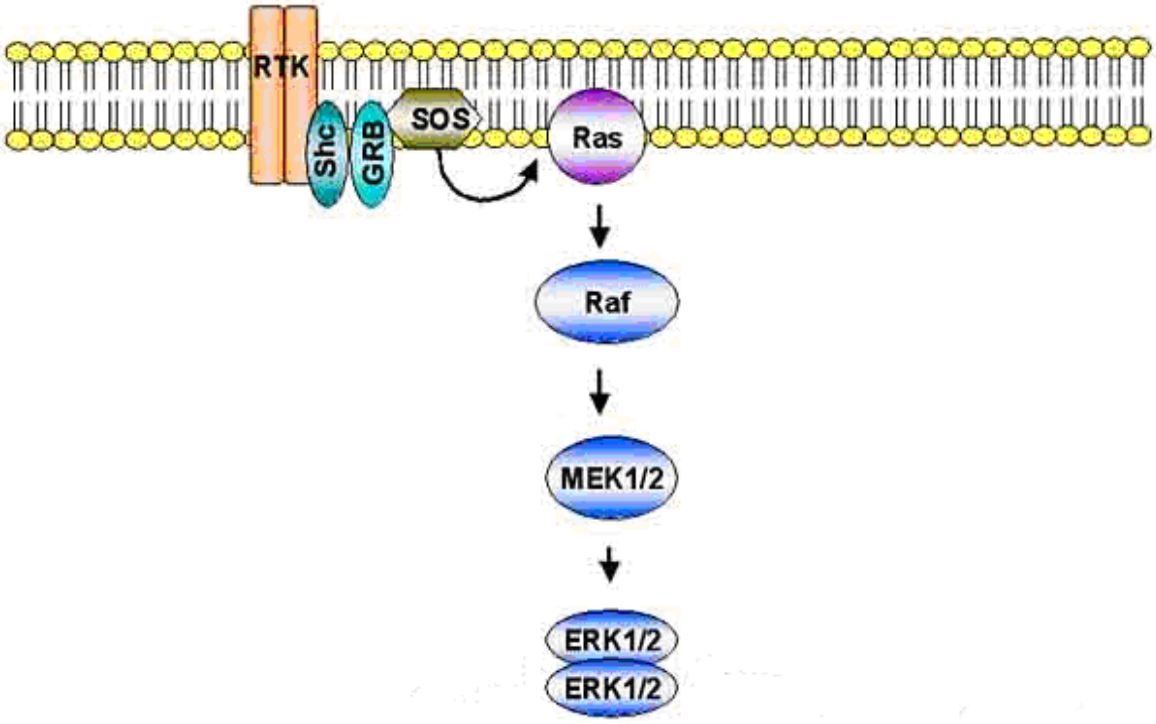

Figure 1.7: RAF/MEK/ERK signalling cascade (Image adapted from (Chin and D'Mello 2005)). Receptor tyrosine kinases (e.g. fibroblast growth factor, epidermal growth factor, and insulin receptors) are stimulated by an extracellular stimulus such as a ligand resulting in activation of the membrane bound Ras-GTPase through the action of the guanine nucleotide exchange factor SOS. RAS then activates RAF, MEK and ERK to change cellular gene expression.

The importance of BRAF signalling in melanoma progression in many tumours has lead to development of specific BRAF targeted therapies now used as adjuvant therapy in the treatment of patients bearing $BRAF^{V600E}$ mutant tumours, however mechanisms of resistance to BRAF specific therapies have been proposed. Several studies have attributed this resistance to several possible mechanisms which ultimately reults in reactivation of the MAPK pathway. These mechanisms include; upregulation of NRAS through activating mutations (Q61K/R) (Nazarian, Shi et al. 2010); activation of MEK1 by the C121S mutation (Wagle, Emery et al. 2011); alternative splicing of the BRAF gene (Poulikakos, Persaud et al. 2011) and increased $BRAF^{V600E}$ gene amplification (Shi, Moriceau et al. 2012). Additionally, more recent findings have suggested that use of BRAF inhibitors may induce high rates of autophagy which may act as a key mechanism of resistance to these drugs (Ahn JH 2013) highlighting the importance of this pathway in therapeutic resistance.

1.4: Autophagy

1.4.1: Autophagy in health and disease

The process of autophagy (literally meaning to eat oneself) was first identified by Clark *et al* in the late 1950's where the formation of autophagosomes was shown in kidney cells of newborn mice by electron microscopy (Clark 1957). It wasn't until 1963 however, that de Duve, who published an article describing the discovery of the lysosome in 1955, coined the word autophagy (Klionsky 2007). Despite its discovery many years ago, the role of autophagy in health and disease has only recently emerged. The main role of autophagy is to maintain cellular energy levels and hence its role in advanced tumours is thought to be a mechansism of maintaining cellular energy and hence survival (Bertout, Patel *et al*. 2008; Zhang, Bosch-Marce *et al*. 2008). However additional evidence has implicated autophagy in a cell death process that is independent of apoptosis (Shimizu, Kanaseki *et al*. 2004; Yu, Alva *et al*. 2004). Inhibition of the pro-survival function of autophagy, or induction of autophagic cell death, is therefore an attractive target for the treatment of many diseases, particularly cancer (Gozuacik and Kimchi 2004; Voss, Senft *et al*. 2010).

Ordinarily, autophagy plays a key physiological role in cells in which the bulk degradation of cytoplasmic macromolecules as well as damaged organelles, such as mitochondria, occurs via the lysosomal system. Three mechanisms have been described; macro-autophagy, micro-autophagy and chaperone-mediated autophagy, which differ in their physiological function, cargo specificity and how the autophagic vesicles (also known as autophagosomes) ultimately transfer their cargo to the lysosomes. Macro-autophagy, termed autophagy from here on, is a highly regulated process which has been shown to be induced during starvation, differentiation, and normal growth control to maintain energy homeostasis and cell survival (Kuma, Hatano et al. 2004; Shintani and Klionsky 2004; Komatsu, Waguri et al. 2005). Autophagy has since been classified by Mizushima as either 'induced' autophagy (which is for production of amino acids under conditions of starvation) or 'basal' autophagy (which is for the constitutive turnover of components of the cytoplasm) (Mizushima 2005; Mizushima 2007). However if autophagy becomes highly accelerated it can promote cell death rather than cell survival, a process known as type II autophagic cell

death. Type II autophagic cell death is easily distinguishable from type I apoptotic cell death (Gozuacik and Kimchi 2004); unlike apoptosis, autophagic cell death does not result in DNA laddering or nuclear fragmentation, with only partial chromatin condensation and the nucleus remaining relatively intact until much later on in the process. An increase in autophagic vesicles and autolysosomes is observed, which does not occur during apoptosis, and caspases (which are an essential component of apoptosis) are not activated during autophagic cell death, further revealing that this takes place independently of apoptosis (Table 1.1). However, whether non-apoptotic cell death is induced by autophagy or simply occurs in the presence of autophagy is still a matter of debate (Kroemer and Levine 2008). To date the most convincing data in support of type II autophagic cell death has involved the prevention of this process by knockdown of key autophagy genes *in vitro* by use of small interfering RNA (siRNA) in which the characteristics of type II autophagic cell death are observed. Lenardo *et al* reported that in L929 fibrosarcoid cells and in U937 monocytoid cells, siRNA knockdown of two essential autophagy genes (Atgs) (*Becn1,* which encodes Beclin-1, or *Atg7*) resulted in blockade of non-apoptotic cell death which had previously been induced as an alternative mechanism of cell death upon caspase 8 inhibition (Yu, Alva *et al*. 2004). Similarly Tsujimoto *et al* reported in 2004 that $Bax^{-/-}Bak^{-/-}$ mouse embryotic fibroblasts undergo non-apoptotic cell death in response to staurosporine or etoposidea, where large-scale autophagic vacuolization was observed (Shimizu, Kanaseki *et al*. 2004). This process is then reduced by knockdown of either *Becn1* or *Atg5* indicating this non-apoptotic cell death is regulated by autophagy (Shimizu, Kanaseki *et al*. 2004). In further support of cell death induced by autophagy, a more recent report by Salazar *et al* showed that cannabinoids (in particular tetrahydrocannabinol (THC)) induced cell death of glioma cells through the stimulation of autophagy by an early endoplasmic reticulum (ER) stress response (Salazar, Carracedo *et al*. 2009), as described in this report as autophagy-mediated cell death. However this process involved stimulation of apoptosis through autophagy rather than autophagic cell death specifically.

Table 1.1: A table detailing a comparison between the two types of programmed cell death. (Gozuacik and Kimchi 2004).

	Type I apoptotic	**Type II autophagic**
Nucleus	Chromatin Condensation	Partial chromatin condensation
	Pyknosis of nucleus	Sometime pyknosis of nucleus
	DNA laddering/nuclear fragmentation	Nucleus intact until late stages
		No DNA laddering
Cytoplasm	Cytoplasmic condensation	Increased autophagic vesicle number
	Ribosome loss from RER	Increased autolysosome number
	Fragmentation of apoptotic bodies	Increased lysosomeal activity
	MOMP	Enlarged Golgi, sometimes ER dilation
	Caspase activation	MOMP may be involved
		Caspase-independent
Cell membrane	Blebbing	Blebbing
Corps clearance	Heterophagy by other cells	Late and occasional heterophagy by other cells
Detection methods	Electron microscopy	Electron microscopy
	Caspase activation tests	LC3 flux assays
	TUNEL staining	Test increased lysosomal activity (Acridine orange)
	Annexin V staining	Test increased long-lived protein degradation
	DNA laddering detection	

1.4.2: Regulation of autophagy

A total of 34 Atg genes have been identified so far in yeast and most of the mammalian orthologues for these have also been identified. To date, 18 of these genes have been shown to be required for the regulation autophagy. Autophagy is characterised by the induction, nucleation, extension and completion of an isolation membrane, or phagophore, to form an autophagosome which ultimately fuses with a lysosome for the degradation of its constituents (Roy and Debnath 2010). The exact origin of the membrane has yet to be completely defined, however recent publications suggest the initial stages of phagophore development occurs at a sub-domain of the endoplasmic reticulum with the formation of a 'cradle' like structure (Hayashi-Nishino, Fujita *et al*. 2009).

The induction of autophagy in mammals occurs via the ULK complex which is composed of FIP200/mAtg13/unc-51-like kinase (ULK). This complex is regulated by the mammalian target of rampamycin (mTOR), a serine/threonine kinase belonging to the phosphatidylinositol 3-kinase-related kinase (PIKK) protein family. mTOR plays a crucial role in maintaining a balance between autophagy and cell growth in response to signals that stimulate autophagy, such as insulin, growth factors (e.g. IGF-1 and IGF-2) and mitogens (Hay and Sonenberg 2004). Two complexes of mTOR have been identified known as mTORC1 (composed of mTOR, GßL, raptor, Deptor and PRAS40) and mTORC2 (composed of mTOR, GßL, rictor, Deptor, Sin1 and PRR5) (Wullschleger, Loewith *et al*. 2006). mTORC1 primarily mediates growth factor signalling in response to the Akt pathway (Hay and Sonenberg 2004) and is involved in the regulation of autophagy. The role of mTORC2 is less clear, although it has been shown to activate the Akt pathway (Sarbassov, Guertin *et al*. 2005) but a role for this protein in autophagy has not been identified. Under normal conditions mTORC1 negatively regulates autophagy by phosphorylating ULK1/2 to prevent autophagy induction (Ganley, Lam *et al*. 2009) (Fig 1.8), however, upon nutrient deprivation it dissociates from the ULK complex. When this occurs ULK1/2 become dephosphorylated resulting in their activation, the phosphorylation of FIP200 and mAtg13 and the initiation of starvation-induced autophagy (Jung, Jun *et al*. 2009). The ULK complex is then localised to the phagophore for the subsequent stages of autophagosome formation.

After autophagy is induced, nucleation of the phagophore occurs, requiring phosphatidylinositol 3-phosphate (PI3P) generation by a class III PI3K complex. This complex

is composed of p150/Vps34/Beclin-1 (Simonsen and Tooze 2009), and recent studies have identified UVRAG, Atg14L and Ambra-1 (activating molecule in Beclin-1 regulated autophagy protein 1) (Liang, Feng et al. 2006; Fimia, Stoykova et al. 2007; Itakura, Kishi et al. 2008; Matsunaga, Saitoh et al. 2009; Zhong, Wang et al. 2009) as binding partners of Beclin-1 which positively regulate its activity in addition to their role in regulating different steps of autophagosome formation and maturation. Elongation occurs after phagophore nucleation and requires two ubiquitin-like systems (Ohsumi 2001; Ohsumi and Mizushima 2004). The first system involves the conjugation of Atg5 to Atg12 via the E1-like activity of Atg7 and the E2-like activity of Atg10. Once this has taken place the Atg5/Atg12 complex binds to Atg16 to form Atg16L, a large multimeric complex which localises to the outer surface of the autophagosome membrane. The second ubiquitin-like system involves cleavage of Atg8 (a ubiquitin-like molecule for which the mammalian homologue is microtubule-associated protein light chain 3 (LC3)) by the protease Atg4. The subsequent exposure of a C-terminal glycine residue is required for conjugation with phosphatidylethanolamine (PE), producing lipidated LC3 (LC3-II) which is necessary for formation of the autophagosome and which can be measured in tissue or cells through detection of the lipidated form of LC3, LC3-II, by immunohistochemistry (IHC) or inmmunoflourescence (IF) (characterised by a punctate staining pattern when LC3-II is localised to the autophagosome), as well as by, immunoblotting (IB) (Kabeya, Mizushima *et al*. 2000; Roy and Debnath 2010). Completion and maturation of the autophagosome membrane is followed by fusion of this membrane structure with a lysosome to form an autophagolysosome, via the action of the lysosomal proteins LAMP1 and LAMP2 as well as Rab7 and UVRAG (Fig 1.8) (Liang, Lee *et al*. 2008).

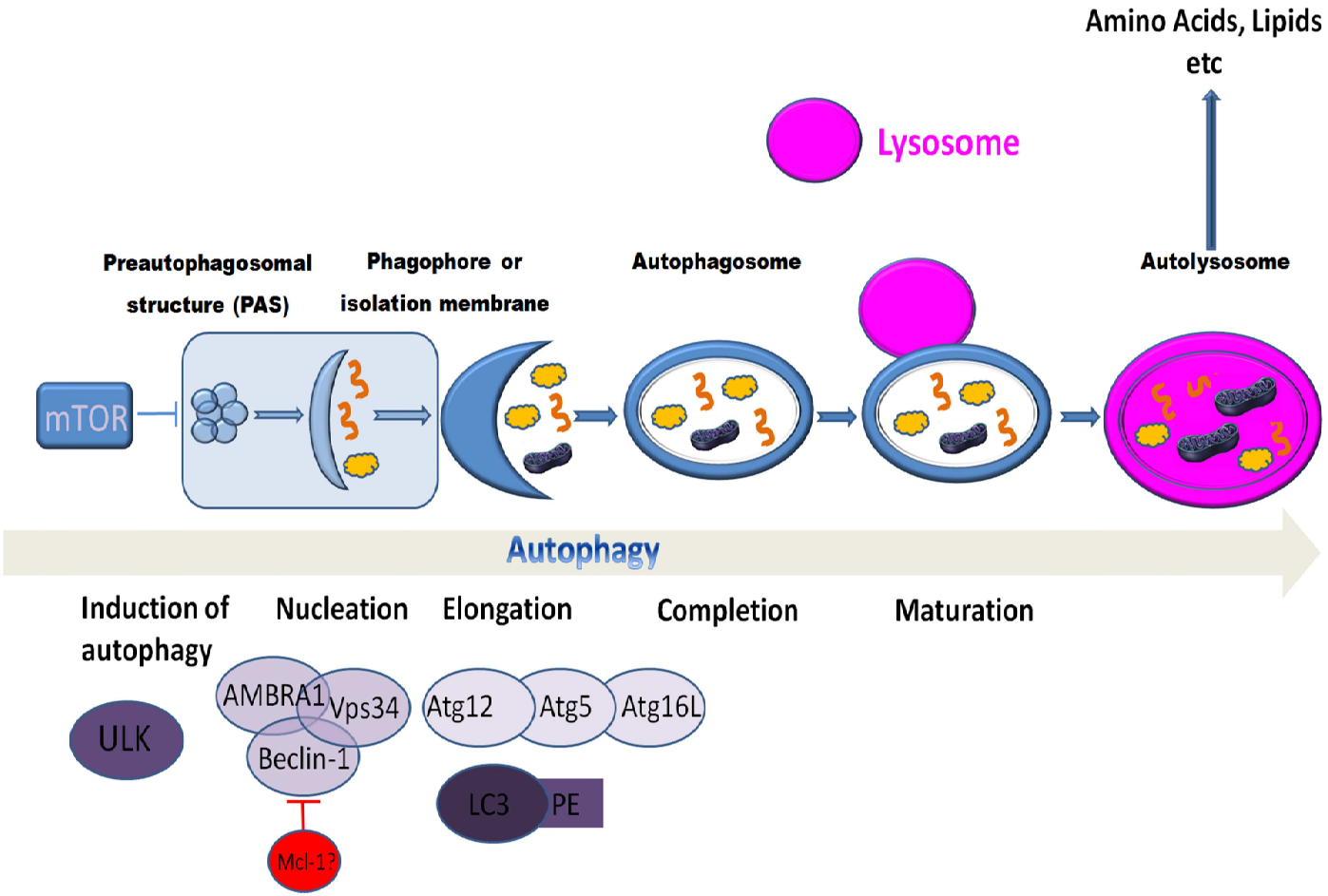

Figure 1.8: Schematic of the autophagy pathway (Roy and Debnath 2010). Autophagy is a complex process involving many steps before the final formation of the autophagolysosomes. These steps are categorized by the induction, nucleation, elongation and completion of an isolation membrane known as a phagophore and the subsequent formation of an autophagosome which eventually fuses with a lysosome to form an autophagolysosome. Induction of autophagy is regulated by the ULK complex. Nucleation is regulated by the Class III PI3K complex. Elongation of the phagophore involves two ubiquitin like complexes; Atg5/Atg12 complex and PE-conjugated Atg8. Completion and maturation are defined by the formation of a completed autophagosome containing the sequestered cargo and its eventual fusion with the lysosome.

1.5: Autophagy in Cancer and Melanoma

Autophagy has been shown to have a paradoxical role in cancer progression, with data supporting both tumour promoting and tumour suppressive functions (Roy and Debnath 2010). Mathew *et al* revealed that, in autophagy-defective cells, an inability to eliminate p62 altered NF-κB regulation and promoted tumourigenesis, suggesting that autophagy suppresses cancer initiation by preventing accumulation of p62 (Mathew, Karp *et al*. 2009). As p62 is often up-regulated in human tumours (Zatloukal, French *et al*. 2007) this points towards a role for defective autophagy in cancer development. Genetic evidence also suggests that the tumour suppressive functions of autophagy act in the early stages of tumour development (Qu, Yu *et al*. 2003; Yue, Jin *et al*. 2003). For example, spontaneous tumour development occurs in *Beclin-1* $^{+}/^{-}$ mutant (Qu, Yu *et al*. 2003; Yue, Jin *et al*. 2003). Conversely, there is evidence that autophagy is used as a survival strategy in established and more advanced tumours, allowing these tumours to maintain an energy supply and survive the harsh microenvironments and stress conditions it encounters in advanced stages (Bertout, Patel *et al*. 2008, Zhang; Bosch-Marce *et al*. 2008). As such, the general opinion is that autophagy suppresses growth early during tumour development but promotes tumour survival at more advanced stages (Figure 1.9, Corazzai, Fimia *et al*). Prevention of the autophagy process for advanced stage cancer therefore represents a viable therapeutic strategy and has included the use of lysosomal inhibitors such hydroxychloroquinone (HCQ), a lysosomotropic drug that raises intralysosomal pH (Poole and Ohkuma 1981) as well as impairing autophagic protein degradation (Amaravadi, Yu *et al*. 2007; Glaumann and Ahlberg 1987). Combined HCQ treatment with several anticancer drugs in preclinical models, is now incorporated into a number of phase I and II clinical trials in tumours associated with high basal levels of autophagy (Garber 2011). However HCQ is nevertheless a non-specific inhibitor of autophagy with both undesirable side-effects as well as off target effects Additionally although responses to HCQ may be effective *in vitro*, this is not always refelected *in vivo* (Swampillai, Salomoni *et al*. 2012) leading to the need for development of more specific autophagy inhibitors. Many autophagy-inducing agents are also currently being used for the treatment of different human cancers, for example Rapamycin which targets and inhibits mTOR as well as 2nd generation mTOR inhibitors (RAD001, CC1-779, and AP23573) (Faivre, Kroemer *et al*. 2006). These chemotherapeutics have shown to result in

significant anti-tumour properties in several cancer models (Faivre, Kroemer *et al*. 2006), though clinical trails of mTOR inhibitors have been generally disappointing, perhaps due to the activation of autophagy and its effect on the maintainance of cell survival. However, cannabinoids, and in particular THC, are able to promote potent cell death mediated by autophagy as a result of ER stress in human glioma cells (Salazar, Carracedo *et al*. 2009), and hence show promise as an alternative approach to exacerbating autophagy to promote tumour cell death.

Evidence for autophagy in melanoma includes the presence of autophagosomes containing melanized melanosomes with 'coarse melanin' in some tumours (Handerson and Pawelek 2003; Lazova, Klump *et al*. 2009) as well as evidence for altered Beclin-1 and LC3 expression in primary melanocytic neoplasms (Miracco, Cevenini *et al*. 2010). P62 expression has also been found to be significantly increased in melanoma compared to naevi (Ellis, *et al*. submitted data to J Invest Dermatol). Furthermore, Atg5 (a key autophagy regulator gene) is often down-regulated in primary melanomas compared to benign nevi, leading to a reduction of basal autophagy as evidenced by a reduced expression of LC3-I/II (Liu, He *et al*. 2013). Liu *et al* also demonstarted in a study of 158 primary melanomas low tumoural Atg5 expression correlated with reduced progression-free survival (Liu, He *et al*. 2013). Additionally, using melanocytes with transduced mutated BRAF, the authors found reducing the expression of Atg5 in these cells promoted proliferation by preventing oncogene-induced senescence suggesting down-regulated Ag5 contributes to tumourigenesis of cutaneous melanoma (Liu, He *et al*. 2013). In metastatic melanoma however, autophagy has been shown to be increased, likely providing these cells with an essential energy source under the nutrient deprived environments encountered (Lazova, Klump *et al*. 2009). Data has also revealed that hyperactivation of BRAF results in induction of autophagy in melanoma through down-regulation of mTOR signalling due to increased phosphorylation of mTOR at serine 2481 (Maddodi, Huang *et al*. 2010), suggesting activated BRAF promotes autophagy. However, Armstrong *et al* reported that although autophagy can be induced beyond basal levels in BRAF wild-type melanoma cells in response to treatment with chemotherapeutic drugs, such agents could not induce autophagy beyond basal levels in melanoma cells bearing mutant BRAF (Armstrong, Corazzari *et al*. 2011), suggesting oncogenic BRAF prevents autophagy induction. Furthermore, use of the BH3 mimetic

ABT737 was able to partially reactivate autophagy in BRAF mutant melanoma cells (Armstrong, Corazzari *et al*. 2011). As ABT737 has previously been shown to activate autophagy through disrupting the interaction between Beclin-1 and anti-apoptotic Bcl-2 protein family members (Maiuri, Criollo *et al*. 2007), these data suggest that resistance to autophagy induction in BRAF mutated melanoma cells is mediated, at least in part, by inhibition of Beclin-1 function.

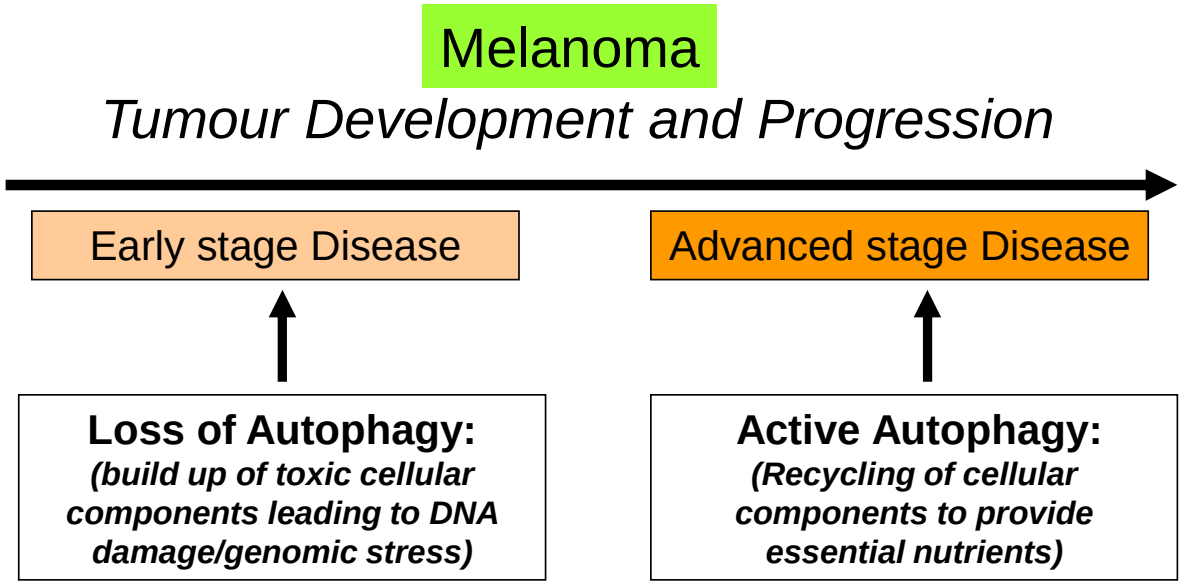

Figure 1.9: The autophagy paradox (Corazzari, Fimia et al. 2013) Loss of autophagy in early disease can result in the build up of toxic cellular components/DNA damage/genomic stress leading to development and progression of tumours. Reacquisition of autophagy in later stages of disease can aid in tumour survival by recycling of cellular components and removal of stress elements at sites of metastasis and hypoxia.

1.6 The Beclin-1 complex

Belcin-1 was first identified as a BH3 only Bcl-2 family member acting as a coiled-coil protein that is involved in autophagy regulation (Liang, Kleeman *et al*. 1998). In canonical autophagy Beclin-1 binds to hVps34/PI(3)K and p150 which it achieves through its evolutionarily conserved domain (Liang, Kleeman *et al*. 1998; Liang, Jackson *et al*. 1999). The hVps34/PI(3)K generates PI(3)P which is required for the regulation of autophagy as confirmed by studies of the PI(3)K inhibitor wortmannin which subsequently inhibited autophagy (Blommaart, Krause *et al*. 1997). Additional proteins have since been discovered to associate with Beclin-1 which aid in the autophagy process including UVRAG, Ambra 1, Bif-1 and Vps15 which have a positive role in autophagy regulation although each may not be in fact be complexed to the same Beclin-1 complex (Fimia, Stoykova *et al*. 2007: Itakura, Kishi *et al*. 2008; Liang, Feng *et al*. 2006; Matsunaga, Saitoh *et al*. 2009; Zhong, Wang *et al*. 2009). Further interactors of Beclin-1 have also been identified resulting in the formation of additional complexes with differing functional roles in autophagy is a specific substrate of the Beclin-1/Ambra 1 complex which functions in the targeting of this complex to the site of autophagosome formation (Matsunaga, Saitoh *et al*. 2009). Atg14 C terminus contains an Barkor/Atg14(L) autophagosome-targeting sequence (BATS) domain which preferentially binds to curved membranes containing PI(3)P and targets the Beclin-1 complex to the isolation membrane (Obara and Ohsumi 2011). Additional Beclin-1 complexes identified contain the interacting protein rubicon (Matsunaga, Saitoh *et al*. 2009). Rubicon binds to a subpopulation of UVRAG complexes separate to Atg14L forming a different Beclin-1 complex to that previously described (Sun Q 2010). Knockdown of Rubicon results in the enhancement of autophagy, especially at the maturation step, as well as enhancement of endocytic trafficking revealing the complex Beclin-1 forms with this protein has a negative role in autophagy regulation. These data also suggest that the Beclin-1-hVps34 complex functions in two different steps of autophagy by altering the subunit composition (Matsunaga, Saitoh *et al*. 2009, Zhong, Wang *et al*. 2009). As such at least three different functional Beclin-1 complexes have been identified; the Beclin-1/Vps34/Vps15/Ambra 1/Atg14L complex; the Beclin-1/Vps34/Vps15/UVRAG complex; and the Beclin-1/Vps34/Vps15/UVRAG/Rubicon complex (Figure 1.10).

Articles have emerged over recent years which highlight additional functions of Beclin-1. A large scale study of protein-protein interactions for many of the key autophagy genes has increased of yet more interactions, providing a platform for future studies (Behrends, Sowa *et al*. 2010). Since its initial discovery, the importance of Beclin-1 in pathways additional to autophagy have also emerged, including its role in cell cycle regulation where Beclin-1 is required for chromosome congression and proper outer kinetochore assembly (Frémont S 2013). Additionally, recent observations show under certain conditions Beclin-1 may be cleaved by caspases thus preventing its pro-autophagic activity (Wirawan, Vande Walle *et al*. 2010). Interestingly further studies also revealed the C-terminal fragment produced as a result of this cleavage event has a function of its own in which it amplifies mitochondrial mediated apoptosis suggesting cross talk between apoptosis and autophagy (Djavaheri-Mergny, Maiuri *et al*. 2010).

Recent data has already shown the ability of Beclin-1 to form multiple complexes with differing functions in autophagy, including its complex with Ambra 1. Ambra 1 is a WD40-containing protein which functions in autophagy as a protein-protein interacting platform (Fimia, Corazzari *et al*. 2012) with the Beclin-1/Vps34 complex. When complexed to these proteins Ambra 1 is involved in the nucleation phase of autophagy causing the formation of phospatidyl inositol 3 kinase rich membranes thus acting as a positive regulator of autophagy (Roy and Debnath, 2010; Fimia *et al*., 2011). Interestingly, members of the Beclin-1/Ambra 1/Vps34 complex associate with each other independently of autophagy induction which suggests and additional regulatory element is needed to stimulate Beclin-1 activity to function in the induction of autophagy (Fimia, Di Bartolomeo *et al*. 2011). A further interaction of Ambra 1 has been identified with dynein light chains 1 and 2 associated with the dynein motor complex where Ambra 1 is bound under conditions of basal autophagy (Di Bartolomeo, Corazzari *et al*. 2010). Under conditions of starvation however, the activity of ULK1 becomes dissociated with mTORC1 resulting in the release Ambra 1 from dynein by phosphorylation. Ambra 1 then localizes to the endoplasmic reticulum to prime autophagosome formation and nucleation thus revealing a role for Ambra 1 in autophagy induction in addition to nucleation. (Di Bartolomeo, Corazzari *et al*. 2010; Fimia, Di Bartolomeo *et al*. 2011).

More recently, autophagy independent interactions with Ambra 1 have been found which are linked to apoptosis. In this context, mitochondrial Bcl-2 has been shown to directly interact with Ambra 1 independently of Beclin-1; an event which is disrupted by the induction of either autophagy or apoptosis (Strappazzon, Vietri-Rudan *et al*. 2011). Ambra 1 and Bcl-2 actually bind to Beclin-1 at the same location suggesting that Ambra 1 competes with Bcl-2 to bind to Beclin-1.

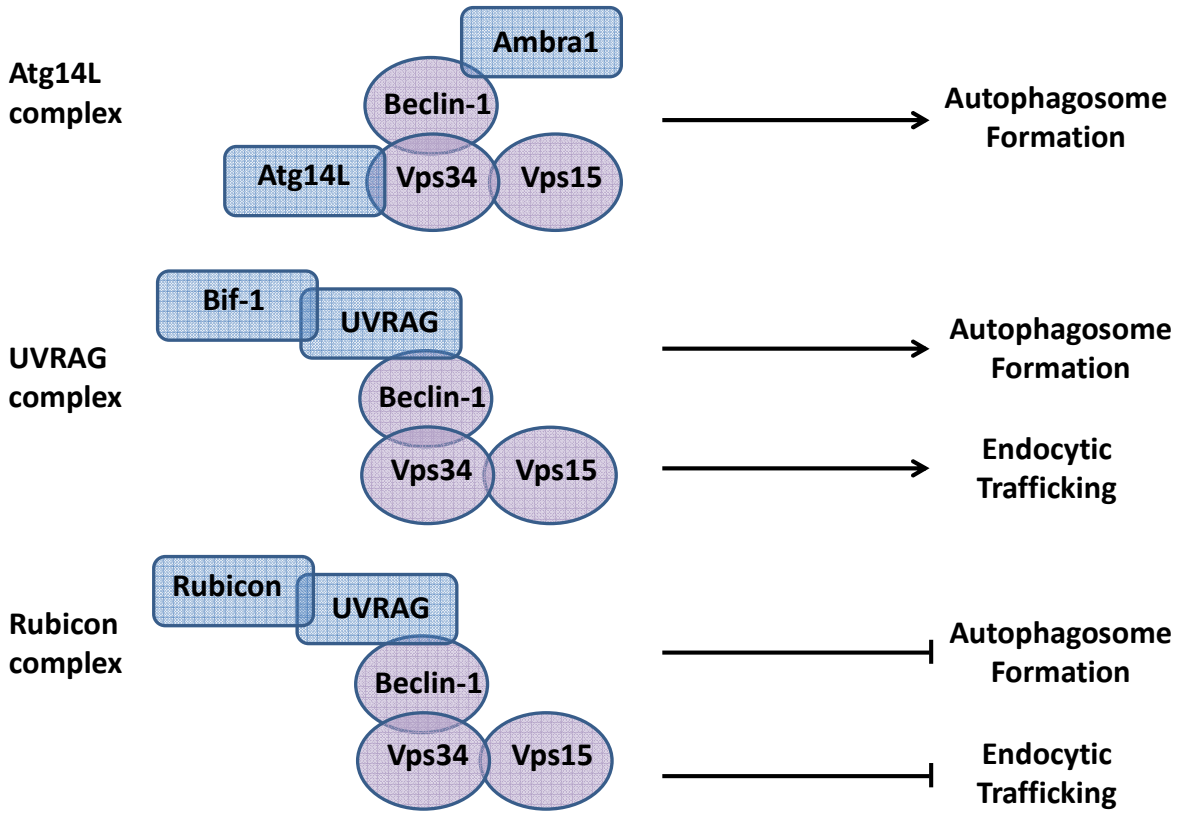

Figure 1.10: Beclin-1 and its complexs. A) Beclin-1/Vps34/Vps15/Ambra 1/Atg14L complex; B) Beclin-1/Vps34/Vps15/UVRAG complex C) Beclin-1/Vps34/Vps15/UVRAG/Rubicon complex

1.7 Apoptosis, Endoplasmic Reticulum Stress and induction by the proteosomal inhibitor, Bortezomib

The word apoptosis, first coined by Kerr, Wyllie and Currie in 1972, was used to describe a distinct form of cell death observed by key morphological changes observed in these studies (Kerr, Wyllie *et al*. 1972). Since then apoptosis has become recognised and accepted as an important mode of cell death involving a highly regulated and genetically determined process for the elimination of cells. Important for homeostasis, apoptosis is crucial for normal development and ageing however, it may also act as a defence mechanism in immune reactions or for the elimination of cells damaged by disease (Norbury and Hickson 2001). However, different cells may die in response to different apoptotic stimuli. Typically, light and electron microscopy are employed to observe the classical morphological features of apoptosis which include cell shrinkage and pyknosis (or chromatin condensation) (Table 1.1) as well as plasma blebbing, karyohexis, apoptotic bodies and budding. Interestingly, inflammatory responses during this process are absent, which has been attributed to three potential possibilities; 1) as apoptotic cells don't release their cellular constituents into surrounding tissue they do not induce an inflammatory response; 2) apoptotic cells are quickly phagocytosed; 3) engulfing cells don't produce any anti-inflammatory cytokines during this process (Savill and Fadok 2000). In terms of biochemical events, several events are also classically observed during the process of apoptosis including protein cleavage, protein cross linking and DNA breakdown to name a few (Hengartner 2000).

In terms of its regulation, apoptosis is prinicipally mediated through two pathways involving either extrinsic and intrinsic mechanisms, however, evidence suggests that these pathways are linked and molecules in one pathway may influence the other (Igney and Krammer 2002). Furthermore, both mechanisms are mediated by caspases, a family of aspartic proteases (Cohen 1997; Rai, Tripathi *et al*. 2005) widely expressed as inactive proenzymes in most cells which when activated initiate a cascade of caspase activation events, amplifying the apoptotic process and leading to rapid cell death. Caspases have been classified into different groups termed initiators (caspases 2, 8, 9 and 10), effectors/executioners (caspases 3, 6 and 7) and inflammatory caspases (caspases 1, 4, and 5) (Cohen 1997; Rai, Tripathi *et al*. 2005). Other caspases have been identified such as;

caspase 11 which regulates apoptosis by acting during septic shock and cytokine maturation; caspase 12 which mediates endoplasmic reticulum specific apoptosis; caspase 13, which was identified as a gene in bovine DNA and caspase 14, found to be highly expressed in embryonic but not in adult tissues (Hu, Snipas *et al*. 1998; Kang, Wang *et al*. 2002; Koenig, Eckhart *et al*. 2001; Nakagawa, Zhu *et al*. 2000)

Apoptosis mediated by extrinsic signalling involves transmembrane receptor-mediated interactions with death receptors, which are members of the tumor necrosis factor (TNF) receptor family (Locksley, Killeen *et al*. 2001). This family of receptor proteins all have cysteine-rich extracellular domains as well as a cytoplasmic domain known as a death domain, typically about 80 amino acids in length (Ashkenazi and Dixit 1998). This death domain is required for the transmission of pro-death intracellular signalling events stimulated by specific extracelluar ligands including; FasL/FasR, TNF-α/TNFR1, Apo3L/DR3, Apo2L/ DR4 and Apo2L/DR5 (Ashkenazi and Dixit 1998 (Ashkenazi and Dixit 1998; Chicheportiche, Bourdo *et al*. 1997; Peter and Krammer 1998; Rubio-Moscardo, Blesa *et al*. 2005; Suliman, Lam *et al*. 2001) of which FasL/FasR and TNF-α/TNFR1 have been the most extensively studied. During extrinsic apoptosis death receptors cluster causing binding of trimeric ligands. Cytoplasmic adapter proteins are then recruited which activate corresponding death domains. An example of this includes the binding of Fas ligand to the Fas receptor causing recruitment of FADD (the Fas adaptor protein) or the binding of TNF ligand to the TNF receptor resulting in binding of the adapter protein TRADD and the recruitment of FADD and RIP (Hsu, Xiong *et al*. 1995). FADD then associates with procaspase 8 due to dimerization of the death domain forming what is known as a death-inducing signaling complex (DISC) which causes the activation of procaspase 8 (Kischkel, Hellbardt *et al*. 1995) and downstream activation of the extrinsic apoptotic pathway. Extrinsic apoptosis may also be activated by the perforin/granzyme pathway, regulated by CD8+ T-cell lymphocyte induced apoptosis. Typically these cells exert there effect through the FasL/FasR ligand receptors (Bratton, Fadok *et al*. 1997) however, they have also been found to induce apoptosis by secretion of perforin (transmembrane pore-forming molecule), typically used by lymphocytes for virally infected cells. Once the pore is formed cytoplasmic granules are released into the target cell through the pore (Trapani and Smyth 2002). Granzyme A and granzyme B represent the principle components of such, both possessing serine enzyme protease activity (Pardo, Bosque *et al*. 2004). Granzyme B activates procaspase 10 and can

additionally cleave ICAD (Inhibitor of Caspase Activated DNAse) (Sakahira, Enari *et al*. 1998) or Bid and thus inducing cytochrome C release (Barry and Bleackley 2002; Russell, 2002) and can also directly activate caspase 3 thus inducing apoptosis without the requirement for mitochondrial disruption (Goping, Barry *et al*. 2003). Granzyme A however, activates apoptosis through caspase independent mechanisms, alternatively activating DNAse NM23-H1, a tumor suppressor gene product (Fan, Beresford *et al*. 2003), which has an important role in preventing cancer development by apoptosis induced by immune surveillance. Granzyme A exhibits its effects on expression of this DNAse by inactivation of the SET complex (composed of; SET, Ape1, pp32, and HMG2) which ordinarily inhibits expression of the NM23-H1 gene, cleavage of which results in expression of this DNAse, and subsequent DNA degradation. The SET complex also functions in chromatin structure and DNA repair (Fan, Beresford *et al*. 2003). As such granzyme A activity exerts its pro-apoptotic effects by inactivation of this complex and thus blocking DNA and chromatin maintenance.

In contrast to extrinsic apoptosis, the intrinsic pathway is mediated by intracellular signals that act directly on targets within the cell and are typically mitochondrial and/or endoplasmic reticulum initiated events. Stimulators of intrinsic apoptosis include the absence of growth factors, hormones and cytokines which typically would supress cell death and apoptosis. As such these stimuli are known as negative regulators. Examples of positive regulators/stimuli include radiation, toxins, hypoxia, viral infections and free radicals. Ultimately however all stimuli cause mitochondrial outer membrane permeabilisation (MOMP), loss of mitochondrial transmembrane potential and opening of the mitochondrial permeability transition pore. This in turn causes the release of several pro-apoptotic mitochondrial constituents including; cytochrome C, Smac/DIABLO and serine proteases and Omi (Cai, Yang *et al*. 1998; Du, Fang *et al*. 2000; Garrido, Galluzzi *et al*. 2006). Once released, these proteins then activate the caspase dependant mitochondrial pathway of cell death. Cytochrome C functions by binding to Apaf-1 and procaspase 9 forming what is known as the apoptosome. Procaspase 9 is then activated stimulating downstream apoptotic events. Smac/DIABLO acts through inhibition of inhibitor of apoptosis (IAP) proteins (van Loo, van Gurp *et al*. 2002; Schimmer 2004) also stimulating pro-apoptotic downstream events. A second group of pro-apoptotic proteins are released from mitochondria during apoptosis that function independently of caspases which include; AIF, endonuclease G and CAD, but this is a late event that occurs when the cell is already committed to cell death. When AIF is

released it translocates to the nucleus causing DNA fragmentation and DNA laddering as well as condensation of peripheral nuclear chromatin (Joza, Susin *et al*. 2001) which are both classic signs of apoptosis. Endonuclease G, like AIF, translocates to the nucleus cleaving nuclear chromatin producing oligonucleosomal DNA fragments (Li, Luo *et al*. 2001). Finally CAD is released from the mitochondria which then also translocates to the nucleus leading to additional oligonucleosomal DNA fragmentation and the final stages of chromatin condensation (Enari M, Sakahira H *et al*. 1998). The control and regulation of these events has mostly been attributed to Bcl-2 protein family members themselves, regulated by the tumour suppressor protein p53, frequently absent or mutated in many tumours. However mitochondrial damage has also been suggested to occur via the Fas death receptor pathway and cleavage of Bid by caspase 8 (Esposti 2002; Li, Ambrosini *et al*. 1998) suggesting cross talk between death receptor and intrinsic apoptosis.

Ultimately both intrinsic and extrinsinc culminate in the final execution phase apoptosis in which execution caspases are activated which in turn activate cytoplasmic endonucleases which degrade nuclear material as well as proteases that degrade the nuclear envelope and cytoskeletal proteins. The execution caspases cleave various substrates including cytokeratins, PARP, the plasma membrane cytoskeletal protein alpha fodrin, the nuclear protein NuMA and others which ultimately causes the morphological and biochemical changes observed in cells undergoing apoptosis (Slee, Adrain *et al*. 2001). Of all the execution caspase, caspase 3, is considered the most important which can be activated by any of the initiator caspases. Caspase 3 functions in apoptosis by specific activation of the Ca^{2+} and Mg^{2+} dependant endonuclease CAD which is usually complexed with its inhibitor ICAD (Bortner, Oldenburg *et al*. 1995). It does this by cleaving ICAD and thus releasing CAD from its inhibitor (Sakahira, Enari *et al*. 1998). Caspase 3 also induces cytoskeletal reorganization and disintegration of the cell into apoptotic bodies. One of the key substrates identified for caspase 3 is gelsolin which is an actin binding protein which acts as a site for actin polymerization. Caspase 3 cleaves gelsolin and these cleaved fragments then cleave the actin filaments causing disruption of the cytoskeleton, signal transduction, intracellular transport and cell division events (Kothakota, Azuma *et al*. 1997). Finally, apoptotic cells are then consumed by surrounding cells such as macrophages by phagocytosis. It is the expression of cell surface markers which aids early phagocytosis which is achieved by movement of typically inward facing phosphatidylinositolserine of the cells lipid bilayer to

the outer membrane of the bilayer (Bratton, Fadok *et al*. 1997). Here it acts as ligand for phagocytes on surface of apoptotic cells. As annexin 5 interacts specifically with phosphatidylinositolserine it is often used an apoptotic marker utilised for analysing apoptosis events experimentally (Arur, Uche *et al*. 2003; van Engeland, Nieland *et al*. 1998). As apoptosis is such a highly regulated process of cell death with no observed inflammatory responses this process has been extensively researched in order to understand its role in health and disease, be it for apoptosis induction in diseases such as cancer or its prevention in neurological diseases such as Parkinson's disease.

Apoptosis is frequently dysregulated in cancer including melanoma. Several mechanisms of resistance have been proposed effecting both the induction of intrinsic and extrinsic apoptosis (Thompson, Scolyer *et al*. 2005). Intrinsic apoptosis resistance has been attributed to over-expression of anti-apoptotic Bcl-2 proteins including Bcl-2, Bcl-lxl and Mcl-1. Additionally extrinsic apoptosis may result from over-expression of c-FLIP protein preventing caspase 8 activation and downstream apoptotic signalling stimulated by this initiator caspase (Xiao, Yang *et al*. 2005). More recently, evidence has highlighted the importance of the endoplasmic reticulum which may act as a potential "gatekeeper" to control the progression of melanoma (Denoyelle, Abou-Rjaily *et al*. 2006), Moreover, agents able to apoptosis through targeted ER stress have been shown to enhance drug induced cell death of melanoma both *in vitro* and *in vivo* (Lovat, Corazzari *et al*. 2008), including the 26S proteosome inhibitor, bortezomib, also know as velacde (Hill, Martin *et al*. 2009). Additionally, little to no adverse effects were observed on normal melanocytes (Fernandez, Verhaegen *et al*. 2005; Lovat, Corazzari *et al*. 2008).

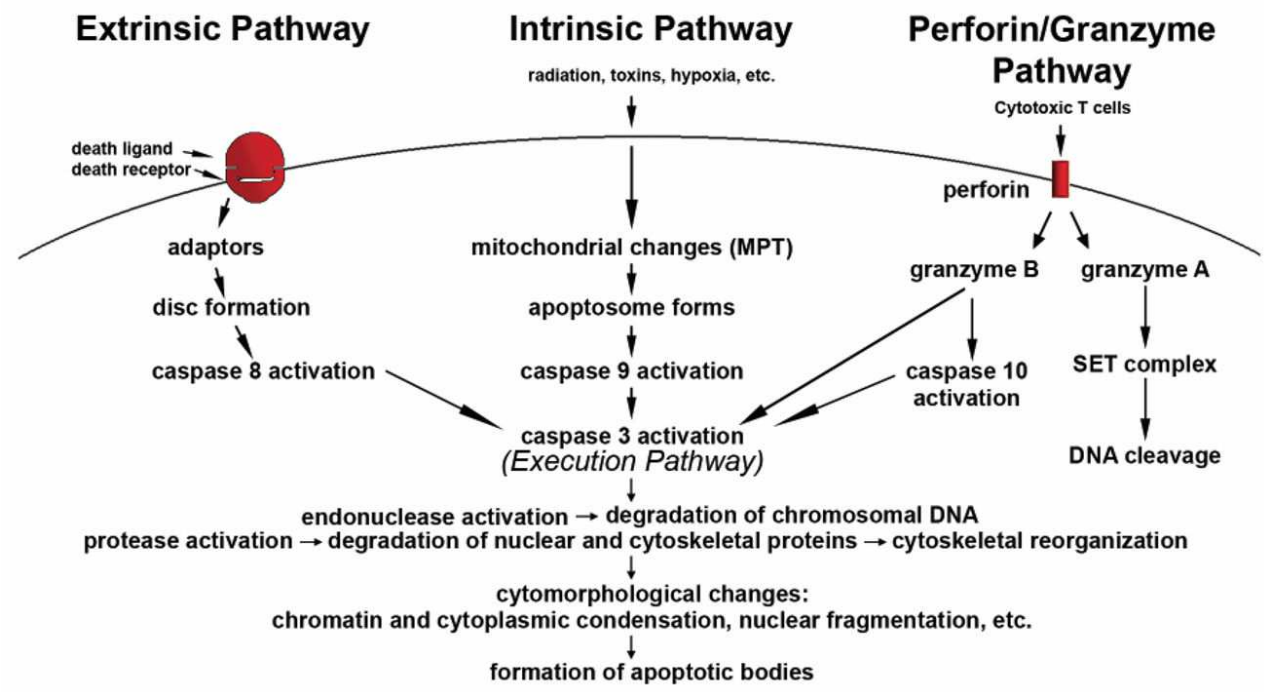

Figure 1.11 Known pathways of apoptosis (Elmore 2007). Two main pathways of apoptosis have been determined known as the extrinsic pathway and the intrinsic pathway. An additional pathway known as the Perforin/Granzyme pathway has also been identified which acts through the intrinsic pathway of apoptosis. The extrinsic pathway is activated through extracellular death ligand interaction with death receptors causing disc formation and activation of capase 8. The intrinsic pathway involves intracellular mitochondrial changes, apoptosome formation and activation of caspase 9. The Perforin/Granzyme pathway involves the release of granzymes via cytotoxic T cells into target cells causing caspase 10 activation of SET complex formation and DNA cleavage to amplify apoptosis. Each pathway converges at the execution phase where caspase 3 is activated and cells are then committed to apoptotic events such as degradation of DNA, nuclear and cytoskeletal protein degradation and formation of apoptotic bodies.

Able to act as a competitive inhibitor of the 26S proteasome preventing degradation of many intracellular proteins, bortezomib may induce the build up of damaged proteins, organelles and alterations in cellular gene expression leading to the ER stress events (Attar, De Angelo *et al*. 2008; Horton, 2007; Ogawa, Tobinai *et al*. 2008; Papandreou, Daliani *et al*. 2004; Richardson, Mitsiades *et al*. 2008; Yin, Zhou *et al*. 2005) . Bortezomib has been shown to induce ER stress events in a variety of cancers including glioblastoma, pancreatic cancer, head and neck squamous cell carcinoma (Brignole, Marimpietri *et al*. 2006; Fribley, Evenchik *et al*. 2006; Fribley, Zeng *et al*. 2004; Fribley, Zeng *et al*. 2004; Nawrocki, Carew *et al*. 2005; Yin, Zhou *et al*. 2005) and has been extensively used in the treatment of multiple myeloma (Jackson, Einsele *et al*. 2005) as well as in the treatment of metastatic melanoma in combination with other chemotherapeutics such as the DNA damaging agent temozolomide. Promising results using human melanoma mouse xenograft models revealed animals receiving combined bortezomib and temozolomide treatment achieved complete remission of palpable tumors after only 30 days of therapy revealing that the chemotherapeutic effects of bortezomib could be augmented using temozolomide which has subsequently led to phase I and phase II trials utilizing this approach (Amiri, Horton *et al*. 2004; Su, Amiri *et al*. 2010) Additionally, more recent studies suggest the BH3 mimetic ABT-737, specifically targeting the anti-apoptotic Bcl-2 family proteins Bcl-2, Bcl-xl, and Bcl-w potentiates bortezomib-induced apoptosis of melanoma (Reuland, Goldstein *et al*. 2012). Interestingly cancer cells treated with bortezomib have also been found to induce a pro-survival autophagic response in order to overcome the adverse effects of proteasome inhibition highlighting how cancer may use autophagy to counter act apoptotic signalling (Armstrong, Corazzari *et al*. 2011)**.** As such the implications of autophagic signalling in cancer as well as its potential link with apoptosis high light its potential as a therapeutic modality.

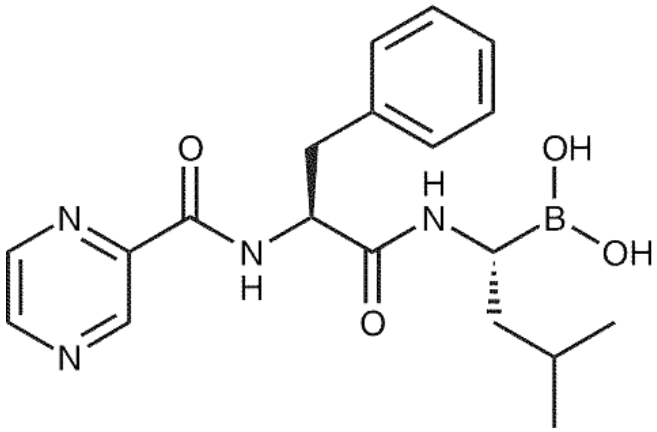

Figure 1.12: Bortezomib chemical structure. The 26S proteasome inhibitor Bortezomib/Velcade.

1.8: The Bcl-2 Family, Myeloid Cell Leukemia-1 Member (Mcl-1), and their Role in Autophagy

The Bcl-2 oncogene was identified from an acute B-cell leukemia cell line as a gene locus on chromosome 18 (band q21) (Tsujimoto, Finger *et al*. 1984) having the ability to increase cellular proliferation as well as inhibiting apoptosis (McDonnell, Deane *et al*. 1989; Vaux 1988). The Bcl-2 family of proteins is composed of both pro-apoptotic members (namely Bax, Bak, PUMA, NOXA, BAD, BID and BIM) and anti-apoptotic members (namely Bcl-2, Bcl-xl, Mcl-1, Bcl-w and A1) which are usually well regulated and kept in a delicate balance in normal tissue homeostasis (Hocker, Singh *et al*. 2008). This protein family is divided into three groups; two pro-apoptotic groups and one anti-apoptotic group, which is based on their conserved regions, known as Bcl-2 homology domains (BH domains). The first pro-apoptotic group contains multi-domain members including Bax and Bak which have BH1, BH2 and BH3 domains, allowing for permeabilization of the mitochondrial membrane, but lack the BH4 domain. The second pro-apoptotic group contains proteins with the BH3 domain only, which are upstream signal detectors that activate Bax and Bak upon activation by pro-apoptotic stimuli. The anti-apoptotic group contains proteins which have conserved BH1, BH2, BH3 and BH4 domains, which form a hydrophobic pocket (Muchmore, Sattler *et al*. 1996) able to bind to the BH3 domains of pro-apoptotic members (typically the BH3 only proteins) (Cheng, Wei *et al*. 2001).

Bcl-2 family members have been implicated as one of the most crucial regulators of apoptosis in melanoma. Often cancers use the RAS/RAF/MEK/ERK pathway to increase expression of anti-apoptotic Bcl-2 family members (most notably Mcl-1, Bcl-2 and Bcl-xl) (Boucher, Morisset *et al*. 2000). As mutant $BRAF^{V600E}$ is common in melanoma, tumours bearing this mutation usually exhibit high levels of ERK1/2 activity which in turn would result in upregulation of these ant-apoptotic members (Balmanno and Cook 2009). Mutant $BRAF^{V600E}$ signalling has also been shown to inhibit BIM (a key pro-apoptotic member) expression in human melanoma cells via sustained over-activation of the RAS/RAF/MEK/ERK signalling pathway resulting in phosphorylation of BIM, targeting this protein for degradation by the proteasome, which further enhances the resistance of melanoma cells to apoptosis (Cartlidge, Thomas *et al*. 2008).

Of particular interest is the anti-apoptotic member Mcl-1, which has been shown to be a critical Bcl-2 family member required for melanoma survival and is highly expressed in both primary and advanced disease (Tang, Tron *et al*. 1998). Additionally Mcl-1 expression has been shown to increase with increasing melanoma disease stage (Zhuang, Lee *et al*. 2007). Activation of ERK1/2 stabilises Mcl-1 by phosphorylation of this protein (Domina, Vrana *et al*. 2004) and Mcl-1 over-expression in pancreatic cancer is correlated with hyper-activation of the ERK1/2 pathway (Boucher, Morisset *et al*. 2000), an occurrence typically seen in melanoma bearing $BRAF^{V600E}$. Resistance to a wide range of traditional and targeted chemotherapeutic agents has been shown to be mediated by Mcl-1 over-expression, possibly due to its uniquely high specificity for suppressing apoptosis induced by BAK (Zhai, Jin *et al*. 2008). Furthermore Mcl-1 has been implicated in the resistance of melanoma to anoikis (a form of apoptosis induced by loss of cellular adhesion or adhesion to an inappropriate cellular matrix), particularly in metastatic disease allowing for tumour survival at distant sites from the primary tumour (Boisvert-Adamo and Aplin 2008).

More recently it has been revealed that, in addition to the role of Bcl-2 family members in apoptosis regulation, this protein family plays a functional role in autophagy. It has been known for several years that Beclin-1 is a BH3-only protein and thus is a binding target of Bcl-2 (Feng, Huang *et al*. 2007), and Erlich *et al* showed that Beclin-1 binds to Bcl-2 and Bcl-xl with a high affinity, and with a weaker interaction to Mcl-1 (Erlich, Mizrachy *et al*. 2007). Therefore Bcl-2 anti-apoptotic proteins are able to inhibit Beclin-1-dependant autophagy, presenting a dual role for anti-apoptotic Bcl-2 members (Fig 1.9). A recent report by Voss *et al* (Voss, Senft *et al*. 2010) demonstrated the pan-Bcl-2 inhibitor ((-)-gossypol), is able to induce caspase-independent cell death in apoptosis resistant malignant glioma cells. As inhibition of Bcl-2 or Bcl-xL alone did not have any effect, Mcl-1 inhibition was therefore identified as a crucial factor mediating caspase-independent cell death in this context (Voss, Senft *et al*. 2010).

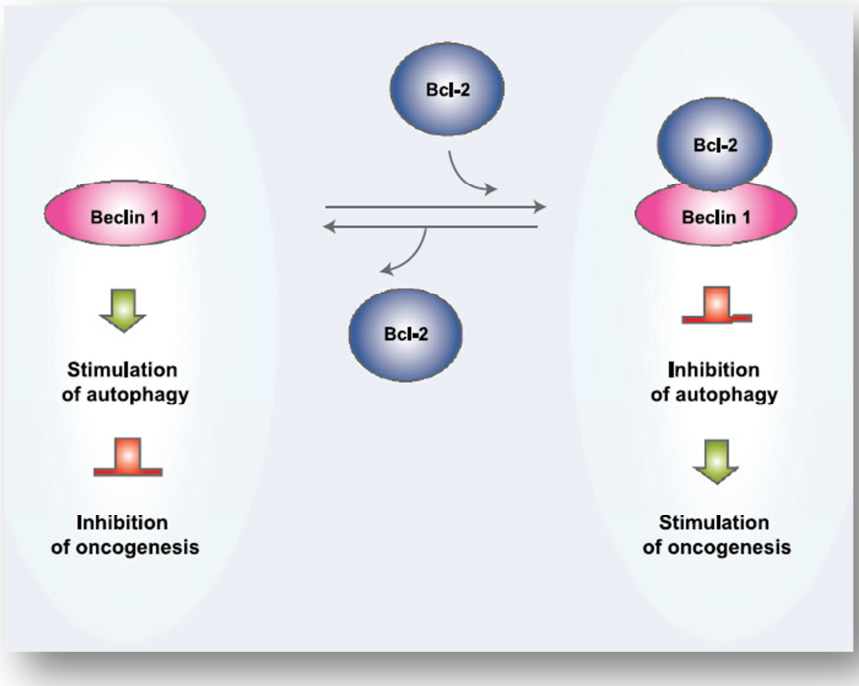

Figure 1.13: Schematic model of the interrelationship between Beclin-1, Bcl-2, autophagy and cancer initiation (Pattingre and Levine 2006). In the absence of Bcl-2 binding to the tumour suppressor Beclin-1, stimulation of autophagy occurs as normal, potentially resulting in the inhibition of oncogenesis. When Bcl-2 is bound to Beclin-1, autophagy is inhibited and thus oncogenesis may be stimulated under these conditions.

The gene encoding Mcl-1 contains 3 exons encoding two splicing variants with opposing roles. The first variant encoded is a polypeptide of 350 amino acids in length and 40 kDa in molecular weight, known as the long form of Mcl-1 (Mcl-1L) (Fig 1.10) which has 35% homology with the C-terminus of Bcl-2 (Kozopas, Yang *et al*. 1993). The Mcl-1L protein possesses Bcl-2 homology domains 1-3 and transmembrane domains that are found in other Bcl-2 family members, as well as a unique PEST sequence (a peptide sequence rich in proline, glutamic acid, serine, and threonine) within its N terminus (Yang, Kozopas *et al*. 1995). The second variant is encoded when the Mcl-1 pre-mRNA undergoes alternative splicing by eliminating exon two resulting in the translation of a polypeptide of 271 amino acids in length with a molecular weight of 35 kDa, known as the short form of Mcl-1 (Mcl-1S) (Fig 1.10) (Bingle, Craig *et al*. 2000). This protein has a 41 residue difference to Mcl-1L at the C-terminus and has only a BH3 domain, much like that of the BH3-only, pro-apoptotic members of the Bcl-2 family. Mcl-1S is therefore able to antagonise the pro-survival role of Mcl-1L, promoting cells to enter apoptosis (Bae, Leo *et al*. 2000; Bingle, Craig *et al*. 2000). In

Basal cell carcinoma (BCC) cells it has been shown that Mcl-1L expression predominates over Mcl-1S, resulting in an increased ratio of Mcl-1L to Mcl-S when compared with primary human keratinocytes (Shieh, Liu *et al*. 2009). Specific targeting of Mcl-1 pre-mRNA by Mcl-1 antisense morpholino oligonucleotides (AMOs) altered the splicing pattern and favoured expression of Mcl-1S mRNA (Shieh, Liu *et al*. 2009). This shift directly induced apoptosis in BCC cells as well as in a human adenocarcinoma gastric cell line. Over-expression of Mcl-1S has also been shown to induce apoptosis in Chinese hamster ovary (CHO) cells and adenocarcinoma human alveolar basal epithelial A549 cells (Bae, Leo *et al*. 2000; Bingle, Craig *et al*. 2000). Thus it would appear that there is a delicate balance between Mcl-1L and Mcl-1S which may determine the fate of many cell types. Interestingly more novel roles for Mcl-1 have also been observed in which a nuclear-localised proteolytic fragment of Mcl-1 has been identified which is able to regulate cell growth by its interaction with cdk 1 (Jamil, Sobouti *et al*. 2005) in which the interaction of nuclear Mcl-1 inhibits the Cdk1 from binding to cyclin B1 thus preventing progression through G2 and M phases of the cell cycle (Jamil, Sobouti *et al*. 2005). These authors further suggest that inhibition of Cdk1 activity accounts for the inhibitory effect of Mcl-1 on cell growth (Jamil, Sobouti *et al*. 2005) and as such further highlights the potential importance of different isoforms of Mcl-1 in cancer growth and survival.

An increased understanding of Bcl-2 anti-apoptotic family members in the regulation of autophagy, suggests these proteins play an essential role in the resistance of malignant cell types to both apoptosis and autophagy. The role of differing Mcl-1 splicing variants in malignant melanoma tumours remains poorly defined and targeting Mcl-1 may therefore be of therapeutic benefit. However, important questions remain regarding the ability of Mcl-1L to inhibit autophagy, and whether Mcl-1L inhibition would promote autophagy and tumour cell survival or autophagic cell death.

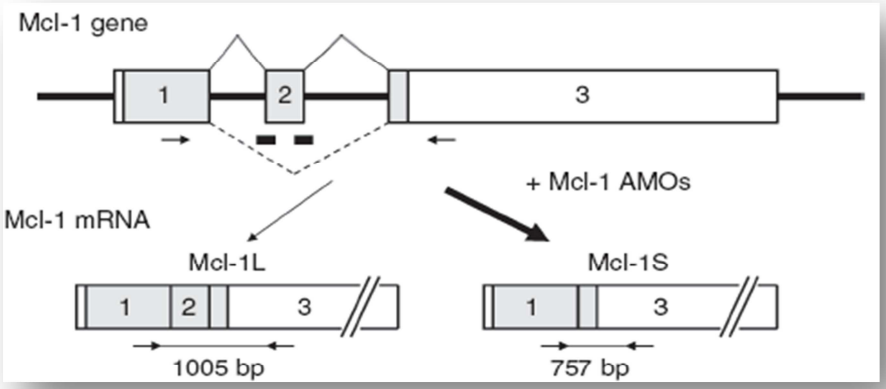

Figure 1.14: Splcing variants of Mcl-1 from the Mcl-1 gene (Shieh, Liu et al. 2009). The first variant encoded from Mcl-1 mRNA is known as Mcl-1L which is 350 amino acids in length and 42 kDa in molecular weight. The second variant is encoded when the Mcl-1 pre-mRNA undergoes alternative splicing by eliminating exon two resulting in the translation of Mcl-1S which is 271 amino acids in length with a molecular weight of 30 kDa.

1.9 Cannabinoids in Autophagy and Cancer

Cannabinoids have been used in medicine for many centuries, particularly in more eastern countries, however since the chemotherapeutic potential of cannabinoids was first suggested in 1998 (Sánchez, Galve-Roperh *et al*. 1998), in which anti-cancer properties of THC was observed in glioma, a rekindled interest for the medicinal application of cannabinoids for their use in the treatment of cancer has developed. The potential use of cannabinoids for the treatment of Glioma in particular, has recently been extensively researched (Duntsch, Divi *et al*. 2006; Salazar, Carracedo *et al*. 2009; Sánchez, Galve-Roperh *et al*. 1998; Sánchez, de Ceballos *et al*. 2001; Velasco, Carracedo *et al*. 2007). As such, several signal transduction pathways and mechanisms of action triggered by cannabinoids have been identified, frequently activated through stimulation of specific cannabinoid receptors found within the plasma membrane. Two cannabinoid receptors have been identified; cannabinoid receptor 1 and 2 (CB1/CBR1 and CB2/CBR2). Cannabinoid receptors are expressed differentially in tissues, with CB1 expression more typically observed in the central nervous system and CB2 expressed in the immune system (Pertwee and Ross 2002). CB receptor expression has however also been identified in other tissues such as lung, liver and the kidney (Galiègue, Mary *et al*. 1995; Sarafian, Montes *et al*. 2008). In addition, the over-expression of both of these receptors has been found in a variety of cancers including Hepatocellular carcinoma (HCC), prostate, breast, lung, pancreatic and lymphoma (Sarfaraz, Afaq *et al*. 2005; Sarfaraz, Adhami *et al*. 2008; Xu, Liu *et al*. 2006). Since this discovery several *in vivo* studies have revealed the upregulation of CB2 in melanoma but not in surrounding tissue with further evidence for CB1 expression through its associated gene CNR1 (Kenessey, Bánki *et al*. 2012; Zhao, Yang *et al*. 2012).

Of all the identified cannabinoids two are typically used for medicinal purposes namely tetrahydrocannabidiol and cannabidiol (Figure 1.15). Although both these agents have been found to have anti-tumoural properties, the underlying mechanisms of action may in fact not be the same. The accumulation of a pro-apoptotic shpingolipid, ceramide, through CB1 activation has been suggested as the mechanism of cannabinoid-induced cell death and in particular in the context of THC-induced apoptosis of glioma (Gómez del Pulgar, Velasco *et al*. 2002). Additionally, strong evidence also supports a key role for autophagy thought to be activated by the eIF2a axis of the ER stress process and resulting in the

increased expression of the stress-regulated proteins p8 and TRB3, and subsequent Akt inhibition resulting in autophagy induction through the inhibition of mTORC1 and ultimately cell death (Carracedo, Gironella *et al*. 2006; Carracedo, Lorente *et al*. 2006; Salazar, Carracedo *et al*. 2009; Salazar, Carracedo *et al*. 2009). Unlike THC however, cannabidiol (CBD), used in the treatment of breast cancer and glioma does not demonstrate any psychoactive effects. Research into the molecular mechanisms of CBD is nevertheless limited and as such the mechanisms through which CBD exerts its anti-cancer activity remain to be fully identified. Evidence has suggested that some cannabinoids can in fact exert their chemotherapeutic action via an additional receptor known as the vanilloid receptor leading to an initial hypothesis that this receptor is crucial for CBD action (Qin, Neeper *et al*. 2008). However, CBD-induced cell death of breast cancer cells appears to be independent of both cannabinoid and vallinoid receptor activation (Shrivastava, Kuzontkoski *et al*. 2011). The lack of a receptor mediated mechanism for CBD is also apparent in studies of CBD-induced inhibition of glioma cell viability in which selective cannabinoid receptor antagonists SR141716 (which targets CB1) or SR144528 (which targets CB2) (Vaccani, Massi *et al*. 2005) were unable to antagonise inhibition of cell viability. Interestingly, however, both autophagy and apoptosis were observed by Electron microscopy following treatment of breast cancer cells with CBD. Additionally similar mechanisms to those observed with THC treatment such as induction of endoplasmic reticulum stress, Akt inhibition and mTOR inihbition have also been observed in breast cancer cells in response to CBD treatment, including decreased levels of phosphorylated mTOR4EBP1, and cyclin D1 (Shrivastava, Kuzontkoski *et al*. 2011). Furthermore, these authors also demonstrated CBD induces several events associated with apoptosis including the reduction of mitochondrial membrane potential, BID translocation to the mitochondria, cytochrome c release into the cytosol, and the activation of the intrinsic apoptotic pathway. CBD also increased the generation of ROS, the inhibition of which blocked both apoptosis and autophagy induction (Shrivastava, Kuzontkoski *et al*. 2011). Although preliminary studies with cannabinoids have been undertaken in melanoma (Blázquez, Carracedo *et al*. 2006; Kenessey, Bánki *et al*. 2012; Scuderi, Cantarella *et al*. 2011; Zhao, Yang *et al*. 2012) there is howver, to date, limited research available as to the potential for THC to treat this disease or whether a similar cytotoxic process.

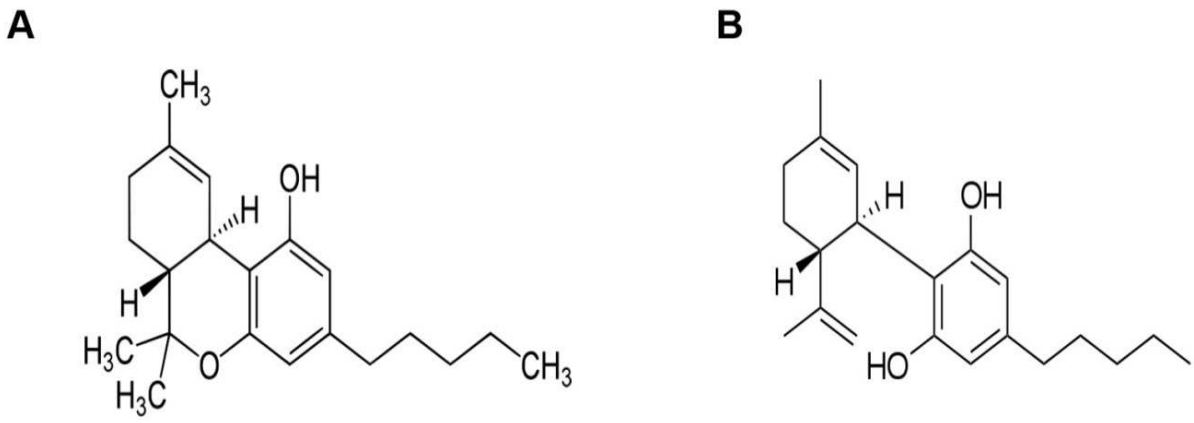

Figure 1.15: Cannabinoid chemical structures. Chemical structure of cannabinoids derived from cannabis *sativa* A) tetrahydricannabidiol and B) cannabidiol.

Observations in melanoma that both cannabinoid receptors CB1 and CB2 are upregulated *in vitro* compared to primary melanocytes lead to the hypothesis that the use of cannabinoids such as THC may offer therapeutic potential (Blázquez, Carracedo *et al*. 2006). Further evidence supporting this concept is given by observations that THC inhibits angiogenesis, tumour growth and metastasis by receptor activation as well as the induction apoptosis (Blázquez, Carracedo *et al*. 2006). Although the majority of studies revealing the molecular mechanisms of THC-induced cell death (and by its homologues) have been identified in glioma, pilot research in prostate and melanoma cancer also suggests similar mechanisms of action in these tumour types (Carracedo, Gironella *et al*. 2006; Carracedo, Lorente *et al*. 2006; Salazar, Carracedo *et al*. 2009). Research in both glioma and prostate cancer cells have highlighted the specific importance of ceramide accumulation for the anti-tumoural effects of cannabinoids (Blázquez, González-Feria *et al*. 2004; Galve-Roperh, Sánchez *et al*. 2000; Gómez del Pulgar, Velasco *et al*. 2002; Mimeault, Pommery *et al*. 2003). The specificity of ceramide accumulation rather than its conversion to complex sphingolipids is also evidenced by a lack of anti-tumoural effects observed with THC treatment when glucosylceramide synthase (the enzyme catalysing rate limiting step of glycosphingolipid biosynthesis) is inhibited (Carracedo, Lorente *et al*. 2006). Further insights using DNA

microarrays have also revealed downstream signalling events resulting from ceramide accumulation. These include the upregulation of several stress-regulated genes including p8, tribbles 3 (TRB3: a mammalian homolog of Drosophila tribbles), C/EBP homologous protein (CHOP) and activating transcription factor 4 (ATF4) (Carracedo, Lorente *et al*. 2006). In this context the authors suggest p8 mediates THC-induced apoptosis and additionally may act as a co-transcription factor, regulating the expression of both CHOP and ATF4 (Carracedo, Gironella *et al*. 2006; Carracedo, Lorente *et al*. 2006). TRB3 expression may thus be regulated by ATF4 and CHOP, key markers of ER stress-induced apoptosis in several tumour types (Ohoka, Yoshii *et al*. 2005; Salazar, Carracedo *et al*. 2009). TRB3 overexpression has been shown to inhibit Akt activation in squamous cell carcinoma derived from tongue tissue (Zhang, Wen *et al*. 2011) and Akt inhibition by TRB3 has also been described in liver tissue in response to starvation whereby TRB3 binding to Akt prevents its kinase activity (Du, Herzig *et al*. 2003). Therefore, as Akt is an essential component of the PI3K/Akt/mTOR pathway in which it inhibits autophagy through activation of mTORC1, its inhibition by TRB3 overexpression would therefore result in the induction of autophagy (Salazar, Carracedo *et al*. 2009).

Collectively these findings suggest that THC-induced cell death occurs as a result of ceramide accumulation and ER stress induction which leads to Akt inhibition and the induction of autophagy, ultimately resulting in cell death (Figure 6.1). The importance for autophagy in this pathway is further highlighted by observations in glioma showing it is essential for the induction of apoptosis (Salazar, Carracedo *et al*. 2009). Inhibition of several autophagy genes such as Atg1, Atg5 and Ambra-1 not only prevented the accumulation of autophagosomses but also inhibited THC-induced cell death revealing a novel and crucial role for this pathway in the ultimate induction of apoptosis (Salazar, Carracedo *et al*. 2009). Furthermore increasing evidence suggests autophagy mediated cell death by cannabinoids may involve the permeabilization of lysosomes (Velasco *et al* unpublished data) however whether or not this is a crucial step in THC-induced cell death remains to be determined.

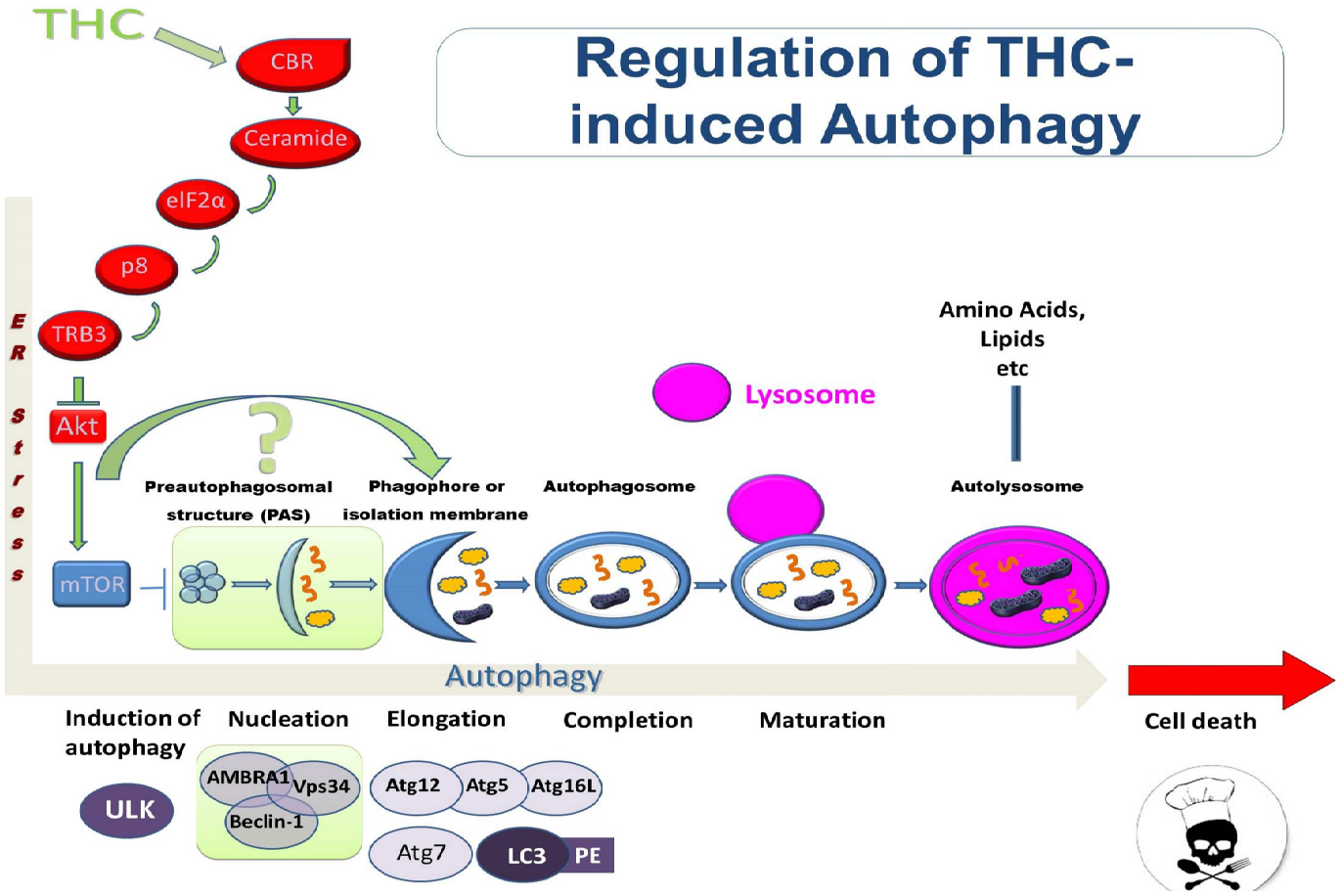

Figure 1.16: Molecular mechanisms induced by THC (Adapted from (Salazar, Carracedo et al. 2009).

THC-induced autophagy and apoptosis involves many steps for either to occur. Initiation of these events occurs by binding of THC to cannabinoid receptors CBR and the accumulation of ceramide leading to activation of the eIF2α axis of the ER stress pathway. P8 and TRB3 overexpression inhibits the Akt/mTOR pathway causing the induction of autophagy which is required for THC-induced cell death.

1.10 Lysosome membrane permeabilization and cell death

Lysosomes were originally identified by De Duve *et al* in 1949 (Nys, Aubert *et al*. 1949) who also coined their name and even in these early points of discovery that these organelles were implicated in cell death leading towards their description as 'suicide bags' (De Duve 1959). Since this initial observation further studies have demonstrated their molecular action in the cell death process revealing the requirement of lysosomal membrane permeabilization (LMP) (Boya and Kroemer 2008; Guicciardi, Deussing *et al*. 2000). LMP occurs when lysosomes become damaged inducing the release of many of the hydrolytic enzymes found within the lysosomal lumen including proteases, lipases, and nucleases (Boya and Kroemer 2008). Many different stimuli from differing pathways can cause lysosomal damage and LMP to occur which include (but is not limited to); p53, Bax, ROS, PLA2, caspases and bacteria and viral proteins (Figure 1.17) (Boya and Kroemer 2008). This release of lysosomal constitutents into the cytoplasm causes indiscriminate degradation within the cell which ultimately causes cell death. Depending on the degree of lysosomal damage and amount of LMP different modes of cell death may occur. Large scale lysosomal damage results in the cytoplasm becoming acidified causing necrosis, however, more typically LMP occurs as a partial and selective process where the released hydrolytic enzymes (particularly proteases) activate apoptosis through mitochondrial outer membrane permeabilization, cytochrome C release and subsequent activation of caspases (Guicciardi, Deussing *et al*. 2000). Most enzymes found within the lysosome function maximally at an acidic pH which is maintained within the lysosomal lumen by vacuolar-type H+-ATPases (V-ATPases) which regulates pH by pumping H+ ions into the lysosome (Izumi, Torigoe *et al*. 2003). As such many of these enzymes become inactive within the relatively neutral pH conditions of the cytoplasm. However, certain proteases including cathepsins remain active at neutral pH (Emert-Sedlak, Shangary *et al*. 2005; Sever, Altintas *et al*. 2007) and as such are able to exert there effects within the cytoplasm. Cathespsins are a family of proteases with approximately a dozen members each with distinguishable characteristics such as structure, catalytic mechanism and their protein substrates. They are typically cysteine, serine and aspartyl proteinases and their expression is often associated with different diseases including cancer (Nomura and Katunuma 2005). Of particular interest is cathepsin B which not only remains active at neutral pH but also has a clear function in apoptosis induction during LMP and is

often linked to cancer progression due to its ability to degrade cellular matrices and aid cell invasion (Nomura and Katunuma 2005). Cathepsin B, once released into the cytoplasm has been found to induce cytochrome C release and apoptosis (Guicciardi, Deussing *et al*. 2000). Interestingly it has been found that not all lysosomes are permeabilized at the same time (Ono, Kim *et al*. 2003) (although the reasons for this remain unknown) and cancer cells are more prone to LMP than normal cells. Conditions within these cells often result in large amounts of reactive oxygen species (ROS) being produced and it is these ROS which are able to induce LMP (Figure 1.17). However this effect is only able to occur in subcellular regions such as near the mitochondria due to the spatially limited range of action of ROS. Another mechanism particularly relevant to cancer cells including melanoma is due to the presence of iron within the lysosomes. Lysosomes are the main reservoir of chelatable iron within the cell which typically accumulates within these compartments upon the degradation of iron-containing proteins such as ferritin and cytochromes. It is iron which catalyses fenton reactions within lysosomes and this produces highly reactive pro-oxidants which may result in damage to the lysosomal membrane particularly if the levels of iron are too great (Link, Pinson *et al*. 1993; Mak and Weglicki 1985) (Figure 1.17). As a further point of interest, THC may also induce fenton reactions which can increase cell death in glioma cells as a result of oxidative stress (Goncharov, Weiner *et al*. 2005). Moreover, a link between melanoma and iron-containing proteins has been demonstrated through the increased expression of ferritin in melanoma cell compared to normal melanocytes, in which increased expression is associated with tumour progression through reduced sensitivity to oxidative stress (Baldi, Lombardi *et al*. 2005).

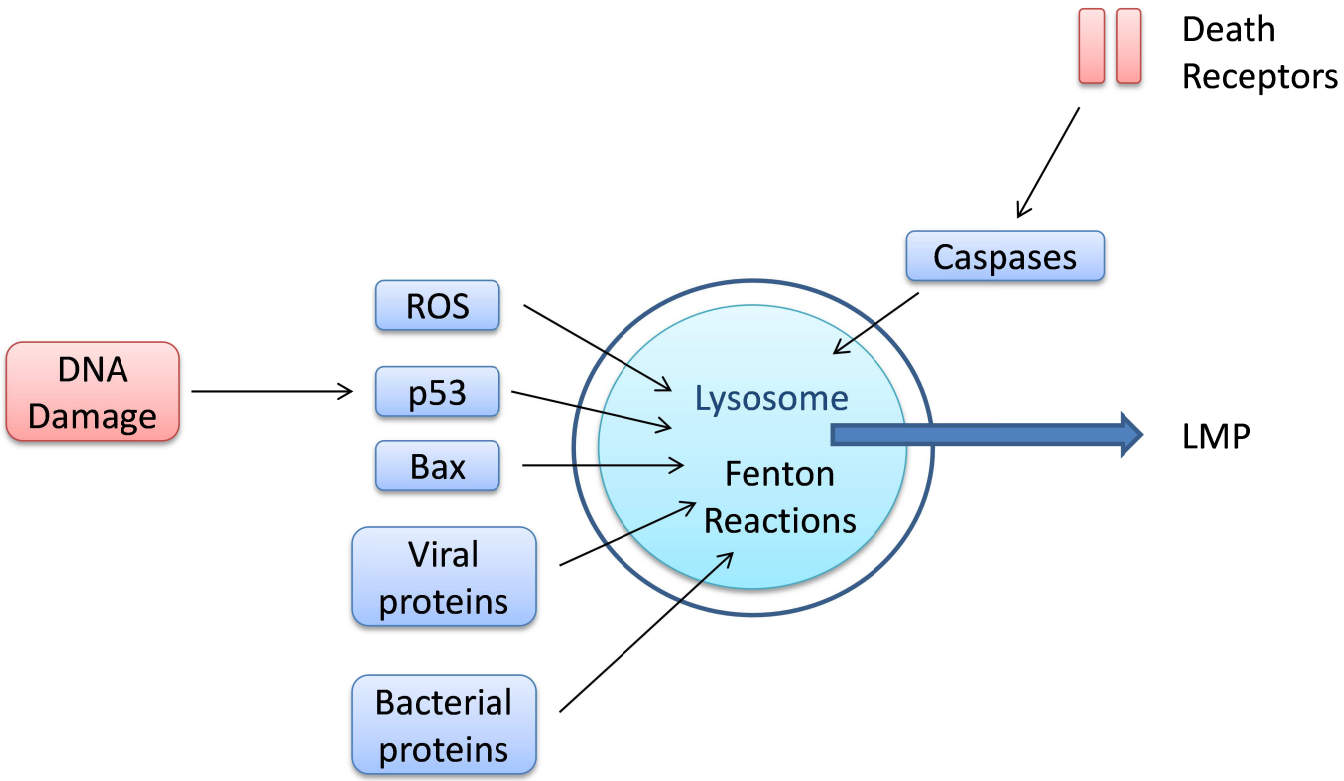

Figure 1.17: Pathways inducing LMP, adapted from (Boya and Kroemer 2008). Several factors inducing LMP have been identified including; bacterial proteins, viral proteins, Bax, p53, death receptor activated caspases, ROS and intracompartmental fenton reactions. Each factor causes the release of lysosomal constituents into the cytoplasm.

1.11 Aims of the Studies

With increasing evidence emerging linking autophagy and apoptosis, including interactions between anti-apoptotic Bcl-2 proteins Beclin-1, as well as both pro-survival and pro-death functions of this pathway in cancer, the importance of autophagy as a potential chemotherapeutic target is becoming ever more apparent. The principle aim of this study was therefore to determine how autophagy modulation in metastatic melanoma may be harnessed for clinical benefit.

To this the specific objectives were to:

- Determine the influence of oncogenic BRAF on Mcl-1 splice variant expression in melanoma and the effect of modulating Mcl-1 expression on drug-induced autophagy and cell death
- Decipher the contribution of autophagy to THC-induced cell death in comparison to canonical autophagy induction in response to bortezomib.
- Identify further potential molecular targets through the utilization of data mining software to determine possible underlying molecular events both upstream and downstream of autophagy induction.
- Determine the contribution of molecular targets identified through data mining software to identify potential molecular links between THC-induced autophagy and cell death.
- Decipher whether THC treatment causes the permeabilization of lysosomes as a mechanism of melanoma cell death and whether autophagy plays a role in this process.

Chapter 2: Materials and Methods

Chapter 2: Methods and Materials

Table of Contents

2.1: Growth and maintenance of melanoma cell lines

Human A375, SKMEL-23, SKMEL-28, CHL-1 and G361 melanoma cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich Co; Poole, UK) supplemented with 10% foetal calf serum (FCS; Sigma-Aldrich) culture medium and maintained in a humidified atmosphere of 5% CO_2 in air at 37°C. Each cell line was cultured continuously for up to 50 passages. A375 and CHL-1 cells stably expressing RFP-GFP-LC3 or A375 cells overexpressing Ambra 1 or β-Gal were cultured as above but only cultured for a maximum of 5 passages.

A375 cells stably transfected using short hairpin Ambra 1 RNA or with a short hairpin Ctrl RNAi (Kindly provided by Dr Marco Corazzari (University of Rome 'Tor Vergata', Italy) were maintained under selection with puromycin (2.5 μM) in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich Co; Poole, UK) supplemented with 10% foetal calf serum (FCS; Sigma-Aldrich). Frozen stocks of replacement cell lines were stored in liquid nitrogen in 10% dimethyl sulfoxide (DMSO, Fisher Scientific; New Jersey, USA) and 90% FCS.

Human primary melanocytes supplied by Professor Reynolds and colleagues were cultured in Medium 254 supplemented with Human Melanocyte Growth Supplement (Life Technologies; Invitrogen; Paisley, UK).

2.2: Treatment of melanoma cells with chemical agents

Prioir to all drug treatments, cells were seeded in 6 well plates (Helena Biosciences) at a density of 1.8 x 10^5 (A375) and 1.5 x 10^5 (CHL-1) cells per well, 12 well plates at a density of 0.71 x 10^5 (A375) and 0.5 x 10^5 (CHL-1) cells per well, or 96 well plates a density of 0.5 x 10^4 cells per well (A375, CHL-1). Cells treated with THC were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 0.5% foetal calf serum (FCS; Sigma-Aldrich) (THC culture medium) and allowed to attach overnight. For all other treatments conditions cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 10% foetal calf serum (FCS; Sigma-Aldrich) and allowed to attach overnight.

MEK inhibitor U0126 (Promega; Southampton, UK) was dissolved in DMSO at a concentration of 10 mM, stored at -20°C for 7 days and added to cell cultures at a final concentration of 10 μM (for 1 or 3 days; Helena Biosciences; Gateshead, UK) to cells seeded at a density of 1.05 x 10^6 cells per flask, with an equal volume of vehicle added to control cells.

Tetrahydrocannabinol (THC) (Kindly provided by Dr Guillermo Velasco; Complutense University, Madrid, Spain) was dissolved in DMSO at a concentration of 100 mM, stored at -20°C and added to cell cultures at 1-10 μM for initial optimisations and at final optimised concentrations of 4.5 μM or 5 μM (for 24 hours).

Bortezomib (Promega; Southampton, UK) initially dissolved in DMSO at a concentration of 10 mM and stored at -80°C was added to cell cultures at final concentrations of 10, 50 and 100nM (for 24 hours). Chloroquine (CQ; Sigma-Aldrich) was dissolved in nanopure H_2O at a concentration of 10 mM, stored under light protected conditions at room temperature and added to cell cultures at a final concentration of 10 μM (for 2 or 24 hours).

Cathepsin B inhibitor (CAO74-me) (Promega; Southampton, UK) was dissolved in DMSO at a concentration of 10 mM, stored at -20°C and added to cell cultures at a final concentration of 10 μM (for 24 hours).

Ceramide synthetase inhibitor (ISP-1) (Enzo life sciences (UK) Ltd, Exeter, UK) was dissolved in DMSO at a concentration of 10 mM, stored at -20°C and added to cell cultures at a final concentration of 5 μM (for 24 hours).

Bafimoycin (Sigma-Aldrich) was dissolved in DMSO at a concentration of 100 mM, stored at -20°C and added to cells cultured at a final concentration of 10 nM (for 24 hours).

The mTOR inhibitor Rapamycin (Sigma-Aldrich) was dissolved in DMSO at a concentration of 100 mM and added to cells cultured at a final concentration of 1 μM (for 24 hours).

The pan caspase inhibitor ZVAD-fmk (benzyloxycarbonyl-V-A-D(OMe)-fluoromethylketone) (Tocris Bioscience, Bristol, UK) was dissolved in DMSO at a concentration of 2 mM, stored at -20°C and added to cell cultures at a final concentration of 20 μM (for 24 hours).

2.3: Reverse-transcriptase polymerase chain reaction

Total RNA was isolated from melanoma cell lines using an RNAeasy Mini Kit according to manufacturer's specifications with included DNase digestion (Qiagen; Hilden, Germany). Reverse transcriptase polymerase chain reaction (RT-PCR) was performed using the Access RT-PCR system (Promega; Madison, USA) using primers for Mcl-1 (5'-GGACACAAAGCCAATGGGCAGGT-3' forward primer, 3'-GCAAAAGCCAGCAGCACATTCCTGA-5' reverse primer) (Gautrey and Tyson-Capper 2012) TRB3 (5'-GGACACAAAGCCAATGGGCAGGT-3' forward primer, 3'-GCAAAAGCCAGCAGCACATTCCTGA-5' reverse primer) or ß-actin (Armstrong, Ruiz *et al*. 2005). 0.15µg of RNA (for Mcl-1 amplification) 0.1µg (for TRB3 amplification) or 10ng (for ß-actin amplification) was used in each reaction. 35 cycles of PCR were performed for Mcl-1 and TRB3 under the conditions 95°C, 30s; 60°C, 1 minute, 72°C, 2 minutes and a final extension at 72°C, 7 minutes. 40 cycles of PCR were performed for ß-actin under the following conditions 95°C, 30s; 65°C, 1 minute; 72°C, 2 minutes; and a final extension at 72°C, 7 minutes. Control reactions (no RNA, no reverse transcriptase and positive control RNA (Promega)) were included in each experiment using the manufacturer's recommended specifications. PCR products were separated on a 2% (w/v) agarose gel and visualised by UV light upon ethidium bromide staining (10mg/ml) (Sigma, UK).

2.4: Immunohistochemistry for Mcl-1 expression in formalin-fixed paraffin-embedded (FFPE) melanoma cell lines

Melanoma cell line pellets were mixed with agar and then fixed in formalin and embedded in paraffin, prior to sectioning onto glass slides (performed by Cellular Pathology, Royal Victoria Infirmary, Newcastle Upon Tyne). 4 µM sections were subsequently de-paraffinised in Histoclear (AGTC Bioproducts; Hessle, UK) for 20 minutes before being rehydrated in 100%, 75% and 50% ethanol prior to distilled water for 5 seconds each. Antigen retrieval was achieved by microwaving in 10 mM sodium citrate (pH 6.0) (Mcl-1L/S antibody, Santa Cruz Biotechnology Inc; Delaware Avenue, USA; sc-819), or for optimisation of an Mcl-1L specific antibody (Abcam; Cambridge, UK; ab 25955) in 10 mM sodium citrate, 10 mM Tris-HCL (pH 7.6), 10 mM Tris-HCL (pH 9.0) or 10 mM citric acid (pH 6.0). Slides were then allowed to cool at room temperature for 20 minutes before washing for 3 minutes in

phosphate buffered saline (PBS; Sigma-Aldrich) containing 0.05% Tween-20 (T; Fisher Scientific). Slides were then incubated in 0.2% Triton X-100 in PBS/T for 10 minutes at room temperature, and washed in PBS/T prior to blocking endogenous peroxidase with 3% hydrogen peroxide (Vector Laboratories, Ltd; Peterborough, UK) in water. After a further 3 washes with PBS/T, sections were incubated in 2% serum in PBS/T (provided in a Vectastain Elite kit (Vector Laboratories, Ltd) for 20 minutes at room temperature. Sections were then incubated with primary antibodies to Mcl-1L/S (diluted 1:1000) or Mcl-1L (Abcam) (diluted 1:1000; 1:2000; 1:5000) diluted in PBS containing 2% bovine serum albumin (BSA) (Alpha Diagnostics; San Antonio, Texas, USA) for 1 hour at room temperature. After washing 3 times with PBS/T, sections were incubated for 30 minutes with biotinylated secondary antibodies (diluted 1:200 in 2% serum/PBS/T) (supplied with Vectorstain Elite kit) before washing 3 times in PBS/T. Sections were then incubated with Vectorstain Elite ABC reagent for 30 minutes at room temperature, washed 3 times with PBS/T, and incubated with VIP solution (Vector Laboratories, Ltd) for 10 minutes before rinsing in tap water for 5 minutes rehydrating in 75% and 100% ethanol for 5 seconds and finally cleaned in Histoclear for 2 minutes before mounting in di-n-butylPhthalate (DPX; Sigma-Aldrich Co). Mcl-1 and Mcl-1L staining was visualised by light microscopy using a Leica DFC310 *FX* microscope (Leica Microsystems; Milton Keynes, UK) and images captured with Leica QWin software.

2.5: Immunohistochemistry for Mcl-1 and Beclin-1 expression in formalin-fixed paraffin-embedded (FFPE) primary tissue

Immunohistochemical analysis of Beclin-1 and Mcl-1 in benign neavi or primary melanoma tissue was performed in formalin-fixed paraffin-embedded sections (prepared by Cellular Pathology, Royal Victoria Infirmary, Newcastle Upon Tyne). 5-6 µm sections were baked at 56°C overnight onto X-tra microscope slides (Leica Microsystems, Milton Keynes, UK). Sections were de-paraffinised in Histoclear (AGTC Bioproducts; Hessle, UK) for 20 minutes before being rehydrated in 100%, 75% and 50% ethanol prior to distilled water for 5 seconds each. Antigen retrieval was achieved by microwaving in 10 mM sodium citrate (pH 6.0) (Mcl-1L/S antibody, Santa Cruz Biotechnology Inc; Delaware Avenue, USA; sc-819; Beclin-1 antibody) for 12 minutes. Slides were then allowed to cool at room temperature for 20 minutes before washing for 3 minutes in phosphate buffered saline (PBS; Sigma-Aldrich)

containing 0.05% Tween-20 (T; Fisher Scientific). Slides were then incubated in 0.2% Triton X-100 in PBS/T for 10 minutes at room temperature, and washed in PBS/T prior to blocking endogenous peroxidase with 3% hydrogen peroxide (Vector Laboratories, Ltd; Peterborough, UK) in water. Endogenous Avidin was then blocked using Avidin solution of an Avidin/Biotin Blocking kit (Vector Laboratories Inc., Burlingame, USA) for 15 minutes, and then washed with PBS/T. Sections were then incubated with Biotin for 15 minutes and then washed with PBS/T. Protein was blocked by incubating sections in 2% blocking serum from an animal specific Vectastain Elite kit (Vector Laboratories Inc., Burlingame, USA) (Mouse for Beclin-1, Rabbit for Mcl-1) for 20 minutes at room temperature. Sections were then incubated with primary antibodies to Mcl-1L/S (diluted 1:1000) or Beclin-1 (BD transduction Lab, Oxford, UK; 612112) (diluted 1:100) diluted in PBS containing 2% bovine serum albumin (BSA) (Alpha Diagnostics; San Antonio, Texas, USA) for 1 hour at room temperature. After washing 3 times with PBS/T, sections were incubated for 30 minutes with animal specific biotinylated secondary antibodies (diluted 1:200 in 2% serum/PBS/T) (supplied with Vectorstain Elite kit) before washing 3 times in PBS/T. Sections were then incubated with Vectorstain Elite ABC reagent for 30 minutes at room temperature, washed 3 times with PBS/T, and incubated with VIP solution (Vector Laboratories, Ltd) for 10 minutes. Slides were rinsed in tap water for 5 minutes and counter stained with haematoxylin (Sigma Diagnostics, St.Louis, USA) for 2 minutes. After counter staining, slides were washed for 10 minutes in tap water with frequent changes. Slides were finally dehydrated using 75% and then 100% ethanol for 5 seconds and sections were cleaned for 2 minutes in histoclear. Slides were allowed to dry and coverslips were mounted with DPX mountant (VWR International Ltd., Poole, UK). Mcl-1 and Beclin-1 staining was visualised by light microscopy using a Zeiss Axioimager II (Carl Zeiss Microscopy, LLC, United States; 490022-0011-000) and images captured with Axioimager software.

2.6: Western blotting

Protein was extracted from melanoma cell lines into cell lysis buffer (0.1 M Tris-HCl (pH7.4), 25 mM NaF, 0.1 M NaCl, 2 mM EDTA (pH8), 1 mM benzamidine, 0.1 mM sodium orthoVanadate, 0.1% Triton-X100) (all supplied by Sigma-Aldrich Co) containing complete mini EDTA-free protease inhibitor cocktail (Roche, Manheim, Germany). Cell lysates were incubated on ice for 20 minutes and then probe sonicated for 3 X 5 seconds at 6 microns (Soniprep150, MSE, UK). Protein concentration was quantified using a commercial Bradford assay using Coomassie reagent (Pierce Biotech, Rockford, IL, USA) according to manufacturer's specifications in a 96-well flat bottomed plate (Helena Biosciences) and absorbance measured at 595nm using a SpectraMAX 250 plate reader (Molecular Devices Ltd; Wokingham, UK). Proteins were denatured in 4x sample buffer (0.25 M Tris-HCl (pH8.0), 8% sodium dodecyl sulphate (SDS), 40% Glycerol, bromophenol blue, 100 µl/ml ß-mercaptoethanol) (all supplied by Sigma-Aldrich) and separated by gel electrophoresis in 4-20% Tris-Glycine pre-cast gels (Life Technologies; Invitrogen) with Tris-Glycine (0.25M Tris Base; 1.92M Glycine) running buffer containing 1% SDS. Protein was then transferred onto Hybond-P transfer membrane (GE healthcare; Little Chalfont, UK) in Tris-Glycine buffer with 10% methanol (Fisher Scientific; Loughborough, UK). Membranes were subsequemtly washed in Tris buffered saline (TBS) containing 5% milk (OXOID; Basingstoke, Hertfordshire, UK) to block exposed hydrophobic sites. Membranes were then incubated with primary antibodies to Mcl-1L/S (1:4000; sc-819, Santa Cruz, Mcl-1L 40 kDa, Mcl-1S 35 kDa), BRAF (1:100,000; sc-5284, Santa Cruz, 86 kDa), phosphorylated ERK1/2 (P-ERK, 1:4000; 4377S, Cell Signalling/NEB (Hitchin, Hertfordshire), 42/44 kDa), ERK1/2 (1:4000; 9102, Cell Signalling/NEB, 42/44 kDa) Beclin-1 (1:2000; BD transduction Lab, 62 kDa), Atg7 (1:1000; sc-8668, Santa Cruz, 60kDa) Cleaved Caspase 3 (1:1000; 9661, Cell Signalling, 19/17 kDa), LC3-I/II (1:5000; 2775, Cell signalling, 18/16 kDa), Ambra 1 (1:1000; 26190002, Novus Biologicals (Cambridge, UK), 139 kDa) and ß-actin as a loading control (1:40,000; AC-74 Sigma Aldrich, 45 kDa), diluted in TBS / 5% non-fat milk (Mcl-1, BRAF, Beclin-1, Atg7, Ambra 1, ß-actin) or 5% BSA (Alpha Diagnostic Intl, Inc; San Antonio, Texas, USA) (P-ERK, ERK, LC3-I/II, Cleaved Caspase 3). To detect proteins, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Vector Laboratories, Ltd, Burlingame, CA, USA) diluted 1:4000 (Mcl-1L/S, P-ERK and ERK), 1:100,000 (BRAF), 1:1000 (Atg7, Ambra 1, Cleaved

Caspase 3), 1:2000 (Beclin-1) 1:5000 (LC3-I/II) or 1:40,000 (ß-actin), and visualised using the ECL-plus system (GE healthcare) and photographic film using a developer (MINIMED90 X-RAY film processor; photon imaging systems, AFP imaging corp., Elmsford, NY, USA). Densitometric quantification of signal intensity was performed using FluorcChem software (Alpha Inntoech; San Leandro, CA, USA) and quantified as described in section 2.13

2.7: Plasmid preparation and restriction enzyme digestion

The expression vectors pEFm-BRAF^{wt} and pEFm-BRAF^{V600E} (provided by Professor Richard Marais, Institute of Cancer Research, London), pcDNA4 or pcDNA3.1-Mcl-1S (Fig. 3.1, provided by Dr Jeng-Jer Shieh, National Hsing University Taichung, Taiwan) were transformed into competent E.coli (OneShot TOP10 chemically competent E.coli, Life Technologies; Invitrogen) using a standard heat shock procedure (according to supplier protocol), and selected by ampicillin (50µg/ml and isolated using a HiSpeed plasmid Maxi kit (Qiagen) according to manufacturer's instructions. pcDNA3.1-Mcl-1S was subsequently digested using HindIII (Roche; Hertfordshire, UK) and EcoRV (Fermentas; St. Leon-Rot, Germany) using SuRE Cut buffer B (Roche) for 2 hours at 37°C and analysed by agarose gel electrophoresis (0.8% agarose).

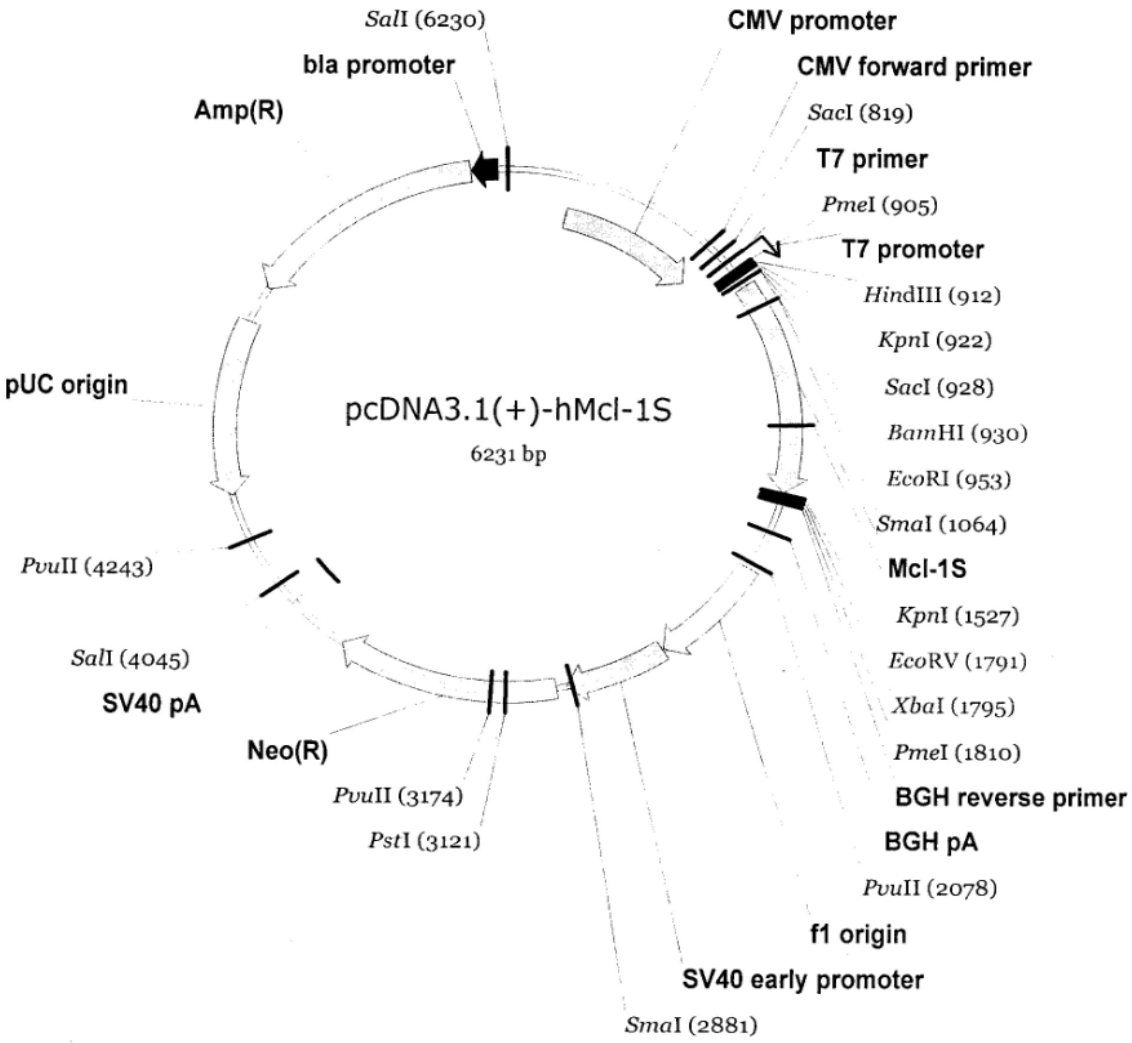

Figure 2.1: pcDNA3.1Mcl-1S plasmid map

2.8: Transient transfection of $BRAF^{V600E}$ and Mcl-1S in human melanoma cells

For over-expression of $BRAF^{wt}$ or $BRAF^{V600E}$, CHL-1 melanoma cells were seeded in 25 cm^2 tissue culture flasks (Helena Biosciences) at a density of 1 x 10^6 and allowed to adhere overnight. Cells were then washed with PBS and the medium replaced with 5 ml of OPTIMEM (Life Technologies; Invitrogen) transfection medium prior to transfection for 6 hours with 5 µg pEFm-$BRAF^{wt}$ and pEFm-$BRAF^{V600E}$ and 15 µl lipofectamine 2000 (Life Technologies; Invitrogen) in accordance with the manufacturer's instructions. Transfection was terminated by replacing the OPTIMEM with culture medium. Cells were re-seeded 20 hours later at a density of 0.5 x 10^6 cells/25 cm^2 flask and harvested after a further 48 hour. BRAF over-expression and downstream activation of ERK was verified by Western blot analysis.

For over-expression of Mcl-1S, A375 cells were seeded in 6-well tissue culture plates (Helena Biosciences) at a density of 4 x 10^5 cells/well and allowed to adhere overnight. Cells were then washed with PBS and the medium replaced with 2 ml of OPTIMEM prior to transfection for 6 hours with 0-5 µg pcDNA3.1-Mcl-1S and 0-8 µl lipofectamine 2000 (Life Technologies; Invitrogen) in accordance with the manufacturers instructions. Cells were re-seeded 20 hours later at a density of 2 x 10^5 cells/well and harvested after a further 48 hour. Mcl-1S over-expression was verified by Western blot analysis.

2.9: Transient RNAi-mediated knockdown of Mcl-1, Beclin-1, Atg7 and TRB3 in melanoma cell lines using reverse transfection.

A375 or CHL-1 cells were transfected using siRNAs; Mcl-1 (M-004501-08-0005) (Thermo Scientific, Loughborough, UK), Beclin-1 (HSS112741, HSS112742) (Life Technologies; Invitrogen, Paisley, UK), Atg7 (HSS116182, HSS173705) (Life Technologies; Invitrogen, Paisley, UK), TRB3 (sc-44426) (Santa Cruz) and validated using negative control siRNA (Qiagen AllStars Negative Control siRNA/Life Technologies; Invitrogen Negative Control Low GC Duplex siRNA); at the point of seeding in a volume of 2 ml culture medium in 6 well plates or 0.8ml culture medium to achieve a desired cell density of 50-80%, which corresponds approximately to 1.5 x 10^5 cells per well for CHL-1 cells and 1.8 x 10^5 cells per

well for A375 cells. For transient transfection, siRNA was transfected using lipofectamine RNAiMAX according to the manufacturers' specifications in 0.5ml (6 well plate) or 0.2ml (12 well plate) serum-free Opti-MEM growth medium (Life Technologies; Invitrogen Ltd) to given final concentrations of 2-4 nM siRNA. The siRNA containing Opti-MEM was then added to 2ml (6 well plates) or 0.8ml (12 well plates) of complete culture medium with the appropriate cell concentrations to a final volume of 2.5ml (6well plates) or 1ml (12 well plates) and incubation was continued overnight before knockdown of the target gene was verified by Western blot analysis 24 hours after transfection or subsequent cell viability, death or autophagy assays performed.

2.10 Cell Viability Assays

MTT *(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)* or MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assays were used as ameasure of celll viability For MTT assays, SK-MEL-28 or A375 cells were seeded at $0.71x10^5$ cells/well and CHL-1 cells seeded at $0.5\ x10^5$ cells/well into 12 flat well tissue culture plates (Corning Inc, Corning, NY) in a volume of 1 ml of culture medium and allowed to adhere overnight. Cells were treated using THC in the presence or absence of CQ, CathBi, Bafilomycin, ZVAD or ISP (see 2.2) or following RNAi-mediated knockdown studies (see 2.9). Cell viability was assessed by the addition of 100µl of Thiazolylbluetetrazolium at a concentration of 5mg/ml and incubation for 4hours with the absorbance measured at 570nm using a SpectraMAX 250 plate reader.

For MTS assays SK-MEL-28, A375, or CHL-1 cells were seeded at $0.5x10^4$ cells/well and cultured in 100µl complete media in a 96 well plate (Corning Inc) and allowed to adhere overnight. Following drug treatment, cell viability, was assessed by the addition of 20 µl of MTS to each well and incubation continued for a further 4hours before absorbance was measured at 490nm using a SpectraMAX 250 plate reader.

2.11 Immunofluorescence

Melanoma cell lines were seeded in 12 well plates (Helena Biosicnces) on sterile glass cover slips at a density of 0.71 x 10^5 (A375, SKMEL-28) or 0.5 x 10^5 (CHL-1) cells per well in a volume of 1ml media and allowed to attach for 24 hours prior to chosen treatment conditions. After treatment, cells were fixed in 100% ice cold methanol for 10 minutes and subsequently washed three times with 1ml PBS for 1 minute. Cells were stored at 4°C for up to two weeks for immunofluorescence analysis.

Analysis of Cyctochourome C release as well as Mcl-1 expression levels in *BRAF*^{V600E} mutated overexpressing CHL-1 cells was performed using the following immunefluorescence protocol. After permeabilisation for 10 min with 0.2% Triton-X100 (Sigma-Aldrich) in phosphate buffered saline (PBS) and two washes with 1ml PBS containing 0.05% Tween 20 (PBS/T; Sigma-Aldrich) cells were immersed in 10% goat serum in 1ml PBS/T for 10 min to minimise non-specific binding before treatment with primary antibodies to BRAF (Santa Cruz), Mcl-1 (Santa Cruz) or Cytochourome C (BD Biosciences). BRAF, and Cytochourome C staining was detected with a secondary Oregon green-conjugated goat anti-rabbit secondary antibody (Life Technologies; Invitrogen Ltd) diluted 1:200 in PBS/T containing 2% bovine serum albumin (BSA; Sigma-Aldrich), Mcl-1 was detected with secondary 633-conjugated goat anti-mouse secondary antibody (Alexfluor,) and cytochrome C staining was detected with a secondary goat anti-rabbit secondary antibody (Life Technologies; Invitrogen Ltd) diluted 1:200 in PBS/T containing 2% bovine serum albumin (BSA; Sigma-Aldrich) for 1 hour at room temperature. Stained sections were mounted in Vector Shield containing DAPI (Vector Laboratories; Peterborough, UK). For Cytochourome C staining RNase was was added for 20 minutes after three 2 minute washes in 1ml PBS-T post secondary antibody incubation before counterstaining nuclei for 15 min with TOTO-3 iodide (Life Technologies; Invitrogen Ltd) diluted 1:7,000 in PBS/T containing 2% BSA. All slides were analysed by confocal microscopy using a Leica TCS SP II laser scanning confocal microscope with images captured and processed using LCS Lite 2.61 software (Leica Microsystems; Heidelberg, Germany).

Analysis of cathepsin B release and co-localisation with lysosomal associated membrane protein 2 (LAMP2) was performed using Immunofluorescence double staining approach for primary antibodies raised from the same species (Mouse). Cells fixed to cover

slips were washed two times with 1ml PBS containing 0.05% Tween 20 (PBS/T; Sigma-Aldrich). The first serum blocking step was performed by immersing fixed cells with 2% goat serum/2% BSA in PBS for 30 minutes to minimise non-specific binding before treatment with mouse anti-cathepsin B antibody (Calbiochem; IM27L) diluted 1:200 in 2% goat serum/2%BSA in PBS for 1 hour at room temperature. After three 2 minute washes with 1ml PBS/T, Cathepsin B staining was detected using Oregon green-conjugated goat anti-mouse secondary antibody (Life Technologies; Invitrogen Ltd) diluted 1:200 in PBS. The second serum blocking was performed by incubating fixed cells for 1 hour using MOM (Mouse on Mouse) reagent (MKB-2213) (Vector Laboratories; Peterborough, UK) (2 drops in 5ml PBS) to block free binding sites of the anti-mouse immunoglobulin. Additional blocking was performed by immersing fixed cells in 2% goat serum/2% BSA in PBS for 30 minutes to further minimise non-specific binding before addition of the second primary antibody mouse antibody LAMP2 (BD; (Oxford, UK) 555803) which was added to the fixed cells diluted 1:200 in 1% goat serum/1% BSA in PBS for 1 hour at room temperature. LAMP2 staining was detected with secondary 633-conjugated goat anti-mouse secondary antibody (Life Technologies; Invitrogen, A-21052) diluted 1:200 for 1 hour at room temperature. Stained cells were washed and dehydrated in 100% ethanol before mounting cover slips onto slides with in Vector Shield containing DAPI (Vector Laboratories) to counterstain cell nuclei. Cells were analysed by confocal microscopy using a Leica TCS SP II laser scanning confocal microscope with images captured and processed using LCS Lite 2.61 software (Leica Microsystems; Heidelberg, Germany).

2.12: Analysis of autophagic flux in dual tagged mRFP-GFP-LC3 melanoma cells

Human A375 and CHL-1 melanoma cell lines stably expressing LC3 fluorescently tagged with red fluorescent protein (RFP) and green fluorescent protein (GFP) (mRFP-GFP-LC3) were kindly provided Dr Marco Corazzari and seeded in 12 well plates at a density of 0.71×10^5 (A375) and 0.5×10^5 (CHL-1) cells per well on cover slips prior to treatment with THC at optimised concentrations (section 2.2) for 18 hours and fixation for 10 minutes with 10% paraformaldehyde. Cells on cover slips were mounted in Vector Shield containing DAPI

(Vector Laboratories). Cellular mRFP-GFP-LC3 fluorescence was visualised using Zeiss AxioImager II (Carl Zeiss Microscopy, LLC, United States; 490022-0011-000) and images captured using the supplied Axioimager software. Autophagy induction was assessed by the puntal staining patterns observed for degradation of LC3-GFP signal and increased LC3-RFP signal. This assay allows for measurement of autophagic flux by pH changes occurring during autophagy in which autophagosomse/lysosome fusion occurs which eliminates signals emitted by GFP resulting in RFP signals alone.

2.13: Molecular visualisation software for Beclin-1 protein-protein interactions

Beclin-1 protein-protein interactions were pooled and identified from several articles using the cytoscpae plugin provided by Biological General Repository for Interaction Datasets (BioGRID) and the data downloaded directly from the BioGRID website for visualisation (http://thebiogrid.org/). Beclin-1 protein-protein interactions were visualised and organised into cellular processes of relevance to the project using an open source bioinformatics software platform Cytoscape (http://www.cytoscape.org/).

2.14: Statistical analysis

Protein expression data were analysed by cell line (CHL-1, SKMEL-23, A375,G361) or transfection type (Control (non-transfected), $BRAF^{WT}$, $BRAF^{V600E}$) with the use of one-way ANOVA and Dunnett's post-hoc correction, using SPSS Release 15 (SPSS Inc.). For Mcl-1L expression analysis, Mcl-1 band intensity was normalised to ß-actin band intensity (Mcl-1/ß-actin) for each cell line and expressed relative to the mean Mcl-1/ß-actin value (across all cell lines) for each individual experiment.

Autophagy induction was determined and statistically analysed using LC3-II In *vitro* protein expression data normalised to ß-actin band intensity (LC3-II/ß-actin) for each cell line expressed relative to the mean LC3-II/ß-actin value (across all cell lines) for each individual experiment. Variation in expression levels were analysed between RNAi-mediated knockdown and treatment conditions in A375 and CHL-1 melanoma cell lines with the use of one-way ANOVA and Dunnett's post-hoc correction, using SPSS Release 15 (SPSS Inc.).

Statistical analysis of all MTT/MTS proliferation data was undertaken using GraphPad Prism 5 (GraphPad Software; San Diego, USA) software and all representative graphs were created using this software. Significance is indicated as P values of $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ *** thouroughout based on a minimum of 3 replicates with the use of one-way ANOVA and Dunnett or Bonferonni post-hoc correction.

Mcl-1 *In vitro* expression levels analysed by immunocytochemistry were determined using Image J analysis software (National Institute of Health; Bethesda, Maryland, USA) and variation in expression levels was established using one-way ANOVA with Dunnett's post-test correction.

Chapter 3: The role of Mcl-1 in autophagy and melanoma

Chapter 3: The role of Mcl-1 in autophagy and melanoma

Table of Contents

3.1 Introduction

Current research suggests tumour cell resistance to chemotherapy-induced apoptosis may be overcome by manipulation of the autophagy pathway (Armstrong, Corazzari *et al*. 2011; Degtyarev, De Mazière *et al*. 2008; Salazar, Carracedo *et al*. 2009; Shrivastava, Kuzontkoski *et al*. 2011). Additionally recent reports have implicated anti-apoptotic members of the Bcl-2 family of proteins (crucial to the regulation of apoptosis) in the regulation of autophagy (Feng, Huang *et al*. 2007), thereby presenting potential therapeutic targets involved in both processes. The anti-apoptotic Bcl-2 family member Mcl-1 is of particular interest as it is highly expressed in both primary and advanced melanoma and has previously been shown to be essential for melanoma survival (Tang, Tron *et al*. 1998). The gene encoding Mcl-1 contains 3 exons encoding two splicing variants, Mcl-1L and Mcl-1S, with opposing roles. Mcl-1L is a polypeptide of 350 amino acids in length and 40 kDa in molecular weight (Kozopas, Yang *et al*. 1993) which possesses Bcl-2 homology domains 1-3 and transmembrane domains that are found in other Bcl-2 family members, as well as a unique PEST sequence (a peptide sequence rich in proline, glutamic acid, serine, and threonine) within its N terminus (Yang, Kozopas *et al*. 1995). Mcl-1S is encoded by alternative splicing of Mcl-1 pre-mRNA which eliminates the second exon resulting in the translation of a polypeptide of 271 amino acids in length with a molecular weight of 35 kDa (Bingle, Craig *et al*. 2000). Mcl-1S protein has a 41 residue difference to Mcl-1L at the C-terminus and only has a BH3 domain, similar to pro-apoptotic BH3-only members of the Bcl-2 family. Mcl-1S is thought therefore to antagonise the pro-survival role of Mcl-1L, promoting cells to enter apoptosis (Bae, Leo *et al*. 2000; Bingle, Craig *et al*. 2000). To date, however, the differential expression of each Mcl-1 splice variant in melanoma has remained undefined, and which may be important in terms of mitogen activated protein kinase (MAPK) signalling (Huang, Huang *et al*. 2000), a pathway frequently constitutively activated in melanoma through hyperactivating mutations in the BRAF protein kinase and which contribute to apoptotic resistance and tumour progression (Davies, Bignell *et al*. 2002; Dong, Phelps *et al*. 2003; Uong and Zon 2009). Activation of ERK1/2 stabilises Mcl-1 by phosphorylation of the protein and Mcl-1 over-expression has been shown to correlate with hyper-activation of ERK1/2 signalling (Boucher, Morisset *et al*. 2000). Additionally Mcl-1L has

been shown to interact directly with Beclin-1 (a BH3 only Bcl-2 family member involved in the nucleation step of autophagy) suggesting a potential role for Mcl-1 in autophagy regulation (Feng, Huang *et al*. 2007).

The aim of this chapter was therefore to characterise the expression levels of Mcl-1 and its splice variants in metastatic melanoma cells *in vitro* and *in vivo* and establish whether there is a relationship between differing splice variant expression and constitutive activation of MAPK signalling, as well as to investigate the effect of manipulating Mcl-1 expression on drug-induced autophagy and cell death in response to the autophagy-inducing therapeutic agents, bortezomib and Tetrahydrocannabinol (THC).

3.2: Results

3.2.1: Mcl-1 mRNA is increased in metastatic melanoma cells compared to expression in primary melanocytes *in vitro.*

Reverse transcription PCR (RT-PCR) was performed in order to analyse the differential expression of Mcl-1L and Mcl-1S in primary human melanocytes as well as in metastatic melanoma cell lines with differing *BRAF* mutational status, specifically $BRAF^{V600E}$ mutated metastatic A375, G361 and $BRAF^{WT}$ CHL-1 and SKMEL-23 cells. A significant increase in Mcl-1L, but not Mcl-1S, mRNA expression was seen in melanoma cell lines compared to primary melanocytes, with the highest expression of Mcl-1L observed in $BRAF^{V600E}$ mutated cells (Figure 3.1 B&C).

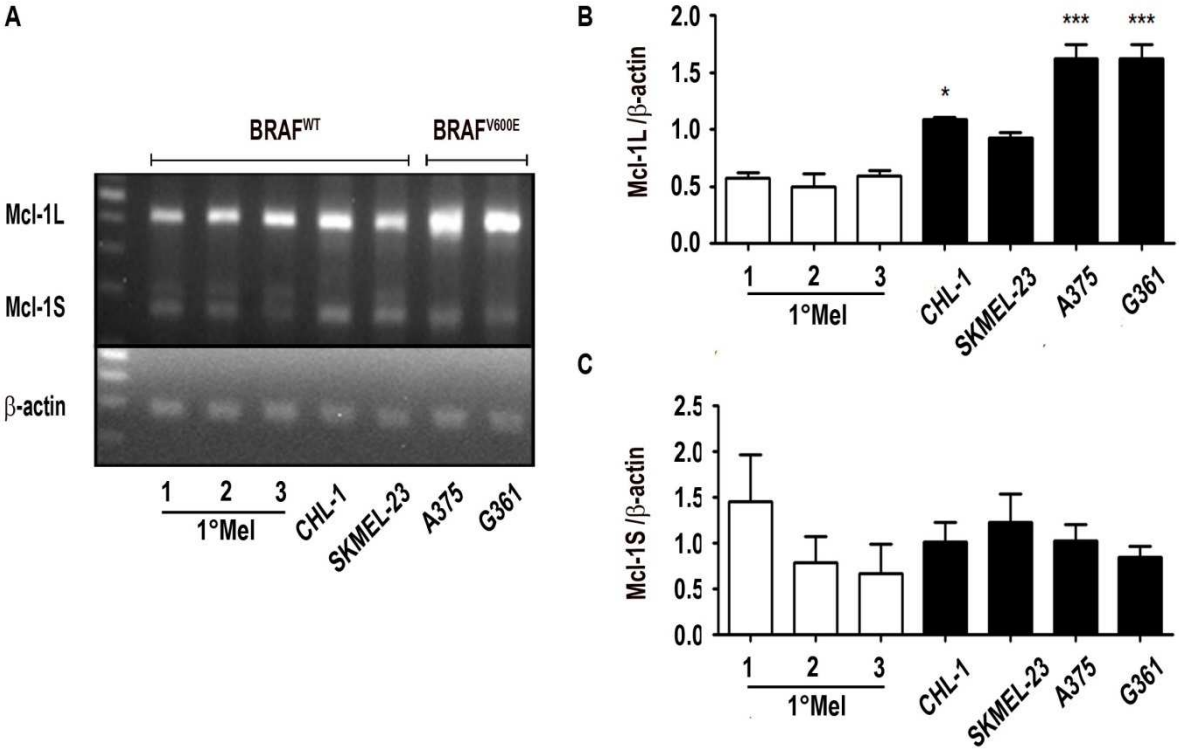

Figure 3.1: Mcl-1L mRNA expression in metastatic melanoma cell lines and primary melanocytes. (A) RT-PCR of Mcl-1L, Mcl-1S and β-actin mRNA expression in primary melanocytes (1° Mel samples 1-3), $BRAF^{WT}$ CHL-1 and SKMEL-23 or *BRAF* mutated A375 and G361 human metastatic melanoma cell lines. Each bar represents (B) Mcl-1L and (C) Mcl-1S band intensity normalised to β-Actin loading control (mean +/- SEM, n=3). Statistics were acquired using one way ANOVA with Dunnet's post-hoc test * $p< 0.05$, *** $p<0.001$ comparing Mcl-1 expression levels in melanoma cell lines to Mcl-1 expression in primary melanocytes.

3.2.2: Mcl-1 protein expression is increased in BRAF mutated compared BRAF wild-type metastatic melanoma cell lines.

Analysis of total Mcl-1 protein expression by immunocytochemistry of formalin-fixed and paraffin-embedded (FFPE) cell pellets derived from metastatic melanoma cell lines revealed increased expression of Mcl-1 in *BRAF*^{V600E} mutated G361 cells compared to expression in *BRAF*^{WT} CHL-1 cells (Figure 3.2).

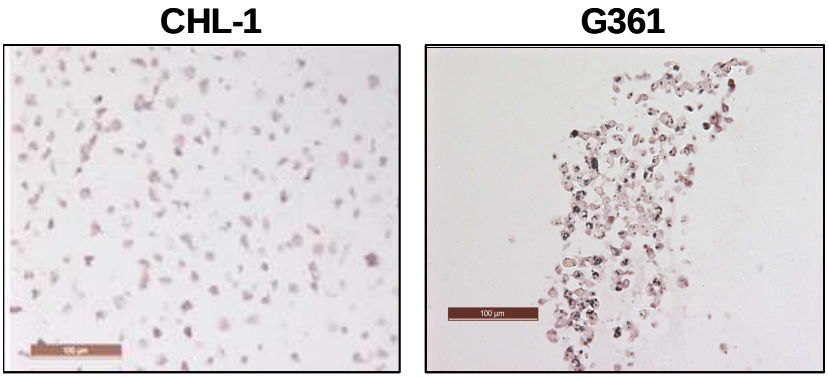

Figure 3.2: Mcl-1 expression is increased in BRAF mutated metastatic melanoma cells. Immunoctyochemical analysis of Mcl-1 expression in *BRAF*^{WT} CHL-1 and *BRAF*^{V600E} mutated G361 metastatic melanoma cell lines.

As it was not possible to measure Mcl-1 splice variant expression by immunohistochemistry using a currently available antibody for Mcl-1L splice variant detection (data not shown), protein was isolated from human metastatic melanoma cell lines CHL-1, SKMEL-23, A375 and G361 and expression of both Mcl-1L and Mcl-1S determined by Western blotting using an antibody able to detect both splice variants.

Results demonstrated significantly increased expression of Mcl-1L in $BRAF^{V600E}$ A375 and G361 cells compared with expression in $BRAF^{WT}$ cell lines, CHL-1 and SKMEL-23 (Figure 3.3B). Additionally, membranes derived from the Western blot analysis of Mcl-1L were over exposed in order to detect Mcl-1S protein. Results demonstrated Mcl-1S expression was detectable in *$BRAF^{V600E}$* mutated A375 and G361 cells while little or no expression was evident in *$BRAF^{WT}$* CHL-1 or SKMEL-23 cells (Figure 3.3A).

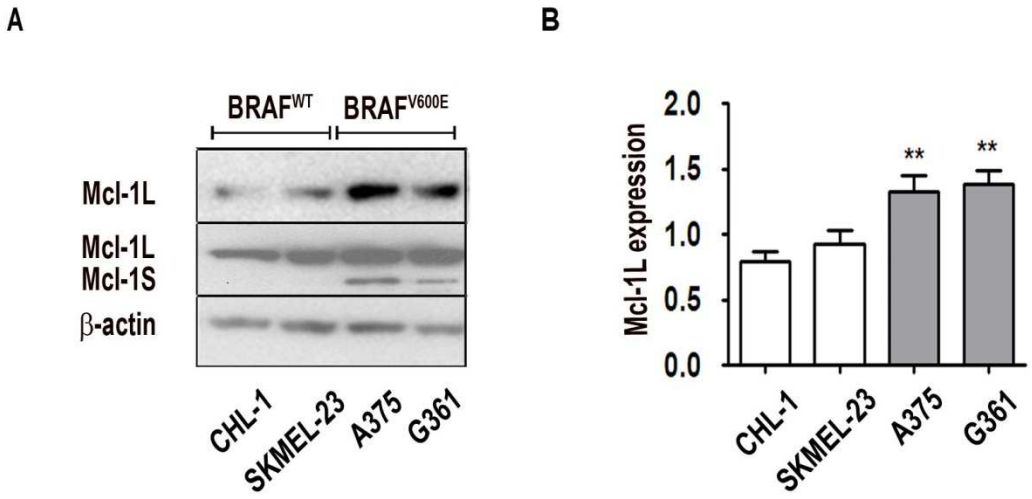

Figure 3.3: Mcl-1L expression is increased in BRAF mutated metastatic melanoma cell lines. (A) Western blot of Mcl-1L, Mcl-1S and β- actin expression in *$BRAF^{WT}$* CHL-1 and SKMEL-23 cells and *$BRAF^{V600E}$* mutated A375 and G361 cells. (B) Each bar represents Mcl-1L band intensity normalised to β-actin and expressed relative to Mcl-1L expression in CHL-1 cells (mean ± SD, n=3). Statistical analysis was performed using one-way ANOVA, with Dunnett's post-hoc test, ** $p < 0.01$ comparing Mcl-1 expression levels in *$BRAF^{V600E}$* melanoma cell lines to expression in *$BRAF^{WT}$* melanoma cell lines.

3.2.3: Oncogenic BRAF signalling increases Mcl-1L expression

In order to further assess the influence of hyperactivated BRAF signalling on the differential expression of Mcl-1L and Mcl-1S, *BRAF*^{WT} or *BRAF*^{V600E} were transiently transfected into BRAF^{WT} CHL-1 cells and levels of Mcl-1L and Mcl-1S expression levels determined by Western blotting. Over-expression of both BRAF^{WT} and BRAF^{V600E} was confirmed by the increased expression of BRAF (Figure 3.4A). Activation of BRAF signalling was confirmed by increased phosphorylation of ERK for BRAF^{WT} and BRAF^{V600E} over-expressing cells, with higher levels of ERK phosphorylation observed in cells over-expressing BRAF^{V600E} (Figure 3.4A). Results demonstrated Mcl-1L expression was increased following over-expression of either BRAF^{V600E} or BRAF^{WT}, and was significantly increased by over expression of BRAF^{V600E} relative to levels in control untransfected CHL-1 cells $p < 0.05$, (Figure 3.4B). Additionally Mcl-1S was also increased following over-expression of either BRAF^{V600E} or BRAF^{WT}, and further increased by over-expression of BRAF^{V600E}, relative to levels in control untransfected cells.

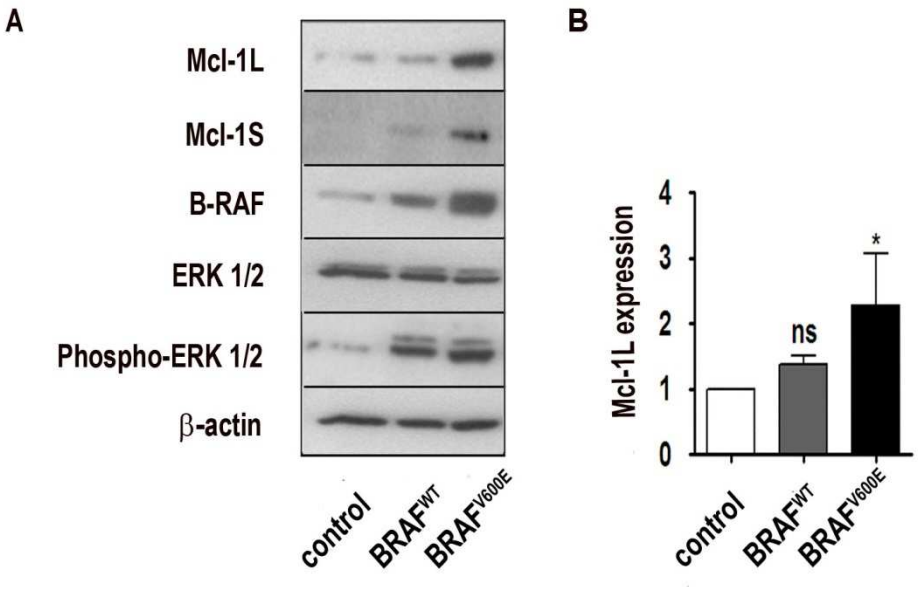

Figure 3.4: Oncogenic BRAF increases Mcl-1L/S expression. (A) Western blot for Mcl-1L/S, BRAF, phosphorylated ERK 1/2 (P-ERK 1/2), ERK 1/2 and β-actin expression in *BRAF* wild-type CHL-1 cells or in CHL-1 cells transiently transfected for 3 days with $BRAF^{WT}$ or $BRAF^{V600E}$. (B) Each bar is relative to Mcl-1L expression levels in non-transfected control CHL-1 cells (mean ± SEM, n=3). Statistics were acquired using one way ANOVA with Dunnett post-hoc test, * $p< 0.05$, ns - not significant comparing $BRAF^{V600E}$ and $BRAF^{WT}$ transfected CHL-1 cells to control un-transfected CHL-1 cells.

The ability of oncogenic BRAF signalling to increase Mcl-1 expression was also confirmed by immunofluorescence (IF) studies. Results demonstrated transfection of $BRAF^{WT}$ CHL-1 cells with $BRAF^{V600E}$ induced a small but significant increase in the expression of Mcl-1, compared to cells transfected with $BRAF^{WT}$ (Figure 3.5). Mcl-1 Nuclear localisation was also observed in cells transfected with $BRAF^{V600E}$.

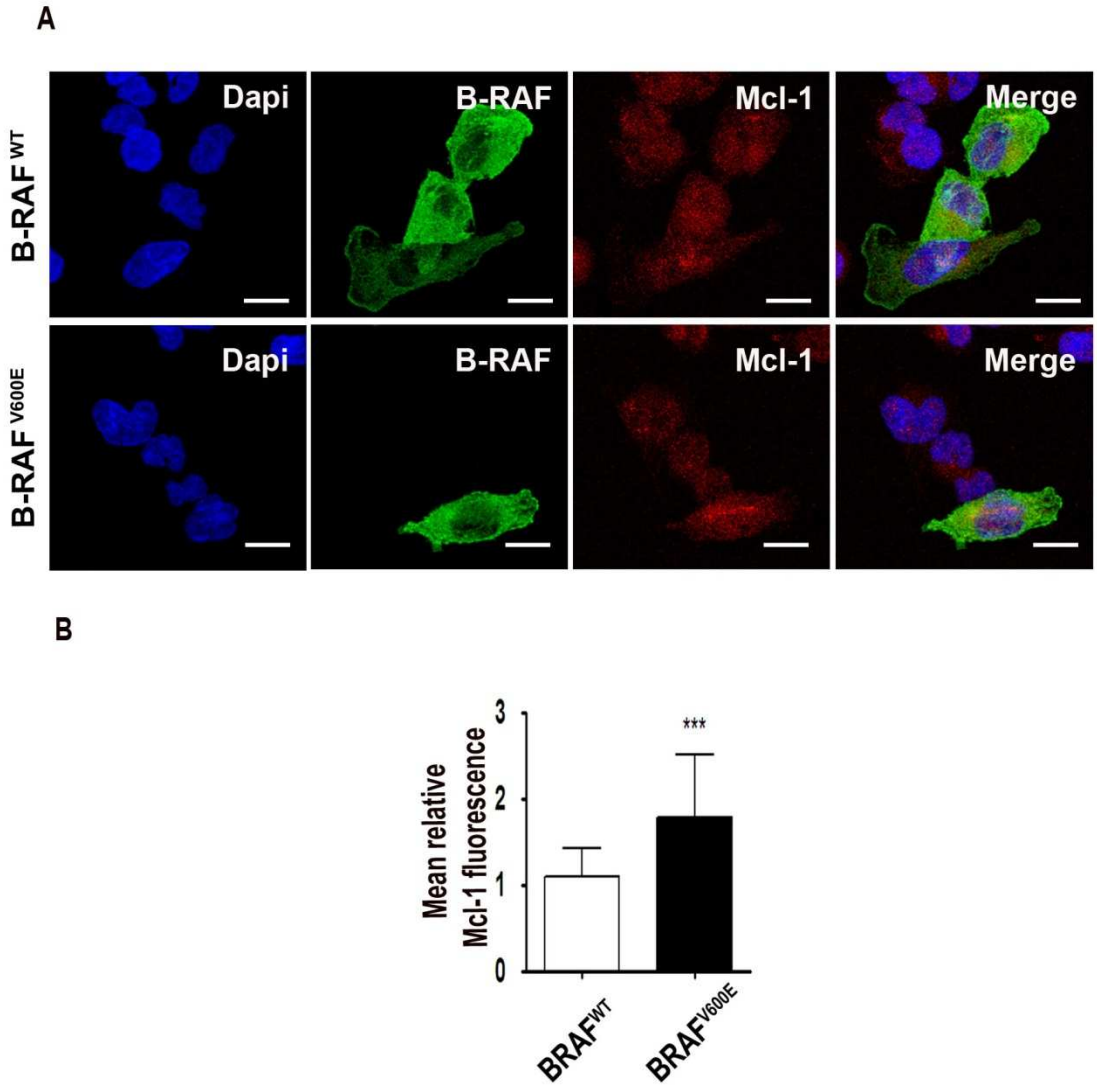

Figure 3.5: Oncogenic BRAF increases Mcl-1 expression compared to wild-type BRAF. (A) Immunofluorescence for Mcl-1 (Red) and BRAF (Green) expression in *BRAF* wild-type in CHL-1 cells transiently transfected for 3 days with $BRAF^{WT}$ or $BRAF^{V600E}$. (B) Relative Mcl-1 expression in CHL-1 cells transiently transfected for 3 days with $BRAF^{WT}$ or $BRAF^{V600E}$. Each bar is a representative of 54 and 52 transfected cells. Statistics were acquired by t-test analysis, *** $p < 0.001$ comparing $BRAF^{V600E}$ transfected CHL-1 cells to $BRAF^{WT}$ transfected control CHL-1 cells.

3.2.4 Expression of Mcl-1L/S in metastatic melanoma tumours *in vivo*

To assess Mcl-1 splice variant expression levels *in vivo*, protein was isolated from metastatic melanoma tumour samples derived from 8 patients with advanced stage melanoma and the expression of Mcl-1L or S evaluated by Western blotting. Each tumour sample was also genotyped by SNP analysis of the most common *BRAF* (V600E and V600D) and *NRAS* mutations (Q61K and Q61R) as previuously described (Hiscutt, Hill *et al*. 2010).

Results demonstrated differential expression of both Mcl-1L and Mcl-1S in melanoma tumours derived from patients however, no correlation with *BRAF/NRAS* mutational status was observed (Figure 3.6). As the data derived was from a panel of only 8 tumours it remains unclear as to whether or not Mcl-1 expression definitively correlates with *BRAF/NRAS* mutational status in melanoma *in vivo*.

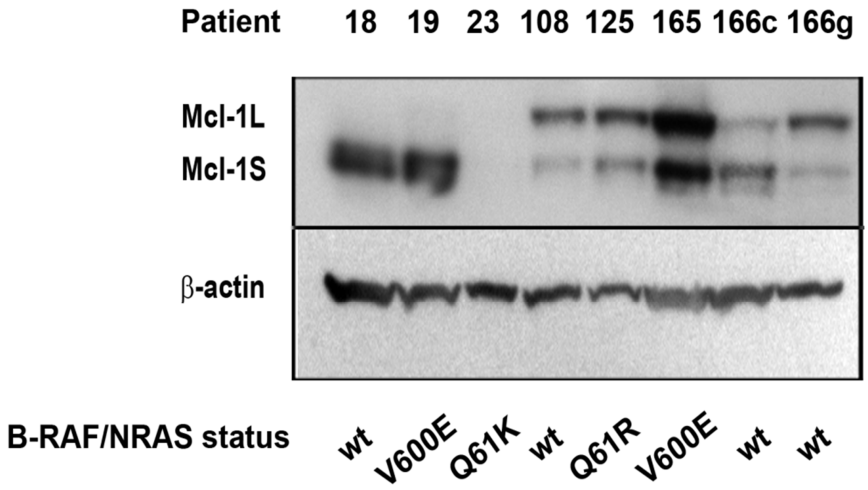

Figure 3.6: Expression of Mcl-1 splice variants in 8 metastatic melanoma tumours. Western blot for Mcl-1L, Mcl-1S and ß-actin in a small panel of *BRAF/NRAS* wild-type (WT), *BRAF* mutated (V600E) or *NRAS* mutated (Q61K) (Q61R) metastatic melanoma tumours.

3.2.5: RNAi-mediated knockdown of Mcl-1 increases THC and bortezomib-induced autophagy and cell death

As activating mutations in *BRAF* increases the expression of both Mcl-1L and Mcl-1S in *vitro,* identification of a functional role for Mcl-1 in the regulation of autophagy and cell death was undertaken. This was achieved by modulation of Mcl-1 expression either through RNAi-mediated knockdown or through transient over-expression of Mcl-1S, prior to determining the effect on drug-induced autophagy and apoptosis in response to bortezomib or Tetrahydrocannabinol (THC). RNAi-mediated knockdown of Mcl-1 was achieved by reverse transfection and autophagy was induced by incubating cells with THC or bortezomib in the presence or absence of chloroquine (CQ). Initial dose response studies of THC-induced inhibition of cell viability were performed in order to find an optimal dose in which THC induces approximately 50% inhibition of cell viability at 24 hours. Treatment of A375 cells with increasing concentrations of THC demonstrated THC-induced approximately 50% inhibition of cell viability between concentrations of 4 and 5 μM (Figure 3.7), and hence this concentration range was adopted for use in all subsequent experiments. RNAi-mediated knockdown of Mcl-1 resulted in enhanced THC-induced inhibition of A375 cell viability which was prevented by combined treatment with chloroquine (Figure 3.8).

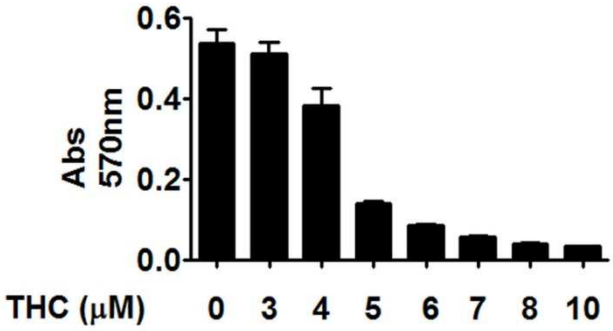

Figure 3.7: THC induces dose-dependent inhibition of A375 cell viability. MTT proliferation assay for cell viability (absorbance 570nm) of A375 cells treated for 24 hours with a dose range of THC (0, 3, 4, 5, 6, 7, 8, 10 μM) or with vehicle control (DMSO). Each bar is the mean +/-SD, n=3.

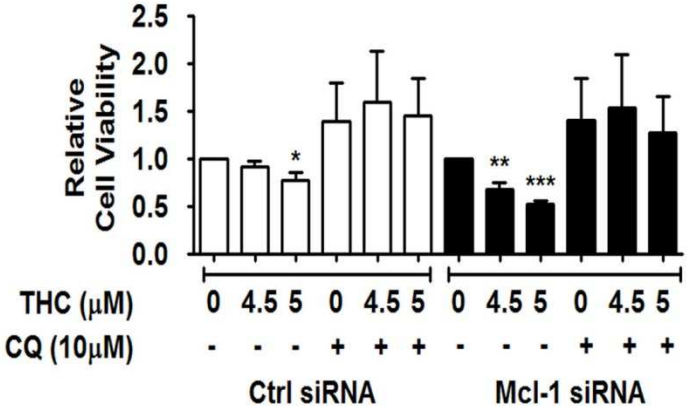

Figure 3.8: RNAi-mediated knockdown of Mcl-1 enhances THC-induced inhibition of A375 cell viability, which is prevented by combined treatment with chloroquine. Relative viability A375 cells following transfection with control siRNA (Ctrl siRNA) or Mcl-1 siRNA for 24 hours and subsequent treatment for 24 hrs with 4.5 μM or 5 μM THC either alone or in combination with 10μM CQ. Each bar is relative to untreated melanoma cells for each siRNA condition (mean +/- SEM, n=3). Statistics were acquired using one way ANOVA with Dunnett post-hoc test, * $p< 0.05$, ** $p< 0.01$, *** $p<0.001$ comparing drug treatments shown to untreated melanoma cells for each siRNA condition alone.

To confirm THC activated autophagy beyond basal levels in A375 cells, conversion of LC3-I to LC3-II (a standard marker of autophagy) was analysed by Western blotting. Combined treatment with chloroquine for the final 2 hours of treatment was used to prevent degradation of LC3-II in the latter stages of autophagy to provide a more accurate indication of autophgaic flux. Results demonstrated THC at concentrations of 4.5 μM or 5 μM induced increased levels of LC3-II in the presence of chloroquine compared to cells treated with either THC or chloroquine alone, demonstrating that THC is able to activate autophagy (Figure 3.9). Furthermore, RNAi-mediated knockdown of Mcl-1 in A375 cells enhanced THC-induced LC3-II levels in the presence of chloroquine compared to the same treatment in cells transfected with ctrl siRNA (Figure 3.9 B). To confirm THC treatment resulted in apoptosis in A375 cells, the levels of caspase 3 cleavage (a downstream marker of both extrinsic and intrinsic apoptosis) were analysed by Western blotting. Results demonstrated THC at concentrations of 4.5 μM or 5 μM induced a modest increase in cleavage of caspase 3 compared to untreated cells in all cases (Figure 3.9). Additionally, RNAi-mediated knockdown of Mcl-1 in A375 cells resulted in substantially increased levels of Cleaved Caspase 3 which were further increased by THC treatment suggesting increased sensitivity of A375 cell to THC in the absence of Mcl-1.

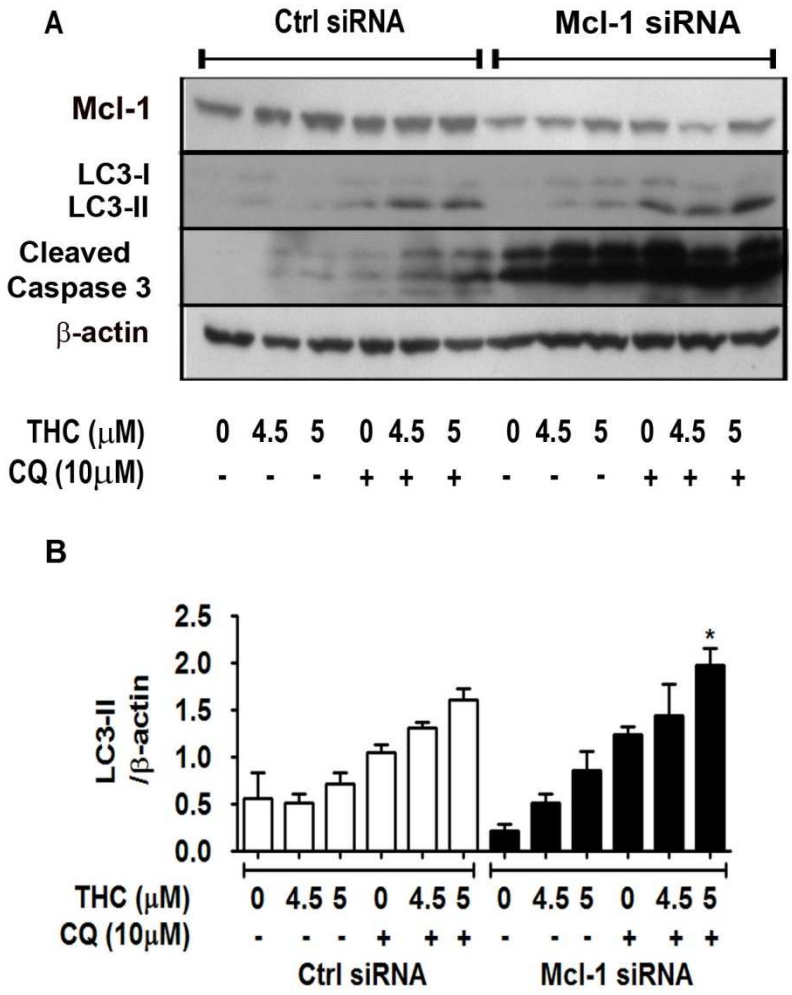

Figure 3.9: RNAi-mediated knockdown of Mcl-1 enhances THC-induced autophagy. (A) Western blot for Mcl-1, LC3-I/LC3-II, Cleaved Caspase 3 and ß-actin in A375 cells treated with THC for 24 hours in the presence or absence of CQ for the final 2 hours of treatment (B) Each bar represents LC3-II band intensity normalised to β-actin band intensity (LC3-II/β-actin) for each treatment condition, and expressed relative to the mean of each individual experiment across multiple experiments (mean ± SD, n=3). Statistics were acquired using one way ANOVA with Dunnett post-hoc test, * $p < 0.05$ comparing drug treatments shown relative to untreated melanoma cells for each siRNA condition alone.

To further assess the underlying effects of pro-death induced autophagy with THC after Mcl-1 knockdown in A375 cells, the pro-survival autophagy inducing agent bortezomib was used to evaluate its effect on the inhibition of cell viability, and the induction of caspase 3 cleavage and LC3-II induction. In line with THC treatment, results also demonstrated treatment of cells with bortezomib for 24 hours inhibited A375 cell viability, which was further enhanced by RNAi-mediated knockdown of Mcl-1 (Figure 3.10). However, in contrast to THC, bortezomib-induced inhibition of cell viability was not prevented by the combined treatment with chloroquine (Figure 3.10).

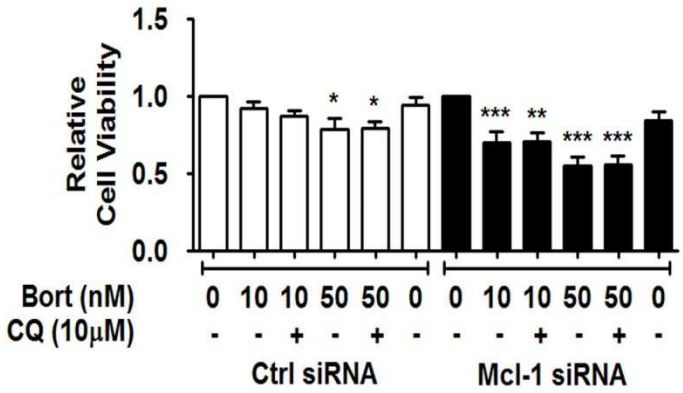

Figure 3.10: RNAi-mediated knockdown of Mcl-1 enhances bortezomib-induced-inhibition of A375 cell viability, which is not prevented by combined treatment with chloroquine. Relative viability of A375 cells following transfection with control siRNA (Ctrl siRNA) or Mcl-1 siRNA for 24 hr and subsequent treatment for 24 hours with 10 or 50nM Bort either alone or in combination with 10μM CQ. Each bar is relative to untreated melanoma cells for each siRNA condition (mean +/- SEM, n=3). Statistics were acquired using one way ANOVA with Dunnett post-hoc test, * $p< 0.05$, ** $p< 0.01$, *** $p<0.001$ comparing drug treatments shown relative to untreated melanoma cells for each siRNA condition alone.

Western blotting also confirmed bortezomib treatment resulted in increased LC3-II expression; however, this was not further enhanced by knockdown of Mcl-1. Increased apoptosis was also observed in response to bortezomib treatment and this was enhanced by RNAi-mediated knockdown of Mcl-1. Treatment with bortezomib also resulted in an increase in Mcl-1 levels in both Ctrl siRNA and Mcl-1 siRNA transfected cells (Figure 3.11).

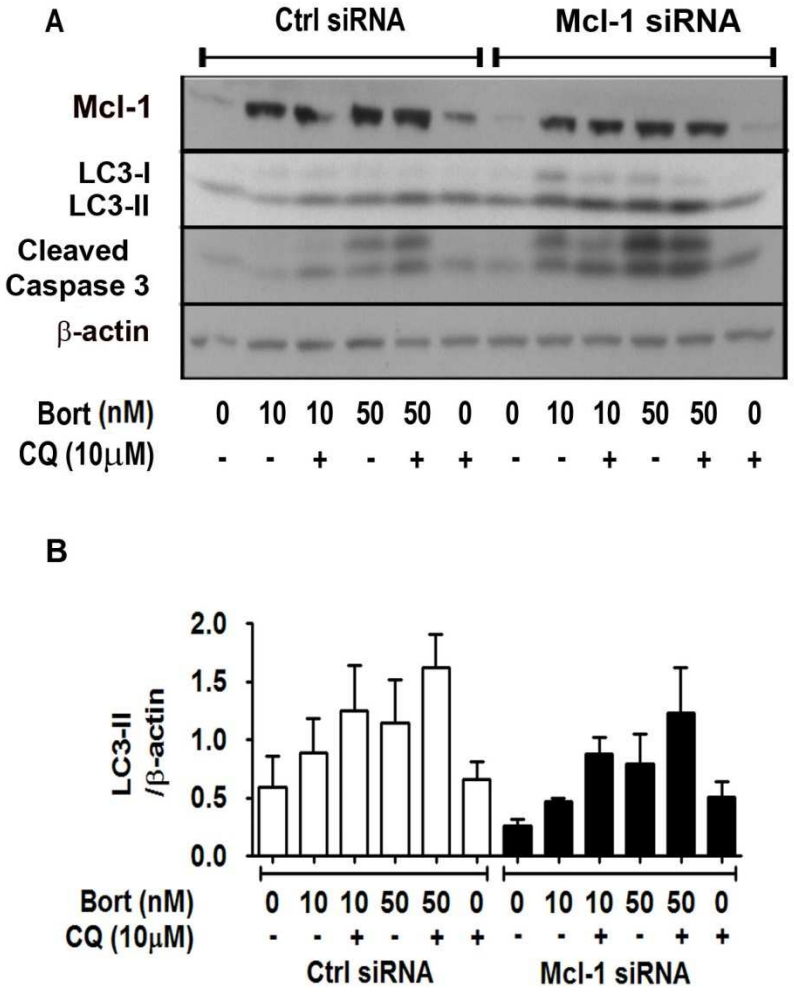

Figure 3.11: Bortezomib treatment induces autophagy which is not enhanced by RNAi-mediated knockdown of Mcl-1. (A) Western Mcl-1, LC3-I/LC3-II, Cleaved Caspase 3 and ß-actin in A375 cells treated with Bort for 8 hours in the presence or absence of CQ for the final 2 hours of treatment. (B) Each bar represents 3 replicates of LC3-II band intensity normalised to β-actin band intensity (LC3-II/β-actin) for each treatment condition, expressed relative to the mean of each individual experiment across multiple experiments (mean ± SD, n = 3).

3.2.6: Effect of Mcl-1S over-expression on A375 induced inhibition of cell viability and induction of autophagy

In order to determine whether inhibition of total Mcl-1 or just Mcl-1L alone is able to promote cell death and autophagy as well as enhance the observed effects of THC on these processes, expression of Mcl-1S was modulated in BRAF mutated A375 melanoma cells. Approximately 50% inhibition of A375 cell viability was observed with over-expression of Mcl-1S alone (data not shown) and although THC significantly inhibited the viability of A375 cells transfected with both control pcDNA4 and Mcl-1S, there was however, no overall increased effect of Mcl-1S over expression on THC-induced inhibition of cell viability (Figure 3.12). Results also demonstrated combined treatment of THC with CQ for 24 hours prevented reduction of cell viability (Figure 3.12).

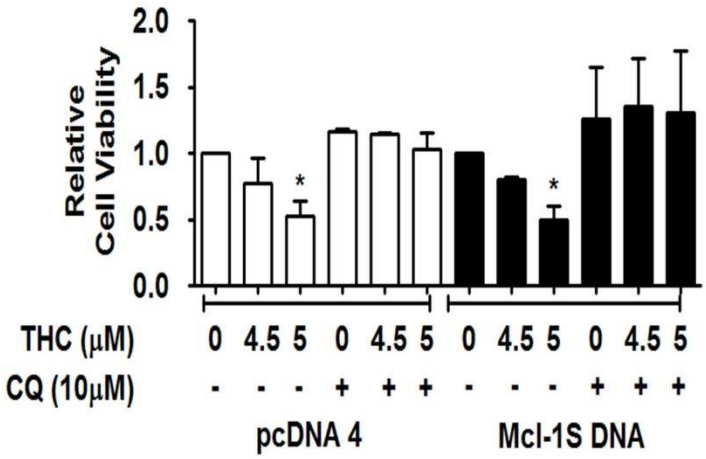

Figure 3.12: THC-induced inhibition of A375 cell viability is not enhanced by Overexpression of Mcl-1S. Relative viability of A375 cells transfected for 24 hrs with pcDNA4 empty vector or pcDNA 3.1-Mcl-1Ss and treated subsequently with 4.5 μM or 5 μM THC either alone or in combination with 10 μM CQ. Each bar is relative to untreated melanoma cells for each siRNA condition (mean +/- SEM, n=3). Statistics were acquired using one way ANOVA with Dunnett post-hoc test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ comparing drug treatments shown relative to untreated melanoma cells for each DNA transfection condition alone.

Interestingly unlike the effects derived by RNAi-mediated knockdown of Mcl-1, over-expression of Mcl-1 S did not increase THC-induced LC3-II expression (Figure 3.13). In fact the data suggests over-expression of Mcl-1S blocked induction of autophagy in response to THC indicating an inhibitory role for Mcl-1S in THC-induced autophagy.

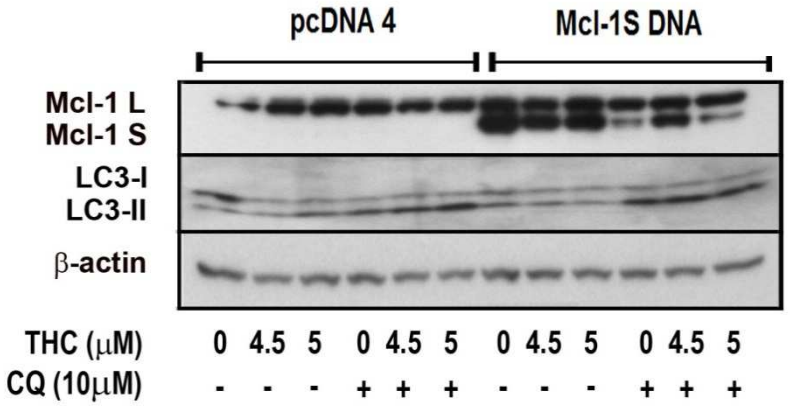

Figure 3.13: Overexpression of Mcl-1S does not enhance THC-induced autophagy. Western blot for Mcl-1L, Mcl-1S, LC3-I/LC3-II, Cleaved Caspase 3 and ß-actin in A375 cells transfected for 24 hrs with pcDNA4 empty vector or pcDNA 3.1-Mcl-1S and treated subsequently with THC (4.5 μM or 5 μM) for 24 hours in the presence or absence of CQ (10 μM) for the final 2 hours of treatment.

3.3: Discussion

Results from this chapter suggest that activating mutations in *BRAF* result in increased expression of anti-apoptotic Mcl-1L and pro-apoptotic Mcl-1S *in vitro*. RT-PCR of the gene encoding Mcl-1 revealed a significant increase in Mcl-1L, but not Mcl-1S, mRNA expression in melanoma cell lines compared to primary melanocytes, with the highest expression of Mcl-1L observed in melanoma cells bearing mutant $BRAF^{V600E}$. Immunocytochemistry of FFPE melanoma cells confirmed total Mcl-1 protein expression was greater in melanoma cells bearing a $BRAF^{V600E}$ mutation compared to expression in $BRAF^{WT}$ cells however, this approach was not suitable for the detection of splice variant expression. Furthermore no correlation was identified in a small panel of patient tumours with *BRAF/NRAS* mutated or *BRAF* wild-type tumours.

Western blotting using an antibody to detect both Mcl-1L and Mcl-1S showed a significantly higher level of Mcl-1L expression in *BRAF* mutated melanoma cell lines compared to expression in *BRAF* wild-type cells supporting the increased mRNA levels found by RT-PCR for Mcl-1L. Increased expression of Mcl-1S protein was also detected, however this was only detectable in *BRAF* mutated cell lines. Mcl-1 has a relatively short half-life and is tightly regulated by the proteasome, however it has been suggested that the stability of Mcl-1 protein is potentially increased by ERK-dependent phosphorylation of the Mcl-1 PEST domain at site $Threonine^{163}$ which could account for the resulting increased expression of both Mcl-1L and Mcl-1S protein observed (Domina, Vrana *et al*. 2004). These data support previous observations (Zhai, Jin *et al*. 2008) suggesting that high expression of anti-apoptotic Mcl-1 in advanced melanoma promotes resistance to chemotherapy-induced apoptosis. Wild-type CHL-1 cells over-expressing $BRAF^{V600E}$ also showed a significant increase in the expression of Mcl-1L (as well as an increase in Mcl-1S) compared to those over-expressing $BRAF^{WT}$ or untransfected CHL-1 cells. Immunofluorescence also demonstrated a small but significant increase in the expression of Mcl-1 following over-expression of $BRAF^{V600E}$ compared to CHL-1 cells over-expressing $BRAF^{WT}$ providing further evidence for a role of mutant *BRAF* in Mcl-1 expression levels. Interestingly, nuclear expression of Mcl-1 was observed in this study which is consistent with previous reports which identified localisation of a proteolytic fragment of Mcl-1 to the nucleus resulting in growth inhibition, likely due to

an inhibitory interaction with cdk 1 (Jamil, Sobouti *et al*. 2005). However, whether nuclear Mcl-1 has any functional impact in melanoma has yet to be defined.

RNAi-mediated knockdown of Mcl-1 alone resulted in decreased viability in A375 cells. This effect was not surprising since previous reports have shown that Mcl-1 is essential for melanoma survival, particularly in advanced stage disease (Tang, Tron *et al*. 1998). However, this also presented a weakness in this study as complete knockdown of the Mcl-1 gene could not be achieved without causing excessive cell death, thereby preventing subsequent studies. However despite this limitation, interesting and useful data were generated from these findings. Studies using THC and bortezomib revealed both agents induced apoptosis as indicated by an increase in Cleaved Caspase 3. Interestingly, treatment with bortezomib also increased the levels of Mcl-1 in both Ctrl siRNA and Mcl-1 siRNA transfected cells. This is most likely due to inhibition of the proteasome by bortezomib thus preventing proteasomal degradation of Mcl-1. Furthermore, results confirmed both THC and bortezomib-induced autophagy as indicated by increased LC3-I/LC3-II conversion. RNAi-mediated knockdown of Mcl-1 resulted in a significant increase in THC and bortezomib-induced inhibition of A375 viability and interestingly combined treatment of THC with chloroquine prevented any loss of THC-induced inhibition of cell viability suggesting autophagy is involved in cell death induced by this agent. These data support previous findings in glioma and pancreatic cancer which also suggest autophagy is crucial to THC-induced apoptosis in these cancers (Salazar, Carracedo *et al*. 2009). No change in the reduction of viability was evident in response to dual treatment with bortezomib and chloroquine suggesting, in contrast to THC, that autophagy induced by bortezomib is not required for bortezomib-induced cell death. Indeed these findings are partially in line with previous findings demonstrating a pro-survival function of bortezomib-induced autophagy in melanoma, however these studies demonstrated knockdown of key autophagy genes (*Beclin-1* or *Atg7*) sensitized $BRAF^{WT}$ cells to bortezomib-induced cell death (Armstrong, Corazzari *et al*. 2011) therefore one would expect a similar effect with dual treatment with chloroquine. It is possible that the time course of treatment and the method chosen to assess viability changes is not a sensitive enough approach to therefore demonstrate sensitisation of melanoma cells to bortezomib-induced cell death in combination with chloroquine. Additionally, more recent studies suggesting chloroquine promotes apoptosis in melanoma cells through inhibiting the degradation of BH3-only protein p53 upregulated

modulators of apoptosis (ie: PUMA) which required far greater concentrations of chloroquine (50μM) to impact on the viability of melanoma cells (Lakhter, Sahu *et al*. 2013). However this effect was likely directly through stimulation of pro-apoptosis rather than solely through inhibition of pro-survival autophagy. Furthermore, the fact that metastatic melanoma A375 cells harbour mutated $BRAF^{V600E}$ may also prevent sensitisation of this cell line to bortezomib when used in combination with chloroquine, in line with previous observations demonstrating autophagy inhibition is only beneficial in $BRAF^{WT}$ cells (Armstrong, Corazzari *et al*. 2011).

Studies of Mcl-1 expression demonstrated over-expression of Mcl-1S alone reduced viability of A375 melanoma cells to a similar extent to that observed by RNAi-mediated knockdown of Mcl-1. Over-expression of Mcl-1S has also been shown to induce apoptosis in Chinese hamster ovary (CHO) cells and adenocarcinoma human alveolar basal epithelial A549 cells (Bae, Leo *et al*. 2000, Bingle, Craig *et al*. 2000) supporting data derived from the present study and a pro-apoptotic role for Mcl-1S. It would appear that over-expression of Mcl-1S directly induces apoptosis of melanoma, in keeping with findings reported in basal carcimona cells in which targeting Mcl-1 pre-mRNA with Mcl-1 antisense morpholino oligonucleotides (AMOs) shifted expression towards Mcl-1S and directly induced apoptosis (Shieh, Liu *et al*. 2009). It is therefore most likely that the resulting apoptosis observed following Mcl-1S over-expression was due to the antagonising role of Mcl-1S on Mcl-1L, which acts in a similar manner to other BH3-only proteins thus inhibiting the anti-apoptotic function of Mcl-1 (Bae, Leo *et al*. 2000; Bingle, Craig *et al*. 2000). However in contrast to RNAi-mediated knockdown of Mcl-1, data reported in this chapter for over-expression of Mcl-1S did not show any significant increase in the sensitivity of A375 cells to treatment with THC. Additionally Mcl-1S over-expression did not enhance autophagy and in fact appeared to prevent autophagy induction by THC in this case. How Mcl-1S prevents THC-induced autophagy and still results in cell death in response to this agent (despite autophagy being required for THC-induced cell death) remains to be seen, however these data suggest the functional effect of total Mcl-1 knockdown may be via loss of Mcl-1L rather than total Mcl-1. Although the basis of autophagy abrogation remains unclear it is possible Mcl-1S is able to interact with key members of the autophagy pathway perhaps by displacement of Beclin-1 from its identified binding partners such as Mcl-1L by Mcl-1S. Again however, the

mechanisms of this effect have yet to be determined with limited published data on Mcl-1 splice variants available.

In summary, data derived from this chapter suggests that activating mutations in *BRAF* result in increased expression of anti-apoptotic Mcl-1L and also Mcl-1S. Additionally, these data suggest Mcl-1 is able to inhibit autophagy to some extent, thus supporting the hypothesis that Mcl-1 amplification, in addition to prevention of apoptosis, may contribute to oncogenesis stimulated by the inhibition of Beclin-1-depedant autophagy. This effect could potentially function in addition to other potential cancer promoting functions, such as a recently reported role for localisation of an amino-terminally truncated isoform of Mcl-1 into the mitochondrial matrix to facilitate mitochondrial fusion, ATP production, membrane potential, respiration and potentially control survival of cancer cells (Perciavalle, Stewart *et al*. 2012). Enhanced sensitivity of melanoma cells with Mcl-1 knockdown in these findings suggest specifically targeting Mcl-1 (with more specific BH3 mimetics for example) may represent an alternative therapeutic approach in combination with novel therapeutics shown to induce both cytotoxic autophagy and apoptosis like THC as well as pro-survival autophagy induced bortezomib. Furthermore, targeting Mcl-1 pre-mRNA with Mcl-1 antisense morpholino oligonucleotides (AMOs) or other alternative splicing inducing agents may also present an alternative approach to apoptosis induction as a further mechanism to exploit for the therapeutic benefit of advanced stage melanoma.

3.4: Summary

- Mcl-1L but not Mcl-1S mRNA expression is increased in melanoma cells compared to primary melanocytes.
- Mcl-1L but not Mcl-1S mRNA expression is further increased in $BRAF^{V600E}$ mutant melanoma cells compared to $BRAF^{WT}$ melanoma cells.
- Activating mutations in *BRAF* increase the expression of both Mcl-1L and Mcl-1S *in vitro*
- No correlation was identified in a small panel of patient tumours for *BRAF/NRAS* mutated tumours and *BRAF* wild-type tumours.
- Mcl-1L is required for the maintenance of melanoma cell viability
- Mcl-1L expression reduced autophagy and cell death in melanoma cells in response to cytotoxic agents THC and bortezomib.
- Over-expression of pro-apoptotic Mcl-1S alone reduced viability of A375 melanoma but did not enhance autophagy or cell death in response to THC.

Chapter 4: The Role of autophagy and Beclin-1 in THC-induced cell death

Chapter 4: The Role of autophagy and Beclin-1 in THC-induced cell death

Table of Contents

4.1: Introduction

The use of cannabinoids in medicine has been applied for many centuries however, since the chemotherapeutic potential of cannabinoids was first suggested in glioma in 1998 (Sánchez, Galve-Roperh *et al*. 1998) extensive research into the medicinal application of these agents has sparked rekindled interest for their use in the treatment of cancer. Arguably the tumour type which has undergone the most intensive research for the chemotherapeutic action of cannabinoids is glioma (Duntsch, Divi *et al*. 2006; Sánchez, de Ceballos *et al*. 2001; Sánchez, Galve-Roperh *et al*. 1998; Velasco, Carracedo *et al*. 2007), revealing several signal transduction pathways triggered by cannabinoids which are activated through stimulation of specific cannabinoid receptors. The accumulation of ceramide through CB1 has been suggested as the likely contender responsible for cell death induced by cannabinoids, particularly THC, in glioma (Gómez del Pulgar, Velasco *et al*. 2002). Additionally, strong evidence exists for autophagy being a key player in this process, thought to be activated by the eIF2α axis of the ER stress process and increased expression of the stress-regulated protein p8 and TRB3, and Akt inhibition resulting in autophagy induction through mTOR inhibition and ultimately cell death (Carracedo, Gironella *et al*. 2006; Carracedo, Lorente *et al*. 2006; Salazar, Carracedo *et al*. 2009; Salazar, Carracedo *et al*. 2009). However, although preliminary studies with cannabinoids have been undertaken in melanoma (Blázquez, Carracedo *et al*. 2006; Kenessey, Bánki *et al*. 2012; Scuderi, Cantarella *et al*. 2011; Zhao, Yang *et al*. 2012) there are limited research available as to the potential for THC to treat this disease or whether a similar cytotoxic process occurs.

The potential for cannabinoid action in melanoma was first speculated by the observation that both cannabinoid receptors CB1 and CB2 are up-regulated in melanoma *in vitro* compared to primary melanocytes (Blázquez, Carracedo *et al*. 2006). Activation of these receptors inhibited tumour growth, angiogenesis, and metastasis as well as promoting the induction of apoptosis, suggesting that CB activation by a cannabinoid such as THC could have similar effects (Blázquez, Carracedo *et al*. 2006). Prevention of angiogenesis with cannabinoids has also been further assessed in the context of cannabinoids (Blázquez, Casanova *et al*. 2003; Casanova, Blázquez *et al*. 2003). Several mechanisms suggest angiogenesis prevention including, inhibition of vascular endothelial cell migration and impacts on cell survival, as shown by altered blood vessel morphology and decreased

expression of pro-angiogenic factors (vascular endothelial growth factor (VEGF and angiopoietin-2) as well as the expression of matrix metalloproteinase-2 in melanoma tumours further supporting the potential therapeutic efficacy for THC and other cannabinoids for melanoma (Blázquez, Casanova *et al*. 2003; Casanova, Blázquez *et al*. 2003). CB receptors are usually expressed differentially with CB1 being expressed more typically in the central nervous system and CB2 expressed in the immune system (Galiègue, Mary *et al*. 1995; Sarafian, Montes *et al*. 2008)) with increased expression of both receptors reported in a variety of cancers (Sarfaraz, Afaq *et al*. 2005; Sarfaraz, Adhami *et al*. 2008; Xu, Liu *et al*. 2006), including melanoma

Results from the previous chapter revealed that treatment of A375 metastatic melanoma cells with THC results in autophagy induction and cell death. The importance of autophagy in THC-induced cell death in glioma is clear as abrogation of this pathway completely prevents cell death in response to THC (Salazar, Carracedo *et al*. 2009). Interestingly studies in glioma have found the autophagy induced in response to THC is via a non-canonical autophagic pathway which is independent of Beclin-1 activity (Salazar, Carracedo *et al*. 2009). The aims of this chapter were therefore to; verify autophagy induction in response to THC in a panel of metastatic melanoma cell lines; determine the contribution of autophagy to THC-induced cell death as well as identify the molecular pathway responsible for cell death; identify similarities and differences between THC-induced molecular events in melanoma with those reported in glioma; and finally, to compare the role of autophagy in THC-induced cell death with that of canonical autophagy induced in response to bortezomib.

4.2: Results

4.2.1: THC induces autophagy in melanoma cells *in vitro*

Observations from the previous chapter revealed induction of autophagy in response to THC in A375 cells. In order to confirm THC-induced autophagy in melanoma cells *in vitro,* conversion of LC3-I to LC3-II was analysed by Western blotting in a panel of metastatic melanoma cell lines (A375, CHL-1 and SKMEL-28) (Figure 4.1 A, B, C respectively), and following combined treatment with chloroquine to prevent LC3-II degradation in the latter stages of autophagy, hence providing a more accurate indication of LC3-II conversion levels. The induction of autophagy by THC treatment was confirmed in all tested cell lines at optimised concentrations of 4.5 μM or 5 μM respectively by the observation of increased LC3-II levels compared to control untreated cells. Additionally autophagy induction was assessed by fluorescence microscopy in A375 or CHL-1 cells stably expressing mRFP-GFP-LC3. Autophagy induction and flux is indicated by the presence of autophagosomes represented as distinct green/yellow puncta and autolysosomes represented as red puncta. Results clearly demonstrated THC-induced autophgaic flux in this context in both A375 and CHL-1 cells compared to control untreated cells (Figure 4.1 D, E).

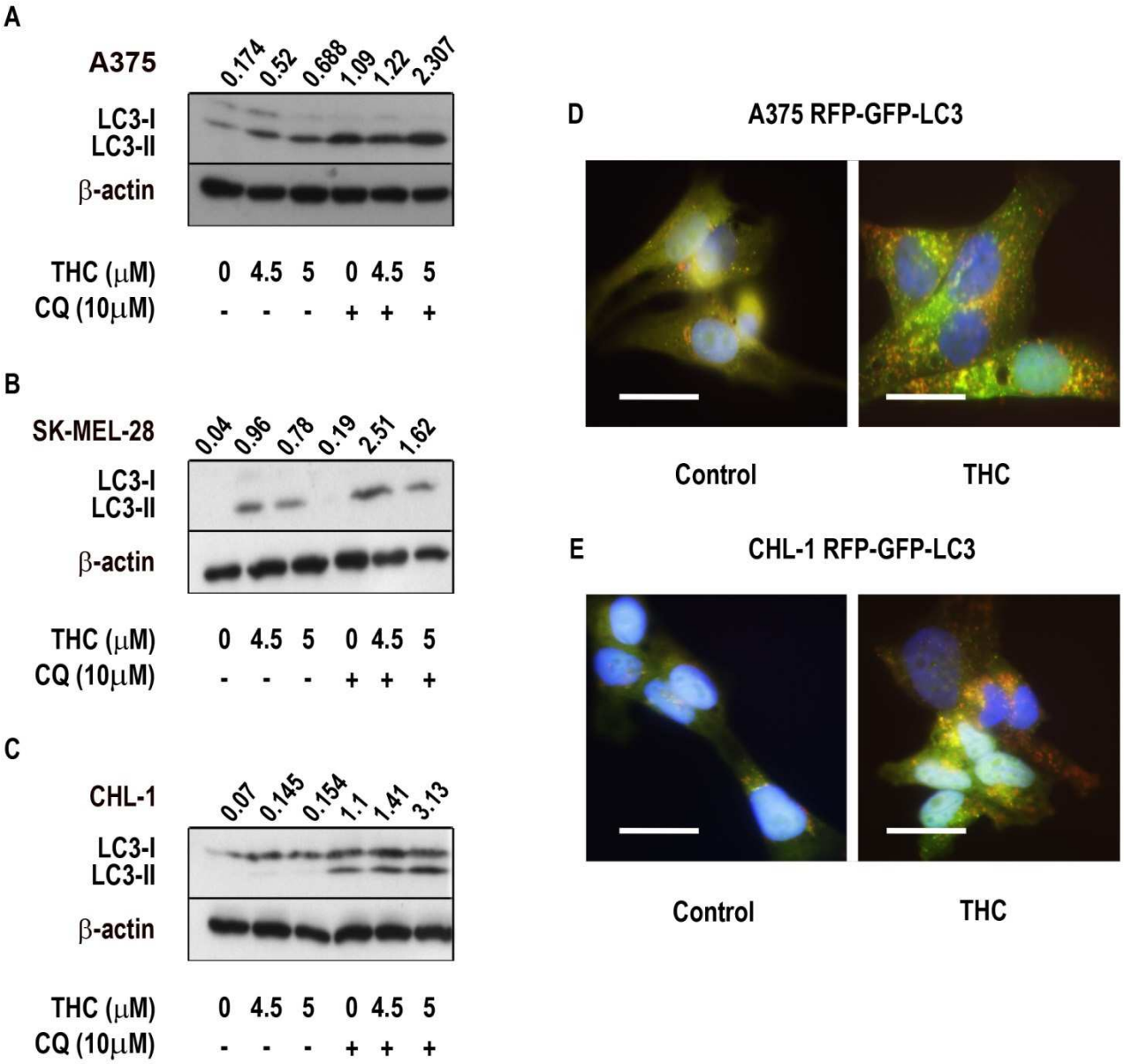

Figure 4.1: THC induces autophagy in melanoma cells in vitro. Western blot for LC3-I/LC3-II and ß-actin in (A) A375, (B) SKMEL-28 and (C) CHL-1 cells treated with 4.5 or 5 μM THC for 24 hours in the presence or absence of 10 μM CQ for the final 2 hours of treatment. Densitometric analysis representing LC3-II expression relative to β-actin loading control is given above each treatment condition. The data expressed are a combination of LC3-II/β-actin normalised relative to the mean of one experiment. Representative fluorescence microscopy from 3 replicate experiments for autophagosome (green/yellow) and autolysosome (red) mRFP-GFP-LC3 puncta in (D) A375 and (E) CHL-1 cells treated with 5 μM THC for 18 hours (scale bar represents a length of 20 μm).

4.2.2: THC-induced cell death is prevented when autophagy is deficient

Previous studies have shown that autophagy is required for cell death in response to THC in other cancer types (Salazar, Carracedo *et al*. 2009; Shrivastava, Kuzontkoski *et al*. 2011). As such, in order to ascertain whether the autophagy induced by THC is crucial for the induction of cell death in melanoma, RNAi-mediated knockdown of *Atg7* (an essential autophagy gene) was achieved by reverse transfection. Treatment with THC for 24 hours reduced viability of metastatic melanoma cell lines (A375, CHL-1), which was prevented by *Atg7* knockdown or treatment with chloroquine (Fig 4.2).

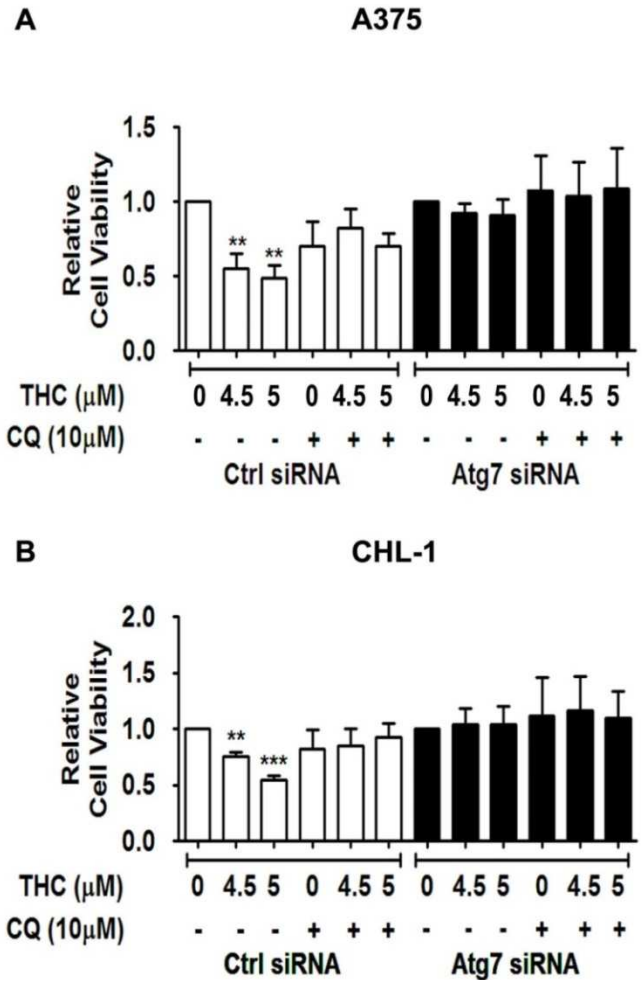

Figure 4.2: RNAi-mediated knockdown of Atg7 prevents THC-induced inhibition of melanoma cell viability. Relative viability of melanoma cell lines (A) A375 and (B) CHL-1 following transfection with control siRNA (Ctrl siRNA) or Atg7 siRNA for 24 hours and subsequent treatment for 24 hours with 4.5 μM or 5 μM THC in the presence or absence of 10 μM CQ. Each bar is relative to untreated melanoma cells for each siRNA condition (mean +/- SEM, n=3). Statistics were acquired using one way ANOVA with Dunnett post-hoc test, ** $p< 0.01$ *** $p< 0.001$ comparing drug treatments relative to untreated melanoma cells for each siRNA condition.

To confirm THC-induced autophagy was prevented by either Atg7 knockdown or co-treatment with chloroquine, conversion of LC3-I to LC3-II was analysed by Western blotting. The induction of autophagy by THC treatment was confirmed in both A375 and CHL-1 cells transfected with Ctrl siRNA by the increased LC3-II levels observed compared to control untreated cells (Figure 4.3 A and 4.4 A), which was significant following treatment with 5 μM THC in combination with chloroquine in both A375 and CHL-1 cells compared to cells treated with chloroquine alone. Although some LC3-II accumulated in untreated Atg7 knockdown cells compared to untreated Ctrl siRNA cells, RNAi-mediated knockdown of Atg7 prevented significant autophagy induction by THC as shown by the non-significant increase in the levels of LC3-II after treatment with THC compared to control untreated cells and the equal levels of LC3-II observed in response to combined treatment with chloroquine (Figure 4.3 B).

To determine whether autophagy deficiency prevented THC-induced cell death and if the cell death observed was apoptosis, levels of Cleaved Caspase 3 were analysed by Western blotting. THC treatment resulted in increased Cleaved Caspase 3 in both A375 and CHL-1 cells following treatment with 4.5 μM or 5 μM concentrations compared to little or no expression in untreated cells this indicating increased apoptosis in response to THC (Figures 4.3 and 4.4). While RNAi-mediated knockdown of Atg7 in CHL-1 cells resulted in increased caspase 3 cleavage, THC treatment did not however, promote caspase 3 cleavage in the absence of Atg7 knockdown in either cell line (Figure 4.4).

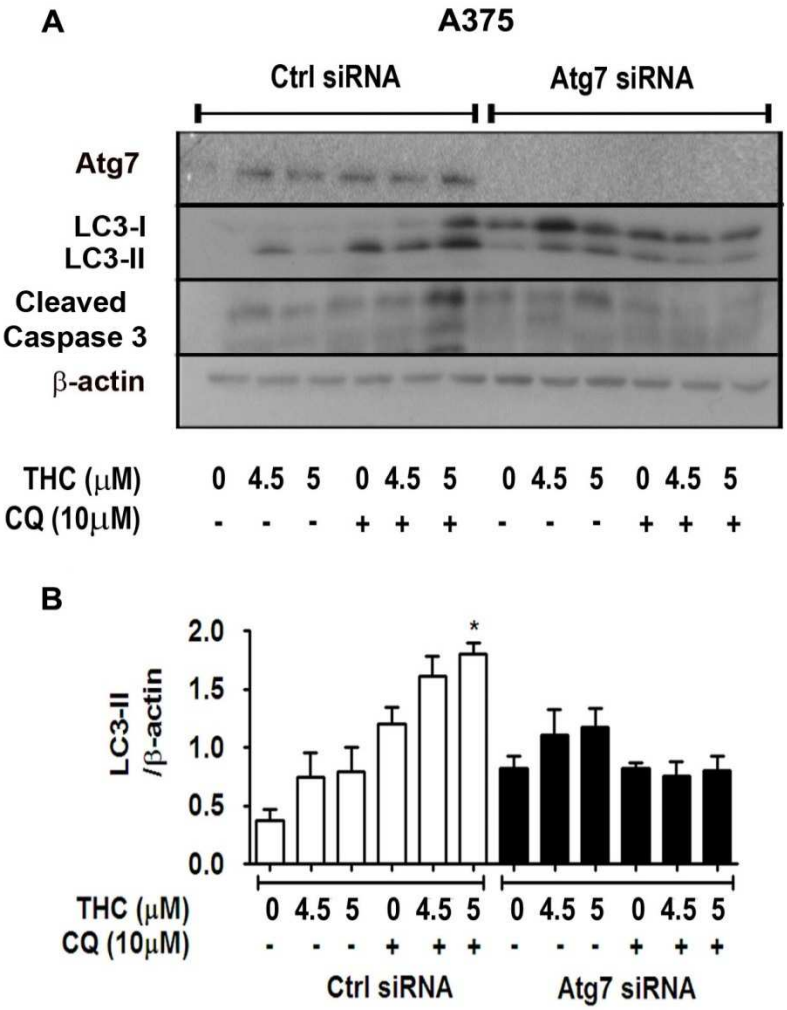

Figure 4.3: RNAi-mediated knockdown of Atg7 prevents autophagy and apoptosis induced by THC. (A) Western blot for Atg7, LC3-I/LC3-II, Cleaved Caspase 3 and ß-actin in A375 cells treated with 4.5 or 5 μM THC for 24 hours in the presence or absence of 10 μM CQ for the final 2 hours of treatment following transfection with control siRNA (Ctrl siRNA) or Atg7 siRNA for 24 hours. (B) Each bar represents 3 replicates of LC3-II band intensity normalised to β-actin band intensity (LC3-II/β-actin) for each treatment condition, and expressed relative to the mean of each individual experiment (mean ± SD, n = 3). Statistics were acquired using one way ANOVA with Dunnett's post-hoc test, * $p < 0.05$ comparing drug treatments to either control DMSO or combined treatment with CQ for each siRNA condition.

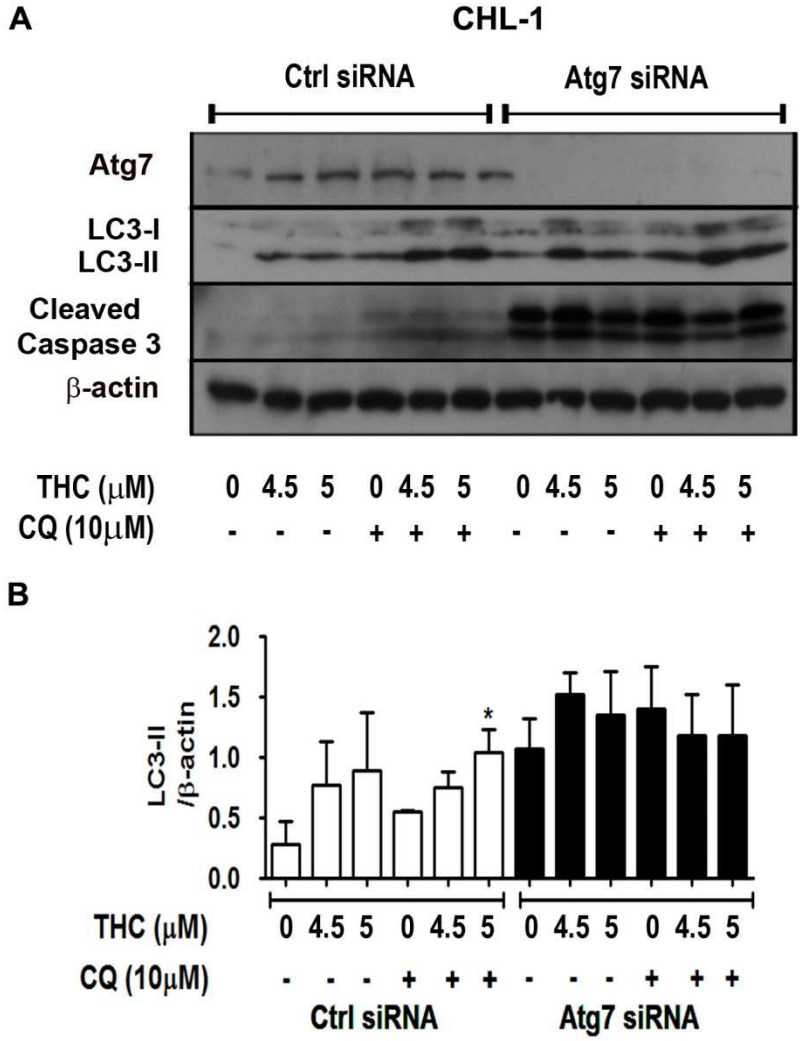

Figure 4.4: RNAi-mediated knockdown of Atg7 prevents autophagy and apoptosis induced by THC. (A) Western blot for Atg7, LC3-I/LC3-II, Cleaved Caspase 3 and ß-actin in CHL-1 cells treated with 4.5 or 5 μM THC for 24 hours in the presence or absence of 10 μM CQ for the final 2 hours of treatment following transfection with control siRNA (Ctrl siRNA) or Atg7 siRNA for 24 hours. (B) Each bar represents 3 replicates of LC3-II band intensity normalised to β-actin band intensity (LC3-II/β-actin) for each treatment condition, and expressed relative to the mean of each individual experiment (mean ± SD, n = 3). Statistics were acquired using one way ANOVA with Dunnett's post-hoc test, * $p < 0.05$ comparing drug treatments to either control DMSO or combined treatment with CQ for each siRNA condition.

The effect of Atg7 knockdown on bortezomib-induced inhibition of cell viability, caspase 3 cleavage and LC3-II induction was also evaluated in A375 and CHL-1 metastatic melanoma cells in order to compare pro-survival autophagy induced by bortezomib with pro-death autophagy activated by THC. In line with THC treatment, results demonstrated treatment of cells with bortezomib for 24 hours inhibited melanoma cell viability. RNAi-mediated knockdown of Atg7 or treatment with chloroquine, in contrast to THC, however, did not effect the inhibition of cell viability in response to bortezomib (Figure 4.5).

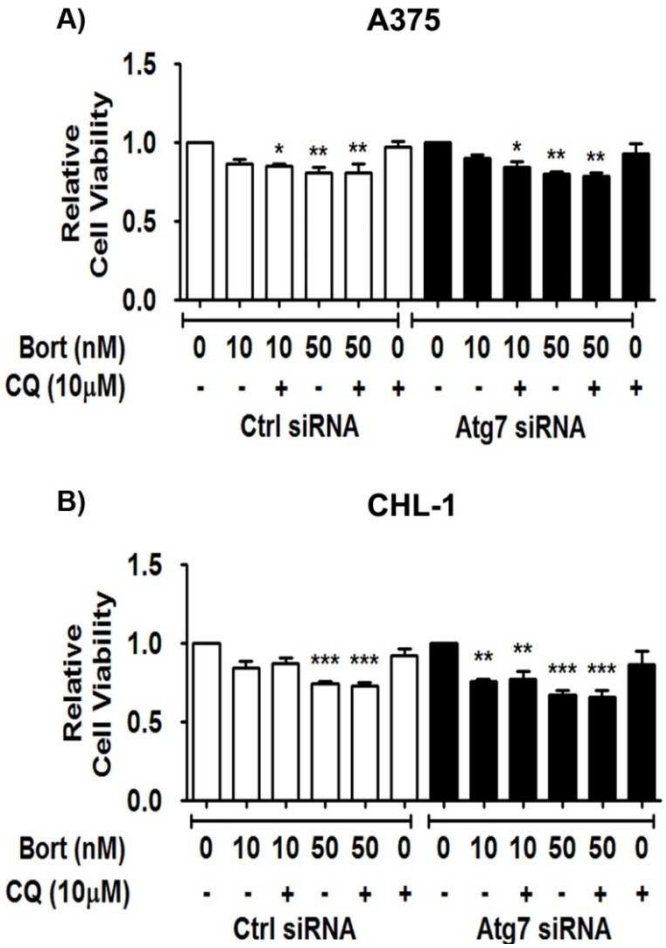

Figure 4.5: Bortezomib-induced inhibition of melanoma cell viability, is not prevented by Atg7 knockdown or combined treatment with Chloroquine. Relative viability of melanoma cell lines (A) A375 and (B) CHL-1 following transfection with control siRNA (Ctrl siRNA) or Atg7 siRNA for 24 hours and subsequent treatment for 24 hours with 10 or 50 nM bortezomib either alone or in combination with 10 μM CQ. Each bar is relative to untreated melanoma cells for each siRNA condition (mean +/- SEM, n=3).Statistics were acquired using one way ANOVA with Dunnett post-hoc test, ** $p< 0.01$, *** $p< 0.001$ comparing drug treatments to untreated melanoma cells for each siRNA condition.

Western blotting also confirmed bortezomib treatment resulted in increased LC3-II expression in both A375 and CHL-1 cells which was partially prevented by Atg7 knockdown, however these data did not reach statistical significance. RNAi-mediated knockdown of Atg7 also increased bortezomib-induced apoptosis, as indicated by increased expression of Cleaved Caspase 3 (Figures 4.6 and 4.7).

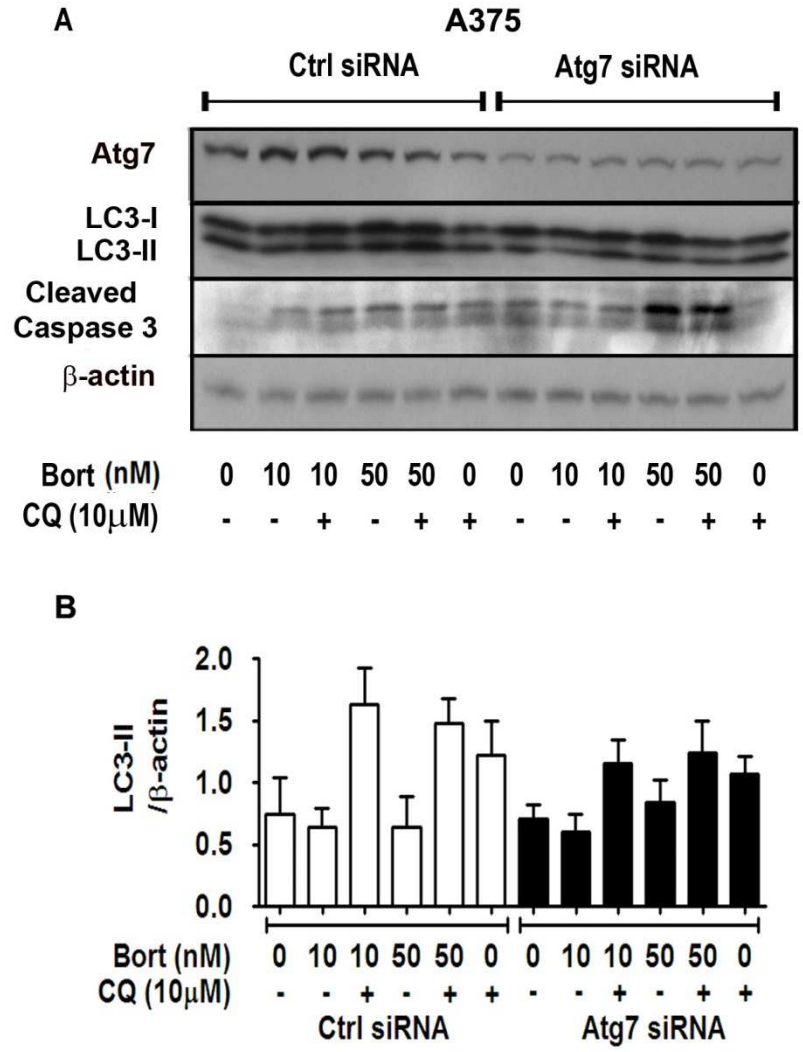

Figure 4.6: Bortezomib-induced autophagy and apoptosis is not prevented by RNAi-mediated knockdown of Atg7 in A375 cells. (A) Western blot for Atg7, LC3-I/LC3-II , Cleaved Caspase 3 and ß-actin in A375 cells treated with 10 or 50 nM Bortezomib (Bort) for 8 hours in the presence or absence of 10 μM CQ for the final 2 hours of treatment following transfection with control siRNA (Ctrl siRNA) or Atg7 siRNA for 24 hours. (B) Each bar represents 3 replicates of LC3-II band intensity normalised to β-actin band intensity (LC3-II/β-actin) for each treatment condition, and expressed relative to the mean of each individual experiment (mean ± SD, n = 3).

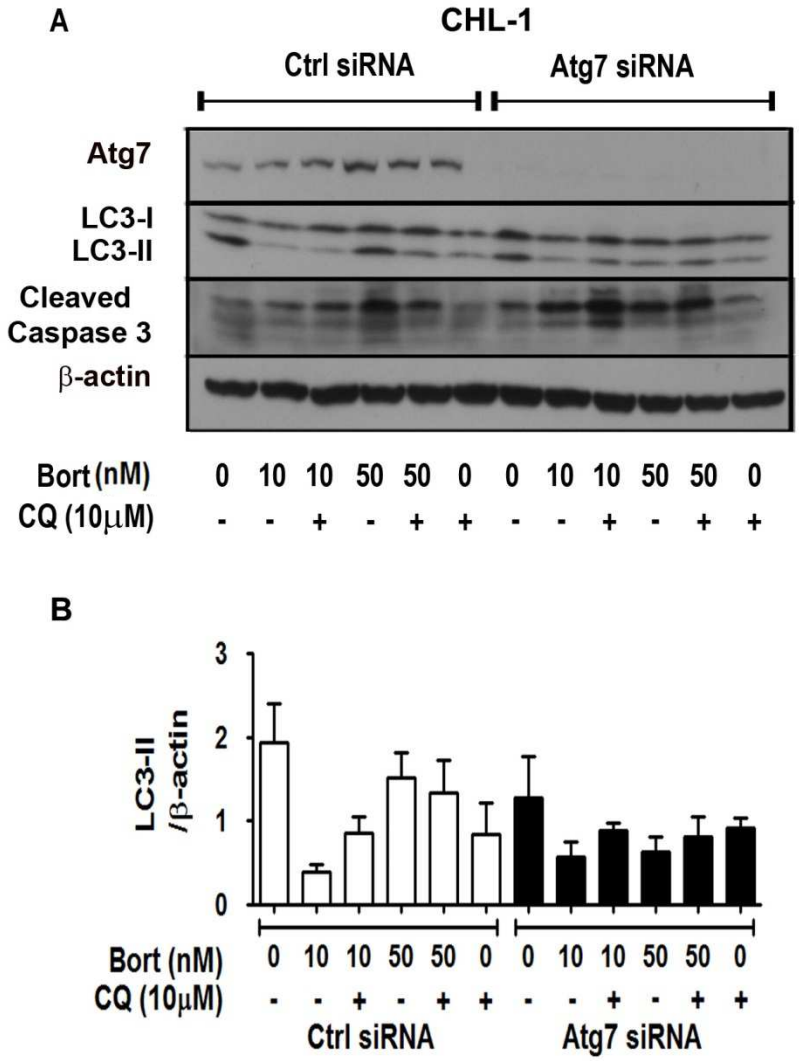

Figure 4.7: Bortezomib-induced autophagy and apoptosis is not prevented by RNAi-mediated knockdown of Atg7 in CHL-1 Cells. (A) Western blot for Atg7, LC3-I/LC3-II , Cleaved Caspase 3 and ß-actin in CHL-1 cells treated with 10 or 50 nM Bortezomib (Bort) for 8 hours in the presence or absence of 10 μM CQ for the final 2 hours of treatment following transfection with control siRNA (Ctrl siRNA) or Atg7 siRNA for 24 hours. (B) Each bar represents 3 replicates of LC3-II band intensity normalised to β-actin band intensity (LC3-II/β-actin) for each treatment condition, and expressed relative to the mean of each individual experiment (mean ± SD, n = 3).

4.2.3: THC-induced cell death is dependent on a class III PI3K, Beclin-1

It has been previously reported that the presence of functional Beclin-1 is not essential for THC to activate autophagy and cell death (Salazar, Carracedo *et al*. 2009). To determine whether this is the case in melanoma and to also provide further evidence for the role of autophagy in THC-induced cell death, RNAi-mediated knockdown of Beclin-1 was achieved by reverse transfection. Treatment with THC for 24 hours reduced viability of A375 and CHL-1 cells, however, unlike in studies of glioma Beclin-1 knockdown prevented THC-induced inhibition of cell viability as did treatment with chloroquine (Fig 4.8).

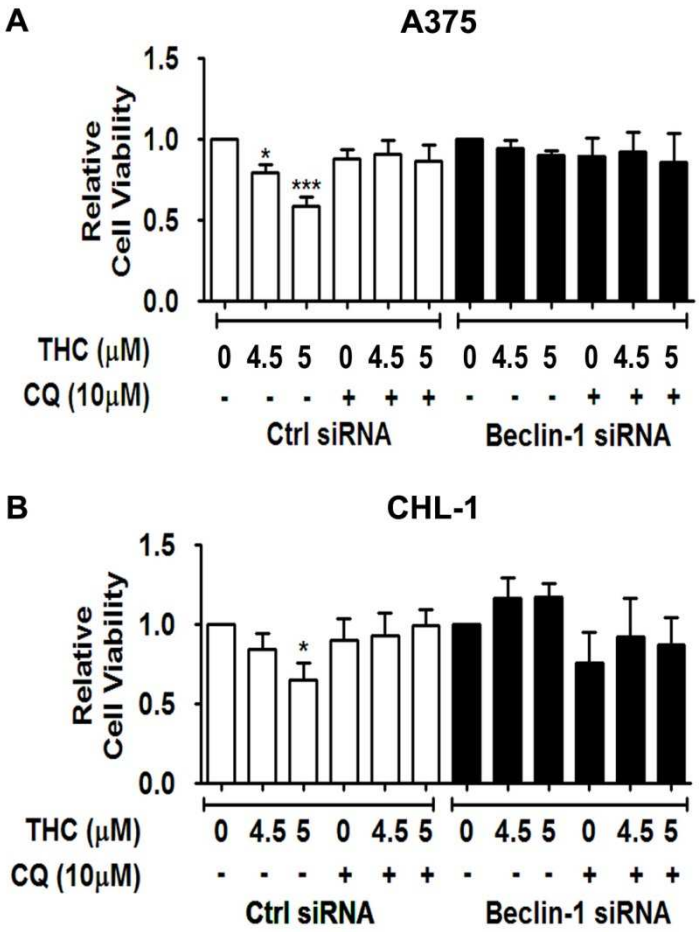

Figure 4.8: RNAi-mediated knockdown of Beclin-1 prevents THC-induced inhibition of melanoma cell viability. Relative viability of melanoma cell lines (A) A375 and (B) CHL-1 following transfection with control siRNA (Ctrl siRNA) or Beclin-1 siRNA for 24 hour and subsequent treatment for 24 hours with 4.5 μM or 5 μM THC in the presence or absence of 10 μM CQ. Each bar is relative to untreated melanoma cells for each siRNA condition (mean +/- SEM, n=3). Statistics were acquired using one way ANOVA with Dunnett post-hoc test, * $p < 0.05$ *** $p < 0.001$ comparing drug treatments shown relative to untreated melanoma cells for each siRNA condition.

THC-induced autophagy was confirmed in each cell line following transfection with Ctrl siRNA which were subsequently treated with 4.5 or 5 μM THC, as evidenced by increased LC3-II levels compared to control untreated cells (Figures 4.9 and 4.10). Furthermore, the increased levels of LC3-II were significant for A375 cells treated with 4.5 μM and 5 μM THC in combination with chloroquine compared to A375 cells treated with chloroquine alone. Interestingly, although RNAi-mediated knockdown of Beclin-1 prevented THC-induced autophagy in CHL-1 cells (Figure 4.10), this effect was only apparent in A375 cells in response to treatment with THC at 5 μM concentrations (Figure 4.9).

To further verify autophagy deficiency prevented THC-induced cell death caspase 3 cleavage was analysed by Western blotting. THC treatment (with 4.5 or 5 μM concentrations) resulted in increased expression of Cleaved Caspase 3 in both A375 and CHL-1 compared to untreated cells indicating increased apoptosis (Figures 4.9 and 4.10). Similarly to Atg7 knockdown, RNAi-mediated knockdown of Beclin-1 in CHL-1 cells resulted in increased caspase 3 cleavage however this was not increased by THC treatment in either CHl-1 or A375 cells in the presence of Beclin-1 knockdown (Figure 4.10).

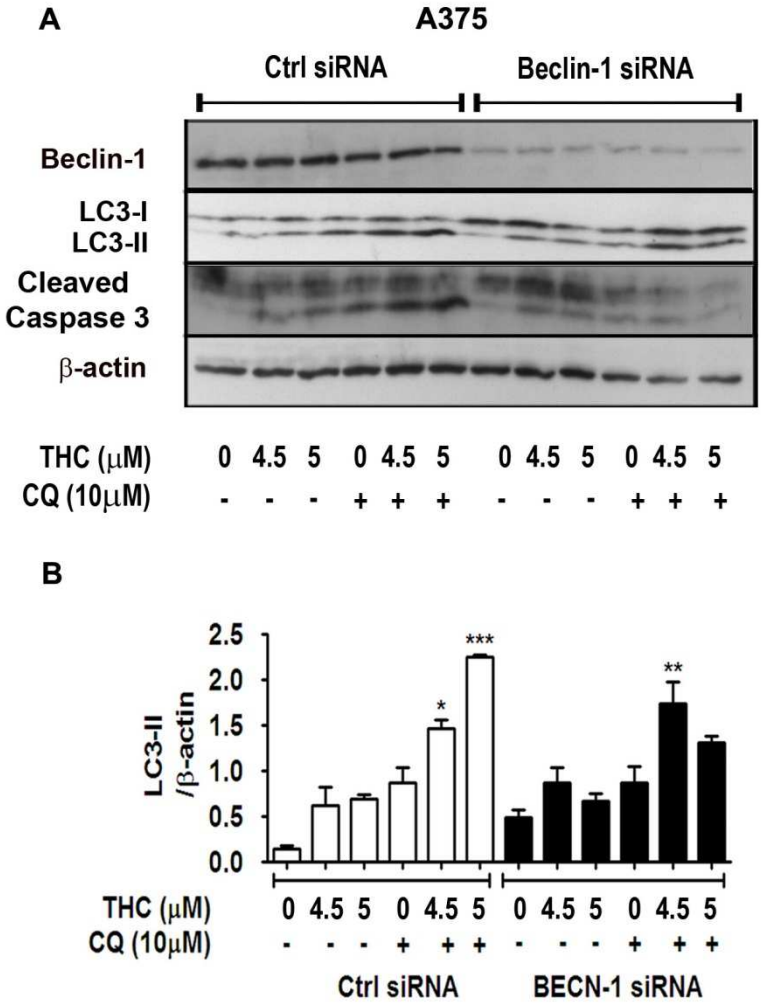

Figure 4.9: RNAi-mediated knockdown of Beclin-1 prevents apoptosis but not autophagy induced by THC. (A) Western blot for Beclin-1, LC3-I/LC3-II, Cleaved Caspase 3 and ß-actin in A375 cells treated with 4.5 or 5 μM THC for 24 hours in the presence or absence of 10 μM CQ for the final 2 hours of treatment following transfection with control siRNA (Ctrl siRNA) or Beclin-1 siRNA for 24 hours. (B) Densitometric analysis representing LC3-II expression relative to β-actin loading control. Each bar represents 3 replicates of LC3-II band intensity normalised to β-actin band intensity (LC3-II/β-actin) for each treatment condition, and expressed relative to the mean of each individual experiment (mean ± SD, n = 3). Statistics were acquired using one way ANOVA with Dunnett post-hoc test, * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$ comparing drug treatments shown to either control DMSO or combined treatment with CQ for each siRNA condition.

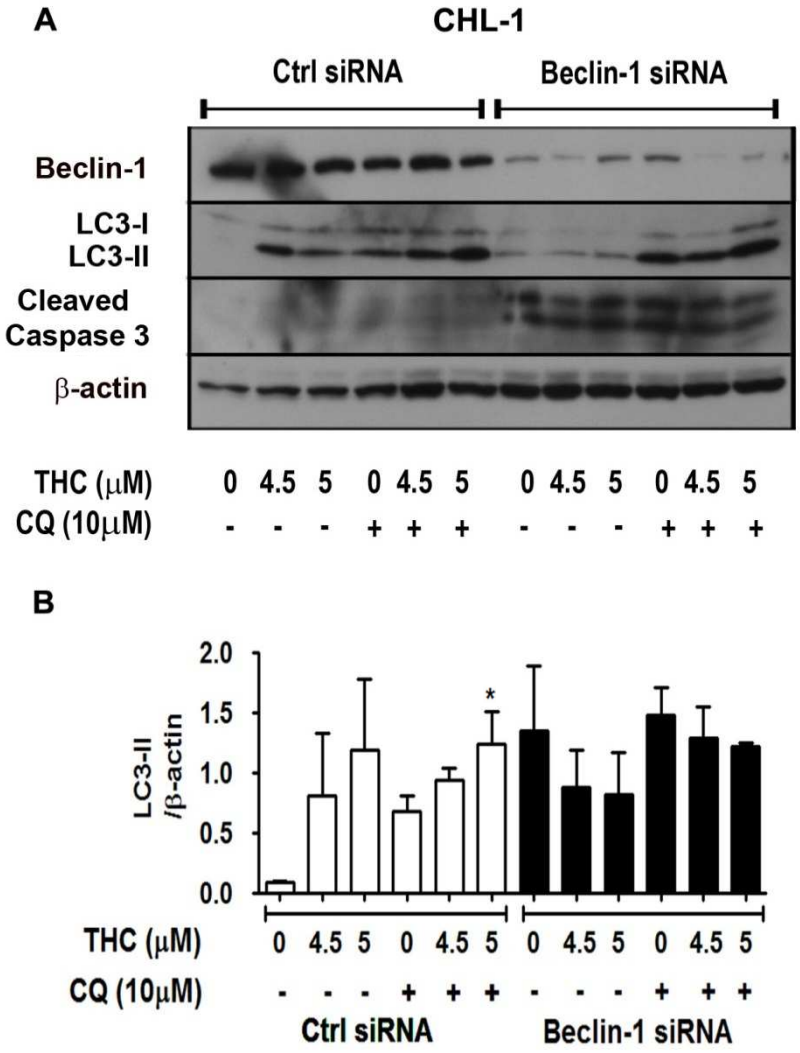

Figure 4.10: RNAi-mediated knockdown of Beclin-1 prevents autophagy and apoptosis induced by THC. (A) Western blot for Beclin-1, LC3-I/LC3-II, Cleaved Caspase 3 and ß-actin in CHl-1 cells treated with 4.5 or 5 µM THC for 24 hours in the presence or absence of 10 µM CQ for the final 2 hours of treatment following transfection with control siRNA (Ctrl siRNA) or Beclin-1 siRNA for 24 hours. (B) Each bar represents 3 replicates of LC3-II band intensity normalised to β-actin band intensity (LC3-II/β-actin) for each treatment condition, and expressed relative to the mean of each individual experiment (mean ± SD, n = 3). Statistics were acquired using one way ANOVA with Dunnett post-hoc test, * p< 0.5 comparing drug treatments shown to either control DMSO or combined treatment with CQ for each siRNA condition.

The effect of Beclin-1 knockdown on bortezomib-induced inhibition of cell viability, caspase 3 cleavage and LC3-II induction was again evaluated in metastatic melanoma cell lines A375 and CHL-1 to compare pro-survival autophagy induced by bortezomib with THC-induced autophagy. In line with THC treatment, results also demonstrated treatment of cells with bortezomib for 24 hours inhibited cell viability of both cell lines. RNAi-mediated knockdown of Beclin-1, in contrast to THC, did not however, prevent bortezomib-induced inhibition of cell viability (Figure 4.11). Additionally, combined treatment with chloroquine did not effect bortezomib-induced inhibition of cell viability with or without knockdown of Beclin-1 (Figure 4.11).

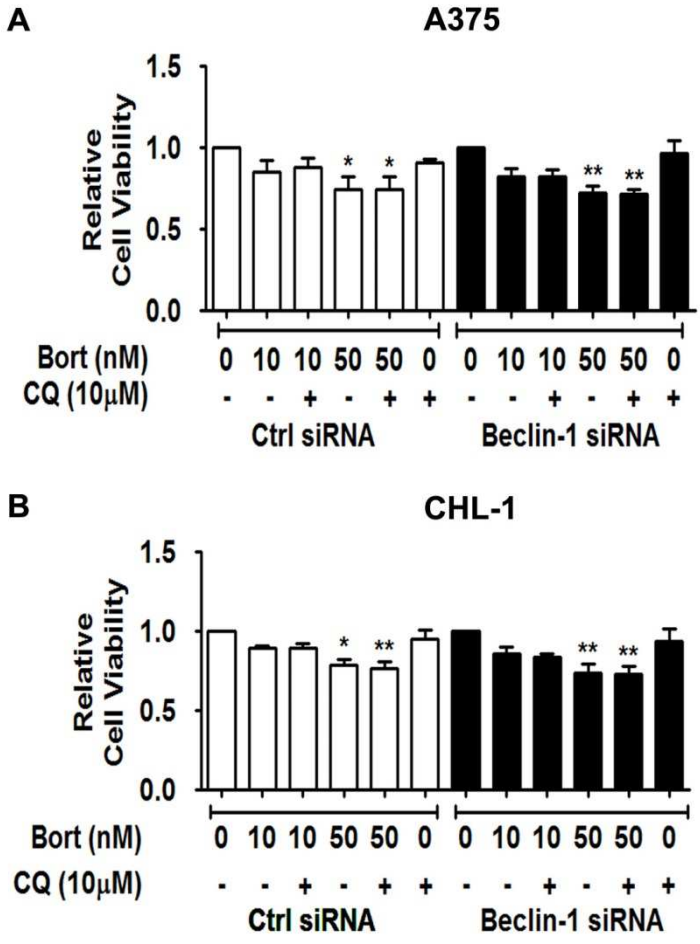

Figure 4.11: bortezomib-induced inhibition of melanoma cell viability, is not prevented by Beclin-1 knockdown or combined treatment with Chloroquine. Relative viability of melanoma cell lines (A) A375 and (B) CHL-1 following transfection with control siRNA (Ctrl siRNA) or Beclin-1 siRNA for 24 hours and subsequent treatment for 24 hours with 10 or 50 nM bortezomib either alone or in combination with 10 μM CQ. Each bar is relative to untreated melanoma cells for each siRNA condition (mean +/- SEM, n=3). Statistics were acquired using one way ANOVA with Dunnett post-hoc test, * p< 0.05, ** p< 0.01, *** p< 0.001 comparing drug treatments shown to untreated melanoma cells for each siRNA condition.

Western blotting also confirmed bortezomib treatment resulted in increased LC3-II expression in both A375 and CHL-1 cells (although not to significant levels in CHL-1 cells) while Beclin-1 knockdown had no inhibitory effects on LC3-II expression (Figures 4.12 and 4.13). Increased apoptosis indicated by the levels of Cleaved Caspase 3 was also observed in response to bortezomib treatment of both cell lines, and was noticeably enhanced by RNAi-mediated knockdown of Beclin-1 in A375 cells followed by treatment with 10nM bortezomib (Figures 4.12 and 4.13). Additionally RNAi-mediated knockdown of Beclin-1 alone induced increased levels of Cleaved Caspase 3 (Figures 4.12 and 4.13).

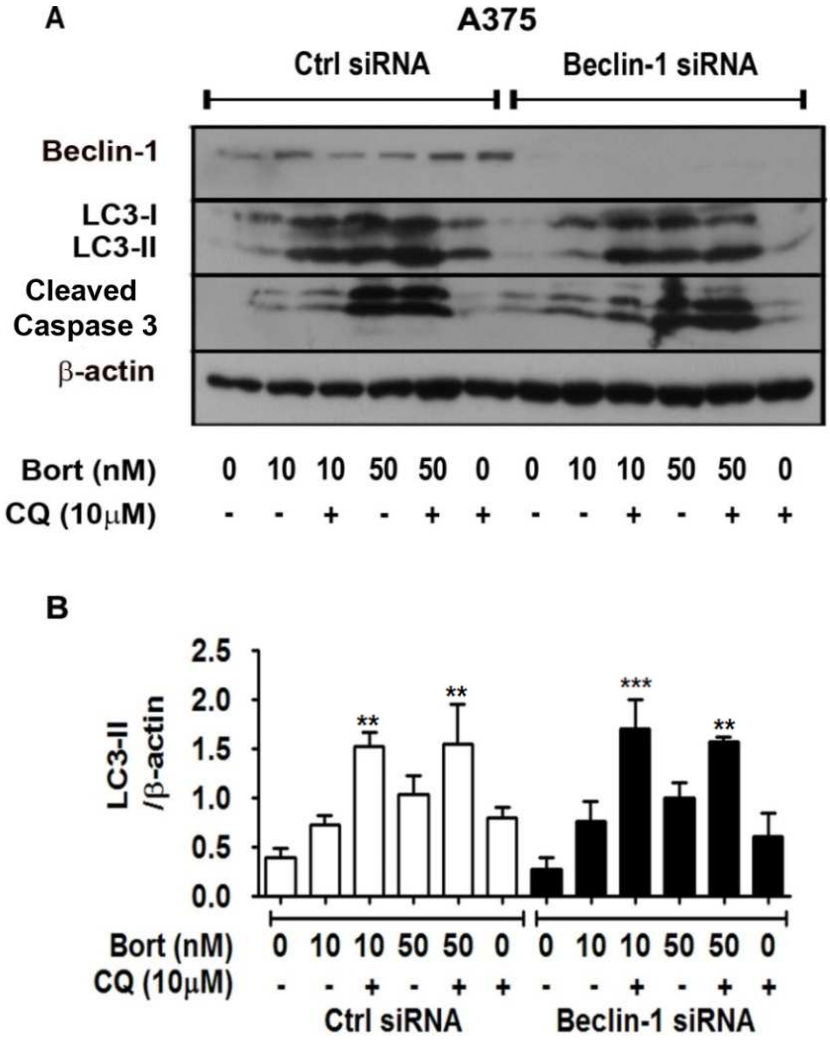

Figure 4.12: Bortezomib treatment induces autophagy and apoptosis in melanoma cells which is not prevented by RNAi-mediated knockdown of Beclin-1. (A) Western blot for Beclin-1, LC3-I/LC3-II , Cleaved Caspase 3 and ß-actin in A375 cells treated with 10 or 50 nM Bortezomib (Bort) for 8 hours in the presence or absence of 10 μM CQ for the final 2 hours of treatment following transfection with control siRNA (Ctrl siRNA) or Beclin-1 siRNA for 24 hours. (B) Each bar represents 3 replicates of LC3-II band intensity normalised to β-actin band intensity (LC3-II/β-actin) for each treatment condition, and expressed relative to the mean of each individual experiment (mean ± SD, n = 3). Statistics were acquired using one way ANOVA with Dunnett post-hoc test ** $p< 0.01$ *** $p< 0.001$ comparing drug treatments shown to either control DMSO or combined treatment with CQ for each siRNA condition.

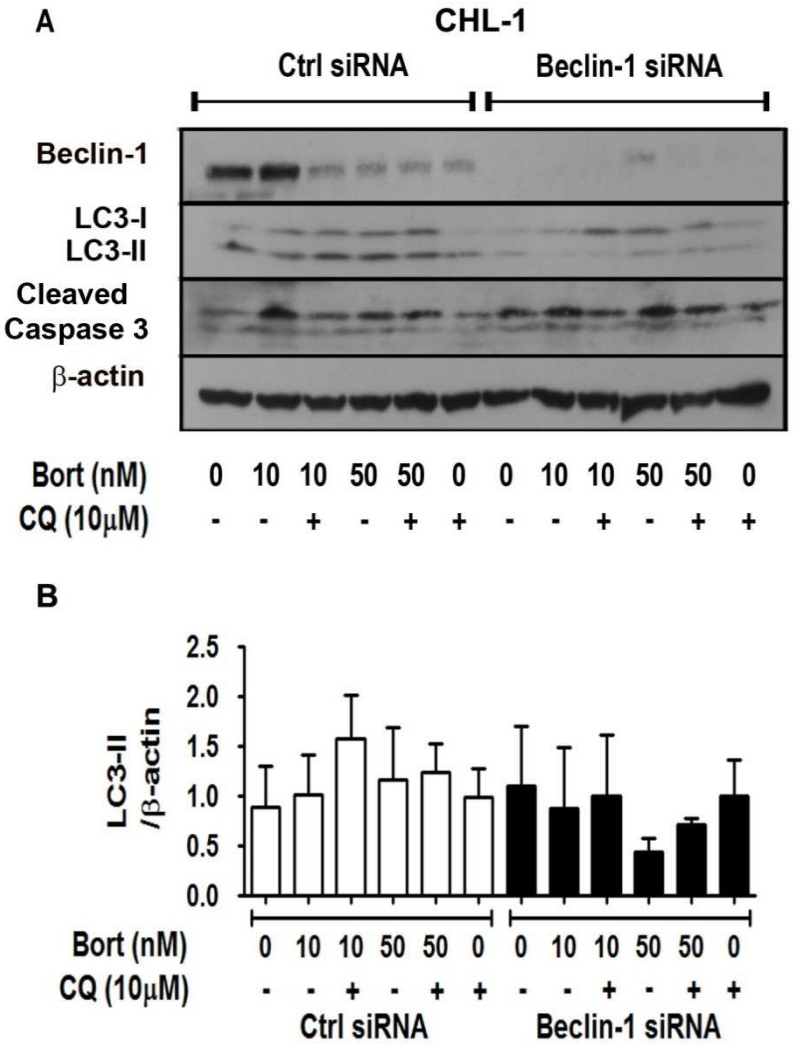

Figure 4.13: Bortezomib treatment induces autophagy and apoptosis in melanoma cells which is not prevented by RNAi-mediated knockdown of Beclin-1. (A) Western blot for Beclin-1, LC3-I/LC3-II , Cleaved Caspase 3 and ß-actin in CHL-1 cells treated with 10 or 50 nM Bortezomib (Bort) for 8 hours in the presence or absence of 10 μM CQ for the final 2 hours of treatment following transfection with control siRNA (Ctrl siRNA) or Beclin-1 siRNA for 24 hours. (B) Each bar represents 3 replicates of LC3-II band intensity normalised to β-actin band intensity (LC3-II/β-actin) for each treatment condition, and expressed relative to the mean of each individual experiment (mean ± SD, n = 3).

4.2.4: Validation for the role of Beclin-1 in canonical autophagy in melanoma cell lines

In normal tissues, Beclin-1 is required for canonical/starvation induced autophagy (Liang, Jackson *et al*. 1999; Pattingre, Tassa *et al*. 2005). However results from these studies reveal autophagy induction in A375 cells in response to both THC and bortezomib even in the absence of this protein. The reasons for this are unclear, however the chosen siRNA's may have off target effects which may have resulted in the unexpected findings observed. In order to confirm the role of Beclin-1 in canonical autophagy, cells were treated with the mTOR inhibitor rapamycin (Li, Wu *et al*. 2013) for 24 hours following Beclin-1 knockdown . Combined treatment with chloroquine was again used in the final two hours of treatment to provide a more accurate indication of LC3-II conversion levels for each treatment condition. Rapamycin treatment resulted in the induction of LC3-II in A375 cells following transfection with either control or Beclin-1 siRNA (Figure 4.14), suggesting autophagy induction is stil occurring even in the absence of Beclin-1 expression.

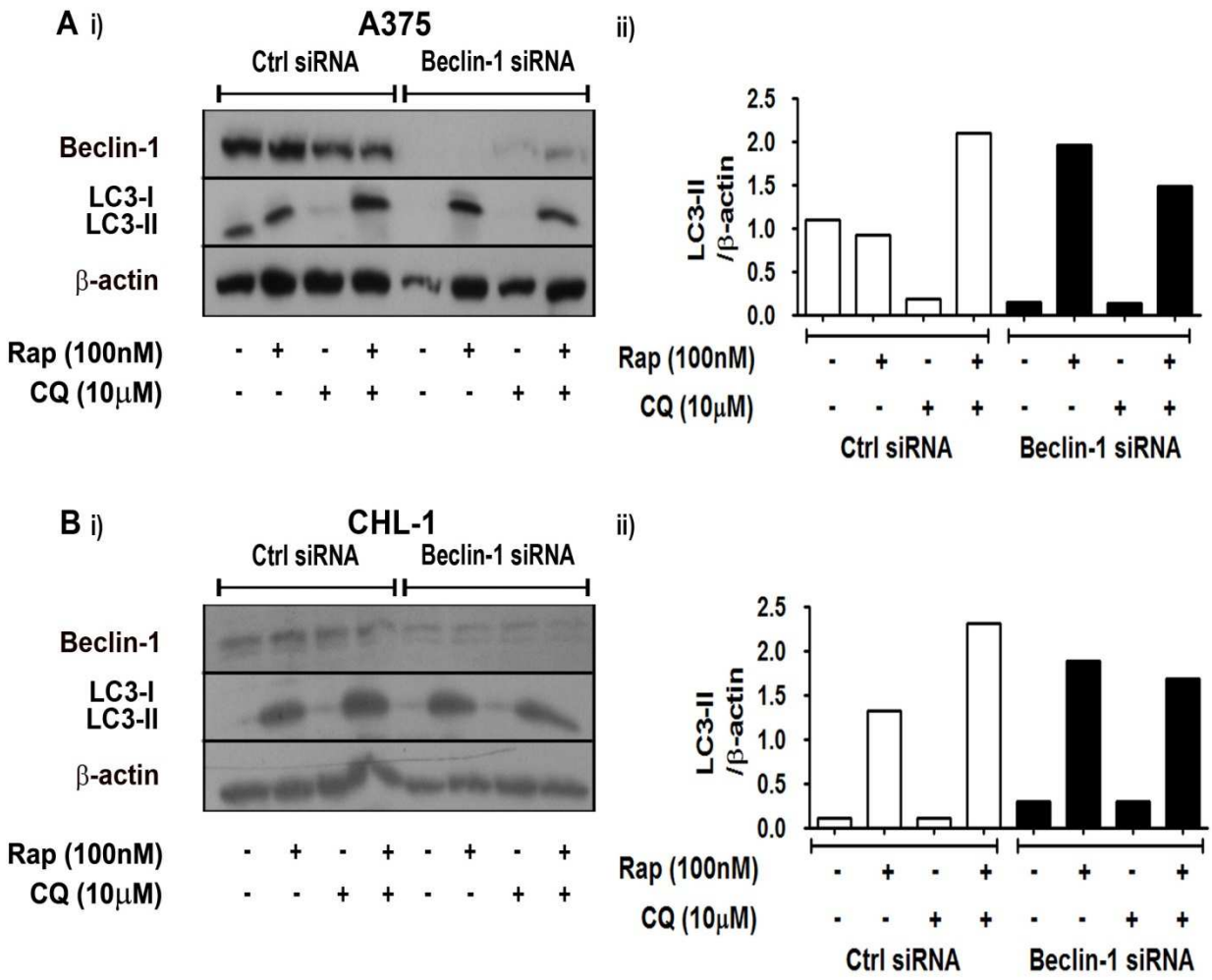

Figure 4.14: RNAi-mediated knockdown of Beclin-1 prevents Rapamycin induced autophagy. Western blot for Beclin-1, LC3-I/LC3-II, and ß-actin in (A i) A375 cells (B i) CHL-1 cells treated with 100 nM Rapamycin (Rap) for 24 hours in the presence or absence of 10 μM CQ for the final 2 hours of treatment following transfection with control siRNA (Ctrl siRNA) or Beclin-1 siRNA for 24 hours. (A ii, B ii) Each bar represents LC3-II band intensity normalised to β-actin band intensity (LC3-II/β-actin) for each treatment condition, and expressed relative to the mean of an individual experiment.

4.2.5: Beclin-1 expression in patient tumours

Results from these studies suggest the requirement of Beclin-1 for the induction of cell death in response to THC. Therefore the expression of Beclin-1 was determined in a panel of primary melanomas in order to determine the potential efficacy of THC treatment *in vivo*. An immunohistochemical assay was optimised to determine Beclin-1 protein expression in formalin-fixed and paraffin-embedded (FFPE) cell pellets derived from metastatic the melanoma cell line CHL-1 or in CHL-1 cells stably overexpressing Beclin-1 (Figure 4.15). Results confirmed increased levels of Beclin-1 in CHL-1 cells overexpressing Beclin-1 compared to control CHL-1 cells.

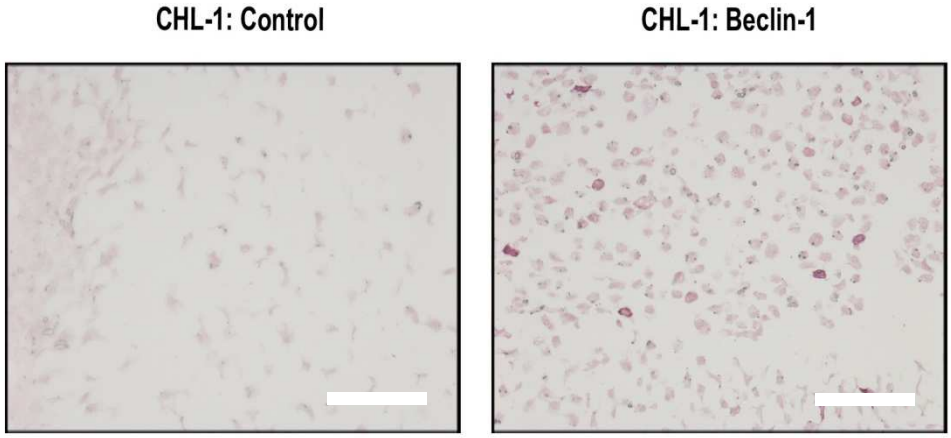

Figure 4.15: Optimisation of Beclin-1 immunohistochemistry. Immunohistochemical analysis of Beclin-1 expression in formalin-fixed paraffin-embedded cell blocks of untransfected control CHL-1 cells and CHL-1 cells over-expressing Beclin-1 (scale bar represents a length of 100 μm).

Primary melanomas derived from 20 patients were subsequently analyzed for the expression of Beclin-1 and scored according to the intensity of staining observed where 0 represents no expression, 1 represents low expression, and 2 represents high expression of Beclin-1 (Figure 4.16). Results showed medium to high expression of Beclin-1 in advanced melanomas while little or no expression was observed in, two out of three early stage tumours. Beclin-1 expression also varied in the analysed normal nevi and no correlation was identified between expression in NRAS/BRAF wild-type and mutant tumours (Table 4.1).

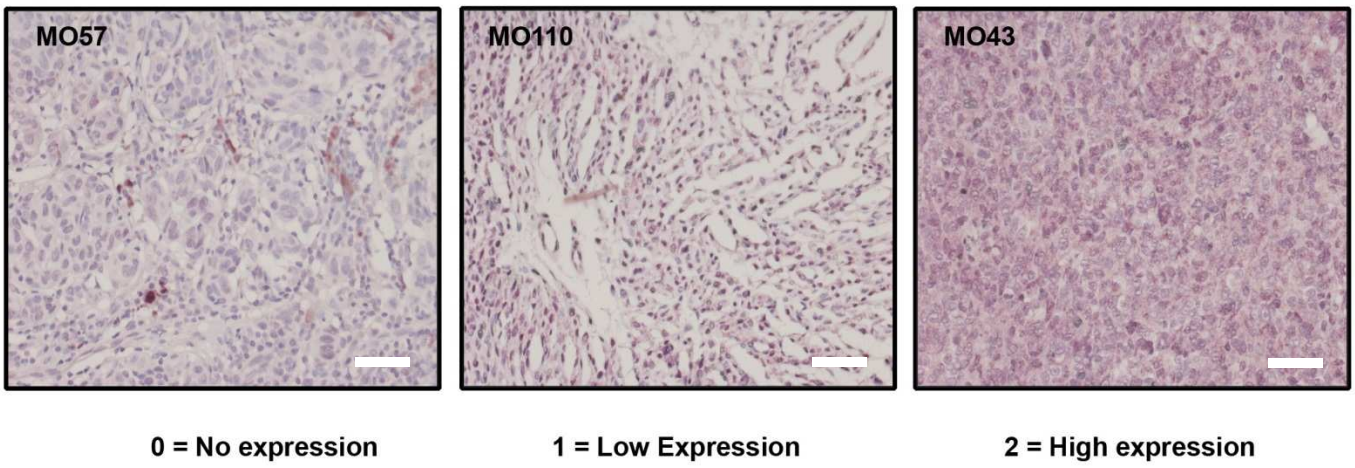

Figure 4.16: Scoring of Beclin-1 expression in patient tissues. Representative images for scoring of immunohistochemical analysis of Beclin-1 expression in formalin-fixed paraffin-embedded patient tissues. 0 represents no expression (example tumour; MO57) 1 represents low expression (example tumour; MO110) and 2 represents high expression (example tumour; MO43) (scale bar represents a length of 100 µm).

Table 4.1: Beclin-1 expression in vivo. Analysis of patient derived tissue: 10 naevi (white), 3 early stage melanomas (yellow) and 7 advanced stage melanomas (red). Expression of Beclin-1 is indicated as no expression, low expression and high expression scored as 0, 1, 2 respectively.

Tumour	Beclin-1 Score	Disease Stage: Diagnosis	Disease Stage: Final	BRAF Mutational status
M004	2	Naevus	Naevus	V600E
M049	0	Naevus	Naevus	WT
M108	0	Naevus	Naevus	V600E
M109	1	Naevus	Naevus	V600E
M110	1	Naevus	Naevus	V600E
M208	0	Naevus	Naevus	V600E
M219	2	Naevus	Naevus	WT
M220	0	Naevus	Naevus	V600E
M222	0	Naevus	Naevus	V600E
M053	1	Naevus	Naevus	V600E
M057	0	I	I	WT
M237	0	I	I	V600E
M130	2	I	I	WT
M043	2	II	III	WT
M074	2	II	II	WT
M077	1	II	IV	V600E
M137	1	II	III	WT
M147	1	II	II	V600E
M155	2	II	IV	V600E
M060	2	II	III	V600E

4.2.6: THC is less cytotoxic to primary melanocytes compared to melanoma cells

It has been suggested that THC causes little cytotoxic effect in normal tissue revealing specificity for anti-tumour activity with relatively harmless potential side effects for surrounding tissues (Chan, Sills *et al*. 1996; Iversen 2005). As melanoma cells are derived from melanocytes the effect of THC on the viability of primary melanocytes was compared with the effects on metastatic melanoma cell lines THC-induced Inhibition of cell viability was observed in A375 and CHL-1 cells in response to treatment for 24 hrs with concentrations of $\geq$ 4 µM with complete inhibition observed using 7 µM or above, whereas concentrations of $\geq$ 7 µM were required to inhibit the viability of primary melanocytes (Figure 4.17).

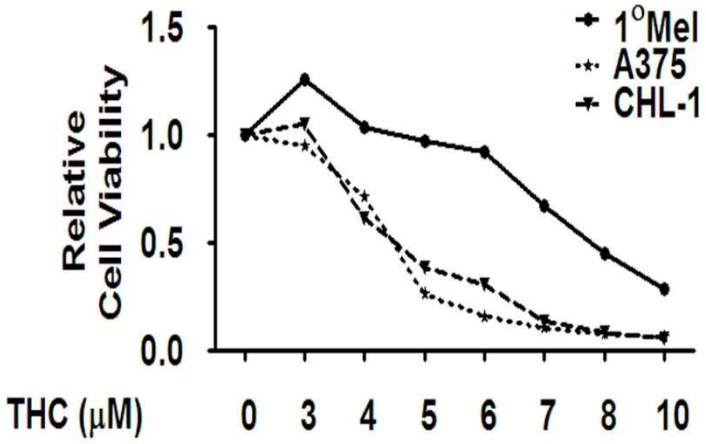

Figure 4.17: Effect of THC on normal melanocyte cell viability. Relative viability of normal melanocytes compared to metastatic melanoma cell lines (A375, CHL-1) in response to 24 hour treatment with increasing doses of THC (0,3,4,5,6,7,8,10 µM) or with vehicle control (DMSO). Each bar is the mean +/- SD, n=2.

4.2.7: THC-induced cell death of metastatic melanoma cell lines is dependent on the activity of caspases

Data from this chapter demonstrates THC induces cleavage of caspase 3 in A375 and CHL-1 cells. However, whether the resulting inhibition of cell viability and observed cell death is due entirely to the activity of caspases required confirmation. Therefore, in order to determine whether THC-induced cell death was caspase-dependant, A375 and CHL-1 cells were treated with the pan caspase inhibitor ZVAD-fmk either alone or in combination with THC. As shown by Figure 4.18, the combined treatment with THC and ZVAD-fmk prevented the significant inhibition of cell viability observed by treatment with THC alone in both melanoma cell lines (Figure 4.18), thereby confirming THC-induced inhibition of cell viability is caspase-dependent.

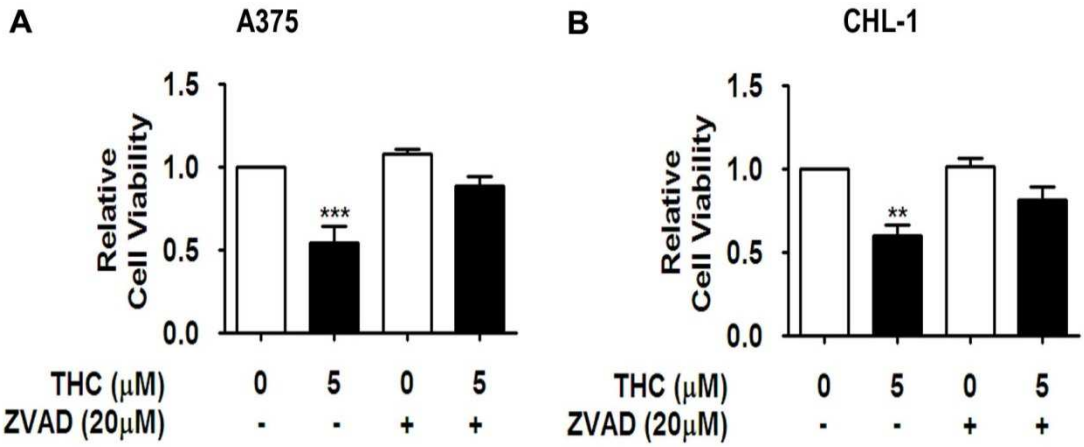

Figure 4.18: THC-induced apoptosis is caspase-dependent. Relative viability of melanoma cells (A) A375 and (B) CHL-1) following treatment for 24 hours with 5 μM THC in the presence or absence of 20 μM ZVAD-fmk (ZVAD). Each bar is relative to vehicle-treated control melanoma cells (mean +/- SEM, n=3). Statistics were acquired using one way ANOVA with Dunnett's post-hoc test, ** $p < 0.01$ *** $p < 0.001$ comparing all treatment conditions to untreated cells.

4.3: Discussion

Results from this chapter demonstrate THC induces autophagy in a panel of melanoma cell lines bearing both mutatant and wild-type *BRAF,* as confirmed by Western blot analysis of LC3-I/II conversion and analysis of autophagic flux by fluorescence microscopy. Strong evidence in the literature suggests THC activates autophagy via inhibition of PI3K/Akt/mTOR signalling and activation of the elF2α pathway in glioma (Salazar, Carracedo *et al*. 2009), with data from this chapter showing for the first time that THC causes autophagy induction in melanoma and leading to the speculation of a similar process of autophagy induction to that identified in glioma. Additionally, studies in glioma highlight the requirement of autophagy for the cytotoxic effect of THC (Salazar, Carracedo *et al*. 2009), and suggest that THC essentially hijacks the autophagy process to promote a cell death outcome. Again, results from this chapter confirm similar effects in melanoma cells as evidenced by observations in which RNAi-mediated knockdown of both *Atg7* and *Beclin-1* prevented THC-induced inhibition of cell viability and further verified by combined treatment wit the lysosomal inhibitor chloroquine (Egger, Huang *et al*. 2013; Shen, Wang *et al*. 2013). Interestingly increased LC3-I levels were observed in A375 cells transfected with Atg 7 siRNA compared to cells transfected Ctrl siRNA however Atg7 knockdown nevertheless prevented significant autophagy induction by THC. It is likely that the knockdown of Atg7 has resulted in the inhibition of autophagy and therefore prevented autophagic flux and LC3 degradation, which could also explain why the addition of chloroquine had no effect on LC3 levels in cells in which Atg7 had been knocked down. Additionally the cell death observed in response to THC was likely via apoptosis, as indicated by increased levels of Cleaved Caspase 3 and the ability of the caspase inhibitor to abrogate THC-induced loss of cell viability.

In studies of glioma, Beclin-1 was not required for THC-induced cell death leading the authors to suggest a non-canonical pro-death autophagic response (Salazar, Carracedo *et al*. 2009). Non-canonical autophagic signalling has also been mentioned demonstrated in breast cancer in which treatment with resveratrol not only induced cell death but also required a Beclin-1-independent autophagy process (Scarlatti, Maffei *et al*. 2008). However the requirement for Beclin-1 in THC-induced cell death in this study reveals noticeable differences for the possible underlying molecular events in melanoma cells treated with THC compared with studies in glioma. Increasing evidence in the literature supports the induction

of intrinsic apoptosis in response to a range of natural and synthetic cannabinoids in which several mechanisms are suggested to be involved. There is evidence for multiple cell signalling events occurring in different cancer types in response to cannabinoids such as increased phosphorylation of BAD, Akt inhibition, increased phosphorylation of ERK and lipid raft signalling events (Galve-Roperh, Sánchez *et al*. 2000; Greenhough, Patsos *et al*. 2007; Salazar, Carracedo *et al*. 2009; Scuderi, Cantarella *et al*. 2011) so it is entirely possible for the underlying molecular events in melanoma to be specific to this tumour type. However, the ultimate outcome in many of these cancers appears to be definitive in that THC ultimately induces cell death at clinically achievable doses. It should be noted cell viability assays involving analysis of formazan dye products (i.e. MTT/XTT/MTS assays) have several limitations when assessing cell death responses to drugs due to underestimations in findings attributed, for example, to the presence of cellular superoxides (Wang, Henning *et al*. 2010; Wang, Yu *et al*. 2011). As THC has been shown to generate reactive oxygen species in cancer cells (Shrivastava, Kuzontkoski *et al*. 2011) this could result in increased formazan dye products being produced indicating the cells are more viable than they are in reality. However the supporting Western data indicating increased apoptosis upon THC treatment support the findings observed using these assays.

As further evidence for the requirement of autophagy for THC-induced cell death in melanoma, RNAi-mediated knockdown of both Atg7 and Beclin-1 prevented THC-induced caspase 3 cleavage in A375 and CHL-1 cells. Two interesting observations occurred from this study however. Firstly, knockdown of either Beclin-1 or Atg7 alone in $BRAF^{WT}$ CHL-1 cells resulted in a markedly increased level of Cleaved Caspase 3 despite preventing any enhancement in response to THC. As autophagy is typically required as a pro-survival response in many normal tissues and tumour types including melanoma (Armstrong, Corazzari *et al*. 2011; Wright, McKee *et al*. 2013; Del Bello, Toscano *et al*. 2013; Greene, Nolan *et al*. 2013; Periyasamy-Thandavan, Jiang *et al*. 2008) and basal autophagy levels are noticeably lower in $BRAF^{WT}$ compared with BRAF mutated melanoma cell lines (Armstrong, Corazzari *et al*. 2011), this may account for the death observed following knockdown of Atg7 or Beclin-1 as autophagy may be more essential to CHL-1 cell survival. Secondly, knockdown of Beclin-1 in A375 cells did not prevent THC-induced autophagy despite preventing cell death in response to THC. The reasons for this observation remain unclear, however increasing evidence for alternative roles of Beclin-1 that are independent of autophagy

which may account for this effect (a point addressed and described in greater detail in proceeding chapters). One possibility could have been a lack of siRNA specificity for the Beclin-1 siRNA. As such studies were undertaken in an attempt to validate the role of Beclin-1 in canonical autophagy in melanoma cell lines using the mTOR inhibitor rapamycin, known to require Beclin-1 for autophagy induction (Armstrong, Corazzari *et al*. 2011; Li, Wu *et al*. 2013). Results demonstrated rapamycin induced autophagy beyond basal levels in A375 and CHL-1 cells transfected with control siRNA. However, rapamycin was also able to induce autophagy in cells transfected with Beclin-1 siRNA. Ultimately these data are hence difficult to interpret in terms of Beclin-1's function in autophagy within these cells in addition to the limited number of experiments performed (n=1) however, combined with observations that THC and bortezomib can still induce autophagy in the absence of Beclin-1 questions the function of this protein in autophagy in A375 and CHL-1 cells.

Although the increased levels of Cleaved Caspase 3 identified in these studies is a reasonable indication for the induction of intrinsic apoptosis in response to THC treatment, the possibility of caspase-independent cell death or necrosis (a process suggested to occur by cannabinoid treatment (Gustafsson, Wallenius *et al*. 2013)) could not be ruled out. In order to determine whether caspase cleavage is essential for THC-induced cell death use of the irreversible pan caspase inhibitor ZVAD-fmk was employed to test its ability to inhibit THC-induced inhibition of cell viability. Treatment with ZVAD-fmk partially prevented THC-induced inhibition of cell viability suggesting the observed cell death is caspase-dependent and providing further evidence for intrinsic apoptosis. As THC has also been shown to induce anti-proliferative effects through its effects on the cell cycle and which ZVAD-fmk can not prevent (Blázquez, Casanova *et al*. 2003; Blázquez, Carracedo *et al*. 2006), this possibility can also not be ruled out.

Although these studies demonstrate the clear cytotoxic effects of THC on melanoma cells *in vitro*, cytotoxicity is nevertheless a matter of concern for many chemotherapeutics resulting in limitations including achievable clinical doses which exert a similar effect *in vivo* as to that seen *in vitro* as well as potentially harmful side effects. Interestingly however, cannabinoids display a particularly good drug safety profile in both animal models and humans with no observed cytotoxic effects on normal tissues (Chan, Sills *et al*. 1996; Grotenhermen 2003; Iversen 2005). In line with these findings treatment with increasing concentrations of THC did not reduce the cell viability of primary melanocytes in response to

doses of THC which were shown to significantly inhibit viability of both A375 and CHL-1 cells. These observations support previous findings comparing stable melanocyte cell lines melan-c and Hermes 2b with melanoma cell lines B16 and A375 in which inhibition of melanoma cell viability was observed in response to cannabinoids (THC and WIN-55,212-2) but for which no significant cytotoxic effect was observed in melanocytes (Blázquez, Carracedo *et al*. 2006), albiet using low 1 µM concentrations, far below the maximal dose used in the current studies of 10 µM. It was once written by the proclaimed father of toxicology, Paracelsus, "Everything is poison. Only the dose makes a thing not a poison" this could therefore be adapted in this case in which such a high dose of THC (far beyond that required to induce cell death in cancer cells) would ultimately result in non-tumour cell cytotoxic effects.

As previously discussed, the possibility of tumour cell death induced by autophagy is an uncommon and novel occurrence as cancer cells typically induce autophagy as a pro-survival response to chemotherapies. In order to determine the difference in pro-death autophagy induced by THC with a more typical pro-survival autophagic response, bortezomib treatment of melanoma cells was compared with the effects of THC on the induction of both autophagy and apoptosis. Bortezomib acts through inhibition of the 26S proteasome and it is reported to induce apoptosis through ER stress of melanoma cells (Corazzari, Lovat *et al*. 2007; Hill, Martin *et al*. 2009). Additionally, bortezomib has little to no adverse effects on normal melanocytes (Fernandez, Verhaegen *et al*. 2005; Lovat, Corazzari *et al*. 2008). However, induction of unfolded protein response (UPR) as well as the induction of pro-survival autophagy may overcome the cellular stress conditions caused by bortezomib (Armstrong, Corazzari *et al*. 2011). Results from the present study demonstrate bortezomib-induced inhibition of cell viability is neither abrogated nor enhanced in combination with chloroquine suggesting that this event is autophagy independant. Additionally, in contrast to previous reports demonstrating Beclin-1 or Atg7 knockdown sensitized $BRAF^{WT}$ cells to bortezomib-induced cell death (Armstrong, Corazzari *et al*. 2011), knockdown of Atg7 and Beclin-1 in CHL-1 cells did not enhance inhibition of cell viability in these studies. However, knockdown of autophagy genes in CHL-1 cells (particularly Beclin-1) resulted in cell death and high levels of Cleaved Caspase 3 levels of which were further increased in response to bortezomib supporting the previous suggestion of a pro-survival function of autophagy in this cell line. It is possible that limitations in the MTS assay used to assess cell viability meant this method was not sensitive enough to determine the differences in cell viability in

response to abrogated autophagy. However, it is clear that autophagy is not required for cell death in response to bortezomib and confirms the differing role autophagy plays in response to different chemotherapeutics.

It should be noted that, although the underlying molecular events for the contribution of Beclin-1 to THC-induced cell death in melanoma have yet to be determined, the requirement for Beclin-1 for THC to cause cell death is more conclusive. As such, if similar findings were to be found *in vivo* the expression of Beclin-1 in patient tumours may potentially determine the stratification of particular patients for treatment with THC. In order to determine this, Beclin-1 expression was assessed in a small cohort of nevi and primary melanomas. However, during the timescale of this research results have since been published which demonstrate a biphasic expression pattern in melanoma (Sivridis, Koukourakis *et al*. 2011). In essence low expression of Beclin-1 in tumours was associated with high breslow depth, ulceration , and increased angiogenesis, whereas high expression of Beclin-1 resulted in increased rate of early deaths and metastatic disease (Sivridis, Koukourakis *et al*. 2011). Although the panel studied in this chapter is relatively small, initial observations indicate similar findings where expression of Beclin-1 is lost in earlier stage disease and some naevi while expression is maintained in more advanced tumours, thus supporting the paradoxical role of autophagy in cancer (Mathew, Karp *et al*. 2009; White and DiPaola 2009; Wu, Coffelt *et al*. 2012). As such one could speculate maintained Beclin-1 expression may predict THC responsiveness *in vivo*. Nevertheless it should be noted that expression of Beclin-1 does not necessarily confer functional activity. In summary, data derived from this chapter demonstrates THC-induced apoptosis of melanoma cells likely depends on autophagy. However a definitive link between autophagy and apoptosis remains elusive and further studies are required to discern this link which is the focus of studies in subsequent chapters. With promising data derived from clinical trials of sativex (a mixture of THC and canibidol) in glioma this research nevertheless enhance our understanding of how to exploit autophagy for the clinical benefit of melanoma and the potential use of cannabinoids to promote effective cell death.

4.4: Summary

- THC induces autophagy in melanoma cells *in vitro.*
- THC-induced autophagy is required for cell death.
- THC-induced cell death is likely mediated via intrinsic apoptosis requiring the activity of caspases.
- Bortezomib-induced cell death is independent of autophagy.
- Expression of Beclin-1 in a small panel of patient tumours indicates increased expression in advanced stage compared to earlier disease stages.
- THC is relatively in toxic to primary melanocytes.

Table 4.2 Summary for the observed effects of THC and bortezomib treatment on autophagy induction, caspase 3 cleavage and inhibition of cell viability in A375 and CHL-1 cells.

Cell line	Treatment	Process	Dependent on Atg7?	Dependent on Beclin-1?
A375	THC	Autophagy induction	Yes	No
		Caspase 3 cleavage	Yes	Yes
		Inhibition of cell viability	Yes	Yes
	* Bort	Autophagy	? partially	No
		Caspase 3 cleavage	↑	↑
		Inhibition of cell viability	No	No
CHL-1	THC	autophagy	Yes	Yes
		Caspase 3 cleavage	Yes	?
		Loss of viability	Yes	Yes
	* Bort	Autophagy induction	? Partially	?
		Caspase 3 cleavage	↑	↑
		Inhibition of cell viability	No	No

*** Bortezomib induces pro-survival autophagy**

Chapter 5: Identification of Beclin-1 protein-protein interactions

Chapter 5: Identification of Beclin-1 protein-protein interactions

Table of contents

5.1: Introduction

Results from the previous chapter demonstrated autophagy is apparently required for THC-induced cell death of metastatic melanoma cells *in vitro,* verified both by the knockdown of two essential autophagy genes (*Beclin-1 and Atg7*) and treatment with chloroquine. Conversely, bortezomib-induced autophagy in metaststic melanoma cells is pro survival. However despite the observed data following knockdown of *Atg7*, knockdown of *Beclin-1* did not prevent THC-induced autophagy despite preventing cell death. Beclin-1 siRNA target specificity was verified using rapamycin thus suggesting an alternative mechanism for Beclin-1 in determining the fate of metastatic melanoma cell lines treated with THC. As such, investigations of known interactions of Beclin-1 were undertaken to further elaborate on potential molecular mechanisms induced by THC involving Beclin-1.

In recent years several publications have revealed interacting partners for many members of the autophagy pathway including Beclin-1. A vast wealth of information revealing potentially crucial protein-protein interactions is readily available in the current literature, however pooling of this data can prove taxing by conventional approaches. Several bioinformatics tools have become available over the years which allow for accurate data mining approaches. One such tool is Cytoscape, an open source bioinformatics software platform used for visualizing molecular interaction networks which has multiple functions enhanced by the use of specific plugins designed by the scientific community (Shannon, Markiel *et al*. 2003; Yeung, Cline *et al*. 2008). In essence a plugin is a set of software components that adds specific abilities to a larger software application for which several are available for the Cytoscape software to advance upon its analytical capabilities (Shannon, Markiel *et al*. 2003). One such plugin used for the visualisation of known protein-protein interactions for a protein of interest has been designed by Biological General Repository for Interaction Datasets (BioGRID) which gathers constantly updated interaction data for several organisms (Homo sapiens included) from several databases; Saccharomyces Genome Database, Flybase, GeneDB, TAIR, and Entrez Gene (Chatr-Aryamontri, Breitkreutz *et al*. 2013). The data provides several useful pieces of information in addition to the identified genetic and protein-protein interactions such as the source of the information, listed by publication, as well as the techniques used to obtain the results allowing for further in depth analysis of each identified interaction.

The overall aim of this chapter was therefore to utilize such software to gain further insight into protein-protein interactions for Beclin-1. This information was then further advanced by categorising identified interacting proteins into key cellular pathways of relevance to the central focus of this thesis, namely autophagy, apoptosis and the cell cycle.

5.2: Results

5.2.1: Protein-protein interactions of Beclin-1

Several publications in recent years have revealed the many genetic and protein interactions for several key members of the autophagy pathway (Behrends, Sowa *et al*. 2010). Beclin-1 is one of such member for which interacting partners have been extensively researched (Chang, Nguyen *et al*. 2010; Chen, Gao *et al*. 2010; Criollo, Niso-Santano *et al*. 2011; Kim, Bennett *et al*. 2011; Liang, Kleeman *et al*. 1998; Platta, Abrahamsen *et al*. 2012; Shi and Kehrl 2010; Wagner, Beli *et al*. 2011; Xu, Liu *et al*. 2011; Yue, Horton *et al*. 2002). The data mining tool cytoscape was hence used in combination with a plug-in provided by BioGRID in order to identify known protein-protein interactions of Beclin-1 and combine all known published data of these interactions. A total of 34 known protein-protein interactions were identified for Beclin-1 (Figure 5.1).

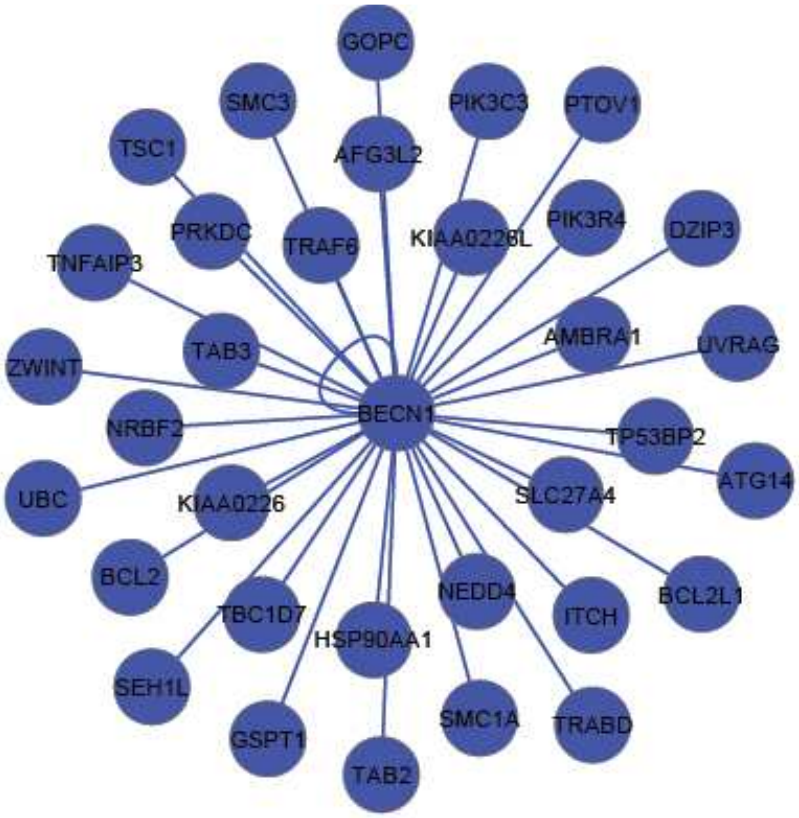

Figure 5.1: Known protein-protein interactions for Beclin-1. The plot shown represents all direct protein-protein interactions identified using BioGRID and visualised in cytoscape.

5.2.2: Categorization of identified protein-protein interactions of Beclin-1

In order to further evaluate initial findings for known Beclin-1 interacting proteins, all identified Beclin-1 protein-protein interactions were categorised according to their biological function and subcategorised according to whether they have a positive or negative regulatory role in these cellular pathways. Many of the proteins identified fit into categories for biological processes relevant to the theme of this thesis, namely those involved in autophagy, the cell cycle and apoptosis (Figure 5.2).

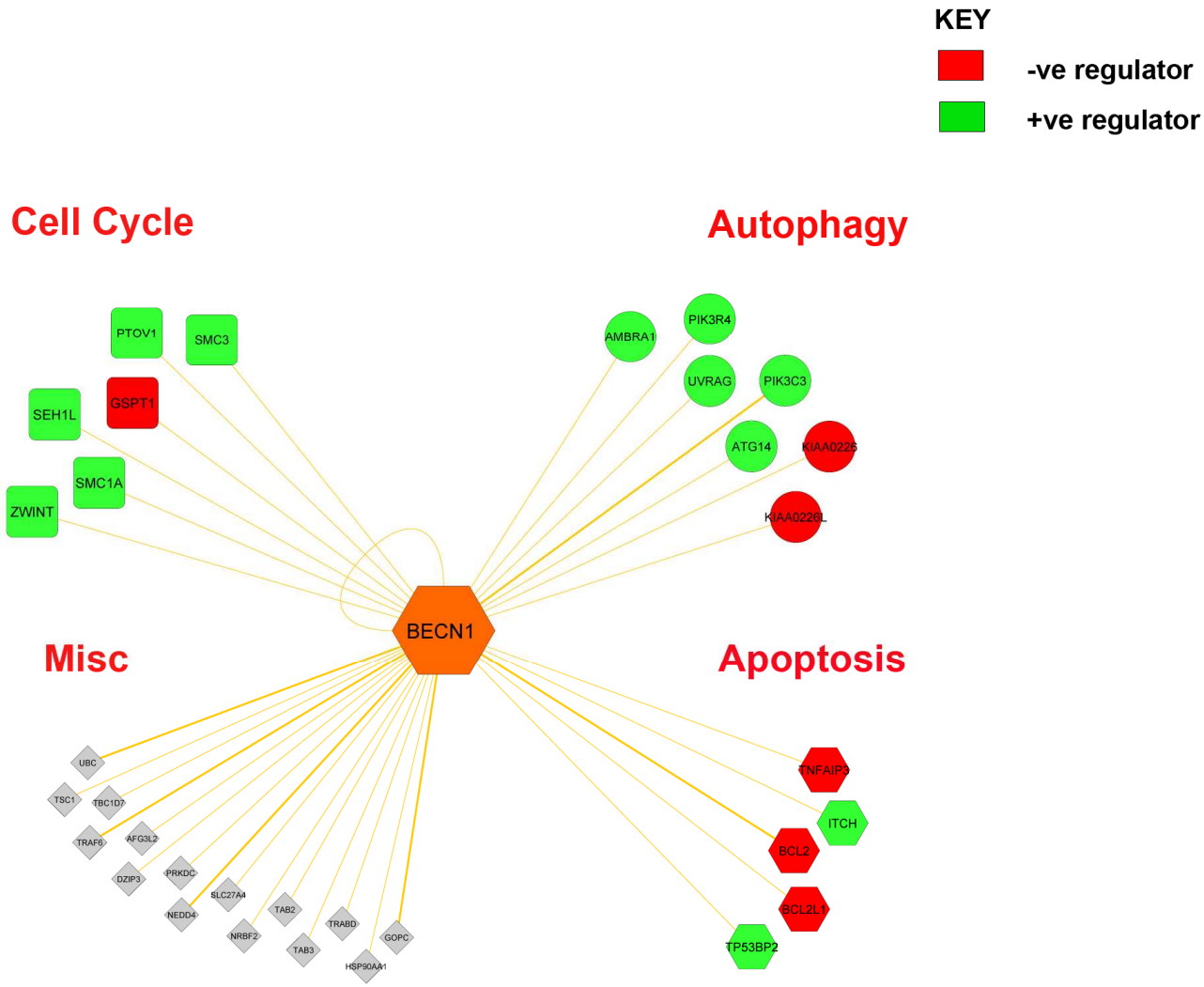

Figure 5.2: Regulatory functions of known Beclin-1 interacting proteins. Proteins are grouped according to their regulatory roles positive (Green) or negative (Red) in several intracellular pathways; Autophagy (Circles), Apoptosis (Hexagons), Cell cycle (Squares), Miscellaneous (Misc, Diamonds).

Seven identified Beclin-1 interacting proteins were found to have a crucial regulatory role in the autophagy pathway. Of these, seven were found to have a positive role in autophagy regulation; Ambra 1, PIK3R4, UVRAG, PIK3C3, Atg14 while two were found to have a negative role; KIAA0226 (also known as Rubicon) and KIAA0226L (Figure 5.3). Individual functions for Beclin-1 interactors key to the regulation of autophagy are outlined in Table 5.1.

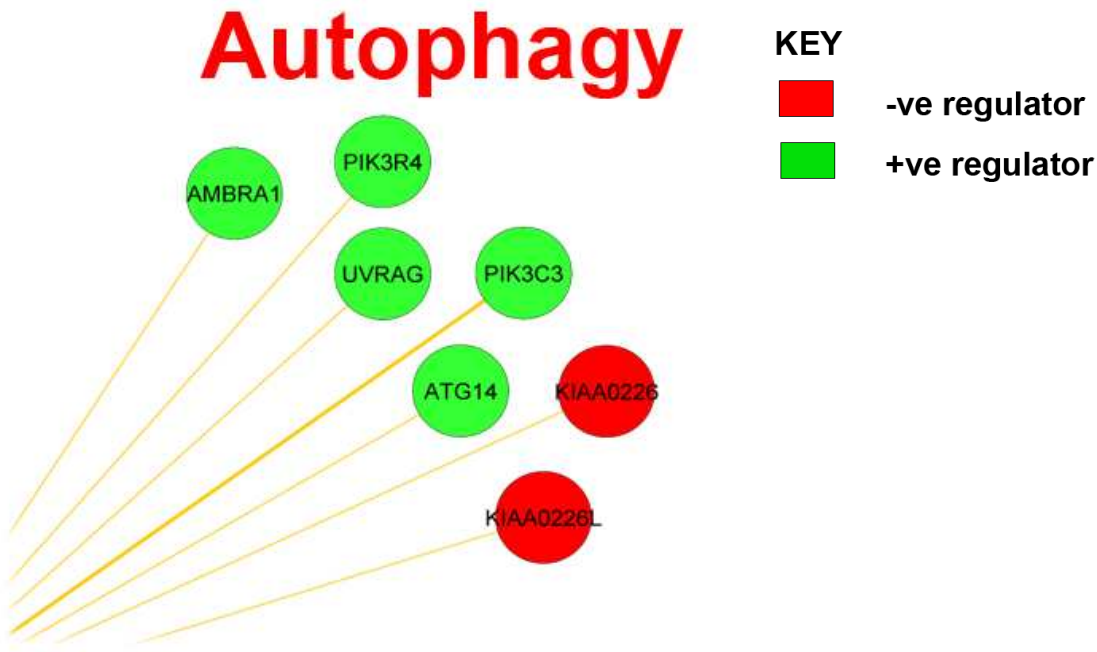

Figure 5.3: Autophagy regulatory proteins which interact with Beclin-1. Proteins are grouped according to their regulatory roles positive (Green) or negative (Red)**.**

Table 5.1: Beclin-1 interacting proteins involved in autophagy regulation

Identified member	Regulatory role	Function
Ambra 1	Positive	Ambra 1 is a positive regulator of Beclin-1-dependent autophagy (Fimia, Stoykova *et al*. 2007). ULK1 activity releases Ambra 1 from dynein which then localizes to the ER and primes autophagosome formation and nucleation (Di Bartolomeo, Corazzari *et al*. 2010). Additionally under normal conditions, a pool of Ambra 1 is bound to Bcl-2 at the mitochondria preventing its role in autophagy however after autophagy is induced, this mitochondrial bound Ambra 1 separates from Bcl-2 and binds to Beclin-1 further enhancing the induction of autophagy. (Strappazzon, Vietri-Rudan *et al*. 2011)
Atg14	Positive	Atg14 is a specific substrate of the Beclin-1 complex which functions in the targeting of this complex to the site of autophagosome formation. Atg14 C terminus contains an Barkor/Atg14(L) autophagosome-targeting sequence (BATS) domain which preferentially binds to curved membranes containing PI(3)P and targets the Beclin-1 complex to the isolation membrane. (Obara and Ohsumi 2011)
KIAA0226 (Rubicon)	Negative	Rubicon binds to a subpopulation of UVRAG complexes separate to Atg14L forming a different Beclin-1 complex (Sun Q 2010). Rubicon was localized at the endosome/lysosome and knockdown of Rubicon caused enhancement of autophagy, especially at the maturation step, as well as enhancement of endocytic trafficking. These data suggest that the Beclin-1-hVps34 complex functions in two different steps of autophagy by altering the subunit composition. (Matsunaga, Saitoh *et al*. 2009; Zhong, Wang *et al*. 2009).
KIAA0226L (RubiconL)	Negative	Functions in the same manner as Rubicon.
PIK3C3	Positive	Regulatory subunit of the PI3K complex also known as Vps34. Vps34 may also regulate membrane trafficking late in the endocytic pathway (Johnson, Overmeyer *et al*. 2006). This protein belongs to the protein kinase Ser/Thr protein kinase superfamily. This subunit, part of a complex composed of regulatory and catalytic subunits, associates with the phosphatidylinositol 3-kinase type 3 (PIK3C3) (Zhou, Takatoh *et al*. 2011).
PIK3R4	Positive	Catalytic subunit of the PI3K complex which is involved in the transport of lysosomal enzyme precursors to lysosomes. Belongs to the PI3/PI4-kinase family and forms a complex with Ambra 1 and Beclin-1. PIK3C3 is also critical for the formation of pre-autophagosomes which are important for degradation of large protein aggregates and organelles (Petiot, Ogier-Denis *et al*. 2000). Finally, PIK3C3 is shown to be required for nutrient/amino acid mediated activation of mTOR signaling in cultured cells (Zhou, Takatoh *et al*. 2011).
UVRAG	Positive	Originally identified as a protein related to UV resistance, this protein promotes autophagy by activation of the Beclin-1-PI(3)KC3 complex and suppresses the proliferation of human colon cancer cells (Liang, Feng *et al*. 2007; Iida, Emi *et al*. 2000). Additionally UVRAG has been found to have a role in the maintenance of chromosome stability independent of autophagy where it localizes at centrosomes and physically associates with CEP63 (Knævelsrud H, Ahlquist T *et al*. 2010; Zhao, Oh *et al*. 2012). Chromosomal aberrations involving this gene are associated with left-right axis malformation and mutations in this gene have been associated with colon cancer (Iida, Emi *et al*. 2000).

Five identified Beclin-1 interacting proteins were found to have a crucial regulatory role in the apoptosis pathway. Of these five, two were found to have a positive role in apoptosis regulation; ITCH and TP53P2 and three were found to have a negative role; Bcl-2, Bcl-2L1, TNFAIP3 (Figure 5.4). Each of these proteins has been extensively studied and has a crucial role in apoptosis regulation for which their individual functions are outlined in Table 5.2.

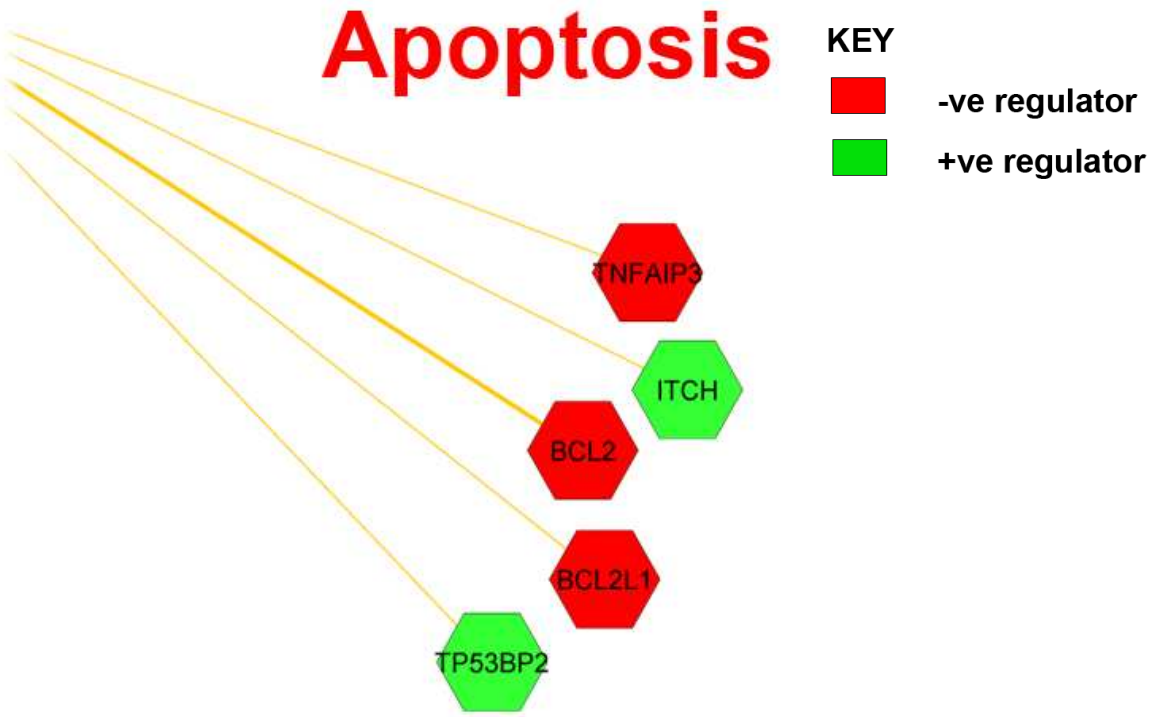

Figure 5.4: Apoptosis regulatory proteins known to interact with Beclin-1. Proteins are grouped according to their regulatory roles positive (Green) or negative (Red)

Table 5.2: Beclin-1 interacting proteins involved in apoptosis regulation

Identified member	Regulatory role	Function
Bcl-2	Negative	The proteins encoded by the Bcl-2 gene family are located at the outer mitochondrial membrane, and have been shown to regulate outer mitochondrial membrane channel (VDAC) opening. VDAC regulates mitochondrial membrane potential, and thus controls the production of reactive oxygen species (ROS) and release of cytochrome C by mitochondria, both of which are the potent inducers of cell apoptosis. (Tsujimoto and Shimizu 2000)
Bcl-2L1	Negative	Same function as Bcl-2.
ITCH	Negative	Itch selectively binds and ubiquitinates p73 but not p53; this results in the rapid proteasome-dependent degradation of p73. P73 is a member of the p53 family of transcription factors and is up-regulated in response to DNA damage thus inducing cell cycle arrest and apoptosis (Rossi M 2005).
TNFAIP3	Positive	This gene was identified as a gene whose expression is rapidly induced by the tumour necrosis factor (TNF). The protein encoded by this gene is a zinc finger protein and ubiquitin-editing enzyme, and has been shown to inhibit NF-kappa B activation as well as TNF-mediated apoptosis (Li, Soetandyo *et al*. 2009). The encoded protein, which has both ubiquitin ligase and deubiquitinase activities, is involved in the cytokine-mediated immune and inflammatory responses. (Li, Soetandyo *et al*. 2009)
TP53P2	Positive	This gene encodes a member of the ASPP (apoptosis-stimulating protein of p53) family of p53 interacting proteins. The protein contains four ankyrin repeats and an SH3 domain involved in protein-protein interactions. It is localized to the perinuclear region of the cytoplasm, and regulates apoptosis and cell growth through interactions with other regulatory molecules including members of the p53 family thus acting as a tumour suppressor (Nakagawa, Koyama *et al* 2000; Samuels-Lev, O'Connor *et al* 2001).

Six identified Beclin-1 interacting proteins were found to have a crucial regulatory role in the cell cycle pathway. Of these six, five were found to have a positive role in cell cycle regulation; PTOV1, SMC3, SEH1L, SMC1A, ZWINT and one was found to have a negative role; GSPT1 (Figure 5.5). Each of these proteins is key to the regulation of the cell cycle for which their individual functions are outlined in table 5.3.

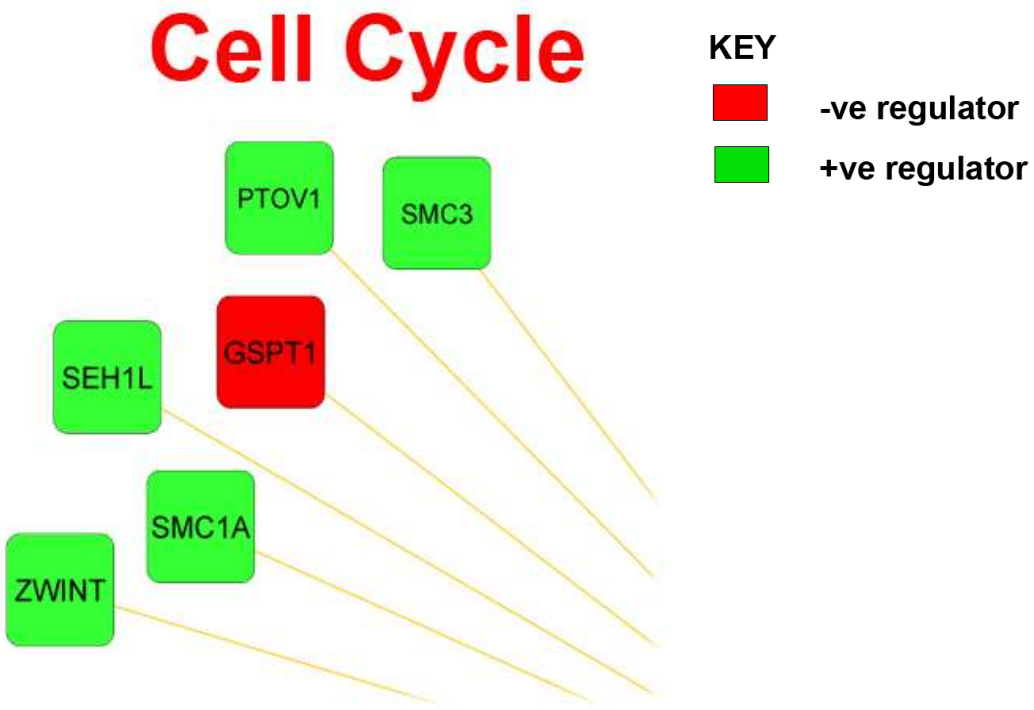

Figure 5.5: Cell cycle regulatory proteins known to interact with Beclin-1. Proteins are grouped according to their regulatory roles positive (Green) or negative (Red)

Table 5.3: Beclin-1 interacting proteins involved in cell cycle regulation

Identified member	Regulatory role	Function
GSPT1	Negative	The GTP protein encoded by this gene belongs to the GTP-binding elongation factor family and was first identified from the cDNA library of human KB cells (Hoshino, Miyazawa *et al*. 1989). It is Involved in translation termination in response to the termination codons UAA, UAG and UGA. Additionally it stimulates the activity of ERF1 and is Involved in regulation of mammalian cell growth. (Hoshino, Miyazawa *et al*. 1989)
PTOV1	Positive	PTOV1 has been shown to promote cell proliferation and as well as stimulate entry into the S phase of the cell cycle (Santamaría, Fernández *et al*. 2003).
SEH1L	Positive	The protein encoded by this gene is part of a nuclear pore complex, Nup107-160. This protein contains WD repeats and shares 34% amino acid identity with yeast SEH1. All constituents of the Nup107-160 complex, including this protein, specifically localize to kinetochores in mitosis. (Glavy, 2007).
SMC1A	Positive	The protein encoded by this gene is part structural maintenance of chromosomes (SMC) protein family. These act as part of the cohesin multiprotein complex which is needed for sister chromatid cohesion (Losada and Hirano 2005). Cohesion of sister chromatids is required for the correct segregation of chromosomes during cell division. The cohesion multiprotein complex is composed partly of two SMC proteins, SMC3 and either SMC1L2 and SMC1A (Anderson DE 2002). Most of the cohesin complexes dissociate from the chromosomes before mitosis, however the complexes at the kinetochore remain. As such SMC1A is thought to be an important part of functional kinetochores (Haering CH 2004). In addition, this protein has been shown to be required for growth of several cancer types (Ma Z 2013; Zhang YF 2013).
SMC3	Positive	This gene belongs to the SMC3 subfamily of SMC proteins. The encoded protein occurs in certain cell types as either an intracellular, nuclear protein or a secreted protein. The nuclear form, known as structural maintenance of chromosomes 3, is a component of the multimeric cohesin complex that holds together sister chromatids during mitosis, enabling proper chromosome segregation (Losada and Hirano 2005).
ZWINT	Positive	ZWINT-1 is involved in kinetochore function as It interacts with ZW10, another kinetochore protein, recruiting It for attachment to unattached kinetochores (Wang, Hu *et al*. 2004; Kops, Kim *et al*. 2005). The encoded protein localizes to prophase kinetochores before ZW10 does and it remains detectable on the kinetochore until late anaphase. It has a uniform distribution in the cytoplasm of interphase cells.

5.3: Discussion

Results from this chapter identified 34 known Beclin-1 protein-protein interactors using a freely available plugin tool provided by The Biological General Repository for Interaction Datasets (BioGRID). Cytoscape, was used to visualize molecular interaction networks. Investigation of individual functions for each of the 34 identified proteins resulted in the categorization of several interactors into three molecular processes relevant to the underlying research focus of this thesis, ie) Autophagy (seven members identified), Apoptosis (five members identified) and the cell cycle (six members identified). Members identified within each biological process were further subdivided into whether they have a positive or negative regulatory role for each of these processes revealing the potential effects an interaction with Beclin-1 may ensue.

Data suggests that core members and conformation of the Beclin-1 complex may both positively and negatively impact on a multitude of cellular pathways. The identification of at least three different PI(3)K complexes in autophagy further supports the possibility of differing roles for Beclin-1 depending on what it is complexed to (Matsunaga, Saitoh *et al*. 2009) although the physiological function of several of the above interactions have yet to be defined. Interestingly a recent article highlighted the importance of Beclin-1 in cell cycle regulation where it is required for chromosome congression and proper outer kinetochore assembly (Frémont S 2013) thus revealing an important role for Beclin-1, independent of autophagy. Several interactions of Beclin-1 with key members of cell cycle regulation were identified in this chapter which are particularly important during mitosis and kinetochore assembly including; SEH1L, SMC3, ZWINT (Glavy, 2007; Kops, Kim et al. 2005; Losada and Hirano 2005; Wang, Hu et al. 2004) further highliting a role for Beclin-1 in cell cycle regulation. Additionally, recent observations show under certain conditions Beclin-1 may be cleaved by caspases thus preventing its pro-autophagic activity (Wirawan, Vande Walle *et al*. 2010). Interestingly further studies have found the C-terminal fragment produced as a result of this cleavage event has a function of its own in which it amplifies mitochondrial mediated apoptosis suggesting cross talk between apoptosis and autophagy (Djavaheri-Mergny, Maiuri *et al*. 2010). Thus, caspase activation (an event shown to occur during treatment with THC) could further drive cells to a point of no return and their entry into apoptosis. As a further point linking cross talk between autophagy and apoptosis, the interaction of Beclin-1 with

the apoptosis-stimulating protein of p53 tumour suppressor family member tumor protein 53-induced nuclear protein 2 (TP53P2, also known as ASPP2) was also highlighted in these studies. TP53P2 is typically found in the perinuclear region of the cytoplasm and its key responsibilities within the cell are the regulation of apoptotsis and cell growth via a multitude of interactions including TP53 and has been shown to enhance DNA binding of TP53 on the promoters of proapoptotic genes *in vivo* (Nakagawa, Koyama *et al* 2000; Samuels-Lev, O'Connor *et al* 2001). Another member of this protein family, TP53P1 (otherwise known as TP53INIP1) has not only been shown to induce apoptosis in pancreatic cancer cells but also promotes autophagy-dependent cell death, highlighting the importance of this protein family as a tumour suppressor mechanism (Gironella, Seux *et al*. 2007; Gommeaux, Cano *et al*. 2007; Seillier, Peuget *et al*. 2012). Seillier *et al* showed knockdown of key autophagy genes Beclin-1 and Atg5 as well as inhibition of caspase activity prevented cell death induced by TP53INIP1 suggesting a cross link between autophagy and apoptosis and implicating the requirement of both processes (Seillier, Peuget *et al*. 2012). Additionally TP53INP1 has also been shown to interact with Atg8-family proteins as well as LC3 to induce autophagy-dependent cell death. Hence, the interaction of Beclin-1 with members of this protein family may also have important implications in this context which are yet to be defined. As such, future insights into the physiological relevance of Beclin-1 and its recently identified protein-protein interactions will undoubtedly help to define further cellular functions in which Beclin-1 is implicated.

Ambra 1 (or activating molecule in Beclin-1 regulated autophagy protein 1) was one of the key autophagy proteins identified as an interactor of beclin-1 and several reports support the role for this protein as a positive regulator of autophagy and a component of the Beclin-1/VPS34 complex (Fimia, Di Bartolomeo *et al*. 2011; Fimia, Stoykova *et al*. 2007; Roy and Debnath 2010). Ambra 1 is a WD40-containing protein allowing it to function as a protein-protein interacting platform (Fimia, Corazzari *et al*. 2012) where its key role in autophagy is in the nucleation phase where it associates with additional members of the Beclin-1 complex such as Atg14 and UVRAG (Di Bartolomeo, Corazzari *et al*. 2010; Fimia, Di Bartolomeo *et al*. 2011; Fimia, Stoykova *et al*. 2007; Itakura and Mizushima 2010; Roy and Debnath 2010). However, other interactions with Ambra 1 have been identified which are independent of autophagy and linked to apoptosis. A recent report demonstrated mitochondrial Bcl-2 interacts directly with Ambra 1 independently of Beclin-1; an event

which is disrupted by the induction of either autophagy of apoptosis (Strappazzon, Vietri-Rudan *et al*. 2011). Interestingly Ambra 1 and Bcl-2 bind to Beclin-1 at the same site and so it is suggested that Ambra 1 competes with the binding of Bcl-2 to Beclin-1 (Strappazzon, Vietri-Rudan *et al*. 2011). As Bcl-2 family members are often highly expressed in melanoma this interaction may be critical to both the regulation of both autophagy and apoptosis in this cancer type. Furthermore, in a similar manner to caspase cleavage of Beclin-1 , Ambra 1 may be irreversibly cleaved and degraded by the activity of caspases and calpains (Corazzari, Fimia *et al*. 2012), key to the promotion of cell death over a pro-survival autophagic response in which the switch is regulated by caspases.

Due to its interaction with Beclin-1, its known role in autophgay (Di Bartolomeo, Corazzari *et al*. 2010; Fimia, Stoykova *et al*. 2007; Itakura, Kishi *et al*. 2008; Simonsen and Tooze 2009; Roy and Debnath 2010) and links with apoptosis (Corazzari, Fimia *et al*. 2012), Ambra 1 was therefore identified as a key protein to further evaluates and in particular the evaluation of its contribution to THC-induced autophagy and cell death in melanoma. Previous studies in glioma have shown that Ambra 1 is essential for THC-induced autophagy and cell death (Salazar, Carracedo *et al*. 2009) and that this process did not require Beclin-1 (personal communication with Guillermo Velasco). Therefore further studies were also undertaken to determine the role of Ambra 1 in THC-induced autophagy and cell death in melanoma in a cell survival versus cell death context.

5.4: Summary

- A total of 34 known Beclin-1 protein-protein interactions were identified using The BioGRID plugin for cytoscape.
- 7/34 identified proteins were key to autophagy of which 5 displayed a positive and 2 a negative regulatory role.
- 5/34 identified 34 proteins were key to apoptosis of which 2 displayed a positive and 3 a negative regulatory role.
- 6/34 identified proteins were key to the cell cycle of which 5 displayed a positive and 1 a negative regulatory role.
- Identified interactions revealed alternative roles for Beclin-1, both dependent and independent of autophagy.
- Ambra 1 was identified as a key interactor of Beclin-1.

Chapter 6: Regulation of Autophagy and Apoptosis by THC-induced molecular pathways in metastatic melanoma *in vitro*

Chapter 6: Regulation of Autophagy and Apoptosis by THC-induced molecular pathways in metastatic melanoma *in vitro*

Table of Contents

6.1: Introduction

Since the chemotherapeutic potential of THC was first suggested extensive research has been undertaken to identify its underlying mechanisms of action in a variety of cancers. While the majority of identified mechanisms induced by THC have predominately been identified in glioma, recent research in prostate and melanoma have also been carried out (Carracedo, Gironella *et al*. 2006; Carracedo, Lorente *et al*. 2006; Salazar, Carracedo *et al*. 2009). Studies in glioma and prostate tumour cells suggest that the anti-tumoural effect of cannabis is due in part to the accumulation of ceramide, a pro-apoptotic sphingolipid (Blázquez, González-Feria *et al*. 2004; Galve-Roperh, Sánchez *et al*. 2000; Gómez del Pulgar, Velasco *et al*. 2002; Mimeault, Pommery *et al*. 2003). Interestingly the conversion of ceramide to complex sphingolipids was not found to be a key event in THC-induced apoptosis evidenced by inhibition of glucosylceramide synthase (the enzyme catalysing rate limiting step of glycosphingolipid biosynthesis) having no preventative effects on THC treatment suggesting it is the accumulation of ceramide itself which exerts the effects observed in response to THC treatment (Carracedo, Lorente *et al*. 2006). In light of these observations, DNA microarrays identified the upregulation of several stress-regulated genes in response to cannabinoid induced ceramide accumulation including p8, tribbles 3 (TRB3: a mammalian homolog of Drosophila tribbles), C/EBP homologous protein (CHOP) and activating transcription factor 4 (ATF4) (Carracedo, Lorente *et al*. 2006). Identification of each of these genes revealed that the stress regulated protein p8 mediates THC-induced apoptosis and may act as a co-transcription factor to regulate the expression of ATF4, CHOP (Carracedo, Gironella *et al*. 2006; Carracedo, Lorente *et al*. 2006). ATF4 and CHOP in turn have been found to regulate expression of TRB3 in response to ER stress events in tumour cells (Ohoka, Yoshii *et al*. 2005). The overexpression of TRB3 often occurs in response to endoplasmic reticulum stress events (Salazar, Carracedo *et al*. 2009) and has been shown to inhibit Akt activation in squamous cell carcinoma derived from tongue tissue (Zhang, Wen *et al*. 2011). The link between TRB3 and Akt inhibition was first identified in liver tissue in which insulin resistance was attributed to TRB3 expression induced during fasting conditions in which TRB3 disrupts insulin signalling by binding directly to Akt preventing its kinase activity and suggesting Akt inhibition by overexpression of TRB3 may be a universal mechanism (Du, Herzig *et al*. 2003). TRB3 inhibition of Akt would then prevent activation of mTORC1 through

the PI2K/Akt/mTOR pathway and thus result in the induction of autophagy (Salazar, Carracedo *et al*. 2009). As such the following molecular pathway has been suggested for THC-induced autophagy in which THC binds to cannabinoid receptors causing the accumulation of ceramide which then leads to activation of the eIF2α axis of the ER stress pathway. These events result in the increased expression of p8 and TRB3 that in turn inhibits the Akt/mTOR pathway causing the induction of autophagy which is required for THC-induced cell death (Figure 6.1).

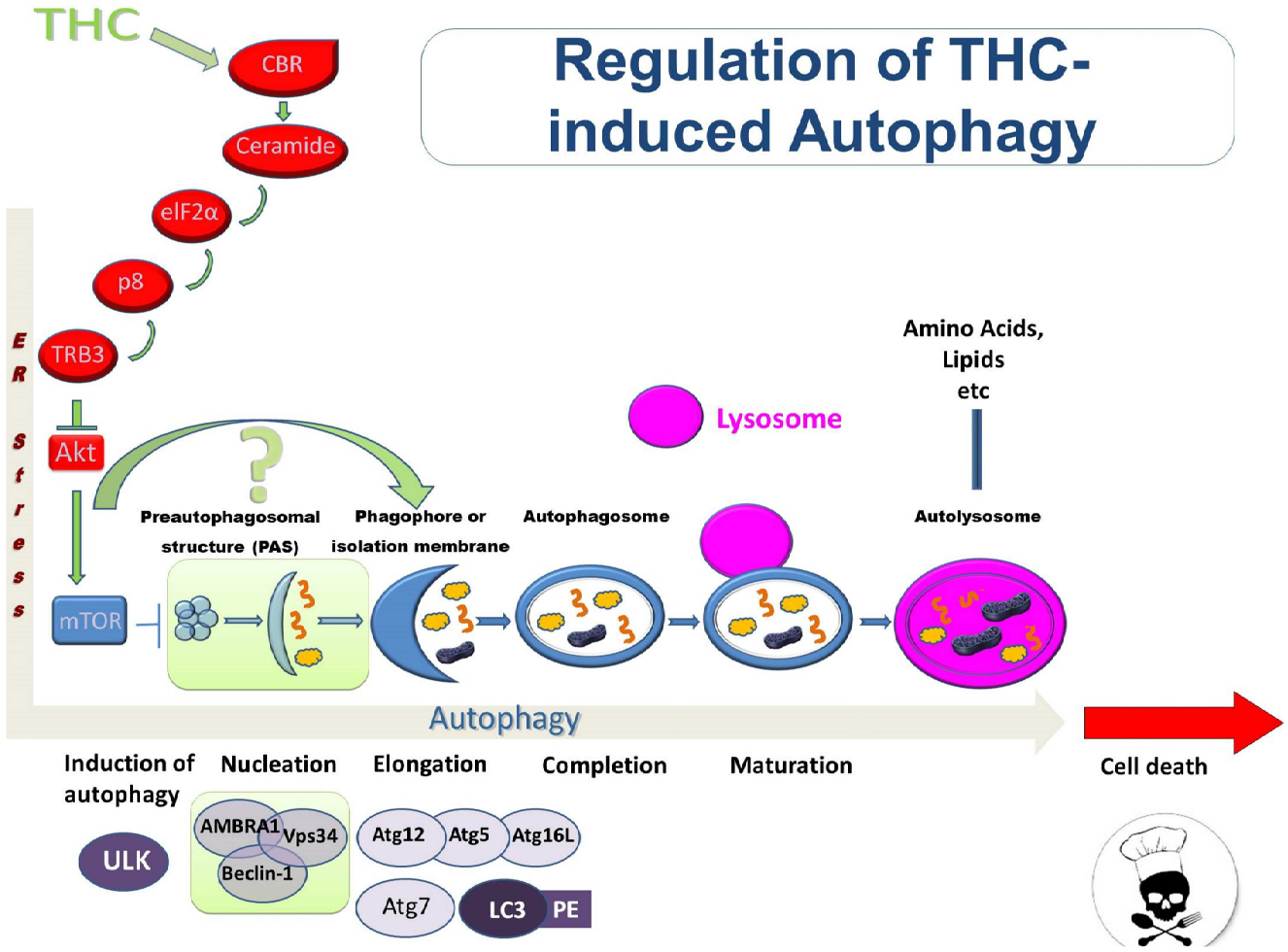

Figure 6.1: Molecular mechanisms induced by THC (Adapted from (Salazar, Carracedo et al. 2009). THC-induced autophagy and apoptosis involves many steps for either to occur. Initiation of these events occurs by binding of THC to cannabinoid receptors CBR and the accumulation of ceramide leading to activation of the eIF2α axis of the ER stress pathway. These events result in increased p8 and TRB3 expression which in turn inhibits the Akt/mTOR pathway causing the induction of autophagy which is required for THC-induced cell death.

As a result of such findings we are now beginning to understand some of the pathways induced by THC which lie upstream of autophagy. The requirement of autophagy for THC-induced cell death is also becoming clearer (Salazar, Carracedo *et al*. 2009). However, although several mechanisms of cell death have been identified in response to THC including mitochondrial outer membrane permeabilization (MOMP) and caspase 3 activation (Casanova, Blázquez *et al*. 2003; Carracedo, Gironella *et al*. 2006; Galve-Roperh, Sánchez *et al*. 2000; Ruiz, Miguel *et al*. 1999; Sánchez, Galve-Roperh *et al*. 1998; Sarfaraz, Afaq *et al*. 2005), the link between THC-induced ER stress, autophagy and apoptosis has yet to be identified. One suggestion is that treatment with THC results in permeabilization of lysosomal membranes in cancer cells (personal communication, G Velasco). Lysosome membrane permabilization (LMP) is an event resulting from damage to lysosomes resulting in the release of several hydrolytic enzymes including proteases, lipases, nucleases and more. Typically most of these enzymes function maximally at acidic pH however certain proteases known as cathepsins (or more specifically cathespin B, D and L) remain active at neutral pH (Emert-Sedlak, Shangary *et al*. 2005; Sever, Altintas *et al*. 2007). The release of lysosomal contents into the cytoplasm causes indiscriminate degradation ultimately resulting in cell death. Depending on the degree of lysosomal damage and LMP different outcomes may ultimately occur as large scale damage may result in acidification of the cytoplasm leading to necrosis of the cell. However, more typically partial and selective LMP occurs in which the proteases activate apoptosis and controlled cell death through MOMP, cytochrome C release and caspase activation (Guicciardi, Deussing *et al*. 2000). The convergence of autophagic pathway with lysosomes in addition to identified ceramide accumulation makes LMP an interesting potential missing link in the understanding of mechanisms of THC-induced apoptosis downstream of autophagy.

As a further point of interest, results from the previous chapter identified Ambra 1 as a key Beclin-1 interacting protein with a positive regulatory role in autophagy. Interestingly Ambra 1 was found to be essential for THC-induced cell death and autophagy in glioma (Salazar, Carracedo *et al*. 2009) which is believed to be independent of Beclin-1 (personal communication with Guillermo Velasco). However, to date, the molecular function of this protein in melanoma or even the requirement of Ambra 1 in autophagy induced by chemotherapeutic agents including THC and bortezomib remains unknown.

Results from previous chapters determined the requirement for autophagy induction by THC in order to induce apoptosis suggesting similar modes of action to those discussed here may be occur in metastatic melanoma cells. Therefore, the aim of this chapter was to firstly asses the requirements for Ambra 1 in THC-induced autophagy and cell death and secondly to verify the underlying molecular events occurring in melanoma which lie both upstream and downstream of autophagy in order to identify a potential link between THC-induced autophagy and cell death in melanoma.

6.2: Results

6.2.1: The role of identified Beclin-1 interacting protein, Ambra 1 during cytotoxic and pro-survival autophagy

Ambra 1 was identified in the previous chapter as a key Beclin-1 interacting protein chosen for both its involvement in autophagy and its previous implication in THC-induced cell death in glioma (Salazar, Carracedo *et al*. 2009). As such, in order to ascertain the importance of Ambra 1 for autophagy and cell death induced by THC in melanoma, the effect of THC on autophagy and apoptsos induction was determined following modulation of Ambra 1 expression in A375 cells. A375 cell stably overexpressing Ambra 1 (rAmbra 1 cells) or stably transfected with a lentiviral shAmbra 1 construct (shAmbra 1 cells) were kindly provided in collaboration with Dr Marco Corazzari, University of Rome, Tor Vergata) together with A375 cells transfected with appropriate control vectors (rβGal, shCtrl). Results demonstrated treatment with THC for 24 hours reduced viability of all transfected A375 cells, which was not affected by the presence or absence of Ambra 1. Treatment with chloroquine also prevented inhibition of cell viability (Figure 6.2).

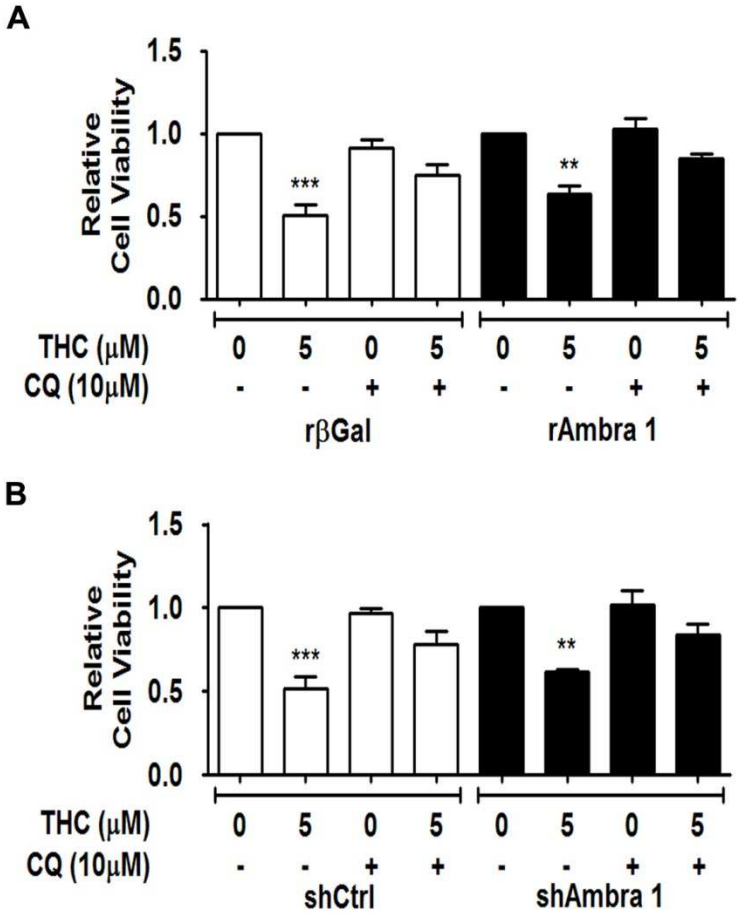

Figure 6.2: THC-induced inhibition of melanoma cell viability is independent of Ambra 1. Relative viability of melanoma cell line A375 stably (A) overexpressing Ambra 1 or (B) knocked down for Ambra 1 after subsequent treatment for 24 hours with 5 μM THC in the presence or absence of 10 μM CQ. Each bar is relative to untreated melanoma cells for each shRNA condition (mean +/- SEM, n=3). Statistics were acquired using one way ANOVA with Dunnett post-hoc test, ** $p < 0.01$ *** $p < 0.001$ comparing drug treatments shown to untreated melanoma cells for each shRNA or overexpression condition alone.

To identify whether Ambra 1 was required for THC-induced autophagy, conversion of LC3-I to LC3-II was analysed by Western blotting in rAmbra 1 and shAmbra 1 A375 cells. Combined treatment with chloroquine was included in the final two hours of treatment in order to prevent LC3-II degradation in the latter stages of autophagy. The induction of autophagy by THC treatment was confirmed at optimised concentrations of 4.5 μM or 5 μM respectively by increased LC3-II levels compared to control untreated cells (Figure 6.3). Overexpression and knockdown of Ambra 1 was confirmed by Western blotting and THC-induced autophagy was maintained in these conditions (Figure 6.3).

To assess whether the presence or absence of Ambra 1 expression was required for THC-induced cell death the level of Cleaved Caspase 3 was analysed by Western blotting. THC treatment resulted in increased Cleaved Caspase 3 in all tested cell lines in response to treatment with concentrations of 4.5 μM or 5 μM compared to untreated cells, indicating increased apoptosis (Figure 6.3). Neither overexpression nor knockdown of Ambra 1 prevented THC-induced apoptosis. As a further point of interest THC treatment alone also appeared to down-regulate the expression of Ambra 1 (Figure 6.3).

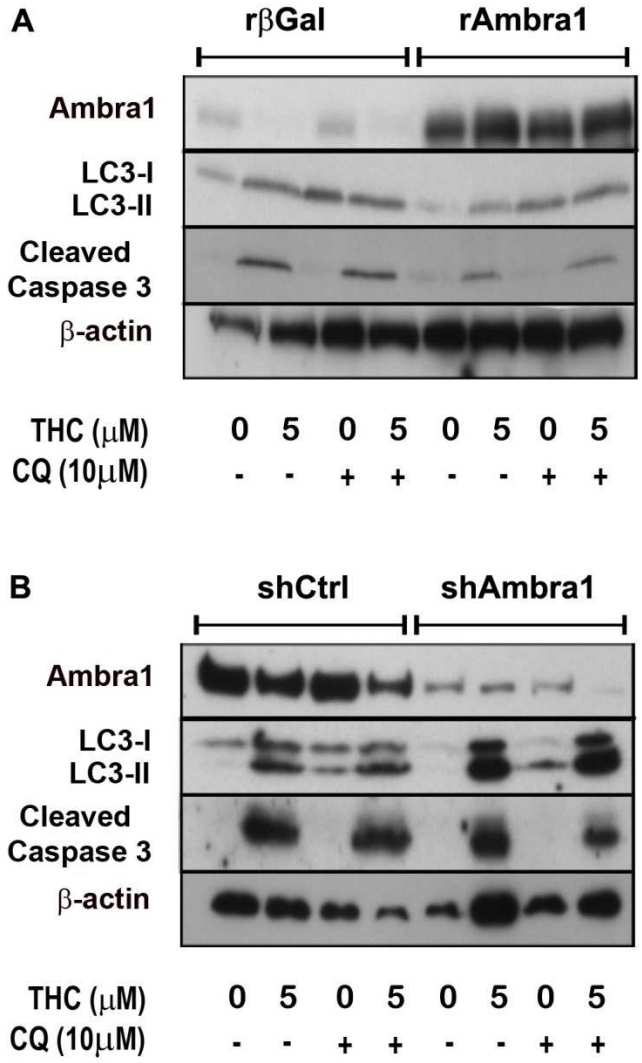

Figure 6.3: THC-induced autophagy and apoptosis are independent of Ambra 1. Western blot for Ambra 1, LC3-I/LC3-II, Cleaved Caspase 3 and ß-actin in A375 cells stably (A) overexpressing Ambra 1 or (B) knocked down for Ambra 1 treated with 5 μM THC for 24 hours in the presence or absence of 10 μM CQ for the final 2 hours of treatment.

The role of Ambra 1 in bortezomib-induced inhibition of cell viability, caspase 3 cleavage and LC3-II induction was also evaluated in rAmbra 1 and shAmbra 1 melanoma cells In line with THC treatment, results also confirmed treatment of cells with bortezomib for 24 hours inhibited melanoma cell viability. Inhibition of cell viability was partially enhanced in shAmbra 1 cells treated with bortezomib compared with shCtrl Cells (45% viability on average compared with 55% viability respectively) while bortezomib treatment of rAmbra 1 over expressing cells resulted in partially reduced inhibition of cell viability compared with rβGal cells (66% viability on average compared with 50% viability respectively) (Figure 6.4). Ultimately however, this difference was not significant and bortezomib-induced inhibition of viability appeared to be independent of Ambra 1. Additionally, combined treatment with chloroquine did not alter any effect of bortezomib on inhibition of cell viability (Figure 6.4).

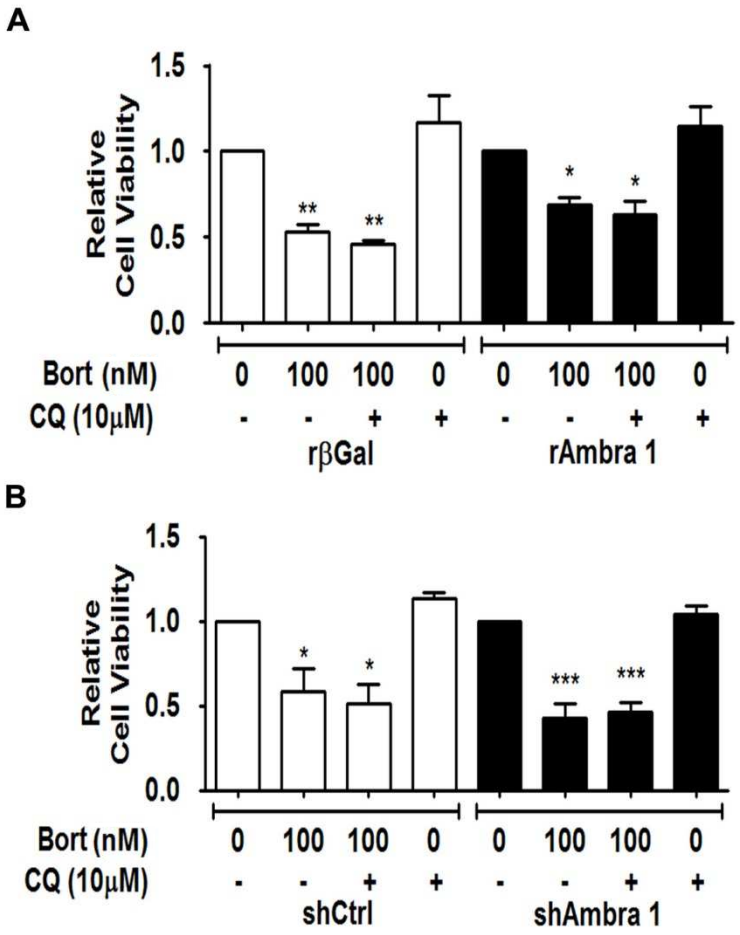

Figure 6.4: Bortezomib-induced inhibition of melanoma cell viability is independent of Ambra 1. Relative viability of melanoma cell line A375 stably (A) overexpressing Ambra 1 or (B) knocked down for Ambra 1 after subsequent treatment for 24 hours with 100nM Bort in the presence or absence of 10μM CQ. Each bar is relative to untreated melanoma cells for each shRNA condition (mean +/- SEM, n=3). Statistics were acquired using one way ANOVA with Dunnett post-hoc test, ** $p< 0.01$ *** $p< 0.001$ comparing drug treatments shown to untreated melanoma cells for each shRNA or overexpression condition alone.

Western blotting also confirmed bortezomib-induced LC3-II expression in all tested melanoma cells which was neither prevented by Ambra 1 knockdown nor enhanced by Ambra 1 overexpression. Increased apoptosis was observed with bortezomib treatment as indicated by increased levels of Cleaved Caspase 3. This effect was further enhanced by Ambra 1 knockdown but Ambra 1 overexpression had no observed effects on the levels of Cleaved Caspase 3 (Figures 6.5).

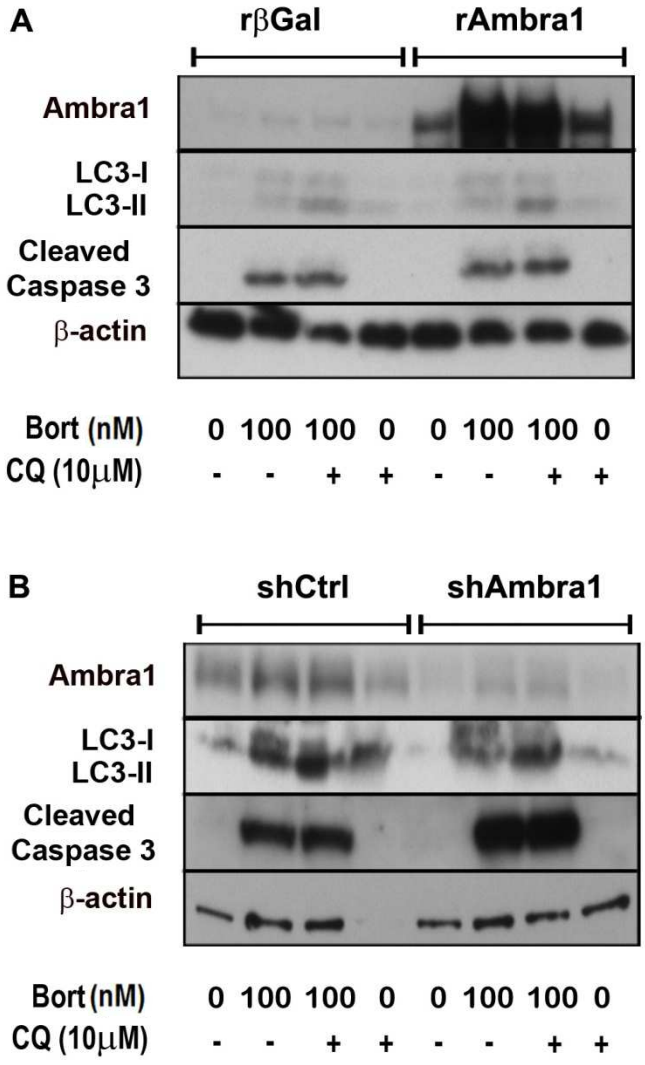

Figure 6.5: Ambra 1 is not required for enhancement of autophagy and apoptosis induced by bortezomib. Western blot for Ambra 1, LC3-I/LC3-II, Cleaved Caspase 3 and ß-actin in A375 cells stably (A) overexpressing Ambra 1 or (B) knocked down for Ambra 1 treated with 100 nM Bort for 24 hours in the presence or absence of 10 μM CQ for the final 2 hours of treatment.

6.2.2: THC-induced events upstream of autophagy induction

The accumulation of ceramide is suggested as an early event after THC treatment and has been verified in glioma by use of the serine palmitoyl transferase-1 inhibitor ISP-1 (also known as myriocin) (Gómez del Pulgar, Velasco *et al*. 2002; Salazar, Carracedo *et al*. 2009). As such combined treatment of THC with ISP-1 can be used in an attempt to prevent THC-induced inhibition of cell viability by preventing ceramide accumulation and thus subsequent downstream events. Although in the present study less reduction in viability was observed in response to the combined treatment of A375 cells with THC and ISP-1 (5 µM) (Figure 6.6 A) no significant difference was found between treatment conditions comparing 6 µM THC alone with 6 µM THC combined with ISP-1 (Figure 6.6 B). As such ISP-1 at 5 µM did not prevent THC-induced loss of viability (Figure 6.6) in A375 melanoma cells.

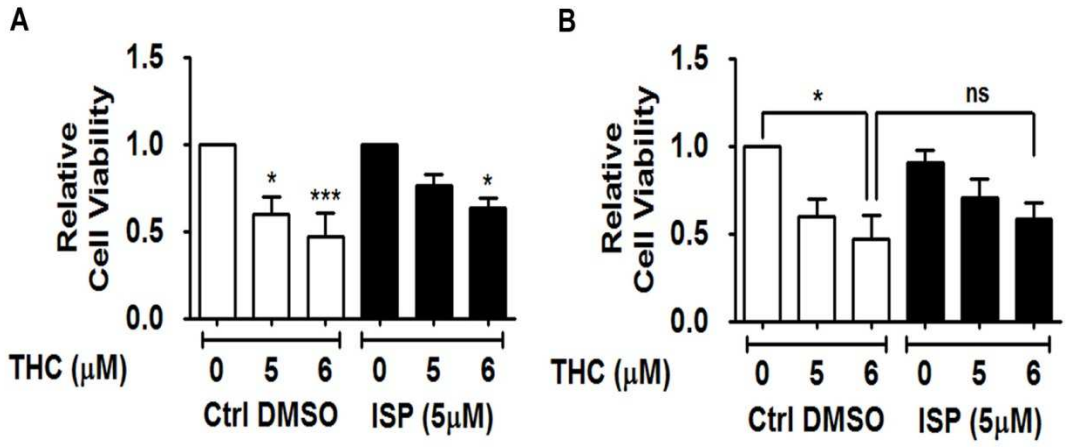

Figure 6.6: Inhibtion of melanoma cell viability with THC is independent of Ceramide accumulation. Relative viability of A375 melanoma cell lines treatment for 24 hrs with 5 or 6 µM THC either alone or in combination with 5 µM ISP. Each bar is relative to melanoma cells untreated with THC for either control DMSO or combined with ISP (mean +/- SEM n=3). (A) Statistics were acquired using one way ANOVA with Dunnett post-hoc test or one way ANOVA * $p< 0.05$, *** $p< 0.001$ comparing treatment with THC relative to untreated melanoma cells for control DMSO or treatment with ISP alone. (B) Statistics were acquired using one way ANOVA with Bonferroni post-hoc test * $p< 0.05$, ns – non-significant comparing drug treatments shown relative to untreated melanoma cells for control DMSO .

TRB3 has is also upregulated in glioma in response to THC resulting in TRB3-dependent autophagy induction and thus cell death in this tumour type (Salazar, Carracedo *et al*. 2009; Salazar, Carracedo *et al*. 2009). Therefore in order to define mechanisms upstream of autophagy crucial for THC-induced cell death, RNAi-mediated knockdown of TRB3 was achieved by reverse transfection. Knockdown was confirmed by RT-PCR and optimal conditions determined by using 2nM TRB3 siRNA (Fig 6.7 A). Treatment with THC for 24 hours reduced viability of A375 cells which was prevented by transient knockdown of TRB3. (Fig 6.7 B)

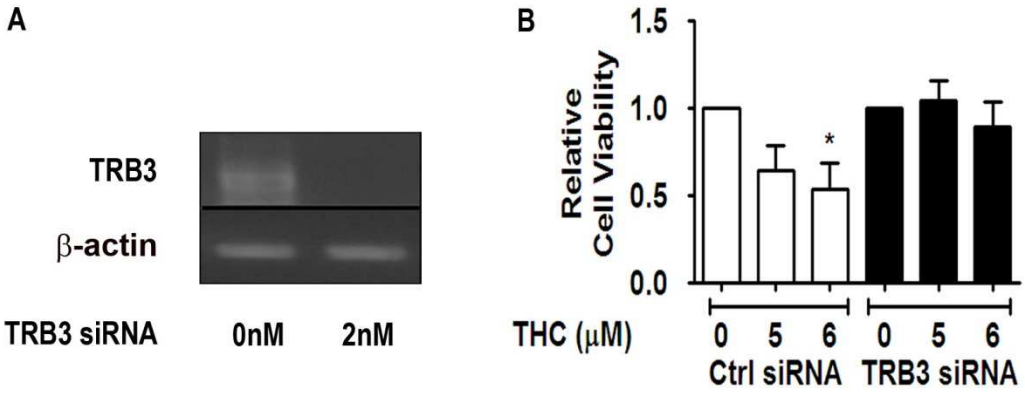

Figure 6.7: RNAi-mediated knockdown of TRB3 prevents THC-induced inhibition of melanoma cell viability. A) mRNA expression levels of TRB3 for ctrl siRNA transfectd A375 cells or cells transfected with 2 nM TRB3 siRNA B) Relative viability of melanoma cell lines (A375) following transfection with control siRNA (Ctrl siRNA) or TRB3 siRNA for 24 hours and subsequent treatment for 24 hours with 5 μM or 6 μM THC. Each bar is relative to untreated melanoma cells for each siRNA condition (mean +/- SEM, n=3). Statistics were acquired using one way ANOVA with Dunnett post-hoc test, * $p<0.05$ comparing drug treatments shown relative to untreated melanoma cells for each siRNA condition.

6.2.3: THC-induced events downstream of autophagy

Although THC has been shown to induce apoptosis in a variety of cancers and autophagy is emerging as a crucial event in this process, the molecular events downstream of autophagy which may result in apoptosis are yet to be fully defined in melanoma. One suggested mechanism for THC-induced apoptosis is the permeabilization of lysosomes resulting in the release of apoptosis inducing proteins such as cathepsins (Velasco *et al* unpublished data). As such to assess the underlying mechanisms for THC-induced apoptosis which lie downstream of autophagy, lysosome membrane permeabilization was assessed by analysing cathepsin B release. Cathepsin B release from lysosomes was confirmed by dual staining of melanoma cells to determine cathepsin B (Green) co-localisation with lysosomal associated membrane protein 2 (LAMP2; Red) and visualised using immunofluorescence and confocal microscopy. Control untreated melanoma cells exhibited a granular fluorescence pattern representing intracellular lysosomes which co-localised with cathepsin B. Treatment with THC resulted in large swollen lysosomal structures. Results also demonstrated cathepsin B release from lysosomes into the cytoplasm as determined by a reduction in co-localisation (yellow) in melanoma cells treated with THC at concentrations high enough to induce apoptosis (Figure 6.8).

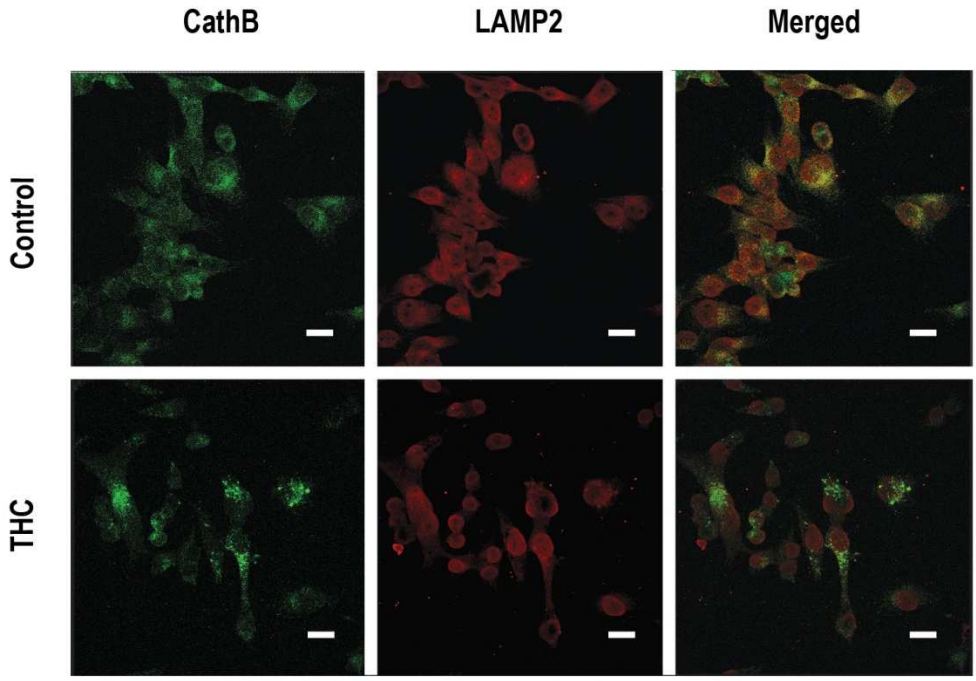

Figure 6.8: THC induces LMP and cathepsin B release from lysosomes. Immunofluorescence for cathepsin B (Green) and LAMP2 (Red) co-localisation in A375 melanoma cells treated with control DMSO or with 5 µM THC for 18 hours (scale bar represents a length of 20 µm).

Bafilomycin should prevent release of cathepsins from the lysosomal lumen as it has been shown to prevent LMP by acting as both an inhibitor of lysosomal acidification and lysosome-autophagosome fusion by inhibition of vacuolar H+ ATPase (V-ATPase) (Yamamoto, Tagawa *et al*. 1998). As such combined treatment with bafilomycin was used in an attempt to prevent THC-induced apoptosis by inhibition of lysosome membrane permeabilization. Treatment with bafilomycin alone did not result in cathepsin B release unlike treatment with THC; however, combined treatment with THC and bafilomycin prevented the release of cathepsin B from lysosomes and the granular staining pattern observed in control untreated cells was retained (Figure 6.9 B). Additionally, combined treatment with THC and bafilomycin prevented THC-induced loss of viability suggesting LMP is involved in cell death (Figure 6.9 A).

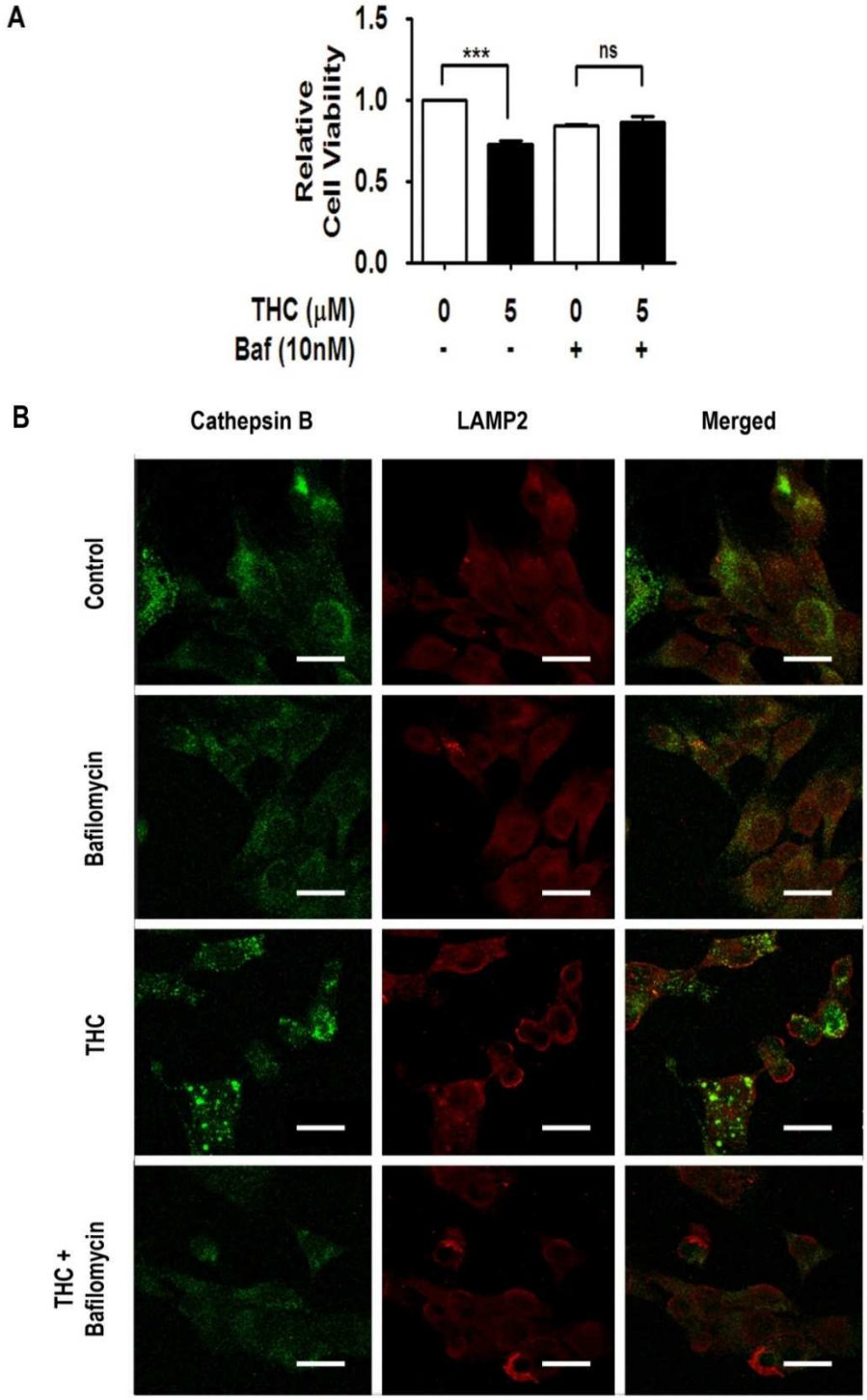

Figure 6.9: Bafilomycin prevents THC-induced cell death and prevention of cathepsin B release from lysosomes. (A) Relative viability of melanoma cell lines (A375) following treatment for 24 hours with 5 µM THC in the presence or absence of 10 nM bafilomycin (Baf). Each bar is relative to melanoma cells untreated with THC using control DMSO (mean +/- SEM, n=3). Statistics were acquired using one way ANOVA with Dunnett post-hoc test, *** $p<0.001$ comparing drug treatments shown relative to untreated melanoma cells for control DMSO or combined treatment conditions. (B) Immunofluorescence for cathepsin B (Green) and LAMP2 (Red) co-localisation with lysosomes in A375 melanoma cells treated for 18 hours individually or in combination with THC and bafilomycin (scale bar represents a length of 20 µm).

6.2.4: Lysosome membrane permeabilization and THC-induced apoptosis in metastatic melanoma

The release of cathepsins from lysosomes has been shown to disrupt mitochondrial integrity leading to an event known as mitochondrial outer membrane permeabilization (MOMP). This in turn results in distinct changes in intracellular mitochondrial staining patterns observed and cytochrome C release (Guicciardi, Deussing *et al*. 2000). In order to determine whether THC-induced apoptosis occurs as a result of MOMP, cytochrome C staining was analysed by immunofluorescence in A375 cells. Control untreated melanoma cells revealed a distinct mitochondrial staining pattern similar to those observed using mitochondrial imagining techniques (Mitra and Lippincott-Schwartz 2010) (Figure 6.10). Treatment with THC resulted in a diffuse staining pattern representing cytochrome C release into the cytoplasm (an early event in intrinsic apoptosis) as well as morphological changes such as cellular shrivelling and damaged nuclei. Additionally nuclear localisation of cytochrome C was also observed (Figure 6.10).

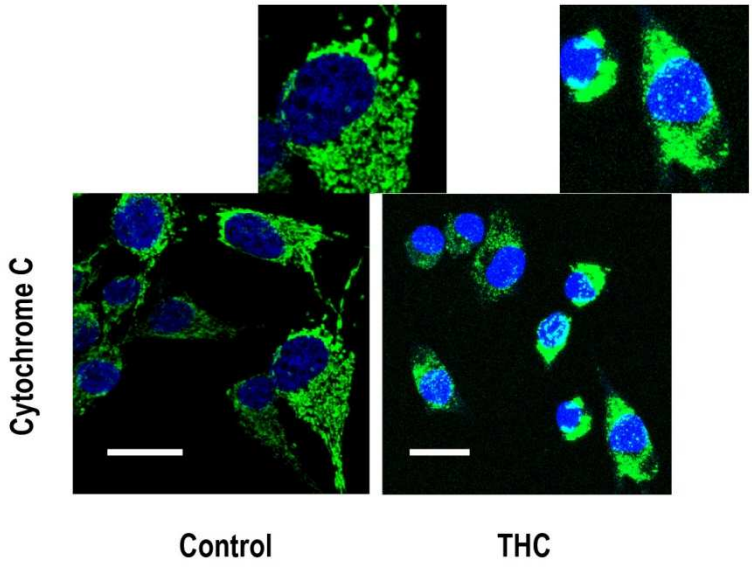

Figure 6.10: THC induces cytochrome C release in A375 melanoma cells. Representative immunofluorescence from 3 replicate experiments for cytochrome C expression in A375 cells treated with (A) DMSO vehicle control or (B) 5 µM THC for 24 hours (Original magnification is 63x, scale bar represents a length of 20 µm).

In order to determine whether cathespsin B was essential for cytochrome C release and subsequent cell death, combined treatment of THC with a cathepsin B inhibitor (CAO74-me) was assessed for its effect on THC-induced inhibition of cell viability and induction of cytochorme C release. Combined treatment of THC with the cathepsin B inhibitor (CAO74-me) partially prevented THC-induced loss of cell viability when THC was used at a concentration of 6 µM (Figure 6.11 A). Treatment with the cathepsin B inhibitor alone however, did not reduce cell viability (Figure 6.11 A) or result in cytochrome C release (Figure 6.10 B). Interestingly combined treatment with THC and the cathepsin B inhibitor for 24 hours revealed a mixed pattern of loss of mitochondrial integrity and cytochrome C release (as observed for cells treated with THC alone) as well as stable mitochondrial staining patterns (as observed in both untreated and cathepsin B inhibitor alone treated cells) (Figure 6.11 B).

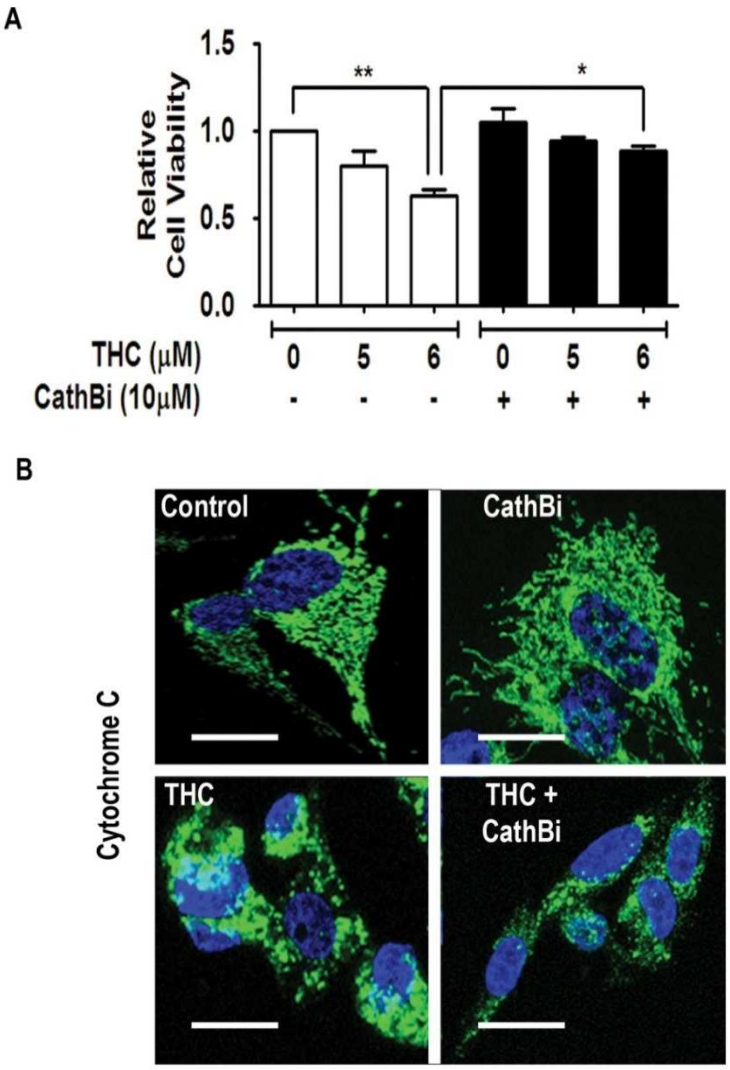

Figure 6.11: Inhibition of cathepsin B activity prevents THC-induced cell death and cytochrome C release. (A) Relative viability of melanoma cell lines (A375) following treatment for 24 hours with 5 µM THC in the presence or absence of 10 µM Cathepsin B inhibitor (CathBi; CAO74-me). Each bar is relative to melanoma cells treated with vehicle only (mean +/- SEM, n=3). Statistics were acquired using one way ANOVA with Bonferroni post-hoc test, * $p<0.05$, ** $p<0.01$ comparing 6 µM THC to untreated melanoma cells for control DMSO or 6 µM THC alone with 6 µM THC combined with CathBi. (B) Representative confocal images from 3 replicate experiments of cathepsin B (Green) and LAMP2 (Red) co-localisation in A375 melanoma cells treated individually or in combination with THC and CathBi (scale bar represents a length of 20 µm).

6.2.5: The role of Autophagy in THC-induced lysosome membrane permeabilization.

Results from previous chapters have revealed an essential role for autophagy in THC-induced cell death in melanoma cells *in vitro*. However, the link between autophagy, lysosome membrane permeabilization and apoptosis remains unclear. In order to determine a requirement for autophagy in THC-induced lysosome membrane permeability, RNAi-mediated knockdown of Atg7 was achieved by reverse transfection in metastatic melanoma cell lines A375 and SKMEL-28. Cathepsin B release from lysosomes was confirmed by dual staining of melanoma cells to determine cathepsin B co-localisation with LAMP2 and visualised using immunofluorescence and confocal microscopy. THC treatment in Ctrl siRNA transfected melanoma cell lines resulted in cathepsin B release and lysosome swelling as previously observed (Figures 6.12, 6.13). Atg7 knockdown alone however did not result in cathepsin B release and prevented THC-induced cathepsin B release in all tested melanoma cell lines compared to THC treated cells transfected with Ctrl siRNA (Figures 6.12, 6.13).

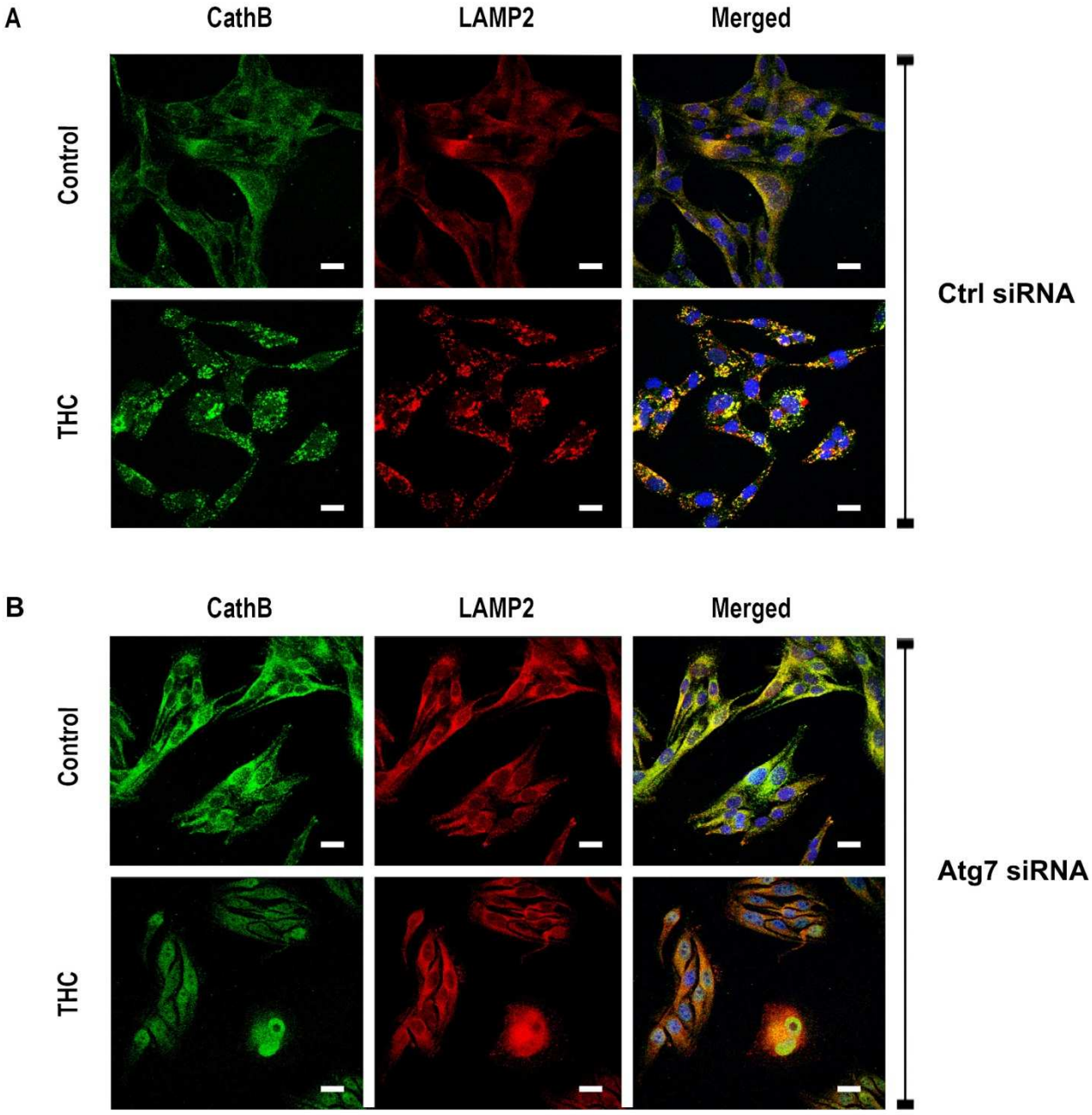

Figure 6.12: Atg7 knockdown prevents THC-induced LMP and cathepsin B release from lysosomes in A375 melanoma cells. Representative image from 3 replicate experiments for immunofluorescence of cathepsin B (Green) and LAMP2 (Red) co-localisation in A375 melanoma cells transfected with (A) Ctrl siRNA or (b) Atg7 siRNA treated for 18 hours with 5 µM THC. Nuclear staining was achieved using DAPI (Blue) (scale bar represents a length of 20 µm).

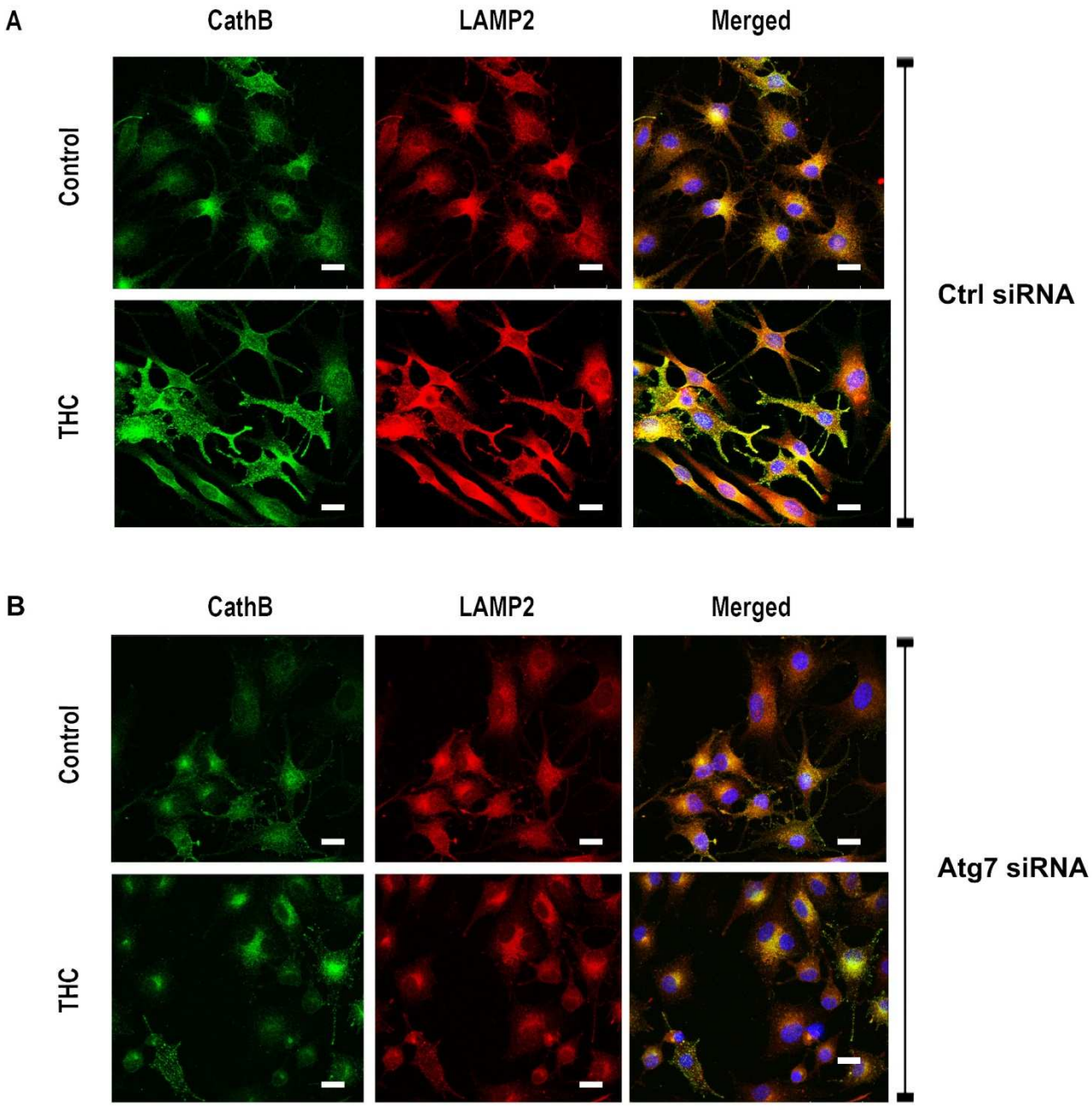

Figure 6.13: Atg7 knockdown prevents THC-induced LMP and cathepsin B release from lysosomes in SKMEL-28 melanoma cells. Representative image from n=3 experiments for immunofluorescence of cathepsin B (Green) and LAMP2 (Red) co-localisation in SKMEL-28 melanoma cells transfected with (A) Ctrl siRNA or (b) Atg7 siRNA treated for 18 hours with 5 µM THC. Nuclear staining was achieved using DAPI (Blue) (scale bar represents a length of 20 µm).

6.3: Discussion

Results from this chapter demonstrate THC is able to induce autophagy and cell death independently of Ambra 1, as verified by inhibition of cell viability, increased LC3-I/II conversion and increased levels Cleaved Caspase 3 observed in A375 cells in which Ambra 1 expression had been stably knocked down. Ambra 1 is a member of the Beclin-1/Vps34 complex which is required for the nucleation phase of autophagy (Fimia, Di Bartolomeo *et al*. 2011; Roy and Debnath 2010) and has been suggested to be a key player in the canonical autophagy pathway where it essentially acts as a protein-protein interacting platform (Fimia, Corazzari *et al*. 2012). However, additional cofactors such as UVRAG and Atg14 can also activate Beclin-1 complex activity independent of Ambra 1 (Itakura, Kishi *et al*. 2008; Simonsen and Tooze 2009) suggesting Ambra 1 is not always necessary for autophagy induction. Interestingly, treatment with THC alone appeared to down-regulate Ambra 1 which could be due to cleavage of Ambra 1 by caspases as described by Corazzari *et al* (Corazzari, Fimia *et al*. 2012). Despite extensive research into the function of Ambra 1 in autophagy most of these studies have been undertake in neuronal cells and cancers such as glioma with little direct evidence about the function of Ambra 1 in melanoma. Results from the present melanoma studies reveal autophagy induction by THC and bortezomib is able to occur independently of Ambra 1, suggesting a non-canonical autophagic process is taking place in which the requirement of Ambra 1 may not be essential.

Ceramide accumulation may also not be required for THC-induced inhibition of cell viability. The accumulation of ceramide occurs as a result of cannabinoid treatment in several cancer types (Blázquez, González-Feria *et al*. 2004; Galve-Roperh, Sánchez *et al*. 2000, Gómez del Pulgar, Velasco *et al*. 2002; Mimeault, Pommery *et al*. 2003) and many studies have revealed that ceramide acts as a mediator of the ER stress response in eukaryotes (Hannun 1996). Its accumulation has been suggested to occur by four means; i) activation of *de novo* synthesis in the endoplasmic reticulum by ceramide synthetase and serine-parlmotyl transferases (SPT) (Chalfant, Rathman *et al*. 2002; Perry, Carton *et al*. 2000) ii) inhibition of ceramide hydrolysis (Lucci, Han *et al*. 1999; Strum, Small *et al*. 1994) iii) activation of sphingomyelin hydrolysis (Senchenkov, Litvak *et al*. 2001) and iv) stimulation of glucosylceramide hydrolysis (Senchenkov, Litvak *et al*. 2001). ISP-1 inhibits ceramide accumulation in glioma by prevention of its *de novo* synthesis which is due to inhibition of

serine palmitoyl transferase-1 (SPT1) suggesting that ceramide accumulation in glioma is required for THC-induced ER stress and subsequent autophagy and apoptosis. However, in the present studies ISP-1 did not prevent THC-induced inhibition of cell viability of melanoma cells. As such, whether ceramide accumulation is occurring via a different mechanism or even if it is required in melanoma for downstream responses induced by THC remains to be verified. It is also possible that the concentration of ISP-1 used in these studies, although adequate to prevent ceramide accumulation in glioma, was inadequate to prevent its accumulation in melanoma cells and hence additional studies are required to truly disregard ceramide accumulation as a mechanism required for THC to exert its effects.

Knockdown of the pseudokinase TRB3 prevented inhibition of melanoma cell viability by THC suggesting that TRB3 is required for THC-induced cell death in melanoma. Increased TRB3 expression as a result of p8 overexpression was identified as a crucial step for THC-induced autophagy and apoptosis in glioma and pancreatic cells, verified both *in vivo* and *in vitro* (Carracedo, Gironella *et al*. 2006; Carracedo, Lorente *et al*. 2006; Salazar, Carracedo *et al*. 2009; Salazar, Carracedo *et al*. 2009). Genetic inhibition of TRB3 in glioma prevented cannabinoid induced autophagy and cell death suggesting this protein is crucial for connecting ER stress events to autophagy and subsequent downstream events in response to THC. The key step is likely TRB3 mediated Akt inhibition which would in turn inhibit mTORC1 activity and induce autophagy. As such it is likely these events are occurring in melanoma as a result of THC treatment (although further studies will be required to verify this).

The ability for THC to induce apoptosis has been extensively shown with several key events associated with apoptosis signalling being identified (Carracedo, Gironella *et al*. 2006; Casanova, Blázquez *et al*. 2003; Galve-Roperh, Sánchez *et al*. 2000; Ruiz, Miguel *et al*. 1999; Sánchez, Galve-Roperh *et al*. 1998; Sarfaraz, Afaq *et al*. 2005) however the release of cytochrome C in response to THC has yet to be verified. Analysis of cytochrome C staining revealed THC treatment caused the release of cytochrome C from mitochondria, resulting in a diffuse staining pattern likely due to MOMP, revealing for the first time a possible mechanism of caspase activation induced by THC in melanoma cells. As a further point of interest, nuclear staining of cytochrome C was observed in A375 cells after THC treatment. Although the amount of research related to this event is limited, nuclear translocation of cytochrome C during apoptosis has been observed previously (Nur-E-Kamal, Gross *et al*.

2004). It was found that cytochrome C released from mitochondria during apoptosis gradually accumulated in the nucleus of HeLa cells which was shown by both immunofluorescence and subcellular fractionation. Interestingly the nuclear localisation of cytochrome C was suggested to be directly involved in the remodelling of chromatin as evidenced by chromatin condensation and the release of acetylated histone H2A into the cytoplasm (possibly by its displacement from chromatin) inducing what the authors termed nuclear apoptosis (Nur-E-Kamal, Gross *et al*. 2004). As such this may also be a mechanism of apoptosis induction occurring as a result of cytochrome C release and nuclear localisation observed in response to THC.

LMP has been speculated as a potential mechanism of apoptosis induction in response to THC (personal communication with Guillermo Velasco) which may lie upstream of apoptotic events, however there is little direct evidence to support this hypothesis. LMP occurs when lysosomes become damaged causing the release of its constituents into the cytoplasm resulting in indiscriminate degradation and ultimately apoptosis or necrosis depending on the scale of lysosomal damage. In these studies treatment with THC resulted in large swollen lysosomal structures and reduced colocalisation of cathepsin B with LAMP2, revealing for the first time that LMP is induced in response to THC. Further supporting evidence was revealed by the combined treatment with THC and bafilomycin (an established LMP inhibitor preventing lysosomal acidification (Yamamoto, Tagawa *et al*. 1998; Yoshimori, Yamamoto *et al*. 1991) where both inhibition of cell viability and cathepsin B release was prevented compared to THC treatment alone. Larger lysosomes (often observed in cancers) have been suggested to be more susceptible to LMP (Glunde, Guggino *et al*. 2003; Ono, Kim *et al*. 2003) and the release of cathepsin B from lysosomes (indicated by reduced co-localisation of cathepsin B with LAMP2) in addition to the swollen lysosomal structures indicates this is happening as a result of THC treatment. Additionally, cathepsin B release has been found to induce cytochrome C release and apoptosis in hepatocytes (Guicciardi, Deussing *et al*. 2000) and as both these events have been shown in the present studies, as well as the inhibition of cytochrome C release by use of a cathepsin B inhibitor, it is likely that a similar mechanism of THC-induced apoptosis occurs in melanoma cells. It should be noted however, that the cathepsin b inhibitor (CAO74-me) had only a partial effect on the release of cytochrome C as a mixed pattern of loss of mitochondrial integrity and cytochrome C were observed. The cathepsin B inhibitor used in these studies results in an

irreversible binding of the inhibitor to cytosolic cathepsin B. As such, eventually the inhibitor will be used up and any further release of cathepsin B would no longer be inhibited which may account for the partial prevention of cytochrome C release observed despite preventing inhibition of THC-induced inhibition of cell viability. In order to prove that this is the case a range of different cathepsin B inhibitor concentrations would have to be applied and analysed to see if full prevention of cytochrome C release could be attained. Additional methodology could also be applied to verify the release of cathepsin B, as well as other cathespins, into the cytosol including immunoblots of subcellular fractions (i.e. cytosolic against lysosomes) (Michallet, Saltel *et al*. 2004) or measure lysosome-impermeable cathepsin substrates such as pepstatin BODIPY (Yin, Stearns *et al*. 2005). Interestingly it has been found that not all lysosomes are permeabilized at the same time (Ono, Kim *et al*. 2003) (although the reasons for this remain unknown) which could account for the lysosomal structures observed which appear intact and are still co-localised with cathepsin B. The cause of LMP by THC has yet to be defined but several observations could account for this event (Emert-Sedlak, Shangary *et al*. 2005; Guicciardi, Deussing *et al*. 2000; Sever, Altintas *et al*. 2007). Cancer cells often exhibit large amounts of ROS and treatment with THC has been shown to cause a dramatic increase in intracellular ROS levels likely due to their release from mitochrondria during their disruption (Goncharov, Weiner *et al*. 2005; Sarafian, Kouyoumjian *et al*. 2003). ROS can induce LMP but only in subcellular regions such as near the mitochondria as ROS have a spatially limited range of action. This may also explain why some lysosomes remain intact during THC treatment and others do not. Another mechanism particularly relevant to cancer cells and melanoma may relate to the presence of iron within the lysosomes. Lysosomes are the main reservoir of chelatable iron which accumulates upon degradation of iron-containing proteins such as cytochromes and ferritin. Iron catalyses fenton reactions within lysosomes producing highly reactive pro-oxidants which may then damage lysosomal membranes if the levels of iron are too great (as found in some pathologies associated with Iron overload (Link, Pinson *et al*. 1993; Mak and Weglicki 1985). Fenton reactions have also been suggested to occur in response to THC treatment, increasing glioma cell death due to oxidative stress (Goncharov, Weiner *et al*. 2005). An interesting link between melanoma and iron-containing proteins has been found with the expression of ferritin within melanoma cells by Baldi and colleagues (Baldi, Lombardi *et al*. 2005). In these studies, authors increased ferritin expression in melanoma cells which

contribute to tumour progression by modulating cell growth and reducing sensitivity to oxidative stress (Baldi, Lombardi *et al*. 2005). Although the level of iron containing proteins has not been assessed in these studies it is possible that the increased levels of ferritin and iron containing proteins in melanoma may contribute to LMP induced by THC. Increased autophagy in response to THC could then potentially result in increased invagination and degradation of such proteins within the lysosomes ultimately leading to LMP, cathepsin B release and apoptosis.

In conclusion THC-induced autophagy and cell death appears to be Ambra 1 independent and additionally the requirement for ceramide accumulation may also be unnecessary for THC-induced cell death, revealing differences in the underlying mechanisms activated by THC in melanoma compared with those found in glioma. Still, prevention of THC-induced inhibition of cell viability upon TRB3 knockdown suggests THC treatment in melanoma follows some of the predicted pathways identified in glioma. Additionally these studies have identified novel findings and specifically that the induction of lysosome membrane permeabilization resulting in the release of cytochrome C contributes to the potency of THC in its induction of autophagy and apoptosis of melanoma (Figure 6.14).

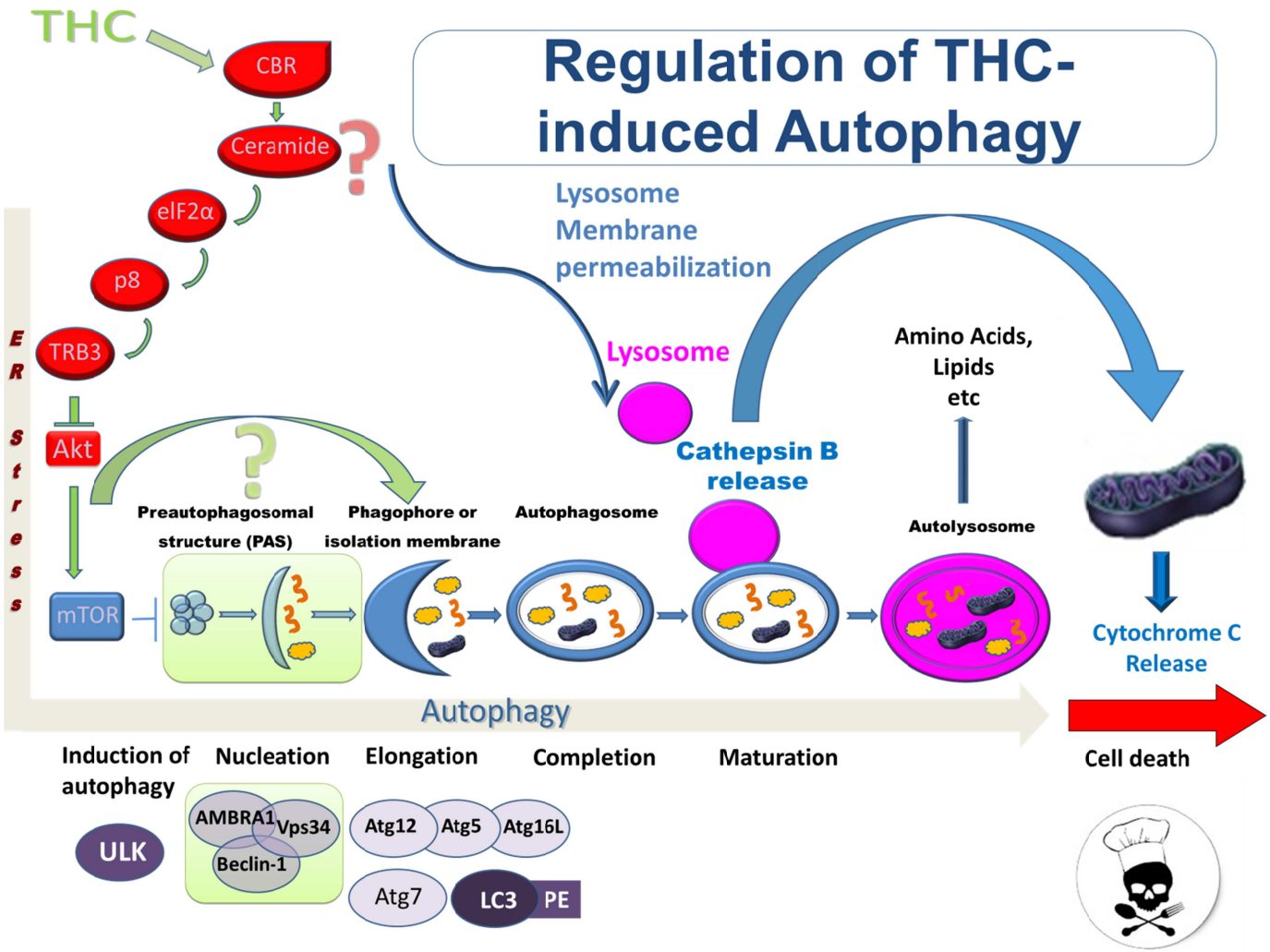

Figure 6.14: Updated molecular mechanisms induced by THC as a result of these studies (Adapted from (Salazar, Carracedo et al. 2009)) An updated version of THC-induced autophagy and apoptosis in melanoma resulting from this thesis. The requirement of ceramide in melanoma for these events remains unknown. TRB3 is required for THC-inducedcell death and autophagy induction occurs in response to this agent. THC causes Lysosome membrane permeabilization and cytochrome C release resulting in apoptosis.

6.4 Summary

- Ambra 1 is not required for THC-induced autophagy and apoptosis in melanoma.
- Treatment of melanoma cells with THC results in cytochrome C release which is a likely cause of apoptosis.
- Treatment with THC results in LMP and cathepsin B release.
- Inhibition of cathepsin B activity prevents THC-induced inhibition of cell viability.
- Bafilomycin treatment prevents LMP and cathespsin B release, as well as prevention of THC-induced inhibition of cell viability.
- Inhibition of cathepsin B partially prevents cytochrome C release, suggesting cathepsin B activity is required for THC-induced apoptosis.
- Autophagy is required for cathepsin B release as knockdown of Atg7 prevented LMP in A375 and SKMEL-28 cells.

Chapter 7: Final Discussion

Chapter 7: Final Discussion

Table of Contents

7.1 Prelude to final discussion

To date cutaneous metastatic melanoma remains largely untreatable most likely attributable to the notorious resistance of these tumours to apoptosis induction by current chemotherapies. As the incidence of this disease has steadily increased globally in the last fifty years particularly in those of European decent (Thompson, Scolyer *et al*. 2005) there is a high demand for developing new treatment regimes to overcome apoptotic resistance. The underlying mechanisms of apoptosis have been extensively studied leading to development of several chemotherapeutics however, due to apoptotic resistance much research focussed on alternative approaches to induce cell death. A rekindled interest in the autophagy pathway has revealed the importance of this pathway in cancer development and survival and as such presents new targeting opportunities for cancer treatment (Qu, Yu *et al*. 2003, Yue, Jin *et al*. 2003). To an extent apoptotic resistance can partially be attributed to the ability of tumours to use autophagy as a pro-survival mechanism, leading to development of several novel therapeutic strategies shown to inhibit autophagy in advanced cancers as a means through which to overcome apoptotic resistance (Bertout, Patel *et al*. 2008; Zhang, Bosch-Marce *et al*. 2008). However, a number of factors impact on the success of this approach. Firstly, the most commonly used lysosomal inhibitor, hydroxychloroquine (HCQ) (Garber 2011) is not a specific inhibitor of autophagy and more-over its use is associated with several off target effects including blockade of autophagy in normal organs such as the muscles and kidneys leading to development of muscle weakness and renal insufficiency (Albay, Adler *et al*. 2005; Kimura, Takabatake *et al*. 2013) in addition to well documented cases of retinoid damage and potential blindness through prolonged HCQ treatment (Mavrikakis, Papazoglou *et al*. 1996; Tehrani, Ostrowski *et al*. 2008). As such more specific autophagy inhibitors are currently in clinical development including Verteporfin, an FDA approved benzoporphyrin derivative used in photodynamic therapy shown more recently to inhibit drug and starvation-induced autophagy even in absence of light activation (Donohue, Tovey *et al*. 2011). Another effective inhibitor is lucanthone, an autophagy inhibitor also found to induce lysosome membrane permeabilization and which possessed significantly greater potency against breast cancer models compared with chloroquine (Carew, Espitia *et al*. 2011). However, a major caveat to inhibiting autophagy is that blockade of autophagy itself may lead to the development of secondary tumours (Qu, Yu *et al*. 2003; Yue, Jin *et al*.

2003). As such, the alternative approach of harnessing autophagy to promote cell death may represent a better therapeutic strategy. In this context recent studies of the antidiabetic drug metformin (Bailey and Turner 1996) demonstrate the potent ability of this agent to inhibit growth of several tumour types, including breast, colorectal and pancreatic cancer (Ben Sahra, Le Marchand-Brustel *et al*. 2010; Liu, Fan *et al*. 2009; Wang, Li *et al*. 2008) concurrent with the induction of autophagy (Buzzai, Jones *et al*. 2007). More recently metformin has also been shown to exert antiproliferative effects on melanoma cells, with little or no effect on normal melanocytes (Tomic 2011). Furthermore, these studies revealed prolonged exposure of melanoma cells to metformin resulted in the induction of autophagy, inhibition of which, through RNAi-mediated knockdown of either Atg5 or LC3-I/II also resulted in decreased apoptosis (Tomic 2011). These studies thus suggest metformin-induced apoptosis is dependent on autophagy, and reveal an alternative mode through which to harness autophgay modulation. Further studies also confirmed the growth inhibitory effects of metformin *in vivo* using B16 mice injected with B16 melanoma cells of human melanoma as well as the induction of both autophagy and apoptosis markers in this mouse model (Tomic 2011). In addition to metformin, evidence also reports the potential of cannabinoids, and in particular tetrahydrocannabidiol (THC), as agents with potent anti-cancer effects derived through the ability to induce autophagy mediated apoptosis in human glioma cells with minimal adverse effects observed in patients (Duntsch, Divi *et al*. 2006; Salazar, Carracedo *et al*. 2009; Sánchez, Galve-Roperh *et al*. 1998; Sánchez, de Ceballos *et al*. 2001; Velasco, Carracedo *et al*. 2007). Furthermore, evidence for the requirement of Autophagy in THC-induced apoptosis of glioma cells is evidenced by *in vitro* studies demonstrating knockdown of Atg1, Atg5 or Ambra 1 prevented apoptosis (Salazar, Carracedo *et al*. 2009).

As several links between autophagy and apoptosis are being identified such as the discovery of direct interactions of anti-apoptotic Bcl-2 proteins with the autophagy regulatory protein Beclin-1 (Feng, Huang *et al*. 2007; Decuypere, Jan *et al*. 2012), the importance of this pathway in cancer development is becoming ever more apparent and hence a target for therapeutic development. This thesis has focussed on the identification of potential avenues to exploit autophagy for the therapeutic benefit of metastatic melanoma and in particular the use of THC. Studies have focussed on the role of Mcl-1 in autophagy

leading to studies of the therapeutic potential of THC and the underlying molecular mechanisms through which it modulates autophagy to promote cell death. In line with previous observations demonstrating Bcl-2 family members are crucial regulators of apoptosis in melanoma and the role of anti apoptotic Mcl-1 (Balmanno and Cook 2009; Boucher, Morisset *et al*. 2000 Tang, Tron *et al*. 1998; Zhai, Jin *et al*. 2008) in autophagy, data derived from this thesis suggests manipulating Mcl-1 splice variant expression may represent a potential novel therapeutic strategy. Secondly, in comparison to the effects of bortezomib-induced apoptosis, a recognised inducer of pro-survival autophagy (Armstrong, Corazzari *et al*. 2011), data demonstrate the ability of THC to induce autophagy mediated apoptosis of melanoma cells, the effect of which is amplified by the induction of lysosomal membrane permabilization, collectively suggesting a novel therapeutic strategy through which to harness autophagy modulation for the clinical management and benefit of metastatic disease.

7.2: Mcl-1 as a therapeutic target in melanoma

7.2.1: Influence of oncogenic BRAF signalling on Mcl-1 splice variant expression

Previous studies have demonstrated the increased expression of anti-apoptotic Bcl-2 protein family members in primary melanomas. In particular the increased expression of Bcl-2 and Bcl-xl has been observed in early stage tumours while increased Mcl-1 expression is frequently observed in more advanced stage tumours (Tang, Tron *et al*. 1998; Zhuang, Lee *et al*. 2007). Additionally Mcl-1 has been shown to be crucial for melanoma survival (Zhuang, Lee *et al*. 2007) and as such, its increased expression likely contributes to the chemoresistance of melanoma. Increased expression of anti-apoptotic Bcl-2 family members (Boucher, Morisset *et al*. 2000) may be mediated by RAS/RAF/MEK/ERK signalling frequently hyper-activated in melanoma by genetic mutations most commonly in BRAF or NRas (Davies, Bignell *et al*. 2002; Balmanno and Cook 2009). Hence it is likely that activated RAS/RAF/MEK/ERK signalling directly influences Bcl-2 family protein expression levels in melanoma. Furthermore, the Bcl-2 protein family plays a functional role in autophagy. As Beclin-1 is a BH3-only protein it is also a binding target of Bcl-2 (Decuypere, Jan *et al*. 2012; Feng, Huang *et al*. 2007), and Erlich *et al* showed that Beclin-1 binds to Bcl-2 and Bcl-xl with a high affinity, and as well as, with an albeit weaker interaction, with Mcl-1 (Erlich, Mizrachy *et al*. 2007). As such, Bcl-2 anti-apoptotic proteins including Mcl-1L are able to inhibit Beclin-1-dependant autophagy.

Mcl-1 encodes two splice variants known as Mcl-1 short (Mcl-1S) and Mcl-1 long (Mcl-1L) with either pro-apoptotic or anti-apoptotic function respectively (Bae, Leo *et al*. 2000; (Bingle, Craig *et al*. 2000; Shieh, Liu *et al*. 2009). Despite identification of high Mcl-1 levels in melanoma the influence of BRAF mutational status on Mcl-1 expression or for the expression of differing Mcl-1 splice variants in melanoma has to date remained poorly defined. Results from this thesis support and extend previous observations revealing activating mutations in *BRAF* result in increased expression of both anti-apoptotic Mcl-1L and pro-apoptotic Mcl-1S *in vitro* (McKee *et al* 2013 *In press*). Results confirmed increased Mcl-1L mRNA and protein expression in melanoma cells bearing mutant $BRAF^{V600E}$. As such this suggests that BRAF/MEK/ERK signalling may contribute to apoptosis resistance through overexpression of anti-apoptotic Mcl-1 (McKee *et al* 2013 *In press*). Mcl-1S mRNA levels on

the other hand were not increased suggesting that increased Mcl-1S protein expression observed in melanoma cells bearing oncogenic BRAF was possibly due to a stabilisation event of the protein rather than increased transcription and/or splicing of the Mcl-1 gene (Domina, Vrana *et al*. 2004).

7.2.2: The role of Mc-1 in autophagy and apoptosis in melanoma

Several anti-apoptotic Bcl-2 family members including Mcl-1 are interacting partners of Beclin-1 (Decuypere, Jan *et al*. 2012; Erlich, Mizrachy *et al*. 2007; Feng, Huang *et al*. 2007) and as such associated with autophagy. Since initial results from the present study demonstrated increased total Mcl-1 expression in A375 and G361 melanoma cells with a significantly higher expression of anti-apoptotic Mcl-1L in BRAF mutated (A375, G361) compared with BRAF wild-type cells (CHL-1, SKMEL-23), further studies were undertaken to determine a functional role of total Mcl-1 in autophagy regulation in response to treatment with the autophagy modulatory agents THC and bortezomib. Both agents induced apoptosis in A375 metastatic melanoma cells as revealed by inhibition of viability and increased levels of Cleaved Caspase 3. Interestingly, bortezomib treatment also increased the levels of Mcl-1L in A375 cells transfected with control and Mcl-1 siRNA. This effect was most likely due to inhibition of the proteasome (the principle mechanism of bortezomib action (Attar, De Angelo *et al*. 2008; Horton, 2007; Ogawa, Tobinai *et al*. 2008; Papandreou, Daliani *et al*. 2004; Richardson, Mitsiades *et al*. 2008; Yin, Zhou *et al*. 2005) and the prevention of proteasomal mediated degradation of Mcl-1. Both THC and bortezomib also induced autophagy as evidenced by increased LC3-II expression compared to expression in untreated A375 cells and initial findings following treatment of A375 cells with either agent in combination with the lysosomal inhibitor chloroquine also suggested THC-induced cell death was dependent on autophagy while bortezomib-induced cell death appeared to be independent of autophagy. However, there were a number of limitations to this approach since even partial knockdown of Mcl-1 expression resulted in significant induction of apoptosis. The analysis of the effect of Mcl-1 knockdown on autophagic signalling was therefore not possible. Nevertheless, these findings further highlight the importance of Mcl-1 for melanoma cell survival *in vitro*. Despite the limitations with the Mc-1 knockdown

studies, partial knockdown of Mcl-1 resulted in the induction of autophagy to some extent as shown by increased levels of LC3-II in Mcl-1 siRNA transfected A375 cells compared to Ctrl siRNA transfected cells, thus supporting the hypothesis that Mcl-1 amplification, in addition to prevention of apoptosis, may contribute to oncogenesis stimulated by the inhibition of Beclin-1-dependent autophagy.

7.2.3 Future potential of Mcl-1 as a therapeutic target

Resistance to apoptosis is one of the key obstacles to overcome in order to develop more effective treatments for cancer. Increased anti-apoptotic Bcl-2 family members in addition to other factors including p53 mutations, BRAF/NRAS mutations or alterations in PI3K/Akt/mTOR signalling events to name but a few, may account for such resistance (Grossman and Altier 2001; Zhai, Jin *et al*. 2008). As such the high levels of anti apoptotic Mcl-1 mRNA observed in the present study in all tested cell lines (CHL-1, SKMEL-23, A375, G361) support previous observations of increased Mcl-1 in advanced melanomas and its association with apoptotic resistance induced by chemotherapy (Zhai, Jin *et al*. 2008). Results showing Mcl-1L expression is further increased in cells lines bearing oncogenic BRAF (A375, G361) compared with BRAF wild-type cells (CHL-1, SKMEL-23) also support the potential contribution of Mcl-1 to the chemoresistance in BRAF mutated tumours. It should be noted however, that melanomas without BRAF activating mutations may still have activated MEK/ERK signalling, as observed in some patient tumours (Passeron, Lacour *et al*. 2011), possibly due to activation of other signalling pathways, as observed for the development of resistance following prolonged treatment with the BRAF inhibitor, vemurafenib (Johannessen, Boehm *et al*. 2010) which may therefore also influence Mcl-1 expression. Of particular note, signalling via the protein signal transducers and activators of transcription 3 (STAT3) has been found to play an important role in melanoma development and progression and has recently been found as an important factor in BRAF inhibitor resistance (Liu, Cao *et al* 2013). STAT3 is a member of a family of seven proteins (STATs1, 2, 3, 4, 5a, 5b, and 6) which are involved in transmission of signals from the plasma membrane to the nucleus in order to modulate transcription of several genes involved in the regulation of key processes in cancer survival such as cell differentiation, proliferation, angiogenesis,

metastasis, and immune responses (Johnston and Grandis 2011; Al Zaid Siddiquee and Turkson 2008). When activated in melanoma STAT3 promotes growth of melanoma cells and survival (Niu, Heller et al 1999; Niu, Bowman et al 2002) and target genes of STAT3 include Mcl-1 and Bcl-xl, increasing their expression and further contributing to melanoma resistance to apoptosis (Zhuang, Lee *et al* 2007). Apoptotic resistance in both BRAF mutated and BRAF wild-type tumours, which may also have activation of alternative signalling pathways and exhibit high anti-apoptotic Mcl-1 expression, may therefore potentially be overcome through the use of specific Mcl-1 inhibitors rather than broad spectrum BH3 mimetics. However, several questions remain unanswered in order to understand the potential of Mcl-1 as a therapeutic target in melanoma including its interaction with Beclin-1 in addition to the influence of alternative pathways of MEK/ERK activation on Mcl-1 expression. As such, future studies could include:

- Studies of the interaction of Mcl-1 with Beclin-1, the prevention of this interaction and the resulting effects on autophagy modulation involving perhaps the expression of a Beclin-1 construct bearing a mutation in the Mcl-1 binding site and the comparison of effect in BRAF mutant and wild-type cells in response to autophagy induction.
- Further studies into the application of BH3 mimetics which specifically target Mcl-1
- Additional studies of the impact of downstream MEK/ERK signalling on Mcl-1 splice variant expression through the use of MEK or ERK specific inhibitors.

7.3 Autophagy modulation as a therapeutic target in melanoma

7.3.1 THC and Bortezomib-induced autophagy

The importance of autophagy in cancer development has highlighted potential new therapeutic targets. Typically treatments have focussed towards inhibiting autophagy including through indirect inhibition with the lysomal inhibitor hydroxychloroquinone (HCQ) (Garber 2011). HCQ raises intralysosomal pH (Poole and Ohkuma 1981) and also impairs autophagic protein degradation (Glaumann and Ahlberg 1987; Levy and Thorburn 2011) and has been combined with several anticancer drugs resulting in its inclusion in a number of phase I and II clinical trials for tumours exhibiting high levels of basal autophagy (Garber 2011). However alternative strategies suggest autophagy may be more appropriately harnessed to induce tumour cell death through the combination of current chemotherapeutics with autophgay inducing agents such as metformin or cannabinoids (Buzzai, Jones *et al*. 2007; Salazar, Carracedo *et al*. 2009; Tomic 2011). Initially autophagy-inducing agents such as mTOR inhibitors (Rapamycin, RAD001, CC1-779 and AP23573) were used for the treatment of different human cancers, for example Rapamycin (Faivre, Kroemer *et al*. 2006) has been shown to exert significant anti-tumour properties in several cancer models (Faivre, Kroemer *et al*. 2006). However, clinical trials of mTOR inhibitors have been disappointing and have consequently led to the search for more effective autophagy inducing agents able to exert a cytotoxic effect (Buzzai, Jones *et al*. 2007; Salazar, Carracedo *et al*. 2009; Tomic 2011). Cannabinoids in particular are able to drive cancer cells to cell death through induction of autophagy (Salazar, Carracedo *et al*. 2009) and in particular THC, the most extensively studied for its medicinal usage (Carracedo, Gironella *et al*. 2006; Carracedo, Lorente *et al*. 2006; Gómez del Pulgar, Velasco *et al*. 2002; Salazar, Carracedo *et al*. 2009; Salazar, Carracedo *et al*. 2009; Sánchez, Galve-Roperh *et al*. 1998). In glioma the underlying mechanism of THC-induced apoptosis appears to result from ceramide accumulation leading to the activation of an ER stress response and subsequent, Akt inhibition, autophagy induction and ultimately cell death (Duntsch, Divi *et al*. 2006; Salazar, Carracedo *et al*. 2009; Sánchez, Galve-Roperh *et al*. 1998; Sánchez, de Ceballos *et al*. 2001; Velasco, Carracedo *et al*. 2007). Results from this thesis demonstrate THC is also able to

induce autophagy in melanoma as evidenced by increased autophagosomes and the induction of autophagic flux in $BRAF^{V600E}$ (A375) and $BRAF^{WT}$ (CHL-1) cells stably expressing RFP-GFP-LC3 as well as through the use of chloroquine, in which THC-induced cell death was prevented. However, the use of a single autophagy inhibitor alone, or even the knockdown of a single key autophagy gene, is not enough to fully support the claim that THC requires autophagy to induce cell death (Mizushima, Yoshimori *et al*. 2010). Combining data derived from THC treatment of melanoma cells in which knockdown of both Beclin-1 and Atg7 expression in A375 and CHL-1 cells prevented THC-induced cells death however, in addition to the observed effects in response to combined treatment with chloroquine provides strong evidence for the requirement of autophagy for apoptosis induction in response to THC. Importantly therefore, these data thus suggest similar mechanisms of THC-induced autophagy and apoptosis are apparent in multiple tumour types (melanoma, glioma, pancreatic). Nevertheless, differences have been observed within this thesis compared to studies in glioma. The most noticeable example of this is for that of the induction of ceramide accumulation, which is reported to be a prerequisite for THC-induced ER stress, autophagy and apoptosis (Gómez del Pulgar, Velasco *et al*. 2002; Salazar, Carracedo *et al*. 2009) in glioma. The importance of this event in glioma was confirmed as ISP (a selective inhibitor of serine palmitoyltransferase, the enzyme that catalyzes the first step of sphingolipid biosynthesis (Ogretmen and Hannun 2004)) prevented THC-induced autophagy and cell death. However, using 5 μM ISP treatment in A375 melanoma cells had little effect on the inhibition of melanoma cell viability. As such, whether ceramide accumulation is occurring via a different mechanism or even if it is required in melanoma for downstream responses induced by THC remains to be verified. However, knockdown of the protein kinase tribbles 3 (a negative regulator of NFκB shown to be required for THC-induced autophagy and cell death in glioma (Salazar, Carracedo *et al*. 2009) did prevent THC-induced inhibition of cell viability indicating the existence of similar downstream signalling events to ceramide accumulation to those reported in studies of THC-induced autophagy and apoptosis of glioma cells (Salazar, Carracedo *et al*. 2009).

Comparing the effects of THC and bortezomib-induced autophagy revealed opposing effects of autophgay induction on cell death of melanoma cells. While knockdown of the autophagy regulatory genes Beclin-1/Atg7 or chloroquine treatment prevented THC-induced inhibition of melanoma cell viability, this had no effect on bortezomib-induced cell

death. Both agents however, were still able to induce autophagy even in the absence of Beclin-1 as shown by increased LC3-II expression in A375 cells in response to subsequent treatment with THC or bortezomib. However, rapamycin treatment still resulted in the induction of LC3-II expression even in the absence of Beclin-1 in both A375 and CHL-1 cells. Collectively these data thus suggest the role of Beclin-1 in autophagy regulation in melanoma may not be canonical and that this protein may in fact exert additional autophgay-independent functions required for THC-induced cell death which are not necessarily required for a similar response to bortezomib. Additionally, these data suggest autophagy induction in melanoma may occur even in the absence of Beclin-1. This hypothesis nevertheless requires validation through use of alternative siRNA sequences to knockdown Beclin-1.

The identified requirement of Beclin-1 in THC-induced cell death of melanoma is also an important factor to consider when stratifying patients for potential treatment. Although the panel of patient tumours studied in this thesis is relatively small, differences in Beclin-1 expression were clearly observed in which tumours exhibited either little or no expression or alternatively high expression of this protein. Furthermore previous publications have also observed a bi-phasic expression pattern of Beclin-1 in patient tumours where Beclin-1 expression was found to be either high or low in different patient tumours (Sivridis, Koukourakis *et al*. 2011). As such this would lead one to speculate that only those patients expressing Beclin-1 may benefit from treatment with THC as Beclin-1 knockdown in A375 and CHL-1 cells prevented THC-induced apoptosis and inhibition of cell viability.

7.3.2 Potential therapeutic applications of autophagy modulation by THC

The importance of autophagy in cancer development and survival is becoming ever clearer, however, targeting autophagy through use of either specific inhibitors is essentially a ‘double edged sword’. On one hand autophagy inhibition may over-come induction by chemotherapeutic drugs as a mechanism to counter act apoptotic signals or to block pro survival autophagic signalling frequently observed in advanced stage tumours (Bertout, Patel *et al*. 2008; Zhang, Bosch-Marce *et al*. 2008). On the other hand however, this approach may prevent the tumour suppressive function of autophagy and facilitate the development of

secondary tumours (Qu, Yu *et al*. 2003; Yue, Jin *et al*. 2003). Alternatively, autophagy induction resulting in apoptosis as observed in response to THC (Salazar, Carracedo *et al*. 2009) may represent a better and more effective strategy. However, as autophagy is typically utilised as a pro-survival mechanism in many tumour types, if the correct balance for beneficial effects of THC as an anti-cancer agent are not achieved this may in theory prove more beneficial to advanced stage tumours rather than cause their demise.

Results from this thesis nevertheless suggest THC may be useful in the promotion of melanoma cell death *in vivo*, as evidenced by its clear effects on the induction of apoptosis and inhibitory effects on cell viability *in vitro.* However, *in vivo* studies are lacking at this stage and hence would be required to further verify the findings *in vitro* and provide further evidence to support its clinical use in the treatment of metastatic melanoma. Despite the lack of *in vivo* data, the potential use of THC in combination with temozolmide, a main-stay chemotherapeutic in the treatment of melanoma, has been shown in glioma to induce synergistic responses (Torres, Lorente *et al*. 2011) highlighting the potential application of THC in combination well established chemotherapeutics for melanoma treatment. As such future studies of THC may include:

- *In vivo* studies of mouse xenografts of human melanoma treated with THC alone or in combination with temozolomide.
- Studies of THC clinical derivatives, such as Sativex (GW Pharmaceuticals Ltd) and the effect of such agents on the induction of autophagy and apoptosis of melanoma *in vitro* and *in vivo.*

7.4 Bioinformatics for identifying Beclin-1 complex protein-protein interactions in melanoma

7.4.1 Protein-protein interactions of Beclin-1

Identification of protein-protein interactions utilizing well established methods such as co-immunoprecipitation pull down assays, bimolecular fluorescence complementation assays and chemical cross-linking approaches are some of the most effective practical approaches to determining direct interactions of a protein of interest (Hu, Chinenov *et al*. 2002; Berggård, Linse *et al*. 2007). This can prove very useful in understanding functional roles as well as providing additional avenues of future research to explore. However it is often easy to overlook articles which have already identified many important interactions of a particular protein of interest. Furthermore, pooling of this data can prove even more taxing by more traditional approaches. To overcome these obstacles, the development of several bioinformatics tools in recent years have provided a mechanism for accurate data mining approaches to provide a platform for gathering such information. The tool adopted by studies in this thesis, Cytoscape, is an open source bioinformatics software platform used for visualizing molecular interaction networks equipped with multiple functions enhanced by the use of specific plugins designed by the scientific community (Shannon, Markiel *et al*. 2003; Yeung, Cline *et al*. 2008). The plugin of choice used to visualise known protein-protein interactions for a protein of interest has been designed by Biological General Repository for Interaction Datasets (BioGRID) which gathers constantly updated interaction data for several organisms (Homo sapiens included) from several databases; Saccharomyces Genome Database, Flybase, GeneDB, TAIR, and Entrez Gene (Chatr-Aryamontri, Breitkreutz *et al*. 2013). The data provides several useful pieces of information in addition to the identified genetic and protein-protein interactions including the source of the information and the techniques used to obtain results and allowing for further in depth analysis of each identified interaction in addition to methodical approaches which can be utilized and adapted for personal experiments. Unexplained findings observed following knockdown of Beclin-1 and subsequent treatment with THC, bortezomib or rapamycin in which autophagy was still induced despite the confirmed reduction in Beclin-1 expression lead to utilization of such software in order to define more specific targets for further analysis and provide further

insight into possible roles for this protein in a variety of cellular pathways. In recent years several publications have revealed interacting partners for many members of the autophagy pathway including Beclin-1 (Behrends, Sowa *et al*. 2010)**.** As such a wealth of information revealing potentially crucial protein-protein interactions for Beclin-1 is already available. Using the BIOGRID plugin on Cytoscape revealed 34 known protein-protein interactions for Beclin-1. Several of these interactions where identified for proteins involved in cellular pathways particularly important to the theme of this project including autophagy and apoptosis as well as the cell cycle the involvement of which may explain more fully the role of Beclin-1 in THC-induced cell death and autophagy.

7.4.2 Ambra 1; an important Beclin-1 interacting protein

Utilising the BIOGRID plugin for cytoscape, Ambra 1 (or activating molecule in Beclin-1 regulated autophagy protein 1) was highlighted as an important Beclin-1 interacting protein. Ambra 1 was chosen to further investigate its contribution to THC-induced autophagy and cell death in melanoma due to several factors; its interaction with Beclin-1, its known role in autophagy (Fimia, Stoykova et al. 2007; Itakura, Kishi et al. 2008; Roy and Debnath 2010; Simonsen and Tooze 2009) and links with apoptosis (Corazzari, Fimia et al. 2012). Furthermore previous studies in glioma have shown that Ambra 1 is essential for THC-induced autophagy and cell death (Salazar, Carracedo et al. 2009) and that this process does not require Beclin-1 (personal communication with Guillermo Velasco, Complutenese University of Madrid, Spain). Interestingly as shown in chapter 6, in contrast to studies of THC-induced autophagy in glioma, Ambra 1 does not appear to be required for autophagy induced by THC in melanoma since stable knockdown of this protein did not prevent THC-induced inhibition of cell viability or cell death. Additionally bortezomib also induced autophagy in the absence of Ambra 1. These data thus further support a non-canonical mechanism of THC-induced autophagy in melanoma in which the classical Beclin-1/Vps34/Ambra 1 complex is not necessarily required for induction of cell death or in the induction of THC or bortezomib-induced autophagy.

7.4.3 Future studies of Beclin-1 functions in melanoma

The identification of Beclin-1 protein-protein interactions has revealed several pathways of interest that Beclin-1 maybe involved in for which there is little information available in the literature to be able to elaborate further. At least three different functional Beclin-1 complexes have been identified thus far in the literature; the Beclin-1/Vps34/Vps15/Ambra 1/Atg14L complex; the Beclin-1/Vps34/Vps15/UVRAG complex; and the Beclin-1/Vps34/Vps15/UVRAG/Rubicon complex which are each involved in autophagy regulation (Matsunaga, Saitoh *et al*. 2009). The variety of complexes that Beclin-1 has already been shown to form in addition to direct interactions with anti-apoptotic Bcl-2 proteins suggests that further studies are required to truly understand the complex functions of this protein within cells be they cancerous or normal. Additional articles have provided evidence for functions of Beclin-1 outside of autophagy (Djavaheri-Mergny, Maiuri *et al*. 2010; Frémont S 2013; Wirawan, Vande Walle *et al*. 2010). Beclin-1's role in cell cycle regulation was suggested to be required for chromosome congression and proper outer kinetochore assembly (Frémont S 2013). Additionally, Beclin-1 can be cleaved by caspases thus preventing its pro-autophagic activity (Wirawan, Vande Walle *et al*. 2010) and the C-terminal fragment produced from this celavage event functions in amplifying mitochondrial mediated apoptosis (Djavaheri-Mergny, Maiuri *et al*. 2010). This, in addition to further interactions identified in this thesis using Cytoscape suggests that Beclin-1 may in fact have several important functions independent of its role in autophagy. As such future studies could address:

- Protein-protein interactions to verify other Beclin-1 interacting proteins revealed in the Cytoscape search and their function in melanoma.
- The functional role of Beclin-1 in the additional cellular pathways identified by the present cytoscape data mining data including those involved in apoptosis and the cell cycle, including investigating the effects of their modulation on apoptosis and cell cycle function.

7.5 THC as an apoptosis inducing agent for melanoma therapy

7.5.1 THC induces intrinsic apoptosis in melanoma

The realisation that cannabinoids induce apoptosis as a mechanism of cell death has lead to intense interest in their clinical usage in cancer therapy. Many articles have highlighted the capability of THC to induce apoptosis (Carracedo, Gironella *et al*. 2006; Carracedo, Lorente *et al*. 200 6; Salazar, Carracedo *et al*. 2009; Salazar, Carracedo *et al*. 2009) with some pilot data suggesting similar effects in melanoma (Blázquez, Carracedo *et al*. 2006; Kenessey, Bánki *et al*. 2012; Scuderi, Cantarella *et al*. 2011; Zhao, Yang *et al*. 2012). Data from this thesis confirms not only is THC able to induce death of melanoma cells but that cell death specifically results from the induction of apoptosis. Treatment with THC resulted in the inhibition of cell viability in all tested cell lines (CHL-1, A375, SKMEL-28) using a dose range of 4-10 μM THC with complete inhibition of cell viability observed by 10 μM concentrations and was paralleled by increased levels of Cleaved Caspase 3, the initiation event for the execution phase of apoptosis. Combined treatment of THC with the pan caspase inhibitor ZVAD-fmk prevented inhibition of melanoma cell viability in both A375 and CHL-1 cells also confirming the induction of apoptosis in melanoma cells by THC. Finally cytochrome C release, an early event in intrinsic apoptosis (Cai, Yang *et al*. 1998, van Loo, van Gurp *et al*. 2002; Schimmer 2004), was also observed in response to THC treatment further suggesting the activation of intrinsic apoptosis signalling mediated through mitochondria. Coupled with observations showing THC treatment had little effect on the cell viability of primary melanocytes with inhibitory effects only observed at much higher doses than required to induce apoptosis of melanoma cells (>7 μM as opposed to >3 μM) these data thus highlight the potential suitability of this agent as therapeutic agent for metastatic melanoma.

7.5.2. Alternative modes of THC induced apoptosis

Additional studies (further discussed in section 7.6) suggest the toxicity of THC is further enhanced through its ability to induce lysosome membrane permeabilization. However, further understanding of the downstream apoptotic events induced by THC will be crucial to elucidating the potential of THC as a chemotherapeutic agent. Data derived from this thesis provide strong evidence for key events in THC-induced intrinsic apoptosis. However, downstream signalling events of caspase 3 cleavage involved in the execution pathway remain to be identified and additional observations such as cytochrome C nuclear localisation observed in THC treated A375 cells by fluorescence microscopy, an event suggested to induce apoptosis directly (Nur-E-Kamal, Gross *et al*. 2004), suggests other signalling mechanisms are occurring which may be required for THC-induced apoptosis. As such, this will require further study to determine the importance of these events in THC-induced apoptosis. Several additional molecular events have been shown to occur in response to THC in other tumour types. The most noteworthy of these events is the induction of reactive oxygen species (observed in THC treated glioma cells) (Marcu, Christian *et al*. 2010) which is an event known to induce apoptosis through several mechanisms including; direct death receptor activation, mitochondrial mediated intrinsic apoptosis induction and JNK mediated extrinsic and intrinsic apoptosis induction to name a few (Circu and Aw 2010; Dhanasekaran and Reddy 2008; Ryter, Kim *et al*. 2007). However, whether these events are occurring in melanoma remain to be seen and the contribution of ROS induction towards THC-induced cell death has not been determined. To further elucidate other signalling events that contribute to THC-induced apoptosis, future studies could include:

- Studies to determine the importance of nuclear localised cytochrome C towards THC-induced apoptosis in melanoma cells.
- Studies to determine the impact of THC treatment on the induction of oxidative stress and the contribution of ROS induction to THC-induced melanoma cell death determined by studies of ROS induction.

- Further studies to determine whether death receptor activation and JNK actiavation are occurring in response to ROS induction would also be important to understand mechanisms of cell death induced by THC.
- Assessment of events downstream of the execution pathway of apoptosis such as degradation of chromosomal DNA, degradation of nuclear and cytoskeletal proteins, formation of apoptotic bodies to confirm intrinsic apoptotic cell death is caused by THC in melanoma.

7.6 Lysosomes; the missing link between THC induced autophagy and apoptosis?

7.6.1: THC induces lysosome membrane permeabilization in melanoma cells

The importance of lysosomes in cell death has long been established. Initially described as 'suicide bags' (De Duve 1959), increased evidence now shows lysosome membrane permeabilization (LMP) is a key event contributing to cell death (Aits and Jäättelä 2013; Boya and Kroemer 2008). LMP results from lysosomal damage causing the release of constituents including hydrolytic enzymes such as proteases, lipases, and nucleases (Aits and Jäättelä 2013; Boya and Kroemer 2008). Damage can be induced by several stimuli such as; p53, Bax, ROS, PLA2, active caspases and bacteria and viral proteins (Boya and Kroemer 2008), resulting in indiscriminate degradation within the cell and ultimately cell death, which depending on the amount of lysosomal damage may result from necrosis (in the case of large scale damage) or apoptosis (where moderate damage is induced). LMP typically occurs as a partial and selective process causing apoptosis via the released hydrolytic enzymes resulting in mitochondrial outer membrane permeabilization, cytochrome C release and caspase activation (Guicciardi, Deussing *et al*. 2000). Results from the present study have observed each of these events in melanoma cells in response to treatment with THC as evidenced by observations demonstrating cathepsin B was no longer co-localised with LAMP2 in A375 or SKMEL-28 cells following treatment with THC (suggesting they are no longer contained with lysosomes); cytochrome C exhibited a cytoplasmic staining pattern in THC treated A375 cells suggesting they have been released from the mitochondria; and activation of caspase 3 was observed in THC treated A375 and CHL-1 cells. Each of these observations suggests LMP is thus a crucial factor contributing to THC-induced apoptosis of melanoma cells.

Additionally, fenton reactions which are known to occur in response to chelatable iron overload within the lysosome, have also been suggested to result from THC treatment of glioma cells resulting in increased cell death mediated by oxidative stress (Goncharov, Weiner *et al*. 2005). Since increased ferritin expression in melanoma cells may contribute to melanoma progression by modulating cell growth and reducing sensitivity to oxidative stress (Baldi, Lombardi *et al*. 2005), which also leads to LMP induction, it is possible therefore that

increased levels of ferritin and iron containing proteins may contribute to LMP induced by THC.

Recycling of cellular components through autophagy utilizes a lysosome based mechanism of degradation in which the autophagosome ultimately fuses with the lysosome to degrade its constituents (Roy and Debnath 2010). As such lysosomes may play a crucial role in cannabinoid-induced apoptosis with increased sphingolipid accumulation in addition to ROS generation further supporting the possibility of THC-induced induction of LMP. Indeed data derived from this thesis demonstrates for the first time LMP induction by THC as shown by release of cathepsin B from lysosomes and the inhibition of this process by bafilomycin. Additionally, prevention of LMP by inhibition of autophagy through Atg7 knockdown in A375 and SKMEL-28 cells was also observed demonstrating THC-induced autophagy leads to LMP and release of cathepsin B. As inhibition of cell viability was also prevented by a cathepsin B inhibitor in A375 cells and the use of this agent in combination with THC partially prevented cytochrome C release it is therefore likely that cathepsin B, at least in part, is a key event in THC-induced apoptosis as a result of LMP.

7.6.2 Future directions for understanding the role of LMP in THC induced apoptosis

Results from the present study reveal the potential importance of cathepsin B release from lysosomes in the induction of apoptosis in melanoma cells in response to THC. However cathepsin B is only one of many proteases leaked from the lysosome during LMP (Emert-Sedlak, Shangary *et al*. 2005; Guicciardi, Deussing *et al*. 2000; Sever, Altintas *et al*. 2007). Many cathespins are active at neutral pH and cathepsin D release has also been shown to induce apoptosis in other tumour types (Emert-Sedlak, Shangary *et al*. 2005; Sever, Altintas *et al*. 2007). Therefore identifying whether additional cathepsins are also released from the lysosome and function in downstream apoptotic signalling events will provide further insight into the impact of LMP in THC-induced apoptosis. Additionally studies of how autophagy may regulate the release of cathepsins from the lysosome will enhance the understanding of autophagy mediated LMP and its requirement or not for THC-induced cell apoptosis or whether it is simply an amplification step in the induction of cell death.

Therefore, to elaborate on these findings and determine more fully the relationship between autophagy and LMP in THC treatment of melanoma cells future studies might entail:

- Further assessment of the functional impact of additional lysosomal cathepsin release (e.g. cathepsin D) on LMP induction in response to THC.
- Real time analysis of autophagosome-lysosomal fusion in relation to THC-induced LMP, cytochrome C release and cell death.
- Additional studies to verify the requirement of autophagy for THC-induced LMP.

7.7 Final remarks

This thesis aimed to identify novel therapeutic strategies through which to harness autophagy modulation for the clinical benefit of metastatic melanoma and has revealed novel potential avenues to exploit for drug development. The discovery that activated BRAF/MEK/ERK signalling causes increased expression of anti-apoptotic Mcl-1L suggests a potential mechanism of apoptotic resistance in melanoma which maybe further enhanced in those tumours bearing BRAF mutations. As such targeting Mcl-1L using specific Mcl-1 inhibitors rather than broad spectrum BH3 mimetics, maybe offers a more effective therapeutic approach, particularly for patients bearing BRAF/NRAS mutant melanomas or for patients who have developed resistance to BRAF-specific inhibitors (Jang and Atkins 2013). Additionally, increased expression of Mcl-1S may offer a potential anti-cancer effect in melanoma cells, possibly through reducing the functional impact of increased Mcl-1L (Shieh, Liu *et al*. 2009). As such targeting Mcl-1 pre-mRNA with Mcl-1 antisense morpholino oligonucleotides to increase Mcl-1S expression may be a further mechanism to exploit. Furthermore data derived from this thesis show in contrast to pro-survival autophagy inducing agents such as bortezomib (Armstrong, Corazzari *et al*. 2011), THC-induced apoptosis of melanoma cells which is dependent on the induction of autophagy. Additionally, the ability of THC to induce LMP in metastatic melanoma cells, which appears to be dependent on autophagic signalling suggests for the first time, a potential link between autophagy and apoptosis through the permeabilization of lysosomes and the release of cathepsin B leading to loss of mitochondrial integrity, cytochrome C release and enhanced cell death. As such the amplified effect of THC-induced autophagy and LMP suggest a novel means through which to increase the potency of this cannabinoid providing a strong rational for its development and clinical use in the treatment of metastatic melanoma. Current clinical trials of sativex (GW Pharmaceuticals Ltd), a 50:50 mixture of THC and CBD in combination with temozolomide are underway for the treatment of recurrent glioma with promising results which together with ongoing studies in melanoma already showing a potent synergistic effect *in vitro* (Hill and Lovat un published data) will hopefully lead the way to a new era of autophagy modulatory therapy for metastatic melanoma with improved clinical outcome.

References

References

Ahn JH, L. M. (2013). "Autophagy-Dependent Survival of Mutant BRAF Melanoma Cells Selected for Resistance to Apoptosis Induced by Inhibitors against Oncogenic BRAF." Biomolecules and Therapeutics **21**(2): 114-120.

Aits, S. and M. Jäättelä (2013). "Lysosomal cell death at a glance." Journal of Cell Science **126**(Pt 9): 1905-1912.

Albay, D., S. G. Adler, J. Philipose, C. C. Calescibetta, S. G. Romansky and A. H. Cohen (2005). "Chloroquine-induced lipidosis mimicking Fabry disease." Modern Pathology **18**(5): 733-738.

Amaravadi, R. K., D. Yu, J. J. Lum, T. Bui, M. A. Christophorou, G. I. Evan, A. Thomas-Tikhonenko and C. B. Thompson (2007). "Autophagy inhibition enhances therapy-induced apoptosis in a Myc-induced model of lymphoma." Journal of Clinical Investigation **117**(2): 326-336.

Amiri, K. I., L. W. Horton, B. J. LaFleur, J. A. Sosman and A. Richmond (2004). "Augmenting chemosensitivity of malignant melanoma tumors via proteasome inhibition: implication for bortezomib (VELCADE, PS-341) as a therapeutic agent for malignant melanoma." Cancer Res **64**(14): 4912-4918.

Anderson DE, L. A., Erickson HP, Hirano T. (2002). "Condensin and cohesin display different arm conformations with characteristic hinge angles." The Journal of Cell Biology **156**(3): 419-424.

Armstrong, J. L., M. Corazzari, S. Martin, V. Pagliarini, L. Falasca, D. S. Hill, N. Ellis, S. Al-Sabah, C. P. F. Redfern, G. M. Fimia, M. Piacentini and P. E. Lovat (2011). "Oncogenic BRAF signalling in melanoma impairs the therapeutic advantage of autophagy inhibition." Clin Cancer Res **17**(8): 2216-2226.

Armstrong, J. L., M. Ruiz, A. V. Boddy, C. P. Redfern, A. D. Pearson and G. J. Veal (2005). "Increasing the intracellular availability of all-trans retinoic acid in neuroblastoma cells." British Journal of Cancer **92**(4): 696-704.

Arur, S., U. E. Uche, K. Rezaul, M. Fong, V. Scranton, A. E. Cowan, W. Mohler and D. K. Han (2003). "Annexin I is an endogenous ligand that mediates apoptotic cell engulfment." Developmental Cell **4**(4): 587-598.

Ashkenazi, A. and V. M. Dixit (1998). "Death receptors: signaling and modulation." Science **281**(5381): 1305-1308.

Atallah, E. and L. Flaherty (2005). "Treatment of metastatic malignant melanoma." Current Treatment Options in Oncology **6**: 185-193.

Attar, E. C., D. J. De Angelo, J. G. Supko, F. D'Amato, D. Zahrieh, A. Sirulnik, M. Wadleigh, K. K. Ballen, S. McAfee, K. B. Miller, J. Levine, I. Galinsky, E. G. Trehu, D. Schenkein, D. Neuberg, R. M. Stone and P. C. Amrein (2008). "Phase I and pharmacokinetic study of bortezomib in combination with idarubicin and cytarabine in patients with acute myelogenous leukemia." Clin Cancer Res **14**(5): 1446-1454.

Bae, J., C. P. Leo, S. Y. Hsu and A. J. Hsueh (2000). "MCL-1S, a splicing variant of the antiapoptotic BCL-2 family member MCL-1, encodes a proapoptotic protein possessing only the BH3 domain." The Jornal of Biological Chemistry **275**(33): 25255-25261.

Bailey, C. J. and R. C. Turner (1996). "Metformin." New England Journal of Medicine **334**(9): 574-579.

Balch, C. M., J. E. Gershenwald, S. J. Soong, J. F. Thompson, M. B. Atkins, D. R. Byrd, A. C. Buzaid, A. J. Cochran, D. G. Coit, S. Ding, A. M. Eggermont, K. T. Flaherty, P. A. Gimotty, J. M. Kirkwood, K. M. McMasters, M. C. Mihm, Jr., D. L. Morton, M. I. Ross, A. J. Sober and V. K. Sondak (2009). "Final version of 2009 AJCC melanoma staging and classification." J Clin Oncol **27**(36): 6199-6206.

Baldi, A., D. Lombardi, P. Russo, E. Palescandolo, A. De Luca, D. Santini, F. Baldi, L. Rossiello, M. L. Dell'Anna, A. Mastrofrancesco, V. Maresca, E. Flori, P. G. Natali, M. Picardo and M. G. Paggi (2005). "Ferritin contributes to melanoma progression by modulating cell growth and sensitivity to oxidative stress." Clinical Cancer Research **11**(9): 3175-3183.

Balmanno, K. and S. J. Cook (2009). "Tumour cell survival signalling by the ERK1/2 pathway." Cell Death Differ **16**: 368-377.

Barry, M. and R. C. Bleackley (2002). "Cytotoxic T lymphocytes: all roads lead to death." Nature Reviews of Immunology **2**(6): 401-409.

Behrends, C., M. E. Sowa, S. P. Gygi and J. W. Harper (2010). "Network organization of the human autophagy system." Nature **466**(7302): 68-76.

Ben Sahra, I., Y. Le Marchand-Brustel, J. F. Tanti and F. Bost (2010). "Metformin in cancer therapy: a new perspective for an old antidiabetic drug?" Molecular Cancer Therapeutics **9**(5): 1092-1099.

Berggård, T., S. Linse and P. James (2007). "Methods for the detection and analysis of protein-protein interactions." Proteomics **7**(16): 2833-2842.

Bertout, J. A., S. A. Patel and M. C. Simon (2008). "The impact of O2 availability on human cancer." Nature reviews. Cancer. **8**: 967-975.

Bevona, C., W. Goggins, T. Quinn, J. Fullerton and H. Tsao (2003). "Cutaneous melanomas associated with nevi." Arch Dermatol **139**: 1620-1624.

Bingle, C. D., R. W. Craig, B. M. Swales, V. Singleton, P. Zhou and M. K. Whyte (2000). "Exon skipping in Mcl-1 results in a bcl-2 homology domain 3 only gene product that promotes cell death." The Jornal of Biological Chemistry **275**(29): 22136-22146.

Blázquez, C., A. Carracedo, L. Barrado, P. J. Real, J. L. Fernández-Luna, G. Velasco, M. Malumbres and M. Guzmán (2006). "Cannabinoid receptors as novel targets for the treatment of melanoma." FASEB Journal **20**(14): 2633-2635.

Blázquez, C., M. L. Casanova, A. Planas, T. Gómez Del Pulgar, C. Villanueva, M. J. Fernández-Aceñero, J. Aragonés, J. W. Huffman, J. L. Jorcano and M. Guzmán (2003). "Inhibition of tumor angiogenesis by cannabinoids." FASEB Journal **17**(3): 529-531.

Blázquez, C., L. González-Feria, L. Alvarez, A. Haro, M. L. Casanova and M. Guzmán (2004). "Cannabinoids inhibit the vascular endothelial growth factor pathway in gliomas." Cancer Research **64**(16): 5617-5623.

Blommaart, E. F., U. Krause, J. P. Schellens, H. Vreeling-Sindelárová and A. J. Meijer (1997). "The phosphatidylinositol 3-kinase inhibitors wortmannin and LY294002 inhibit autophagy in isolated rat hepatocytes." European Journal of Biochemistry **243**(1-2): 240-246.

Boissy, R. E. (2003). "Melanosome transfer to and translocation in the keratinocyte." Exp Dermatol **12 Suppl 2**: 5-12.

Boisvert-Adamo, K. and A. E. Aplin (2008). "Mutant BRAF mediates resistance to anoikis via Bad and Bim." Oncogene **27**(23): 3301-3312.

Bortner, C. D., N. B. Oldenburg and J. A. Cidlowski (1995). "The role of DNA fragmentation in apoptosis." Trends in Cellular Biology **5**(1): 21-26.

Bos, J. L. (1989). "ras oncogenes in human cancer: a review." Cancer Research **49**(17): 4682-4689.

Boucher, M. J., J. Morisset, P. H. Vachon, J. C. Reed, J. Lainé and N. Rivard (2000). "MEK/ERK signaling pathway regulates the expression of Bcl-2, Bcl-X(L), and Mcl-1 and promotes survival of human pancreatic cancer cells." Journal of Cellular Biochemistry **79**(3): 355-369.

Boya, P. and G. Kroemer (2008). "Lysosomal membrane permeabilization in cell death." Oncogene **27**(50): 6434-6451.

Bratton, D. L., V. A. Fadok, D. A. Richter, J. M. Kailey, L. A. Guthrie and P. M. Henson (1997). "Appearance of phosphatidylserine on apoptotic cells requires calcium-mediated nonspecific flip-flop and is enhanced by loss of the aminophospholipid translocase." Journal of Biological Chemistry **272**(42): 26159-26165.

Bratton, D. L., V. A. Fadok, D. A. Richter, J. M. Kailey, L. A. Guthrie and P. M. Henson (1997). "Appearance of phosphatidylserine on apoptotic cells requires calcium-mediated nonspecific flip-flop and is enhanced by loss of the aminophospholipid translocase." Journal of Biological Chemistry **272**(42): 26159-26165.

Brignole, C., D. Marimpietri, F. Pastorino, B. Nico, D. Di Paolo, M. Cioni, F. Piccardi, M. Cilli, A. Pezzolo, M. V. Corrias, V. Pistoia, D. Ribatti, G. Pagnan and M. Ponzoni (2006). "Effect of bortezomib on human neuroblastoma cell growth, apoptosis, and angiogenesis." J Natl Cancer Inst **98**(16): 1142-1157.

Buzzai, M., R. G. Jones, R. K. Amaravadi, J. J. Lum, R. J. DeBerardinis, F. Zhao, B. Viollet and C. B. Thompson (2007). "Systemic treatment with the antidiabetic drug metformin selectively impairs p53-deficient tumor cell growth." Cancer Research **67**(14): 6745-6752.

Cai, J., J. Yang and D. P. Jones (1998). "Mitochondrial control of apoptosis: the role of cytochrome c." Biochimica et Biophysica Acta **1366**(1-2): 139-149.

Carew, J. S., C. M. Espitia, J. A. n. Esquivel, D. Mahalingam, K. R. Kelly, G. Reddy, F. J. Giles and S. T. Nawrocki (2011). "Lucanthone is a novel inhibitor of autophagy that induces cathepsin D-mediated apoptosis." Journal of Biological Chemistry **286**(6): 6602-6613.

Carracedo, A., M. Gironella, M. Lorente, S. Garcia, M. Guzman, G. Velasco and J. L. Iovanna (2006). "Cannabinoids induce apoptosis of pancreatic tumor cells via endoplasmic reticulum stress-related genes." Cancer Res **66**(13): 6748-6755.

Carracedo, A., M. Lorente, A. Egia, C. Blazquez, S. Garcia, V. Giroux, C. Malicet, R. Villuendas, M. Gironella, L. Gonzalez-Feria, M. A. Piris, J. L. Iovanna, M. Guzman and G. Velasco (2006). "The stress-regulated protein p8 mediates cannabinoid-induced apoptosis of tumor cells." Cancer Cell **9**(4): 301-312.

Cartlidge, R. A., G. R. Thomas, S. Cagnol, K. A. Jong, S. A. Molton, A. J. Finch and M. McMahon (2008). "Oncogenic BRAF(V600E) inhibits BIM expression to promote melanoma cell survival." Pigment Cell Melanoma Res **21**(5): 534-544.

Casanova, M. L., C. Blázquez, J. Martínez-Palacio, C. Villanueva, M. J. Fernández-Aceñero, J. W. Huffman, J. L. Jorcano and M. Guzmán (2003). "Inhibition of skin tumor growth and angiogenesis *in vivo* by activation of cannabinoid receptors." The Journal of Clinical Investigations **111**(1): 43-50.

Chalfant, C. E., K. Rathman, R. L. Pinkerman, R. E. Wood, L. M. Obeid, B. Ogretmen and Y. A. Hannun (2002). "De novo ceramide regulates the alternative splicing of caspase 9 and Bcl-x in A549 lung adenocarcinoma cells. Dependence on protein phosphatase-1." Journal of Biological Chemistry **227**(15): 12587-12595.

Chan, P. C., R. C. Sills, A. G. Braun, J. K. Haseman and J. R. Bucher (1996). "Toxicity and carcinogenicity of delta 9-tetrahydrocannabinol in Fischer rats and B6C3F1 mice." Fundamental and Applied Toxicology **30**(1): 109-117.

Chang, N. C., M. Nguyen, M. Germain and G. C. Shore (2010). "Antagonism of Beclin-1-dependent autophagy by BCL-2 at the endoplasmic reticulum requires NAF-1." EMBO J **29**(3): 606-618.

Chatr-Aryamontri, A., B. J. Breitkreutz, S. Heinicke, L. Boucher, A. Winter, C. Stark, J. Nixon, L. Ramage, N. Kolas, L. O'Donnell, T. Reguly, A. Breitkreutz, A. Sellam, D. Chen, C. Chang, J. Rust, M. Livstone, R. Oughtred, K. Dolinski and M. Tyers (2013). "The BioGRID interaction database: 2013 update." Nucleic Acids Research **41:**(D1): D816-823.

Chen, D., F. Gao, B. Li, H. Wang, Y. Xu, C. Zhu and G. Wang (2010). "Parkin mono-ubiquitinates Bcl-2 and regulates autophagy." Journal of Biological Chemistry **285**(49): 38214-38223.

Cheng, E. H., M. C. Wei, S. Weiler, R. A. Flavell, T. W. Mak, T. Lindsten and S. J. Korsmeyer (2001). "BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis." Molecular Cell **8**(3): 705-711.

Chicheportiche, Y., P. R. Bourdo, H. Xu, Y. M. Hsu, H. Scott, C. Hession, I. Garcia and J. L. Browning (1997). "TWEAK, a new secreted ligand in the tumor necrosis factor family that weakly induces apoptosis." Journal of Biological Chemistry **272**(51): 32401-32410.

Chin, L., G. Merlino and R. A. DePinho (1998). "Malignant melanoma: modern black plague and genetic black box." Genes Dev **12**(22): 3467-3481.

Chin, P. C. and S. R. D'Mello (2005). "Brain chemotherapy from the bench to the clinic: targeting neuronal survival with small molecule inhibitors of apoptosis." Frontiers in Bioscience **10**: 552-568.

Circu, M. L. and T. Y. Aw (2010). "Reactive oxygen species, cellular redox systems, and apoptosis." Free Radical Biology and Medicine **48**(6): 749-762.

Clark, S. L. J. (1957). "Cellular differentiation in the kidneys of newborn mice studies with the electron microscope." The Journal of Biophysiocal and Biochemical Cytology **3**: 349-362.

Cohen, G. M. (1997). "Caspases: the executioners of apoptosis." Biochemical Journal **326**(Pt 1): 1-16.

Corazzari, M., G. M. Fimia, P. Lovat and M. Piacentini (2013). "Why is autophagy important for melanoma? Molecular mechanisms and therapeutic implications." Seminars in Cancer Biology **In Press**.

Corazzari, M., G. M. Fimia and M. Piacentini (2012). "Dismantling the autophagic arsenal when it is time to die: concerted Ambra 1 degradation by caspases and calpains." Autophagy **8**(8): 1255-1257.

Corazzari, M., P. E. Lovat, J. L. Armstrong, G. M. Fimia, D. S. Hill, M. Birch-Machin, C. P. Redfern and M. Piacentini (2007). "Targeting homeostatic mechanisms of endoplasmic reticulum stress to increase susceptibility of cancer cells to fenretinide-induced apoptosis: the role of stress proteins ERdj5 and ERp57." Br J Cancer **96**(7): 1062-1071.

Costin, G. E. and V. J. Hearing (2007). "Human skin pigmentation: melanocytes modulate skin color in response to stress." Faseb J **21**(4): 976-994.

Criollo, A., M. Niso-Santano, S. A. Malik, M. Michaud, E. Morselli, G. Mariño, S. Lachkar, A. V. Arkhipenko, F. Harper, G. Pierron, J. C. Rain, J. Ninomiya-Tsuji, J. M. Fuentes, S. Lavandero, L. Galluzzi, M. C. Maiuri and G. Kroemer (2011). "Inhibition of autophagy by TAB2 and TAB3." EMBO J **30**(24): 4908-4920.

Dankort, D., D. P. Curley, R. A. Cartlidge, B. Nelson, A. N. Karnezis, W. E. Damsky, M. J. You, R. A. DePinho, M. McMahon and M. Bosenberg (2009). "Braf(V600E) cooperates with Pten loss to induce metastatic melanoma." Nature Genetics: 544-552.

Davies, H., G. R. Bignell, C. Cox, P. Stephens, S. Edkins, S. Clegg, J. Teague, H. Woffendin, M. J. Garnett, W. Bottomley, N. Davis, E. Dicks, R. Ewing, Y. Floyd, K. Gray, S. Hall, R. Hawes, J. Hughes, V. Kosmidou, A. Menzies, C. Mould, A. Parker, C. Stevens, S. Watt, S. Hooper, R. Wilson, H. Jayatilake, B. A. Gusterson, C. Cooper, J. Shipley, D. Hargrave, K. Pritchard-Jones, N. Maitland, G. Chenevix-Trench, G. J. Riggins, D. D. Bigner, G. Palmieri, A. Cossu, A. Flanagan, A. Nicholson, J. W. Ho, S. Y. Leung, S. T. Yuen, B. L. Weber, H. F. Seigler, T. L. Darrow, H. Paterson, R. Marais, C. J. Marshall, R. Wooster, M. R. Stratton and P. A. Futreal (2002). "Mutations of the BRAF gene in human cancer." Nature **417**(6892): 949-954.

De Duve, C. (1959). "Lysosomes, a new group of cytoplasmic particles. ." In: Hayashi T (ed). Subcellular Particles. **Ronald Press Co.: New York.**

Decuypere, J. P., B. Jan, P. J.B. and G. Bultynck (2012). "Regulation of the Autophagic Bcl-2/Beclin-1 Interaction." Cells **1**(3): 284-312.

Degtyarev, M., A. De Mazière, C. Orr, J. Lin, B. B. Lee, J. Y. Tien, W. W. Prior, S. van Dijk, H. Wu, D. C. Gray, D. P. Davis, H. M. Stern, L. J. Murray, K. P. Hoeflich, J. Klumperman, L. S. Friedman and K. Lin (2008). "Akt inhibition promotes autophagy and sensitizes PTEN-null tumors to lysosomotropic agents." The Journal of Cell Biology **183**(1): 101-116.

Denoyelle, C., G. Abou-Rjaily, V. Bezrookove, M. Verhaegen, T. M. Johnson, D. R. Fullen, J. N. Pointer, S. B. Gruber, L. D. Su, M. A. Nikiforov, R. J. Kaufman, B. C. Bastian and M. S. Soengas (2006). "Anti-oncogenic role of the endoplasmic reticulum differentially activated by mutations in the MAPK pathway." Nat Cell Biol **8**(10): 1053-1063.

Dhanasekaran, D. N. and E. P. Reddy (2008). "JNK signaling in apoptosis." Oncogene **27**(48): 624562-624551.

Dhomen, N., J. S. Reis-Filho, S. da Rocha Dias, R. Hayward, K. Savage, V. Delmas, L. Larue, C. Pritchard and R. Marais (2009). "Oncogenic Braf induces melanocyte senescence and melanoma in mice." Cancer Cell **15**: 294-303.

Di Bartolomeo, S., M. Corazzari, F. Nazio, S. Oliverio, G. Lisi, M. Antonioli, V. Pagliarini, S. Matteoni, C. Fuoco, L. Giunta, M. D'Amelio, R. Nardacci, A. Romagnoli, M. Piacentini, F. Cecconi and G. M. Fimia (2010). "The dynamic interaction of Ambra 1 with the dynein motor complex regulates mammalian autophagy." Journal of Cell Biology **191**(1): 155-168.

Djavaheri-Mergny, M., M. C. Maiuri and G. Kroemer (2010). "Cross talk between apoptosis and autophagy by caspase-mediated cleavage of Beclin-1." Oncogene **29**(12): 1717-1719.

Domina, A. M., J. A. Vrana, M. A. Gregory, S. R. Hann and R. W. Craig (2004). "Mcl-1 is phosphorylated in the PEST region and stabilized upon ERK activation in viable cells, and at additional sites with cytotoxic okadaic acid or taxol." Oncogene **23**(31): 5301-5315.

Dong, J., R. G. Phelps, R. Qiao, S. Yao, O. Benard, Z. Ronai and S. A. Aaronson (2003). "BRAF oncogenic mutations correlate with progression rather than initiation of human melanoma." Cancer Research **63**: 3883-3885.

Donohue, E., A. Tovey, A. W. Vogl, S. Arns, E. Sternberg, R. N. Young and M. Roberge (2011). "Inhibition of autophagosome formation by the benzoporphyrin derivative verteporfin." Journal of Biological Chemistry **286**(9): 7290-7300.

Du, C., M. Fang, Y. Li, L. Li and X. Wang (2000). "Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition." Cell **102**(1): 33-42.

Du, K., S. Herzig, R. N. Kulkarni and M. Montminy (2003). "TRB3: a tribbles homolog that inhibits Akt/PKB activation by insulin in liver." Science **300**(5625): 1574-1577.

Duntsch, C., M. K. Divi, T. Jones, Q. Zhou, M. Krishnamurthy, P. Boehm, G. Wood, A. Sills and B. M. n. Moore (2006). "Safety and efficacy of a novel cannabinoid chemotherapeutic, KM-233, for the treatment of high-grade glioma." Journal of Neuro-oncology **77**(2): 143-152.

Egger, M. E., J. S. Huang, W. Yin, K. M. McMasters and L. R. McNally (2013). "Inhibition of autophagy with chloroquine is effective in melanoma." The Journal of Surgical Research **doi: 10.1016/j.jss.2013.04.055**.

Elder, D. E. (1987). "Metastatic melanoma." In Pigment cell: pp. 182 Karger, Basel, Switzerland.

Ellis, R., J. Armstrong, N. Kirkham, T. Ness, S. Horswell and P. Lovat (2012). "p62: a novel diagnostic and prognostic biomarker of malignant melanoma." Journal of Investigative Dermatology **132**(S124-136).

Elmore, S. (2007). "Apoptosis: A Review of Programmed Cell Death." Toxicologic Pathology **35**(4): 495-516.

Emert-Sedlak, L., S. Shangary, A. Rabinovitz, M. B. Miranda, S. M. Delach and D. E. Johnson (2005). "Involvement of cathepsin D in chemotherapy-induced cytochrome c release, caspase activation, and cell death." Molecular Cancer Therapeutics **4**(5): 733-742.

Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A and N. S. (1998). "A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD." Nature **391**(6662): 43-50.

Erickson, C. A. and M. V. Reedy (1998). "Neural Crest Development: The Interplay between Morphogenesis and Cell Differentiation " Current Topics in Developmental Biology **40**: 177-209.

Erlich, S., L. Mizrachy, O. Segev, L. Lindenboim, O. Zmira, S. Adi-Harel, J. A. Hirsch, R. Stein and R. Pinkas-Kramarski (2007). "Differential interactions between Beclin-1 and Bcl-2 family members." Autophagy **3**(6): 561-568.

Esposti, M. D. (2002). "The roles of Bid." Apoptosis **7**(5): 433-440

Faivre, S., G. Kroemer and E. Raymond (2006). "Current development of mTOR inhibitors as anticancer agents." Nature reviews. Drug Discovery. **5**: 671-688.

Fan, Z., P. J. Beresford, D. Y. Oh, D. Zhang and Lieberman (2003). "Tumor suppressor NM23-H1 is a granzyme A-activated DNase during CTL-mediated apoptosis, and the nucleosome assembly protein SET is its inhibitor." Cell **112**(5): 659-672.

Feng, W., S. Huang, H. Wu and M. Zhang (2007). "Molecular basis of Bcl-xl's target recognition versatility revealed by the structure of Bcl-xl in complex with the BH3 domain of Beclin-1." Journal of Molecular Biology **372**(1): 223-235.

Fernandez, Y., M. Verhaegen, T. P. Miller, J. L. Rush, P. Steiner, A. W. Opipari, Jr., S. W. Lowe and M. S. Soengas (2005). "Differential regulation of noxa in normal melanocytes and melanoma cells by proteasome inhibition: therapeutic implications." Cancer Research **65**(14): 6294-6304.

Fimia, G. M., M. Corazzari, M. Antonioli and M. Piacentini (2012). "Ambra 1 at the crossroad between autophagy and cell death." Oncogene **doi: 10.1038**.

Fimia, G. M., S. Di Bartolomeo, M. Piacentini and F. Cecconi (2011). "Unleashing the Ambra 1-Beclin-1 complex from dynein chains: Ulk1 sets Ambra 1 free to induce autophagy." Autophagy **7**(1): 115-117.

Fimia, G. M., A. Stoykova, A. Romagnoli, L. Giunta, S. Di Bartolomeo, R. Nardacci, M. Corazzari, C. Fuoco, A. Ucar, P. Schwartz, P. Gruss, M. Piacentini, K. Chowdhury and F. Cecconi (2007). "Ambra 1 regulates autophagy and development of the nervous system." Nature **447**(7148): 1121-1125.

Flaherty, K. T., J. R. Infante, A. Daud, R. Gonzalez, R. F. Kefford, J. Sosman, O. Hamid, L. Schuchter, J. Cebon, N. Ibrahim, R. Kudchadkar, H. A. r. Burris, G. Falchook, A. Algazi, K. Lewis, G. V. Long, I. Puzanov, P. Lebowitz, A. Singh, S. Little, P. Sun, A. Allred, D. Ouellet, K. B. Kim, K. Patel and J. Weber (2012). "Combined BRAF and MEK inhibition in melanoma with BRAF V600 mutations." The New England Journal of Medicine **367**(18): 1694-1703.

Frémont S, G. A., Galloux M, Janvier K, Karess RE, Berlioz-Torrent C. (2013). "Beclin-1 is required for chromosome congression and proper outer kinetochore assembly." EMBO Reports **14**(4): 364-372.

Fribley, A., Q. Zeng and C. Y. Wang (2004). "Proteasome inhibitor PS-341 induces apoptosis through induction of endoplasmic reticulum stress-reactive oxygen species in head and neck squamous cell carcinoma cells." Mol Cell Biol **24**(22): 9695-9704.

Fribley, A. M., B. Evenchik, Q. Zeng, B. K. Park, J. Y. Guan, H. Zhang, T. J. Hale, M. S. Soengas, R. J. Kaufman and C.-Y. Wang (2006). "Proteasome inhibitor PS-341 induces apoptosis in cisplatin-resistant squamous cell carcinoma cells by induction of noxa." J Biol Chem **281**: 31440-31447.

Galiègue, S., S. Mary, J. Marchand, D. Dussossoy, D. Carrière, P. Carayon, M. Bouaboula, D. Shire, G. Le Fur and P. Casellas (1995). "Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations." European Journal of Biochemistry **232**(1): 54-61.

Galve-Roperh, I., C. Sánchez, M. L. Cortés, T. Gómez del Pulgar, M. Izquierdo and M. Guzmán (2000). "Anti-tumoral action of cannabinoids: involvement of sustained ceramide accumulation and extracellular signal-regulated kinase activation." Nature Medicine **6**(3): 313-319.

Ganley, I. G., D. H. Lam, J. Wang, X. Ding, S. Chen and X. Jiang (2009). "ULK1.ATG13.FIP200 Complex Mediates mTOR Signaling and Is Essential for Autophagy." J Biol Chem **284**: 12297-12305.

Garber, K. (2011). "Inducing indigestion: companies embrace autophagy inhibitors." Jounal National Cancer Institute **103**(9): 708-710.

Garnett, M. J. and R. Marais (2004). "Guilty as charged: BRAF is a human oncogene." Cancer Cell **6**: 313-319.

Garrido, C., L. Galluzzi, M. Brunet, P. E. Puig, C. Didelot and G. Kroemer (2006). "Mechanisms of cytochrome c release from mitochondria." Cell Death and Differentiation **13**(9): 1423-1433.

Gautrey, H. L. and A. J. Tyson-Capper (2012). "Regulation of Mcl-1 by SRSF1 and SRSF5 in cancer cells." PLoS One **7**(12): 1-12.

Gironella, M., M. Seux, M. J. Xie, C. Cano, R. Tomasini, J. Gommeaux, S. Garcia, J. Nowak, M. L. Yeung, K. T. Jeang, A. Chaix, L. Fazli, Y. Motoo, Q. Wang, P. Rocchi, A. Russo, M. Gleave, J. C. Dagorn, J. L. Iovanna, A. Carrier, M. J. Pébusque and N. J. Dusetti (2007). "Tumor protein 53-induced nuclear protein 1 expression is repressed by miR-155, and its restoration inhibits pancreatic tumor development." Proc Natl Acad Sci U S A. **104**(41): 16170-16175.

Glaumann, H. and J. Ahlberg (1987). "Comparison of different autophagic vacuoles with regard to ultrastructure, enzymatic composition, and degradation capacity--formation of crinosomes." Experimental and Molecular Pathology **47**(3): 346-362.

Glavy JS, K. A., Cristea IM, Berke IC, Boehmer T, Blobel G, Chait BT. (2007). "Cell-cycle-dependent phosphorylation of the nuclear pore Nup107-160 subcomplex." Proc Natl Acad Sci U S A **104**(10): 3811-3816.

Glunde, K., S. E. Guggino, M. Solaiyappan, A. P. Pathak, Y. Ichikawa and Z. M. Bhujwalla (2003). "Extracellular acidification alters lysosomal trafficking in human breast cancer cells." Neoplasia **533-545**(5): 6.

Goding, C. R. (2007). "Melanocytes: The new Black." The International Journal of Biochemistry and Cell Biology **39**: 275-279.

Gómez del Pulgar, T., G. Velasco, C. Sánchez, A. Haro and M. Guzmán (2002). "De novo-synthesized ceramide is involved in cannabinoid-induced apoptosis." Biochemical Journal **363**(Pt 1): 183-188.

Gommeaux, J., C. Cano, S. Garcia, M. Gironella, S. Pietri, M. Culcasi, M. J. Pébusque, B. Malissen, N. J. Dusetti, J. L. Iovanna and A. Carrier (2007). "Colitis and colitis-associated cancer are exacerbated in mice deficient for tumor protein 53-induced nuclear protein 1." Molecular Cell Biology **27**(6): 2215-2228.

Goncharov, I., L. Weiner and Z. Vogel (2005). "Delta9-tetrahydrocannabinol increases C6 glioma cell death produced by oxidative stress." Neuroscience **134**(2): 567-574.

Goping, I. S., M. Barry, P. Liston, T. Sawchuk, G. Constantinescu, K. M. Michalak, I. Shostak, D. L. Roberts, A. M. Hunter, R. Korneluk and R. C. Bleackley (2003). "Granzyme B-induced apoptosis requires both direct caspase activation and relief of caspase inhibition." Immunity **18**(3): 355-365.

Gozuacik, D. and A. Kimchi (2004). "Autophagy as a cell death and tumor suppressor mechanism." Oncogene **23**: 2891-2906.

Greene, L. M., D. P. Nolan, D. Regan-Komito, G. Campiani, D. C. Williams and D. M. Zisterer (2013). "Inhibition of late-stage autophagy synergistically enhances pyrrolo-1,5-benzoxazepine-6-induced apoptotic cell death in human colon cancer cells." International Journal of Oncology.

Greenhough, A., H. A. Patsos, A. C. Williams and C. Paraskeva (2007). "The cannabinoid delta(9)-tetrahydrocannabinol inhibits RAS-MAPK and PI3K-Akt survival signalling and induces BAD-mediated apoptosis in colorectal cancer cells." Int J Cancer **121**(10): 2172-2180.
Grichnik, J. M. (2008). "Melanoma, Nevogenesis, and Stem Cell Biology." Journal of Investigative Dermatology **128**: 2365-2380.

Grossman, D. and D. C. Altier (2001). "Drug resistance in melanoma: mechanisms, apoptosis, and new potential therapeutic targets." Cancer Metastasis Review **20**(1-2): 3-11.

Grotenhermen, F. (2003). "Pharmacokinetics and pharmacodynamics of cannabinoids." Clinical Pharmatokinetics **42**(4): 327-360.

Guicciardi, M. E., J. Deussing, H. Miyoshi, S. F. Bronk, P. A. Svingen, C. Peters, S. H. Kaufmann and G. J. Gores (2000). "Cathepsin B contributes to TNF-alpha-mediated hepatocyte apoptosis by promoting mitochondrial release of cytochrome c." Journal of Clinical Investigations **106**(9): 1127-1137.

Gustafsson, S. B., A. Wallenius, H. Zackrisson, D. Popova, L. Plym Forshell and S. O. Jacobsson (2013). "Effects of cannabinoids and related fatty acids upon the viability of P19 embryonal carcinoma cells." Archives of Toxicology **95**(2): 197-203.

Guzmán, M., M. J. Duarte, C. Blázquez, J. Ravina, M. C. Rosa, I. Galve-Roperh, C. Sánchez, G. Velasco and L. González-Feria (2006). "A pilot clinical study of Delta9-tetrahydrocannabinol in patients with recurrent glioblastoma multiforme." British Journal of Cancer.

Haering CH, S. D., Nishino T, Helmhart W, Nasmyth K, Löwe J. (2004). "Structure and stability of cohesin's Smc1-kleisin interaction." Molecular Cell **152**(6): 951-964.

Handerson, T. and J. M. Pawelek (2003). "Beta1,6-branched oligosaccharides and coarse vesicles: a common, pervasive phenotype in melanoma and other human cancers." Cancer Research **63**(17): 5363-5369.

Hannun, Y. A. (1996). "Functions of ceramide in coordinating cellular responses to stress." Science **274**(5294): 1855-1859.

Hay, N. and N. Sonenberg (2004). "Upstream and downstream of mTOR." Genes and development **18**: 1926-1945.

Hayashi-Nishino, M., N. Fujita, T. Noda, A. Yamaguchi, T. Yoshimori and A. Yamamoto (2009). "A subdomain of the endoplasmic reticulum forms a cradle for autophagosome formation." Nat Cell Biol **11**(12): 1433-1437.

Hemesath, T. J., E. R. Price, C. Takemoto, T. Badalian and D. E. Fisher (1998). "MAP kinase links the transcription factor Microphthalmia to c-Kit signalling in melanocytes." Nature **391**: 298-301.

Hengartner, M. O. (2000). "The biochemistry of apoptosis." Nature **407**(6805): 770-776.

Hill, D. S., S. Martin, J. L. Armstrong, R. Flockhart, J. J. Tonison, D. G. Simpson, M. A. Birch-Machin, C. P. Redfern and P. E. Lovat (2009). "Combining the endoplasmic reticulum stress-inducing agents bortezomib and fenretinide as a novel therapeutic strategy for metastatic melanoma." Clin Cancer Res **15**(4): 1192-1198.

Hiscutt, E. L., D. S. Hill, S. Martin, R. Kerr, A. Harbottle, M. Birch-Machin, C. P. Redfern, S. Fulda, J. L. Armstrong and P. E. Lovat (2010). "Targeting X-Linked Inhibitor of Apoptosis Protein to Increase the Efficacy of Endoplasmic Reticulum Stress-Induced Apoptosis for Melanoma Therapy." J Invest Dermatol **130**: 2250-2258.

Hocker, T. L., M. K. Singh and H. Tsao (2008). "Melanoma genetics and therapeutic approaches in the 21st century: moving from the benchside to the bedside." Journal of investigative Dermatology **128**(11): 2575-2595.

Hoshino, S., H. Miyazawa, T. Enomoto, F. Hanaoka, Y. Kikuchi, A. Kikuchi and M. Ui (1989). "A human homologue of the yeast GST1 gene codes for a GTP-binding protein and is expressed in a proliferation-dependent manner in mammalian cells." EMBO Journal **8**(12): 3807-3814.

Hsu, H., J. Xiong and D. V. Goeddel (1995). "The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation." Cell **81**(4): 495-504.

Hu, C. D., Y. Chinenov and T. K. Kerppola (2002). "Visualization of interactions among bZIP and Rel family proteins in living cells using bimolecular fluorescence complementation." Molecular Cell **9**(4): 789-798.

Hu, S., S. J. Snipas, C. Vincenz, G. Salvesen and V. M. Dixit (1998). "Caspase-14 is a novel developmentally regulated protease." Journal of Biological Chemistry **273**(45): 29648-29653.

Huang, H. M., C. J. Huang and J. J. Yen (2000). "Mcl-1 is a common target of stem cell factor and interleukin-5 for apoptosis prevention activity via MEK/MAPK and PI-3K/Akt pathways." Blood **96**(5): 1764-1771.

Igney, F. H. and P. H. Krammer (2002). "Death and anti-death: tumour resistance to apoptosis." Nature Reviews of Cancer **2**(4): 277-288.

Iida, A., M. Emi, R. Matsuoka, E. Hiratsuka, K. Okui, H. Ohashi, J. Inazawa, Y. Fukushima, T. Imai and Y. Nakamura (2000). "Identification of a gene disrupted by inv(11)(q13.5;q25) in a patient with left-right axis malformation." Human Gnentics **106**(3): 277-287.

Itakura, E., C. Kishi, K. Inoue and N. Mizushima (2008). "Beclin-1 Forms Two Distinct Phosphatidylinositol 3-Kinase Complexes with Mammalian Atg14 and UVRAG." Mol. Biol. Cell **19**(12): 5360-5372.

Itakura, E., C. Kishi, K. Inoue and N. Mizushima (2008). "Beclin-1 forms two distinct phosphatidylinositol 3-kinase complexes with mammalian Atg14 and UVRAG." Molecular Biology of the Cell **19**(12): 5360-5372.

Itakura, E. and N. Mizushima (2010). "Characterization of autophagosome formation site by a hierachical analysis of mammalian Atg proteins." Autophagy **6**(6): 764-776.

Ito, S. and K. Wakamatsu (2008). "Chemistry of mixed melanogenesis--pivotal roles of dopaquinone." Photochemistry and Photobiology **84**: 582-592.

Iversen, L. (2005). "Long-term effects of exposure to cannabis." Current Opinion in Pharmacology **5**(1): 69-72.

Izumi, H., T. Torigoe, H. Ishiguchi, H. Uramoto, Y. Yoshida, M. Tanabe, T. Ise, T. Murakami, T. Yoshida, M. Nomoto and K. Kohno (2003). "Cellular pH regulators: potentially promising molecular targets for cancer chemotherapy." Cancer Treatment Reviews **29**(6): 541-549.

Jackson, G., H. Einsele, P. Moreau and J. S. Miguel (2005). "Bortezomib, a novel proteasome inhibitor, in the treatment of hematologic malignancies." Cancer Treat Rev **31**(8): 591-602.

Jamil, S., R. Sobouti, P. Hojabrpour, M. Raj, J. Kast and V. Duronio (2005). "A proteolytic fragment of Mcl-1 exhibits nuclear localization and regulates cell growth by interaction with Cdk1." Biochemical Journal **387**(Pt 3): 659-667.

Jang, S. and M. B. Atkins (2013). "Which drug, and when, for patients with BRAF-mutant melanoma?" Lancet Oncology **14**(2): e60-69.

Johannessen, C. M., J. S. Boehm, S. Y. Kim, S. R. Thomas, L. Wardwell, L. A. Johnson, C. M. Emery, N. Stransky, A. P. Cogdill, J. Barretina, G. Caponigro, H. Hieronymus, R. R. Murray, K. Salehi-Ashtiani, D. E. Hill, M. Vidal, J. J. Zhao, X. Yang, O. Alkan, S. Kim, J. L. Harris, C. J. Wilson, V. E. Myer, P. M. Finan, D. E. Root, T. M. Roberts, T. Golub, K. T. Flaherty, R. Dummer, B. L. Weber, W. R. Sellers, R. Schlegel, J. A. Wargo, W. C. Hahn and L. A. Garraway (2010). "COT drives resistance to RAF inhibition through MAP kinase pathway reactivation." Nature **468**(7326): 968-972.

Johnson, E. E., J. H. Overmeyer, W. T. Gunning and W. A. Maltese (2006). "Johnson EE, Overmeyer JH, Gunning WT, Maltese WA." Journal of Cell Science **119**(Pt 7): 1219-1232.

Johnston PA, Grandis JR. (2011) “STAT3 signaling: anticancer strategies and challenges.” Mol Interv. **11**(1): 18-26.

Joza, N., S. A. Susin, E. Daugas, W. L. Stanford, S. K. Cho, C. Y. Li, T. Sasaki, A. J. Elia, H. Y. Cheng, L. Ravagnan, K. F. Ferri, N. Zamzami, A. Wakeham, R. Hakem, H. Yoshida, Y. Y. Kong, T. W. Mak, J. C. Zúñiga-Pflücker, G. Kroemer and J. M. Penninger (2001). "Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death." Nature **410**(6828): 549-554.

Jung, C. H., C. B. Jun, S.-H. Ro, Y.-M. Kim, N. M. Otto, J. Cao, M. Kundu and D.-H. Kim (2009). "ULK-Atg13-FIP200 Complexes Mediate mTOR Signaling to the Autophagy Machinery." Mol. Biol. Cell **20**(7): 1992-2003.

Kabeya, Y., N. Mizushima, T. Ueno, A. Yamamoto, T. Kirisako, T. Noda, E. Kominami, Y. Ohsumi and T. Yoshimori (2000). "LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing." Embo J **19**(21): 5720-5728.

Kang, S. J., S. Wang, K. Kuida and J. Yuan (2002). "Distinct downstream pathways of caspase-11 in regulating apoptosis and cytokine maturation during septic shock response." Cell Death and Differentiation **9**(10): 1115-1125.

Kasper, B., V. D'Hondt, P. Vereecken and A. Awada (2007). "Novel treatment strategies for malignant melanoma: a new beginning?" Critical Reviews in Oncology and Hematology. : 16-22.

Kenessey, I., B. Bánki, A. Márk, N. Varga, J. Tóvári, A. Ladányi, E. Rásó and J. Tímár (2012). "Revisiting CB1 receptor as drug target in human melanoma." Pathology Oncology Research **18**(4): 857-866.

Kerr, J. F., A. H. Wyllie and A. R. Currie (1972). "Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics." British Journal of Cancer **26**(4): 239-257.

Kim, W., E. J. Bennett, E. L. Huttlin, A. Guo, J. Li, A. Possemato, M. E. Sowa, R. Rad, J. Rush, M. J. Comb, J. W. Harper and S. P. Gygi (2011). "Systematic and quantitative assessment of the ubiquitin-modified proteome." Molecular Cell **44**(2): 325-340.

Kimura, T., Y. Takabatake, A. Takahashi and Y. Isaka (2013). "Chloroquine in cancer therapy: a double-edged sword of autophagy." Cancer Research **73**(1): 3-7.

Kischkel, F. C., S. Hellbardt, I. Behrmann, M. Germer, M. Pawlita, P. H. Krammer and M. E. Peter (1995). "Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor." EMBO journal **14**(22): 5579-5588.

Klinac, D., E. S. Gray, M. Millward and M. Ziman (2013). "Advances in personalized targeted treatment of metastatic melanoma and non-invasive tumor monitoring." Frontiers in Oncology **3**(54).

Klionsky, D. J. (2007). "Autophagy: from phenomenology to molecular understanding in less than a decade." Nat Rev Mol Cell Biol **8**: 931-937.

Knævelsrud H, Ahlquist T, Merok MA, Nesbakken A, Stenmark H, Lothe RA and S. A. (2010). "UVRAG mutations associated with microsatellite unstable colon cancer do not affect autophagy." Autophagy **6**(7): 863-870.

Koenig, U., L. Eckhart and E. Tschachler (2001). "Evidence that caspase-13 is not a human but a bovine gene." Biochemical and Biophysical Research Communications **285**(5): 1150-1154.

Kollias, N., R. M. Sayre, L. Zeise and M. R. Chedekel (1991). "Photoprotection by melanin." J Photochem Photobiol B **9**(2): 135-160.

Komatsu, M., S. Waguri, T. Ueno, J. Iwata, S. Murata, I. Tanida, J. Ezaki, N. Mizushima, Y. Ohsumi, Y. Uchiyama, E. Kominami, K. Tanaka and T. Chiba (2005). "Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice." The Jornal of Cell Biology **169**: 425-434.

Kops, G. J., Y. Kim, B. A. Weaver, Y. Mao, I. McLeod, J. R. r. Yates, M. Tagaya and D. W. Cleveland (2005). "ZW10 links mitotic checkpoint signaling to the structural kinetochore." Journal of Cell Biology **169**(1): 49-60.

Kothakota, S., T. Azuma, C. Reinhard, A. Kippel, J. Tang, K. Chu, T. J. MGarry, M. W. Kirschner, K. Koths, D. J. Kwiatkowski and L. T. Williams (1997). "Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis." Science **278**(5336): 294-298.

Kozopas, K. M., T. Yang, H. L. Buchan, P. Zhou and R. W. Craig (1993). "Mcl-1, a gene expressed in programmed myeloid cell differentiation, has sequence similarity to Bcl-2." Proceedings of the National Academy of Sciences **90**(8): 3516-3520.

Kroemer, G. and B. Levine (2008). "Autophagic cell death: the story of a misnomer." Nature reviews. Molecular Cell Biology **9**(12): 1004-1010.

Kuma, A., M. Hatano, M. Matsui, A. Yamamoto, H. Nakaya, T. Yoshimori, Y. Ohsumi, T. Tokuhisa and N. Mizushima (2004). "The role of autophagy during the early neonatal starvation period." Nature **432**: 1032-1036.

Lakhter, A. J., R. P. Sahu, Y. Sun, W. K. Kaufmann, E. J. Androphy, J. B. Travers and S. R. Naidu (2013). "Chloroquine Promotes Apoptosis in Melanoma Cells by Inhibiting BH3 Domain-Mediated PUMA Degradation." The Journal of Investigative Dermatology **doi: 10.1038/jid.2013.56**.

Land, E. J. and P. A. Riley (2000). "Spontaneous redox reactions of dopaquinone and the balance between the eumelanic and phaeomelanic pathways." Pigment Cell Res **13**(4): 273-277.

Lazova, R., V. Klump and J. Pawelek (2009). "Autophagy in cutaneous malignant melanoma." J Cutan Pathol **37**(2): 256-268.

Levy, J. M. and A. Thorburn (2011). "Targeting autophagy during cancer therapy to improve clinical outcomes." Pharmacology & Therapeutics **131**(1): 130-141.

Li, F., G. Ambrosini, E. Y. Chu, J. Plescia, S. Tognin, P. C. Marchisio and D. C. Altieri (1998). "Control of apoptosis and mitotic spindle checkpoint by survivin." Nature **396**(6711): 580-584.

Li, L., N. Soetandyo, Q. Wang and Y. Ye (2009). "The zinc finger protein A20 targets TRAF2 to the lysosomes for degradation." Biochemica et Biophysica Acta **1793**(2): 346-353.

Li, L. Y., X. Luo and X. Wang (2001). "Endonuclease G is an apoptotic DNase when released from mitochondria." Nature **412**(6842): 95-99.

Li, X., D. Wu, J. Shen, M. Zhou and Y. Lu (2013). "Rapamycin induces autophagy in the melanoma cell line M14 via regulation of the expression levels of Bcl-2 and Bax." 5 **1**: 167-172.

Liang, C., P. Feng, B. Ku, I. Dotan, D. Canaani, B. H. Oh and J. U. Jung (2006). "Autophagic and tumour suppressor activity of a novel Beclin-1-binding protein UVRAG." Nature Cell Biology **8**(7): 688-699.

Liang, C., P. Feng, B. Ku, B. Oh and J. U. Jung (2007). "UVRAG. A new palyer in autophagy and tumor cell growth." Autophagy **3**: 69-71.

Liang, C., J. S. Lee, K. S. Inn, M. U. Gack, Q. Li, E. A. Roberts, I. Vergne, V. Deretic, P. Feng, C. Akazawa and J. U. Jung (2008). "Beclin-1-binding UVRAG targets the class C Vps complex to coordinate autophagosome maturation and endocytic trafficking." Nature Cell Biology **10**: 776-787.

Liang, X. H., S. Jackson, M. Seaman, K. Brown, B. Kempkes, H. Hibshoosh and B. Levine (1999). "Induction of autophagy and inhibition of tumorigenesis by beclin-1." Nature **402**: 672-676.

Liang, X. H., L. K. Kleeman, H. H. Jiang, G. Gordon, J. E. Goldman, G. Berry, B. Herman and B. Levine (1998). "Protection against fatal Sindbis virus encephalitis by beclin, a novel Bcl-2-interacting protein." Journal of Virology **72**(11): 8586-8596.

Lin, J. Y. and D. E. Fisher (2007). "Melanocyte biology and skin pigmentation." Nature **445**(7130): 843-850.

Link, G., A. Pinson and C. Hershko (1993). "Iron loading of cultured cardiac myocytes modifies sarcolemmal structure and increases lysosomal fragility." The Journal of Laboratory and Clinical medicine **121**(1): 127-134.

Lister, J. A., C. P. Robertson, T. Lepage, S. L. Johnson and D. W. Raible (1999). "nacre encodes a zebrafish microphthalmia-related protein that regulates neural-crest-derived pigment cell fate." Development **126**: 3757-3767.

Liu, B., Z. Fan, S. M. Edgerton, X. S. Deng, I. N. Alimova, S. E. Lind and A. D. Thor (2009). "Metformin induces unique biological and molecular responses in triple negative breast cancer cells." Cell Cycle **8**(13): 2031-2040.

Liu F, Cao J, Wu J, Sullivan K, Shen J, Ryu B, Xu Z, Wei W, Cui R. (2013) "Stat3-targeted therapies overcome the acquired resistance to vemurafenib in melanomas." J Invest Dermatol **133**(8): 2041-9. doi: 10.1038/jid.2013.32.

Liu, H., Z. He, T. von Rütte, S. Yousefi, R. E. Hunger and H. U. Simon (2013). "Down-Regulation of Autophagy-Related Protein 5 (ATG5) Contributes to the Pathogenesis of Early-Stage Cutaneous Melanoma." Science Translational Medicine **5**(202): 202ra123.

Locksley, R. M., N. Killeen and M. J. Lenardo (2001). "The TNF and TNF receptor superfamilies: integrating mammalian biology." Cell **104**(4): 487-501.

Losada, A. and T. Hirano (2005). "Dynamic molecular linkers of the genome: the first decade of SMC proteins." Genes & Developmemt **19**(11): 1269-1287.

Lovat, P. E., M. Corazzari, J. L. Armstrong, S. Martin, V. Pagliarini, D. Hill, A. M. Brown, M. Piacentini, M. A. Birch-Machin and C. P. Redfern (2008). "Increasing melanoma cell death using inhibitors of protein disulfide isomerases to abrogate survival responses to endoplasmic reticulum stress." Cancer Res **68**(13): 5363-5369.

Lucci, A., T. Y. Han, Y. Y. Liu, A. E. Giuliano and M. C. Cabot (1999). "Modification of ceramide metabolism increases cancer cell sensitivity to cytotoxics." International Journal of Oncology **15**(3): 541-546.

Ma Z, L. M., Li K, Fu Y, Liu X, Yang D, Zhao Y, Zheng J, Sun B. (2013). "Knocking down SMC1A inhibits growth and leads to G2/M arrest in human glioma cells." Int J Clin Exp Pathol **6**(5): 862-869.

Maddodi, N., W. Huang, T. Havighurst, K. Kim, B. J. Longley and V. Setaluri (2010). "Induction of Autophagy and Inhibition of Melanoma Growth *In vitro* and *In vivo* by Hyperactivation of Oncogenic BRAF." J Invest Dermatol **In Press**.

Maiuri, M. C., A. Criollo, E. Tasdemir, J. M. Vicencio, N. Tajeddine, J. A. Hickman, O. Geneste and G. Kroemer (2007). "BH3-only proteins and BH3 mimetics induce autophagy by competitively disrupting the interaction between Beclin-1 and Bcl-2/Bcl-X(L)." Autophagy **3**(4): 374-376.

Mak, I. T. and W. B. Weglicki (1985). "Characterization of iron-mediated peroxidative injury in isolated hepatic lysosomes." Journal of Clinical Investigations **75**(1): 58-63.

Marcu, J. P., R. T. Christian, D. Lau, A. J. Zielinski, M. P. Horowitz, J. Lee, A. Pakdel, J. Allison, C. Limbad, D. H. Moore, G. L. Yount, P. Y. Desprez and S. D. McAllister (2010). "Cannabidiol enhances the inhibitory effects of delta9-tetrahydrocannabinol on human glioblastoma cell proliferation and survival." Molecular Cancer Therapeutics **9**(1): 180-189.

Mathew, R., C. M. Karp, B. Beaudoin, N. Vuong, G. Chen, H. Y. Chen, K. Bray, A. Reddy, G. Bhanot, C. Gelinas, R. S. Dipaola, V. Karantza-Wadsworth and E. White (2009). "Autophagy suppresses tumorigenesis through elimination of p62." Cell **137**(6): 1062-1075.

Matsunaga, K., T. Saitoh, K. Tabata, H. Omori, T. Satoh, N. Kurotori, I. Maejima, K. Shirahama-Noda, T. Ichimura, T. Isobe, S. Akira, T. Noda and T. Yoshimori (2009). "Two Beclin-1-binding proteins, Atg14L and Rubicon, reciprocally regulate autophagy at different stages." Nature Cell Biology **11**(4): 385-396.

Mavrikakis, M., S. Papazoglou, P. P. Sfikakis, G. Vaiopoulos and K. Rougas (1996). "Retinal toxicity in long term hydroxychloroquine treatment." Annals of the Rheumatic Diseases **55**(3): 187-189.

McDonnell, T. J., N. Deane, F. M. Platt, G. Nunez, U. Jaeger, J. P. McKearn and S. J. Korsmeyer (1989). "bcl-2-immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation." Cell **57**(1): 79-88.

McGill, G. G., M. Horstmann, H. R. Widlund, J. Du, G. Motyckova, E. K. Nishimura, Y. L. Lin, S. Ramaswamy, W. Avery, H. F. Ding, S. A. Jordan, I. J. Jackson, S. J. Korsmeyer, T. R. Golub and D. E. Fisher (2002). "Bcl-2 regulation by the melanocyte master regulator Mitf modulates lineage survival and melanoma cell viability." Cell **109(6)**: 707-719.

Michallet, M. C., F. Saltel, M. Flacher, J. P. Revillard and L. Genestier (2004). "Cathepsin-dependent apoptosis triggered by supraoptimal activation of T lymphocytes: a possible mechanism of high dose tolerance." Journal of Immunology **172**(9): 5405-5014.

Michaloglou, C., L. C. Vredeveld, M. S. Soengas, C. Denoyelle, T. Kuilman, C. M. van der Horst, D. M. Majoor, J. W. Shay, W. J. Mooi and D. S. Peeper (2005). "BRAFE600-associated senescence-like cell cycle arrest of human naevi." Nature **436**(7051): 720-724.

Miller, R. W. and C. S. Rabkin (1999). "Merkel cell carcinoma and melanoma: etiological similarities and differences." Cancer Epidemiology, Biomarkers & Prevention **8**: 153-158.

Mimeault, M., N. Pommery, N. Wattez, C. Bailly and J. P. Hénichart (2003). "Anti-proliferative and apoptotic effects of anandamide in human prostatic cancer cell lines: implication of epidermal growth factor receptor down-regulation and ceramide production." Prostate **56**(1): 1-12.

Miracco, C., G. Cevenini, A. Franch, P. Luzi, E. Cosci, V. Mourmouras, I. Monciatti, S. Mannucci, M. Biagioli, M. Toscano, D. Moretti, R. Lio and D. Massi (2010). "Beclin-1 and LC3 autophagic gene expression in cutaneous melanocytic lesions." Human Pathology **In Press**.

Mitra, K. and J. Lippincott-Schwartz (2010). "Analysis of mitochondrial dynamics and functions using imaging approaches." Current Protocols in Cellular Biology **Chapter 4** (Unit 4.25): 1-21.

Mizushima, N. (2005). "The pleiotropic role of autophagy: from protein metabolism to bactericide." Cell Death and Differentiation **12**.
Mizushima, N. (2007). "Autophagy: process and function." Genes and Development **21**: 2861-2873.

Mizushima, N., T. Yoshimori and B. Levine (2010). "Methods in mammalian autophagy research." Cell **140**(3): 313-326.

Muchmore, S. W., M. Sattler, H. Liang, R. P. Meadows, J. Harlan, Y. HS., D. Nettesheim, B. S. Chang, C. B. Thompson, S. L. Wong, S. L. Ng and S. W. Fesik (1996). "X-ray and NMR structure of human Bcl-xl, an inhibitor of programmed cell death." Nature **381**(6580): 335-341.

Nakagawa H, Koyama K, Murata Y, Morito M, Akiyama T, Nakamura Y (2000). “APCL, a central nervous system-specific homologue of adenomatous polyposis coli tumor suppressor, binds to p53-binding protein 2 and translocates it to the perinucleus.” Cancer Res. **60**(1): 101-5.

Nakagawa, T., H. Zhu, N. Morishima, E. Li, J. Xu, B. A. Yankner and J. Yuan (2000). "Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta." Nature **403**(6765): 98-103.

Nawrocki, S. T., J. S. Carew, M. S. Pino, R. A. Highshaw, K. Dunner, Jr., P. Huang, J. L. Abbruzzese and D. J. McConkey (2005). "Bortezomib sensitizes pancreatic cancer cells to endoplasmic reticulum stress-mediated apoptosis." Cancer Res **65**(24): 11658-11666.

Nazarian, R., H. Shi, Q. Wang, X. Kong, R. C. Koya, H. Lee, Z. Chen, M. K. Lee, N. Attar, H. Sazegar, T. Chodon, S. F. Nelson, G. McArthur, J. A. Sosman, A. Ribas and R. S. Lo (2010).

"Melanomas acquire resistance to BRAF(V600E) inhibition by RTK or NRAS upregulation." Nature **468**(7326): 973-977.

Nishimura, E. K., S. A. Jordan, H. Oshima, H. Yoshida, M. Osawa, M. Moriyama, I. J. Jackson, Y. Barrandon, Y. Miyachi and S. Nishikawa (2002). "Dominant role of the niche in melanocyte stem-cell fate determination." Nature **416**: 854-860.

Niu G, Bowman T, Huang M, Shivers S, Reintgen D, Daud A, Chang A, Kraker A, Jove R, Yu H. 2002 “Roles of activated Src and Stat3 signaling in melanoma tumor cell growth.” Oncogene **21**(46): 7001-7010

Niu G, Heller R, Catlett-Falcone R, Coppola D, Jaroszeski M, Dalton W, Jove R, Yu H. 1999 “Gene therapy with dominant-negative Stat3 suppresses growth of the murine melanoma B16 tumor in vivo.” Cancer Res. **59**(20): 5059-63.

Nomura, T. and N. Katunuma (2005). "Involvement of cathepsins in the invasion, metastasis and proliferation of cancer cells." The Journal of Medical Investigation **52**(1-2): 1-9.

Norbury, C. J. and I. D. Hickson (2001). "Cellular responses to DNA damage." Annual Reviews of Pharmacologcial Toxicology **41**: 367-401.

Nur-E-Kamal, A., S. R. Gross, Z. Pan, Z. Balklava, J. Ma and L. F. Liu (2004). "Nuclear translocation of cytochrome c during apoptosis." Journal of Biological Chemistry **279**(24): 24911-24914.

Nys, A., X. Aubert and C. De Duve (1949). "The distribution of glycogen in the liver of rabbits." Biochemical Journal **45**(3): 245.

Obara, K. and Y. Ohsumi (2011). "Atg14: a key player in orchestrating autophagy." International Journal of Cell Biology **2011**(713435).

Ogawa, Y., K. Tobinai, M. Ogura, K. Ando, T. Tsuchiya, Y. Kobayashi, T. Watanabe, D. Maruyama, Y. Morishima, Y. Kagami, H. Taji, H. Minami, K. Itoh, M. Nakata and T. Hotta (2008). "Phase I and II pharmacokinetic and pharmacodynamic study of the proteasome inhibitor bortezomib in Japanese patients with relapsed or refractory multiple myeloma." Cancer Sci **99**(1): 140-144.

Ogretmen, B. and Y. A. Hannun (2004). "Biologically active sphingolipids in cancer pathogenesis and treatment." National Reviews in Cancer **4**: 604-616.

Ohoka, N., S. Yoshii, T. Hattori, K. Onozaki and H. Hayashi (2005). "TRB3, a novel ER stress-inducible gene, is induced via ATF4-CHOP pathway and is involved in cell death." EMBO J **24**: 1243-1255.

Ohsumi, Y. (2001). "Molecular dissection of autophagy: two ubiquitin-like systems." Nature reviews. Molecular Cell Biology **2**(3): 211-216.

Ohsumi, Y. and N. Mizushima (2004). "Two ubiquitin-like conjugation systems essential for autophagy." Seminar in Cell and Developmental Biology **15**(2): 231-236.

Ono, K., S. O. Kim and J. Han (2003). "Susceptibility of lysosomes to rupture is a determinant for plasma membrane disruption in tumor necrosis factor alpha-induced cell death." Molecular Cellular Biology **23**(2): 665-676.

Papandreou, C. N., D. D. Daliani, D. Nix, H. Yang, T. Madden, X. Wang, C. S. Pien, R. E. Millikan, S. M. Tu, L. Pagliaro, J. Kim, J. Adams, P. Elliott, D. Esseltine, A. Petrusich, P. Dieringer, C. Perez and C. J. Logothetis (2004). "Phase I trial of the proteasome inhibitor bortezomib in patients with advanced solid tumors with observations in androgen-independent prostate cancer." J Clin Oncol **22**(11): 2108-2121.

Pardo, J., A. Bosque, R. Brehm, R. Wallich, J. Naval, A. Müllbacher, A. Anel and M. M. Simon (2004). "Apoptotic pathways are selectively activated by granzyme A and/or granzyme B in CTL-mediated target cell lysis." Journal of Cellular Biology **167**(3): 457-468.

Passeron, T., J. P. Lacour, M. Allegra, C. Ségalen, A. Deville, A. Thyss, D. Giacchero, J. P. Ortonne, C. Bertolotto, R. Ballotti and P. Bahadoran (2011). "Signalling and chemosensitivity assays in melanoma: is mutated status a prerequisite for targeted therapy?" Experimental Dermatology **20**(12): 1030-1032.

Pattingre, S. and B. Levine (2006). "Bcl-2 Inhibition of Autophagy: A New Route to Cancer?" Cancer Research **66**: 2885-2888.

Pattingre, S., A. Tassa, X. Qu, R. Garuti, X. H. Liang, N. Mizushima, M. Packer, M. D. Schneider and B. Levine (2005). "Bcl-2 antiapoptotic proteins inhibit Beclin-1-dependent autophagy." Cell **122**(6): 927-939.

Patton, E. E., H. R. Widlund, J. L. Kutok, K. R. Kopani, J. F. Amatruda, R. D. Murphey, S. Berghmans, E. A. Mayhall, D. Traver, C. D. Fletcher, J. C. Aster, S. R. Granter, A. T. Look, C. Lee, D. E. Fisher and L. I. Zon (2005). "BRAF mutations are sufficient to promote nevi formation and cooperate with p53 in the genesis of melanoma." Curr Biol **15**(3): 249-254.

Perciavalle, R. M., D. P. Stewart, B. Koss, J. Lynch, S. Milasta, M. Bathina, J. Temirov, M. M. Cleland, S. Pelletier, J. D. Schuetz, R. J. Youle, D. R. Green and J. T. Opferman (2012). "Anti-apoptotic Mcl-1 localizes to the mitochondrial matrix and couples mitochondrial fusion to respiration." Nature Cell Biology **14**(6): 575-583.

Periyasamy-Thandavan, S., M. Jiang, Q. Wei, R. Smith, X. M. Yin and Z. Dong (2008). "Autophagy is cytoprotective during cisplatin injury of renal proximal tubular cells." Kidney International **74**(5): 631-640.

Perry, D. K., J. Carton, A. K. Shah, F. Meredith, D. J. Uhlinger and Y. A. Hannun (2000). "Serine palmitoyltransferase regulates de novo ceramide generation during etoposide-induced apoptosis." Journal of Biological Chemistry **275**(12): 9078-9084.

Pertwee, R. G. and R. A. Ross (2002). "Cannabinoid receptors and their ligands." Prostaglandins Leukot Essent Fatty Acids **66**(2-3): 101-121.

Peter, M. E. and P. H. Krammer (1998). "Mechanisms of CD95 (APO-1/Fas)-mediated apoptosis." Current Opinions in Immunology **10**(5): 545-551.

Petiot, A., E. Ogier-Denis, E. F. Blommaart, A. J. Meijer and P. Codogno (2000). "Distinct classes of phosphatidylinositol 3'-kinases are involved in signaling pathways that control macroautophagy in HT-29 cells." The Journal of Biological Chemistry **275**(2): 992-998.

Platta, H. W., H. Abrahamsen, S. B. Thoresen and H. Stenmark (2012). "Nedd4-dependent lysine-11-linked polyubiquitination of the tumour suppressor Beclin-1." The Biochemical Journal **441**(1): 399-406.

Pollock, P. M., U. L. Harper, K. S. Hansen, L. M. Yudt, M. Stark, C. M. Robbins, T. Y. Moses, G. Hostetter, U. Wagner, J. Kakareka, G. Salem, T. Pohida, P. Heenan, P. Duray, O. Kallioniemi, N. K. Hayward, J. M. Trent and P. S. Meltzer (2003). "High frequency of BRAF mutations in nevi." Nat Genet **33**(1): 19-20.

Poole, B. and S. Ohkuma (1981). "Effect of weak bases on the intralysosomal pH in mouse peritoneal macrophages." Journal of Cell Biology **90**(3): 665-669.

Poulikakos, P. I., Y. Persaud, M. Janakiraman, X. Kong, C. Ng, G. Moriceau, H. Shi, M. Atefi, B. Titz, M. T. Gabay, M. Salto, K. B. Dahlman, M. Tadi, J. A. Wargo, K. T. Flaherty, M. C. Kelley, T. Misteli, P. B. Chapman, J. A. Sosman, T. G. Graeber, A. Ribas, R. S. Lo, N. Rosen and D. B. Solit (2011). "RAF inhibitor resistance is mediated by dimerization of aberrantly spliced BRAF(V600E)." Nature **480**(7377): 387-390.

Qin, N., M. P. Neeper, Y. Liu, T. L. Hutchinson, M. L. Lubin and C. M. Flores (2008). "TRPV2 is activated by cannabidiol and mediates CGRP release in cultured rat dorsal root ganglion neurons." Journal of Neuroscience **28**(24): 6231-6238.

Qu, X., J. Yu, G. Bhagat, N. Furuya, H. Hibshoosh, A. Troxel, J. Rosen, E. L. Eskelinen, N. Mizushima, Y. Ohsumi, G. Cattoretti and B. Levine (2003). "Promotion of tumorigenesis by heterozygous disruption of the Beclin-1 autophagy gene." J Clin Invest **112**(12): 1809-1820.

Rai, N. K., K. Tripathi, D. Sharma and V. K. Shukla (2005). "Apoptosis: a basic physiologic process in wound healing." International Journal of Lower Extremity Wounds. **4**(3): 138-144.

Reuland, S. N., N. B. Goldstein, K. A. Partyka, S. Smith, Y. Luo, M. Fujita, R. Gonzalez, K. Lewis, D. A. Norris and Y. G. Shellman (2012). "ABT-737 synergizes with Bortezomib to kill melanoma cells." Biology Open **1**(2): 92-100.

Richardson, P. G., C. Mitsiades, R. Schlossman, I. Ghobrial, T. Hideshima, N. Munshi and K. C. Anderson (2008). "Bortezomib in the front-line treatment of multiple myeloma." Expert Rev Anticancer Ther **8**(7): 1053-1072.

Rossi M, D. L. V., Munarriz E, Green DR, Liu YC, Vousden KH, Cesareni G, Melino G. (2005). "The ubiquitin-protein ligase Itch regulates p73 stability." The EMBO journal **24**(4): 836-848.

Roy, S. and J. Debnath (2010). "Autophagy and Tumourigenesis." Seminars in Immunopathology **32**(4): 383-396.

Rozanowska, M., T. Sarna, E. J. Land and T. T.G (1999). "Free radical scavenging properties of melanin interaction of eu- and pheo-melanin models with reducing and oxidising radicals." Free Radic Biol Med. **26**(5-6): 518-525.

Rubinstein, J. C., M. Sznol, A. C. Pavlick, S. Ariyan, E. Cheng, A. Bacchiocchi, H. M. Kluger, D. Narayan and R. Halaban (2010). "Incidence of the V600K mutation among melanoma patients with BRAF mutations, and potential therapeutic response to the specific BRAF inhibitor PLX4032." Journal of Translational Medicine **8**(67).

Rubio-Moscardo, F., D. Blesa, C. Mestre, R. Siebert, T. Balasas, A. Benito, A. Rosenwald, J. Climent, J. I. Martinez, M. Schilhabel, E. L. Karran, S. Gesk, M. Esteller, R. deLeeuw, L. M. Staudt, J. L. Fernandez-Luna, D. Pinkel, M. J. Dyer and J. A. Martinez-Climent (2005). "Characterization of 8p21.3 chromosomal deletions in B-cell lymphoma: TRAIL-R1 and TRAIL-R2 as candidate dosage-dependent tumor suppressor genes." Blood **106**(9): 3214-3222.

Ruiz, L., A. Miguel and I. Díaz-Laviada (1999). "Delta9-tetrahydrocannabinol induces apoptosis in human prostate PC-3 cells via a receptor-independent mechanism." FEBS Letters **458**(3): 400-404.

Ryter, S. W., H. P. Kim, A. Hoetzel, J. W. Park, K. Nakahira, X. Wang and A. M. Choi (2007). "Mechanisms of cell death in oxidative stress." Antioxidants and Redox Signalling **9**(1): 49-89.

Sakahira, H., M. Enari and S. Nagata (1998). "Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis." Nature **391**(6662): 96-99.

Salazar, M., A. Carracedo, I. J. Salanueva, S. Hernández-Tiedra, A. Egia, M. Lorente, P. Vázquez, S. Torres, J. L. Iovanna, M. Guzmán, P. Boya and G. Velasco (2009). "TRB3 links ER stress to autophagy in cannabinoid anti-tumoral action." Autophagy **5**: 1048-1049.

Salazar, M., A. Carracedo, I. J. Salanueva, S. Hernández-Tiedra, M. Lorente, A. Egia, P. Vázquez, C. Blázquez, S. Torres, S. García, J. Nowak, G. M. Fimia, M. Piacentini, F. Cecconi, P. P. Pandolfi, L. González-Feria, J. L. Iovanna, M. Guzmán, P. Boya and G. Velasco (2009). "Cannabinoid action induces autophagy-mediated cell death through stimulation of ER stress in human glioma cells." J Clin Invest **119**(5): 1359-1372.

Samuels-Lev Y, O'Connor DJ, Bergamaschi D, Trigiante G, Hsieh JK, Zhong S, Campargue I, Naumovski L, Crook T, Lu X (2001) “ASPP proteins specifically stimulate the apoptotic function of p53.” Mol Cell **8**(4): 781-794.

Sánchez, C., M. L. de Ceballos, T. Gomez del Pulgar, D. Rueda, C. Corbacho, G. Velasco, I. Galve-Roperh, J. W. Huffman, S. Ramón y Cajal and M. Guzmán (2001). "Inhibition of glioma growth *in vivo* by selective activation of the CB(2) cannabinoid receptor." Cancer Research **61**(15): 5784-5789.

Sánchez, C., I. Galve-Roperh, C. Canova, P. Brachet and M. Guzmán (1998). "Delta9-tetrahydrocannabinol induces apoptosis in C6 glioma cells." FEBS letters **436**(1): 6-10.

Santamaría, A., P. L. Fernández, X. Farré, P. Benedit, J. Reventós, J. Morote, R. Paciucci and T. M. Thomson (2003). "PTOV-1, a novel protein overexpressed in prostate cancer, shuttles between the cytoplasm and the nucleus and promotes entry into the S phase of the cell division cycle." The American Journal of Pathology **162**(3): 897-905.

Sarafian, T., C. Montes, A. Harui, S. R. Beedanagari, S. Kiertscher, R. Stripecke, D. Hossepian, C. Kitchen, R. Kern, J. Belperio and M. D. Roth (2008). "Clarifying CB2 receptor-dependent and independent effects of THC on human lung epithelial cells." Toxicology and Applied Parmacology **231**(3): 282-290.

Sarafian, T. A., S. Kouyoumjian, F. Khoshaghideh, D. P. Tashkin and M. D. Roth (2003). "Delta 9-tetrahydrocannabinol disrupts mitochondrial function and cell energetics." American Journal of Physiology and Lung Cell Molecular Physiology **284**(2): L298-306.

Sarbassov, D. D., D. A. Guertin, A. S.M. and D. M. Sabatini (2005). "Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex." Science **307**(5712): 1098-1101.

Sarfaraz, S., V. M. Adhami, D. N. Syed, F. Afaq and H. Mukhtar (2008). "Cannabinoids for cancer treatment: progress and promise." Cancer Research **68**(2): 339-342.

Sarfaraz, S., F. Afaq, V. M. Adhami and H. Mukhtar (2005). "Cannabinoid receptor as a novel target for the treatment of prostate cancer." Cancer Research **65**(5): 1635-1641.

Sarkisian, C. J., B. A. Keister, D. B. Stairs, R. B. Boxer, S. E. Moody and L. A. Chodosh (2007). "Dose-dependent oncogene-induced senescence *in vivo* and its evasion during mammary tumorigenesis." Nature Cell Biology **9**: 493-505.

Savill, J. and V. Fadok (2000). "Corpse clearance defines the meaning of cell death." Nature **407**(6805): 784-788.

Scarlatti, F., R. Maffei, I. Beau, P. Codogno and R. Ghidoni (2008). "Role of non-canonical Beclin-1-independent autophagy in cell death induced by resveratrol in human breast cancer cells." Cell Death and Differentiation **15**(8): 1318-1329.

Schimmer, A. D. (2004). "Inhibitor of apoptosis proteins: translating basic knowledge into clinical practice." Cancer Research **64**(20): 7183-7190.

Scuderi, M. R., G. Cantarella, M. Scollo, L. Lempereur, M. Palumbo, G. Saccani-Jotti and R. Bernardini (2011). "The antimitogenic effect of the cannabinoid receptor agonist WIN55212-2 on human melanoma cells is mediated by the membrane lipid raft." Cancer Letters **310**(2): 240-249.

Seillier, M., S. Peuget, O. Gayet, C. Gauthier, P. N'Guessan, M. Monte, A. Carrier, J. L. Iovanna and N. J. Dusetti (2012). "TP53INP1, a tumor suppressor, interacts with LC3 and ATG8-family proteins through the LC3-interacting region (LIR) and promotes autophagy-dependent cell death." Cell Death and Differentiation **19**(9): 1525-1535.

Senchenkov, A., D. A. Litvak and M. C. Cabot (2001). "Targeting ceramide metabolism--a strategy for overcoming drug resistance." Journal of the National Cancer Institute **93**(5): 347-357.

Sensi, M., G. Nicolini, C. Petti, I. Bersani, F. Lozupone, A. Molla, C. Vegetti, D. Nonaka, R. Mortarini, G. Parmiani, S. Fais and A. Anichini (2006). "Mutually exclusive NRASQ61R and BRAFV600E mutations at the single-cell level in the same human melanoma." Oncogene **25**(24): 3357-3364.

Sever, S., M. M. Altintas, S. R. Nankoe, C. C. Möller, D. Ko, C. Wei, J. Henderson, E. C. del Re, L. Hsing, A. Erickson, C. D. Cohen, M. Kretzler, D. Kerjaschki, A. Rudensky, B. Nikolic and J. Reiser (2007). "Proteolytic processing of dynamin by cytoplasmic cathepsin L is a mechanism for proteinuric kidney disease." Journal of Clinical Investigations **117**(8): 2095-2104.

Shah, M., A. Bhoumik, V. Goel, A. Dewing, W. Breitwieser, H. Kluger, S. Krajewski, M. Krajewska, J. Dehart, E. Lau, D. M. Kallenberg, H. Jeong, A. Eroshkin, D. C. Bennett, L. Chin, M. Bosenberg, N. Jones and Z. A. Ronai (2010). "A role for ATF2 in regulating MITF and melanoma development." PLoS Genetics **6**(12).

Shannon, P., A. Markiel, O. Ozier, N. S. Baliga, J. T. Wang, D. Ramage, N. Amin, B. Schwikowski and T. Ideker (2003). "Cytoscape: a software environment for integrated models of biomolecular interaction networks." Genome Research **13**(11): 2498-2504.

Shen, C., W. Wang, L. Tao, B. Liu, Z. Yang and H. Tao (2013). "Chloroquine blocks the autophagic process in cisplatin-resistant osteosarcoma cells by regulating the expression of p62/SQSTM1." Int J Mol Med **doi: 10.3892/ijmm.2013.1399**.

Shi, C. S. and J. H. Kehrl (2010). "TRAF6 and A20 regulate lysine 63-linked ubiquitination of Beclin-1 to control TLR4-induced autophagy." Science signaling **3**(123).

Shi, H., G. Moriceau, X. Kong, R. C. Koya, R. Nazarian, G. M. Pupo, A. Bacchiocchi, K. B. Dahlman, B. Chmielowski, J. A. Sosman, R. Halaban, R. F. Kefford, G. V. Long, A. Ribas and R. S. Lo (2012). "Pre-existing MEK1 exon 3 mutations in V600E/KBRAF melanomas do not confer resistance to BRAF inhibitors." Cancer Discovery **2**(5): 414-424.

Shieh, J. J., K. T. Liu, S. W. Huang, Y. J. Chen and T. Y. Hsieh (2009). "Modification of alternative splicing of Mcl-1 pre-mRNA using antisense morpholino oligonucleotides induces apoptosis in basal cell carcinoma cells." Journal of investigative dermatology **129**: 2497-2506.

Shimizu, S., T. Kanaseki, N. Mizushima, T. Mizuta, S. Arakawa-Kobayashi, C. B. Thompson and Y. Tsujimoto (2004). "Role of Bcl-2 family proteins in a non-apoptotic programmed cell death dependent on autophagy genes." Nature Cell Biology **6**(12): 1221-1228.

Shintani, T. and D. Klionsky (2004). "Autophagy in health and disease: a double-edged sword." Science **306**: 990–995.

Shrivastava, A., P. M. Kuzontkoski, J. E. Groopman and A. Prasad (2011). "Cannabidiol induces programmed cell death in breast cancer cells by coordinating the cross-talk between apoptosis and autophagy." Mol Cancer Ther **10**(7): 1161-1172.

Shrivastava, A., P. M. Kuzontkoski, J. E. Groopman and A. Prasad (2011). "Cannabidiol induces programmed cell death in breast cancer cells by coordinating the cross-talk between apoptosis and autophagy." Molecular Cancer Therapeutics **10**(7): 1161-1172.
Shrivastava, A., P. M. Kuzontkoski, J. E. Groopman and A. Prasad (2011). "Cannabidiol induces programmed cell death in breast cancer cells by coordinating the cross-talk between apoptosis and autophagy." Molecular Cancer Therapeutics **10**(7): 1161-1172.

Simonsen, A. and S. A. Tooze (2009). "Coordination of membrane events during autophagy by class III PI3-kinase complexes." Journal of Cellular Biology **186**(6): 773-782.

Sivridis, E., M. I. Koukourakis, S. E. Mendrinos, A. Karpouzis, A. Fiska, C. Kouskoukis and A. Giatromanolaki (2011). "Beclin-1 and LC3A expression in cutaneous malignant melanomas: a biphasic survival pattern for beclin-1." Melanoma Research **21(3)**: 188-195.

Slee, E. A., C. Adrain and S. J. Martin (2001). "Executioner caspases 3, 6 and 7 perform distinct non-redundant roles during the execution phase of apoptosis." Journal of Biological Chemistry **276**: 7320-7326.

Steingrimsson, E., N. G. Copeland and N. A. Jenkins (2004). "Melanocytes and the microphthalmia transcription network." Annual review of genetics **38**: 365-411.

Strappazzon, F., M. Vietri-Rudan, S. Campello, F. Nazio, F. Florenzano, G. M. Fimia, M. Piacentini, B. Levine and F. Cecconi (2011). "Mitochondrial Bcl-2 inhibits Ambra 1-induced autophagy." EMBO J **30**(7): 1195-1208.

Strum, J. C., G. W. Small, S. B. Pauig and L. W. Daniel (1994). "1-beta-D-Arabinofuranosylcytosine stimulates ceramide and diglyceride formation in HL-60 cells." Journal of Biological Chemistry **269**(22): 15493-15497.

Su, Y., K. I. Amiri, L. W. Horton, Y. Yu, G. D. Ayers, E. Koehler, M. C. Kelley, I. Puzanov, A. Richmond and J. A. Sosman (2010). "A phase I trial of bortezomib with temozolomide in patients with advanced melanoma: toxicities, antitumor effects, and modulation of therapeutic targets." Clin Cancer Res **16**(1): 348-357.

Suliman, A., A. Lam, R. Datta and R. K. Srivastava (2001). "Intracellular mechanisms of TRAIL: apoptosis through mitochondrial-dependent and -independent pathways." Oncogene **20**(17): 2122-2133.

Sullivan, R. J. and K. T. Flaherty (2013). "Resistance to BRAF-targeted therapy in melanoma." European Journal of Cancer **49**(6): 1297-1304.

Sun Q, W. W., Wong KN, Tan I, Zhong Q. (2010). "Rubicon controls endosome maturation as a Rab7 effector." Proc Natl Acad Sci U S A **107**(45): 19338-19343.

Swampillai, A. L., P. Salomoni and S. C. Short (2012). "The role of autophagy in clinical practice." Clinical Oncology **24**(6): 387-395.

Tang, L., V. A. Tron, J. C. Reed, K. J. Mah, M. Krajewska, G. Li, X. Zhou, V. C. Ho and M. J. Trotter (1998). "Expression of apoptosis regulators in cutaneous malignant melanoma." Clinical Cancer Research **4**: 1865-1871.

Tehrani, R., R. A. Ostrowski, R. Hariman and W. M. Jay (2008). "Ocular toxicity of hydroxychloroquine." Seminars in Opthalmology **23**(3): 201-209.
Thompson, J. F., R. A. Scolyer and R. F. Kefford (2005). "Cutaneous Melanoma." Lancet **365**: 687-701.

Tomic,T. Botton, T. Cerezo, M. Robert, G. Luciano, F. (2011). "Metformin inhibits melanoma development through autophagy and apoptosis mechanisms." Cell Death and Disease **2**(9).

Torres, S., M. Lorente, F. Rodríguez-Fornés, S. Hernández-Tiedra, M. Salazar, E. García-Taboada, J. Barcia, M. Guzmán and G. Velasco (2011). "A combined preclinical therapy of cannabinoids and temozolomide against glioma." Molecular Cancer Therapeutics **10**(1): 90-103.

Trapani, J. A. and M. J. Smyth (2002). "Functional significance of the perforin/granzyme cell death pathway." Nature Reviews of Immunology **2**(10): 735-747.

Tsujimoto, Y., L. R. Finger, J. Yunis, P. C. Nowell and C. M. Croce (1984). "Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation." Science **226**(4678): 1097-1099.

Tsujimoto, Y. and S. Shimizu (2000). "VDAC regulation by the Bcl-2 family of proteins." Cell Death and Differentiation **7**(12): 1174-1181.

Uong, A. and L. I. Zon (2009). "Melanocytes in Development and Cancer." Journal of Cell Physiology **222**: 38-41.

Vaccani, A., P. Massi, A. Colombo, T. Rubino and D. Parolaro (2005). "Cannabidiol inhibits human glioma cell migration through a cannabinoid receptor-independent mechanism." British Journal of Pharmacology **144**(8): 1032-1036.

van Engeland, M., L. J. Nieland, F. C. Ramaekers, B. Schutte and C. P. Reutelingsperger (1998). "Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure." Cytometry **31**(1): 1-9.

van Loo, G., M. van Gurp, D. B., S. M. Srinivasula, I. Rodriguez, E. S. Alnemri, K. Gevaert, J. Vandekerckhove, W. Declercq and P. Vandenabeele (2002). "The serine protease Omi/HtrA2 is released from mitochondria during apoptosis. Omi interacts with caspase-inhibitor XIAP and induces enhanced caspase activity." Cell Death and Differentiation **9**(1): 20-26.

Vance, K. W. and C. R. Goding (2004). "The transcription network regulating melanocyte development and melanoma." Pigment cell research **17**: 318-325.

Vaux, D. L. C., S. Adams, J.M. (1988). "Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells." Nature **335**(6189): 440-442.

Velasco, G., A. Carracedo, C. Blázquez, M. Lorente, T. Aguado, A. Haro, C. Sánchez, I. Galve-Roperh and M. Guzmán (2007). "Cannabinoids and gliomas." Molecular Neurobiology **36**(1): 60-67.

Voss, V., C. Senft, V. Lang, M. W. Ronellenfitsch, J. P. Steinbach, V. Seifert and D. Kögel (2010). "The pan-Bcl-2 inhibitor (-)-gossypol triggers autophagic cell death in malignant glioma." Molecular Cancer Research **8**: 1002-1016.

Wagle, N., C. Emery, M. F. Berger, M. J. Davis, A. Sawyer, P. Pochanard, S. M. Kehoe, C. M. Johannessen, L. E. Macconaill, W. C. Hahn, M. Meyerson and L. A. Garraway (2011). "Dissecting therapeutic resistance to RAF inhibition in melanoma by tumor genomic profiling." Journal of Clinical Oncology **29**(22): 3085-3096.

Wagner, S. A., P. Beli, B. T. Weinert, M. L. Nielsen, J. Cox, M. Mann and C. Choudhary (2011). "A proteome-wide, quantitative survey of *in vivo* ubiquitylation sites reveals widespread regulatory roles." Molecular and Cellular Proteomics **10**(10).

Wang, H., X. Hu, X. Ding, Z. Dou, Z. Yang, A. W. Shaw, M. Teng, D. W. Cleveland, M. L. Goldberg, L. Niu and X. Yao (2004). "Human Zwint-1 specifies localization of Zeste White 10 to kinetochores and is essential for mitotic checkpoint signaling." Journal of Biological Chemistry **279**(52).

Wang, L. W., Z. S. Li, D. W. Zou, Z. D. Jin, J. Gao and G. M. Xu (2008). "Metformin induces apoptosis of pancreatic cancer cells." World Journal ofd Gasteroenterology **14**(47): 7192-7198.

Wang, P., S. M. Henning and D. Heber (2010). "Limitations of MTT and MTS-based assays for measurement of antiproliferative activity of green tea polyphenols." PloS One **5**(4): e10202.

Wang, S., H. Yu and J. K. Wickliffe (2011). "Limitation of the MTT and XTT assays for measuring cell viability due to superoxide formation induced by nano-scale TiO2." Toxicology *in vitro* **25**(8): 2147-2151.

White, E. and R. S. DiPaola (2009). "The double-edged sword of autophagy modulation in cancer." Clin Cancer Res **15**(17): 5308-5316.

Wirawan, E., L. Vande Walle, K. Kersse, S. Cornelis, S. Claerhout, I. Vanoverberghe, R. Roelandt, R. De Rycke, J. Verspurten, W. Declercq, P. Agostinis, T. Vanden Berghe, S. Lippens and P. Vandenabeele (2010). "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 and Disease **1**(e18): doi: 10.1038.

Wright, T. J., C. McKee, M. A. Birch-Machin, R. Ellis, J. L. Armstrong and P. E. Lovat (2013). "Increasing the therapeutic efficacy of docetaxel for cutaneous squamous cell carcinoma through the combined inhibition of phosphatidylinositol 3-kinase/Akt signalling and autophagy." Clinical Experimental Dermatology **38**(4): 421-423.

Wu, W. K., S. B. Coffelt, C. H. Cho, X. J. Wang, C. W. Lee, F. K. Chan, J. Yu and J. J. Sung (2012). "The autophagic paradox in cancer therapy." Oncogene **31**(8): 939-953.

Wullschleger, S., R. Loewith and M. N. Hall (2006). "TOR signaling in growth and metabolism." Cell **124**(3): 471-484.

Xiao, C., B. F. Yang, J. H. Song, H. Schulman, L. Li and C. Hao (2005). "Inhibition of CaMKII-mediated c-FLIP expression sensitizes malignant melanoma cells to TRAIL-induced apoptosis." Exp Cell Res **304**(1): 244-255.

Xu, C., J. Liu, L. C. Hsu, Y. Luo, R. Xiang and T. H. Chuang (2011). "Functional interaction of heat shock protein 90 and Beclin-1 modulates Toll-like receptor-mediated autophagy." FASEB Journal **25(8):2700-10**(8): 2700-2710.

Xu, X., Y. Liu, S. Huang, G. Liu, C. Xie, J. Zhou, W. Fan, Q. Li, Q. Wang, D. Zhong and X. Miao (2006). "Overexpression of cannabinoid receptors CB1 and CB2 correlates with improved prognosis of patients with hepatocellular carcinoma." Cancer Genetics and Cytogenetics **171**(1): 31-38.

Yamamoto, A., Y. Tagawa, T. Yoshimori, Y. Moriyama, R. Masaki and Y. Tashiro (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 Structure and function **23**(1): 33-42.

Yang, T., K. M. Kozopas and R. W. Craig (1995). "The intracellular distribution and pattern of expression of Mcl-1 overlap with, but are not identical to, those of Bcl-2." The Jornal of Cell Biology **128**(6): 1173-1184.

Yeung, N., M. S. Cline, A. Kuchinsky, M. E. Smoot and G. D. Bader (2008). "Exploring biological networks with Cytoscape software." Current Protocols in Bioinformatics **Chapter 8:Unit 8.13**.

Yin, D., H. Zhou, T. Kumagai, G. Liu, J. M. Ong, K. L. Black and H. P. Koeffler (2005). "Proteasome inhibitor PS-341 causes cell growth arrest and apoptosis in human glioblastoma multiforme (GBM)." Oncogene **24**(3): 344-354.

Yin, L., R. Stearns and B. González-Flecha (2005). "Lysosomal and mitochondrial pathways in H2O2-induced apoptosis of alveolar type II cells." Journal of Cellular Biochemistry **94**(3): 433-445.

Yoshimori, T., A. Yamamoto, Y. Moriyama, M. Futai and Y. Tashiro (1991). "Bafilomycin A1, a specific inhibitor of vacuolar-type H(+)-ATPase, inhibits acidification and protein degradation in lysosomes of cultured cells." Journal of Biological Chemistry **266**(26): 17707-17712.

Yu, L., A. Alva, H. Su, P. Dutt, E. Freundt, S. Welsh, E. H. Baehrecke and M. J. Lenardo (2004). "Regulation of an ATG7-Beclin-1 program of autophagic cell death by caspase-8." Science **304**(5676): 1500-1502.

Yue, Z., A. Horton, M. Bravin, P. L. DeJager, F. Selimi and N. Heintz (2002). "A novel protein complex linking the delta 2 glutamate receptor and autophagy: implications for neurodegeneration in lurcher mice." Neuron **35**(5): 921-933.

Yue, Z., S. Jin, C. Yang, A. J. Levine and N. Heintz (2003). "Beclin-1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor." Proc Natl Acad Sci U S A **100**(25): 15077-15082.

Al Zaid Siddiquee, K. Turkson, J. “STAT3 as a target for inducing apoptosis in solid and hematological tumors.” Cell Res. **18**(2): 254-67.

Zatloukal, K., S. W. French, C. Stumptner, P. Strnad, M. Harada, D. M. Toivola, M. Cadrin and M. B. Omary (2007). "From Mallory to Mallory-Denk bodies: what, how and why?" Experimental Cell Research **313**(10): 2033-2049.

Zhai, D., C. Jin, Z. Huang, A. C. Satterthwait and J. C. Reed (2008). "Differential regulation of Bax and Bak by anti-apoptotic Bcl-2 family proteins Bcl-B and Mcl-1." The Jornal of Biological Chemistry **283**: 9580-9586.

Zhang, H., M. Bosch-Marce, L. A. Shimoda, Y. S. Tan, J. H. Baek, J. B. Wesley, F. J. Gonzalez and G. L. Semenza (2008). "Mitochondrial autophagy is an HIF-1-dependent adaptive metabolic response to hypoxia." Journal of Biological Chemistry **283**: 10892-10903.

Zhang, J., H. J. Wen, Z. M. Guo, M. S. Zeng, M. Z. Li, Y. E. Jiang, X. G. He and C. Z. Sun (2011). "TRB3 overexpression due to endoplasmic reticulum stress inhibits Akt kinase activation of tongue squamous cell carcinoma." Oral Oncology **47**(10): 934-939.

Zhang YF, J. R., Li JD, Zhang XY, Zhao P, He M, Zhang HZ, Sun LP, Shi DL, Zhang GX, Sun M. (2013). "SMC1A knockdown induces growth suppression of human lung adenocarcinoma cells through G1/S cell cycle phase arrest and apoptosis pathways *in vitro*." Oncology Letters **5**(3): 749-755.

Zhao, Z., S. Oh, D. Li, D. Ni, S. D. Pirooz, J. H. Lee, S. Yang, J. Y. Lee, I. Ghozalli, V. Costanzo, J. M. Stark and C. Liang (2012). "A dual role for UVRAG in maintaining chromosomal stability independent of autophagy." Developmental Cell **22**(5): 1001-1016.

Zhao, Z., J. Yang, H. Zhao, X. Fang and H. Li (2012). "Cannabinoid receptor 2 is upregulated in melanoma." The Journal of Cancer Research and Therapeutics **8**(4): 549-554.

Zhong, Y., Q. J. Wang, X. Li, Y. Yan, J. M. Backer, B. T. Chait, N. Heintz and Z. Yue (2009). "Distinct regulation of autophagic activity by Atg14L and Rubicon associated with Beclin-1-phosphatidylinositol-3-kinase complex." Nature Cell Biology **11**(4): 468-476.

Zhou, X., J. Takatoh and F. Wang (2011). "The mammalian class 3 PI3K (PIK3C3) is required for early embryogenesis and cell proliferation." PloS One **6**(1): 1-10.

Zhuang, L., C. S. Lee, R. A. Scolyer, S. W. McCarthy, X. D. Zhang, J. F. Thompson and P. Hersey (2007). "Mcl-1, Bcl-xl and Stat3 expression are associated with progression of melanoma whereas Bcl-2, AP-2 and MITF levels decrease during progression of melanoma." Mod Pathol **20**(4): 416-426.

Published manuscripts, abstracts & submissions arising from this work

List of published manuscripts arising from this thesis

TJ Wright, **CS McKee**, MA Birch-Machin, R Ellis, JL Armstrong and PE Lovat (2013)
"Improving the therapeutic efficacy of docetaxel for cutaneous squamous cell carcinoma through the combined inhibition of PI3-K/Akt signalling and autophagy"
Clinical and Experimental Dermatology 2013 Jun; **38**(4):421-3

CS McKee, D Hill, C Redfern, J Armstrong, PE Lovat (2013)
"Oncogenic BRAF signalling increases Mcl-1 expression in cutaneous metastatic melanoma"
Experimental Dermatology Sep 30; doi: 10.1111/exd.12254.

List of published abstracts arising from this thesis

CS McKee, PE Lovat and JL Armstrongstrong
Role of Mcl-1 in the resistance of BRAF mutated Melanoma to autophagy
British Journal Dermatology 2011; 164(4): 936

RA Ellis, JL Armstrong, **CS McKee**, N Kirkham, T Ness, S Horswell, S Tooze and PE Lovat
Biomarkers of defective autophagy in melanoma
British Journal Dermatology 2011; 164(4): 936

CS McKee , G Velasco, JL Armstrong and PE Lovat
Cannabinoids hijack the autophagy pathway to promote Melanoma cell death
ECDO 2012 p120

CS McKee , G Velasco, JL Armstrong and PE Lovat
Cannabinoids hijack the autophagy pathway to promote Melanoma cell death
NEPG 2012