Cross talk between CD271 expressing tumour subpopulations and autophagy in the drug resistance, invasion and survival of cutaneous metastatic melanoma

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Abstract

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Abstract

Cutaneous malignant melanoma remains an increasing world health problem. Although targeted therapy to hyper-activated MAPK signalling has significantly increased survival for patients with BRAF/NRAS mutant melanomas, the development of acquired resistance is inevitable and associated with the emergence of chemo-resistant subpopulations expressing the neurotrophin receptor CD271. The relationship between drug-induced CD271 expressing subpopulations and autophagy however, remains undefined. The central aim of the present study was to define the relationship between CD271 (both constitutive and drug-induced) expression and autophagy, and the impact of CD271 inhibition or autophagy modulation on the potential re-sensitisation of BRAF mutant metastatic melanoma cells to MEK inhibition.

Semi-quantitative immuno-histochemical analysis (IHC) of CD271 expression in primary cutaneous melanomas of different AJCC stage, revealed a significant stepwise increase in expression in advanced, stage III melanomas, paralleled with biphasic expression of p62 and consistent with the paradoxical role of autophagy in cancer. Additionally, chemical inhibition of autophagy with chloroquine or a specific Vps34 inhibitor, selectively inhibited cell viability CD271 positive but not negative subpopulations, isolated from BRAF mutant melanoma cell line, collectively suggesting constitutive CD271 expression is associated with an increase in basal pro-survival autophagy.

Prolonged exposure of $BRAF^{V600E}$ mutant melanoma cells to the MEK 1/2 specific inhibitor trametinib resulted in increased CD271 expression, LC3-II accumulation and reduced p62 expression, again highlighting the relationship between CD271 expression and autophagy activation. Genetic or chemical inhibition of CD271 in trametinib-induced drug-resistant BRAF mutant melanoma subpopulations resulted in significant inhibition of cell viability and re-sensitisation to trametinib-induced apoptosis. Furthermore, Inhibition of autophagy with chloroquine/ Vps34 inhibitor also resulted in reduced colony forming capacity, invasion and the re-sensitization of CD271 expressing BRAF mutant subpopulations to the cytotoxic effects of trametinib *in vitro*, with Vps34 inhibition additionally promoting trametinib-induced death and the prevention of tumour invasion and dissemination of trametinib-induced drug resistant subpopulations in a zebra fish xenograft model.

Attempts to harness cytotoxic autophagy to overcome drug-induced resistance to trametinib additionally revealed treatment of drug-induced resistant CD271 expressing BRAF mutant melanoma subpopulations with 9-tetrahydrocannabinol (THC) resulted in significant inhibition of cell viability.

Collectively these data underpin the intimate relationship between trametinib-induced CD271 drug-resistant subpopulations and pro-survival autophagy and suggest the targeting of CD271 or indirect clinical modulation of autophagy (either through selective inhibition or the harnessing of its cytotoxic effects induced by THC) may provide valuable therapeutic strategies through which to overcome the resistance of BRAF/NRAS mutant metastatic melanoma to MEK inhibition.

Dedication

This work is dedicated to Foteini Alexopoulou

My mum who taught me to work hard and be dedicated, to be kind and caring and to never stop pursuing my dreams.

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This work has been jointly funded through Cancer Research UK, The North Eastern Research Skin Fund and the National Institute for Cancer Research.

My biggest thank you goes to my main supervisor, Professor Penny Lovat. Penny has always supported me, believing in my abilities and, more importantly, has come to be a true friend and mentor. She supported me during my transition from a research-naïve clinical dermatology registrar to a clinical academic and was always there to offer advice and support whenever I needed it. She sacrificed a lot of her precious personal and professional time to help me and every other member of her group, her 'family' as she always refers to us, and this will never be forgotten. I really appreciate all the hard work and time she has spent being there for me as a supervisor, and friend, and I hope we will be there for each other in the future. Thank you Penny!

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Doing my PhD in the Dermatological Sciences lab has been an amazing experience. I cannot be more grateful for the help and support I received from all the current and past students and post-docs in the lab. I would like to thank Dr Carole Todd who is the fountain of knowledge in the lab - I would have never found anything in the lab without you! I would like to also thank Dr Martina Ellias for all her help doing and analysing experiments, and, for her calming influence during times of panic! I would like to personally thank Dr Ashleigh McConnel who stood by me as a true friend and encouraged me to continue when I thought I was never going to make it! Ashleigh's friendship, humour and kindness made her friendship one of the most precious achievements during my research years. I hope we will have a lot more years of friendship to come. Thank you Ash!

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the past twelve years and I could have never done this PhD without you. I am genuinely so grateful to have you in my life. I love you so much!

Declaration

This thesis is submitted for the degree of Doctor of Philosophy at Newcastle University. The research was performed in the Department of Dermatological Sciences under the supervision of Professor Penny Lovat and Professor Ruth Plummer. 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.

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Abbreviations

µl - microlitre

µm – micrometer

µM – micromolar

˚C – Degrees Centigrade

1˚ - Primary

ABCB5 - ATP-binding cassette subfamily B member 5

ACTH – adrenocorticotropic hormone

Akt – Ak Thymoma

AJCC - American Joint Committee On Cancer

AML- Acute Myeloid Leukaemia

α-MSH - α-melanocyte stimulating hormone

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

ANOVA - Analysis of Variance

ARAF - V-Raf Murine Sarcoma Viral Oncogene Homolog A1

Atg - Autophagy Related Protein

BECN-1 - Beclin-1

BP - Base pair

BRAF - v-Raf Murine Sarcoma Viral Oncogene Homolog B1

BSA - Bovine Standard Albumin

cAMP - Cyclic adenosine monophosphate

CDK4 – Cyclin-Dependent kinase 4

CDKN2A - Cyclin-Dependent Kinase Inhibitor 2A

cDNA – Complementary DNA

CD271 – p75 NTR (Nerve Growth Factor Receptor)

CEACAM - Carcinoembryonic Antigen-Related Cell Adhesion Molecule

cm – Centimeter

CML – Chronic Myeloid Leukaemia

CNR1 - Cannabinoid Receptor Gene 1

CO_2 - Carbon dioxide

CRAF - v-Raf Murine Sarcoma Viral Oncogene Homolog C1

CREB - cAMP response element-binding protein

CSC – Cancer Stem Cells

Ct – Cycle Threashold

CTLA-4 - Cytotoxic T Lymphocyte Associated Antigen-4

Ctrl - Control

CQ - Chloroquine

DAPI - 4',6-diamidino-2-phenylindole

DMEM - Dulbecco's Modified Eagle's Medium

DMSO - Dimethyl Supfoxide

DNA - Deoxyribonucleic Acid

dNTP - Deoxyribonucleotide triphosphate

DOPA - Dihydroxyphenylalanine

EDTA - Ethylenediaminetetraacetic Acid

EGF - Epidermal Growth Factor

ELISA – Enzyme Linked Immunosorbent Assay

EMT - Epithelial to Mesenchymal Transition

ERK - Extracellular Signal-Regulated Kinase

FCS - Foetal Calf Serum

FDA - Food and Drug Administration

FFPE - Formalin-Fixed Paraffin-Embedded

FGF - Fibroblast Growth Factor

GAPDH - Glyceraldehyde 3-phosphate dehydrogenase

GM-CSF - Granulocyte-Macrophage Colony-Stimulating Factor

GNA11 - Guanine Nucleotide-Binding Protein Subunit Alpha-11

GNAQ - Guanine Nucleotide-Binding Protein Subunit Alpha- Q

GTP - Guanosine Triphosphate

HCl - Hydrochloric Acid

HCQ – Hydroxychloroquine

HGF/SF - Hepatocyte Growth Factor-Scatter Factor

HIF-1α - Hypoxia-Inducible Factor 1-Alpha

Hr - Hour

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

IB - Immunoblotting

IHC – Immunohistochemistry

IF – Immunofluorescence

ILP - Isolated limb Perfusion

INF- α - Interferon-Alpha

IL-2 – Interleukin-2

IGF - Insulin Growth Factor

IGF1R - Insulin-Like Growth Factor 1 (IGF-1) Receptor

JARID-1B - jumonji/ARID1 (JARID1) histone 3 K4 (H3K4)

JNK - Jun N-terminal Kinase

kDa – Kilodalton

KDR - Kinase Insert Domain Receptor

Ki67- Antigen KI-67

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

LC3 - Microtubule-Associated Protein Light Chain 3

LDH - Lactate Dehydrogenase

LN – Lymph Node

MAGE - M*elanoma*-Associated antigens

MAPK - Mitogen-Activated Protein Kinase

MART-1 - *Melanoma*-associated antigen recognized by T cells

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

MCAM - Melanoma Cell Adhesion Molecule

MCSF-1 - Macrophage Colony-Stimulating Factor

MEK - Mitogen Activated Protein Kinase Kinase

MET - MET proto-oncogene, receptor tyrosine kinase

MITF - Microphthalmia-Associated Transcription Factor

Min - Minutes

ml – Millilitre

MLPA - Multiple Ligation Probe Amplification

mM – Millimolar

MMP - Matrix Metalloproteinases

mRNA - Messenger Ribonucleic Acid

mTOR - Mammalian Target of Rapamycin

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

NaCl - Sodium Chloride

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

nM – Nanomolar

NGF – Nerve Growth Factor

NICE – The National Institute for Health and Care Excellence

NOD - Non-obese diabetic

NRAS - Neuroblastoma Rat Sarcoma Viral Oncogene Homolog

NT – Non-targeting

OS - Overall Survival

p53 - Protein 53

p62 - Nucleoporin p62

PAS - Pre-Autophagosome Structure

PBS - Phosphate Buffered Saline

PBS/T - Phosphate Buffered Saline and Tween

PCR - Polymerase Chain Reaction

PCD-1 - Programmed Cell Death Receptor-1

PDGF - Platelet-Derived Growth Factor

PDX – Patient Derived Xenograft

PFS- Progression Free Survival

PD-1 – Programmed Death cell protein 1

PD-L1 – Programmed Death Ligand-1

PDGFR - Platelet-Derived Growth Factor Receptor

PDGFRB - Platelet-Derived Growth Factor Receptor-β

PE – Phosphatidylethanolamine

PEDF - Pigment Epithelium-Derived Factor

PFS – Progression Free Survival

PI3K - Phosphatidylinositol 3-Kinase

PKA – Protein Kinase A

PLGF - Placental Like Growth Factor

PLX – PLX4720 (BRAF inhibitor)

PMA - Phorbol 12-myristate 13-acetate

POMC - Pro-Opiomelanocortin

P/S – Penicillin Streptomycin

PTEN - Phosphatase and Tensin Homolog Deleted on Chromosome 10

q-PCR - Quantitative Real-time Polymerase Chain Reaction

RAF - V-Raf Murine Sarcoma Viral Oncogene Homolog

RAG - Recombination Activating Gene

RAS - Rat Sarcoma

Rap – Rapamycin

Rb - Retinoblastoma

RGP – Radial Growth Phase

ROS - Reactive Oxygen Species

RTK – Receptor Tyrosine Kinase

RT-PCR – Reverse Transcription Polymerase Chain Reaction

SCID - Severe combined immunodeficiency

SD - Standard Deviation

SDS - Sodium Dodecyl Sulfate

SEM - Standard Error of the Mean

Shc - Src Homology 2 Domain Containing

shRNA - Short-Hairpin Ribonucleic Acid

siRNA - Short Interfering Ribonucleic Acid

SLNB - Sentinel Lymph Node Biopsy

SRS – Stereotactic Radiosurgery

STAT3 - Signal Transducer and Activator of Transcription 3

TAM – Tumour Associated Macrophage

Tram - Trametinib

T+PLX – Trametinib and PLX4720

T+CQ – Trametinib and Chloroquine

T+THC – Trametinib+ Tetrahydrocannabinol

TBS – Tris Buffered Saline

TERT - Telomerase Reverse Transcriptase

TRK - Tyrosine Receptor Kinase

TGF-β - Transforming Growth Factor β

THC – Tetrahydrocannabinol

TNF-α - Tumour Necrosis Factor-α

TNM – Tumour, Node, Metastasis Staging

TP53 – p53 Gene

TYR – Tyrosinase

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

UV – Ultraviolet

UVA – Ultraviolet A Radiation

UVB - Ultraviolet B Radiation

UVR – Ultraviolet Radiation

UVRAG - Ultraviolet Radiation Resistance-Associated Gene Protein

VEGF – Vascular Endothelial Growth Factor

VEGFR – Vascular Endothelial Growth Factor Receptor

Vps34 - Vacuolar Protein Sorting 34

VGP - Vertical Growth Phase

WT - Wild-Type

Chapter 1

Introduction

Chapter 1 Introduction

1.1 Melanocytes, melanogenesis and melanomagenesis

1.1.1 Melanocyte origin and melanogenesis

Melanocytes are an important evolutionary step in human development and play significant roles in both photo protection and providing pigmentation to skin, eyes and hair. Originating from neural crest stem cells, melanocytes are a heterogeneous group of cells that can be found in a variety of human structures such as the epidermis, hair, and iris but also in the inner ear, nervous system and heart (Brito and Kos, 2008). Melanocytes derive from pluripotent neural crest stem cells that arise from the dorsal neural crest, between the surface ectoderm and neural plate (Mackenzie *et al.*, 1997) before the melanocyte precursor, the unpigmented and undifferentiated melanoblast, migrate, proliferate and differentiate en route to their eventual destinations. In the skin, melanocytes reside at the basal layer of the epidermis and interact through their dendritic processes with 30-40 epidermal keratinocytes at a ratio of 1:10 (Park *et al.*, 2009). Contact between the melanocyte dendrites and epidermal keratinocytes enables the transfer of melanin into keratinocytes, determining the skin colour. In the skin, melanin has several properties; mainly photoprotection by UVR absorption and light scattering, a protective role from DNA damage by free radical de-activation and therefore preventing genomic instability (Park *et al.*, 2009). The skin is also the main barrier to the external environment and melanocytes provide, apart from photoprotection, thermoregulation by melanin prduction (Lin and Fisher, 2007). The degree of melanin production in each individual and their response to UV radiation, is one of the most useful predictors of skin cancer risk in the general population.

Melanogenesis, the production of melanin, is regulated by a number of genetic factors and cell signalling pathways. Melanogenesis is primarily regulated by the α-melanocyte stimulating hormone (α-MSH) and its receptor MC1R via the cAMP/PKA-signalling cascade (Kondo and Hearing, 2011). The pro-opiomelanocortin (POMC) gene regulates the production of α-MSH, adrenocorticotropic hormone (ACTH) and β-endorphin, all of which are inductors

of pigmentation (Kondo and Hearing, 2011). Upon α-MSH stimulation through MC1R, increasing levels of intracellular cAMP lead to activation of the transcription factor microphalmia associated transcription factor (MITF), which is essential for pigment synthesis and production (Gaggioli *et al.*, 2003). Melanin synthesis requires a number of enzymatic processes (Cichorek *et al.*, 2013) (Figure 1-1) with the final melanin products divided into two groups, pheomelanin and eumelanin. Whereas the ratio of pheomelanin to eumelanin determines skin pigmentation, pheomelanin itself does not determine skin pigmentation. Eumelanin with its better photo protecting properties provides higher resistance to UVR and ability to reactive oxygen species neutralization (Abdel-Malek *et al.*, 2010; Bertolotto, 2013), potentially explaining consistent results observed across different epidemiological studies that highlight malignant melanoma as a disease of primarily fair skinned individuals and that individuals with darker skin have a higher protective effect towards the harmful effects of UVR.

Melanin production is an important physiologic response to UV radiation. The skin produces a biphasic response to UVR with initial melanin production resulting from the redistribution of existing epidermal melanin reserve and a delayed response with an increase in melanin synthesis and transfer to keratinocytes, a result which is obvious within hours to days following UVR exposure (Park *et al.*, 2002).

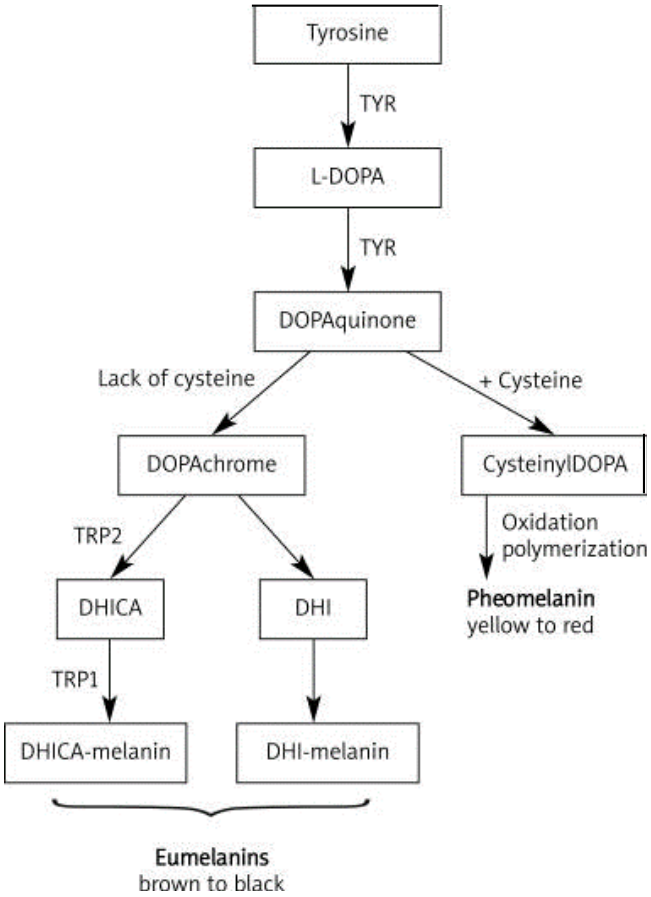


Figure 1-1 Schematic representation of melanin synthesis in melanocytes during melanogenesis

Enzymatic cascade leading to melanin production. (Cichorek *et al.*, 2013)

1.1.2 Melanomagenesis

Although the exact origin of malignant melanoma is not known, it is widely accepted multiple genetic and environmental factors contribute to the malignant transformation of melanocytes. The consecutive acquisition of genetic mutations has been proposed to contribute to the development of malignant melanoma from melanocytes though a stepwise process from melanocyte proliferation, immortalization and malignant transformation, expansion in a radial growth phase followed by vertical expansion to ultimately metastatic spread (Figure 1-2).

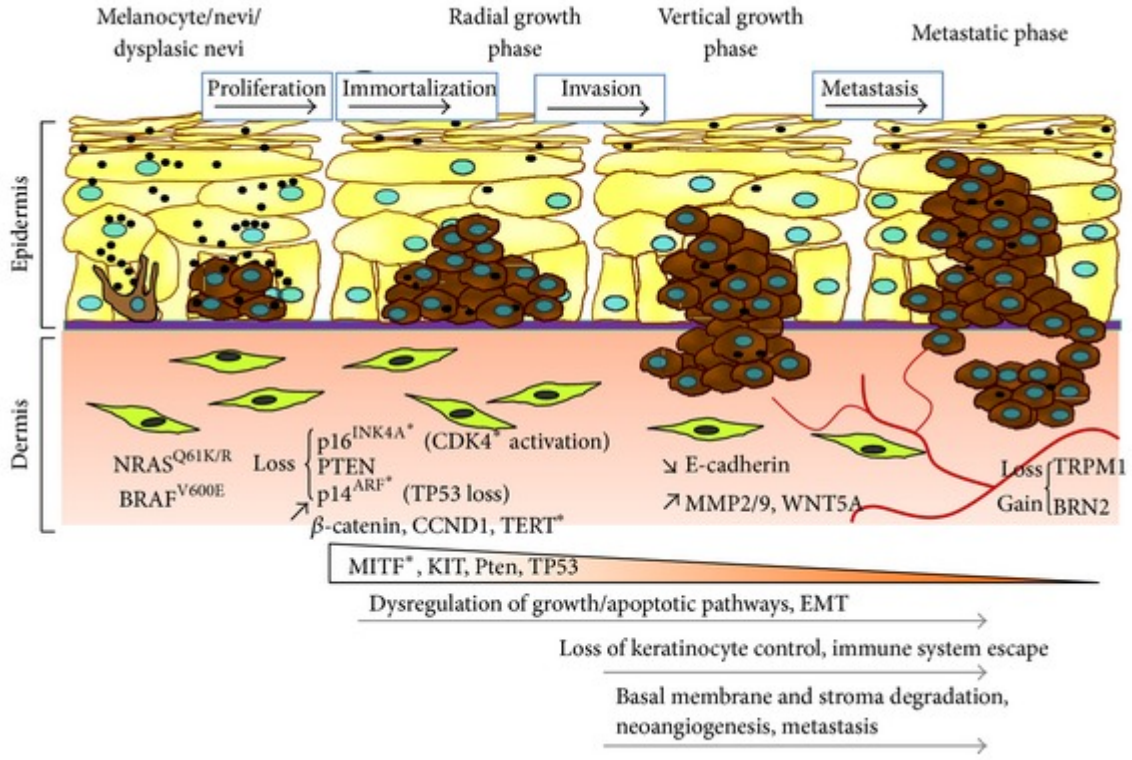


Figure 1-2: Hypothetical model of Melanoma development.
Proposed model for melanomagenesis in which melanomas arise from malignant transformation of melanocytes which proliferate and acquire malignant potential before they invade initially in a radial and then in a vertical fashion followed by metastatic spread. (Bertolotto, 2013)

Ultraviolet radiation (UVR) is associated with specific signature mutations directly affecting nucleotide base pairing in DNA (Wei *et al.*, 2003) and is therefore considered as one of the major risk factors for the development of a number of skin cancers including malignant melanoma. Short UV wavelengths are particularly harmful to the susceptible pyrimidine bases resulting in characteristic transition mutational changes, including for example, a TT→CC switch (Kanjilal *et al.*, 1993). Apart from a direct mutagenic effect of UVR on DNA, UVR may also indirectly cause melanocyte transformation via the production of reactive oxygen species (ROS). Nucleotides are extremely susceptible to free radical injury and oxidation of nucleotide bases promoting base mispairing leading to mutagenesis (Schulz *et al.*, 2000). Nevertheless linked to melanomagenesis, the development of melanoma however, is not always a result of UV-induced mutations, but can also result from germline mutations leading to familial melanoma, with the most extensively described mutations in the cyclin dependent kinase inhibitor 2A (CDKN2A), accounting for approximately 20-30% of all cases of familial melanoma. The CDKN2A tumour suppressor gene encodes two proteins, p16 (INK4a) and p14 (ARF) (Escamez *et al.*, 2013) and although germline mutations are strongly associated with melanoma, sporadic mutations may also occur. Familial melanoma is also associated with germline mutations in CDK4, the catalytic subunit of a protein kinase involved in controlling progression through the G1 phase of the cell cycle (Zuo *et al.*, 1996).

Another low penetrance gene extensively reported for its role as pigment regulator and its role in melanomagenesis is MC1R, where variants in the MC1R allele present in individuals with skin type I (Box *et al.*, 1997) are significantly more common in melanoma conferring a relative disease risk of 3.9 (Ichii-Jones *et al.*, 1998), and reinforcing the pivotal role of MC1R mutations in both pigmentation and melanoma.

The microphthalamia-associated transcription factor (MITF) is located to chromosome 3p12.3–14.1 in humans and is a key transcription factor in melanin biosynthesis. Mutations in MITF play key roles in a number of other processes including the survival, growth, and differentiation of melanocytes, retinal pigment epithelium, osteoclasts, and other hematopoietic lineages (Haq and Fisher, 2011). Although germline mutations in MITF are uncommon, the presence of an E318K variant is associated with multiple primary melanomas

and a family history of melanoma. The E318K variant corresponds to a gain of function associated with loss of a SUMOylation site at codon 318 (Miller *et al.*, 2005).

Further attempts to determine the mutational burden in cutaneous and other forms of malignant melanoma have also led to the discovery of activating mutations in cKIT most commonly affecting the region of the receptor with L576P in exon 11 (Beadling *et al.*, 2008), found in approximately 40% of mucosal and 35% of acral melanomas (Curtin *et al.*, 2006), and indicating different melanoma subtypes might harbour different biological and mechanistic tools to promote tumour growth.

Apart from a number of genetic factors described in familial melanoma, several deregulated signalling mechanisms have been implicated in melanoma development and progression. Abnormal activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway can result to a cascade of biological events, from cell growth and proliferation to survival and migration. In addition, the PI3K/AKT pathway is a crucial regulator of angiogenic pathways and therefore is a major contributor to tumour initiation, progression and metastasis (Carnero *et al.*, 2008). Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) is a phosphatidylinositol phosphate phosphatase that is frequently inactivated in human cancers. Selective activation of Akt3 promotes survival in 40–60% of non-familial melanoma (Stahl *et al.*, 2004) which contributes to the phenotypic plasticity of cancer cells by controlling Epithelial–mesenchymal transition (Larue and Bellacosa, 2005) and mediating tumour invasion. Interestingly, mutations in the tumour suppressor gene TP53 also play a role in melanomagenesis and tumour progression. TP53 gene encodes the transcription factor p53 which is activated in response to exogenous stresses including DNA damage or hypoxia, and regulates many genes involved in cell cycle regulation, cell growth and apoptosis (Vousden and Prives, 2009). Mutations of TP53 are found in approximately 50% of all human cancers; however in melanoma the incidence is lower with only 1–5% of primary melanomas harboring mutated p53 (Giglia-Mari and Sarasin, 2003). TP53 has also been associated with melanomagenesis and synergizes with mutated BRAF to promote UVR-mediated melanomagenesis (Viros *et al.*, 2014), however its role in spontaneous melanomas of non-sun exposed sites is enigmatic.

Strikingly, the most common deregulated signalling pathway in cutaneous melanoma is the Mitogen-activated protein kinase (MAPK) pathway, constitutively activated in up to 70% of all tumours by hyper activating mutations in the BRAF protein kinase, further details of which are more extensively described in 1.2.4.

1.2 **Cutaneous malignant melanoma**

1.2.1 Clinical characteristics of cutaneous malignant melanoma

Cutaneous melanoma is a significant world health problem with an increasing incidence (

Figure 1-3). Malignant melanoma affects people of all ages, sexes and ethnic groups and is one of the most common causes of cancer related deaths in young adults in the UK. Epidemiological data have recorded in excess of 230,000 new diagnoses of malignant melanoma worldwide (Organisation, 2015) with 2148 deaths from malignant melanoma documented in 2012 in the UK alone (*Cancer Research UK*, 2015).

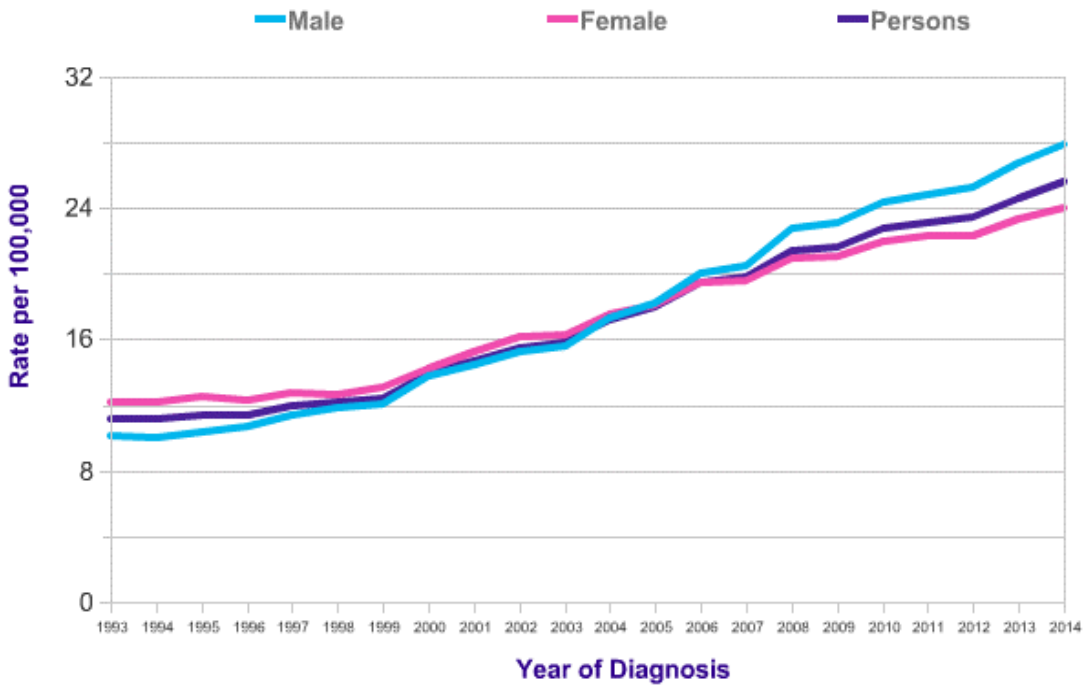
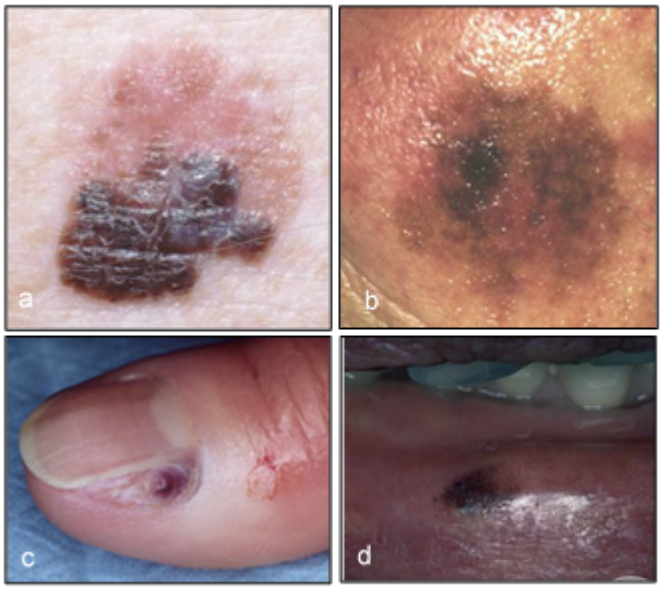


Figure 1-3: Cutaneous melanoma incidence trend in the UK
Incidence trend of cutaneous melanoma. Melanoma incidence rates have increased overall for all of the broad age groups in the UK. www.cancerresearchuk.org (*Cancer Research UK*, 2015)

Cutaneous malignant melanoma presents with different clinical characteristics and may arise either *de novo* or from a pre-existing naevus (Friedman and Rigel, 1985) and which is categorized according to anatomical location and histological characteristics (Figure 1-4). Clinically, melanoma presents as an irregular, growing and changing lesion which in the

majority of cases are pigment producing lesions but occasionally present as rare variants such as in the case of amelanotic melanomas (Cohen-Solal *et al.*, 2002) and which can be challenging to diagnose both clinically and histologically. Certain criteria have been developed to help both patients and clinicians in the diagnosis of malignant melanoma. The most widely used is the ABCDE criteria (Asymmetry, Border irregularity, Colour variation, Diameter >6 mm, Evolution) which is a simple method to raise suspicion of a malignant lesion and urge patients to seek medical advice (Abbasi *et al.*, 2004). Melanoma in situ is widely regarded as a pre-malignant lesion in which the atypical proliferation of melanocytes is confined to the dermo-epidermal junction without breaching the basement membrane (Tannous *et al.*, 2000) whereas melanomas become invasive when atypical melanocytes proliferate in a vertical plane and therefore invade through the basement membrane to the dermis and subcutaneous



fat (Smoller, 2006).

Figure 1-4: Clinical subtypes of malignant melanoma
Clinical subtypes of cutaneous malignant melanoma. a. cutaneous malignant melanoma, b. Lentigo maligna melanoma, c. subungual melanoma, d. mucosal melanoma

Histologically, melanoma development is characterized by three distinct phases; an *in situ*, a radial and subsequent vertical growth phase. Transformed melanocytes are thought to first proliferate above the basement membrane, followed by invasion to the papillary dermis (in situ and invasive radial growth phases of melanoma) (Guerry *et al.*, 1993). The radial growth

phase is characterized by enlargement of the tumour at its periphery. Subsequently cells proliferate in a vertical fashion, characterizing the vertical growth phase, initially reported as the development of a 'new' population of tumour cells within melanomas that grow in a perpendicular manor or radial growth phase (Clark *et al.*, 1984; Rodrigo Arrangoiz, 2016) (Figure 1-5).

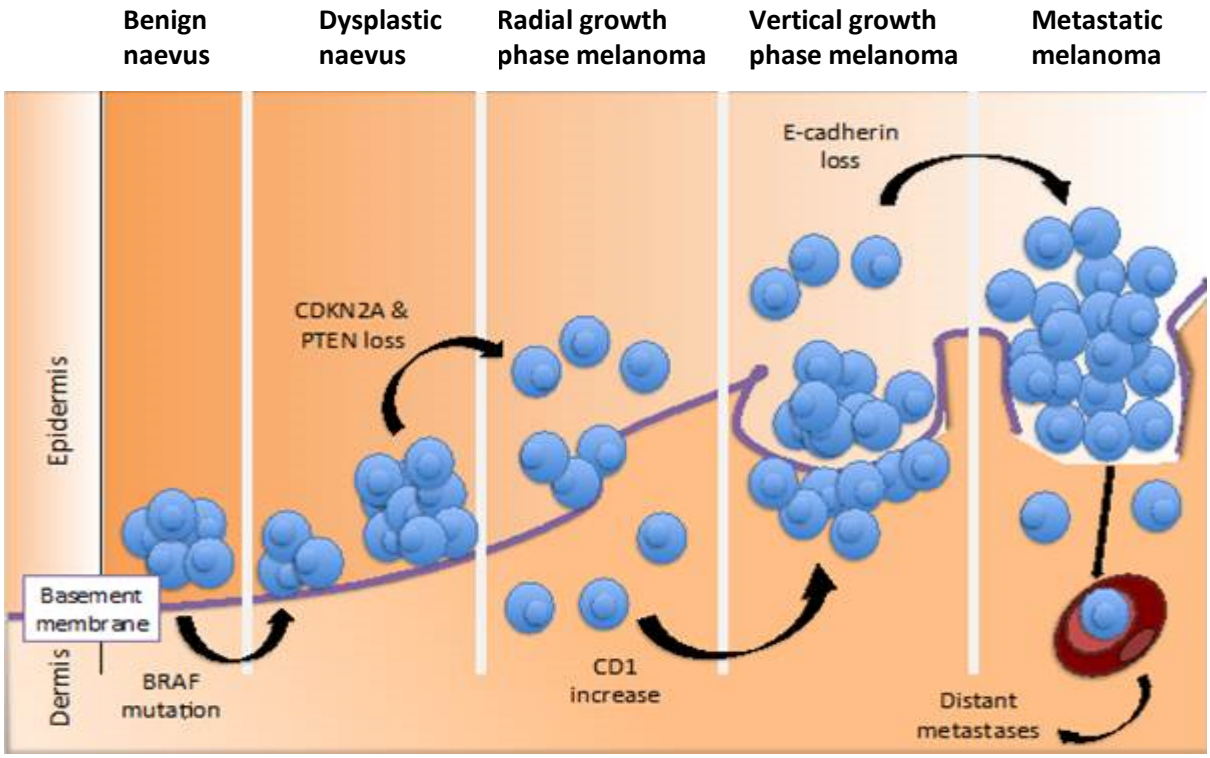


Figure 1-5: Proposed growth phases of malignant melanoma
Malignant melanoma develops from benign naevi through the accumulation of genetic mutation and the deregulation of cell signalling pathways. Initially tumour growth follows a radial growth phase, followed by a vertical growth phase before the detachment of malignant cells from the primary site and development of metastatic disease. (Rodrigo Arrangoiz, 2016)

The observation that thicker tumours are associated with poor prognosis led to the development of stratification methods to assess the risk of developing systemic disease. The first attempt to correlate tumour thickness with risk of metastasis was made by Clark (Clark *et al.*, 1969). Since then several studies (Barnhill *et al.*, 1996; Schuchter *et al.*, 1996) have highlighted Breslow thickness as a more sensitive prognostic biomarker for melanoma which remains an integral part of the current and generally accepted means of melanoma

classification according to the American Joint Committee on cancer (AJCC) staging criteria, further described in 1.2.3.

1.2.2 Melanoma risk factors

A large number of potential determinants or risk factors for malignant melanoma have been proposed. The strongest association that has been proposed in a number of epidemiological studies is the association between melanoma development and UVR exposure both in childhood and during adult life (Richards *et al.*, 2011; Lo and Fisher, 2014). Studies have suggested a consistent lower incidence of melanoma in residents of areas with low UV environment compared to residents in areas with high UV environment (Whiteman *et al.*, 2001). This could explain the significant higher incidence of malignant melanoma in the sub-tropical areas like Australia, where a fair skinned population is subjected to high environmental UVR for the vast majority of the year, making UVR the most important environmental factor associated with melanoma development (Marks, 2002). As discussed above, the presence of light hair, eyes and skin type I-II (easy burning history and poor ability to tan in response to sunlight) is also positively correlated with melanoma development (Gandini *et al.*, 2005). Other significant risk factors include the number of melanocytic naevi. The presence of >100 melanocytic lesions carries a relative risk of 7.6 compared to individuals with <10 melanocytic lesions with the distribution of actinic lentigines also associated with a higher incidence of malignant melanoma (Garbe *et al.*, 1994). In addition, a family history of melanoma increases the risk of developing malignant melanoma by almost 3 fold (Holman and Armstrong, 1984), reinforcing the theory of a potential genetic mediators in melanoma development. Again as discussed above, genetic signatures that have been associated with increased risk of familial melanoma include mutations in CDKN2A (Nobori *et al.*, 1994) and the melanocortin-1receptor (*MC1R*) gene, (Taylor *et al.*, 2015). Despite the recent increase in knowledge of melanoma identification and a significant increase in patient awareness about melanoma development and its association with UV exposure, melanoma however, remains the most deadly form of skin cancer and one of the major causes of death by cancer in young individuals (*Cancer Research UK*, 2015).

1.2.3 Prognosis and staging of malignant melanoma

Malignant melanoma carries a variable prognosis, which differs significantly according to disease stage for which The American Joint Committee on cancer (AJCC) staging criteria (revised in 2009) is the most universally accepted means of melanoma categorization and which provides a prognostic indicator according to specific disease parameters (Balch *et al.*, 2009a) (Table 1.1). Such parameters include 'Breslow' depth, the presence of ulceration as well as the degree of tumour spread to regional or distant metastatic sites. Specifically, the Breslow depth described by Alexander Breslow is a measure of tumour thickness and invasion, (Breslow, 1970) which alone has been strongly correlated to disease progression. The presence of ulceration (the loss of epidermal integrity overlying a melanoma) in a resected primary melanoma provides additional prognostic information, adversely correlating with outcome (Balch *et al.*, 2009a). However, it remains unclear whether ulceration represents the effect of an intrinsically more aggressive tumour on the epidermis, or whether any tumour that becomes ulcerated results in an increased likelihood of spread (Eggermont *et al.*). Primary melanoma proliferation as defined by the mitotic rate is also an independent prognostic biomarker incorporated into AJCC staging where an increased rate correlates with decreased survival and constitutes the second strongest predictor of survival following tumour thickness. A large number of mitotic cells indicates high metabolic cell activity and implies the ability of these tumours to enlarge and potentially metastasize (Francken *et al.*, 2004) (Table 1-1).

AJCC stage	Disease characteristics	5-year survival (%)
IA	Tumour ≤ 1mm, no ulceration	95
IB	Tumour ≤ 1mm, with ulceration or Tumour 1.01-2.0mm without ulceration	89-91
IIA	Tumour 1.01-2.0mm with ulceration or Tumour 2.01-4mm without ulceration	77-79
IIB	Tumour 2.01- 4.0mm with ulceration or tumour > 4mm without ulceration	63-67
IIC	Tumour > 4mm with ulceration	45
IIIA	Tumour of any thickness without ulceration and 1-3 positive lymph nodes with micrometastasis	63-70
IIIB	Tumour of any thickness with ulceration and 1-3 positive lymph nodes with micrometastasis, or Tumour of any thickness without ulceration and 1-3 positive lymph nodes with macrometastasis	46-59
IIIC	Tumour of any thickness with ulceration and 1-3 lymph nodes positive with macrometastasis, or tumour of any thickness and >4 involved lymph nodes	24-29
IV	Presence of any distant metastasis regardless of tumour thickness or status of regional lymph nodes	7-19

Table 1-1: AJCC stage and associated prognosis (Adapted from Balch et al (Balch et al., 2009b)

Histological characteristics of cutaneous melanomas according to the AJCC staging system and associated 5-year survival

Taking into consideration the above points the AJCC staging criteria are thus divided into stages, where by stage I-II is characterized by histological features and tumour size in the absence of any loco-regional metastatic disease, whereas stage III is characterized by the presence of nodal metastases and stage IV by the presence of distant metastatic sites. Nodal metastatic burden is further stratified by the presence of micro metastases or macro metastases and the number of involved lymph nodes (Table 1-2). Collectively indicating that the metastatic nodal burden also has a prognostic impact on patient survival (White et al., 2002).

N stage	Number of metastatic nodes
N0	0
N1	1
N2	2-3
N3	>4 metastatic nodes, or matted nodes, or in transit metastases/satellites with metastatic nodes

Table 1-2: Nodal classification of current AJCC staging system (Balch et al., 2009b)

Metastatic disease is sub-categorised into M1a which describes metastatic loci in distant skin, subcutaneous or nodal metastases, M1b in lung metastases and M1c characterised by the presence of any other visceral metastases (Balch *et al.*, 2009a). Finally, serum levels of lactate dehydrogenase (LDH) are also highlighted by the AJCC staging criteria, providing a surrogate marker of disease burden in patients with stage IV disease where elevated levels are correlated with reduced 1 and 2-year overall survival rates (Balch *et al.*, 2009a). The histological characteristics of melanoma included in the AJCC staging criteria are able to capture a large proportion of high risk tumours, however, there is still a number of seemingly 'low' risk tumours that still go on to metastasize and therefore combined efforts have concentrated in identifying novel mechanisms and biomarkers that can more accurately predict which tumours are innately more aggressive. As a result, the AJCC staging criteria for melanoma is under re-evaluation with the proposed 8th edition likely coming into clinical practice with effect from early 2018. The AJCC 8th edition will incorporate a significant change in melanoma staging which will include the classification of stage IA tumours if the tumour Breslow thickness is less than 0.8 mm (previously 1 mm in 7th AJCC edition, 2009) and with no ulceration whereas stage IB will be classified the tumours with Breslow thickness of 0.8-1.0 mm or <0.8 mm with ulceration (Amin *et al.*, 2017; Jeffrey E. Gershenwald, 2017). Additionally, the tumour mitotic rate will be removed from the classification of stage I tumours. Another

important change in the new AJCC staging criteria is the change in the nodal classification, in which 4 nodal classifications instead of 3 present are proposed. Additionally, the term micro metastasis will be replaced with the term 'clinically occult disease as detected by sentinel lymph node biopsy (SLNB)' (Jeffrey E. Gershenwald, 2017).

Another potential determinant of a patients' prognosis is the presence of lymph node metastases. Sentinel node biopsy is a minimally invasive staging procedure performed with the use of blue dye and radiolabelled particles and is used to identify the primary (i.e., sentinel) node or nodes containing melanoma cells and hence can be an early indicator of metastatic spread (Takeuchi and Kitagawa, 2012). Sentinel lymph node biopsy may give an early indication of melanoma metastases (Faries *et al.*, 2017) but their use is controversial and is not universally adopted.

Although the AJCC staging criteria provide a useful combination of both histological and clinical markers to inform prognosis, metastatic disease evolves with an extensive repertoire of deregulated cellular signalling and therefore further developments to aid stratification according to specific tumoural characteristics are of crucial importance to both improved prognosis and the development of more efficacious personalised therapies.

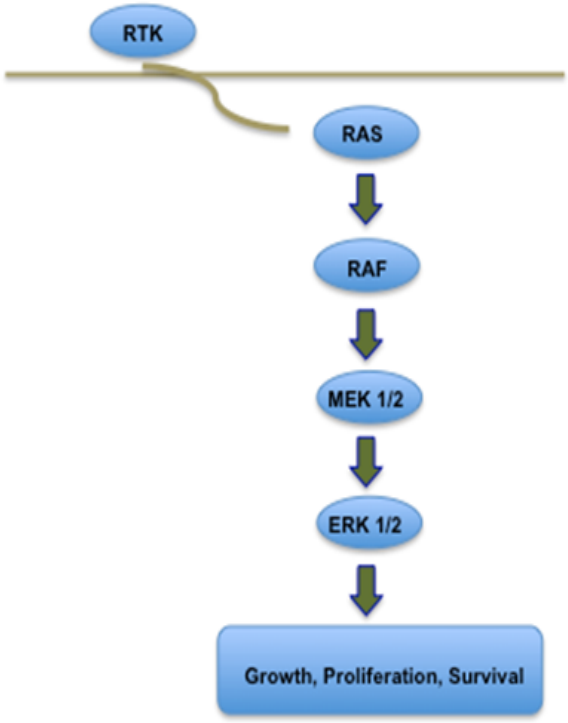
1.2.4 Oncogenic BRAF signalling

Perhaps the best characterized deregulated cellular signalling mechanism linked to the development of melanoma is the constitutive activation of the mitogen-activated protein kinase (MAPK) signalling pathway mediated by hyper activating mutations NRAS or BRAF. The MAPK (

Figure 1-6) is an important cellular signalling pathway linking extracellular signals to promote cell growth, proliferation, differentiation, migration and apoptosis (Dhillon *et al.*, 2007; Wellbrock and Arozarena, 2016). MAPK activation results from the stimulation of receptor tyrosine kinases (RTKs) activating the MAPK cascade in a multistep process. Several growth factors have been implicated into the activation of RTK like structures, such as membrane-bound peptide/protein hormones including nerve growth factor (NGF), platelet-derived growth factor (PDGF), fibroIntroblast growth factor (FGF), epidermal growth factor (EGF), and insulin (Margolis and Skolnik, 1994). RTKs include epidermal growth factor receptor (EGFR), platelet-derived growth factor receptors, fibroblast growth factor receptors (FGFRs), vascular endothelial growth factor receptors (VEGF), Met (hepatocyte growth factor/scatter factor (HGF/SF) (Sebolt-Leopold and Herrera, 2004). G-protein coupled receptors, activated mainly by the epidermal growth factor receptor (EGFR), activate Ras by triggering guanosine triphosphate (GTP) loading of the Ras GTPase (Pierce *et al.*, 2001). These then recruit Raf kinases to the plasma membrane, leading to sequential activation of MEK1/2 and phosphorylation of ERK1/2 (Dhillon *et al.*, 2007) and the translocation of activated ERK to the nucleus which subsequently activate transcription factors and alter gene expression to promote cell growth, differentiation or proliferation (Zhang and Liu, 2002) (Figure 1-6).

Activating mutations in NRAS are present in approximately 15-20% of cutaneous melanomas (Davies *et al.*, 2002). The most common mutations are found in codon 61, with a substitution of glutamine for lycine (Q61K) which represents approximately 48% of all NRAS mutations and the substitution of glutamine for arginine (Q61R) in approximately 36% (Ellerhorst *et al.*, 2011).

The Raf family comprise of serine/theonine kinases (C-Raf, A-Raf and B-Raf) that phosphorylate and activate the MEK1 and MEK2 dual-specificity protein kinases initiating the MAPK cascade (Schreck and Rapp, 2006). Approximately 60% of all cutaneous melanomas harbour a mutation in BRAF which leads to the constitutive activation of MAPK signalling and thus enhanced tumour growth and proliferation. A number of BRAF mutations have been reported in melanoma and of those 90% of observed mutations are located at codon 600 (Davies *et al.*, 2002) with 90 % being of a single nucleotide mutation resulting in substitution



of glutamic acid for valine ($BRAF^{V600E}$: nucleotide 1799 T > A; codon GTG > GAG). The second commonest mutation is $BRAF^{V600K}$ substituting lysine for valine, that represents 5-6 % (GTG > AAG), followed $BRAF^{V600D}$ (GTG > GAT) (Forbes *et al.*, 2001).

Figure 1-6: Schematic representation of the MAPK pathway
Activation of the MAPK pathway is a result of stimulation of receptor tyrosine kinases (RTK) activating a cascade of molecules leading to the final activation of ERK 1/2 and tumour cell growth and survival.

1.2.5 Current and novel treatment approaches for metastatic malignant melanoma

Although surgical excision is the treatment of choice for primary localized melanoma, the successful treatment of patient with metastatic disease remain in consistent, likely linked to the extensive range of molecular defences against immunological and cytotoxic attack, exerted by such tumours that render them notoriously unresponsive to conventional chemotherapy and which has led to a large body of research focused on the development of more specific and more efficacious targeted therapies and immunomodulatory treatments.

1.2.5.1 Chemotherapy

Prior to the instigation of novel targeted therapies and immunotherapy, metastatic disease was traditionally treated with standard chemotherapies which included and still does, the use of dacarbazine (Lens and Eisen, 2003), temozolomide (Middleton *et al.*, 2000), alone or in combination with other agents including cisplatin (Glover *et al.*, 1987) or fotemustine (Avril *et al.*, 2004). Limited response of 15% with the alkylating agent dacarbazine (Lui *et al.*, 2007) with few long-term clinical responders have led to the use of temozolomide, the oral analogue of dacarbazine which reportedly better penetrates the blood-brain barrier. However, the objective response rate of temozolomide is limited to 14% with a median overall survival of only 7.7 months (Middleton *et al.*, 2000) and hence other agents have been used to treat patients with metastatic melanoma including platinum analogues (cisplatin and carboplatin) (Glover *et al.*, 1987) and antimicrotubular agents such as paclitaxel and vinblastine (Rao *et al.*, 2006), all of which have had limited success as single agents. Nevertheless despite numerous trials of such agents alone or in combination which have to date, have not shown any significant effect on over all patient survival (Del Prete *et al.*, 1984; Glover *et al.*, 1987; Legha *et al.*, 1989; Margolin *et al.*, 1998), these agents are still used to treat patients who have failed treatment with targeted therapies or immunotherapy.

1.2.5.2 Radiation therapy

Radiation therapy (whole-brain irradiation and/or stereotactic radiosurgery) is also used in some metastatic melanoma treatment regimens and can be especially useful in patients with intracranial metastases, as most systemic therapies have limited penetration through the blood-brain barrier (Breneman *et al.*, 1997) (Rades *et al.*). In particular, stereotactic radiosurgery (SRS) has significantly improved clinical outcomes in patients with limited brain metastases (1-4 lesions) (Soliman *et al.*, 2016). However, in patients with extensive metastatic disease, WBRT is still used (Dyer *et al.*, 2014).

1.2.5.3 Immunotherapy

While most chemotherapy has a cytotoxic anti-cancer effect through causing DNA damage or manipulation of cell cycle and apoptosis, immunotherapies rely on augmentation of immune responses that naturally target cancer cells (Tjin *et al.*, 2011). Ipilimumab was the first immunotherapy to be FDA approved for the management of patients with metastatic melanoma (Mansh, 2011), which functions by targeting the Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4) receptor to enhance T-cell activity (Walunas *et al.*, 1994). Shown to me more beneficial than the melanoma vaccine gp100 , with an associated improved effect on patient overall survival (Hodi *et al.*, 2010), Ipilimumab has been used as first line treatment for patients with $BRAF^{WT}$ metastatic malignant melanoma with low volume disease until the recent introduction of anti-PD1 agents. PD-1 (programmed death-1) is an important immune checkpoint receptor present in activated T-cells. The associated PD-1 ligand (PDL-1) is expressed on the surface of cancer cells or stromal cells (Topalian *et al.*, 2012a) and the interaction between PD-1 and PDL-1 (Figure 1-7) inhibits T cell function and promotes immunosuppression. Inhibition of this interaction has been reported to enhance immune responses to cancer cells and improve antitumor immune activity (Pardoll, 2012; Melero *et al.*, 2013).

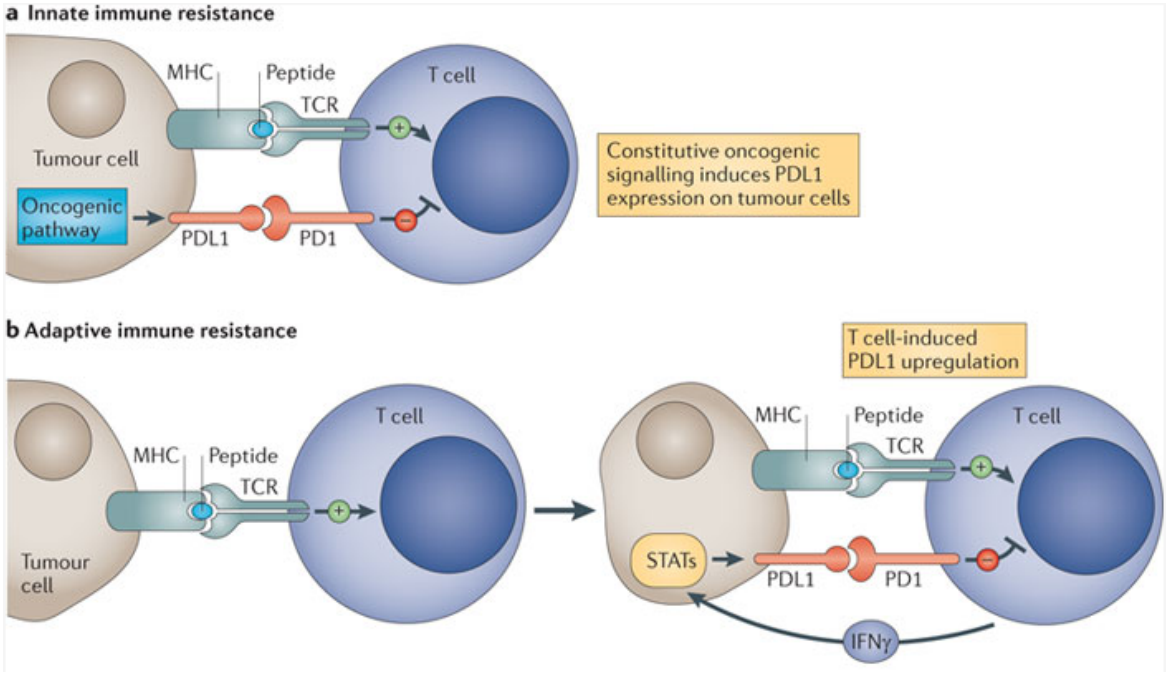


Figure 1-7: PD-1 and PDL-1 interaction (Taken from Pardoll et al)
A.Oncogenic signalling can upregulate PDL1 which upon interaction with PDL-1 expressed in the surface of T-cells results in inhibition of local antitumour T cell-mediated responses. B. PDL-1 expression can be induced by inflammatory signals, like interferon- γ (INF- γ) which is produced by tumour micro environmental factors like tumour infiltrating immune cells (Pardoll, 2012)

Clinically, there is accumulating evidence to support the use of PD-1 and PDL-1 inhibitors in patients with advanced melanoma with the use of PD-1 inhibitors shown to be superior to the use of Ipilimumab alone (Postow *et al.*; Robert *et al.*; Topalian *et al.*, 2012b; Hamid *et al.*, 2013). However, recent evidence suggests that combination immunotherapy with the PD-1 inhibitor nivolumab and ipimilumab show superior effects with an objective response rate of 61% in the combination group compared to 11% in patients treated with ipilimumab alone (Larkin *et al.*, 2015). Nevertheless, combination immunotherapy is associated with a number of adverse effects, including life-threatening autoimmune colitis observed in 44% of patients (Larkin *et al.*, 2015) and hepatitis in 17% of patients, which coupled with the observed development of acquired resistance in some patients limits clinical efficacy (Amaral and Garbe, 2016; Restifo *et al.*, 2016) and emphasises the continued need for more effective immunomodulatory therapies for metastatic melanoma.

1.2.5.4 Targeted therapies

Perhaps, the greatest advance in the management of malignant melanoma over the past decade has been the development of inhibitors to constitutively activated MAPK signalling, either with BRAF specific inhibitors, targeting $BRAF^{V600E}$ or through the use of MEK specific inhibitors to inhibit downstream MAPK activation (Stadler *et al.*, 2015),(Figure 1-8).

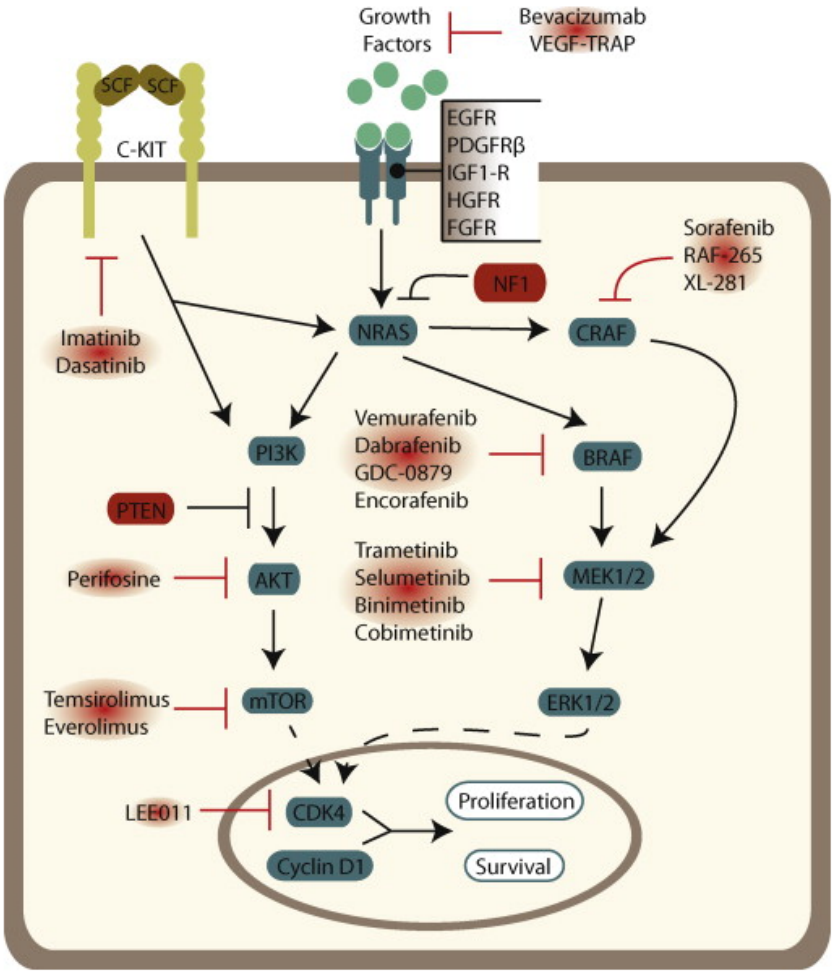


Figure 1-8: Signalling pathways mediating tumour cell growth and targeted therapeutic targets

Schematic representation of signalling pathways mediating melanoma cell growth and proliferation and potential targets. Taken from Stadler et al (Stadler *et al.*, 2015).

Vemurafenib is a potent and effective inhibitor of mutant B-RAF^{V600E} (Chapman *et al.*, 2011; Sullivan and Flaherty, 2013; Menzies and Long, 2014), demonstrating for the first time enhanced overall and progression free survival for patients bearing BRAF^{V600E} mutant melanomas (Chapman *et al.*, 2011). Dabrafenib, another BRAF specific inhibitor, is a reversible and potent ATP-competitive inhibitor that selectively inhibits the BRAF^{V600E} kinase (Laquerre *et al.*, 2009) and like Vemurafenib has been shown to be efficacious in improving progression free survival (5.1 months in the dabrafenib group vs 2.7 months in dacarbazine group) (Hauschild *et al.*, 2012) in patients with metastatic melanoma. However, although both vemurafenib and dabrafenib have revolutionized melanoma therapy, their efficacy is limited by the development of acquired resistance (Sosman *et al.*, 2012), and the resultant reactivation of MAPK signaling. In an attempt to overcome this effect, down-stream MEK inhibitors have been developed including the MEK1/2 specific inhibitor trametinib, which have been used alone or in combination with BRAF inhibitors. The combination has shown an improved effect on progression free survival for patients with BRAF mutant melanoma over a BRAF inhibitor alone (Flaherty *et al.*, 2012; Larkin *et al.*, 2014; Long *et al.*, 2014; Luke *et al.*, 2017). MEK inhibitors alone also target constitutively activated NRAS in patents with NRAS mutations. The combination of dabrafenib plus trametinib has been shown to provide a significant survival benefit in patients with BRAF mutant metastatic melanoma with 3 year overall survival of 44% in the combination group compared to 32% in the dabrafenib alone group (Long *et al.*, 2014) and therefore these exciting data have triggered a fast-track adoption of the combination of dabrafenib plus trametinib in patients with BRAF mutant metastatic melanoma by NICE (NICE, 2016). However, accumulating evidence suggests that acquired resistance to combination targeted therapies, although later than monotherapy alone, is also a major reason for patient mortality (Welsh *et al.*, 2016). The need for a greater understanding of the mechanisms that promote resistance to targeted therapies and the development of more efficacious therapeutic strategies is therefore of paramount importance.

1.2.5.5 Resistance mechanisms to targeted therapies

Although BRAF/MEK specific inhibitors initially block tumour growth and tumour cell proliferation, paradoxical resistance may result in the acceleration of tumour growth. Several mechanisms mediating tumour resistance have bene proposed, broadly divided into two categories of intrinsic and acquired resistance (Luke and Hodi, 2012; Luke and Hodi, 2013) (Figure 1-9).

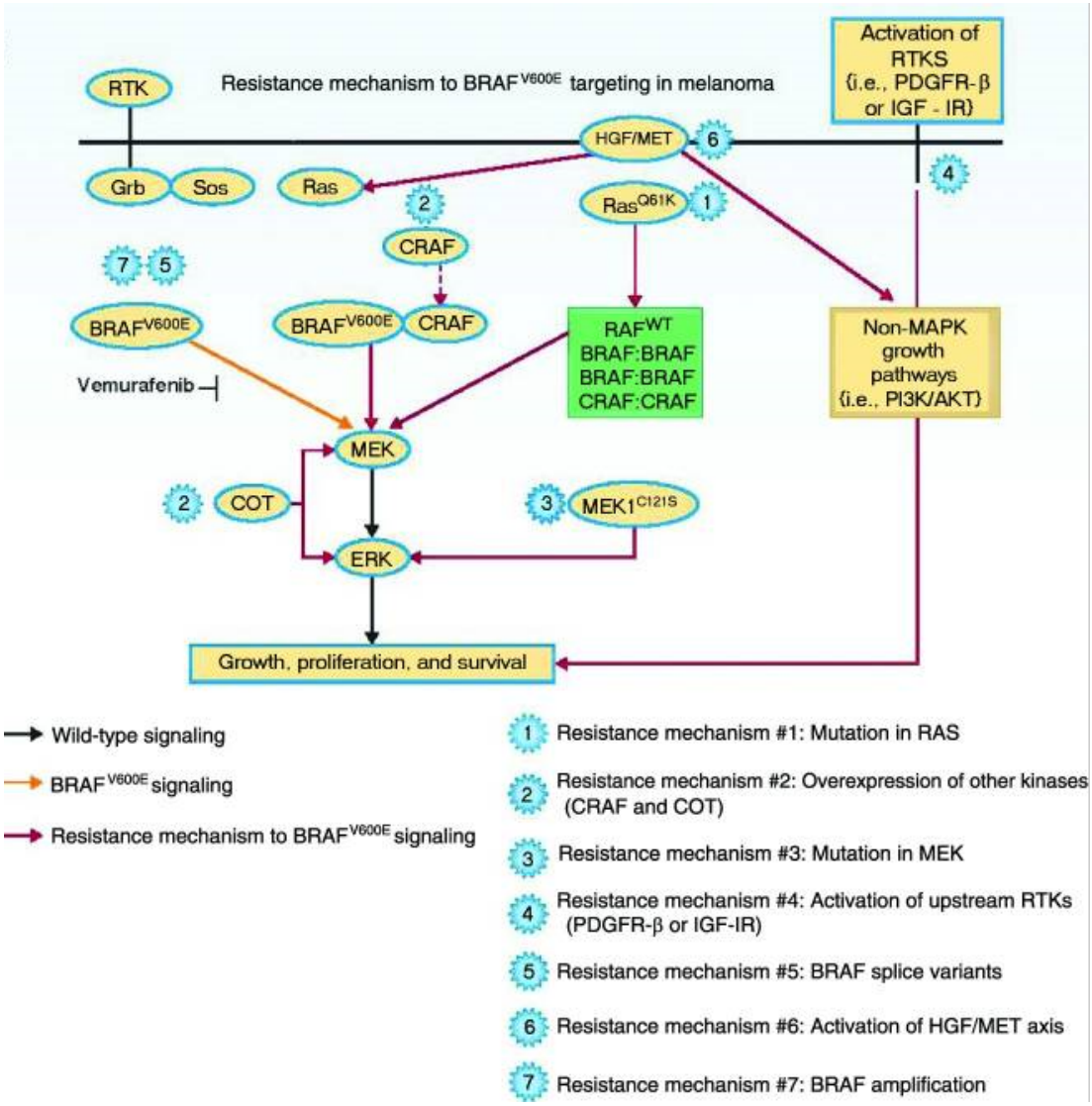


Figure 1-9: Mechanisms of resistance to BRAF inhibitors
A number of resistance mechanisms to BRAF inhibition have been described including 1. Secondary mutations in NRAS, 2. Overexpression of other kinases such as COT, 3. Secondary mutation in MEK, 4. Activation of upstream receptor tyrosine kinases, 5. Splice variants of BRAF, 6. Activation of HGF/cMET *or 7. BARF amplification. Taken from Luke and Hodi (Luke and Hodi, 2013).*

One of the first intrinsic mechanisms of BRAF/MEK inhibitor mediated-resistance described is the up regulation of cell cycle regulator levels like cyclin D. *In vitro* studies in melanoma cell lines with increased levels of cyclin D1 demonstrated resistance to BRAF inhibitors (Smalley *et al.*, 2008). Interestingly, cyclin D1 amplification is present in 15-20% BRAF mutant melanomas, suggesting this mechanism is clinically relevant and may explain the observed resistance seen in patients treated with a BRAF inhibitor (Sauter *et al.*, 2002). Loss of PTEN, a major regulator of the phosphotidylinositol-3-kinase (PI3K) (Sansal and Sellers, 2004) may also explain intrinsic resistance to BRAF/MEK inhibition. (Paraiso *et al.*, 2011). Finally, recent evidence highlights the importance of the hepatocyte growth factor (HGF)-cMET axis in development of BRAF inhibitor acquired resistance indicating that the cross-talk amongst several receptor tyrosine kinases that are co-expressed in tumour cells could explain the development of both intrinsic and acquired resistance (Wilson *et al.*, 2012).

Acquired resistance to BRAF inhibition may also result from the development of secondary mutations in BRAF which block binding of BRAF inhibitors and thus allowing homo and heterodimerization with CRAF or ARAF and therefore paradoxical hyper-activation of the MAPK pathway (Hatzivassiliou *et al.*, 2010) (Whittaker *et al.*, 2010; Poulikakos *et al.*, 2011; Sanchez-Laorden *et al.*, 2014). Downstream activation of MEK and ERK could also result in the up regulation of non-MAPK pathways including COT, which can mediate activation of MEK independently of RAF (Johannessen *et al.*, 2010b). Finally, the up regulation of other receptor tyrosine kinase receptor signalling pathways has been reported to play a role in acquired BRAF/ MEK inhibitor associated drug resistance. Up regulation of platelet derived growth factor receptor beta (PDGFRβ) and insulin-like growth factor 1 receptor (IGF-1R) in this context have been reported to activate the PI3K-AKT-mammalian target of rapamycin (mTOR) resulting in cell proliferation (Nazarian *et al.*, 2010).

Pertinent to the current study, accumulating evidence supports the concept that melanoma resistance to targeted therapies may be the result of emerging subpopulations within the heterogeneously populated tumours that emerge through resistance to both chemotherapy and targeted therapies and are defined by high phenotypic plasticity (Sztiller-Sikorska *et al.*, 2014) and referred to as cancer 'stem-like cells' (Shakhova and Sommer, 2013), multidrug resistance or induced drug tolerant cells (Ravindran Menon *et al.*, 2014).

1.3 **Cancer stem cells**

Although the precise origin of melanoma remains unknown, recent evidence suggests the accumulation of genetic and epigenetic mutations in melanocytes enables the acquisition of stem cell-like capacities via somatic reprogramming (Shakhova and Sommer, 2013) . The cancer stem cell theory was first described in the context of acute myeloid leukaemia (AML) when it was suggested that isolated susceptible cells in the hematopoietic hierarchy were responsible for leukemic transformation (Bonnet and Dick, 1997). Since then there has been an ongoing debate about the origin and existence of cancer stem like cells in solid tumours. Two theories for tumour development have been proposed, the stochastic and the cancer stem cell theory. The stochastic theory proposes that every cell has the potential to initiate tumourigenesis, provided they accumulate tumorigenic genetic alterations (Michor *et al.*, 2004) while the cancer stem cell theory suggests that there is only a small subpopulation within solid tumours have the selective ability to maintain tumour growth via self-renewal and promoting tumour heterogeneity through maintaining differentiation (Reya *et al.*, 2001) (Figure 1-10). The cancer stem cell model was first introduced in the context of malignant melanoma in 2005 with the identification of a subpopulation of melanoma cells able to differentiate under appropriate conditions into multiple cell lineages, including melanocytic, adipocytic, osteocytic, and chondrocytic lineages (Fang *et al.*, 2005a). From a therapeutic perspective, the cancer stem cell concept has significant implications as the targeting of such subpopulations may be required to achieve long-term disease free survival (Visvader and Lindeman, 2008). The targeting of cancer stem cells has attempted by either directly targeting the expression of cell surface markers such as CD133 using small molecules or specific antibodies (Swaminathan *et al.*, 2013) or by targeting signalling pathways cancer stem cells use to survive and promote tumour migration. (Der-Yang *et al.*, 2013).

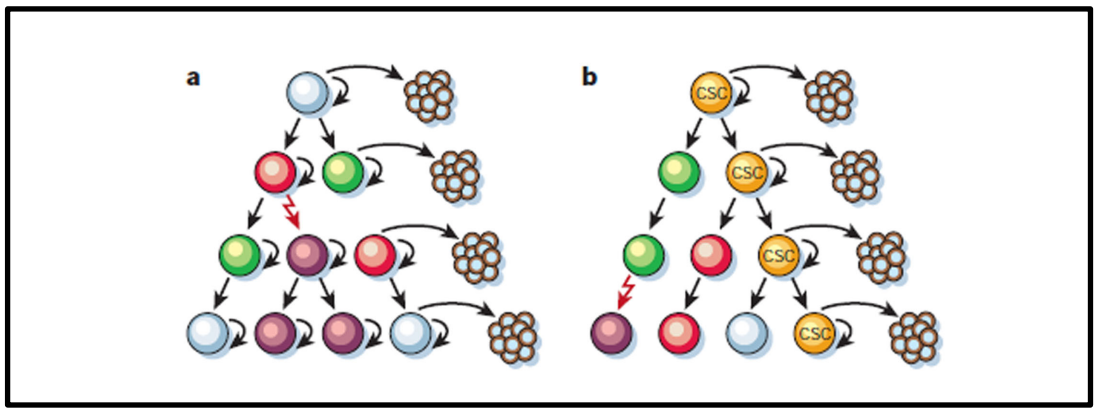


Figure 1-10: Proposed models for tumourigenesis
a. Stochastic model of solid tumour development. Any cell is capable to produce and maintain tumour growth following genetic alterations. b. Only certain cells (CSC) are tumorigenic and have the ability to proliferate and maintain tumour growth. (Adapted from (Reya *et al.*, 2001)

The cancer stem cell (CSC) hypothesis provides an attractive cellular mechanism to account for the therapeutic resistance (Figure 1-11) and the aggressive behaviour of many tumours. The CSC theory hypothesizes that tumour relapse is a result of the existence of CSC that escape anti-cancer therapies. One explanation that has been proposed is that anti-cancer therapies only target differentiated tumour cells within the tumour bulk, while sparing the CSC subpopulations that are able to escape chemotherapy-induced death by down regulating tumour specific surface markers and reverting to a non-differentiated phenotype (Bose *et al.*, 2011; Abdullah and Chow, 2013) and therefore re-instating tumour bulk. Nevertheless, contrary to the AML stem cell cancer model, cancer stem cells in solid tumours are regarded as impure populations, reflected by the poor overlap between reported stem cell markers in pancreatic and breast cancer (Visvader and Lindeman, 2008) and with varying frequencies of expression within the same tumour type, making cancer stem cell research a controversial and difficult area to research.

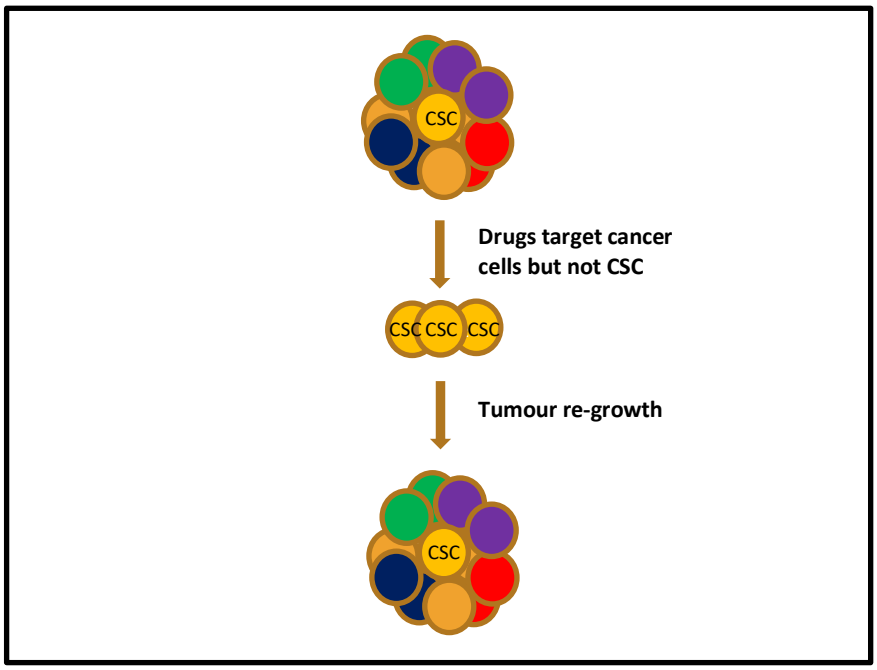


Figure 1-11: Proposed model for tumour chemo resistance
Both traditional chemotherapeutics and targeted therapies are effective in targeting the non-cancer stem-like cells. As a result, the tumour bulk is initially reduced but when resistance develops, the tumour bulk is reconstituted by differentiation and proliferation of tumour cells that have stem-like capabilities.

1.3.1 Cancer stem-like cell surface marker expression

The increased expression of several surface markers has been associated with increased tumourigenic potential and resistance to both standard chemotherapeutics and targeted therapies as well as a cancer stem cell phenotype. The expression of ABCB5 (ATP-binding cassette subfamily B member 5) which acts as an ATP-dependent pump to transport compounds out of the cell (Frank *et al.*, 2003) has been associated with the development of chemo resistant melanoma cells when cultured in the presence of both standard chemotherapeutics such as doxorubicin (Frank *et al.*, 2005a) or targeted agents including BRAF inhibitors (Chartrain *et al.*, 2012). Interestingly, ABCB5 +ve melanoma subpopulations isolated from patient derived tissue and xenotransplanted into NOD/SCID mice ABCB5 +ve melanoma cells demonstrated greater tumorigenic capacity compared to the ABCB5 -ve subpopulations with greater ability to re-establish tumour heterogeneity (Schatton *et al.*, 2008). Additionally,

immunohistochemical analysis of ABCB5 expression in benign naevi, primary melanomas and melanoma metastases demonstrated ABCB5 expression is higher in primary and metastatic MMs compared with benign melanocytic naevi, with increased expression in thick primary compared to thin primary melanomas also noted and collectively suggesting ABCB5 as a molecular marker of neoplastic disease progression (Frank *et al.*, 2005b; Gambichler *et al.*, 2016).

CD133 has also been described as a putative cancer stem cell marker in both melanoma and neurological malignancies (Singh *et al.*, 2004). Higher CD133 expression has been observed in malignant melanomas compared to benign naevi (Klein *et al.*, 2006) with reports of CD133 +ve cells also being resistant to Caffeic acid phenethyl ester (CAPE)-mediated by up regulation of ABCB5 expression (El-Khattouti *et al.*, 2015).

Nestin, a class VI intermediate filament protein, was one of the first neural crest stem cell markers to be described (Lendahl *et al.*, 1990). Nestin expression is variable in malignant melanoma but a stepwise increase in immunohistochemical expression has been associated with increasing AJCC disease stage progression (Akiyama *et al.*, 2013c). Interestingly, the presence and isolation of circulating melanoma cells that express Nestin has also been associated with poor prognosis (Fusi *et al.*, 2011) while knockdown of nestin in melanoma cell lines inhibits tumour cell migration and invasion (Akiyama *et al.*, 2013b), collectively indicating that Nestin plays an important role in melanoma progression and invasion.

SOX10 is a transcription factor important for the development of neural crest derived tissues and melanocytes (Harris *et al.*, 2010) also seems to play a crucial role in melanoma cell proliferation and survival where expression inversely correlates with both tumour thickness and disease stage (Agnarsdottir *et al.*, 2010). SOX10 expression also serves as a reliable marker melanoma metastases in sentinel lymph node samples (Willis *et al.*, 2015). Moreover, SOX10 inhibition in melanoma cell lines results in reduced proliferation and increased cell death through binding to the Melanoma Inhibitory Activity (MIA) promoter and therefore resulting in reduced MIA expression, considered responsible for the invasion-promoting role of SOX10 (Graf *et al.*, 2014b). Finally, the downregulation of SOX10 has also been associated with activation of TGF-b signalling, leading to upregulation of EGFR and platelet-derived growth

factor receptor-β (PDGFRB) and the resistance of melanoma cells to the cytotoxic effects of BRAF and MEK inhibitors (Sun *et al.*, 2014).

More recently JARID1B melanoma subpopulations with stem-like properties have been identified. JARID1B (KDM5B/PLU-1/RBP2-H1 is a member of the highly conserved family of jumonji/ARID1 (JARID1) histone 3 K4 (H3K4) (Roesch *et al.*, 2005) and although JARID1B is minimally expressed in normal tissue, in cancer cells it functions as a transcriptional regulator of oncogenes (Scibetta *et al.*, 2007; Roesch *et al.*, 2010). Specifically in melanoma, JARID1B, has been shown to display increased self-renewal capacity, without having any significant effect on tumour initiation. Interestingly, knock-down of JARID1B leads to an initial acceleration of tumor growth, suggesting JARID1B-positive subpopulations are crucial for tumor growth. Expression of JARID1B however, does not follow a cancer stem cell model as JARID1B-negative cells can become positive, and irrespective of selection are tumorigenic (Roesch *et al.*, 2010). JARID1B positive cells are also reported to emerge as multidrug tolerant cells, for which blockade of the mitochondrial respiratory chain in drug resistant JARID1B expressing cells has been shown to overcome intrinsic drug resistance in melanoma (Roesch *et al.*, 2013) and collectively suggesting that JARID1B expression in melanoma is possibly more associated with drug resistance rather than tumour initiation and is therefore unlikely a true cancer stem cell marker.

Finally CD271 has also been described as a stem cell marker (Boiko *et al.*, 2010a; Cheli *et al.*, 2014a), the expression of which has also been linked to MEK or BRAF-inhibitor induced resistance in melanoma (Ravindran Menon *et al.*, 2014; Gray *et al.*, 2015)

1.3.2 CD271 expression in normal tissue

CD271 (p75NTR), a member of the tumour necrosis factor (TNF) receptor superfamily is a transmembrane protein that binds to the nerve growth factor (NFG) (Johnson et al., 1986) which plays a key role in a number of different biological systems, including the immune, vascular and nervous systems (Liepinsh et al., 1997a). CD271 has been extensively described in the neuroscience literature where variable expression is found throughout normal life but whose expression is increased during developmental periods and with dramatic reduction

during adulthood (Gall and Isackson, 1989; Chen et al., 2009). Associated with different signal transduction pathways including cell survival and apoptosis, CD271 through interaction with the TrK (tyrosine receptor kinase) receptor (Lad et al., 2003) results in the enhancement of NGF mediated neurite growth and in neuronal cell survival (Lad et al., 2003). Downstream mechanisms involved in CD271 signalling in neuronal cells also include extracellular signal-regulated kinase (ERK), nuclear factor-kappa B (NF-κB) and Jun N-terminal kinase (JNK) (Carter et al., 1996; Xue et al., 2000; Yeiser et al., 2004). CD271 acts as a signal transducer to either induce or inhibit apoptosis via activation of different signalling pathways (

Figure 1-12). Upon binding of NGF to CD271 recruited cytoplasmic adaptor proteins recruit the tumor necrosis factor receptor member TRAF6 which can in turn activate the inhibitor of the kappaB kinase (IKK) and thus the canonical NF-Kb signalling (Khursigara et al., 1999) pathway. Alternatively, this interaction can activate c-Jun N-terminal kinase (JNK) (Casademunt et al., 1999) which phosphorylates the transcription factor c-Jun which subsequently mediates apoptosis by upregulating the expression of pro-apoptotic genes.

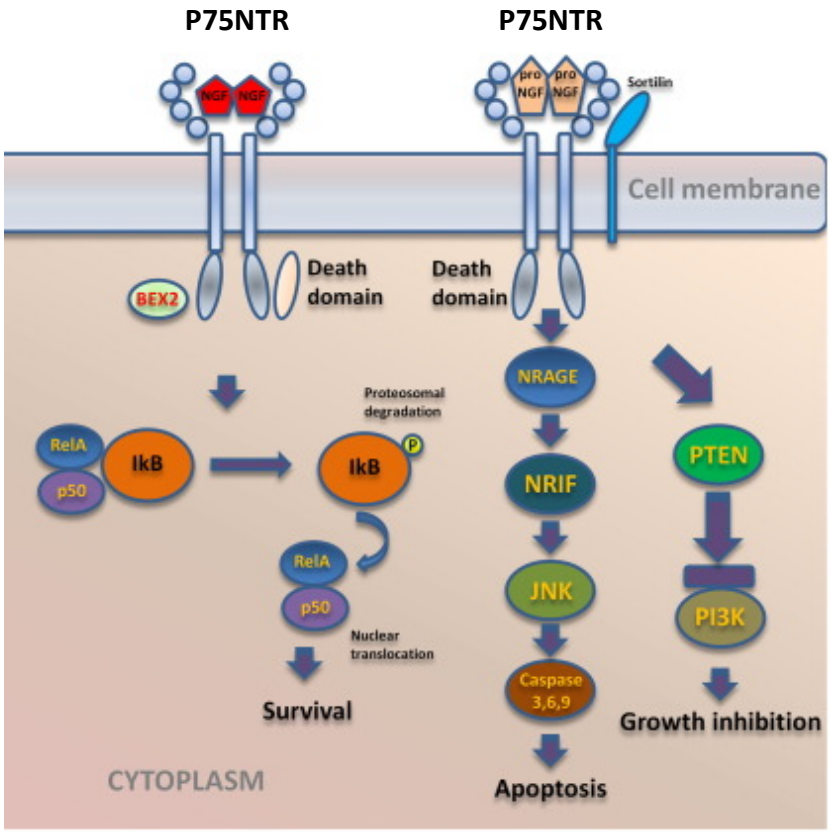


Figure 1-12: CD271 (p75NTR) can induce or inhibit apoptosis in normal cells
CD271 can promote cell survival via activation of the NF-kB pathway or promote apoptosis through activation of JNK in normal cells. (Adapted from Demir *et al (Demir et al., 2016)*

1.3.2 CD271 subpopulations in melanoma

CD271 expressing melanoma cells have been proposed as a tumourigenic and chemo resistant subpopulation. Isolation of CD271 +ve and –ve melanoma cells from metastatic patient derived tumours and xenotransplantation into T, B, and NK deficient Rag2−/− γc−/− mice (RG) mice, results in the engraftment of 70% of CD271+ cells compared to 7% of CD271− ve subpopulations (Boiko *et al.*, 2010b). However, other studies highlight the unstable nature of CD271 *in vitro* and the inconsistency in the tumourigenic potential of CD271 positive cells (Boyle *et al.*, 2016). In addition, literature has reported the emergence of CD271 +ve subpopulations in line with the development of acquired resistance to both targeted therapies and standard chemotherapy (Civenni *et al.*, 2011; Ravindran Menon *et al.*, 2014; Redmer *et al.*, 2014b). CD271 expression *in vivo* is also associated with poor melanoma patient survival (Beretti *et al.*, 2015). Furthermore, increased CD271 expression is observed in metastatic melanoma deposits to the brain (Guo *et al.*, 2014b) indicating that CD271 expression may serve not only as a marker of resistance to treatment but also as a predictive biomarker. However, recent evidence supports the hypothesis that there are different subpopulations within CD271 +ve melanoma expressing cells with different capabilities and tumourigenic potential; although there is a transient slow-growing population within CD271 +ve melanoma cells that are highly tumorigenic, a fast growing/ CD271 +ve population exhibits poor tumorigenic ability (Cheli *et al.*, 2014b). Interestingly, melanoma subpopulations expressing CD271 exhibit lower surface expression of melanoma specific antigens such as TYR, MART and MAGE (Boiko *et al.*, 2010b) possibly indicating a de-differentiated phenotype and supporting the hypothesis that CD271 +ve melanoma subpopulations have a stem-like phenotype. This ability of CD271 +ve melanoma subpopulations to down regulate expression of melanoma antigens (Furuta *et al.*, 2014) could also potentially explain the resistance of melanomas to immune mediated death using standard and newer immunotherapies (Robert *et al.*, 2015b). The fact that the process of autophagy has been reported to be required for the maintenance of stem cell and tumourigenicity of breast cancer stem like cells (Gong *et al.*, 2013) suggests however that this mechanism may also be key to the survival and chemo resistance of melanoma specific subpopulations with stem like properties.

1.3.3 Targeting cancer stem cells as a therapeutic approach for cancer therapy

The identification of cancer stem cells has introduced the concept of targeting these specific subpopulations to overcome resistance to chemotherapy and targeted therapies. The cancer stem cell model comprises an attractive framework to explain acquired resistance through the emergence of cancer stem like subpopulations that harness different pathways to escape the effects of chemotherapy and targeted agents (Figure 1-13).

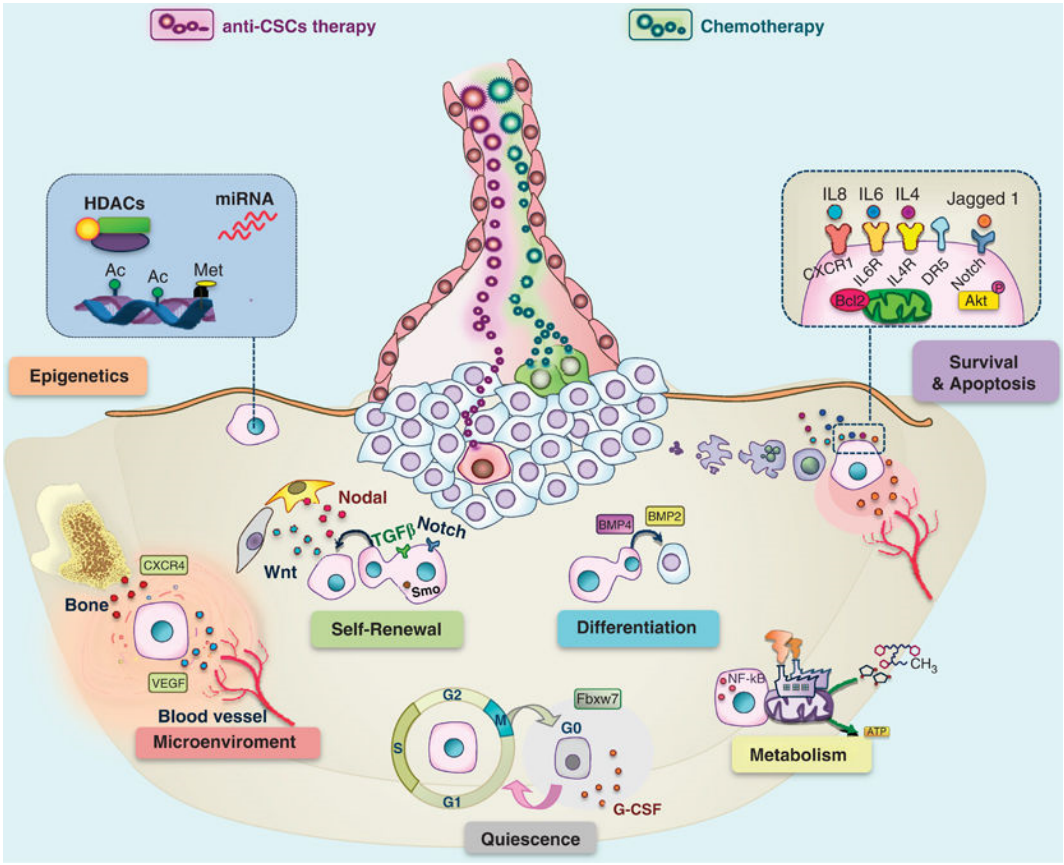


Figure 1-13: Mechanisms of survival of cancer stem cells to chemotherapy
Combination of standard therapy (green) with targeted cancer stem cell therapy targeting specific pathways (purple). Taken from Vidal *et al* (Vidal *et al.*, 2014).

Targeting cancer stem cells has been an attractive research field and two different approaches have been adopted; either targeting the cancer stem cells directly or targeting the signalling pathways key to the survival of such subpopulations.

Targeting cancer stem cell surface markers with either monoclonal antibodies or small molecule inhibitors in combination with standard chemotherapy agents has been shown to be beneficial in head and neck squamous cell carcinomas (Chen *et al.*, 2017) or prostate cancer by targeting CD44 (Liu *et al.*, 2011). Interestingly, targeting signalling pathways crucial to stem cell maintenance and survival such as the Wnt pathway (Zhao *et al.*, 2007), Notch pathway (Hovinga et al., 2010) or the Hedgehog signalling pathway (Zhao *et al.*, 2009) have also all been associated with a reduction in cancer stem cell survival proposing further new avenues for combinatorial therapeutic options that may improve current strategies targeting generic mechanisms of rapid cell growth and survival.

However, it is still not clear which approach is likely to be the most beneficial in the clinical setting and given melanoma is a very heterogeneous cancer, targeting one subpopulation may not be sufficient to overcome acquired resistance that likely equally allows for the emergence of other subpopulations expressing differential stem cell surface markers supported by differential signalling mechanism, such as autophagy.

1.4 **Autophagy**

Autophagy represents the principal catabolic process for the lysosomal mediated degradation and recycling of damaged organelles and excess proteins (Roy and Debnath, 2010) which plays a crucial role in the maintenance of cellular homeostasis. To date, three major types of autophagy have been described: macro autophagy, micro autophagy and chaperone-mediated autophagy (Boya *et al.*, 2013). Macro autophagy involves the sequestration of unwanted products within cytosolic compartment into double-membrane vesicles with the formation of the double-membrane bound phagophore and autophagosome (Feng *et al.*, 2014), while micro autophagy involves the degradation of unwanted products though flap like lysosomal projections and protrusions that facilitate sequestration by direct interlysosomal engulfment of cytosolic material (Mijaljica *et al.*, 2011). On the other hand chaperone mediated autophagy involves the direct transfer of targeted molecules into the lysosomal lumen through a dedicated protein translocation complex and chaperone proteins present on either side of the lysosomal membrane (Cuervo and Wong, 2014). Collectively these processes function to maintain correct degradation and recycling of damaged organelles and proteins, promoting cellular homeostasis and energy. Sensitive to changes in the nutrient environment macroautophagy (from here on referred to as autophagy) is activated under situations of nutrient or oxidative stress (Figure 1-14).

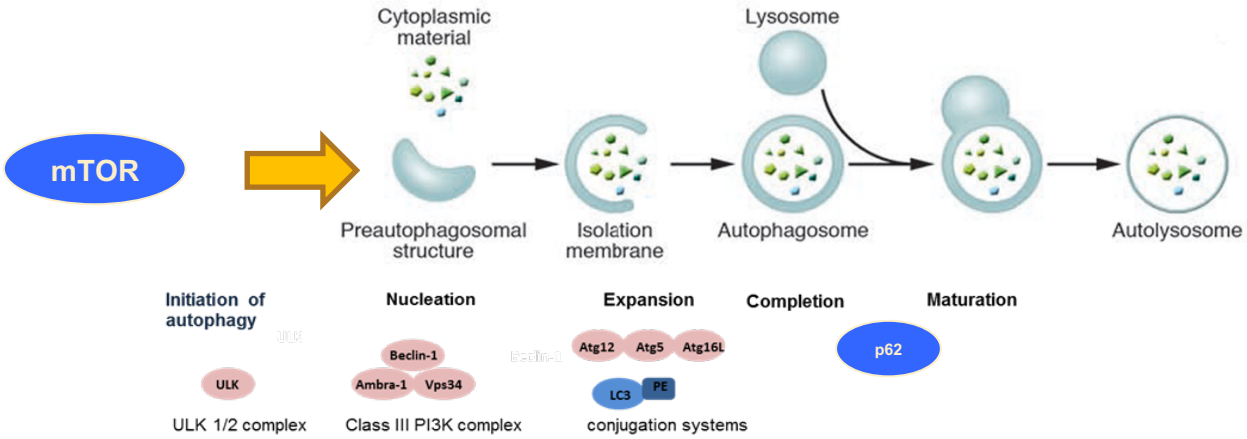


Figure 1-14: Schematic representation of the autophagic process (Professor Penny Lovat)
Autophagy induction is regulated by mTOR and activation leads to a cascade of events including formation of the isolation membrane around cytoplasmic material to be degraded, elongation, autophagosome formation, maturation and finally fusion with the lysosome to form an autolysosome. This process is regulated by key proteins, protein kinase complexes and conjugation systems: mTOR a central regulator of autophagy which is sensitive to changes in nutrient availability, the ULK1/2 and beclin-1 complex and the Atg 12-5-16L and LC3 conjugation systems key to the expansion of the isolation membrane and the recruitment of proteins and organelles to be degraded. Image supplied by Professor Penny Lovat

Activation initiates a cascade of events characterized by an induction phase involving the development of a double isolation membrane or phagophore, followed by maturation of the autophagosome containing cytoplasmic material to be degraded and finally completion of the process characterized by fusion of the autophagosome with a lysosome and the lysosomal degradation/recycling of cytoplasmic material (Tooze and Yoshimori, 2010).

1.4.1 Autophagy regulation

A number of signalling pathways are involved in the regulation and induction of autophagy. Primarily, autophagy induction is regulated through the mammalian target of rapamycin complex 1 (mTORC1) which coordinates the activation of a cascade of key complexes and events (Jung *et al.*, 2009). Upon autophagy induction, the activation of the ULK1/2 complex mediates the initial nucleation phase followed by activation of the Beclin-1 complex (comprising Beclin-1/Atg 6, Ambra-1 and Vps34) controlled by Vps34, a class III PI3 kinase

leading to the formation of a pre-autophagosomal structure. The elongation of the isolation membrane is regulated by the Atg12-Atg5 complex then recruits additional membranes (Kuma *et al.*, 2002) by further interacting with ATG16L in order to expand the isolation membrane. Once the autophagosome has been formed, these autophagy regulatory proteins are released back to the cytoplasm (Mizushima *et al.*, 1999) with the lipidation of LC3 (Atg8) (Ichimura *et al.*, 2000) enabling the recruitment of proteins and organelles to the growing autophagosome (Burman and Ktistakis, 2010). The final maturation step is regulated by the Vps34/ Beclin-1/ UV irradiation resistance-associated gene (UVRAG) complex (Itakura *et al.*, 2008), allowing the fusion of the autophagosome with a lysosome, forming the autolysosome and allowing the enzymatic degradation of the cytosolic content. Key events associated with autophagy induction include the degradation of p62 (also known as sequestosome-1/SQSTM1). P62 is a multidomain adaptor protein which interacts with both ubiquitinated proteins and LC3, allowing for its inclusion within the autophagosome (Moscat and Diaz-Meco, 2009), followed by its degradation after formation of the autophagolysosome.

1.4.2 Autophagy and cancer

The role of autophagy in cancer is complex. While the removal of unwanted organelles and proteins is key to the prevention of tumourigenesis, the accumulation of damaged organelles or excess proteins is genotoxic thereby promoting tumourigenesis. So although autophagy is predominately accepted as a survival mechanism, in the context of cancer, autophagy can both suppress and drive tumourigenesis; the so called “autophagy paradox” (White *et al.*, 2010). Several studies have shown autophagy to be impaired or inhibited in cancer, mediated for example by loss of beclin-1 expression which has been described in the context of a number of human cancers (Ahn *et al.*, 2007; Cai *et al.*, 2014; Rohatgi *et al.*, 2015). Supporting autophagy deregulation in cancer, specific studies of Beclin 1 loss have shown knockdown in murine models increases tumourogenicity (Qu *et al.*, 2003) whereas Beclin 1 overexpression prevents tumour development (Liang *et al.*, 1999). However, paradoxically autophagy may also aid and promote tumour survival at later disease stages through the recycling of cellular components to produce essential nutrients and sustain cellular energy (Ma *et al.*, 2011;

Sandilands *et al.*, 2012) collectively indicating that deregulated autophagy leads to tumour development, whereas in already established cancers autophagy provides an important source of energy though the recycling of cellular components to maintain and promote tumour survival in times of nutrient stress.

AMPK is a major cellular energy sensor activated by ATP and nutrient depletion to maintain energy homeostasis, directly activates autophagy (Hardie, 2011) and has been shown to be a key event in autophagy m52- ediated survival of lung (William *et al.*, 2012) and colorectal (Baba *et al.*, 2010). There is also increasing evidence in the literature to suggest AMPK activated by glucose starvation or ATP depletion to sustain the metabolic and energetic cellular demands in the micro environment may provide the link to the activation of autophagy (Cantó *et al.*, 2009) though inactivation of m-TORCH1 (Hardie, 2011) or direct phosphorylation of ULK1 (Kim *et al.*, 2011a).

Accumulating evidence suggests that autophagy is also deregulated in melanoma. Immunohistochemical expression of LC3-II is increased in malignant melanoma compared to benign naevi (Lazova *et al.*, 2010; Lazova *et al.*, 2012). Further evidence also suggests that the down regulation of ATG5 contributes to melanomagenesis (Liu *et al.*, 2013b), while down regulation of ATG6/Beclin-1 has been associated with melanoma progression (Sivridis *et al.*, 2011). More recently, the immunohistochemical expression of p62 also appears to reflect the paradoxical role of autophagy in melanoma where increased expression was consistent with autophagy being blocked in early stage disease, while its reduction in late stage disease was consistent with autophagy reactivation (Ellis *et al.*, 2014b).

1.4.3 Autophagy and stem-like cells

Stem cells have several capabilities including self-renewal, longevity and differentiation. These processes require a strict regulation of cellular homeostasis and remodelling and there is accumulating evidence that autophagy plays a crucial role in stem cell survival. The role of autophagy in embryonic stem cells has been investigated in detail and is essential for the very early stages of embryogenesis. Knockdown of ATG5 in fertilized oocytes results in their failure

to divide beyond the 4- to 8-cell stages if fertilized by Atg5-null sperm, and therefore failure to undergo more advanced stages of the differentiation processes (Tsukamoto *et al.*, 2008). Autophagy seems to also play a role in differentiation and pluripotency of haematopoietic stem cells (Orford and Scadden, 2008) and neuronal stem cells (Zhang *et al.*, 2008) .

Interestingly, autophagy also seems to play a crucial role in the maintenance and survival of cancer stem-like cells (CSC). CSCs consist of specific subpopulation of cancer cells that may exhibit some characteristics of stem cells, being capable of self-renewal and differentiation that is responsible for the heterogeneity in bulk tumours (Magee *et al.*, 2012). The effects of autophagy in cancer stem-like cells may be related to characteristic micro environmental factors affecting the growth and development of cancer cells and which include hypoxia and nutrient deprivation. The ability of certain subpopulations within tumours to survive under these micro environmental stresses may lead to specific expansion of CSC subpopulations in favour of non-CSC tumour cells. This theory is supported by evidence suggesting that inhibition of autophagy with the lysosomal inhibitor chloroquine is sufficient to suppress the development of breast cancer stem like cell spheroid formation and ex vivo invasion into autologous breast stroma (Espina and Liotta, 2011) indicating that subpopulations of cancer stem-like cells lose their ability to survive upon inhibition of autophagy. In addition, autophagy seems to play a crucial role in the tumourigenicity of breast cancer stem like cells (Gong *et al.*, 2013) and invasion in glioblastoma stem like cells (Galavotti *et al.*, 2013).

Finally, autophagy plays a role not only in the maintenance and invasion of stem like cells but also contributes to chemo resistance in certain tumour types. During chemotherapy, cancer cell clones are subjected to stressful environmental factors allowing the survival of only some cell populations. As described above, autophagy seems to be an important mechanism in the ability of selected cell clones to evade the effects of chemotherapeutic and targeted agents, making autophagy inhibition an attractive therapeutic strategy to potentiate the effects of chemotherapy. To this aim, combination treatments of standard therapies with lysosomal inhibitors like chloroquine, able to indirectly inhibit autophagy have shown considerable promise for a number of tumours including hepatocellular cancer (Guo *et al.*, 2012), pancreatic cancer (Yang *et al.,* 2011), breast cancer (Zarzynska, 2014) and CML (Yu *et al.*, 2012) as well as melanoma (Amaravadi *et al.*, 2011; Molenaar *et al.*, 2017).

1.4.4 Targeting autophagy in cancer therapy

Given the pivotal role of autophagy in resistance to chemotherapy and targeted therapies, there is increasing evidence to support the targeting of autophagy to enhance therapeutic response to cytotoxic agents. Chloroquine and hydroxychloroquine have been used to treat malaria for many years before the effects on autophagy were fully established. Chloroquine acts via inhibiting lysosome acidification, therefore blocking the late stages of autophagy (Geng *et al.*, 2010) wit it use documented in a number of clinical trials in combination with chemotherapy to overcome acquired drug resistance (Schmukler *et al.*, 2014), including in combination with 5-fluouracil in gallbladder carcinomas (Liang *et al.*, 2014), erlotinib in non-small cell lung cancer (Zou *et al.*, 2013) or imatinib (a TKI) in the case of chronic myeloid leukaemia (Bellodi *et al.*, 2009). Inhibition of autophagy has also been shown to be beneficial in combination with radiation therapy. In gliomas, glioma stem-like cells expressing CD133 associated with resistance to radiation therapy demonstrate higher levels of autophagic activity (Lomonaco *et al.*, 2009), where combined autophagy inhibition revealed more extensive DNA double-strand breaks in such radio- resistant sub populations compared to cells that have been treated with radiation therapy alone (Ito H, 2005). Similarly, blocking autophagy *in vitro* with 3MA, which blocks the formation of the autophagosome (Wu *et al.*, 2010), or genetically using an siRNA approach results in radio sensitization of a number of human cancer cell lines (Apel *et al.*, 2008). However, chloroquine is not a specific autophagy inhibitor, leading to the recent development of more specific inhibitors including those targeting the Vps34 complex, currently in clinical trials in combination with mTOR inhibitors for patients with renal cell carcinoma (Pasquier, 2015).

Interestingly, although autophagy inhibition may increase the efficacy of cytotoxic agents, the induction of cytotoxic autophagy represents a promising alternative (Josset *et al*., 2013; Ren *et al.*, 2013), including the use of cannabinoids such as Δ(9)-tetrahydrocannabinol (THC), recently shown to promote autophagy mediated apoptosis in melanoma *in vitro* and in *in vivo* melanoma mouse models (Salazar *et al.*, 2009; Armstrong *et al.*, 2015b; Giglio *et al.*, 2015; Velasco *et al.*, 2016b). THC derived from Cannabis sativa exerts a variety of biological effects

by mimicking endogenous substances, the endocannabinoids, which function via activation of the cannabinoid receptors CB1 and CB2 (Howlett et al., 2002), the engagement of which leads to a cascade of events including the induction of pro-apoptotic sphingolipid ceramide and reduced tumour cell proliferation *in vitro* (Velasco *et al.*, 2016b). Clinical trials of THC have also shown promising results in advanced glioma (Guzmán *et al.*, 2006), while in melanoma, pre-clinical data suggest that THC equally promotes autophagy mediated apoptosis (Armstrong *et al.*, 2015b), further supporting the concept of harnessing autophagy exacerbation with THC might as a viable therapeutic option for patients with advanced cancer.

1.5 **Models of melanoma metastasis**

1.5.1 Murine models for melanoma metastasis

Molecular heterogeneity in human melanoma has impaired efforts to efficiency and consistently target melanoma cells and improve patient survival. Although clinical characteristics of melanoma provide an indication for the identification of high risk individuals, many of the mechanisms mediating tumour initiation, progression and metastasis remain unknown. While molecularly targeted therapies show great promise for melanoma patients, the development of acquired resistance has highlighted the need for more efficacious treatment strategies. To evaluate the effect of such treatment approaches, establishing accurate animal models that recapitulate human cutaneous melanoma progression is of crucial importance. To further reinforce findings deriving from *in vitro* data, in vivo models have been developed to provide clinically relevant information about tumour dissemination and metastasis as well as efficacy of treatments for localised and metastatic disease. In melanoma research, murine models have been extensively used to model metastasis and assess response to different agents. However, murine models of metastatic melanoma require the recipient's innate immune system to be severely compromised. The most commonly used murine model for melanoma metastasis is the nude, NOD (non-obese diabetic), SCID (severe combined immunodeficiency) and RAG (recombination activating gene) strains, which harbour single or multiple genetic mutations and exhibit varying degrees of innate and

adaptive immune deficiencies (Kuzu *et al.*, 2015). However, the use of these models are limited by a low metastatic burden and the fact that this degree of immunodeficiency is not a true representation of the human immune system (Vandamme, 2014). Thus, there is an urgent need for the development of effective and efficient *in vivo* model systems that recapitulate human melanomas so that improve our understanding of the biology of melanoma.

1.5.2 Zebrafish model for melanoma metastasis

As a result, to address the need for clinically relevant *in vivo* models that better recapitulate the metastatic process, alternative *in vivo* models have been developed including the use of a zebrafish model of metastasis. The use of an *in vivo* zebrafish model of metastasis has the advantage of small number of cells required for transplantation, easy tracking of transplanted cells because of the transparency of zebrafish embryos allows for real-time observation of the migration of tumour cells and formation of secondary metastatic deposits as well as the possibility to perform large scale experiments with high reproducibility (Marques *et al.*, 2009; Eguiara *et al.*, 2011; Zhao *et al.*, 2011). As a result, zebrafish models have become increasingly popular in cancer research. Zebrafish models have been used to model a number human cancers including haematological malignancies (Langenau *et al.*, 2003) and solid tumours such as melanoma (Dovey *et al.*, 2009) or rhabdomyosarcoma (Ignatius *et al.*, 2012). Future directions in the zebrafish field include the simultaneous manipulation of a number of genes which can contribute to cancer heterogeneity by using sophisticated gene editing techniques such as Crisp/Cas9 which would allow for the assessment of hundreds of candidate genes which cannot be achieved in mouse models (Jao *et al.*, 2013). Additionally, zebrafish can allow for large scale chemical screening and also allow for the more accurate recapitulation of cancer metastasis. Finally zebrafish can be used to study the epigenetic contribution to cancer development and metastasis by rapid large-scale trans-genesis which will be a key method to determine the temporal dynamics of such changes, which will differ from purely genetic changes seen in many tumour types(Ceol *etal.*,2011)

1.6 **Hypothesis, aims and objectives**

Given the evidence for the presence of CD271 expressing sub-populations and their contribution to melanoma survival or the emergence of CD271 expressing sub populations as a potential key mechanism mediating the resistance of NRAS/BRAF mutant melanoma to BRAF/MEK inhibitor therapy and the likely contribution of key survival signalling mechanisms such as autophagy, this leads to the central hypothesis of the present thesis that:

Autophagy plays a crucial role in the survival and invasion of CD271 expressing melanoma subpopulations and the promotion of tumour progression.

To test this hypothesis the principle objectives were to

- Define the association between constitutive CD271 expression by melanoma cells and autophagy
- Define the association between MEK inhibitor-induced CD271 expression in drug resistant melanoma subpopulations and autophagy
- Evaluate potential therapeutic approaches to inhibit CD271 or autophagy as a means through which to re-sensitize BRAF mutant melanoma to the cytotoxic effects of trametinib.

Chapter 2

Materials and Methods

Chapter 2 Materials and Methods

2.1 Cohort of primary cutaneous melanomas and metastatic lymph nodes

Full ethical permission for the use of patient tissue in these studies, including benign naevi, primary cutaneous melanomas or metastatic lymph nodes was obtained from Newcastle and Tyneside 1 research ethics committee (reference: NRES 08/H0906/95+5_Lovat).

A retrospective cohort of 22 formalin-fixed paraffin-embedded (FFPE) malignant melanomas of eventual American Joint Committee on Cancer (AJCC) stage I, II or III at the time of analysis and 5 benign naevi were obtained from the Histopathology department at the Royal Victoria Infirmary, Newcastle upon Tyne.

A second cohort of 9 formalin-fixed paraffin-embedded (FFPE) primary cutaneous melanomas and patient matched FFPE metastatic lymph nodes (7 matched eventual AJCC stage III melanomas and patient matched lymph node melanoma metastases), or 2 normal lymph nodes (used for experimental purposes as controls) was obtained in collaboration with Mr Jong Kim (department of Plastic surgery, University Hospital of North Durham), and Dr Paul Barrett (Department of Pathology, University Hospital of North Durham, Durham, UK) from the histopathology department at the University Hospital of North Durham.

A third cohort of 11 formalin-fixed paraffin-embedded (FFPE) metastatic melanoma lymph nodes was also obtained from the Histopathology department at the Royal Victoria Infirmary, Newcastle upon Tyne.

2.2 Growth and Maintenance of Human Cutaneous Melanoma Cell Lines, genetically modified melanoma cell lines and melanocytes

Cutaneous human metastatic cell lines WM35 (a generous gift from Professor Meenhard Herlyn, The Wistar Institute, Philadelphia, USA), A375 (American Type Culture Collection (ATCC), Manassas, USA), SK-mel-28, (ATCC) WM164 (ATCC) (all B-RAF V600E mutant), WM266-4 (ATCC) (BRAF^{V600D} mutant), SK-mel-23, CHL-1, C8161 (a generous gift from Professor

Meenhard Herlyn, The Wistar Institute, Philadelphia, USA), Mel 505 (ATCC) (BRAF Wild Type) and WM-1361 (NRAS mutant) were grown and maintained in Dulbecco's modified Eagles Medium (DMEM, Lonza, Vervies, Belgium) supplemented with 10% foetal calf serum (FCS, Sigma, St Louis, U.S.A) and 5% penicillin streptomycin (P/S, Lonza, Vervies, Belgium) (complete media) at 37^{o}C in a humidified atmosphere of 5% CO_2 in air. Cell line mutational status information was obtained from the ATCC website and the Wistar Institute. A summary table of all known mutation is presented in Table 1 (

Table 2-1). All cell lines were cultured for up to a maximum of 50 passages (at which point fresh cells were taken from frozen stocks stored in 90% FCS/10% DMSO in liquid N_2) and passaged by detaching cells with trypsin/EDTA (Lonza, Vervies, Belgium) as previously described (Armstrong *et al.*, 2015a). Cell lines were used at approximately 70% confluency for all experimental procedures. The authentication of all cell lines was verified by the assessment of BRAF and NRAS mutational status using commercial Single Nucleotide Polymorphism (SNP) genotyping assays (TaqMan SNP genotyping assays, Applera, Europe BV, UK) for the presence of the most frequent mutations observed in melanomas; $NRAS^{G61L}$, $NRAS^{G61A}$, $BRAF^{V600E}$ or $BRaf^{V600D}$ mutation as previously described (Hiscutt *et al.*, 2010). In addition, to confirm melanocytic lineage, all cell lines were evaluated for the expression (using immunofluorescence or western blot analysis) of the melanocyte differentiation marker Melan A, again as previously described (Hiscutt *et al.*, 2010). Experiments using BRAF or MEK inhibitor treatment of melanoma cells were performed in BRAF mutant cell lines (A375, WM35, SK-mel-28 or WM266-4).

Genetically modified human cutaneous melanoma cell lines (all previously modified in the Lovat lab or modified as part of the current study as described below); WM266-4 RFP-GFP-LC3B, A375 histone 2B RFP, WM35 histone 2B RFP, A375 stably overexpressing CD271 or WM35 stably overexpressing CD271, were grown and maintained using Dulbecco's modified Eagles Medium supplemented with 10% foetal calf serum and 5% penicillin streptomycin, at 37^{o}C in a humidified atmosphere of 5% CO_2 in air, again for up to a maximum of 50 passages.

Human primary melanocytes were derived from redundant foreskin supplied by the Department of Urology, Freeman Hospital, Newcastle upon Tyne, UK and processed and supplied by colleagues in dermatological sciences, Institute of Cellular Medicine, Newcastle

University as previously described (PhD thesis Dr Ashleigh McConnell 2016). Full ethical permission for the use of surplus human skin tissue was obtained (NRES, Newcastle and Tyneside 1 REC reference 08/H0906/95). Isolated primary melanocytes were subsequently grown and maintained in melanocyte 254 medium (Life Technologies, Paisley, U.K), supplemented with human melanocyte growth supplement (Life Technologies, Paisley, U.K) containing bovine pituitary extract (BPE) (0.2% v/v), fetal bovine serum (0.5% v/v), recombinant human insulin-like growth factor-I (1 µg/ml), bovine transferrin (5 µg/ml)basic fibroblast growth factor (3 ng/ml),Hydrocortisone (0.18 µg/ml) and Heparin (3 µg/ml) Phorbol 12-myristate 13-acetate (PMA) (10 ng/ml), for up to a maximum of 5 passages before being

	BRAF	NRAS	c-KIT	PTEN	CDKN2A	p53
A375	V600E	WT	—	—	—	—
WM35	V600I	WT	WT	WT	WT	WT
SK-mel-28	V600E	WT	WT	Yes	WT	WT
WM164	V600E	WT	WT	WT	Yes	Yes
WM266-4	V600D	WT	—	Hemizygous deletion	Yes	—
SK-mel-23	WT	WT	—	—	—	—
CHL-1	WT	WT	Yes	WT	—	—
C8161	WT	WT	—	—	—	—
Mel 505	WT	WT	—	—	—	—
WM-1361	WT	Yes	WT	—	Yes	WT

used either for experimental purposes or stored for future use in 90% FCS (Sigma) and 10% Dimethyl sulfoxide (DMSO) in a liquid nitrogen storage facility.

Table 2-1: Summary of all known mutations in melanoma cell lines used
Summary of the most common mutations found in melanoma cell lines for all cell lines used in this Thesis. Information obtained from the ATCC website and The Wistar's institute website.

2.3 Chemical and Drug treatment of Cutaneous Melanoma Cell lines *in vitro*

PLX4720 (Plexxikon Inc., Berkeley, U.S.A) is a selective small molecule inhibitor of $BRAF^{V600E}$ which selectively blocks the ATP-binding site of oncogenic $B\text{-}RAF^{V600E}$ (Sievert *et al.*, 2013). PLX4720 was diluted to a stock concentration of in 10 mM in DMSO and stored at -20°C with further dilution in culture media to given final concentrations for treatment of melanoma cells

in vitro at given time periods. For experiments in which melanoma cells were treated for 9 days with 3 μM of PLX4720, the media was changed every 3 days, each time replacing with fresh media containing 3 μM of PLX4720 prior to the assessment of cell viability or effect on differential protein expression by Western Blotting.

Trametinib (GSK 112021B, GSK, Brentford, UK) is a reversible, allosteric inhibitor of mitogen-activated extracellular signal regulated kinase 1 (MEK1) and MEK2 activation (Borthakur *et al.*, 2016). Trametinib was dissolved in DMSO at a concentration of 10 mM and stored in aliquots at -80°C, prior to further dilution in cell culture medium and use at final concentrations of 16 or 32 nM to treat melanoma cells *in vitro* for 3, 6, 9, 12, 24 or 42 days. For experimental procedures exceeding 3 days, the media was replaced every subsequent 3 days, with fresh complete media containing the appropriate given concentration of trametinib. For *in vivo* experiments in a zebra fish xenograft of human metastatic melanoma, trametinib was added directly to the water environment (E3 media) in which fish were maintained in a final concentration of 16 nM at 33^{o}C in an atmosphere of 5% O_2, 5% CO_2 for 72 hours.

Chloroquine (CQ, Sigma-Aldrich, Poole, UK) a lysosomal inhibitor, was diluted to a stock concentration of 10 mM in distilled water, filter sterilized and stored at room temperature protected from light. Chloroquine was added to melanoma cell cultures at a final concentration of 10 μM, either continuously for 24/48 hours to cultures pre-exposed to trametinib or PLX4720 for up to 9-days and prior to the assessment of cell viability or clonogenic potential or for the final 2 hours of given experiments prior to the assessment of LC3 I-II autophagic flux by western blotting.

Vps34 inhibitor PIK-III (Stratech, Suffolk, UK) is a selective inhibitor of VPS34 that inhibits autophagy and *de novo* lipidation of LC3 binding to that binds a unique hydrophobic pocket not present in related kinases such as PI (3)Kα (Dowdle *et al.*, 2014). PIK-III was dissolved in DMSO to stock concentrations of 10 mM and stored at -20^{o}C. For experimental purposes PIK-III was further diluted in complete culture media and used to treat melanoma cells *in vitro* at final concentrations of 1, 5 or 10 μM for 24 or 48 hours. For *in vivo* experiments in a zebra fish xenograft of human metastatic melanoma, PIK III was added directly to the water environment (E3 media) in which fish were maintained at a final concentration of 5 μM at 33^{o}C in an atmosphere of 5% O_2, 5% CO_2 for 72 hours.

Tetrahydrocannabinol (THC) (kindly supplied by Dr Guillermo Velasco, Complutense University of Madrid, Spain) was dissolved in DMSO to a final concentration of 100 mM and stored at -20^{o}C. THC was added to melanoma cell cultures *in vitro* at final concentrations of 4.5 or 5 µM in low serum (0.5% FCS) DMEM for 24 hours as previously described (Armstrong *et al.*, 2015b) before analysis of effects on cell proliferation or protein expression by western blotting.

The CD271 inhibitor TAT-PEP5 (Merk Millipore, Massachusetts, USA) is a small molecule inhibitor which inhibits the interaction of p75NTR with Rho-GDI *in vitro*. TAT-PEP5 was dissolved in DMSO and stock concentrations of 1 mM, stored in -80^{o}C. For treatment of melanoma cells, TAT-PEP5 was further diluted in complete DMEM culture medium and added to cultures at final concentrations of 0.01, 0.1,1,5 or 10 µM for 72 hours.

The CD271 inhibitor, Ro 08-2750 (Tocris Bioscience, Bristol, UK) is a small molecule which inhibits the binding of NGF to p75NTR (Niederhauser *et al.*, 2000). Ro 08-2750 was dissolved in DMSO to stock concentrations of 10 mM and stored at -20^{o}C before being subsequently further diluted into fresh media to a final concentration of 1, 5 or 10 µM for 72 hours.

Recombinant human Nerve growth factor b (b-NGF, Peprotech, New Jersey, USA) is a neurotrophic factor that plays a crucial role in the development and preservation of nervous system (Söderström *et al.*, 1990), used in the present studies to investigate the contribution of exogenous NGF in trametinib-induced CD271 expressing melanoma subpopulation proliferation and invasive potential. NGF was reconstituted in filter sterilized 0.1% Bovine Serum Albumin (BSA, Alpha Diagnostic, Texas, USA) to a concentration of 200 µg/ml and stored at -20^{o}C . For experimental treatment purposes, NGF stock solutions were further diluted in complete culture media and added to melanoma cell cultures at final concentrations of 0.01 ng/ml- 1mg/ml for 72 hours.

Dorsomorphin (Dorsomorphin (Compound C), Stratech Scientific Limited, Newmarket, Suffolk, UK), a potent, reversible, selective AMPK inhibitor (Dai *et al.*, 2013) was dissolved in DMSO to stock concentrations of 5 mM and stored at -80^{o}C. Compound C was subsequently diluted in complete culture medium and added to melanoma cell cultures at final concentrations of 5 µM for given incubation times.

2.4 Isolation of CD271+ve and –ve subpopulations using M. Biotec magnetic columns

CD271 positive and negative subpopulations were isolated from 1×10^7 primary WM35 or metastatic A375 or SK-Mel-28 melanoma cells using M. Biotec magnetic columns (Miltenyi Biotec Ltd, Auburn, USA). Fresh wash/suspension buffer, (2 mM Ethylenediaminetetraacetic acid (EDTA), 0.5% Bovine serum albumin (BSA) in PBS) was prepared for each separation and kept on ice for the duration of the experiment. Following centrifugation of melanoma cells at 310 g for 5 minutes at room temperature and aspiration of the cell supernatant, cell pellets were resuspended in 10mls of sterile PBS in a biosafety cabinet and passed through a 30 µm CellTrics mesh (Miltenyi Biotec Pre-Separation Filters) to achieve a single cell suspension. Cells were then centrifuged again for 5 minutes at 310 g before re-suspension in 80 µl of fresh ice-cold wash/resuspension buffer containing 10 µl of human FcR blocking reagent (Miltenyi Biotec Ltd). 10 µl of CD271-PE antibody (Miltenyi Biotec Ltd) was then added to the suspension prior to incubation for 10 minutes at 4°C. 1 ml of fresh wash/resuspension ice-cold buffer was subsequently added to each cell suspension before further centrifugation at 310 g or 5 minutes, re-suspension in 70 µl of fresh ice-cold buffer and the addition of 10 µl of FcR blocking reagent and 20 µl of anti-PE microbeads (Miltenyi Biotec, Auburn, USA). The cell suspension was then incubated for another 15 minutes at 4°C before 1 ml of fresh ice-cold wash/re-suspension buffer was added to the suspension and further centrifugation at 310 g for 5 minutes. A separation magnet (MiniMACS Separator Miltenyi Biotec) was assembled in a biosafety cabinet and magnetic separation columns (MS Columns Miltenyi Biotec) attached to the magnetic stand. Magnetic columns were pre-prepared by application of 500 µl of fresh ice-cold wash/re-suspension buffer, allowed to pass through the magnet into a 15 ml falcon centrifugation/ test tube (SuperClear™ Ultra-High Performance Centrifuge Tube, VWR International LLC, Radnor, USA) which was then discarded. The cell suspension was then added directly to the magnetic column and CD271 –ve subpopulations collected into a fresh 15 ml falcon tube with 3×500 µl of fresh ice-cold wash/resuspension buffer. Following removal of the magnetic column CD271 positive subpopulations were finally eluted with 1ml of fresh buffer with the application of a column plunger to expel CD271 +ve cells into a fresh 15 ml falcon centrifuge tube. At the end of the process, parental or isolated CD271 +ve or -ve cells

were used directly or following culture for 3-9 days in complete DMEM, to derive cell lysates for Western Blotting, or fresh cell pellets were processed by the Histopathology department at the Royal Victoria Infirmary to derive formalin fixed and paraffin embedded cell blocks.

2.5 **Flow Cytometry**

2.5.1 Flow cytometry of Annexin V or Propidum Iodide (PI) labelled melanoma cells as a measure of apoptosis

The induction of apoptosis was confirmed using an Annexin V- Fluoroscein (FITC)/ Propidium iodide (PI) detection kit (ab14085, Abcam, Cambridge, UK). Upon initiation of apoptosis, phosphatidylserine translocates from the cell plasma membrane to the cell surface. Fluorescent antibodies to Annexin V can selectively conjugate to phosphatidylserine thereby allowing the quantification of the levels of apoptosis (Logue *et al.*, 2009). Counter staining with propidium iodide (PI, which binds to the fragmented DNA of apoptotic cells (Riccardi and Nicoletti, 2006) was performed to detect the number of viable cells.

A375 cells were seeded at a density of 2×10^5 well in tissue culture grade flat bottomed 6 well plates (Corning Incorporated, New York, USA) in a final volume of 3 mls of complete culture medium (DMEM/10% FCS/1%P/S) and allowed to attach overnight at 37^{o}C. The media was subsequently aspirated and replaced with either fresh complete culture media or media containing 16 nM trametinib and incubation continued for 3, 6, 9, 14, 28 or 42 days. Cells were then detached with trypsin/EDTA (as described in section 2.2) before centrifugation at 310 g for 5 minutes, followed by a washing step in 1 ml PBS and further centrifugation at 310 g for another 5 minutes. The supernatant was then aspirated and the cell pellet resuspended in 500 µl of 1 × Binding buffer from the Annexin V/PI kit with the addition of 5 µl Annexin V-FITC and 5 µl of propidium iodide prior to continued incubation at room temperature for 5 minutes in the dark. Annexin V binding and PI staining were detected using a FACS-Canto II (8 Colour) flow cytometer (BD biosciences, San Jose, USA) and data analysed using FlowJo analysis software for flow cytometry (FlowJo, Ashland, Oregon, USA). Viable cells with intact cell membrane are impermeable to PI whereas dead and dying cells are permeable to PI.

Therefore viable cells are both Annexin V and PI negative, whereas early apoptotic cells are Annexin V positive and PI negative and dead cells are both Annexin V and PI positive.

2.5.2 Flow cytometry sorting of melanoma cells overexpressing red fluorescent protein (RFP)

A375 and WM35 cells were stably transfected to express Histone H2B monomeric red fluorescent protein or RFP tagged CD271 as described in section 2.10. Transfected cells were cultured in complete DMEM in the presence of G418 at a concentration of 500 µg/ml to maintain selection of transfected cells. Following detachment with trypsin/EDTA, 1×10^6 cells were pelleted by centrifugation at 310 g for 5 minutes followed by washing in 1 ml PBS and further centrifugation at 310 g for 5 minutes. Cells were then resuspended in 1 ml FCS and passed through a 30 µm CellTrics mesh (Miltenyi Biotec Pre-Separation Filters) to achieve a single cell suspension before transfer to a FACS round bottom tube and incubation on ice prior to sorting RFP positive cells using a FACS-Aria (BD biosciences, San Jose, USA) flow sorting flow cytometer with the help of Dr Andrew Filby (Flow Cytometry Core Facility, Institute of Cellular Medicine, Newcastle University). Auto fluorescence was compensated for using untransfected WM35 or A375 cells respectively. Flow sorted RFP expressing cells were collected into a sterile tube and then returned to culture in complete DMEM for given time periods.

2.6 **Real time reverse transcriptase polymerase chain reaction (qPCR)**

qPCR was used to quantify CD271 mRNA expression in primary melanocytes, human metastatic cutaneous melanoma cell lines WM35, A375, WM266-4, SK-mel-28, WM164, SK-mel-23, CHL-1, C8161, WM-1361 and Mel 505. qPCR was also used to verify siRNA mediated knockdown of CD271 in WM35 human metastatic melanoma cells. All cell lines were seeded at a density of $2x10^5$ cells per well in 3 mls of complete DMEM per well, in 6 well flat bottom cell culture plates for 24 hours. Cells were then washed with ice cold PBS prior to RNA extraction using a ReliaPrep™ RNA Cell Miniprep System (Promega, Madison, Wisconsin, U.S.A) as per the manufacturer's instructions. The extracted RNA was quantified using a NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Waltham, USA) and stored at -80°C. mRNA was then converted to single-stranded cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Vilnius, Lithuania) as per manufacturer's instructions (Table 2-2). Transcription reactions in a total volume of 20 µl/sample were subsequently subjected to reverse transcription using a Gene Amp PCR system 9700 thermal cycler (Applied Biosystems, California, USA) with cycles of 10 minutes at 25 °C, 120 minutes at 37 °C, 5 minutes at 85 °C then 4 °C ∞). cDNA was then stored at 4 °C for short term periods of up to two weeks or at -20 °C for longer term storage.

High Capacity Reverse Transcription	
10X RT Buffer	2µl
25X dNTP Mix (100mM)	0.8 µl
10X RT Random Primers	2 µl
Multiscribe Reverse Transcriptase	0.5 µl
RNase Inhibitor	0.2 µl
Nuclease-free Water	4.5 µl
RNA (same concentration in every reaction)	10 µl

Table 2-2: Constituents of a Single Reverse Transcription Reaction

Following cDNA conversion, qPCR was then performed to determine the relative expression levels of CD271 mRNA, relative to 18s mRNA, used as a house keeping control. PCR reaction mixes were prepared using TaqMan Universal PCR Master Mix (Applied Biosystems, Warrington, UK), and pre-designed Gene Expression Assays for CD271 (NGFR human Hs00609976_m1, TaqMan, ThermoFischer Sienctific, Waltham, USA) (Table 2-3) or 18s as a housekeeping control gene (Integrated DNA Technologies, Leuven, Belgium) (

CD271 (NGFR) TaqMan® Gene Expression (single reaction volumes)	
Master mix (x2)	5 µl
Nuclease free Water	2.5 µl
CD271 TaqMan® Gene Expression Assay (FAM reporter-MGB quencher probe)	0.5 µl
cDNA	2 µl

Table 2-4).

Table 2-3: Constituents of a single PCR reaction using CD271 (NGFR) TaqMan® Gene Expression Assay

18s TaqMan® Gene Expression (single reaction volumes)	
Master mix (x2)	5 µl
Nuclease free Water	2.35 µl
18s Forward & Reverse Primers	0.2 µl
18s FAM-TAMRA Probe	0.25 µl
cDNA	2 µl

Table 2-4: Constituents of a single PCR reaction using 18s TaqMan® Gene Expression Assay

2 µl cDNA was added to 8 µl of PCR reaction mix in each well of a 96 well MicroAmp Fast Optical Reaction Plate (Applied Biosystems, California, USA). All samples were added in duplicate including a non-template control (H_2O only). Plates were then sealed with optical film and centrifuged at 161G for 30 seconds before qPCR analysis using a StepOne Plus Real-

Time PCR instrument (Applied Biosytems, California USA) with 40 cycles of: 2 minutes at 50°C hold, 10 minutes at 95°C hold, 15 seconds at 95°C denature, and 1 minute 60°C anneal/extend. mRNA expression analysis was performed using the StepOne Soft wear V2.3 (Applied Biosystems, California, U.S.A). Data analysis was performed using a comparative C_T method ($2^{-\Delta\Delta C_T}$) as previously described (Schmittgen and Livak, 2008).

2.7 Immunohistochemistry for CD271 and p62 expression in Formalin Fixed Paraffin Embedded melanoma cell pellets and primary melanoma tissue

5 µM formalin fixed and paraffin embedded tissue sections derived from cell pellets of human primary cutaneous melanoma cell line WM35 cultured in the presence or absence of 16 nM Trametinib for 9 days were used to optimise the immunohistochemical expression of CD271 according to standard methodology (Ellis *et al.*, 2014c). Optimal antigen retrieval (10 mM Tris-HCl (pH 9) and primary antibody concentrations (1:50 in PBS/2%BSA) were pre-determined and then used to determine semi quantitative expression of CD271 in a cohort of 47 FFPE primary naevi/melanomas (5 benign naevi, 5 AJCC stage I, 16 AJCC stage II, 9 AJCC stage III and patient matched metastatic melanoma lymph nodes and an additional cohort of 12 metastatic melanoma lymph nodes) and correlation with autophagy status as determined by IHC of p62 expression as previously described (Ellis *et al.*, 2014d).

5 µm FFPE sections were initially baked onto X-tra microscope slides (Leica Microsystems, Milton Keynes, UK) at 60°C overnight. Sections were then de-paraffinised in Histo-Clear (Fisher Scientific, Loughborough, UK) for 20 minutes at room temperature before dehydration through 100%, 75%, and 50% ethanol and washing in distilled water, each for 5 seconds. To optimise antigen retrieval, sections were immersed in 10 mM Tris-HCl (pH 7.6), 10 mM Tris-HCl (pH 9) or 10 mM Sodium Citrate (pH 6) pre-heated in a microwave at full power for 12 minutes before being allowed to cool for 20 minutes at room temperature. Tissue was then marked using an ImmEdge hydrophobic pen (Vector Laboratories Inc., Burlingame, USA. Sections were then rehydrated in PBS containing 0.05% Tween20 (TWEEN® 20, Sigma-Aldrich Co. LLC, U.S.A, PBS/T) for 3 minutes and subsequently incubated in 0.2% Triton X-100 (Sigma,

St. Louis, USA) in PBS/T for 10 minutes at room temperature. Endogenous peroxidase was then blocked by immersion in 3% H_2O_2 in water for 10 minutes, followed by a washing step in PBS/T. Protein blocking was performed by incubation in 2% goat serum in PBS/T (2 drops in 5 ml) from a Vectastain Elite kit (Vector, Peterborough, UK), before a further wash in PBS/T, and subsequent incubation with primary anti-human CD271 monoclonal antibody (Abcam Biochemicals, Cambridge, UK; ab31251); diluted 1:50 in 2% BSA/PBS, or primary anti-p62 antibody (Santa Cruz Biotechnology, USA;SQSTM1 Antibody (D-3): sc-28359) diluted 1:50 in 2% BSA/PBS overnight at 4^{o}C. Sections were then washed three times with PBS/T and incubated with anti-mouse 2° antibody diluted 1:200 in 2% serum/PBS/T from a Vectastain Elite kit for 30 minutes at room temperature. Following three washes with PBS/T sections were then incubated with ABC reagent from a Vectastain Elite kit (pre-mixed 30 minutes prior to use) at room temperature for 30 minutes, again washed three times in PBS/T, and then incubated with VIP solution (Vector Laboratories Inc., Burlingame, USA) for 10 minutes at room temperature prior to counterstaining with haematoxylin (Thermo Fisher Scientific, UK) for 2 minutes, and rinsing in tap water for 10 minutes. Sections were finally dehydrated through 75% and 100% ethanol for 5 seconds and incubated in histoclear for 2 minutes, before air drying and mounting with a coverslip and DPX mounting medium (VWR International Ltd., Poole, UK). Sections were then imaged by light microscopy and representative images captured using a Zeiss Axio Imager microscope (Carl Zeiss Microscopy New York, U.S.A). Sections following CD271 or p62 staining were digitally imaged using an automated slide scanner (Leica SCN400 digital slide scanner (Leica Biosystems, Milton Keynes, UK)), Newcastle University Biobank. CD271 or p62 staining intensity was calculated using the Leica digital slide scanner at ×20 magnification using a semi quantitative calculation of percentage staining intensity of 10 representative areas and statistical comparison using a one-way ANOVA test with Tukey's multiple comparison test.

2.8 Immunofluorescence for the expression of CD271 and p62 in Cutaneous Human Melanoma Cell lines

Melanoma cells were seeded onto glass cover slips (22x22 mm) at a density of $5x10^4$ cells /well of 6 well tissue culture plates (Corning Incorporated, New York, USA), covered with 3 mls of complete culture medium and allowed to attach overnight before the culture medium was aspirated and adherent cells washed twice in PBS for 5 minutes and subsequently fixed with ice-cold paraformaldehyde (4%) in PBS for 15 minutes on ice. Cells were then washed three times with PBS and subsequently incubated in 0.2% Triton X-100 (Sigma, St. Louis, USA) in PBS/T for 10 minutes before a blocking step with 2% goat serum (Sigma, Dorset, UK) in PBS/T for 30 minutes at room temperature. Coverslips were then transferred onto a glass slide and marked using an ImmEdge hydrophobic pen (Vector Laboratories Inc., Burlingame, USA). Slides were incubated with 100 µl of a primary anti-human CD271 monoclonal antibody (Abcam Biochemicals, Cambridge, UK; ab31251) or anti-p62 (Santa Cruz Biotechnology Inc. USA) all diluted 1:50 in 2% BSA and 2% goat serum (Sigma, Dorset, UK) in PBS or in PBS/T alone as a negative control at 4°C overnight. Cells were then washed in PBS/T three times before incubation with a fluorescent secondary antibody Alexa Fluor® 488 Goat Anti-Mouse IgG (Life Technologies, Eugene, Oregon USA) or Alexa Fluor® 568 Goat Anti-Mouse IgG (Life Technologies, Eugene, Oregon USA) to detect primary CD271 and p62 binding. All fluorescent secondary antibodies were diluted 1:250 in 2% BSA and 2% goat serum in PBS with the addition of diamidino-2-phenylindole dye (DAPI, Thermo Fisher Scientific, Thermo Fisher Scientific, Illinois, U.S.A) diluted 1:1000 prior to1 hour incubation at room temperature. Finally, cells were washed with PBS/T three times before mounting with a cover slip using hard set anti-fade mounting medium (Vector labs, Burlingame, California, USA). Fluorescent intensity was visualised and representative images captured using a Leica TCS SP2 UV confocal microscope and analysis with LC2 2.61 software (Leica Microsystems GmbH Heidelberg).

2.9 **Western Blotting**

Western blotting was carried out using standardized methodology (Armstrong *et al.*, 2015a). Briefly, following trypsinisation of melanoma cells and collection of cells and the entire supernatant (to ensure any detached cells following drug treatment were also captured) cells were washed in PBS and pelleted by centrifugation into microfuge tubes before the addition of 50-500 µl of ice cold cell lysis buffer (0.1 M Tris-HCl pH 7.4, 25 mM NaF, 0.1 M NaCl, 2 mM EDTA (pH 8), 1 mM benzamidine, , 0.1% Triton-X100 and 0.1 mM sodium orthoVanadate) for approximately 50,000 to 2 X 10^6 cells, containing 150 µl/ml of freshly added protease inhibitor cocktail (Promega, Southampton, UK). Protein was extracted by sonication with a probe sonicator (Soniprep 150, MSE, UK) for 2 pulses of 5 seconds each at amplitude of 7 microns. Protein concentration was then determined using a Bradford protein quantification assay (Pierce Biotech, Rockford, USA) according to the manufacturers specifications with protein absorbance measured at 595 nm using a SpectraMAX 250 plate reader (Molecular Devices Ltd. UK). 10 µg of protein lysate was diluted at a ratio of 1:3 in 4x sample buffer (250 mM Tris HCL (pH 8), 8% sodium dodecyl sulphate (SDS), 40% glycerol, 10% β-mercaptoethanol and bromophenol blue). Samples were then denaturated at 95^{o}C for 10 minutes on a heat block. Protein separation was performed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with 4-20% tris-glycine gels (Bio-Rad, UK) in a (SDS) running buffer (25 mM Tris base,190 mM glycine 0.1% SDS) and incorporating Bench MarkTM Prestained Protein Ladders (Invitrogen, Paisley, UK; 10748-010). Proteins were then transferred onto a PVDF membrane (Bio-rad, UK) using a Bio-rad Turbo-blotter (1.3A, 25V; Bio-Rad, UK) for 15 minutes before membranes were blocked with 5% non-fat milk (OXOID ltd, Baisingstoke, UK) in Tris-buffered saline (Tris base and NaCl in Millipore water, pH 7.6;TBS) for 1 hour at room temperature. Membranes were then incubated with primary antibodies (Table 2-5) over night at 4^{o}C, before washing three times in TBS with 0.05%Tween 20 (TBS/T) for 15 minutes each and incubation with relevant secondary antibodies; anti-rabbit (Vector Laboratories, Burlingame, USA) for the detection of CD271, LC3 I/II, cleaved caspase 3, cleaved PARP, ERK1/2, phospho ERK1/2, phospho AMPKa, total AMPK, GAPDH and Nestin or anti-mouse secondary antibody (Vector Laboratories, Burlingame, USA) to detect p62, ABCB5, Jarid-1B, SOX10 and β-actin. All secondary antibodies were diluted 1:5000 in 5% non-fat milk in TBS/T.

Membranes were then again washed in TBS/T three times for 15 minutes each before protein expression was visualized using the ECL-plus system (Clarity Western ECL-Substarte, Bio-Rad, USA) and either photographic film (Super RX-N, Fujifilm) or Odyssey FC Image studio soft-wear (Li-cor Biosciences, Lincoln, Nebraska, U.S.A). Photographic film was developed using a developer (MINIMED90 X-Ray film processor; photon imaging systems, AFP imaging corp., Elmsford, NY, U.S.A).

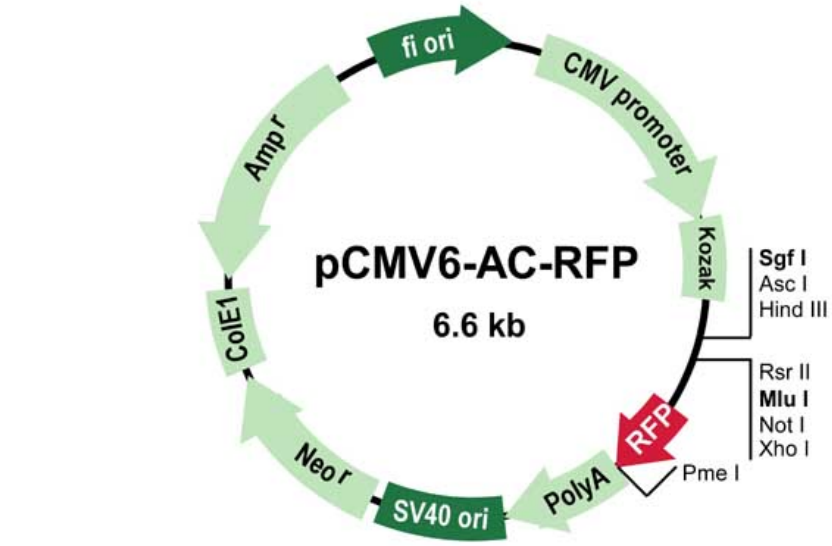
Antibody	Supplier	Species	Dilution	Diluent	Molecular weight (kDa)
CD271	Cell signaling (D8A8) #4201)	Rabbit monoclonal antibody	1:5000	5% non-fat milk in TBS	75kDa
LC3 I-II	Cell Signaling (#2775)	Rabbit monoclonal antibody	1:2000	5% BSA in TBS	14-16kDa
p62	Santa Cruz Biotechnology (sc-28359)	Mouse monoclonal antibody	1:1000	5% non-fat milk in TBS	62kDa
β-actin	Sigma-Aldrich (AC-74)	mouse monoclonal antibody	1:40000	5% non-fat milk in TBS	45kDa
Cleaved Caspase 3	Cell Signaling (#9664)	Rabbit monoclonal antibody	1:1000	5% non-fat milk in TBS	17-19kDa
Cleaved PARP	Cell Signaling (Asp214)	Rabbit polyclonal antibody	1:500	5% non-fat milk in TBS	89kDa
ABCB5	Abcam (ab140667)	Mouse monoclonal antibody	1:1000	5% non-fat milk in TBS	90kDa
KDM5B Jarid-1B	Abcam (ab56759)	Mouse monoclonal antibody	1:1000	5% non-fat milk in TBS	190kDa
Nestin	Abcam (ab105389)	Rabbit monoclonal antibody	1:1000	5% non-fat milk in TBS	177kDa
Sox10	Sigma-Aldrich (SAB1402361)	mouse monoclonal antibody	1:1000	5% non-fat milk in TBS	37kDa
p44/42 MAPK (ERK1/2)	Cell Signaling (#9102S)	Rabbit monoclonal antibody	1:5000	5% non-fat milk in TBS	42-44kDa
Phospho-p44/42 MAPK (Phospho –ERK1/2)	Cell Signaling (#4370)	Rabbit monoclonal antibody	1:5000	5% non-fat milk in TBS	42-44kDa
Phospho-AMPKα	Cell Signaling (#4188)	Rabbit monoclonal antibody	1:2000	5% non-fat milk in TBS	62kDa

Table 2-5: List of primary antibodies used for western blot analysis

2.10 **Lentiviral transduction of human melanoma cell lines with histone 2B-RFP construct or over expression of CD271**

The Histone H2B monomeric red fluorescent protein lentiviral vector (pHIV-H2BmRFP, Addgene plasmid # 18982) was transformed into competent E.Coli (One Shot® TOP10 Chemically Competent E. coli, Invitrogen) following a standard recommended heat shock procedure as per the manufacturer instructions (Moore *et al.*, 2010). Bacterial colonies grown on Lysogeny broth (LB) agar plates containing 50 µg/ml ampicillin (Sigma Aldridge, Poole, UK) were selected and expanded in LB broth (Life Technologies, Paisley, UK). Plasmid DNA was purified using maxi Prep kits (QIAGEN Kit, Venlo, Limburg, Netherlands) according to the manufacturer's specifications. 20 µg of lentiviral plasmid DNA was then co-transfected with 5 µg of an expression plasmid for the viral envelope (pMD2.G) and 15 µg packaging plasmid (pCMVdelta8.91) into HEK293T cells at 80% confluency by standard calcium precipitation (Armstrong *et al.*, 2015a) method. The following day the transfection media was removed and cells washed with PBS before the addition of fresh complete culture media and incubation at 37°C continued for 72 hours. Lentiviral containing supernatant was then harvested and centrifuged at 310 g for 15 minutes before filter sterilization through 0.45 µm filters and storage at -80^{o}C prior to use. Human melanoma cell lines (5×10^{4} cells/well of a 6 well plate) were spin transduced with 2 mls of the viral supernatant supplemented with polybrene (4 µg ml^{-1}) at 310 g for 90 minutes. The viral supernatant was then removed, cells washed with PBS before returning to culture in normal growth culture media (DMEM, 10% FCS, PS). Flow cytometry was used to select for RFP expression using a FACS Aria cell sorter and representative images captured using a confocal microscope (Leica TCS SP2 confocal microscope, Leica microsystems) as described in section 2.5.2.

For stable overexpression of CD271, a mammalian vector with C-terminal tRFP tagFP (pCMV6-AC plasmid vector containing RFP tagged CD271, Origene PS100034, Rockville, Maryland, USA) (Figure 2-1) was transformed into competent E.Coli (One Shot® TOP10 Chemically Competent E. coli, Invitrogen) following a standard recommended heat shock procedure as per the manufacturer instructions (Moore *et al.*, 2010). Bacterial colonies grown on Lysogeny broth (LB) agar plates containing 50 µg/ml ampicillin (Sigma Aldridge, Poole, UK) were selected and expanded in LB broth (Life Technologies, Paisley, UK). Plasmid DNA was purified using maxi Prep (QIAGEN Kit, Venlo, Limburg, Netherlands) according to the manufacturer's specifications.



Schematic of the multiple cloning sites:

pCMV6-AC-RFP

```
                                                                  Kozak
                                                                  Consensus
                    EcoR I          BamH I Kpn I      RBS             Sgf I      Asc I
CTATAGGGCGGCCGGGAATTCGTCGACTGGATCCGGTACCGAGGAGATCTGCCGCCGCGATCGCCGGCGCGCCAGATCT

  Hind III          Rsr II     Mlu I          Not I      Xho I          RFP Tag
CAAGCTTAACTAGTTAGCGGACCG ACG CGT ACG CGG CCG CTC GAG ATG AGC GAG CTG--- --- ---
                           T   R   T   R   P   L   E   M   S   E   L   -   -   -

                                    Pme I     Fse I
--- GGG CAC AGA TGA GTT TAA ACGGCCGGCCGCGG
 -   G   H   R Stop  -  Stop
```

Figure 2-1: Map of pCMV6-AC plasmid vector.
To assess the effect of CD271 overexpression, melanoma cells were transfected with a CD271 overexpressing construct tagged with a C-terminal tRFP tag.

Following DNA purification, a reverse transfection approach was adopted using LTX Lipofectamine (Lipofectamine® LTX with Plus™ Reagent, Thermo Fischer Scientific, Waltham, USA). A tranfection mixture was prepared by mixing 1 µg of DNA with 500 µl DMEM (10% FCS but without antibiotics) in a 1.5 ml Eppendorf tube. 5 µl of PLUS reagent was then added to the mixture followed by a 5-minute incubation at room temperature before the addition of 10 µl of Lipofectamine LTX and a further 30-minute incubation at room temperature. The transfection mixture was then dispersed in one well of a flat bottomed 6 well plate (Corning Incorporated, New York, USA). A cell suspension of 3.5×10^5 A375 or WM35 cells was then added dropwise to the transfection mixture before continued incubation at 37^{o}C for 6-8 hours. The transfection was then terminated by carefully aspirating the transfection mixture and replacing with complete DMEM before again continued incubation for a further 48 hours. Transfected cells were then continuously selected through neomycin resistance by the addition of 500 µg/ml G418. RFP expressing clones were verified by visualization with a Leica TCS SP2 UV confocal microscope and LC2 2.61 analysis software. This technique however resulted in low transfection efficiency and therefore an alternative overexpression approach was used using Xfect Transfection Reagent (Clontech, Mountain View, California, USA).

For CD271 over expression using Xfect, A375 or WM35 cells were seeded at a density of 2×10^5 cells per well of a flat bottom 6 well plate before allowing cells to attach overnight prior to replacing the media with 1 ml of fresh complete media and the addition of pre mixed transfection mixtures for 4 hours. Pre prepared transfection mixtures for each transfection comprised 0.4 µl of Xfect polymer transfection reaction buffer, 2 µg of cDNA and Xfect reaction buffer to a total volume of 100 µl, which was premixed and incubated for 10 minutes at room temperature before brief centrifugation and dropwise addition to cell cultures. The transfection reaction was subsequently terminated by gentle aspiration of the transfection mixture and the addition of 2 mls of fresh complete media to each well. Transfected cells were then continuously selected by the addition of 500 µg/ml G418 prior to confirmation of RFP expression by visualization using a Leica TCS SP2 UV confocal microscope and LC2 2.61 software.

2.11 Stable (ShRNA) and Transient knockdown (SiRNA) of CD271 in human melanoma cell lines

Silencing mediated knockdown of CD271 was performed using either a short hairpin RNA (shRNA) or Small Interfering RNA (siRNA) approach. The incorporation of shRNA into host cells through lentiviral infection allows for stable integration of shRNA and long-term knockdown of the targeted gene (Taxman *et al.*, 2010). However, an alternative approach of using silencing RNA, which functions by oligonucleotides interfering with the expression of target genes by degradation of the mRNA and therefore preventing translation of the target gene (Rao *et al.*, 2009) was also used.

2.11.1 ShRNA- mediated knockdown of CD271

ShRNA-mediated knockdown of CD271 in melanoma cells was performed in collaboration with Dr Paola Giglio and Dr Marco Corazzari, University of Rome 'Tor Vergata', Italy using lentiviral transduction of a shRNA targeting CD271 with five different clones targeting different regions of CD271 (SHCLNG-NM_002507, NGFR MISSION shRNA Bacterial Glycerol Stock nerve growth factor receptor, Sigma Aldrich, St Louis, USA, Table 2-6).

ShRNA plasmid sequences		
Clone	**Sequence**	**Region**
Clone 1	CCGGGCACTGTAGTAAATGGCAATTCTCGAGAATTGCCATTTACTACAGTGCTTTTTG	3UTR
Clone 2	CCGGCCGAGCACATAGACTCCTTTACTCGAGTAAAGGAGTCTATGTGCTCGGTTTTTG	CDS
Clone 3	CCGGCCTCCAGAACAAGACCTCATACTCGAGTATGAGGTCTTGTTCTGGAGGTTTTTG	CDS
Clone 4	CCGGGACAACCTCATCCCTGTCTATCTCGAGATAGACAGGGATGAGGTTGTCTTTTTG	CDS
Clone 5	CCGGGCCTACGGCTACTACCAGGATCTCGAGATCCTGGTAGTAGCCGTAGGCTTTTTG	CDS

Table 2-6: Sequences of five differing ShRNA's targeting CD271.

Plasmid vectors ShRNA to CD271 or scrambled control sequence vectors were transformed into competent E.Coli following a standard recommended heat shock procedure as per the manufacturer instructions. Bacterial colonies grown on Lysogeny broth (LB) agar plates containing 50 µg/ml ampicillin (Sigma Aldridge, Poole, UK) were selected and expanded in LB broth (Life Technologies, Paisley, UK). Plasmid DNA was purified using maxi Prep (QIAGEN Kit, Venlo, Limburg, Netherlands) according to the manufacturer's specifications. 10 µg of lentiviral plasmid vector (shRNA-pLKP ERp57, Sigma Aldrich, St. Louis, Missouri, USA) was then co-transfected with 2.5 µg of an expression plasmid for the vesicular stomatitis virus G protein and psPAX2 plasmid, containing gag, pol and rev genes into HEK293T cells at 80% confluency by standard calcium precipitation (Armstrong *et al.*, 2015a) method. The following day the transfection media was removed and cells washed with PBS before the addition of fresh complete culture media and continued culture at 37°C for 48 hours. The lentiviral particle containing supernatant was then harvested and centrifuged at 310 g for 15 minutes before filter sterilization through 0.45 µm filters and storage at -80°. A375 cells (2×10^5 cells in 10cm tissue culture dish) were transduced with 5mls of the viral supernatant supplemented with polybrene (4 µg ml^{-1}) for 6- 8 hours, before aspiration of the lentiviral supernatant, the addition of fresh complete DMEM media and further culture for 48 hours prior to confirmation of CD271 knockdown by qPCR as described in section 2.6.

2.11.2 SiRNA- mediated knockdown of CD271

A375 or WM35 cells were seeded at a density of $1.5x10^5$ cells/well, in 6 well culture plates in 3ml of complete culture medium and allowed to attach overnight at 37°C. Three transfection mixtures were prepared, one containing 2.5 µl of lipofectamine RNAiMax reagent (Invitrogen, Carlsbad, California, U.S.A) in 125 µl Opti-MEM 1x reduced serum medium (life technologies, New York, U.S.A)/ well of a six well plate, one containing pre-siRNA mixtures consisting of 2.5 µl of ON-TARGETplus Human NGFR siRNA (SMART pool siRNA, Dharmacon, GE lifesciences, Lafayette, Colorado, U.S.A) and one containing Stealth RNAi™ siRNA Negative Control (none target) (Invitrogen, Carlsbad, California, U.S.A) in 125 µl Opti-MEM1x reduced serum medium (Life technologies, U.S.A). Following incubation for 30 minutes, lipofectamine and siRNA

mixtures in OPTIMEM were combined and incubation at room temperature continued for a further 15 minutes.

Following removal of the media from the adhered cells to be transfected and a brief wash in PBS, the media was replaced with 1 ml Opti-MEM1x reduced serum medium and 250 µl of each respective siRNA/ lipofectamine mixture (added dropwise) and incubation at 37^{o}C continued for 6 hours. The transfection was then terminated by replacing the transfection solution with 3 mls of fresh complete DMEM media and transfection efficiency and CD271 knockdown verified by qPCR or western blotting after 24, 48 or 72 hours as described in section 2.6 and 2.9 respectively.

2.12 **Cell Viability Assays**

All melanoma cell lines were seeded in complete culture media at a density of 5 or 2.5×10^{3} cells/well in a volume of 100 µl per well of a 96-flat well plate (Corning Incorporated, New York, U.S.A), with at least four replicates per experimental condition, prior to being allowed to attach overnight at 37^{o}C. The cell culture media was then replaced with 100 µl of fresh media containing a given drug/drug combination before incubation was continued for a further 24 or 48 hours. Cell viability was assessed by the addition of 20 µl of Aqueous NonRadioactive Cell Proliferation Assay (3- (4,5- dimethylthiazol- 2- yl)- 5-(3- carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium, inner salt; MTS, CellTitre 96, Promega, Southampton, UK) to each well (including blank wells containing just media) for 4 hours and colorimetric detection by measuring absorbance at 490 nm using a Spectra Max 250 plate reader (Molecular Devices).

Alternatively to assess cell viability using MTT (Thiazolyl Blue Tetrazolium Bromide, Sigma-Aldrich, St Louis, USA) melanoma cells were seeded at a density of 5×10^{4} cells per well in a 12-flat well tissue culture plate (Corning Inc, Corning, NY, USA) and allowed to adhere overnight, prior to drug treatment for given times and the assessment of cell viability by the addition of 100 µL of Thiazolyl Blue Tetrazolium Bromide at a concentration of 5 mg/ml and continued incubation for 4-hours before aspiration and replacement with 300 µL of Isoproplanol/ Hydrochloric acid (HCL) solution (5 ml HCL in 500 ml Isopropranol) for 5 minutes and the measurement of absorbance at 570 nm using a Spectra Max 250 plate reader (Molecular Devices).

2.13 **Colony forming Assay**

To assess the ability of melanoma cells to undergo division and develop colonies following treatment with either trametinib, chloroquine, PIK-III or combined treatment with trametinib with either chloroquine or PIK-III, a colony forming assay was performed. A375 or Sk-mel-28 cells were initially treated with trametinib (16 nM) for 9 days before subsequent treatment for 48 hours with either trametinib alone, chloroquine (10 µM) alone, PIK-III (5 µM) alone or combination of trametinib with chloroquine or PIK-III. Drug treated or control untreated cells were then detached with trypsin EDTA before three replicates of 100, 250, 500 or 1000 cells in 7 mls of fresh complete media were subsequently seeded into a 10 cm tissue culture dish (Corning Inc., Corning, USA), dispersed evenly and incubated at 37^{o}C for 2 weeks. At the end of the 2 week period, the cell culture media was aspirated and colonies were gently washed in PBS before fixing in 5mls Carnoy's fixative (100 mls acetic acid in 300 mls methanol) for 5 minutes. The Carnoy's fixative was then aspirated and plates were washed in PBS before the addition of 5 mls of 0.4% Crystal Violet (1% Crystal Violet V5265, Sigma Aldrich, St Louis, USA) for 5 minutes. The excess crystal violet was then washed off by careful use of a fine flow of tap water to prevent disturbance of the colonies. The number of colonies were then counted using a colony counter (ColCount^{TM} Oxford Optronics, Abingdon, UK) and the colony efficiency and percentage of cell survival calculated using Equation 2.1 and Equation 2.2 as below:

$$\%\ cloning\ efficiency = \left(\frac{colonies\ counted}{cells\ seeded}\right) x100 \qquad \text{Equation 2.1}$$

$$\%\ survival = \left(\frac{drug\ treated\ cell\ cloning\ efficiency}{control\ cell\ efficiency}\right) x100 \qquad \text{Equation 2.2}$$

2.14 **Collagen Invasion Assay**

Collagen Invasion assays were used to model melanoma cell invasion *in vitro* (De Wever *et al.*, 2014) and specifically the invasive capacity of the human BRAF mutant melanoma cell line, WM35 following long exposure to Trametinib (16 nM) for 9 days and subsequent drug treatment. The wells of 96 well TC grade plates (Corning Incorporated, New York, USA) were pre-coated with 100 µl/well 1.5% low melting point agarose (Agarose, low gelling temperature, A-9414, Sigma-Aldrich Co. LLC, USA), dissolved in PBS by microwave heating for follow 1-3 minutes) for 2 hours, prior to the addition of 5×10^3 control or trametinib treated WM35 cells to each well is a volume of 200 µl/well and culture for 72 hours.

Collagen mix was prepared (Table 2-7) by pre-mixing collagen (Collagen I Bovine Protein (A1064401, Thermo Fisher Scientific, Paisley, UK)), H_2O, Modified Eagle's Medium (EMEM 12-684F, Lonza, Vervies, Belgium) and L-Glutamine (200 mM L-Glutamine solution G7513, Sigma-Aldrich Co. LLC, U.S.A) on ice followed by the addition of 7.5% $NaHCO_3$ and FCS. 200 µl of resulting collagen mix was then added to each well of a 24 well plate and allowed to set at 37°C for 5 minutes.

Collagen mix for collagen invasion assay	
(volumes per 4 well of a 24 well plate)	
Collagen I	1008 µl
H2O	846 µl
EMEM	224 µl
L-Glutamine	20 µl
NaHCO3	68 µl
FCS	248 µl

Table 2-7: Constituents of collagen mix for collagen invasion assay for 4 wells of a 24 well plate

Spheroids were then visualised using a stereomicroscope and 3 spheroids per condition were harvested using a 1 ml pipette tip into a 1.5 ml Eppendorf tube (Eppendorf UK Limited, Stevenage, UK) and allowed to settle by gravity. The excess media was then carefully aspirated before spheroids were resuspended in 300 µl of the collagen mix and carefully transferred

onto the collagen coated wells with continued incubation at 37°C for 15 minutes. 1 ml of complete DMEM containing trametinib, TAT-PEP5, RO-08, NGF or PIK III (Vps34 inhibitor) was then added to each well. Spheroids were visualised using a Nikon A1R confocal microscope and images were captured after 0, 2, 4 and 7 days continued incubation. Spheroid invasion was analysed using Volocity 3D Image Analysis Software (Volocity 6.3, High performance 3D imaging software, PerkinElmer, Waltham, Massachusetts, USA).

2.15 **CellTiter-Glo® Luminescent Cell Viability Assay**

A commercial CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, USA) was used to measure cellular ATP release and therefore estimate the number of viable cells cell viability in WM35 and A375 cells subjected to treatment with trametinib. ATP released from cells is a recognized marker of viable cells. Once cells lose their membrane integrity, the cellular ability of ATP synthesis quickly diminishes and endogenous ATPases degrade any remaining cytoplasmic ATP (Kleijn *et al.*, 2016). Untreated control or WM35 and A375 melanoma cells pre-treated with 16 nM trametinib for 8 days were seeded at a density of 5×10^3 cells/well in a volume of 100 µl per well of an opaque walled 96 flat well plate (Corning® opaque 96 well plates, Sigma-Aldrich Co. LLC, U.S.A), with at least four replicates per experimental condition and four replicates to be used as a positive control, prior to culture overnight at 37°C, to allow cell attachment. Cell culture media was then replaced with 100 µl of fresh media ± 16 nM Trametinib before incubation was continued for a further 24 hours. ATP release was assessed by the addition of 100µl of CellTiter-Glo reagent per well (including to some wells containing media alone, to measure the background luminescence). For induction of ATP release in the positive control cells, cells were additionally treated with 10 µl of 10% sodium dodecyl sulphate (SDS) (sodium dodecyl sulphate, 436143, Sigma Aldrich, St Louis, USA) in de-ionized water for 2 minutes at room temperature on an orbital shaker before the luminescence measured as light intensity of the rate of catalysis by luciferase was recorded using a luminometer (GloMax® Explorer, Promega, Wisconsin, USA).

2.16 Enzyme-linked immunosorbent assay (ELISA) to detect endogenous levels of phosphorylated AMPKα

Cutaneous melanoma cell lines, A375 and WM35 were initially seeded in 7 mls of complete media in 10 cm tissue culture dishes at a density of 2×10^6 in case of trametinib treatment or 2 $x10^5$ in the case of untreated controls in duplicate conditions. Following overnight attachment of cells at 37° C, the cell media was replaced with fresh complete culture media in the presence or absence of 16 nM of trametinib and culture continued for 9 days, changing the media ± trametinib every 3 days. Following 9 days the media of untreated cells was aspirated and replaced with fresh media containing 10 mM hydrogen peroxide (H_2O_2) (30% Hydrogen peroxide solution in H_2O, Sigma Aldrich, St Louis, USA) before continued culture for 10 minutes at which point the media was aspirated from all conditions (untreated control, untreated control plus H_2O_2 for 10 minutes or 9 day trametinib treatment) and cells subsequently washed in ice cold PBS. The tissue culture dishes were then placed on ice prior to the addition of 800 µL in case of untreated control +/- H_2O_2 cells or 500 µL of ice cold Elisa Lysis buffer (PathScan Sandwich ELIZA lysis buffer, Cell Signalling, Danvers, Massachusetts, USA) and the detachment of cells using cell scrapers (25 cm cell scraper, Sarstedt, Newton, USA). Cell lysates were then incubated on ice for 30 minutes before protein concentration was determined using a commercial Bradford assay as described in section 2.9. 10 µg of protein from each sample was then used to determine the level of endogenous pAMPKa.

A commercial sandwich enzyme-linked immunosorbent assay (ELISA), PathScan® Phospho-AMPKα (Thr172) Sandwich ELISA Kit #7959 (Cell Signalling, Danvers, USA) was used to detect pAMPKa secretion as per manufacturer's specifications. Absorbance was measured at 450 nm using a Spectra Max 250 plate reader (Molecular Devices, Workingham, UK) as previously described. *In vitro* pAMPKa secretion by cell lines was expressed as a mean of three individual experiments for cell lines and analysed by two-way ANOVA analysis of variance with Tukey's post hoc correction.

2.17 Use of a zebra Fish xenograft model of human metastatic melanoma to study melanoma invasion in vivo.

To evaluate the potential efficacy of autophagy inhibition on the prevention of systemic invasion on trametinib-induced resistant melanoma cells *in vivo,* a zebrafish model of human metastatic melanoma was developed in collaboration with Dr Bill Chaudhry (Institute of Genetic Medicine, Newcastle University) and Dr David Hill (Institute of Cellular Medicine, Newcastle University). Although murine models have been extensively used to monitor systemic melanoma invasion, zebrafish models are increasingly used to model cancer development and dissemination (Lieschke and Currie, 2007), with advantages including the ability to follow developing pathologies using real time imaging (Santoriello and Zon, 2012).

Transparent transgenic Casper *(flk1:GFP) tagged* zebrafish embryos, which lack skin pigment and express green fluorescent tagged vasculature were used throughout the present studies.

Studies describing how the optimal environmental temperature and oxygen conditions were derived for the growth and monitoring of melanoma invasion *in vivo* using the described zebra fish xenograft are further described in chapter 5.

Adult zebrafish colonies were maintained in a dedicated fish facility at the Institute of Genetic Medicine, Newcastle University with approximately 30 adult fish/tank. Zebrafish were paired for breeding before the eggs were collected 24 hours later, cleaned and incubated in 1×E3 media (50x E3 medium: 5.0 mM NaCl (14.6g), 0.17 mM KCl (0.65g), 0.33 mM CaCl (2.20 g), 0.33 mM MgSO4 (4.05 g) in 1 lt H_2O) at 28.5^oC, 21% O_2 and 0.04% CO_2 for 48 hours.

2.17.1 Preparation and DiI labelling of melanoma cells for inoculation into zebra fish embryos

A375 cells were cultured in a T75 tissue culture flask (Corning® 75 cm² U-shape cell culture flask, canted neck, Corning, New York, USA) in 10 mls of complete DMEM or DMEM containing 16 nM trametinib for 9 or 42 days before the media was aspirated and replaced with 5 mls of complete DMEM media containing 25 µl 1,1'-Dioctadecyl-3,3,3',3'-

Tetramethylindocarbocyanine Perchlorate (DiI) dye (DiI Stain (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate ('DiI'; $DiIC_{18}$(3), D282, Invitrogen, California, USA) for 20 minutes at 37^{o}C . The media was then aspirated and cells washed three times with warm complete DMED for 10 minutes before detaching with trypsin/EDTA as previously described. 500 µl of 15 µm Fluorospheres (FluoSpheres® Polystyrene Microspheres, 15 µm, blue fluorescent (365/415), Thermo Fischer Scientific, Massachusetts, USA) were transferred into a 15 ml sterile plastic centrifuge tube before the addition of 10 mls sterile PBS and centrifugation at 310 g for 5 minutes. The supernatant was then aspirated and mixed with the detached melanoma cells before the combined cell/ fluorosphere mix was centrifuged again at 310 g for 5 minutes and the pellet finally resuspended in 100 µl complete DMEM, prior to inoculation into zebra fish embryos as described below.

2.17.2 Injection of human melanoma cells in the yolk sac of 2 day post fertilisation zebrafish embryos

Viable two-day post fertilization zebrafish embryos were selected, transferred into a 10 cm petri dish and anaesthetized in an E3/tricaine containing solution (46 mls E3 media / 4 mls tricaine (Stock: 400 mg tricaine (Ethyl 3-aminobenzoate methanesulfonate, Sigma Aldrich, Missouri, USA), 0.26 g Tris, 100 ml E3. pH 7) and prior to embedding the embryos in a mix of E3 media and low melting point agarose prepared as follows: 250 mg of low melting point agarose (Sigma-Aldrich, USA) was dissolved in 25 mls of E3 media by microwave heating and cooled to 35^{o}C before the addition of 1 ml of tricaine and transfer to the lid of a 10 cm petri dish and embedding of the anaesthetized zebrafish embryos. To facilitate embedding, the excess agarose/tricaine was removed to allow zebrafish to be embedded into a thin layer of agarose. Microinjection needles (Glass capillaries made of borosilicate glass 3.3, with filament, Hilgenberg, Malsfeld, Germany) were initially pulled with a needle puller (Sutter instruments Co - Model P-97 micropipette puller) at a setting of Heat=638, Pull=100, Velocity=80, Delay=200 before attachment to a pump injector (Nanoliter 2000, Microprocessor controlled nanoliter injection system, World Precision Instruments, Hertfordshire, UK) and the needle being broken to a diameter of 30-40 µm. DiI labelled cells/ fluorospheres were then aspirated

into the microinjection needle prior to injection of 20 nl of cell/flourosphere solution into the inferior part of the yolk sac of anaesthetized zebrafish embryos (

Figure 2-2)

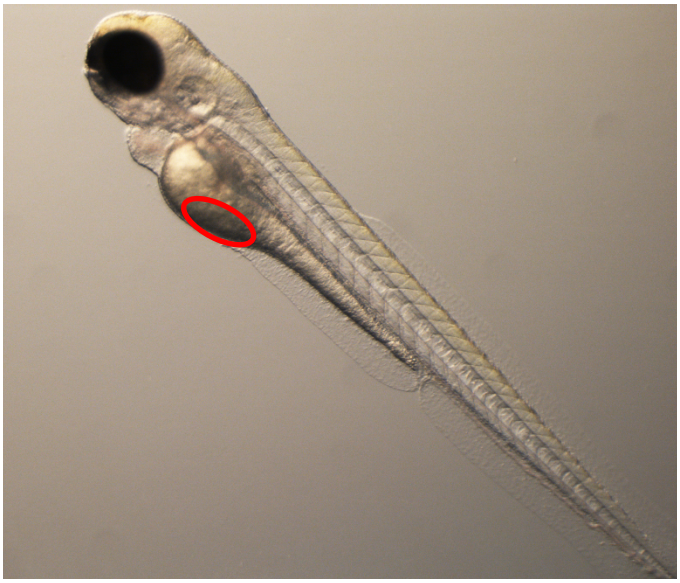


Figure 2-2: Two day post fertilization* Tg(flk1:GFP) *zebrafish embryo.
Marked injection site (red circle) in the inferior part of the yolk sac.

Following injection, fish were examined for the presence of melanoma cells within the yolk sac using a fluorescent stereo Leica M165 FC microscope (Leica, Wetzlar, Germany) through a dsRed filter. Fish with cells outside the injection site, at multiple sites or without cells were discarded and suitable fish were carefully taken out of the agarose/tricaine solution and placed individually into one well of a 24 well plate containing 1 ml of E3 media ± 16 nM trametinib, 5 µM PIK III or combined trametinib/PIK III and incubation at 33°C, in an atmosphere of 5%O_2, 5% CO_2 for 72 hours.

2.17.3 Confocal microscopy imaging zebrafish xenografts

To monitor tumour invasion and dissemination, Zebrafish were then carefully removed and transferred into a glass bottomed Wilko dish in a small amount of agarose/tricaine solution and orientated with the injection site facing towards the bottom of a Wilko glass bottomed

dish (35 mm glass bottom culture dish, 10 mm glass window, MatTek Corporation, Ashland, USA). The surrounding agarose was allowed to solidify before the addition of 3-5 mls of E3 media containing tricaine and images captured using an inverted Nikon A1R confocal microscope at 460 nm (blue beads), 488 nm (green blood vessels) and 564 nm (red cells) and analysed using Velocity 3D Image Analysis Software (Velocity 6.3, High performance 3D imaging software, PerkinElmer, Waltham, Massachusetts, USA).

2.18 **Statistics**

Throughout this thesis data were analysed using Statistical software Graph Pad Prism 7 (Graph Pad Prism version 7, Graph Pad, San Diego, CA USA). All data were assessed for normality using a Shapiro-Wilk Test. In datasets where there were not enough data points to use a Shapiro-Wilk Test, normality was not assumed and non-parametric tests were applied. P values of P< 0.05 *, P< 0.01 **, P< 0.001 ***, P<0.0001 **** were regarded as statistically significant throughout the thesis and values P > 0.05 were regarded non-significant (ns).

In *vitro* protein expression data was acquired using Odyssey FC Image studio soft-wear (Li-cord) and expression intensity normalized to the corresponding loading control (β-actin or GAPDH). Densitometry expression data for each protein analysed were presented as the mean of three individual replicate experiments and analysed by one-way analysis of variance (ANOVA) with Tukey's post hoc correlation relative to the experimental control condition set to 1 in chapters 3 and 4, or relative to the mean of all experimental conditions in chapter 5.

Data deriving from all MTS, MTT and ATP release proliferation assays were reported as the mean of a minimum of four replicates over 3 individual experiments using a one-way ANOVA with Tukey's multiple comparison tests. Results from MTS cell viability assay in isolated CD271 +ve and –ve subpopulations treated for 24 hours with either vehicle control, chloroquine 10 µM or the Vps34 inhibitor PIK III 5 µM were analysed using a Mann Whitney U non-parametric T test.

To quantify basal CD271 mRNA *in vitro* across a panel of cell lines and primary melanocytes as well as CD271 mRNA expression following Si RNA and ShRNA –mediated knockdown of CD271,

CD271 mRNA expression was determined by q PCR as described in section 2.6. Mean CD271 Ct values were normalized to 18S control Ct value data were analysed by one-way ANOVA with Tukey's post hoc correlation. Normalized CD271 mRNA expression between CD271 siRNA and non-target siRNA were compared by Students T-Test.

In *vitro* immunofluorescent expression of CD271 in WM266-4 cells expressing dual tagged LC3 RFP/GFP was compared to a null primary negative control and mean fluorescent intensity analysis derived using ImageJ image analysis software. Immunofluorescent expression was expressed as mean fluorescence per cell (relative to DAPI) and compared using Mann-Whitney U test.

In *vitro* immunohistochemical expression of CD271 and p62 in primary melanomas of different AJCC stage, benign naevi and metastatic melanoma lymph nodes were assessed using an automated slide scanner (Leica SCN400 digital slide scanner) and staining intensity calculated using the Leica digital slide scanner at ×20 magnification and expressed semi quantitatively as the mean percentage staining intensity of 10 representative areas. Results were compared using one way ANOVA test with Tukey's multiple comparison test.

Spheroid collagen invasion of WM35 control or trametinib treated or A375 trametinib resistant cells was analysed using Volocity 3D Image Analysis Software. The maximum diameter of the spheroid was measured and compared to the respective control for each time point. Results were expressed as the mean (± SD) of the absolute increase in spheroid diameter at each time point compared to the respective untreated control. Results compared by two-way ANOVA test with Sidak multiple comparison test.

Chapter 3

Constitutive expression of CD271 in melanoma is associated with an increase in pro-survival basal autophagy

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Chapter 3 Constitutive expression of CD271 in melanoma is associated with an increase in pro-survival basal autophagy

3.1 Introduction

The recent identification of melanoma subpopulations that are involved in tumour progression, metastasis and resistance to targeted therapies has significantly changed the way melanoma biology is considered. Malignant melanoma is a very heterogeneous cancer with complex tumour biology and with diverse survival mechanisms that orchestrate a cascade of events leading to tumour resistance to therapies and escape from immune-mediated death.

Over a decade ago Herlyn *et al* first described the observation that some melanoma cells grow in specialized stem cell growth media as melanospheres and isolated cells deriving from these melanospheres could be differentiated under appropriate conditions into multiple cell lineages, such as melanocytic, adipocytic, osteocytic, and chondrocytic, implicating a direct stem cell heritage of a subpopulation of melanoma cells (Fang *et al.*, 2005b) and therefore paving the way for the investigation of melanoma cells with stem like properties.

Melanoma cells expressing stem cell markers such as ABCB5, CD271, SOX10 and Jarid-1B have recently been described to be involved in melanoma progression and resistance to targeted therapies. However, there is also increasing controversy about the importance of these subpopulations in tumour initiation and their contribution to tumour resistance.

CD271 (p75 NTR) is a low-affinity p75 neurotrophin receptor that has the ability to bind to the nerve growth factor (NGF) (Liepinsh *et al.*, 1997b). Although the role of CD271 in neural crest development is well understood, in the skin, CD271 interacts with several ligands and co-receptors to mediate a variety of cellular functions and the direct role of CD271 in this context is still to be fully elucidated (Pincelli, 2017).

The role of CD271 in neural crest cell development and the neural crest origin of melanocytes and melanoma cells has prompted the investigation of CD271 in human cutaneous melanoma.

The tumorigenic potential of CD271 expressing melanoma cells has been explored in several studies. Boiko *et al* first explored the hypothesis that highly expressing CD271 melanoma cells are more tumorigenic *in vivo* by isolating CD271 +ve and –ve melanoma cells from patient samples and transplanting them into T, B, and NK deficient Rag2−/− γc−/− mice (RG) mice. Results demonstrated the increased tumorigenic potential of isolated CD271 +ve cells compared to CD271 –ve cells indicating that CD271 expression per se demonstrates increased tumourigenicity *in vivo* (Boiko *et al.*, 2010a). However, subsequent studies have not been able to reproduce these findings and recent work by Quintana et al demonstrated that patient derived CD271 +ve and CD271 -ve cells exhibited similar tumorigenic potential once engrafted as single cells in nude mice (Quintana *et al.,* 2010), with additional studies demonstrating CD271 +ve and –ve cells derived from patient tumour and transplanted into patient derived xenografts assays revealed similar ability to for either subset to initiate tumour development. However, genetic studies have highlighted the significant change in CD271 expression in secondary tumours derived from either CD271 +ve or –ve subpopulations indicating a rapid re-equilibration of marker-defined cell populations in these tumours and highlighting the unstable nature of CD271 expressing populations in this context (Boyle *et al.*, 2016).

Further evidence for an association between CD271 expression and melanoma progression is supported by studies of the immunohistochemical expression of CD271, demonstrating expression levels as high as 60% in advanced melanomas, correlated with high risk clinical and dermoscopic characteristics (Beretti *et al.*, 2015) and thus indicating CD271 expression in primary melanomas is associated with poor prognosis. Interestingly, CD271 expression is also increased in melanoma associated cerebral metastases and other visceral metastases further indicating that constitutive CD271 expression may indeed play a role in melanoma progression (Guo *et al.*, 2014a).

These conflicting results highlight the complexity of CD271 expression and interactions, the possible unstable nature of CD271 de novo expression in melanoma but also indicate the potential association of CD271 expression with melanoma progression and metastasis. However, the mechanisms mediating the survival of these subpopulations and their ability to promote tumour migration, progression and metastasis remains poorly defined. The possible

interplay between autophagy and tumour cell survival (Lei *et al.)* in constitutively expressed CD271 positive melanoma subpopulations leads to the aims of the current chapter; to define the interplay between constitutive expression of CD271 in primary cutaneous melanomas *in vivo* and metastatic melanoma cells lines *in vitro* with autophagy, whether or not CD271 expression is related to the expression of other neuroectodermal stem cell markers and to investigate the effect of autophagy inhibition in the survival of CD271 +ve expressing melanoma subpopulations.

3.2 **Results**

3.2.1 CD271 expression is increased in advanced stage in primary cutaneous melanoma and is associated with active autophagy

To confirm previously published data suggesting increased CD271 expression in advanced melanomas and further assess any potential correlation with autophagic activity, the immunohistochemical expression of CD271 was determined in a cohort of 5 benign naevi, 34 metastatic cutaneous malignant melanomas (5 AJCC stage I, 15 AJCC stage II, 7 AJCC stage III) and 7 melanoma lymph node metastases. Initial experiments focused on the optimization of antigen retrieval and primary antibody conditions to reveal CD271 expression in formalin fixed and paraffin embedded tissue (FFPE). Since previous studies reported relatively low levels of CD271 expression in primary melanomas (Gray *et al.*, 2015), immunohistochemical expression was pre-optimised using FFPE cell blocks derived from WM35 cells treated with 16 nM trametinib for 9 days (Figure 3-1: Optimization of an Immunohistochemical assay to detect CD271 expression in FFPE melanoma cell pellets (Figure 3-1). Results demonstrated enhanced expression of CD271 in FFPE WM35 cells following treatment with trametinib compared to untreated control cells. Noteworthy also was the presence of positive CD271 staining in WM35 parental cells, in line with the presence of small numbers of these subpopulations within the parental phenotype prior to trametinib treatment (Figure 3-1). Antigen retrieval optimisation was carried out using sodium citrate (10 mM, pH 6), Tris HCL pH7.6 and Tris HCL pH9, in which, as shown by Figure 3-1, revealed a clear difference in staining intensity between CD271 expression in cell pellets derived from WM35 control cells and the increased staining intensity of CD271 positive cells following treatment with trametinib and revealed using Tris HCL pH 9 as the antigen retrieval buffer and an antibody dilution of 1:100. However, in subsequent analysis of FFPE melanoma tissue primary antibody dilutions of 1:100 failed to detect CD271 expression and hence a more concentrated dilution of 1:50 was adopted for subsequent analysis of expression in primary tissue (Figure 3-2).

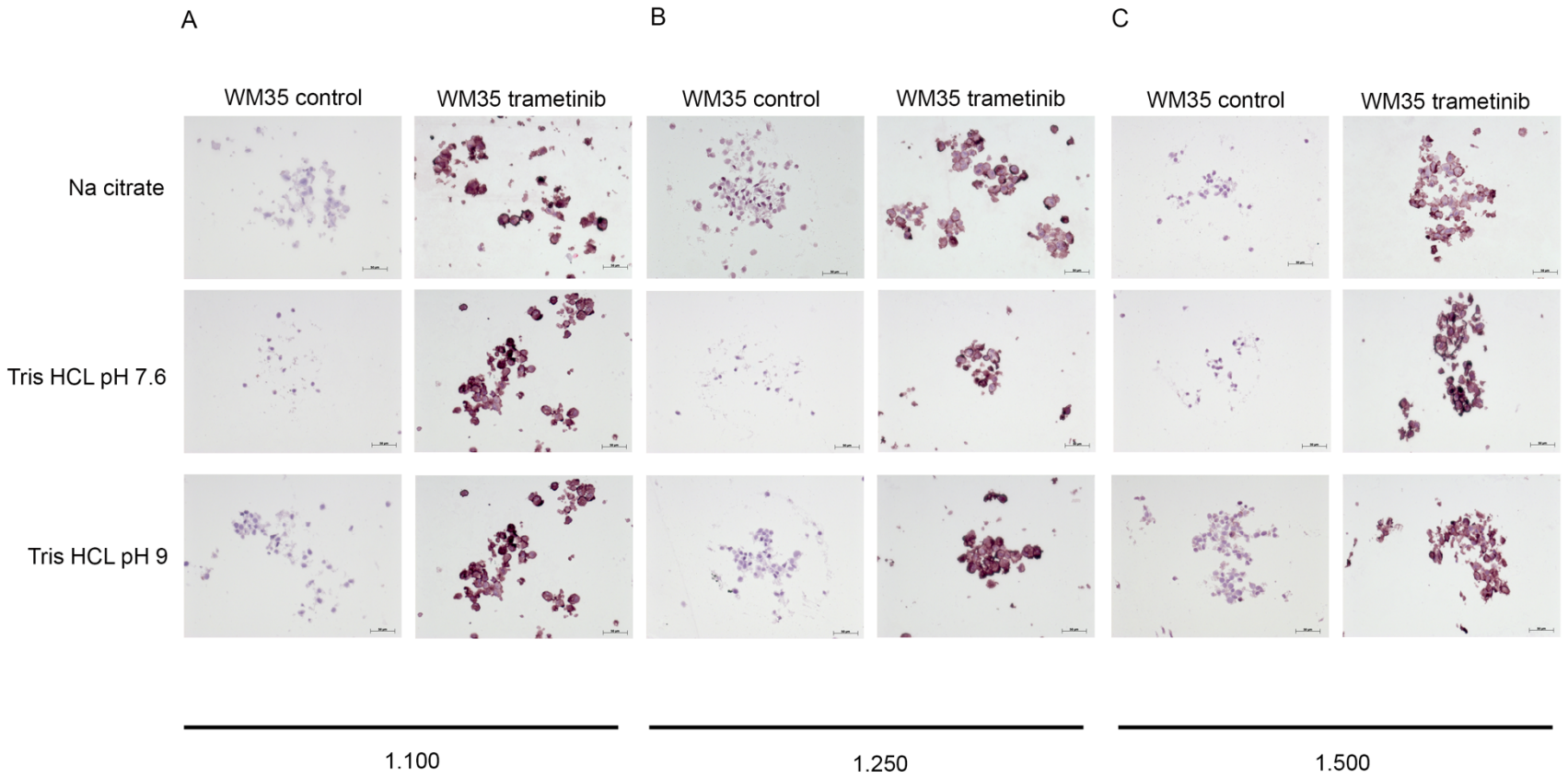


Figure 3-1: Optimization of an Immunohistochemical assay to detect CD271 expression in FFPE melanoma cell pellets

Representative photomicrographs for the immunohistochemical expression of CD271 (abcam, ab3125) in FFPE WM35 cells (WM35 control) or in FFPE WM35 cells following 9 days treatment with 16 nM trametinib (WM35 trametinib) to induce CD271 expression and revealed by antigen retrieval performed in Na Citrate (10 mM, pH 6) (Na Citrate), Tris HCL pH 7.6 or pH 9 and using three different primary CD271 antibody concentrations: 1.100 (A.), 1.250 (B.) and 1.500 (C). Images were acquired at x20 magnification. Scale bar = 50 µM

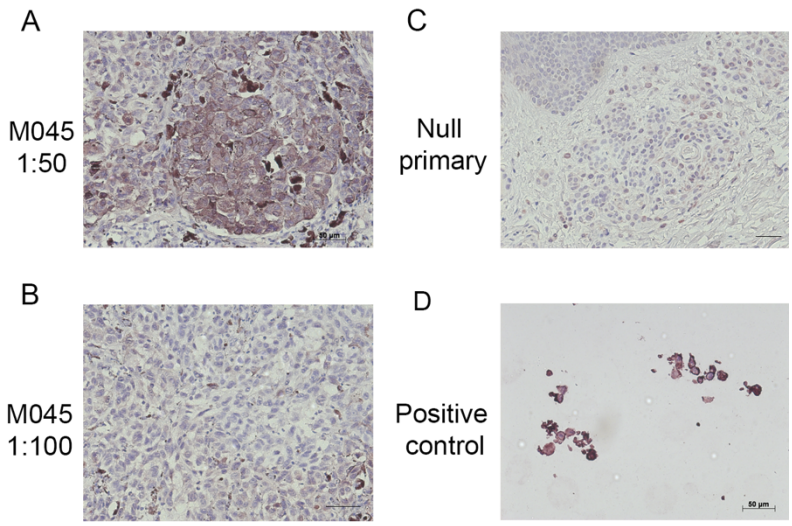


Figure 3-2: 1:50 is the optimal CD271 antibody dilution for the detection of CD271 expression in FFPE melanoma tissue

Representative photomicrographs for the immunohistochemical expression of CD271 (abcam, ab3125) in advanced stage IV melanoma (M045) at 1:50 antibody dilution (A) (M045 1:50), 1:100 antibody dilutions (B) (M045 1:100), null primary staining (C) (Null primary) or in FFPE WM35 cell pellet following 9 days treatment with 16 nM trametinib (D) (Positive control) using Tris HCL pH 9 as antigen retrieval. Images were acquired at x20 magnification. Scale bar = 50 µM

Using optimized conditions for immunohistochemical CD271 detection, subsequent experiments demonstrated increased expression of CD271 in primary cutaneous stage III melanomas compared to benign nevi (Figure 3-3), with analysis in 5 benign naevi and 27 primary cutaneous melanomas of differing AJCC stages (5 AJCC stage I, 15 AJCC stage II and 7 AJCC stage III) consistently revealing low levels of CD271 expression in benign naevi, and AJCC stage I and II melanomas compared to significantly increased expression in advanced stage melanomas (One way ANOVA test with Tukey's multiple comparison test, **P<.0.01, ***P<0.001, ****P<0.0001 (Figure 3-4). These findings were in line with previously published studies highlighting increased CD271 expression in advanced melanomas and the association with more aggressive disease (Ellis *et al.*, 2014a; Beretti *et al.*, 2015).

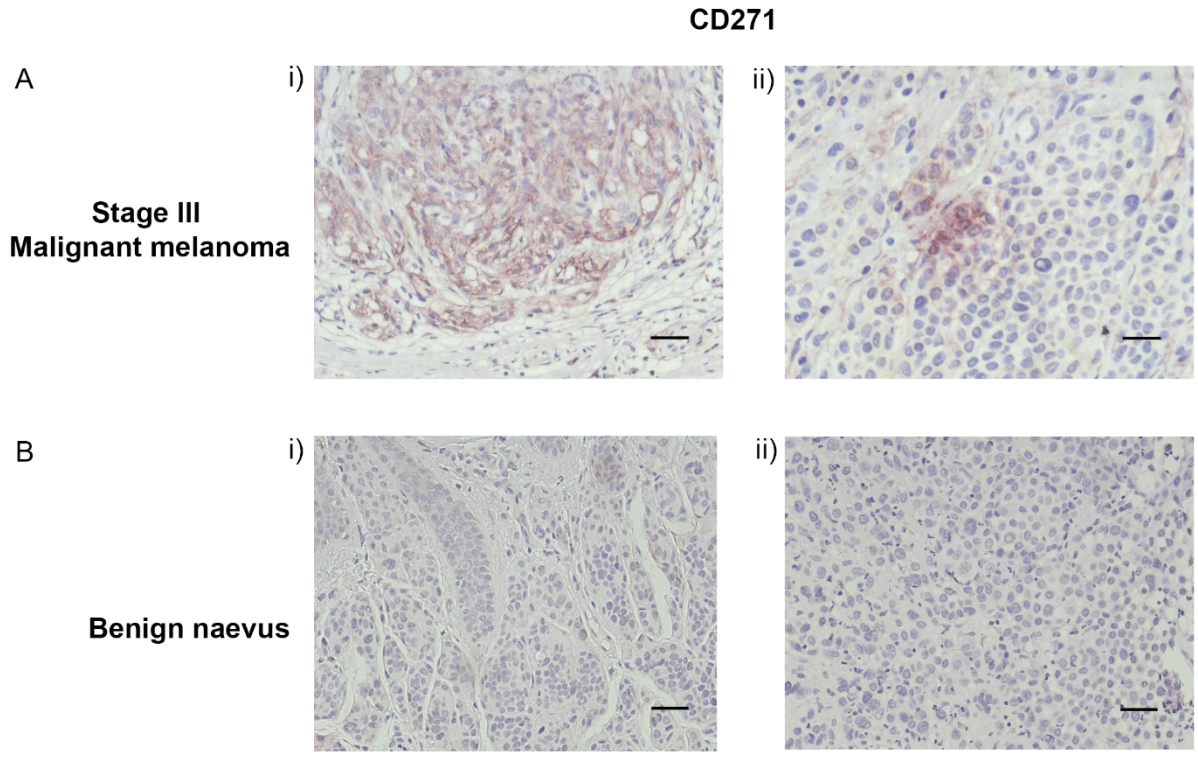


Figure 3-3: CD271 expression is increased in stage III malignant melanomas compared to benign naevi

Representative photomicrographs for the immunohistochemical expression of CD271 (ab3125, diluted 1:50 in A) a primary melanoma (with eventual AJCC stage IV disease) or B) a benign naevus. A i and B i x10 magnification, A ii and B ii x20 magnification. Scale bar = 50 µm (A I and B i) or 100 µm (A ii and B ii).

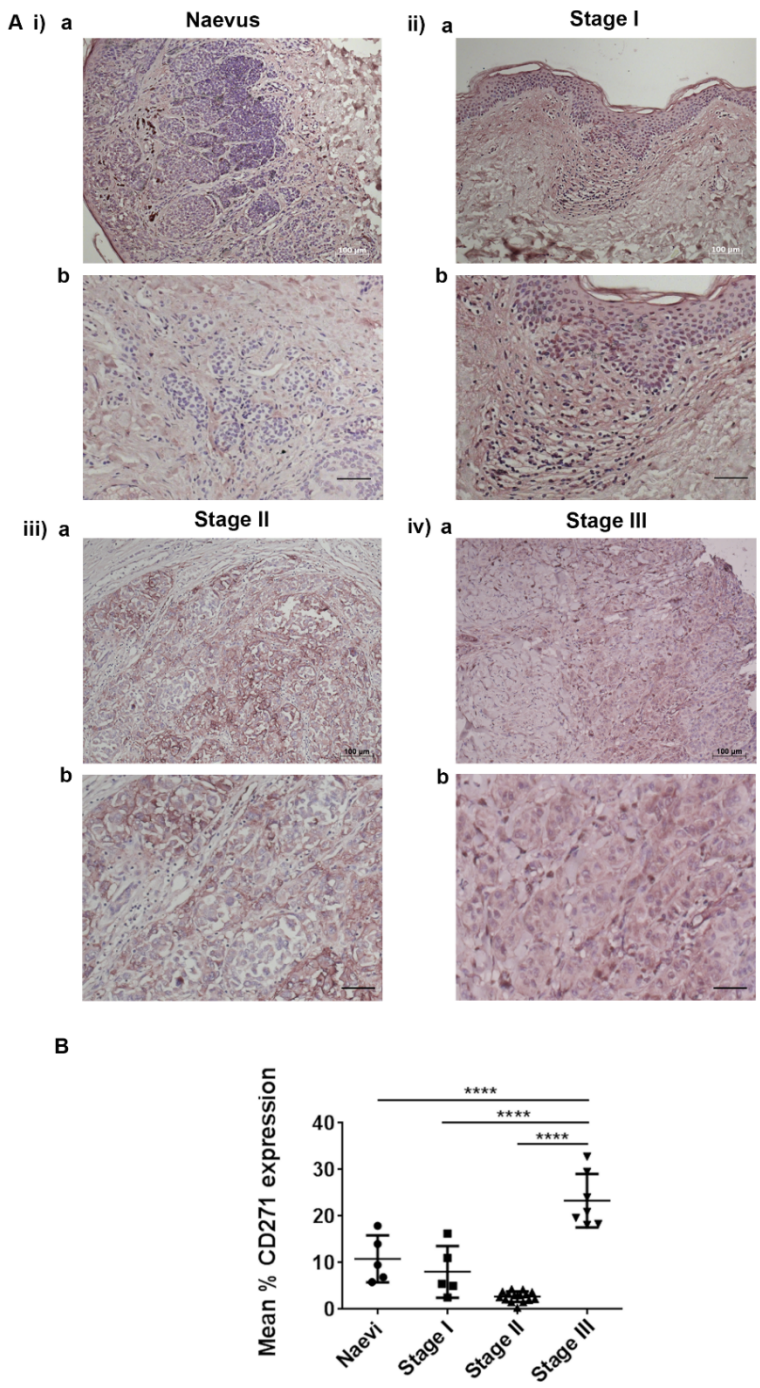


Figure 3-4: Immunohistochemical expression of CD271 is increased in stage III melanomas
Representative photomicrographs for the immunohistochemical expression of CD271 in a benign naevus (i), an AJCC stage I (ii), stage II (ii) or stage III melanoma (iv). B. Mean % CD271 expression is 5 naevi, 5 AJCC stage I, 17 AJCC stage II and 7 AJCC stage III melanomas. I, ii, iii, iv a: x 10magnification, scale bar = 100 µm. I, ii, iii, iv b: x 20magnification, scale bar = 50 µm. Each point is the mean CD271 expression of a minimum of 5 fields of vision. Horizontal lines represent median CD271 expression. **P<.0.01, ***P<0.001, ****P<0.0001

To evaluate a potential association between CD271 expression *in vivo* and autophagic activity, the expression of CD271 in 5 benign naevi and 27 primary cutaneous melanomas of differing AJCC stages (5 AJCC stage I, 16 AJCC stage II and 6 AJCC stage III) was correlated with the immunohistochemical expression of p62, determined using pre-standardized methodology (Ellis *et al.*, 2014a). Results demonstrated the bi phasic expression of p62, with consistently low levels detected in benign naevi and AJCC stage I primary melanomas while expression significantly increased in AJCC stage II melanomas (One way ANOVA with Tukey's post hoc correlation) for the comparison of median p62 expression in benign naevi or AJCC stage I primary melanomas with median expression in primary AJCC stage II melanomas, ** $P < 0.01$ (Figure 3-5). Expression of p62 in AJCC stage III melanomas was however, significantly reduced compared to expression in AJCC stage II primary melanomas (One way ANOVA with Tukey's multiple comparison test for the comparison of mean p62 expression in AJCC stage II primary melanomas with mean expression in primary AJCC stage III melanomas, *$P<0.05$, Figure 3-5), consistent with the paradoxical role of autophagy in cancer and suggesting AJCC stage III melanomas in which CD271 expression was increased are associated with an increase in basal autophagy (as reflected by reduced levels of p62 (Figure 3-5B).

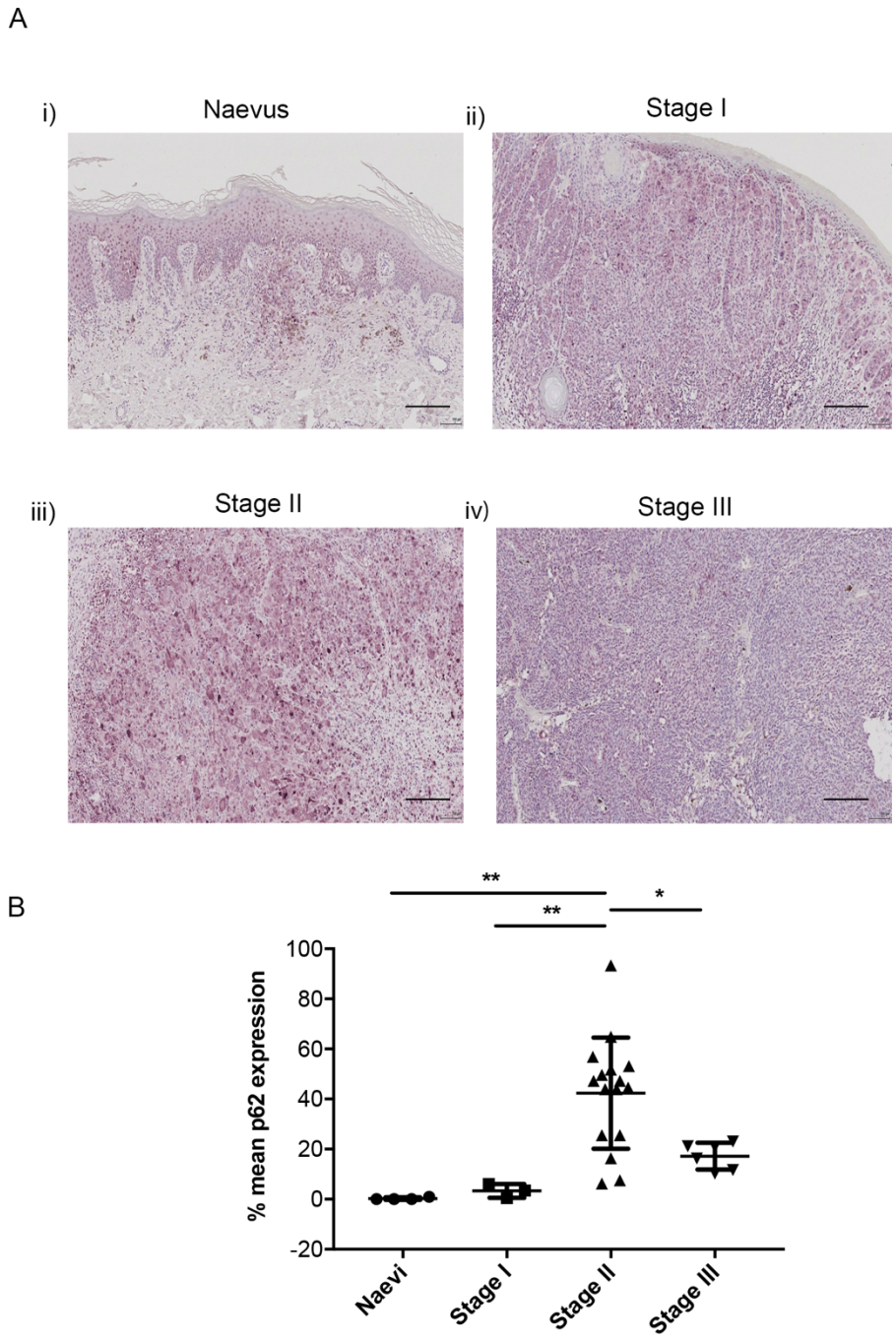


Figure 3-5: p62 expression in primary melanomas is increased in stage II melanomas and decreases in stage III melanomas

A.Representative photomicrographs for the immunohistochemical expression of p62 in a benign naevus (i), a primary AJCC stage I (ii), stage II (iii) or stage III (iv) melanoma. B. Mean % p62 expression in 5 benign naevi, 5 AJCC stage I, 17 AJCC stage II and 7 AJCC stage III (iv) primary melanomas. Each point is the mean % p62 expression of a minimum of 5 fields of vision. Horizontal lines represent median expression levels of CD271 expression. * P< 0.05** P < 0.01.

Further analysis revealed a significant correlation between decreased CD271 and increased p62 expression in primary AJCC stage II melanomas (Spearman correlation coefficient R2: 0.0569, P= 0.04, Figure 3-6A). However, although a trend for a correlation between reduced p62 and increased CD271 expression was evident in AJCC stage III melanomas, this was not in fact significant (Spearman correlation coefficient R2: 0.0135, P= 0.49, Figure 3-6B). Nevertheless, collectively these data highlight a relationship between increased CD271 expression and an increase in basal autophagic activity in more advanced primary melanomas *in vivo*.

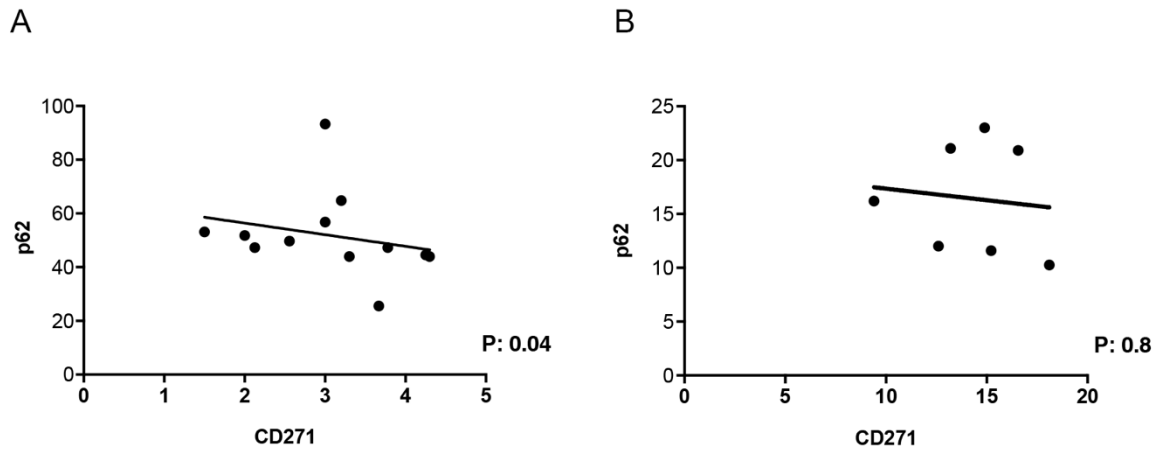


Figure 3-6: Increased CD271 expression in stage II primary melanomas is associated with decreased p62 expression

Scatter plots of mean % CD271 and p62 expression in AJCC stage II (A.) or stage III (B.) melanomas. Each point represents the mean % CD271/p62 expression in an individual primary melanoma derived from 10 representative fields of vision P= 0.04, Spearman correlation coefficient R^2: 0.0569, P=0.49, Spearman correlation coefficient R^2: 0.0135)

To further evaluate the relationship between constitutive CD271 expression and autophagy status in melanoma, studies of CD271 expression were further undertaken in a panel of primary melanocytes and human melanoma cell lines derived from both primary and metastatic tumours, as well as in isolated CD271 +ve and –ve subpopulations derived from the metastatic melanoma cell lines WM35 and SK-mel-28 with additional correlative studies of CD271 expression in melanoma *in vitro* with autophagy status as determined by p62 or LC3-II expression.

3.2.2 Basal CD271 mRNA and protein expression is variable in human melanoma cell lines and primary melanocytes

Analysis of both basal CD271 protein and mRNA expression levels in primary melanocytes derived from two independent donors and a cohort of 10 melanoma cell lines revealed variable expression with no particular trend for any change in expression between melanoma cell lines bearing a BRAF mutation (WM35, A375, SK-mel-28, WM164 (all $BRAF^{V600E}$ mutant), WM266-4 ($BRAF^{V600D}$ mutant), an NRAS mutation (WM-1361), or those wild-type for BRAF/ NRAS (SK-mel-23, CHL-1, C8161, Mel 50) (Figure 3-7).

Furthermore, CD271 mRNA expression levels did not correlate with levels of protein expression, with higher levels of CD271 mRNA observed for example, in WM266-4 and CHL-1 cells, indicating CD271 mRNA may not be translated and highlighting the potential for complex post translational modifications, consistent with reported literature (Vogel and Marcotte, 2012).

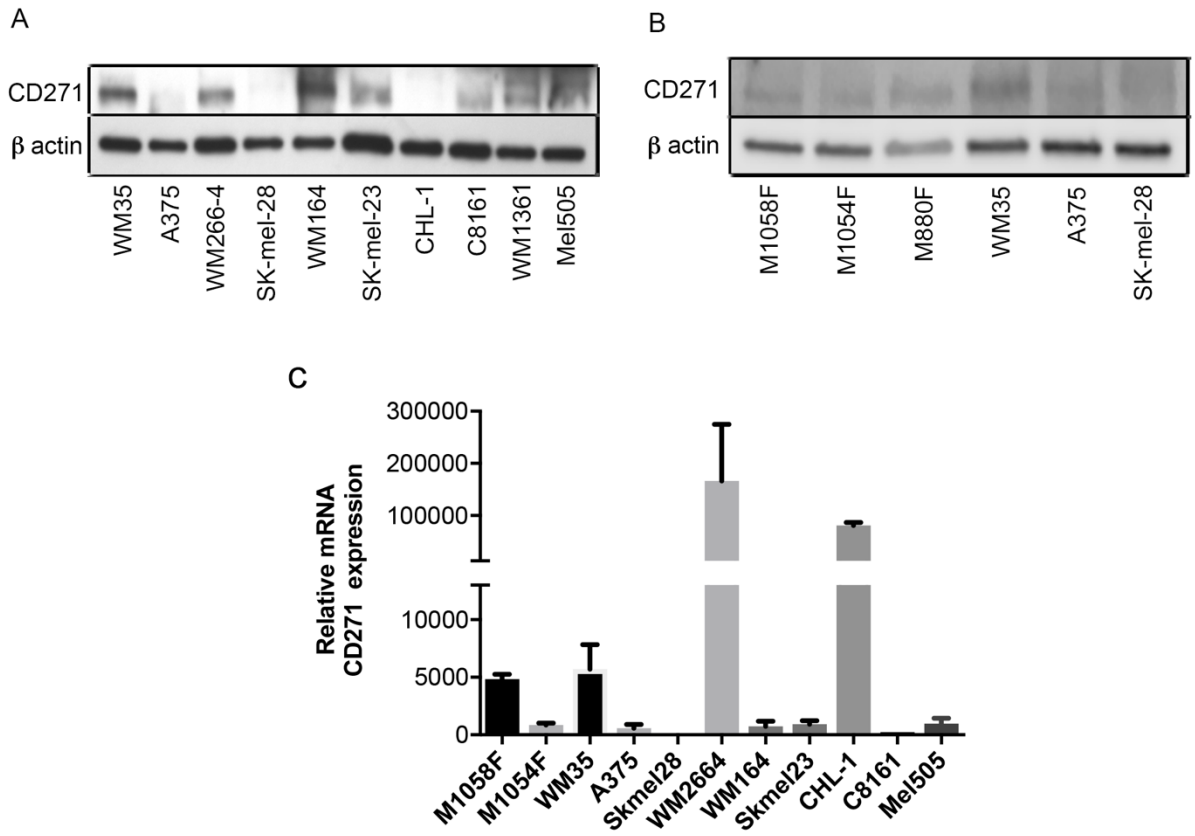


Figure 3-7: Expression of CD271 mRNA and protein varies in human primary melanocytes and melanoma cell lines.

A.Representative western blot for basal CD271 expression (75kDa) and β actin (42kDa) in three primary melanocyte samples (M1058F, M1054F and M880F) and BRAF mutant cell lines (WM35, A375 and SK-mel-28). B. Representative western blot for basal CD271 expression (75kDa) and β actin (42kDa) in included WM35, A375, SK-mel-28, WM164 (all BRAF mutant), WM266-4 (BRAF ^{V600D} mutant), SK-mel-23, CHL-1, C8161, Mel 505 (all BRAF Wild Type) and WM-1361 (NRAS mutant) cell lines. C. Relative mRNA expression of CD271 (relative to 18S loading control). Each bar is the mean of three replicate experiments in all cell lines/melanocytes.

3.2.3 Constitutive CD271 expression in isolated CD271 expressing melanoma cells *in vitro* is associated with increased autophagic activity but is not associated with the expression of other neuro ectodermal stem cell markers

To further delineate a potential association between constitutive CD271 expression and autophagy, CD271 positive and negative subpopulations were initially isolated from either WM35 or SK-mel-28 melanoma cells using Miltenyi Biotec magnetic columns prior to the analysis of CD271 expression by western blotting (Figure 3-8) or associated expression of LC3II or p62 as standard markers of autophagic activity (Figure 3-9). Results demonstrated the increased expression of CD271 in isolated subpopulations from either cell line, although expression of CD271 was only consistently significant in CD271 +ve subpopulations derived from WM35 cells (One way ANOVA with Tukey's multiple comparison test **P<0.01, (Figure 3-8A).

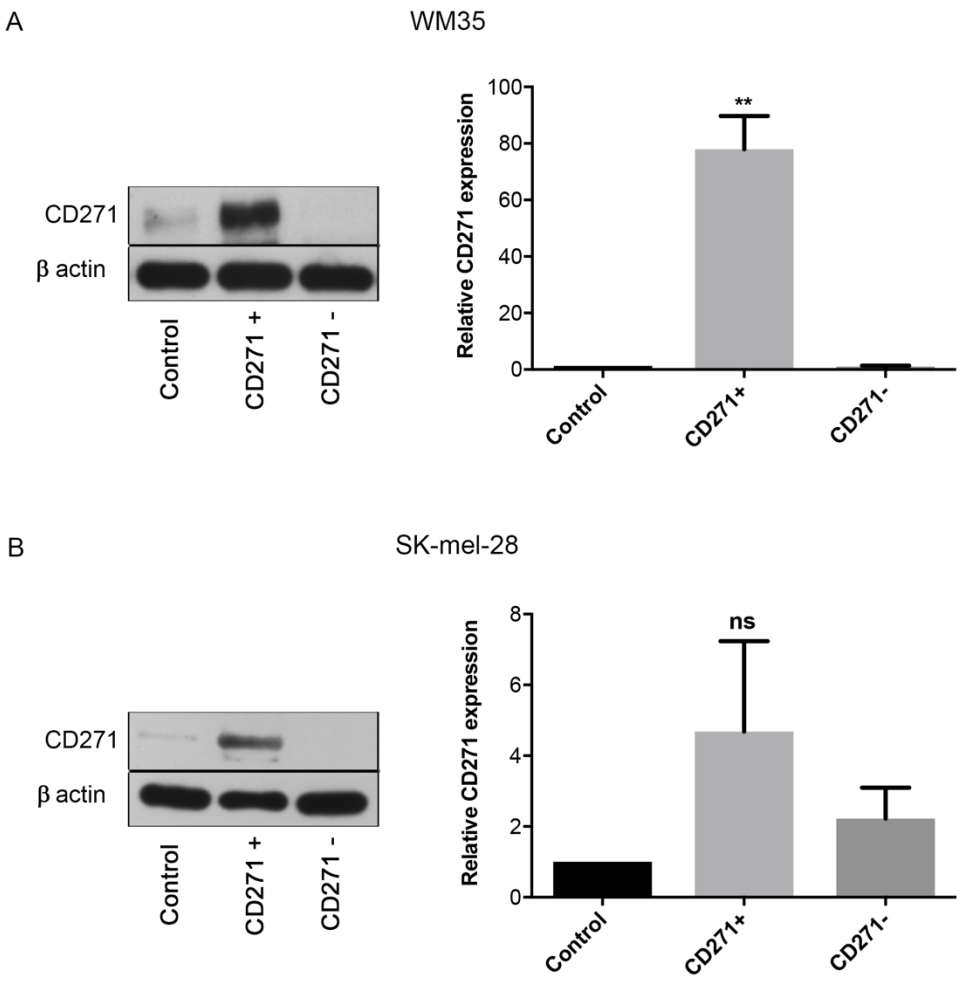


Figure 3-8: Isolation of CD271 subpopulations from melanoma cell lines confirmed by constitutive expression of CD271

Representative western blot for CD271 expression in WM35 (A) and SK-mel-28 (B) subpopulations following isolation using magnetic microbead-tagged CD271 antibodies and columns, and relative CD271 expression in CD271 positive and negative subpopulations derived from WM35 (A) or SK-mel-28 (B) cells. Each bar represents the mean of 3 replicate experiments relative to β actin loading control. **P<0.01

Furthermore, the expression of CD271 in CD271 +ve isolated subpopulations derived from WM35 cells was associated with a significant reduction in p62 expression (One way ANOVA with Tukey's multiple comparison test, **P< 0.01, (Figure 3-9 Ai and iii) and a non-significant trend for the expression of LC3-II compared to a reduction in expression in CD271 –ve subpopulations (Figure 3-9 Ai and ii). Similarly, in isolated CD271 positive subpopulations derived from SK-mel-28 cells, there was a trend for an associated increase in LC3 II and a

decrease in p62 expression, although this was not statistically significant (Figure 3-9B). Conversely, there was no significant change in either LC3 II or p62 expression in CD271 negative subpopulations compared to expression in wild type SK-mel-28 cells (Figure 3-9B). Although suggesting a potential association of increased autophagic activity with constitutive CD271 expression, experiments using chloroquine (added to given treatment conditions for the final 2 hours) to block autophagic flux were undertaken to confirm any association.

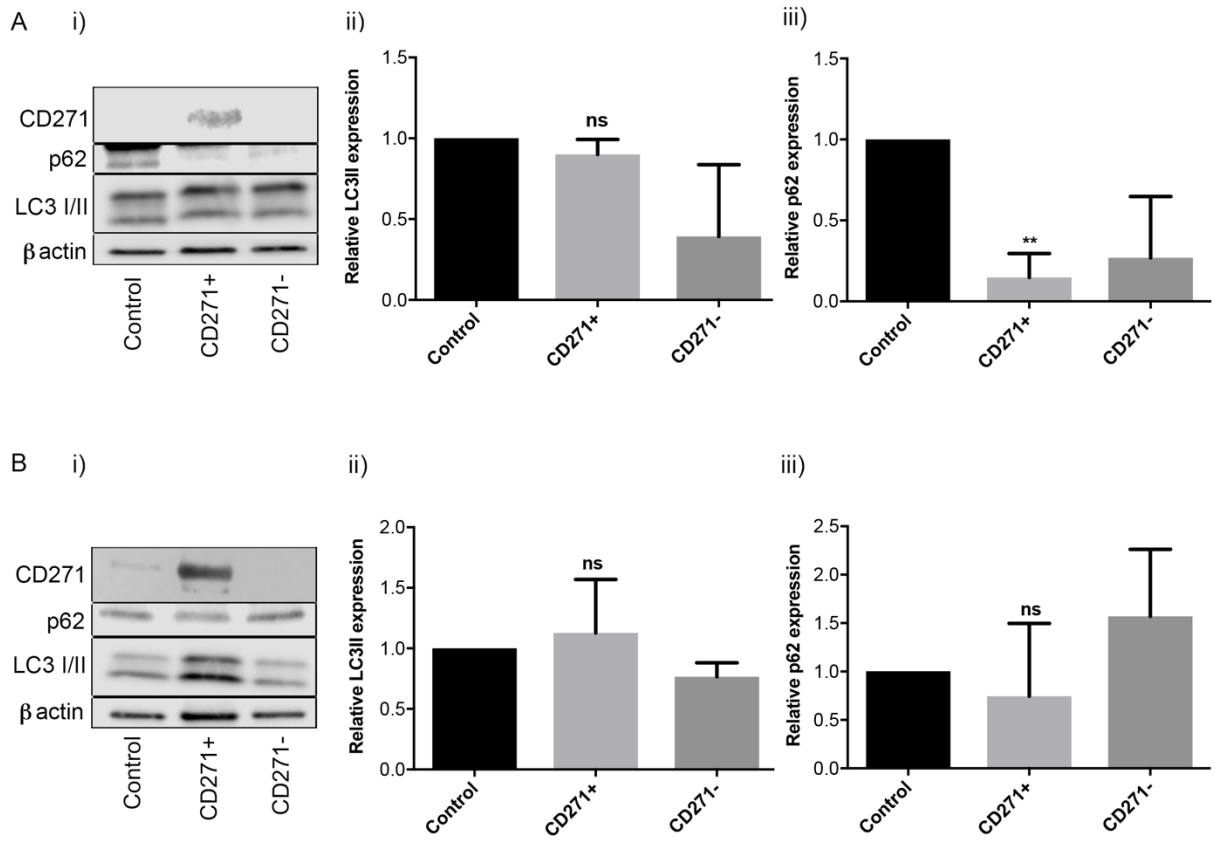


Figure 3-9: Constitutive expression of CD271 is associated with decreased p62 expression
Ai and Bi, Representative western blots for CD271 (75kDa), LC3 I/II (16-18kDa), p62 (62kDa) and β actin (loading control, 42kDa) expression or relative LC3 II (A and B ii) or p62 (A and B iii) expression in wild-type (control), CD271 positive (CD271 +) or CD271 negative (CD271 -) subpopulations isolated from WM35 (A) or SK-mel-28 (B) cells. A/B ii or iii; Each bar represents the mean of 3 replicate experiments expressed relative to the mean of each individual experiment (mean ± SD, N = 3) **P<0.01

Previous studies have shown that the expression of CD271 *in vitro* is unstable (Boyle *et al.*, 2016). The potential for the rapid re-equilibration of isolated CD271 expressing melanoma subpopulations may thus be a potential limiting factor in subsequent functional studies of such subpopulations with further culture or use in xenograft models *in vivo*. To investigate the stability of CD271 expression in isolated melanoma subpopulations, isolated subpopulations derived from WM35 cells were cultured for 3 or 6 days under standard conditions (37°C, 21% O_2 and 5% CO_2) prior to the analysis of CD271 expression as well as LC3 I/II expression, to correlate any impact on basal autophagy status. Results demonstrated the significant increase in CD271 expression (One way ANOVA test with Tukey's multiple comparison test, ** P<0.01),
(

Figure 3-10A) immediately following magnetic micro bead selection but which was reduced following 3 days of culture and further declined after day 6 continued culture (

Figure 3-10). LC3 II expression on the other hand was increased in CD271 positive WM35 subpopulations compared to CD271 negative subpopulations immediately following isolation and remained at a similar level in CD271 positive subpopulations after 3 and 6 days of continued culture (

Figure 3-10).

Collectively these data thus confirmed the unstable nature of CD271 expression after 3 days culture but interestingly the continued increase in basal autophagic activity over 6 days in isolated CD271 positive melanoma sub populations despite the gradual decline in CD271 expression. Nevertheless, since CD271 expression in isolated melanoma subpopulations was stable for at least 3 days, this enabled subsequent experiments to investigate the invasive potential of such subpopulations (described in Figure 3-13, section 3.2.5) within this time frame. To investigate the functional impact of CD271 expression over longer time periods, for example, on tumour invasive potential, CD271 was over expressed in human melanoma cell lines and these studies are described in chapter 4.

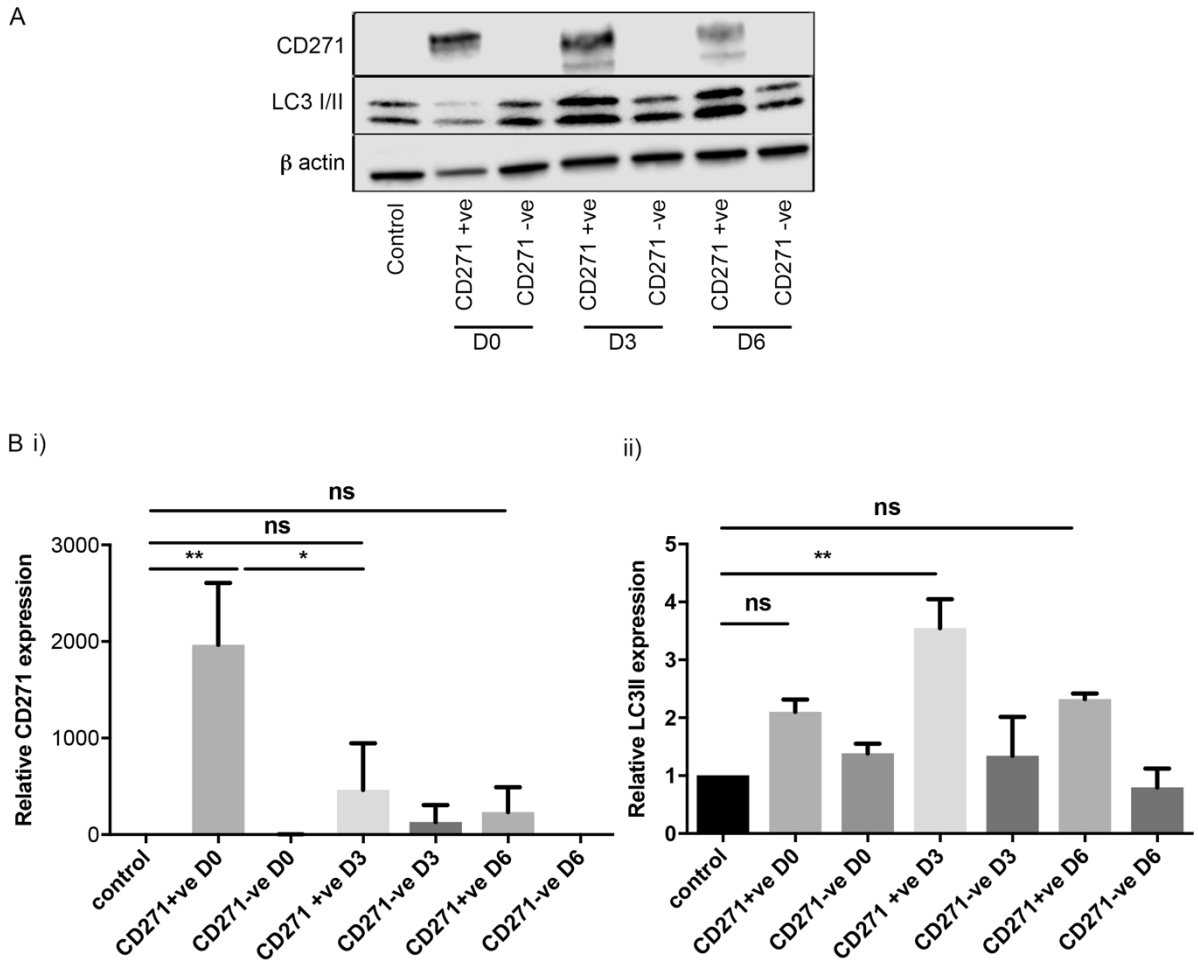


Figure 3-10: CD271 expression in isolated CD271 positive subpopulations declines over time
A.Representative western blot of CD271 (75 kDa), LC3 I/II (16-18 kDa), β actin (42 kDa, loading control) in WM35 melanoma cells (control) or in CD271 +ve and –ve subpopulations following magnetic microbead selection and continued culture for 0, 3 or 6 days. B. Relative CD271 (i) and LC3 II (ii) protein expression in isolated WM35 CD271 +ve or –ve subpopulations following 0 (D0), 3 (D3), or 6 (D6) days continued culture, relative to β actin protein expression. Each bar represents the mean of 3 replicates for each cell line, normalized to β actin and expressed relative to the mean control of each individual experiment (mean ± SD, N = 3) (*p<0.05, **p<0.01)

To further investigate the potential correlation between CD271 expression and increased autophagic activity, CD271 positive and CD271 negative subpopulations isolated from WM35 cells, stably expressing red fluorescent protein were grown as 3D spheroids prior to incorporation into collagen gels and the analysis of CD271 and p62 expression by immunofluorescence. Results revealed by day 7, CD271 negative subpopulations acquired

CD271 expression in the centre of the spheroid while p62 expression was observed only at the periphery of the spheroid where its expression was increased compared to expression in the centre (Figure 3-11) indicating active autophagy in the nutrient deprived core of the spheroid where CD271 expression is acquired. Conversely, CD271 expression was retained in CD271 positive subpopulations where in this case there was no observable distinct pattern of p62 expression indicating similar autophagic activity throughout these spheroids (Figure 3-11).

Taken together these data highlight the dynamic regulation of CD271 expression and a correlation with reduced p62 expression, further supporting observations of increased autophagic activity within CD271 positive melanoma subpopulations.

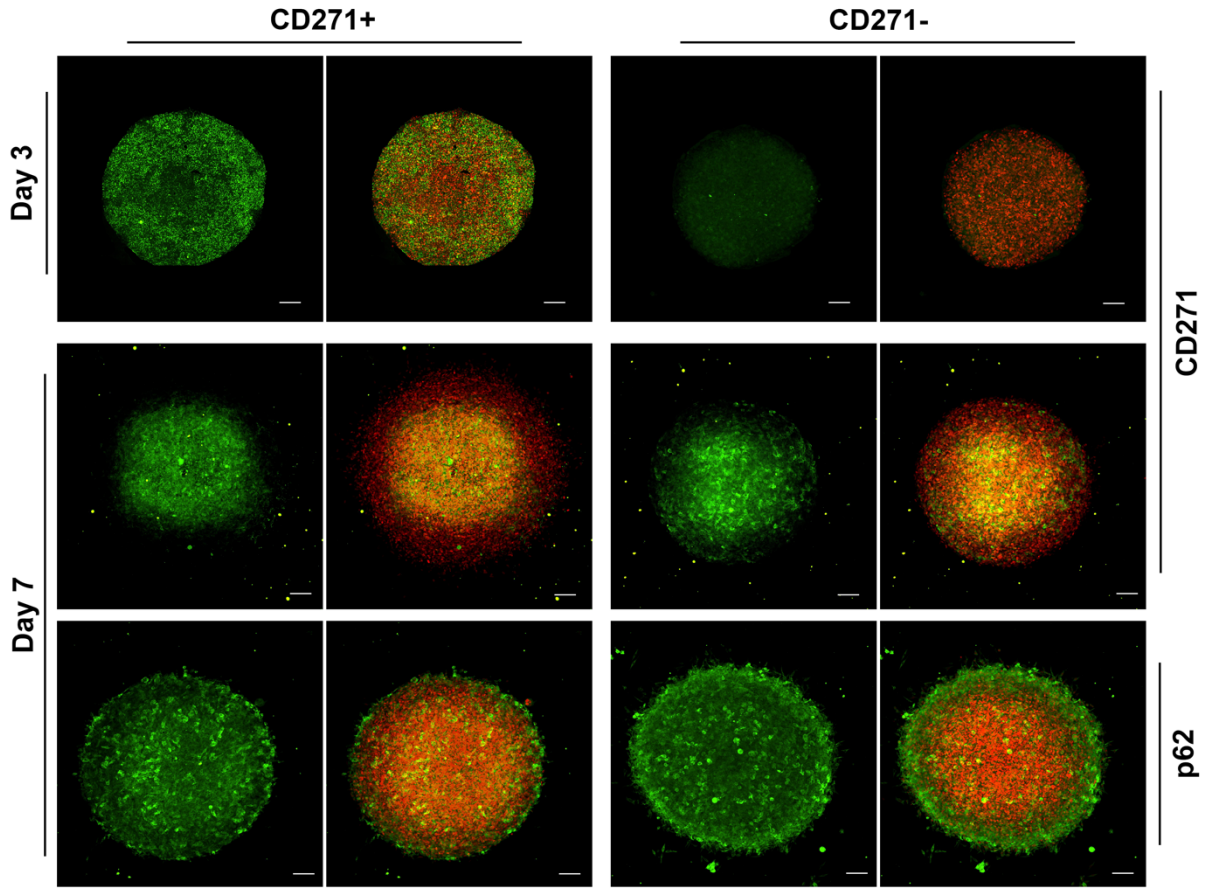


Figure 3-11 CD271 expression in melanoma spheroids is associated with reduced p62 expression in the centre of spheroid
Immunofluorescence images for the expression of CD271 and p62 expression (green fluorescence) in CD271 positive and negative WM35 spheroids stably expressing red fluorescent protein. Scale bar 100 µm.

3.2.4 Constitutive CD271 expression is not associated with the expression of other neuro ectodermal stem cell markers

Recent published literature highlights CD271 as a putative stem cell marker and its associated expression with other neuroectodermal stem cell markers (Roesch *et al.*, 2010; Cheli *et al.*, 2014a; Ladstein *et al.*, 2014; Zhang and Herlyn, 2014). However, there is conflicting evidence as to whether CD271 in melanoma serves as a stem cell marker or whether its expression is more associated with the emergence of chemo resistance to targeted therapies and conventional chemotherapy (Chartrain *et al.*, 2012; Ravindran Menon *et al.*, 2014). To investigate the potential association and overlap between the expression of CD271 in isolated CD271 positive and negative subpopulations derived from WM35 cells and the expression of other stem cell markers, expression of SOX10, Jarid-1B and Nestin in selected subpopulations were determined by Western blotting. Results demonstrated poor overlap and no significant correlation between CD271 expression in enriched CD271 subpopulations with either Nestin, Jarid-1B or SOX 10 expression (Figure 3-12) with in fact, a trend for increased Nestin expression in CD271 negative sub populations (Figure 3-12), suggesting CD271 expression is isolated melanoma subpopulations may not be a marker of stem ness or a stem cell phenotype.

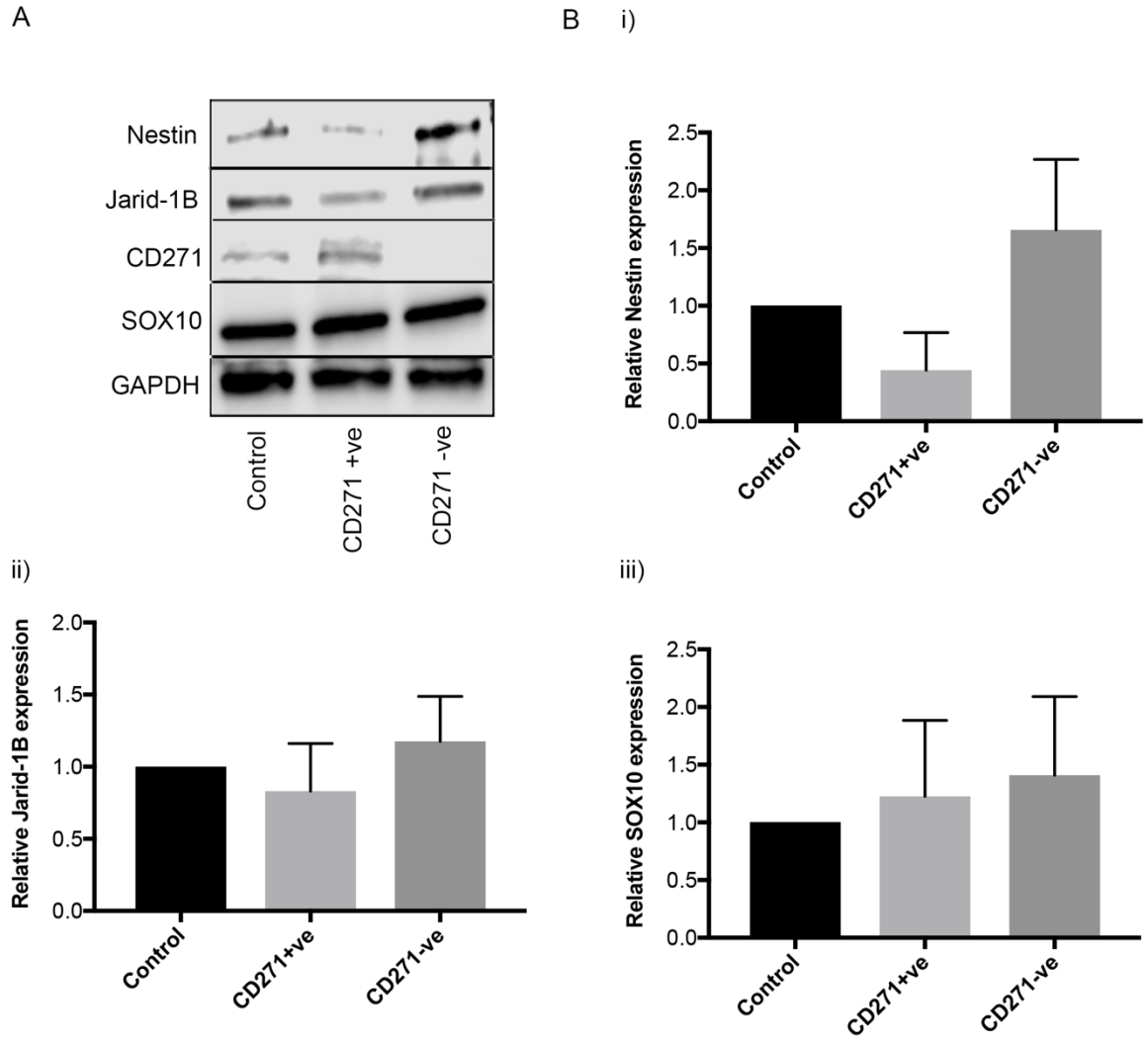


Figure 3-12: CD271 expression in isolated melanoma subpopulations is not associated with the expression of neuroectodermal stem cell markers.

A.Representative western blot for the expression of Nestin (177 kDa), Jarid-1B (190 kDa), SOX 10 (37 kDa) or GAPDH (37 kDa) in WM35 cells (control) or CD271 positive (CD271 +ve) and negative (CD271 – ve) subpopulations selected from WM35 cells. B. Relative Nestin, Jarid-1B or SOX10 expression in CD271 positive and negative WM35 subpopulations, relative to GAPDH expression. Each bar is the mean of 3 biological replicates for each cell line, expressed relative to the mean control of each individual experiment (mean ± SD, N = 3)

3.2.5 Constitutive expression of CD271 is associated with reduced spheroid expansion *in vitro*

In addition to the ongoing debate about the role of CD271 expressing melanoma subpopulations in melanoma initiation, the role of CD271 expression in tumour invasion and metastasis is also poorly defined. Several recent publications highlight the presence of distinct subpopulations within the CD271 expressing melanoma subpopulations that exhibit tumourigenic and invasive properties in contrast to the bulk of the CD271 positive subpopulation that remains less invasive compared to CD271 negative subpopulations (Cheli *et al.*, 2014a).

To investigate the spheroid expansion of constitutive CD271 expression in cutaneous melanoma, isolated CD271 positive and negative subpopulations derived from WM35 or SK-mel-28 cells (stably expressing RFP) were grown as 3D spheroids prior to incorporation into collagen gels and the monitoring of spheroid expansion over 96 hours. Results demonstrated a significant reduction in spheroid expansion of both WM35 and SK-mel-28 CD271 positive (CD271 +ve) compared to CD271 negative (CD271 -ve) subpopulations after 96 hours (Two-way ANOVA with Sidak multiple comparison test, ****$p<0.0001$) (Figure 3-13) suggesting, in line with published literature, that constitutive CD271 expression does not necessarily confer a more invasive phenotype (Saltari *et al.*, 2016) and that the role of CD271 is possibly more associated with chemo resistance to targeted therapies/chemotherapy.

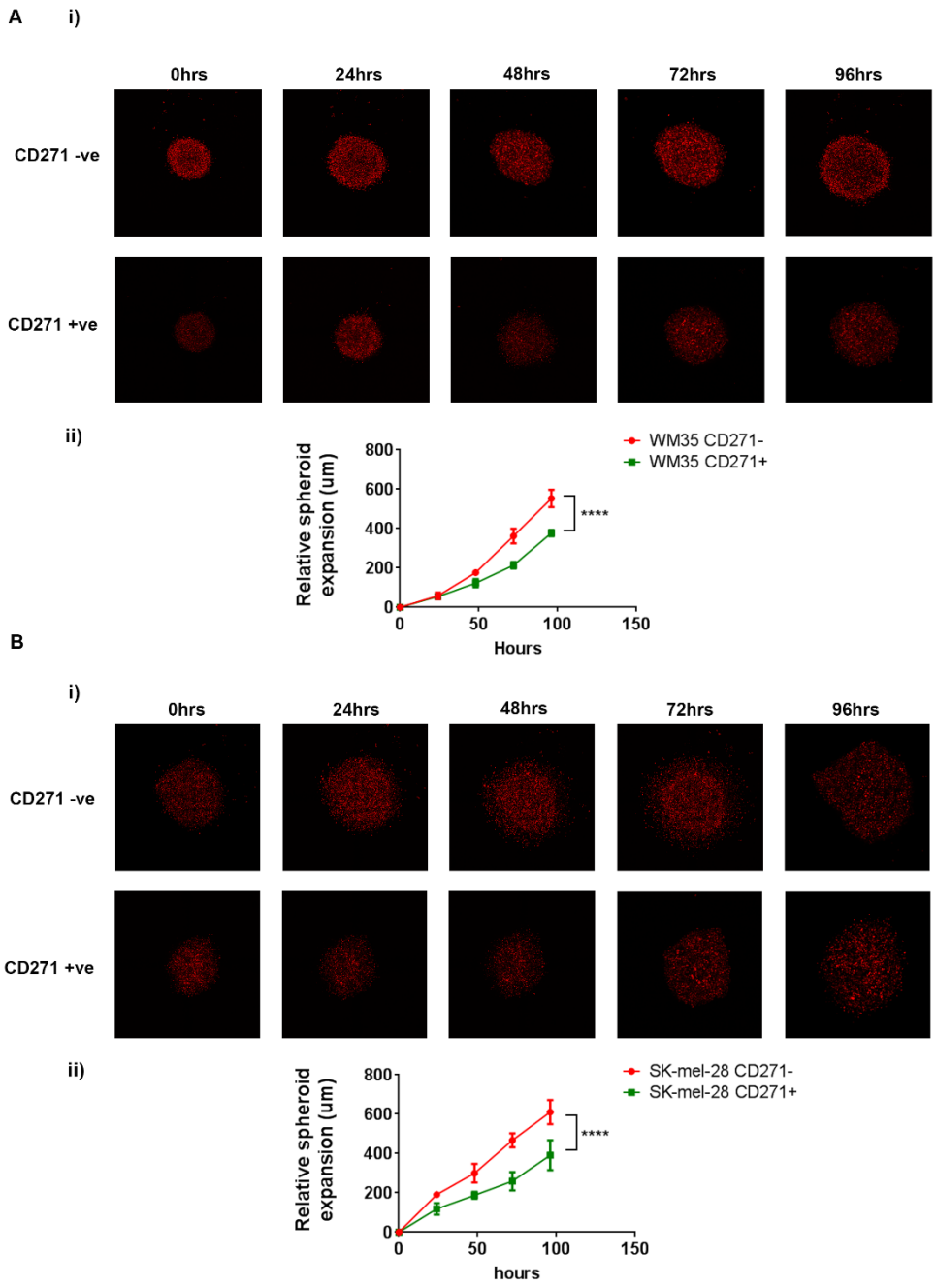


Figure 3-13: Spheroid derived from CD271 positive melanoma subpopulations exhibit reduced collagen expansion compared to CD271 negative subpopulations

Representative immunofluorescence images of spheroids derived from WM35 RFP (A i) or Sk-mel-28 (B i) CD271 negative (CD271 –ve) or positive (CD271+ve) subpopulations in collagen gels. Scale bar + or Images were acquired as a total magnification of X20. A and B ii; Relative spheroid size (µm) of WM35 (A ii) or SK-mel-28 (B ii) CD271 negative (red line) or positive (green line) spheroids (relative to initial spheroid size at time 0 hours) over 96 hours. Each bar is the mean of 3 replicate experiments ± SD. ****P<0.0001

3.2.6 Chemical inhibition of autophagy with chloroquine or selective Vps34 inhibitors inhibit the viability of isolated CD271 positive melanoma subpopulations *in vitro*

Data presented above highlight the association between constitutive CD271 expression and increased autophagy. To further investigate this link and whether autophagy in this context plays a pro-survival role, contributing to the survival of CD271 positive melanoma subpopulations, autophagic activity in selected/isolated melanoma subpopulations was blocked either by treatment with the lysosomal inhibitor chloroquine of with a specific Vps34 inhibitor, PIK III.

Treatment of isolated CD271 positive and negative subpopulations from WM35, SK-mel-28 or A375 BRAF mutant melanoma cells with 10 μM of chloroquine for 24 hours resulted in the significant inhibition of cell viability in CD271 positive with little or no effect on the cell viability of CD271 negative subpopulations derived from either cell line (One-way ANOVA, Tukey's multiple comparison test, **P<0.01, ****P<0.0001 (Figure 3-14).

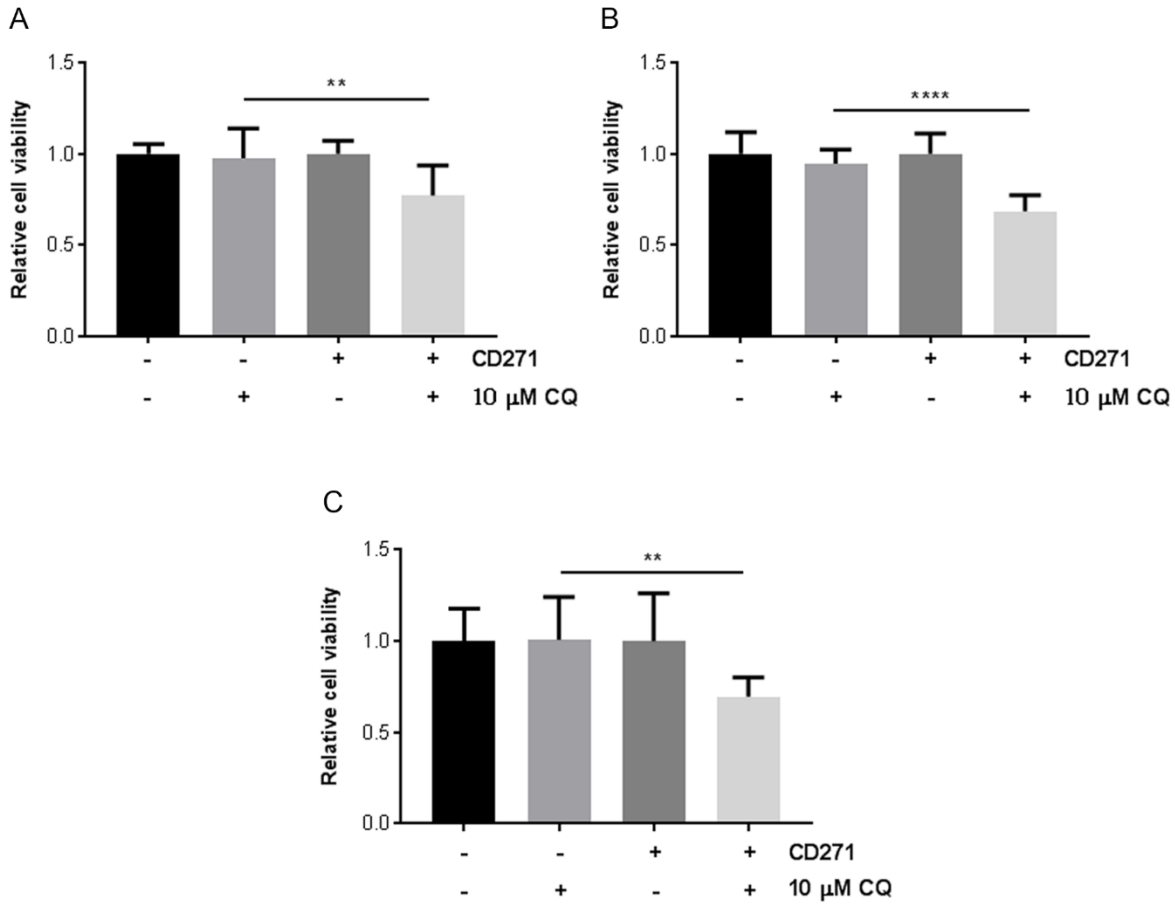


Figure 3-14: Chloroquine inhibits the cell viability of melanoma CD271 expressing subpopulations

Relative cell viability of WM35 (A), SK-mel-28 (B), A375 (C) isolated CD271 positive or negative subpopulations in the presence or absence of treatment with 10 μM chloroquine for 24 hours (relative to control untreated CD271 positive or negative cell viability). Each bar is the mean cell viability of 12 replicates from 3 independent experiments + SD. **P<0.01, *****P<0.0001.

To further define whether the observed effect on cell viability was a dose dependent effect of chloroquine on the isolated cells rather than a direct effect of autophagy inhibition, further dose response studies of chloroquine were performed over clinically achievable concentrations. Results demonstrated isolated CD271 positive WM35 and A375

subpopulations treated with 1, 5 or 10 μM chloroquine for 24 hours resulted in consistently inhibited cell viability, again with little or no effect observed in CD271 negative subpopulations form either cell line in response to treatment with chloroquine at any dose (one way ANOVA with Tukey's multiple comparison test *P<0.05, **P<0.01) (

Figure 3-15). Taken together these data thus further support a pro-survival role for autophagy in melanoma subpopulations constitutively expressing CD271.

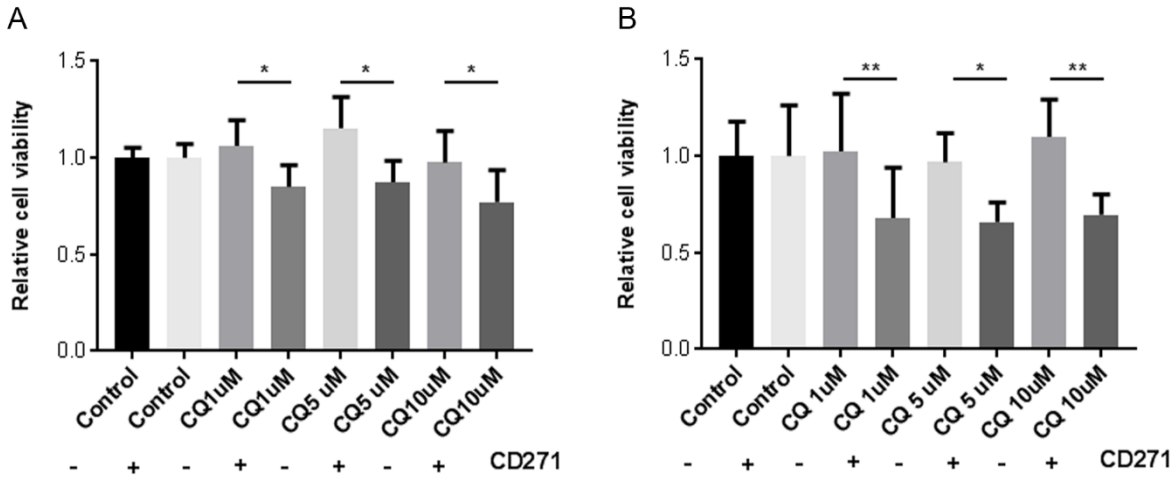


Figure 3-15 Chloroquine selectively inhibits cell viability of CD271 expressing melanoma subpopulations

Relative cell viability of WM35 (A) or A375 (B) isolated CD271 positive or negative subpopulations (relative to control untreated CD271 positive or negative cell viability) following treatment for 24 hrs in the presence or absence of 1, 5 or 10 μM chloroquine. Each bar is the mean cell viability of 12 replicates from 3 independent experiments + SD. *P<0.05, **P<0.01.

To confirm chloroquine-mediated inhibition of CD271 expressing melanoma subpopulation cell viability was specific to autophagy inhibition, isolated CD271 positive or negative WM35 or A375 melanoma subpopulations were treated in the presence of PIK III, a selective and novel inhibitor of Vps34 enzymatic function, essential for the lipidation of LC3 and degradation of autophagy substrates such as p62 and mitochondria (Dowdle *et al.*, 2014). Control wild-type WM35 or A375 melanoma cells were first treated for 24 hours in the presence or absence of dose reducing concentrations of PIK III; 10, 5, 1 μM or 100 or 10 nM for 24 hrs prior to the assessment of cell viability. Results demonstrated the significant inhibition of cell viability in both cell lines following 24-hour treatment with higher concentrations of 10 μM PIK III (one-way ANOVA analysis of variance with Tukey's multiple comparison test, **P<0.01 for the

inhibition of WM35 and ****P<0.0001 for the inhibition of A375 cell viability, Figure 3.16). However, at concentrations of 5 μM and below PIK III had no significant effect on the inhibition of the cell viability in either cell line (Figure 3-16).

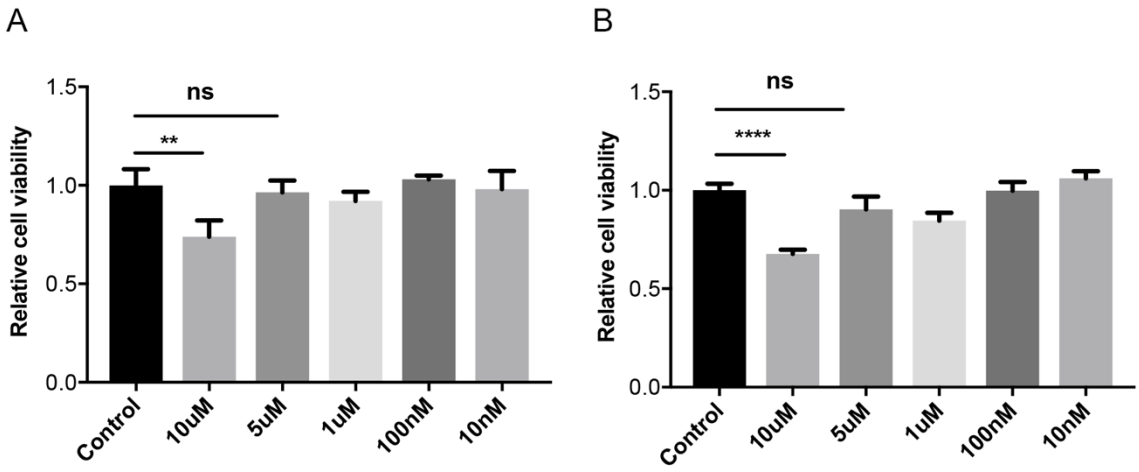


Figure 3-16: Dose-dependent inhibition of melanoma cell viability in vitro by the Vps34 inhibitor PIK III

Relative cell viability of WM35 (A) or A375 (B) in the absence (control) or presence of treatment for 24 hrs with 10, 5 or 1 μM or 100, 10 nM PIK III (relative to DMSO vehicle control treated cells). Each bar is the mean cell viability of 12 replicates from 3 independent experiments + SD. **P<0.01, ****P<0.0001.

To evaluate the potential for Vps34 inhibition to inhibit basal autophagy in wild-type WM35 or A375 melanoma cells, each cell line was treated in the presence or absence of 5 μM of PIK III for up to 24 hours, prior to western blotting for p62 and LC3 /II expression. In WM35 cells, results demonstrated a significant decrease in basal LC3 II expression following treatment with PIK III for 2 or 4 hours (One way ANOVA with Tukey's multiple comparison test, *P <0.05, ***P<0.001) which then increased after 24 hours continuous treatment (Figure 3-17 A i and ii). Similar effects were also observed in A375 cells (Figure 3.17 B i and ii) although PIK III-induced inhibition of LC3 II was only significant after 2 hours treatment. PIK III-induced p62 expression on the other hand revealed a trend for increased expression in a time dependent manner in both WM35 and A375 cells, although induction was only significant in WM35 cells following 24 hrs treatment with PIK III (One way ANOVA with Tukey's multiple comparison test, *P <0.05, ***P<0.001, Figure 3-17 A iii). Interestingly it has been reported that PIK III inhibits the formation of autolysosomes and the lipidation of LC3 and therefore basal levels of

either LC3 I or II may appear increased (Dowdle *et al.*, 2014) likely due to continued LC3 transcription, and noteworthy in the present study this was corroborated by the apparent increase in LC3 I expression, particularly in WM35 cells following treatment with PIK II for 2-24 hrs (Figure 3-17 Ai), as well as in A375 cells (Figure 3-17 Bi). Collectively nevertheless, these data suggest that concentrations of 5 μM PIK III inhibit autophagy at 24 hours.

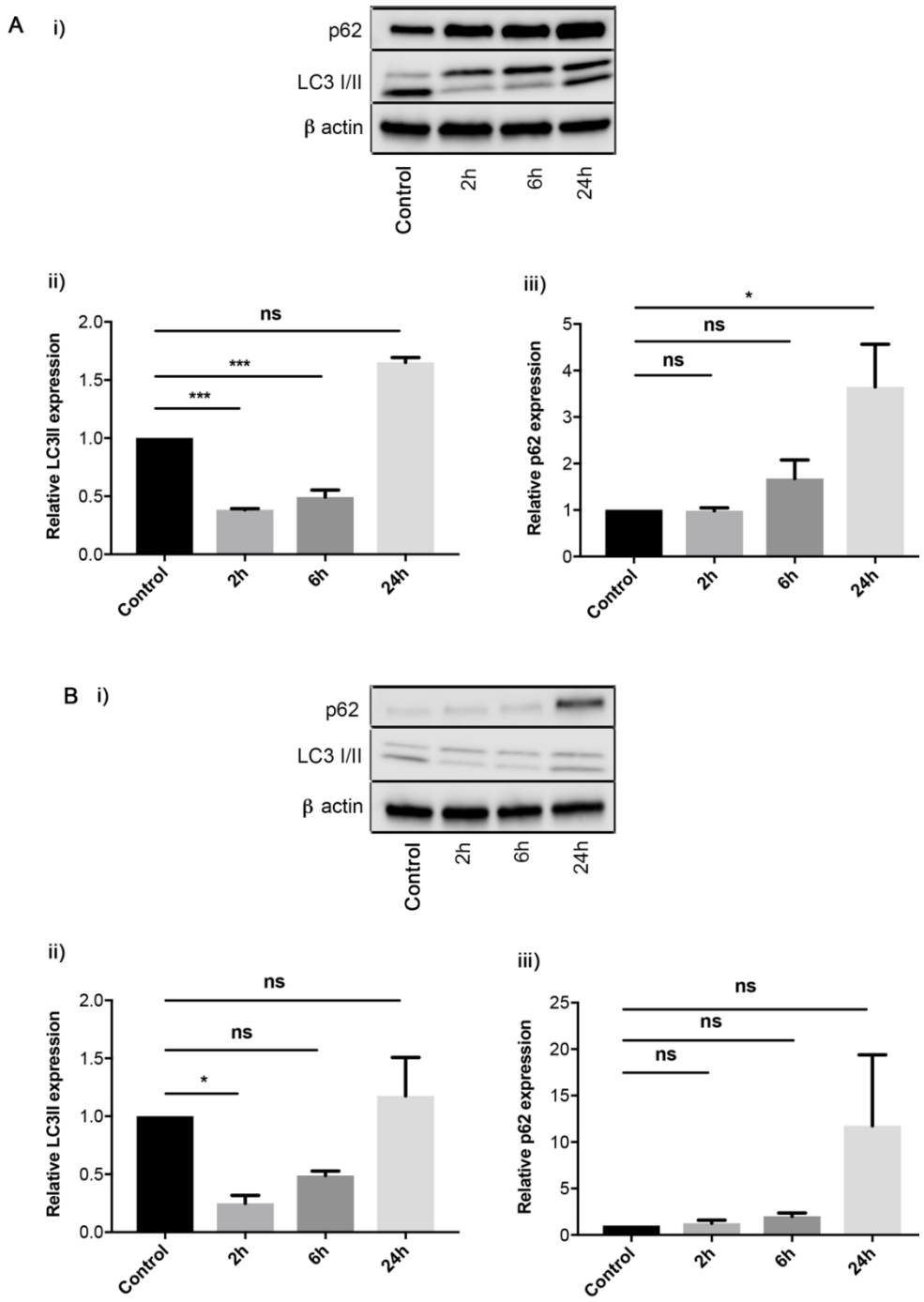


Figure 3-17: PIK III inhibits basal autophagy in melanoma cells in vitro in a time dependent manner

Representative western blots for the expression of p62 (kDa size), LC3 I/II (kDa sizes), or β-actin loading control (kDa sizes) in WM35 (Ai) or A375 (Bi) melanoma cells (control) or following treatment with 5 μM PIK III for 2, 4 or 24 hours. A or B I or ii; Relative LC3 II (A/B ii) or p62 expression (A/B iii, relative to β actin protein expression) in WM35 (A) or A375 (B) melanoma cells (control) or following 2, 6 or 24 hr treatment with 5 μM PIK III. Each bar is the mean of 3 biological replicates for each cell line, and expressed relative to the mean control of each individual experiment (mean ± SD, N = 3, *P<0.05, ***P<0.001

Finally, to assess the effect of specific autophagy inhibition on the cell viability of isolated CD271 positive and negative melanoma subpopulations *in vitro*, CD271 positive and negative subpopulations derived from either WM35 or A375 cells were treated in the presence or absence of 5 μM PIK III for 24 hours prior to the assessment of cell viability. Results demonstrated PIK III induced inhibition of both CD271 positive and negative subpopulations derived from either cell line (Figure 3-18), however, a significant reduction in the cell viability of CD271 positive cells compared to CD271 negative cells was observed in both WM35 and A375 subpopulations (one-way ANOVA analysis of variance with Tukey's multiple comparison test **P<0.01 for A375, ***P<0.001 for WM35) (Figure 3-18). Collectively these data thus highlight the intimate relationship between constitutive expression of CD271 by melanoma subpopulations and increased basal autophagy and suggest CD271 positive subpopulations depend on pro-survival autophagy for survival.

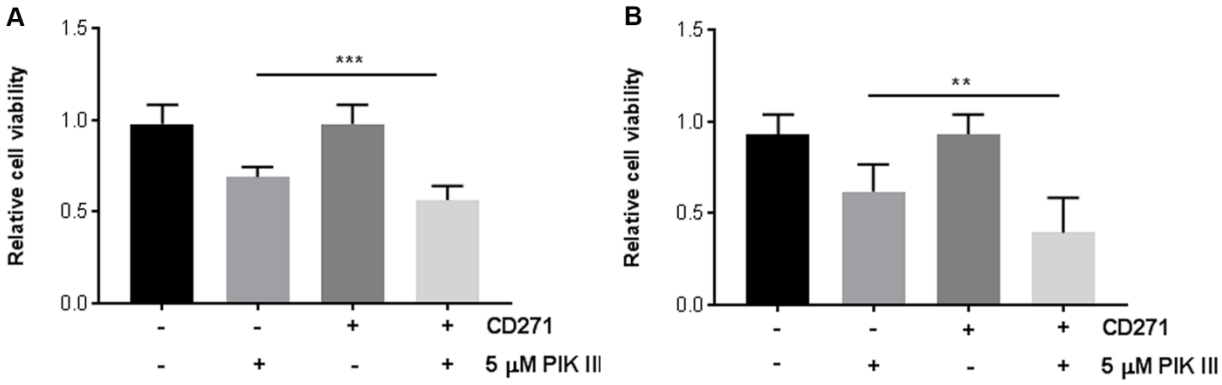


Figure 3-18: Autophagy blockade by Vps34 specific inhibition inhibits the cell viability of CD271 expressing melanoma subpopulations

Relative cell viability of WM35 (A) or A375 (B) isolated CD271 positive and negative subpopulations following treatment for 24 hrs with 5 μM PIK III (data expressed relative to control untreated cells). Each bar is the mean cell viability of 12 replicates from 3 independent experiments ± SD. **P<0.01, ***P<0.001.

3.3 **Discussion**

3.3.1 Constitutive expression of CD271 *in vitro* and *in vivo*, a marker of an aggressive melanoma phenotype?

To date, the only well-established predictors for melanoma progression and metastasis are those included in current AJCC staging, namely tumour Breslow thickness, ulceration and the presence of mitoses (Charles *et al.*, 2009). However, these markers do not capture all high-risk melanomas and occasionally melanomas with indolent tumoural characteristics still go on to develop metastatic disease with associated poor prognosis, thereby emphasizing the acute need for credible biomarkers able to identify high risk tumour subsets. Recent literature highlights the presence of subpopulations within melanomas that exhibit a more aggressive phenotype and express neural crest stem cell markers. A number of these melanoma subpopulations have been described including those expressing neuroectodermal stem cell markers such as Nestin, SOX10, Jarid-1B and ABCB5 (Roesch *et al.*, 2010; Chartrain *et al.*, 2012; Ladstein *et al.*, 2014; Zhang and Herlyn, 2014), while expression of the neural crest stem cell marker CD271 has been linked to both melanoma progression as well as resistance to targeted therapies (Ravindran Menon *et al.*, 2014; Beretti *et al.*, 2015). The observations of high levels of constitutive CD271 expression in metastatic melanoma tissue compared to expression observed in early AJCC stage primary melanomas that did not progress in the present study, are thus consistent with the association of such subpopulations with metastasis. These data therefore highlight constitutive CD271 expression as a marker of advanced, stage III melanomas. However, as CD271 expression was very low in benign naevi, stage I and stage II melanomas, the expression of CD271 could not serve as an early prognostic biomarker but the presence of increased CD271 expression could signify more advanced and aggressive melanomas.

Data form the present study additionally highlighted the variable expression of CD271 expression in primary melanocytes, primary and metastatic melanoma cell lines *in vitro*, consistent with previous findings reporting a diverse range in CD271 expression from 3-70% and a cell line dependent effect (Gray *et al.*, 2015). Interestingly, current data also

demonstrated the disproportionate expression of CD271 at the mRNA and protein level, a phenomenon previously linked to protein translation efficiency, protein half-life and protein turnover (Vogel and Marcotte, 2012) and factors which perhaps explain the current mismatch between protein and mRNA expression observed between many cell lines. Phenomenon which was particularly pronounced in WM164 cells demonstrating high expression of CD271 at the protein level but which was not accompanied by increased expression at the mRNA level or in CHL-1 cells where high levels of CD271 mRNA level were not translated at the protein level.

As mentioned, recent literature suggests CD271 as a putative stem cell marker in melanoma (Boiko *et al.*, 2010a), although there is conflicting evidence for an association between CD271 expression and other stem cell markers and the significance of such an association if any, in melanomagenesis (Boiko *et al.*, 2010a; Quintana *et al.*, 2010) and melanoma progression (Beretti *et al.*, 2015). A recent publication by Redmer *et al* suggests only a fraction of CD271 positive cells are present in melanoma tumours *in vivo* which co-express the associated gene and stem cell marker, CD133 (Redmer *et al.*, 2014a). Nevertheless, Redmer *et al* reported the constitutive expression of CD271 prevented melanoma cell differentiation and hence these studies support CD271 expression by melanomas as a stem like marker. Another study by Cheli *et al* also highlighted the poor overlap between the expression of CD271 *in vitro* and *in vivo* and the co- expression of another neuroectodermal stem cell marker, ABCB5 (Cheli *et al.*, 2014a). Interestingly, this group also confirmed the association of CD271 expression with an increase in the expression of other markers of 'stem ness', including expression of CD271 *per se* by melanoma cells is associated with a stem –like phenotype. Other stem like markers that have been described to be associated with melanoma progression and resistance to chemotherapy and targeted therapies include Nestin, a marker neural progenitor cells important for the development of the central nervous system and which is associated with more aggressive cutaneous melanomas (Ladstein *et al.*, 2014). Jarid-1B, a member of the highly conserved family of jumonji/ARID1 (JARID1) histone 3 K4 (H3K4) demethylases is also associated with a chemo resistant slow cycling melanoma subpopulation (Roesch *et al.*, 2010). However, co-expression of CD271 with either of these stem cell markers still remains

inconclusive controversial. Data reported in the present chapter however, demonstrated a poor association between CD271 expression and the co-expression of other stem cell markers; Jarid-1b, nestin and SOX10, suggesting constitutive CD271 expression may not in fact be linked to a melanoma stem like phenotype. Nevertheless, since the present study did not evaluate the co-expression of CD271 with these markers at a single cell level, the derived results should be interpreted with some caution as a mixed population may have been present *in vitro* and studies within isolated CD271 positive and negative melanoma subpopulations *in vitro* skewing the derived data. Thus, the correlation between constitutive CD271 expression by melanoma with a stem cell phenotype remains somewhat enigmatic.

Concerning a link between constitutive CD271 expression by melanoma subpopulations and the contribution or role of such subpopulations to melanoma invasion and tumourigenicity, data derived from the present chapter suggest melanoma cells constitutively expressing CD271 exhibit reduced spheroid expansion *in vitro* compared to their CD271 negative counterparts. These data are consistent with recent reports demonstrating CD271 negative cells are more invasive *in vitro* in collagen based invasion assays as well as in skin equivalent models and *in vivo* in a zebrafish xenograft model (Saltari *et al.*, 2016). However, additional studies by Cheli *et al* add further complexity to the role of CD271 in melanoma tumourigenecity, describing alternative subcategorization of CD271 positive and negative melanoma and the existence of a sub sets expressing high levels of CD271 and which are characterized by their slow growth but highly tumorigenic potential or subpopulations which express low levels of CD271 but which display a high proliferation rate with low tumorigenic potential (Cheli *et al.*, 2014a).

Having made the association between constitutive CD271 expression and a more aggressive but less invasive melanoma phenotype, and in line with previous studies suggesting a pro-survival role of autophagy in cancer stem cell (Maycotte *et al.*, 2015b) (Yang *et al.*, 2015b) survival, few studies however have investigated the potential association, if any, between constitutive CD271 expression and autophagy.

3.3.2 Linking constitutive CD271 expression with autophagy

Previous data from the Lovat group has shown the biphasic expression of p62 is consistent with the paradoxical role of autophagy in cancer (Ellis *et al.*, 2014a). Autophagy functions to remove degraded cellular debris; failure to execute this function results in the accumulation of toxic cellular waste, thereby driving tumourigenesis (Mathew *et al.*, 2007) while autophagy activation in the hypoxic environment of solid tumours such as melanoma serves to promote tumour survival and metastatic development (Ma *et al.*, 2014). Consistent with the studies by Ellis *et al* (Ellis *et al.*, 2014a), data derived from the present study revealed expression of p62 in naevi and primary AJCC stage I melanomas was reduced compared to expression in AJCC stage II and III. These observations thus support a potential association between the constitutive expression of CD271 by some melanoma subpopulations within a more aggressive melanoma and increased autophagic activity. However, in the present study this correlation was only statistically significant in AJCC stage II tumours and not in stage III. The lack of statistical significance in this context however, may reflect the small sample size of stage III tumours accessed and therefore further studies in an expanded cohort of AJCC stage III melanomas are required to provide a clear and more representative analysis and the validation of a potential correlation between constitutive CD271 expression and increased autophagic activity *in vivo*.

Breast and pancreatic cancer stem cell research has additionally highlighted the pivotal role of autophagy in the survival of breast (Maycotte *et al.*, 2015b) and pancreatic (Yang *et al.*, 2015b) cancer stem cells. Although results from the present study demonstrated a trend for increased basal autophagic activity in isolated CD271 positive compared to negative subpopulations *in vitro*, this association was not in fact significant. It should be noted however, that studies to support associated increased LC3-II expression in isolated CD271 melanoma subpopulations *in vitro* were performed in the absence of the addition of chloroquine to block autophagic flux (Loos *et al.*, 2014) and hence the incorporation of such experiments in future studies is required to more definitively validate the association between constitutive CD271 expression and the apparent increase in basal autophagy in such subpopulations *in vitro*. As

well as reporting a potential association between CD271 expression and increased basal autophagy in isolated melanoma subpopulations, the present study also revealed the plasticity and instability of CD271 expression in melanoma was more pronounced in 3D spheroids culture in which CD271 negative subpopulations acquired CD271 expression in the centre of the spheroid while p62 expression was observed only at the periphery of the spheroid, indicating active autophagy in the nutrient deprived core of the spheroid where CD271 expression is acquired. Conversely, CD271 expression was retained in CD271 positive subpopulations in which there was no observable distinct pattern of p62 expression and indicating similar autophagic activity throughout these spheroids. Collectively these data thus further support the notion that constitutive CD271 expression by melanoma cells *in vitro* is associated with increased autophagic activity. However, there is increasing evidence to support the influence of micro environmental factors on stem cell development and metastatic potential (Scott *et al.*, 2014). A recent paper by Menon *et al,* investigating the role of micro environmental variables such as hypoxia and nutrient deprivation on CD271 expression (Ravindran Menon *et al.*, 2014) demonstrated the induction of CD271 expression in response to pronounced hypoxia (5 or 1% O_2) as well as glucose deprivation indicating an early stress response to hostile micro environmental factors. Results from the present studies highlight the unstable nature of CD271 expression, and hence stability may depend on the hypoxic environment and nutrient availability additionally shown in other studies to influence the induction of CD271 expression (Ravindran Menon *et al.*, 2014).

There is increasing evidence associating cancer stem cells with the activation of pro-survival autophagy (Mowers *et al.*, 2017). However, there is little evidence to suggest the blockade of autophagy in melanoma stem like cells may present a viable approach to prevent the survival of such subpopulations or influence melanomagenesis. Data from the present study demonstrated blocking autophagy in isolated CD271 positive melanoma subpopulations *in vitro* with the lysosomal inhibitor chloroquine, resulted in the consistent inhibition of cell viability of such subpopulations derived from multiple cell lines, thereby supporting the notion that CD271 positive subpopulations, which as discussed above may not represent a true stem like subpopulation, nevertheless use autophagy to maintain survival. However, chloroquine is

not a specific autophagy inhibitor and hence additional studies of autophagy inhibition in melanoma subpopulations constitutively expressing CD271 were undertaken using a selective inhibitor of the Vps34 complex. Results demonstrated the Vps34 inhibitor, PIK III blocked both autophagy and the cell viability of wild-type WM35 and A375 melanoma cells as well as CD271 positive subpopulations derived from either cell line compared to CD271 negative subpopulations, collectively further supporting the hypothesis that melanoma cell subpopulations constitutively expressing CD271 use autophagy as a pro-survival mechanism.

Collectively results from this chapter highlight the association of CD271 expression with more aggressive melanomas and the correlation of CD271 expression with increased autophagic activity both *in vitro* and *in vivo*. Contrary to the original hypothesis of the present study it seems collectively however, that constitutive expression of CD271 expression by melanoma subpopulations is associated with reduced spheroid expansion, raising the question as to whether drug-induced CD271 melanoma subpopulations represent an inherently different phenotype which play a differing role in melanoma invasion and progression. These questions are addressed in the following chapters to the present thesis.

3.4 **Summary**

- Constitutive expression of CD271 is increased in advanced primary AJCC stage III melanomas compared to AJCC stage I, II or benign naevi

- Constitutive expression of CD271 in AJCC stage II melanomas is inversely correlated with p62 expression indicating CD271 expression *in vivo* is associated with increased basal autophagy

- Primary melanocytes, primary and metastatic melanoma cell lines display variable CD271 mRNA and protein expression

- CD271 positive melanoma subpopulations isolated from metastatic melanoma cell lines *in vitro* display increased basal autophagy

- Constitutive expression of CD271 by melanoma cells *in vitro* does not correlate with the expression of other neuroectodermal stem cell markers

- Spheroid derived from CD271 positive melanoma subpopulations isolated from metastatic melanoma cell lines *in vitro* exhibit reduced spheroid expansion in collagen gels than their CD271 negative counterparts

- Inhibition of autophagy with chloroquine or a specific inhibitor of Vps34 results in significant reduction in cell viability of CD271 positive compared to CD271 negative melanoma subpopulations isolated from melanoma cell lines *in vitro.*

Chapter 4

Drug-induced or overexpression of CD271 in melanoma is associated with increased pro-survival autophagy and reduced spheroid collagen expansion

Chapter 4 Drug-induced or overexpression of CD271 in melanoma is associated with increased pro-survival autophagy and reduced collagen expansion

4.1 Introduction

Although the prognosis for early AJCC stage melanomas is excellent, for patients with malignant AJCC stage IV disease, prognosis remains very poor with continued 5 year survival rates of 10-20% (http://www.cancerresearchuk.org/about-cancer/melanoma/survival, 2016). The recent identification of driver mutations in BRAF, present in approximately 60% of all melanomas (Ascierto *et al.*, 2012) has unveiled a new era in targeted therapy for patients with advanced BRAF mutant melanoma. BRAF and subsequently MEK inhibitors have been developed to target activated MAPK signalling, showing significant benefit in progression free and overall survival for patients with stage IV metastatic melanoma (Chapman *et al.*, 2011; Long *et al.*, 2014). However, the early development of acquired resistance to BRAF inhibitors (usually established after 6-9 months of treatment initiation (Shi *et al.*, 2014) which although may delayed by up to 12 months through combined therapy with novel generation MEK specific inhibitors (Welsh *et al.*, 2016), contributes to disease progression and impacts on patient survival. Much research has focused on mechanisms mediating the resistance of melanoma to BRAF/MEK inhibition and novel approaches through which to overcome such acquired resistance (Aplin *et al.*, 2011) and given the complexity of a heterogeneous cancer type with different histological and biological characteristics, it is therefore perhaps not surprising that multiple mechanisms have been proposed, including the secondary upregulation of N-RAS (Nazarian *et al.*, 2010), activation of receptor tyrosine kinases (RTK) such as IGF-1R (Villanueva *et al.*, 2011) and the emergence of chemo resistant subpopulations expressing stem cell surface markers such as CD271 (Ravindran Menon *et al.*, 2014), ABCB5 (Chartrain *et al.*, 2012) and Jarid-1B (Roesch *et al.*, 2013).

Recently CD271 expressing melanoma subpopulations have emerged as one of the major subpopulations associated with the resistance of melanoma to BRAF and MEK inhibition

(Ravindran Menon *et al.*, 2014). CD271 is a neurotrophin receptor expressed in normal tissues and involved in neuronal development (Dechant and Barde, 2002), however, the precise role of CD271 expression in the drug-induced resistance of melanoma is not fully understood. As revealed in chapter 3, constitutive CD271 expression by melanoma cell lines is associated with an increase in autophagic activity and accumulating evidence further highlights CD271 expressing melanoma subpopulations as an early drug-induced tolerant population that emerges under stressful micro environmental conditions such as hypoxia and nutrient deprivation (Ravindran Menon *et al.*, 2014), key factors in autophagy activation and suggesting autophagy may also play an integral role in the survival of drug resistant CD271 expressing melanoma subpopulations (Pursiheimo *et al.*, 2008), (Young *et al.*, 2009). As a major cellular energy sensor activated by ATP and nutrient depletion to maintain energy homeostasis, AMPK may directly activate autophagy (Hardie, 2011), shown to be a key event in autophagy mediated survival of lung (William *et al.*, 2012) and colorectal (Baba *et al.*, 2010) cancer and hence raises the question as to whether AMPK activation may also initiate pro-survival autophagy in drug-resistant CD271 expressing melanoma subpopulations.

Results reported in the previous chapter further highlighted the reduced invasive capacity of constitutively CD271 expressing melanoma subpopulations. It is well known that CD271 as a nerve growth factor receptor is stimulated by NGF to promote neuronal cell survival *in vivo* (Johnson *et al.*, 1986) and increasing evidence supports the role for NGF in tumour cell proliferation and invasion. In pancreatic cancer cells, over expression of NGF is associated with increased perineural cancer invasion (Zhu *et al.*, 2002) while *in vitro* exogenous NGF treatment of melanoma increases tumour cell invasion in a dose dependent manner (Herrmann *et al.*, 1993). It is therefore possible that the invasive capacity of CD271 drug resistant subpopulations is supported by NGF or other factors independently of autophagy. Nevertheless, given the association of constitutive CD271 expression by melanoma cells with increased basal autophagy activity and recent literature describing the association of increased autophagy with BRAF/MEK inhibitor-induced CD271, autophagy is clearly linked to the emergence of drug resistant CD271 expressing melanoma subpopulations. Whether autophagy is activated as a consequence of drug-induced CD271 expression (or by over

expression of CD271) in melanoma or whether its activation leads to the induction of CD271 expression in BRAF/MEK inhibitor drug-resistant subpopulations is unclear and leads to the aims of the present chapter of defining the association between autophagy activation and CD271 induction in melanoma cells in the presence or absence of BRAF or MEK inhibition, and the cascade of events and relative contribution of autophagy to the survival and invasive potential of CD271 expressing melanoma subpopulations.

4.2 **Results**

4.2.1 Drug-induced resistance of melanoma to BRAF and MEK inhibition *in vitro* is associated with an increase in CD271 expression and basal autophagy

To confirm the association between BRAF and MEK inhibitor-induced CD271 (Ravindran Menon et al., 2014; Lehraiki et al., 2015; Fallahi-Sichani et al., 2017) and an increase in basal autophagy BRAF mutant melanoma cells, WM35, SK-mel-28 or A375 cells were treated continuously over a long-term period of 9 days with 3 µM PLX4720 (BRAF inhibitor) or 16 nM trametinib (MEK inhibitor), prior to western blot analysis of CD271, p62 and LC3 I/II expression. Initial pilot experiments of PLX4720 in SK-mel-28 cells revealed the dose dependent induction of CD271 paralleled by the induction of LC3 I/II (

Figure 4-1).

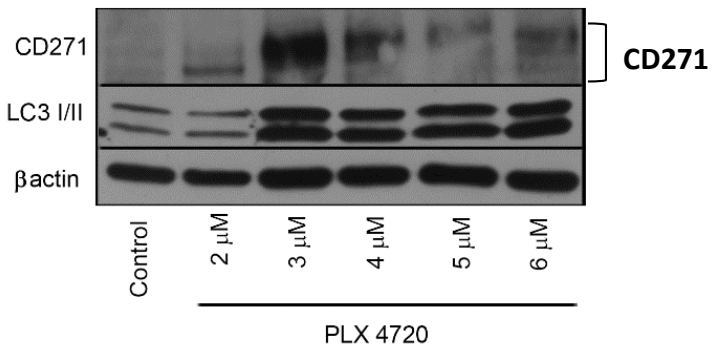


Figure 4-1: PLX4720 increases CD271 and LC3 I/II in BRAF mutant SK-mel-28 cells in a dose-dependent manner.

Representative Immunoblot for the expression of CD271 (75 kDa), LC3I/II (16-18 kDa) or β actin loading control (45 kDa) in SK-mel-28 cells (control) or in SK-mel-28 cells following treatment with 2-6 μM PLX 4720 for 9 days (n=1)

Given treatment with 3 μM PLX4720 induced maximal CD271 and LC3 I/II expression following 9 days treatment, this concentration was used in subsequent experiments to establish the optimal time point for BRAF inhibitor- induced CD271 induction and associated changes in autophagic activity. Experiments performed by Dr David Hill using BRAF mutant A375 cells treated with 3 μM PLX4720 for 3, 6, 9 or 12 days prior to western blot analysis of CD271or p62 expression. Results demonstrated a time-dependent increase in CD271 expression by PLX4720 which peaked at 12 days (

Figure 4-2 which was again paralleled by an increase in autophagic activity as evidenced by a reduction in p62 expression (

Figure 4-2).

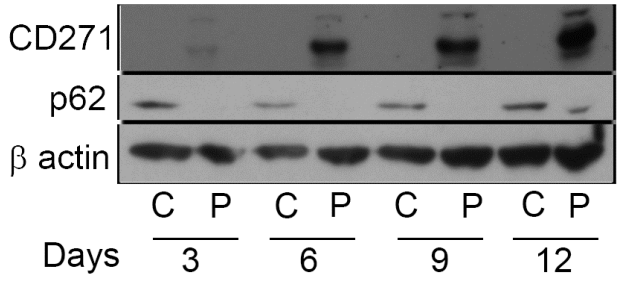


Figure 4-2: PLX4720 induces a time dependent increase in CD271 and decrease in p62 in BRAF mutant A375 cells

Representative Immunoblot for the expression of CD271 (75 kDa), p62 (62 kDa) or β actin loading control (45 kDa) in untreated control A375 (C) or in A375 cells following treatment with 3 μM PLX 4720 (P) for 3, 6, 9 or 12 days (n=1)

Increasing evidence supports the clinical benefits of dual inhibition of activated MAPK signalling in patients with BRAF or NRAS mutant metastatic melanoma (Robert *et al.*, 2015a) and there has been a change in clinical practice to the use of combination targeted therapy

since the NICE approval of Dabrafenib (BRAF inhibitor) and Trametinib (MEK inhibitor) for the management of patients with stage IV BRAF mutant melanoma.

To assess the impact of MEK inhibition on CD271 expression and autophagy, BRAF mutant A375, WM35 and SK-mel-28 cells were treated continuously with 16 nM trametinib for 9 days prior to the analysis of CD271, LC3 I/II and p62 expression. Results demonstrated the significant induction of CD271 in all cell lines following treatment with trametinib for 9 days (Figure 4-3 A, B, C i and ii, unpaired Student's T test ***P < 0.001 for WM35 and *P < 0.05 for Sk-mel-28 and A375 cells), which was associated with the concurrent increase of lipidated LC3-II expression (Figure 4-3 A, B, C i and ii, Student's T test **P < 0.01 for WM35 and *P < 0.05 for Sk-mel-28) and significantly decreased expression of p62 (Figure 4-3 A, B, C i and ii, Student's T test, ****P < 0.0001 for WM35 and A375 and *P < 0.05 for SK-mel-28) indicating a concurrent increase in basal autophagic activity with the induction of CD271 expression.

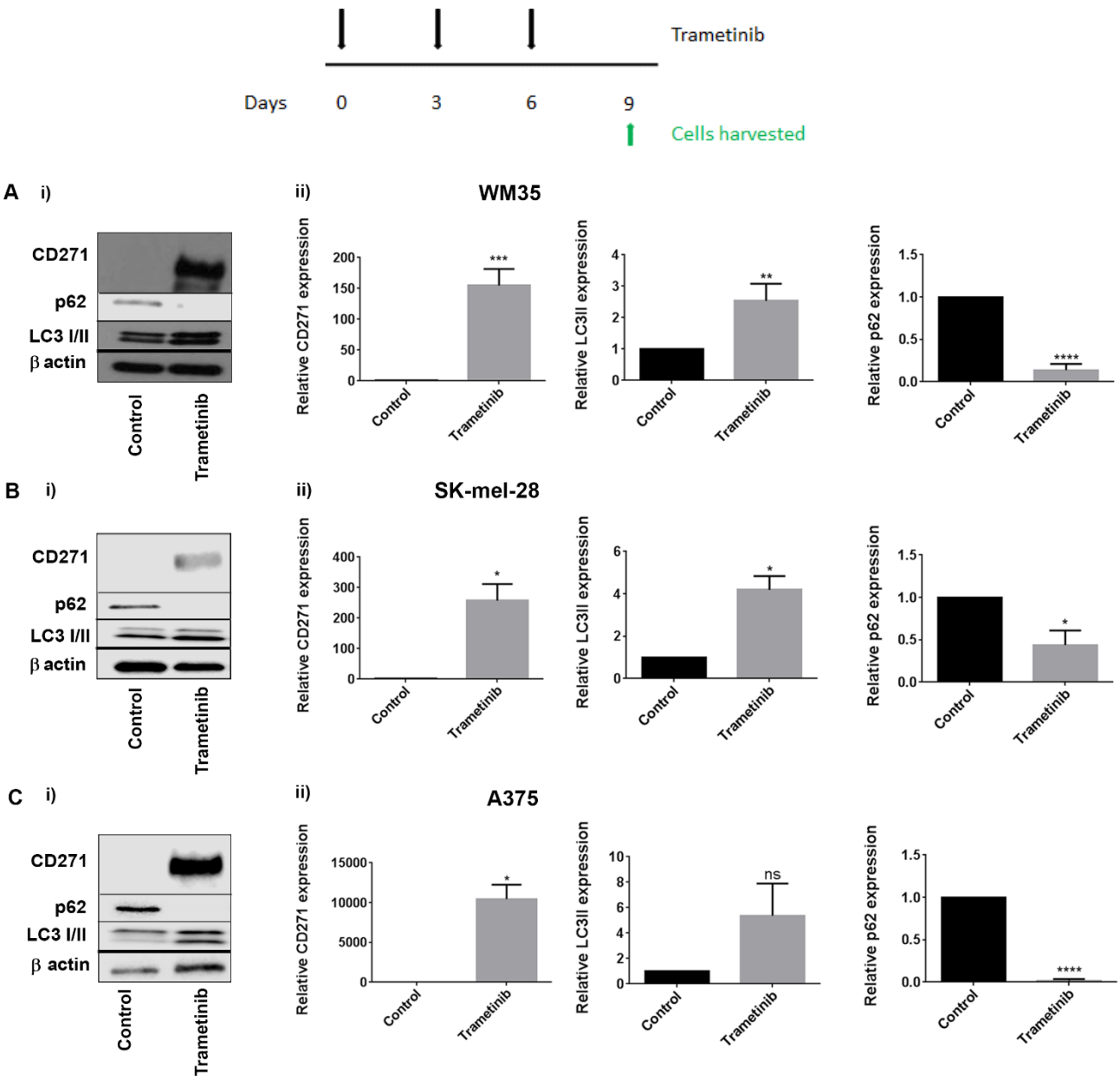


Figure 4-3 Chemoresistance of SK-mel-28, WM35 and A375 melanoma cells to trametinib is associated with the induction of CD271 expressing subpopulations and increased basal autophagy.

Representative immunoblots for the expression of CD271 (45 kDa), LC3I/II (16 kDa), p62 (62 kDa) or β actin loading control (45 kDa) in WM35 (A (i), control), SK-mel-28 (B (i), control) and A375 (C (i), control) cells or following treatment with 16 nM trametinib for 9 days or relative expression levels of CD271, LC3-II or p62 relative to β actin in 3 individual experiments (A(ii), B(ii), C(ii)). Each bar is the mean of 3 experiments ± SD. *P < 0.05, **P<0.01***P<0.001, ****P<0.0001

Although the detection of LC3 II induction and p62 degradation by western blotting are key indicators of autophagy activity they do not reflect true LC3 autophagic flux, a key and universally accepted measure of autophagy (Corazzari *et al.*, 2015). To confirm the observed increase in autophagy in trametinib-induced CD271 expressing melanoma subpopulations LC3 flux was determined in BRAF mutant WM266-4 cells previously engineered to stably express LC3-RFP-GFP and allowing the simultaneous detection of autophagosomes as well as autolysosomes. As shown in Figure 4-4, treatment of WM266-4 cells with 16 nM trametinib for 9 days resulted in the significant induction of CD271 expression (depicted by pink fluorescence, Figure 4-4 B and C, unpaired Students T test, ***P < 0.001, Figure 4-4 D) as well as the significant induction of LC3 flux (depicted by the presence of red fluorescence only, Figure 4-4 A & C) and reflected by a significant decrease in mean GFP fluorescence intensity (Figure 4-4 D, unpaired Students T test, **P < 0.01) and a significant increase in mean RFP fluorescence intensity (Figure 4-4 D, unpaired Students T test, *P < 0.05), further emphasizing the association between drug resistant CD271 expressing melanoma sub populations and increased autophagy.

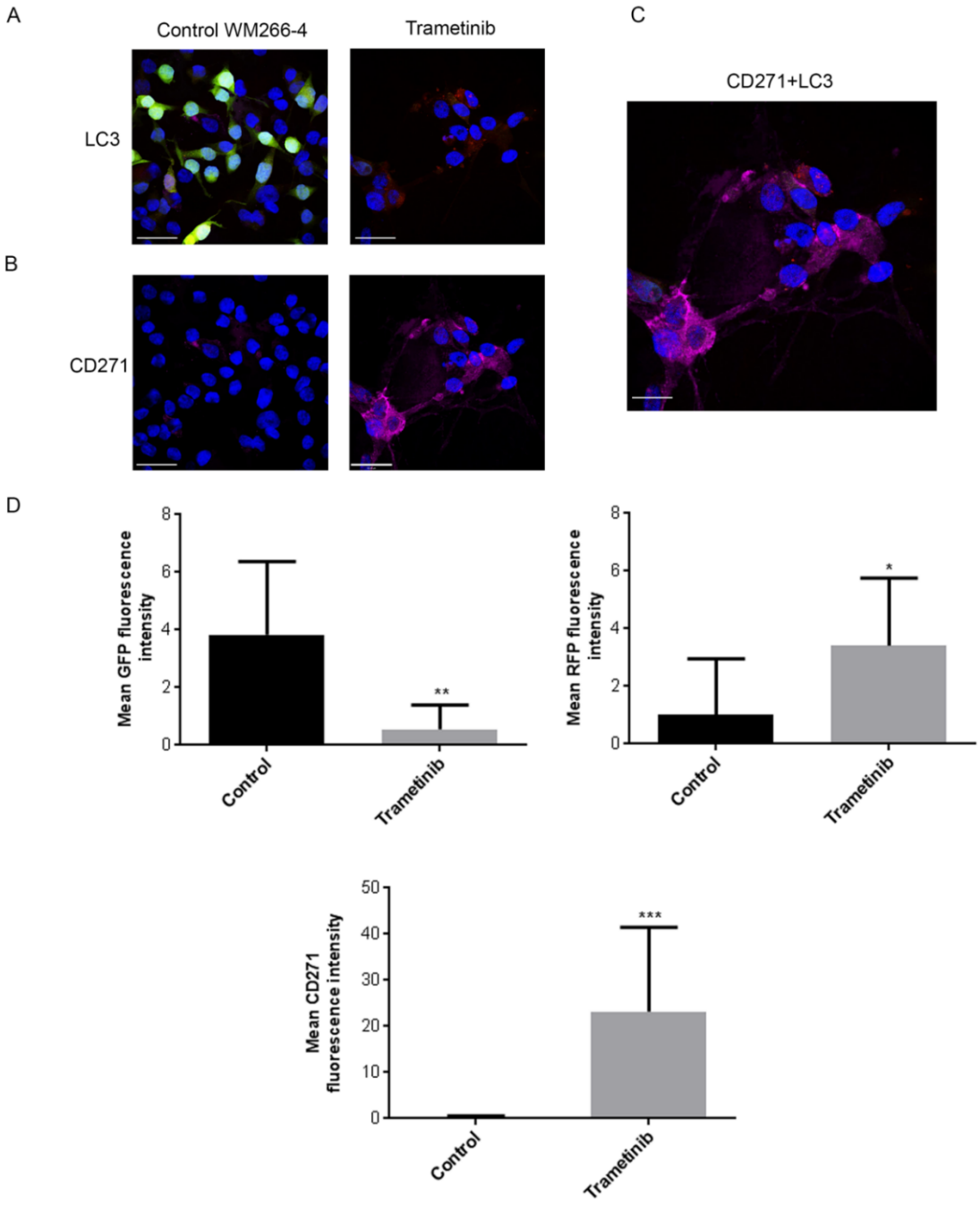


Figure 4-4: Trametinib-induced CD271 expression is associated with increased autophagic flux

Representative immunofluorescence of WM266-4 mRFP-GFP-LC3 cells depicting A: basal LC3 green fluorescence or B: CD271 expression (pink fluorescence) in control WM266-4 cells or increased LC3 flux (reflected by decreased green and increased red fluorescence, in A) or CD271 expression (B) in cells following 9 days treatment with 16nM trametinib. (C). Merged images of LC3 flux and CD271 induction as indicated by the presence of pink (CD271) and red dots in trametinib treated WM266-4 cells. (D) Mean CD271, RFP and GFP fluorescence intensity in RFP-GFP-LC3 WM266-4 cells following treatment with 16nM trametinib compared to untreated control cells (*P < 0.05, *** P<0.001). Blue fluorescence indicates DAPI labelled cell nuclei, Scale bar = 50 µm

4.2.2 Prolonged treatment of human A375 melanoma cells with trametinib results in transient induction of CD271 expression and a concurrent increase in autophagic activity

Recent literature highlights the transient expression of CD271 in melanoma subpopulations in response to treatment with BRAF/MEK inhibitors (Ravindran Menon *et al.*, 2014) suggesting CD271 may only be expressed as a transient survival mechanism until tumour repopulation and regrowth are re-established, at which point CD271 may not be required for tumour maintenance. To investigate this possibility and any associated changes in autophagy, A375 cells were grown in the presence of 16 nM trametinib for 3, 6, 9, 12, 24 or 42 days prior to the analysis of CD271, LC3 I/II, p62, Cleaved PARP, pERK, total ERK or GAPDH expression by western blotting. Results demonstrated an increase in trametinib-induced CD271 expression at day 6 which peaked at day 9 and then returned to baseline expression from day 14, which was accompanied by a concurrent increase in trametinib-induced autophagy as reflected by an increase LC3 II and a decrease in p62 expression (Figure 4-5 A & B). LC3 II expression increased over time and peaked at 14 days followed by a reduction in expression from day 24 which was, however, increased compared to baseline expression (Figure 4-4 A, B). P62 expression was reduced at day 3, 6 and 9 indicating an induction n autophagic activity and then steadily increased from day 14 to day 42 (Figure 4-4 A, B). PARP cleavage was also induced by trametinib reflecting increased apoptotic cell death at day 3 and 6 which then decreased after 14 days treatment (Figure 4-5 A & B). Interestingly, trametinib also abolished pERK expression following 3 days treatment, but after 6 days treatment, levels began to increase, in line with the development of drug resistance (Figure 4-5A & B). However, ongoing long-term treatment with trametinib for more than 14 days resulted in reduced pERK expression indicating fully drug-resistant A375 subpopulations may not depend on the MAPK pathway to promote tumour development and growth (Figure 4-5 A, B). Collectively these data highlight the transient induction of CD271 in response to trametinib which is lost after 14 days and is accompanied by an induction in autophagic activity peaking between 9 and 14 days. The subsequent decline in autophagic activity in trametinib treated cells, albeit still above base line even in the absence of CD271 expression additionally supports current

literature in other tumour types demonstrating the induction of pro-survival autophagy by inhibitors of activated MAPK signalling (Goulielmaki *et al.*, 2016).

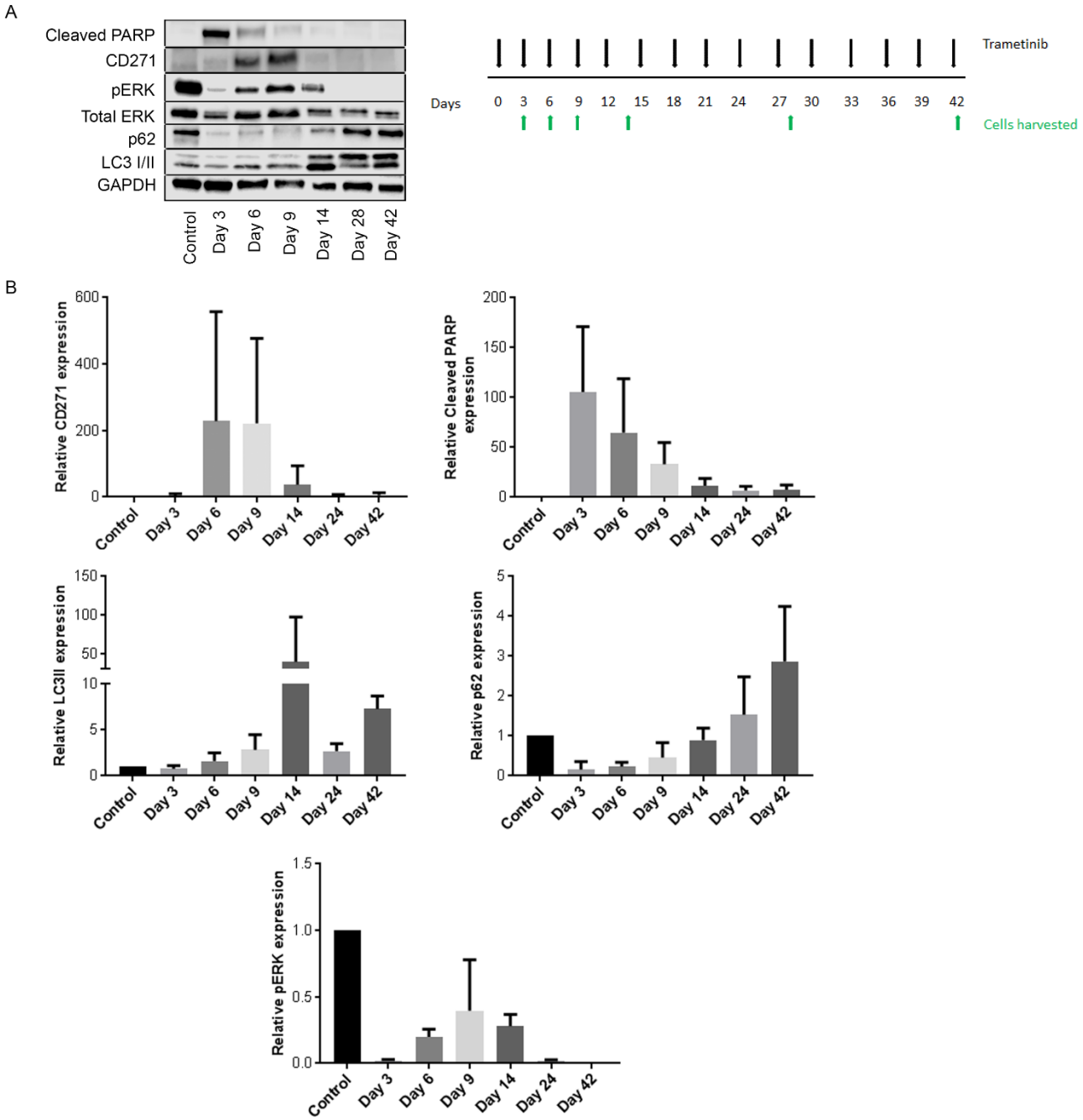


Figure 4-5 Trametinib induces transient CD271 expression by melanoma cells in vitro

A: Representative immunoblots for the expression of Cleaved PARP (89 kDa), CD271 (45 kDa), pERK (42-44 kDa), total ERK (42-44 kDa), p62 (62 kDa), LC3I/II (16-18 kDa), or GAPDH (37 kDa) loading control in A375 control cells (Control) or in cells treated with 16 nM trametinib for 3, 6, 9, 14, 24 and 42 days
B: Relative expression of CD271, Cleaved PARP, LC3-II or p62 relative to GAPDH loading control in 3 individual experiments of A375 cells treated in the absence (control) or presence of 16nM trametinib for 3-42 days . Each bar is the mean of 3 experiments ± SD (NS P>0.05).

Further experiments to confirm these findings were performed by Dr David Hill, Newcastle University. Flow cytometry analysis of annexinV/PI stained cells over a time-course treatment of A375 cells for 28 days with 16 nM trametinib, demonstrated significant trametinib-induced apoptosis up to 14 days post-treatment, however, by day 28 most cells were resistant to the cytotoxic effects of trametinib (one-way ANOVA with Tukey's multiple comparison post hoc correlation *p<0.05, **p<0.01, ***p<0.001), (

Figure 4-6A). Gating of live, annexinV/PI stained A375 cells that were co-stained with anti-CD271 antibody indicated trametinib induced a time-dependent increase in CD271 expression, peaking after 9 days treatment (Figure 4-6 B, C).

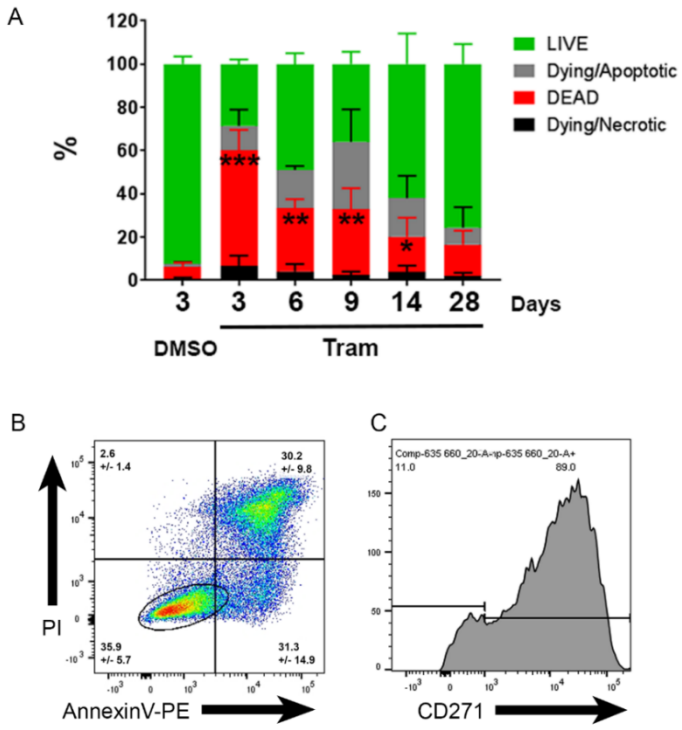


Figure 4-6 Trametinib induces apoptotic cell death and CD271 expressing melanoma subpopulations

A.Flow cytometry of AnnexinV-PE/PI stained A375 melanoma cells for percentage Live, Dead, Dying/Apoptotic or Dying/Necrotic following treatment in the presence or absence of 16 nM trametinib for 3, 6, 9, 14 or 28 days (percentages did not significantly change following DMSO treatment, which shown at day 3 only for clarity). Each bar is the mean of 3 experiments ± SD (*P<0.05, **P<0.01, ***P<0.001).B. Representative flow cytometry plots of A375 cells stained with AnnexinV-PE/PI/anti-CD271 following treatment for 9 days with 16 nM trametinib. Each bar is the mean of 3 experiments ± SD. Oval gate shown for Live cells was used to quantify CD271 expression, shown in right panel, which was negative for null-primary control. C. Histogram of CD271 staining of gated live cells

Collectively these data further demonstrate a transient induction of CD271 in melanoma cells in response to trametinib, with a concurrent increase in autophagy.

Although improving progression free survival over and above monotherapy with BRAF inhibitors, the development of acquired resistance to combined BRAF/MEK inhibitor therapy, remains the main reason for treatment discontinuation, with similarly to monotherapy with BRAF inhibitors, similar mechanisms of drug-induced resistance proposed including the activation of pro-survival PI3K-mTOR signalling and immune alterations (Welsh *et al.*, 2016). To determine the impact of combined BRAF and MEK inhibition on CD271 expression in drug resistant subpopulations and associated autophagy activity, WM35 and A375 cells were treated in the presence of 16 nM trametinib and 2 µM PLX4720 for 14 days prior to the analysis of CD271, p62 and LC3 I/II expression by Western blotting. Incubation with combined trametinib/PLX 4720 was extended to 14 days as initial pilot experiments revealed no induction of CD271 expression in either cell line by combined treatment at 9 days (data not shown), in line with clinical data indicating resistance to BRAF/MEK inhibition occurs later than in patients treated with monotherapy BRAF or MEK inhibitors. Results demonstrated the induction of CD271 in both WM35 and A375 cells by single agent trametinib or PLX4720 or by combined trametinib/PLX4720, although the combination therapy resulted in lower levels of CD271 induction at 14 days indicating that CD271-associated acquired resistance to combination therapy requires longer exposure to the inhibitors (Figure 4-7 A and B), which is in line with clinical observations that acquired resistance in patients receiving combination therapy develops later compared to monotherapy treatment with each drug individually. CD271 induction was accompanied in both cell lines by a concurrent decrease in p62 expression and increased LC3 II expression, again to varying degrees (Figure 4-7 A and B). Collectively these data confirm previous studies indicating an association between BRAF/MEK inhibitor (as monotherapy or combination therapy) induced drug resistant CD271 expressing subpopulations and an associated increase in basal autophagy in such subpopulations. However, whether autophagy is induced as a consequence of drug resistance leading to CD271 induction in resistant subpopulations or whether CD271 induction in drug resistant

subpopulations itself results in autophagy activation is unclear and is further addressed in section 4.2.6, 4.2.7 and 4.2.8 by modulating the expression of CD271.

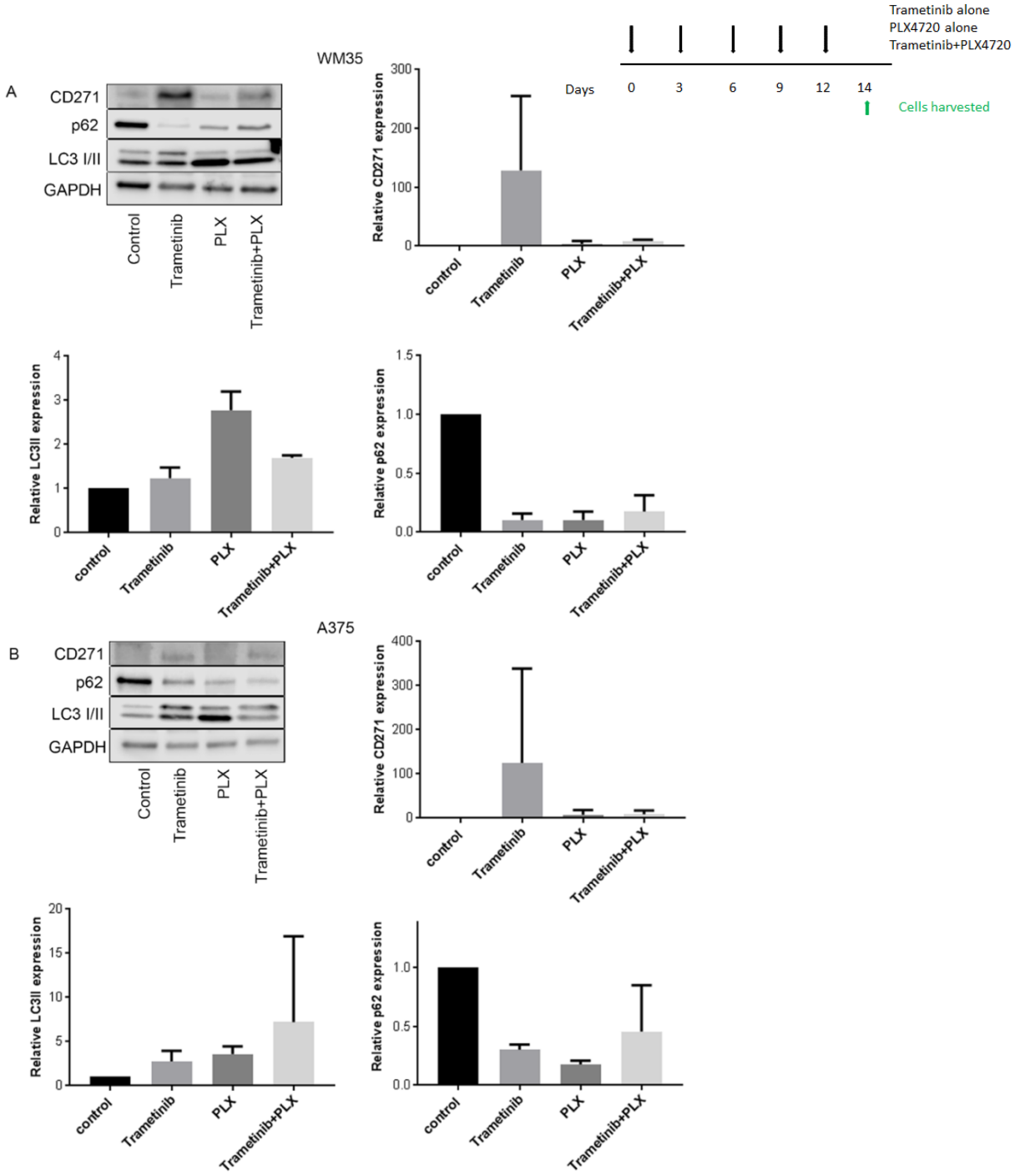


Figure 4-7: Combined treatment of melanoma cells with trametinib and PLX4720 results in induction of CD271 expression and autophagy

Representative immunoblots or relative expression of CD271 (45 kDa), p62 (62 kDa), LC3I/II (16-18 kDa), or GAPDH (37 kDa) loading control or relative to GAPDH in 3 individual experiments in WM35 (A) or A375 (B) cells in the presence or absence (control) of treatment for 14 days with 16nM Trametinib, 3 µM PLX4720 (PLX) or combined Trametinib+PLX. Each bar is the mean of 3 experiments ± SD.

4.2.3 Trametinib-induced CD271 expression *in vitro* is not consistently associated with the induced expression of other stem cell markers

BRAF or MEK inhibitor- induced resistant melanoma subpopulations are reported to express a variety of stem cell markers including ABCB5 (Frank *et al.*, 2005b), Nestin (Akiyama *et al.*, 2013a), SOX 10 (Graf *et al.*, 2014a) and Jarid1B (Roesch *et al.*, 2013). To investigate whether the potential induction of these stem cell markers is associated with MEK inhibitor-induced CD271 expression in drug resistant melanoma subpopulations, SK-mel-28, WM35 and A375 cells were treated in the presence or absence of 16 nM Trametinib for 9 days prior the analysis of ABCB5, Jarid-1B, SOX10 and Nestin by western blotting. Results confirmed the induction of CD271 in each cell line while interestingly however, an apparent trend for trametinib-induced downregulation of ABCB5 expression in SK-mel-28 and A375 cells was observed, with expression increased in WM35 cells (Figure 4-8). Nevertheless, the only significant change in ABCB5 expression was a down regulation in expression in response to trametinib in SK-mel-28 cells (unpaired Student's T-test, *P<0.05 (Figure 4-8).

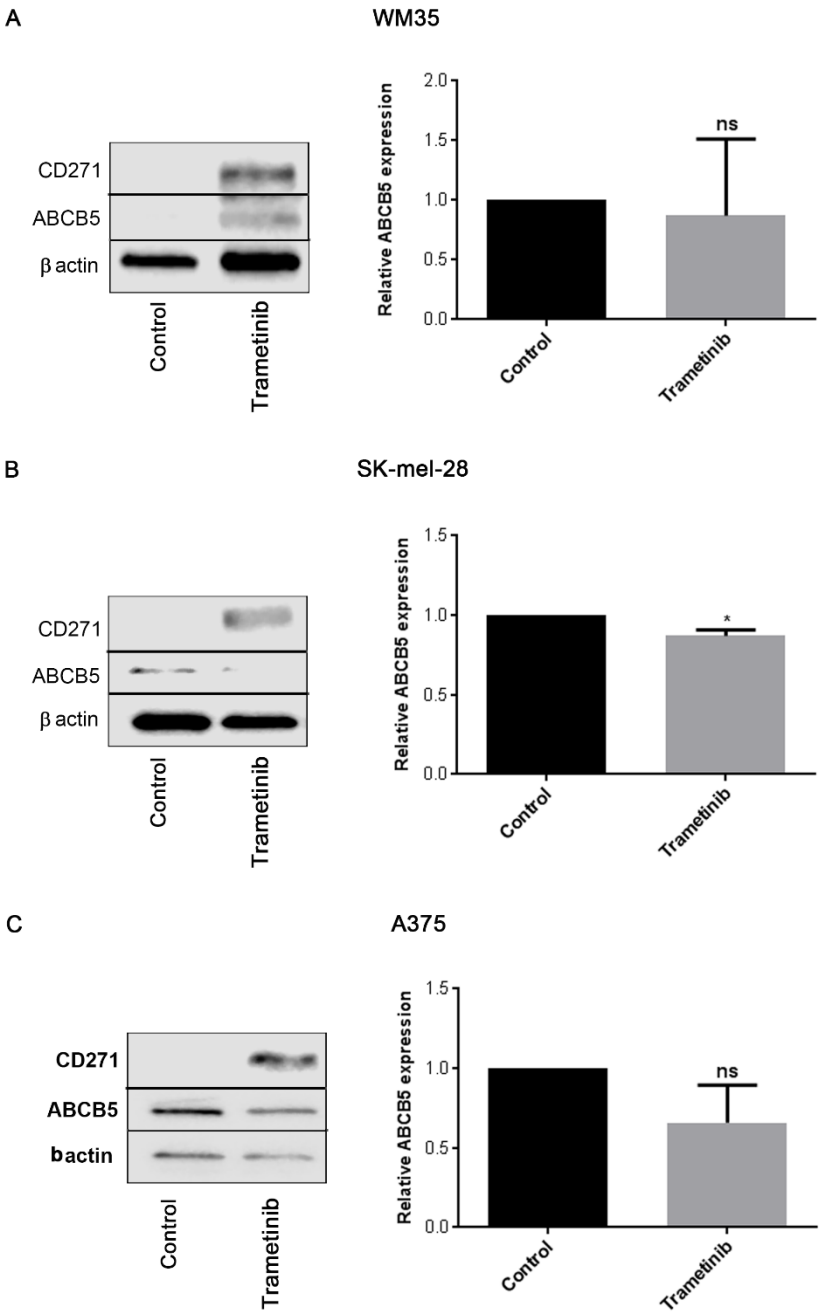


Figure 4-8: Trametinib-induced CD271 Expression in melanoma cells is associated with variable changes in ABCB5 expression

Representative immunoblots for CD271 (45 kDa), ABCB5 (90 kDa), or β actin loading control (45 kDa) or Relative ABCB5 expression (relative to β actin loading control) in WM35 (A), SK-mel-28 (B) and A375 (C) cells following treatment with vehicle DMSO control or 16 nM trametinib for 9 days. Each bar is the mean of 3 experiments ± SD Statistics were acquired using unpaired t test (*P < 0.05).

Conversely, trametinib-induced CD271 was associated with a trend for the consistent induction of both Jarid 1B (Figure 4-9) and SOX 10 (Figure 4-10) in all cell lines, although findings were only significant for trametinib-induced SOX 10 induction in drug resistant CD271 expressing A375 melanoma subpopulations (unpaired Student's T-test, ****P<0.0001, Figure 4-10). Studies of Nestin expression following treatment of SK-mel-28, WM35 or A375 cells for 9 days treatment with 16nM trametinib, revealed a trend for down regulation in drug -induced CD271 expressing subpopulations (Figure 4-11) in all cell lines. However, again this effect was only significant in SK-mel-28 cells (unpaired t test, ***P<0.001, Figure 4-11).

Collectively these data highlight differential co-expression of neuroectodermal stem cell markers with the expression of CD271 in trametinib resistant melanoma subpopulations, and suggest that in this context, CD271 is not a marker of stemness.

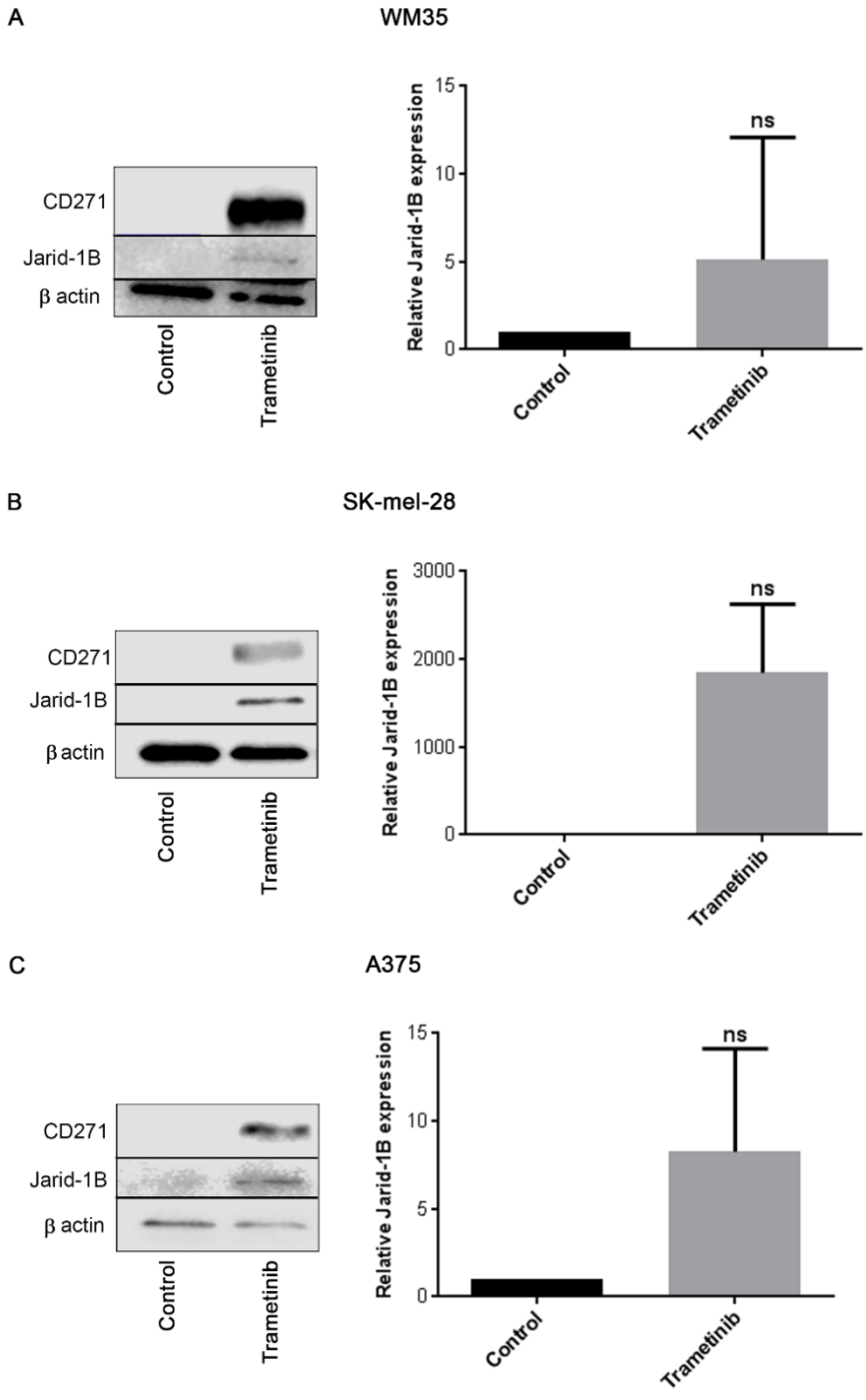


Figure 4-9: Trametinib-induced CD271 Expression in melanoma cells is associated with a trend for increased Jarid-1B expression.

Representative immunoblots for CD271 (45 kDa), Jarid-1B (190 kDa) or β actin loading control (45 kDa) or Relative Jarid-1B expression (relative to β actin loading control) in WM35 (A), SK-mel-28 (B) and A375 (C) cells following treatment with vehicle DMSO control or 16 nM trametinib for 9 days. Each bar is the mean or 3 experiments ± SD.

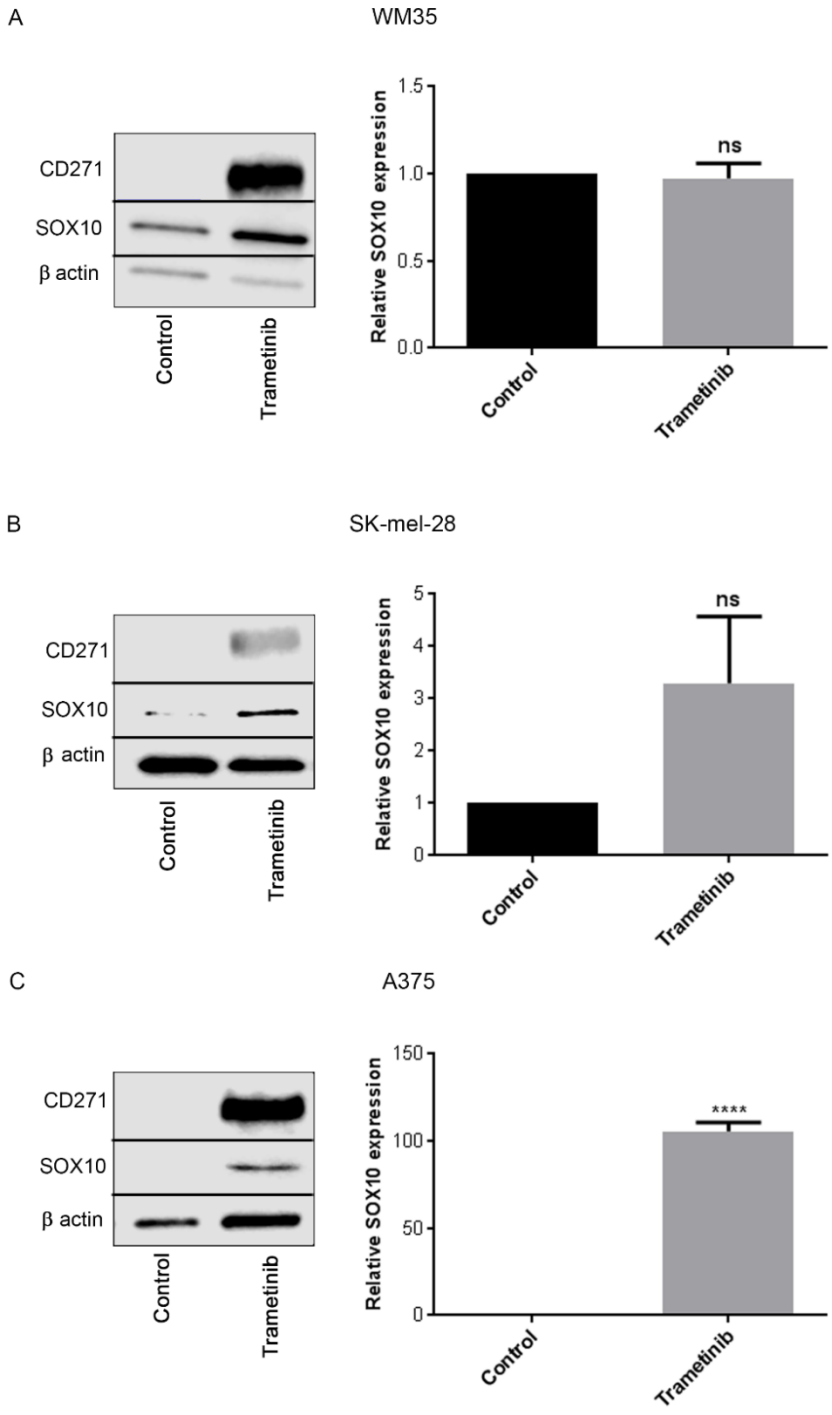


Figure 4-10: Trametinib-induced CD271 Expression in melanoma cells is associated with increased SOX 10 expression

Representative immunoblots for CD271 (45 kDa), SOX 10 (37 kDa), or β actin loading control (45 kDa) or Relative SOX 10 expression (relative to β actin loading control) in WM35 (A), SK-mel-28 (B) and A375 (C) cells following treatment with vehicle DMSO control or 16 nM trametinib for 9 days. Each bar is the mean or 3 experiments ± SD (****P < 0.0001).

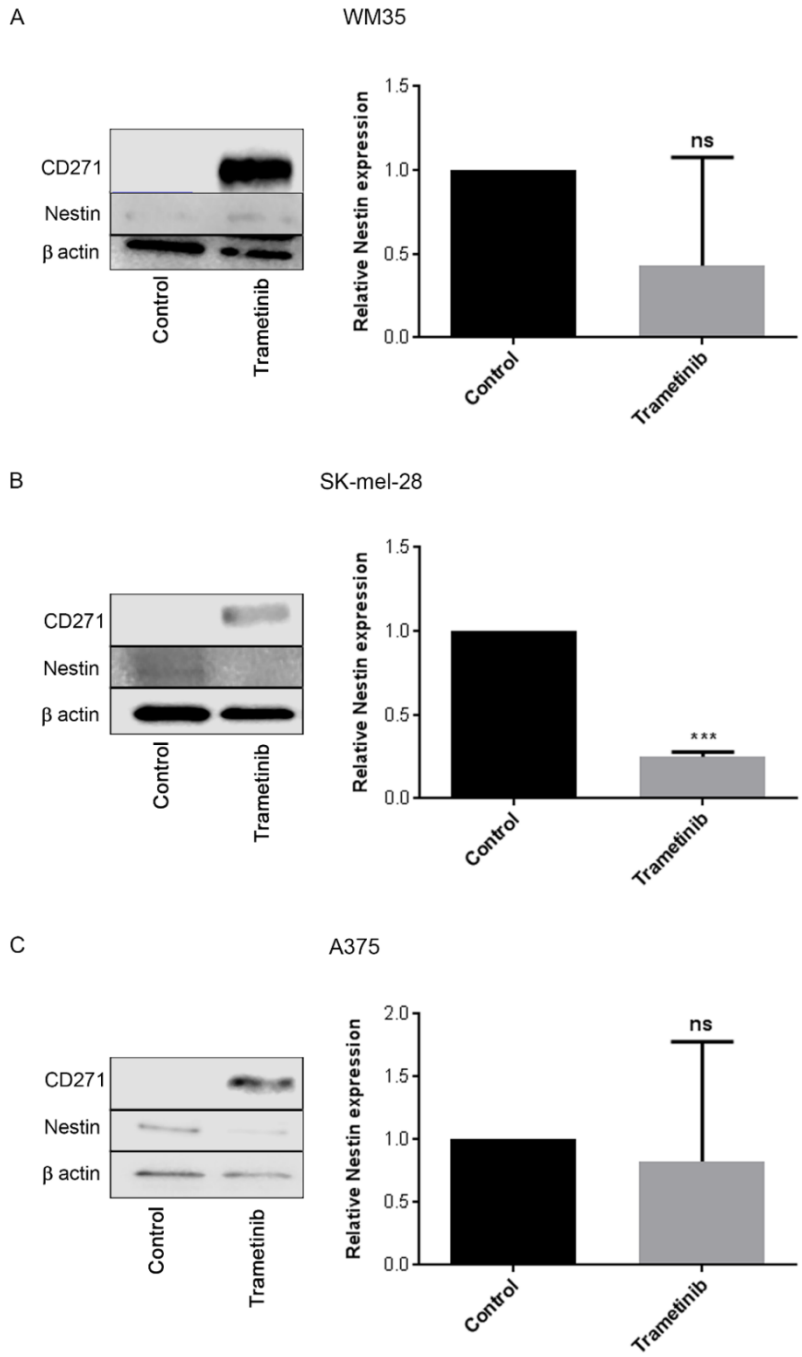


Figure 4-11: Trametinib-induced CD271 Expression in melanoma cells is associated with the down regulation of Nestin expression

Representative immunoblots for CD271 (45 kDa), Nestin (177 kDa), or β actin loading control (45 kDa) or Relative Nestin expression (relative to β actin loading control) in WM35 (A), SK-mel-28 (B) and A375 (C) cells following treatment with vehicle DMSO control or 16 nM trametinib for 9 days. Each bar is the mean or 3 experiments ± SD. Statistics were acquired using unpaired t test, ***P < 0.001.

4.2.4 Trametinib-induced drug resistance is associated with reduced cellular ATP levels and increased pAMPK activation

Increasing evidence highlights the crosstalk between the AMPK signalling and autophagy. Under conditions of nutrient deprivation, AMPK acts as a metabolic checkpoint and inhibits cellular growth and proliferation to sustain hostile micro environmental conditions (Mihaylova and Shaw, 2011). One of the major pathways though which AMPK mediates a reduction in cell proliferation is through the inhibition of the mammalian target of rapamycin complex 1 (mTORC1) pathway, resulting in the activation of autophagy. To further investigate the association between drug induced CD271 expression and the induction of pro-survival autophagy in trametinib-induced drug resistant melanoma subpopulations, BRAF mutant A375 and WM35 cells were treated with 16 nM of trametinib for 9 days prior to the analysis of ATP release and the endogenous levels of pAMPK. Results demonstrated that treatment with trametinib for 9 days resulted in significant reduction in cellular ATP release by both cell lines (one-way ANOVA with Tukey's multiple comparison test, ***P < 0.001, ****P<0.0001), (

Figure 4-12).

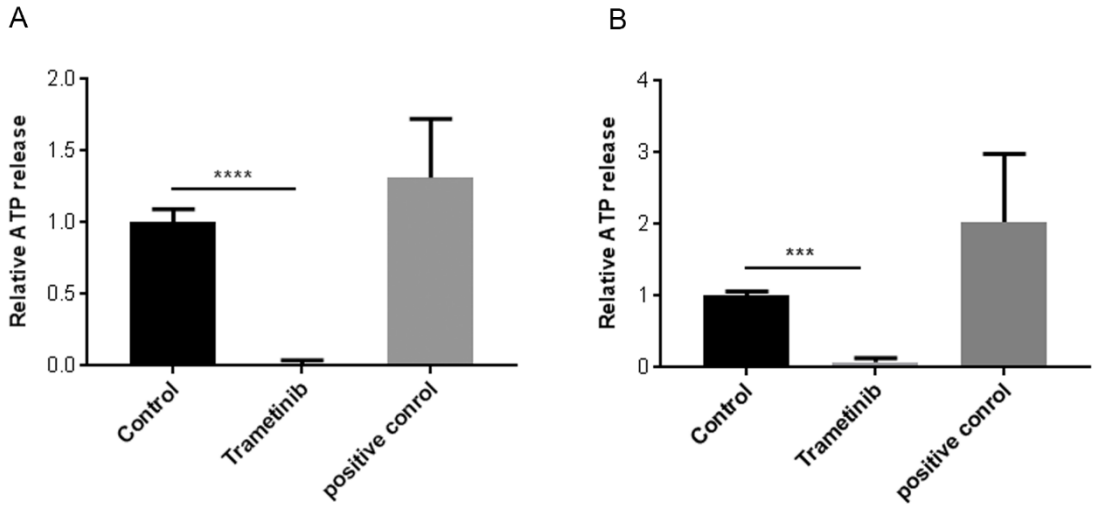


Figure 4-12 Trametinib treatment results in reduced ATP release in WM35 and A375 melanoma cells.

Relative ATP release (relative to ATP release by control vehicle DMSO treated cells) by WM35 (A) or A375 (B) cells or following treatment for 9 days with 16 nM of trametinib. Cells previously treated with 16 nM of trametinib and further treated with 10% SDS for 2 minutes to potentiate ATP release were used as positive control for each experiment. Each bar is the mean or 3 experiments ± SD (***P < 0.001, ****P<0.0001)

To further evaluate whether trametinib-induced reduction in cellular ATP levels is associated with AMPK activation, WM35 and A375 cells were again treated with 16 nM trametinib for 9 days prior to evaluation of pAMPK levels using a commercial ELISA assay. Results demonstrated a significant increase in pAMPK in both cell lines cells treated with 16 nM trametinib for 9 days compared to cells treated with vehicle control (one-way ANOVA, with Tukey's multiple comparison test, *P < 0.05 for the effect of trametinib treatment in WM35 cells and **P < 0.01 for the effect in A375 cells, (Figure 4-13).

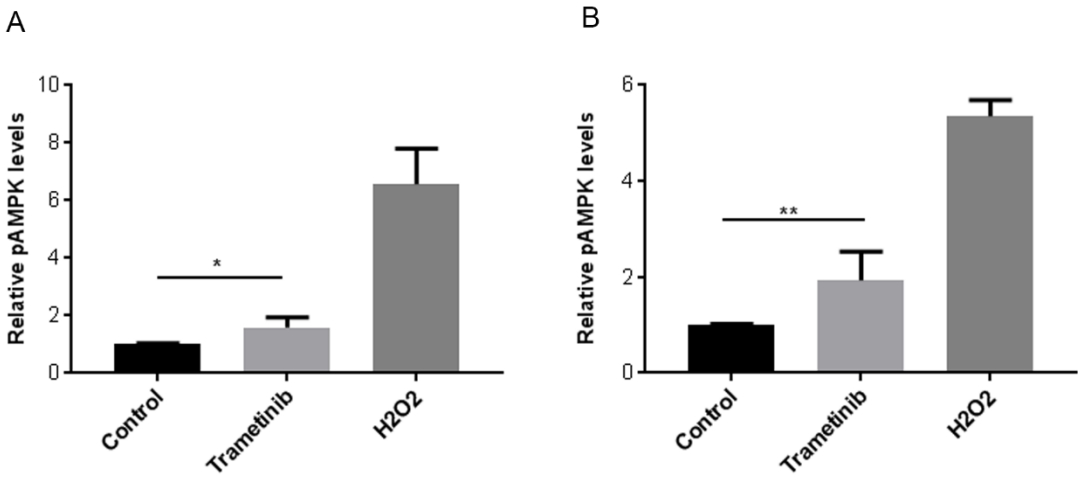


Figure 4-13: Trametinib treatment results in increased AMPK activation in WM35 and A375 melanoma cells.

Relative pAMPK levels in WM35 (A) or A375 (B) cells following treatment for 9 days with DMSO vehicle control or 16 nM of trametinib. Cells previously treated with 16 nM of trametinib and further treated with 10 mM hydrogen peroxide for 10 minutes were used as positive control for each experiment. Each bar is the mean or 3 experiments ± SD (*P < 0.05, **P<0.01).

Collectively these data indicate trametinib–induced drug resistance in melanoma is accompanied by reduced ATP release and increased AMPK activation, providing evidence for the mechanism by which pro-survival autophagy is activated in MEK inhibitor drug resistant melanoma subpopulations.

4.2.5 Spheroids derived from trametinib resistant CD271 expressing melanoma BRAF mutant subpopulations display reduced collagen expansion compared to untreated BRAF mutant melanoma cells

Results from chapter 3 demonstrated isolated CD271 positive subpopulations derived from BRAF mutant melanoma cell lines displayed reduced collagen expansion compared to their CD271 negative counterparts, in line with recent observations reporting the expression of CD271 by melanoma sub populations is associated with a less invasive phenotype (Saltari *et al.*, 2016). To confirm if the same phenomenon is also true in drug-resistant CD271 expressing melanoma subpopulations WM35 H2B RFP cells were treated with 16 nM trametinib or vehicle control prior to incorporation into collagen gels and the assessment of spheroid expansion over 7 days. Results demonstrated significantly reduced spheroid expansion by WM35 cells pre-treated with trametinib compared to vehicle control treated cells (Two-way ANOVA test with Sidak post hoc correction, ***P<0.001, (Figure 4-14).

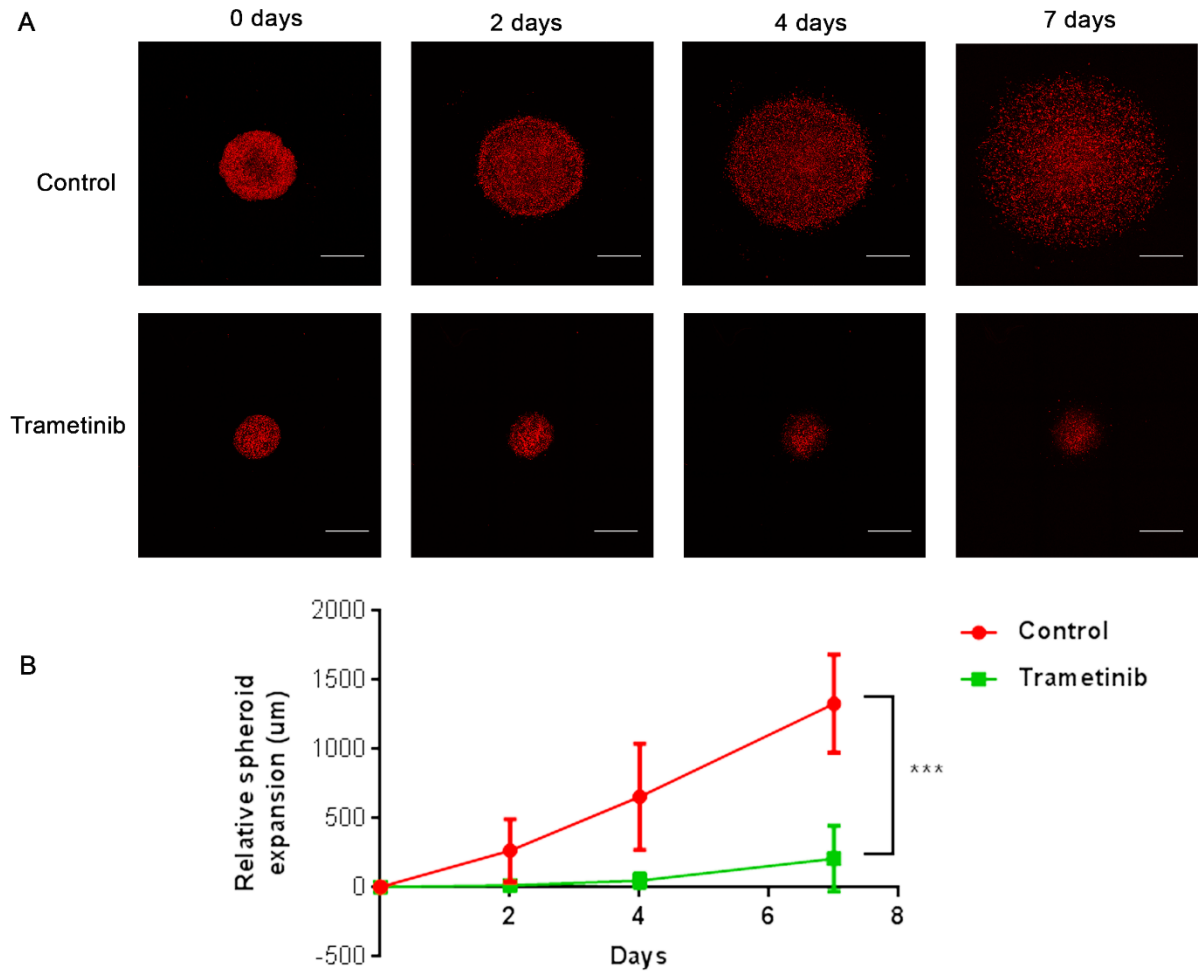


Figure 4-14: Spheroids derived from trametinib-induced drug resistant WM35 cells display reduced collagen expansion compared to untreated WM35 cells.
A: Representative immunofluorescence images of WM35 H2B RFP spheroid size following treatment with DMSO vehicle control or 16nM trametinib for 9 days and incorporation into collagen gels for 0, 2, 4 or 7 days or B: Relative invasion (µm, relative to initial spheroid size at day 0) of WM35 H2B RFP vehicle control treated cells or cells previously treated for 9 days with 16nM trametinib over 7 days. Each point represents the mean relative size of 3 replicate experiments ±SD, ***P<0.001, Scale bar 100 µm

These data were also in line with findings from previous chapter highlighting the association between constitutive CD271 expression and reduced spheroid collagen expansion and suggesting as with melanoma cell populations constitutively expressing CD271 that drug-induced CD271 expression in melanoma is associated with a less invasive phenotype and that other mechanisms may be required to promote tumour cell invasion which are further

explored in section 4.2.9 or that loss of transient drug-induced CD271 subsequently enhances tumour invasion.

4.2.6 Stable overexpression of CD271 increases basal autophagy in melanoma *in vitro* and results in reduced tumour invasion and no effect on susceptibility to trametinib-induced cell death

To further investigate the link between drug-induced CD271 expression and increased autophagy and in an attempt to investigate if autophagy is activated as a consequence of drug-induced CD271 expression, CD271 was stably over expressed in BRAF mutant melanoma cells prior to the analysis of autophagy status and the effect on cell viability or invasive potential.

Initial experiments using Lipofectamine to stably transfect a mammalian pCMV6-AC plasmid vector containing RFP tagged CD271 into A375 cells using G418 antibiotic resistance for selection resulted in low transfection efficacy (

Figure 4-15).

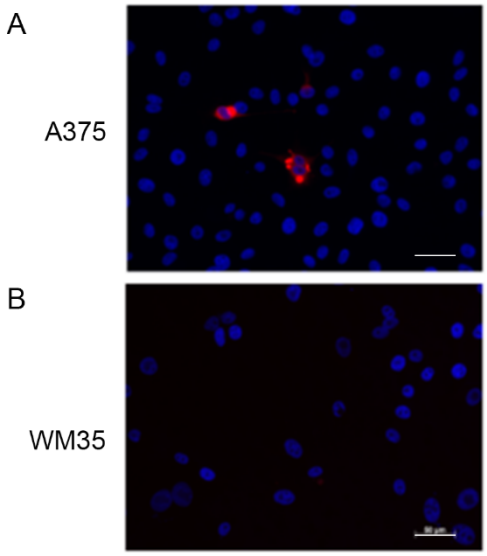


Figure 4-15: Transfection of CD271 into A375 cells results in low transfection efficacy
Representative immunofluorescence image of A375 (A) and WM35 (B) cells transfected with 1 µg of pCMV6-AC plasmid vector containing RFP tagged CD271 and selected using 500 µg/ml G418. Transfected CD271 is depicted as red and DAPI as blue. Blue fluorescence indicates DAPI labelled cell nuclei, Scale bar 50 µm

To increase the transfection efficacy, the same CD271 expression vector or empty vector control was therefore transfected into BRAF mutant A375 and WM35 cells using Xfect Transfection Reagent, which resulted in substantially increased transfection efficiency (

Figure 4-16). Following G418 selection, CD271 cells were then sorted by flow cytometry to obtain pure CD271 expressing subpopulations prior to their use in subsequent experiments.

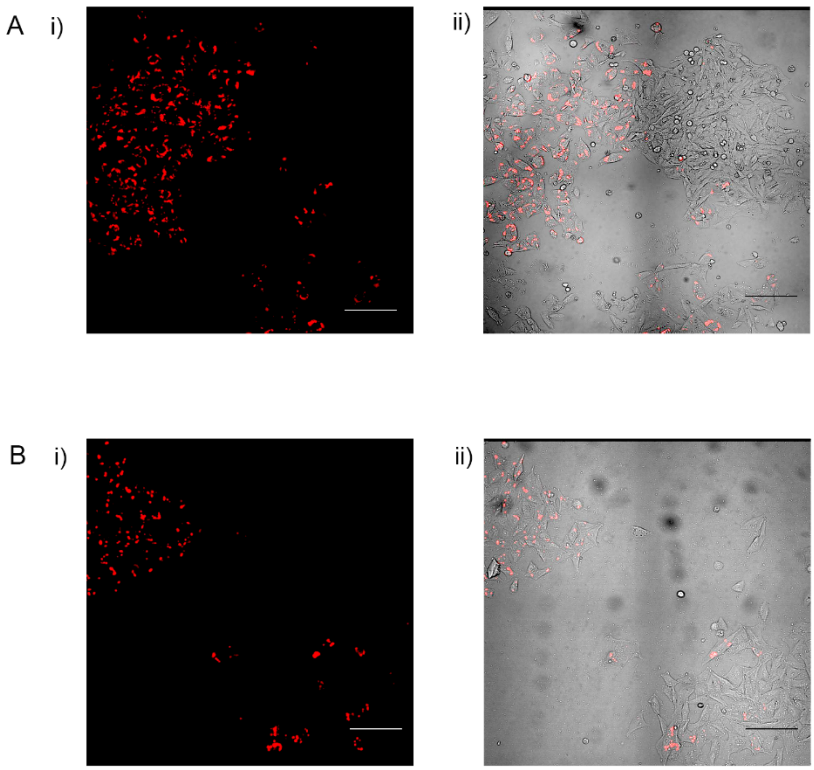


Figure 4-16: Transfection of CD271 with XFect polymer results in high transfection efficiency
Representative immunofluorescence image of A375 (A) and WM35 (B) cells transfected with 1 µg of pCMV6-AC plasmid vector containing RFP tagged CD271 and selected by continuous treatment with 500 µg/ml G418. Cells stably expressing CD271 are depicted as red (A i, B i), bright field and RFP channel overlay (A ii, B ii). Scale bar 100 µm

Analysis of LC3 I/II and p62 in WM35 cells stably overexpressing CD271 revealed as expected resulted the increased expression of CD271 (Figure 4-17 A and B) compared to expression in cells transfected with empty vector control. WM35 cells stably expressing CD271 also displayed increased expression of LC3 II and reduced p62 protein expression indicating an increased level of basal autophagy, although this effect was not significant (Figure 4-17 A, B and C).

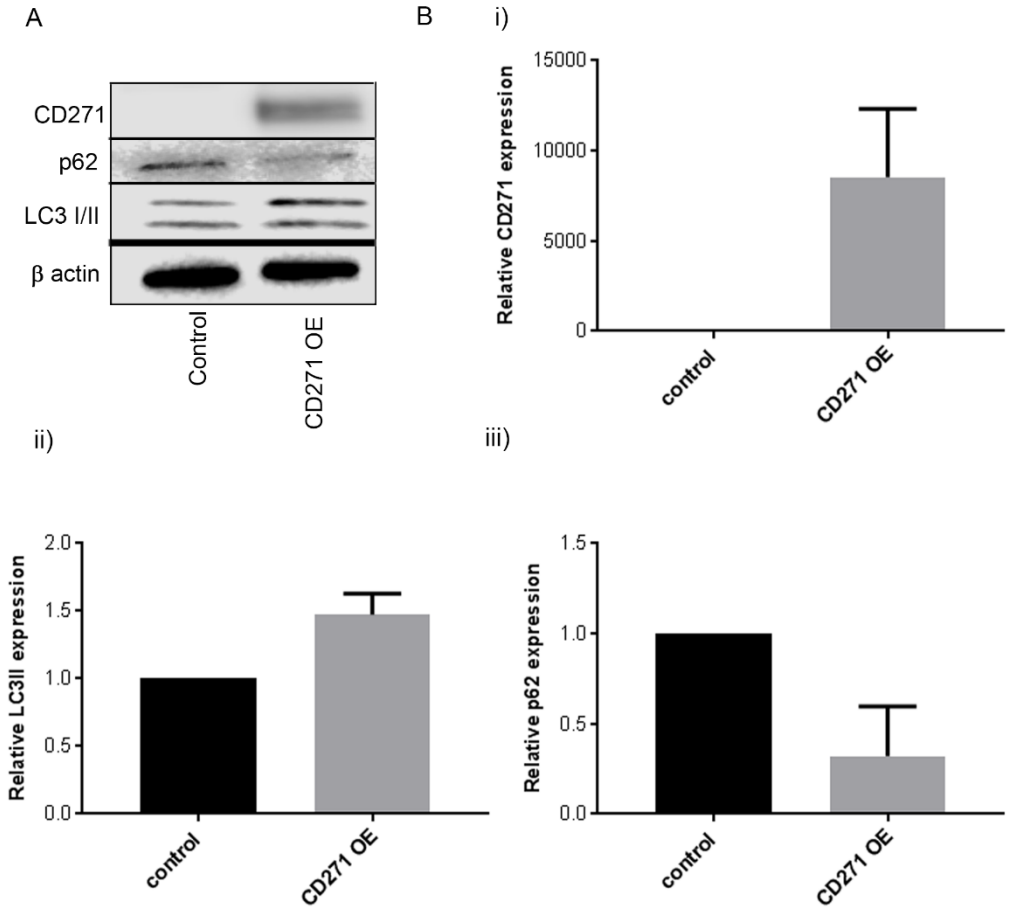


Figure 4-17: Overexpression of CD271 in WM35 cells is suggestive of an increase in basal autophagy

A: Representative western blot for the expression of CD271 (75 kDa), p62 (62 kDa), LC3 I/II (16, 18 kDa) and β actin loading control (42 kDa) in WM35 cells stably transfected with an empty vector control or pCMV6-AC plasmid vector containing RFP tagged CD271 stably overexpressing CD271 (CD271 OE). B: Relative expression of CD271 (i), LC3 II (ii) and p62 (iii) (relative to β actin protein expression) in control or CD271 OE cells. Each bar represents the mean of 3 replicates for each cell line, and expressed relative to the mean of each individual experiment (mean ± SD, N = 3).

In line with reports demonstrating CD271 expressing melanoma cells display increased resistance to BRAF and MEK inhibitors (Lehraiki *et al.*, 2015) and to investigate the cytotoxic effects of MEK inhibition in melanoma cells in which CD271 had been over expressed, WM35 and A375 cells stably expressing CD271 were treated with trametinib for 24, 48 or 72 hours or 9 days prior to evaluating the effect on cell viability. While treatment of either WM35 (Figure 4-18 5 A) or A375 (Figure 4-18 B) transfected with empty vector control or stably expressing

CD271 with trametinib resulted in time-dependent inhibition of cell viability up to 72 hrs, trametinib-induced inhibition of cell viability was partially blocked in both cell lines following 9 days exposure (

Figure 4-18) in both control and cells over expressing CD271. In addition, there were no observed differences in trametinib-induced inhibition of cell viability between control and CD271 over-expressing cells following 24, 48 or 72 hrs treatment in either cell line (

Figure 4-18). However, contrary to published literature (Lehraiki *et al.*, 2015), and albeit in one cell line, WM35 cells stably expressing CD271 were significantly more sensitive to trametinib-induced inhibition of cell viability following 9 days treatment compared to control cells (One way ANOVA with Tukey's post hoc correlation, ****P<0.0001,

Figure 4-18).

Collectively however, these data suggest over expression of CD271 in BRAF mutant melanoma cells does not significantly alter their sensitivity to the cytotoxic effects of MEK inhibition.

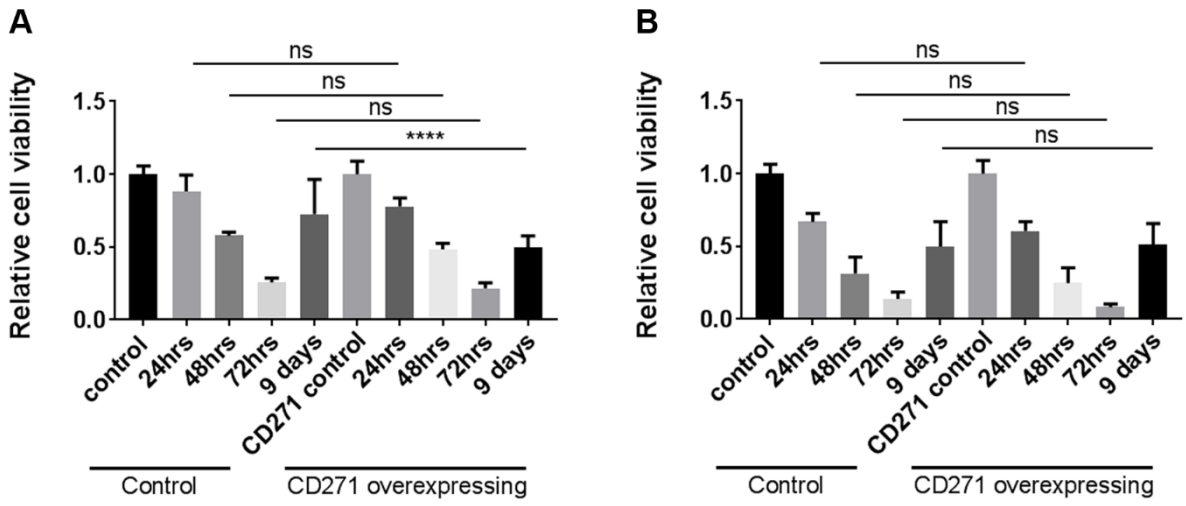


Figure 4-18: CD271 overexpression does not increase resistance of WM35 or A375 cells to the cytotoxic effects of trametinib

Relative cell viability of WM35 (A) or A375 (B) transfected with empty vector control (control) or stably expressing CD271 (CD271 overexpressing) and treated with DMSO vehicle control (Control) or 16 nM of trametinib for 24, 48, 72 hours or 9 days. Each bar represents the mean of 3 replicates for each cell

line, and expressed relative to the mean of each individual experiment (mean ± SD, N = 3) (NS P>0.05, ****P<0.0001).

To investigate the potential effects of CD271 overexpression in melanoma cells on spheroid expansion, a mixed population of WM35 spheroids containing stably expressing CD271 (and tagged with RFP) or not (non-red) were incorporated into collagen gels prior to assessing the relative spheroid size at 24, 48 and 72 hours. Similarly to results derived with isolated melanoma cells constitutively expressing CD271, results demonstrated reduced spheroid collagen expansion of CD271 overexpressing cells (expressing RFP) compared to control cells (Not overexpressing, non-red) (Figure 4-19 A and B).

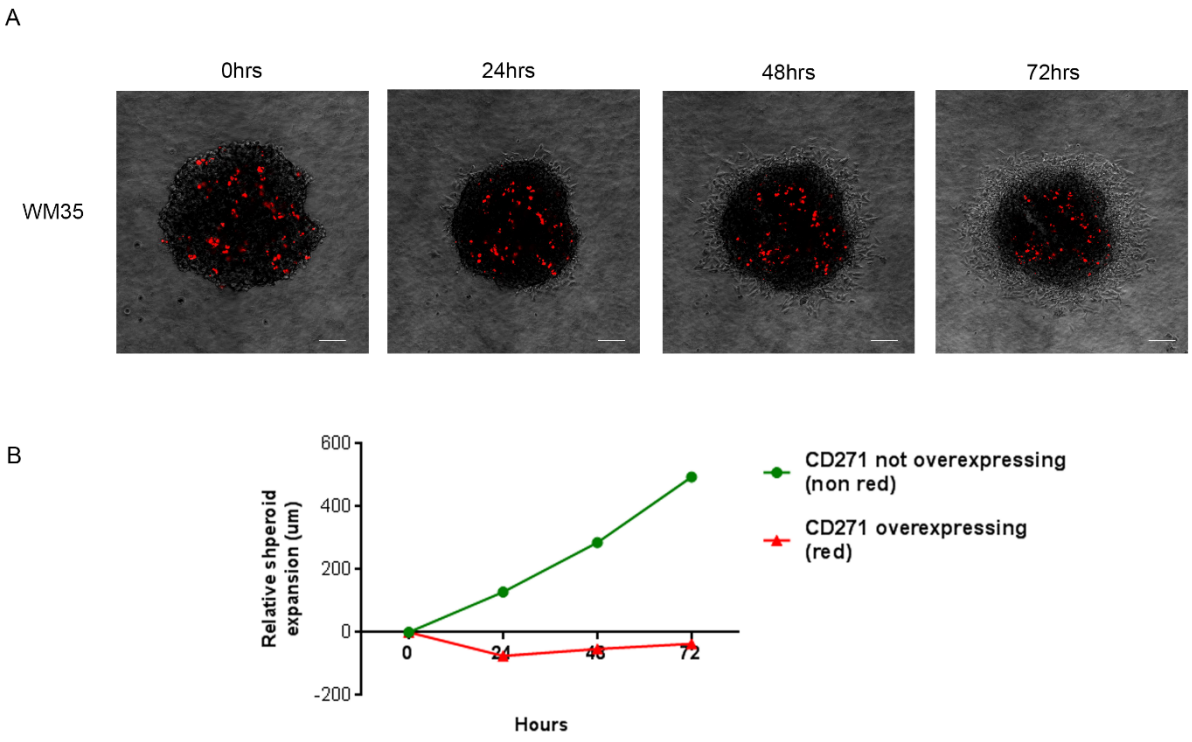


Figure 4-19: Over expression of CD271 in WM35 cells reduces spheroid expansion compared to WM35 not overexpressing cells

A: Representative immunofluorescence images of a mixed population containing WM35 CD271 overexpressing (RFP expressing cells) or non CD271 overexpressing (non-red) cells in a collagen invasion assay. B: Relative invasion (μm) of control (green line) or CD271 over expressing (red line) WM35 cells (relative to initial spheroid size at time 0) over 72 hrs (n=1)

Interestingly rather than demonstrating a diffuse membranous pattern, the cellular localization of CD271 following transfection with CD271 RFP demonstrated a more lobular intracellular pattern

Figure 4-15

Figure 4-16) questioning whether increased autophagy in this context is induced as a cellular stress response. Previous studies have highlighted the crosstalk between autophagy and ER stress (Hoyer-Hansen and Jaattela, 2007) and hence to investigate whether the increase in autophagic activity observed in the CD271 overexpressing populations was a direct effect of CD271 on autophagic activity rather than CD271 localization leading to ER stress, the mRNA expression of a panel of ER stress genes was evaluated in control and CD271 over expressing A375 cells (Figure 4-20). Results revealed there was no significant increase in the expression of Xbp1, ATF4, ATF6, TRB3 or CHOP mRNA expression in A375 cells stably expressing CD271 RFP compared control un-transfected cells (Figure 4-20), indicating that the observed autophagy induction in the CD271 overexpressing subpopulation is not as a result of ER stress activation and therefore more likely directly linked to CD271 overexpression.

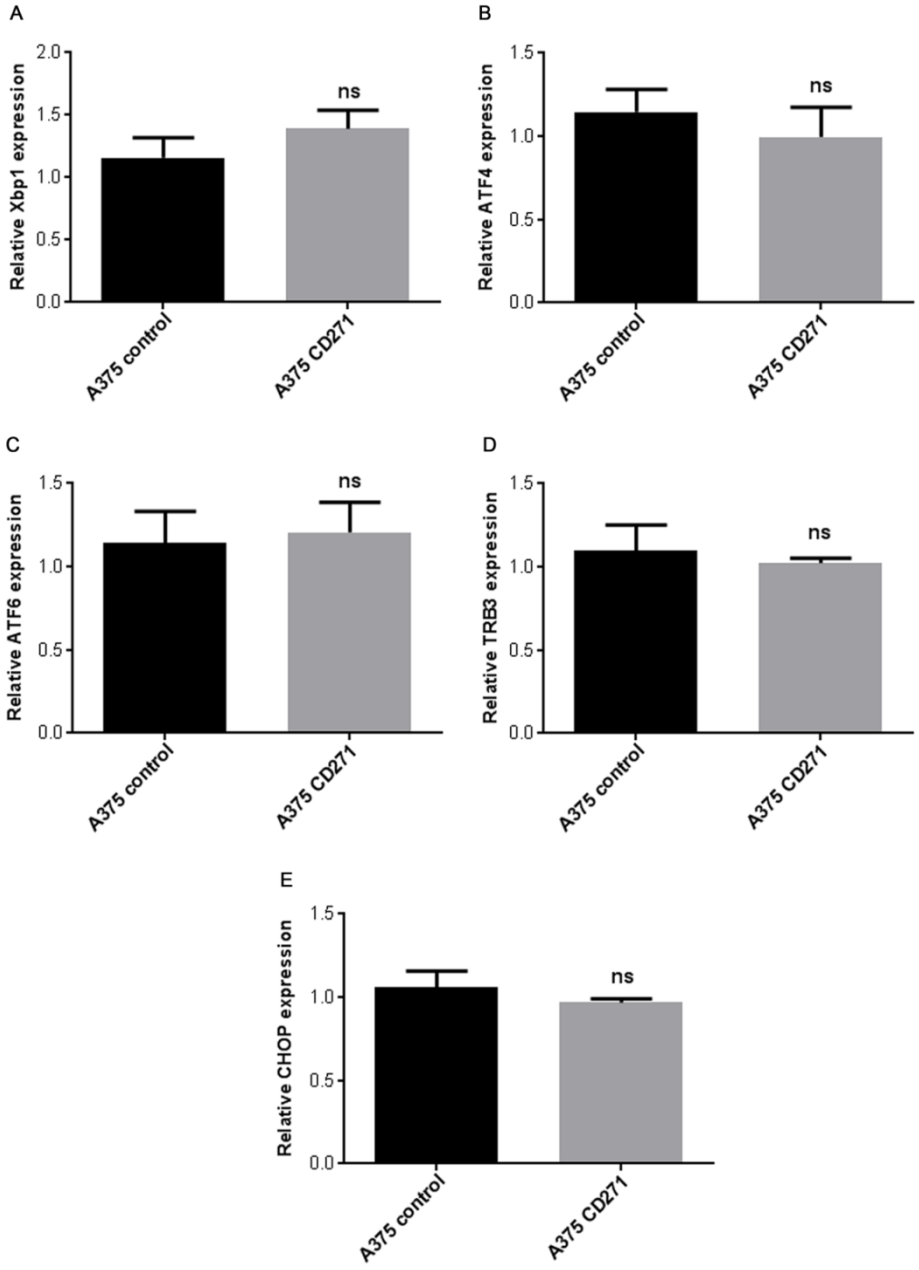


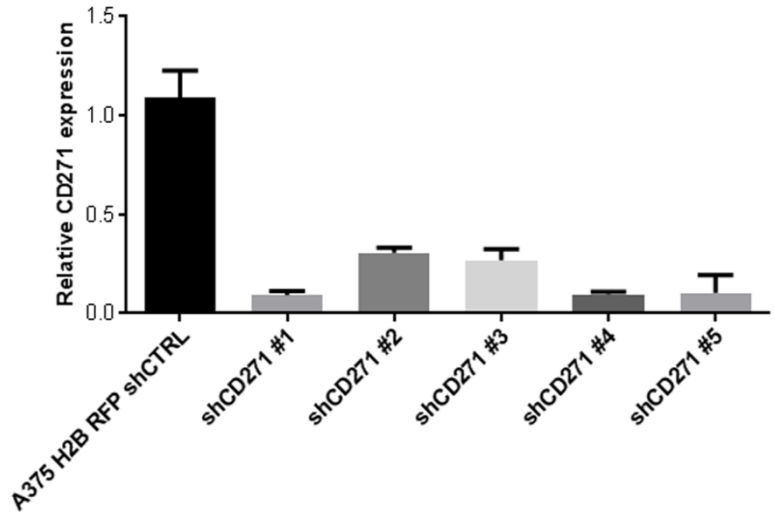
Figure 4-20: Over-expression of CD271 does not up regulate ER stress gene expression
Relative mRNA expression of Xbp1 (A), ATF4 (B), ATF6 (C), TRB3 (D) or CHOP (E) in A375 cells (A375 control) or in A375 cells stably expressing CD271-RFP (A375 CD271). Each bar is the mean of three replicates (±SD) relative to L34 loading control (NS P>0.05).

However, given the conflicting results on the functionality of CD271 overexpression and the cellular localisation of CD271 protein following overexpression of CD271, the alternative approach of CD271 knockdown was adopted to evaluate the effect of abrogating CD271 expression on autophagy and response to trametinib treatment.

4.2.7 Stable knockdown of CD271 is incompatible with melanoma cell survival *in vitro*

To investigate the impact of abrogating CD271 expression on autophagy, an initial approach to knockdown CD271 stably was undertaken in A375 cells using lentiviral shRNA vectors (Moore *et al.*, 2010). Results demonstrated the effective reduction of mRNA levels of CD271 in 5 derived clones (

Figure 4-21) 2 days after transfection. However, although viable 3 days after transfection, within 7 days each clone died, consistent with reports indicating total abrogation of CD271 expression is incompatible with cell viability (Lehraiki *et al.*, 2015) and that melanoma cells



likely require some expression of CD271 for maintained cell proliferation and survival.

Figure 4-21: mRNA CD271 expression in A375 cell clones following lentiviral ShRNA mediated knockdown.

Relative mRNA expression of CD271 following shRNA mediated knockdown of CD271 in A375 H2B RFP cells or transfection with an shRNA control (shCTRL) 2 days after transfection . Each bar is the mean of three replicates (±SD) relative to L34 loading control and normalized to shCTRL.

Since stable knockdown of CD271 was incompatible with melanoma cell viability subsequent studies were undertaken using a transient approach with siRNAs to CD271 in order to further

determine the effect of abrogating CD271 expression on autophagic activity and invasive potential.

4.2.8 siRNA mediated knockdown of basal CD271 expression sensitizes melanoma cells to the cytotoxic effects of trametinib, has no effect on basal autophagy and a trend for reduction in pAMPK expression

To further investigate the effect of CD271 knockdown on melanoma cell viability, autophagic activity and sensitivity to trametinib-induced cell death, an alternative approach was adopted to transiently knockdown CD271.

Transient siRNA-mediated knockdown of CD271 was initially performed in WM35 cells which, as shown in chapter 3, display increased basal CD271 expression. Results confirmed the reduction of CD271 expression following transfection with CD271 siRNA for 24 hrs (1 day) which continued to significantly decline 4 days post transfection (One way ANOVA with Tukey's post hoc correlation, ***P<0.001, (

Figure 4-22 A and Bi). Conversely, basal expression levels of LC3-II were not significantly altered following siRNA mediated knockdown of CD271 and incubation for 1 or 4 days (
Figure 4-22A and Bii), suggesting that the level of knockdown of CD271 may not have been sufficient enough to observe any effect on a predicted reduction on autophagic activity.

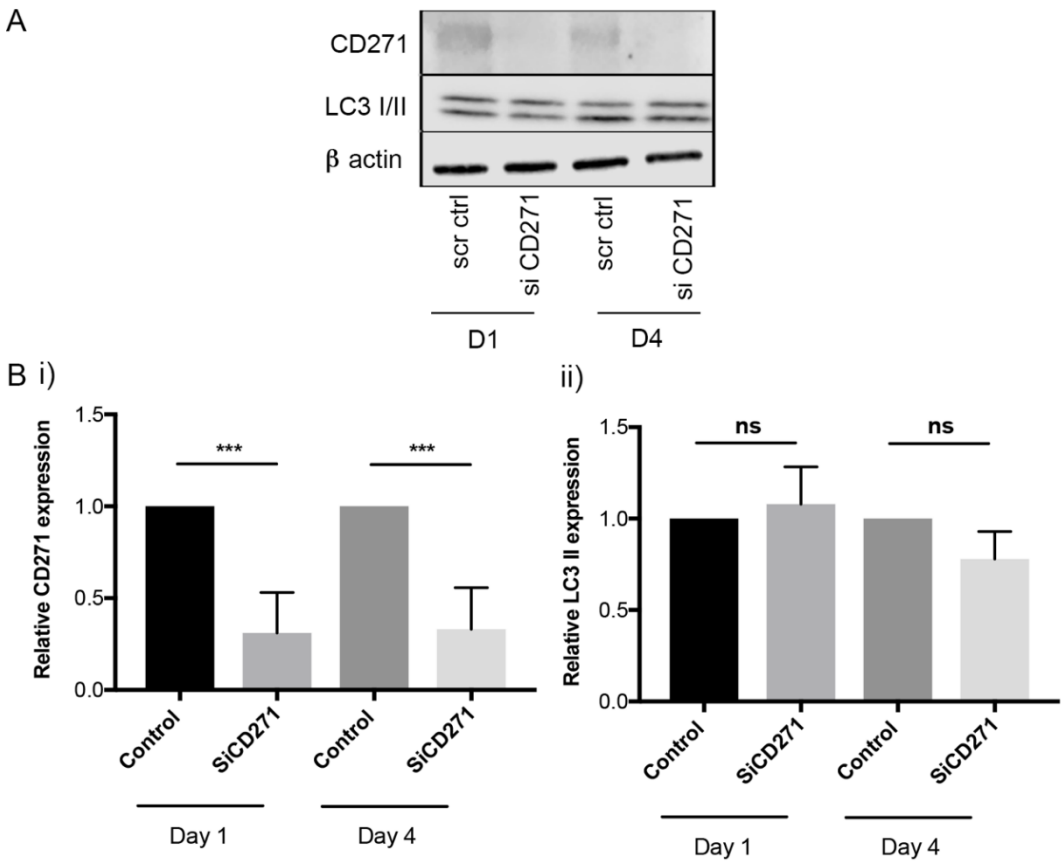


Figure 4-22: siRNA mediated knockdown of CD271 has no effect on basal expression levels of LC3 II in WM35 melanoma cells.

A, Representative immunoblots for the expression of CD271 (75 kDa), LC3 I/II (16,18 kDa) or β actin loading control (45 kDa) in WM35 cells following siRNA mediated knockdown of CD271 and continued culture for 1 or 4 days. B. Relative CD271 (i) or LC3 II (ii), expression levels relative to β actin in 3 individual experiments. Each bar is the mean or 3 experiments ± SD (***P<0.001).

To assess the effect of siRNA mediated knockdown of CD271 on the cytotoxic effects of trametinib, WM35 cells were transfected with control siRNA or CD271 siRNA for 24 hours prior to treatment with 16 nM trametinib for 24 or 72 hours and the assessment of cell viability. Results demonstrated a significant reduction of WM35 cell viability in cells transfected with CD271 siRNA compared to cell transfected with control siRNA (One way ANOVA with Tukey's post hoc correlation, ****P<0.0001), (Figure 4-23), suggesting a possible association between CD271 knockdown and a decrease in autophagic activity. Nevertheless, given the low levels of CD271 expression in the parental cells, to further investigate the effect of CD271 knockdown

on autophagic activity, siRNA to CD271 was performed on 9 day trametinib pre-treated cells in order to induce CD271 expression prior to knockdown.

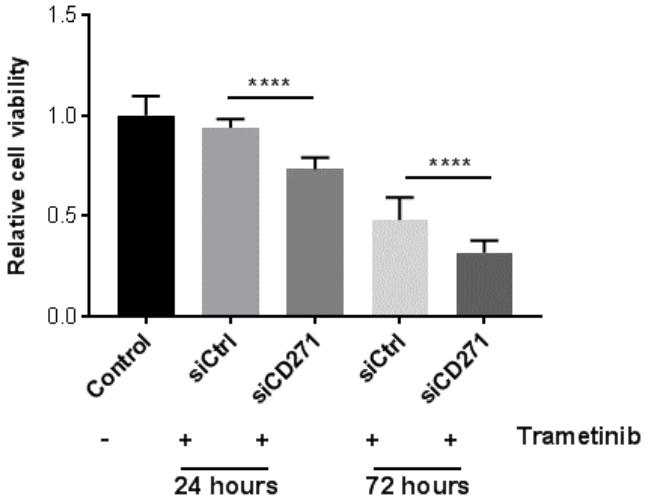


Figure 4-23: siRNA mediated knockdown of CD271 in WM35 cells sensitises cells to trametinib.

Relative cell viability of control WM35 cells transfected with either a scrambled control siRNA (siCtrl) or CD271 siRNA to CD271 (siCD271) following treatment for 24 or 72 hrs with 16nM trametinib (Trametinib), Each bar is the mean +/- SD of 3 independent experiments with 4 technical replicates ****P < 0.0001.

To assess the impact of CD271 knockdown on both autophagic activity and the cell viability of trametinib-induced drug resistant melanoma cells, WM35 and A375 cells were treated in the presence of 16 nM trametinib for 9 days prior to siRNA mediated knockdown of CD271 for 72 hours and the assessment of the effect on cell viability, as well as CD271 expression and autophagic activity by western blotting. Results demonstrated the significant reduction in CD271 expression following siRNA mediated knockdown of CD271 in WM35 cells pre-treated for 9 days with trametinib compared to untreated control cells (Figure 4-24**Error! Reference source not found.** A and Bi, Student's t-test, * P <0.05) paralleled by a consistent but non-significant increase in LC3 II expression, and significant reduction in p62 expression (Figure 4-24 A and B ii, iii, Student's t-test * P <0.05) and a trend for the reduction in pAMPK expression (Figure 4-24A, B iv, Student's t-test) Results also demonstrated the significant reduction in CD271 expression following siRNA mediated knockdown of CD271 in A375 cells pre-treated for 9 days with trametinib compared to untreated control cells (Figure 4-25**Error! Reference source not found.** A and Bi, Student's t-test, * P <0.05) paralleled by a non-significant increase in LC3 II expression, and significant reduction in p62 expression (Figure

4-25 A and B ii, iii, Student's t-test, * P <0.05) and a trend for the reduction in pAMPK expression (Figure 4-25, B iv, Student's t-test).

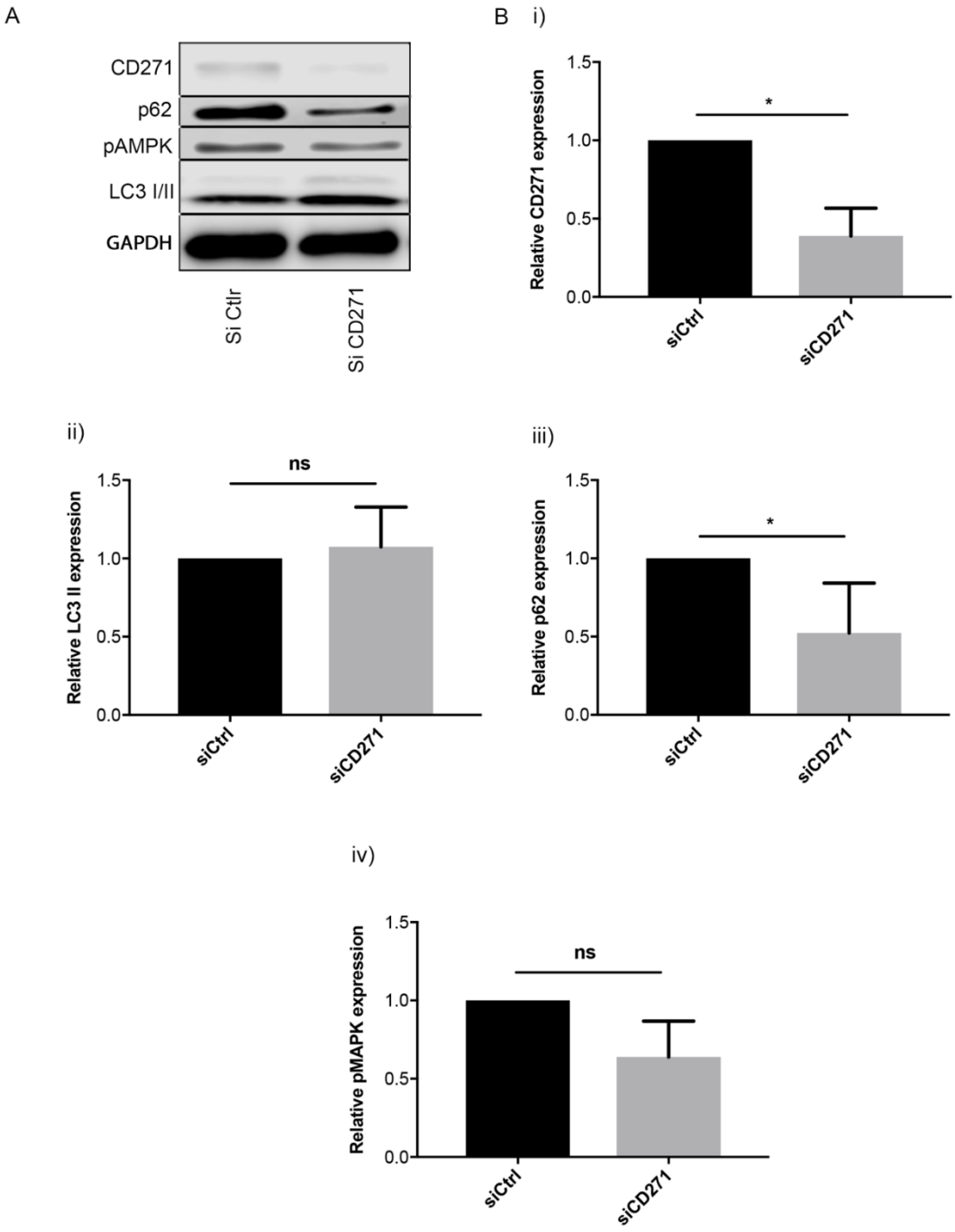


Figure 4-24: SiRNA mediated CD271 knockdown in trametinib-induced CD271 expressing WM35 cells is associated with increased autophagic activity and reduced in pAMPK expression

A.Representative immunoblots for the expression of CD271 (75 kDa), LC3 I/II (16,18 kDa, p62 (62 kDa), pAMPK (62 kDa) or GAPDH loading control (38 kDa) in trametinib-induced CD271 expressing WM35 cells following siRNA mediated knockdown of CD271 for 3 days. B.Relative CD271 (i) or LC3 II (ii), p62 (III) or pAMPK (iv) expression levels relative to GAPDH in 3 individual experiments. Each bar is the mean or 3 experiments ± SD (*P<0.05)

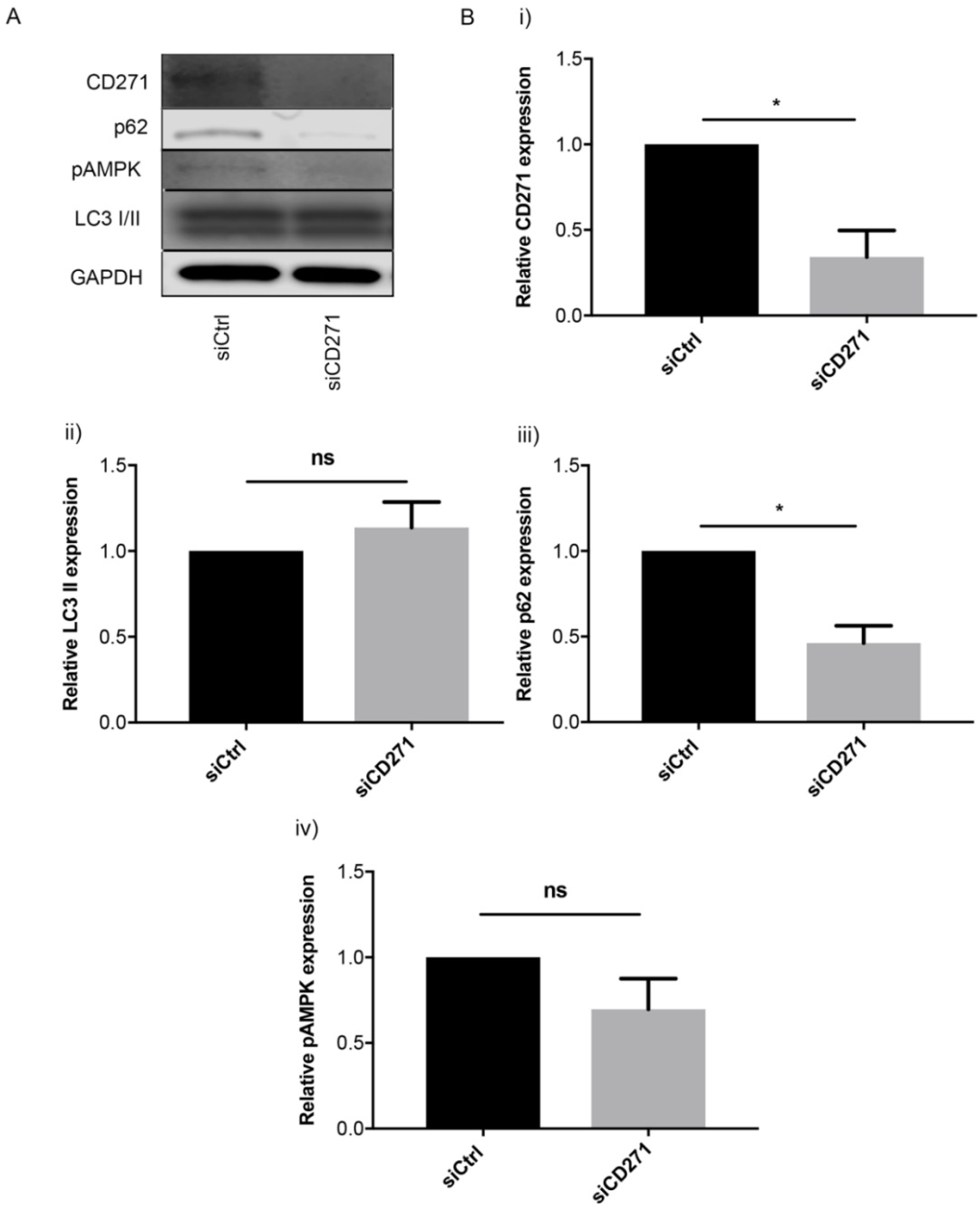


Figure 4-25: SiRNA mediated CD271 knockdown in trametinib induced CD271 expressing A375 cells is associated with increased autophagic activity and reduced in pAMPK expression
A.Representative immunoblots for the expression of CD271 (75 kDa), LC3 I/II (16,18 kDa, p62 (62 kDa), pAMPK (62 kDa) or GAPDH loading control (38 kDa) in trametinib induced CD271 expressing A375 cells following siRNA mediated knockdown of CD271 for 3 days. B.Relative CD271 (i) or LC3 II (ii), p62 (III) or pAMPK (iv) expression levels relative to GAPDH in 3 individual experiments. Each bar is the mean or 3 experiments ± SD (*P<0.05).

In the context of the effects of CD271 knockdown in trametinib-induced drug resistant A375 or WM35 cells, results demonstrated CD271 knockdown induced a significant reduction in cell viability of both A375 and WM35 cells pre-treated with 16nM trametinib for 9 days compared to either control untreated cells or either cell line transfected control siRNA (One way ANOVA with Tukey's multiple comparison test, ***P<0.001, ****P<0.0001, (Figure 4-26).

Collectively, these data suggest knockdown of CD271 further re-sensitizes melanoma cells to the cytotoxic effects of trametinib. However, despite the expected reduction in autophagic activity following CD271 knockdown, siRNA to CD271 is associated with a trend for increased in autophagic activity. It is possible that this effect could be a result of the partial knockdown achieved using an siRNA approach and further experiments using more robust and modern techniques such as using the CRISP/Cas9 technology might be able to further decipher the association between CD271 expression and autophagy.

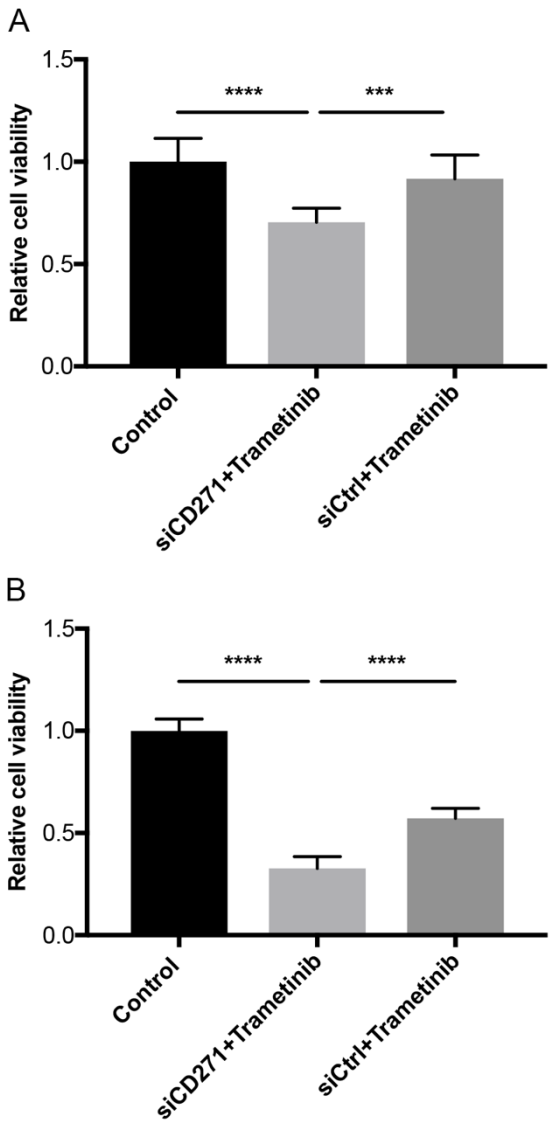


Figure 4-26: siRNA-mediated knockdown of CD271 in trametinib induced drug resistant WM35 and A375 cells re-sensitises cells to the cytotoxic effects trametinib.

Relative cell viability of WM35 (A) or A375 (B) cells treated for 9 days with 16nM trametinib (control) +/- subsequent transfection with siCtrl or siCD271 RNA and treatment for a further 72 hrs with 16nM trametinib (Trametinib), Each bar is the mean +/- SD of 3 independent experiments each containing 4 technical replicates. ****P < 0.0001.

4.2.9 Treatment of trametinib-induced drug resistant CD271 expressing melanoma subpopulations with exogenous NGF increases tumour proliferation but with no effect on tumour spheroid expansion *in vitro*

Studies have suggested that NGF stimulation is required to induce invasion of CD271 expressing melanoma subpopulations (Herrmann *et al.*, 1993). Coupled with observations from the present study demonstrating that spheroids derived from CD271 expressing melanoma cells display reduced collagen expansion compared to subpopulations lacking CD271 expression, this suggests other stimuli such as NGF may be required to promote spheroid growth of Trametinib-induced drug resistant CD271 expressing melanoma sub populations. To test this hypothesis A375 and WM35 cells were first cultured in the presence or absence 16 nM trametinib for 9 days (to induce CD271 expression) prior to treatment with a dose range of exogenous NGF (1 mg/ml-0.01 nm/ml) for 3 days and assessment of the effect on cell viability. Results demonstrated a significant increase in trametinib-induced drug resistant WM35 or A375 cells subsequently treated with 1 mg/ml NGF compared to untreated control cells (one-way ANOVA analysis of variance with Tukey's multiple comparison post hoc correction *P<0.05, Figure 4-27). However, there was no significant effect of NGF when used at concentrations below 1 mg/ml (Figure 4-27).

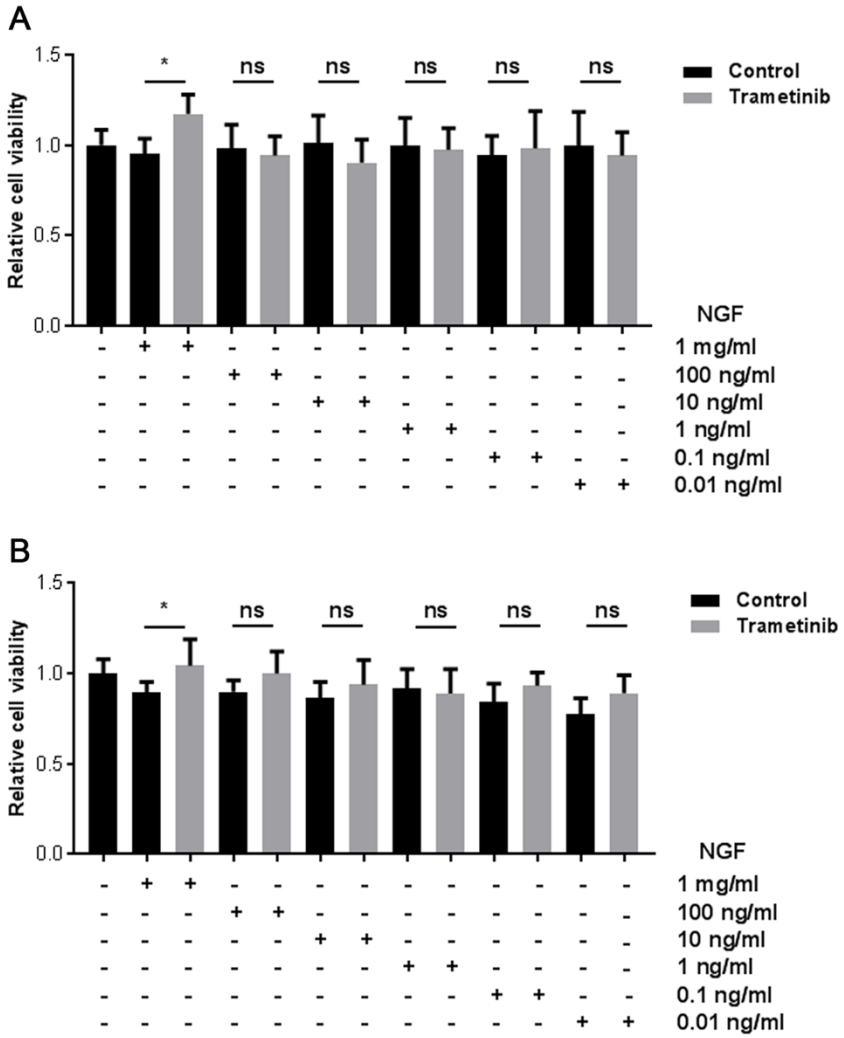


Figure 4-27: NGF promotes the proliferation of trametinib-resistant WM35 and A375 subpopulations.

Relative cell viability of WM35 (A) or A375 (B) cells pre-treated for 9 days with DMSO vehicle control or 16 nM trametinib (Trametinib) and subsequently treated for 72 hours in the presence or absence of a dose range of NGF (0.01ng/ml to 1 mg/ml NGF). Each bar is the mean of 3 replicate experiments, with 4 number of technical replicates/experiment, relative to DMSO untreated control ±SD (* P<0.05).

Next, to investigate the effect of exogenous NGF treatment on the spheroid size of trametinib-resistant (CD271 expressing) melanoma subpopulations, WM35 H2B RFP control spheroids or spheroids derived from cells pre-treated for 9 says with 16 nM trametinib and following subsequent treatment for 72 hrs in the presence or absence of 1 mg/ml NGF were incorporated into collagen gels prior to monitoring tumour spheroid expansion over 7 days. Results demonstrated firstly, that there was no apparent increase in the spheroid expansion of untreated WM35 control cells compared to control WM35 cells treated with NGF (Figure 4-28 A) (Two-way ANOVA test with Sidak post hoc correlation, NS P>0.05).

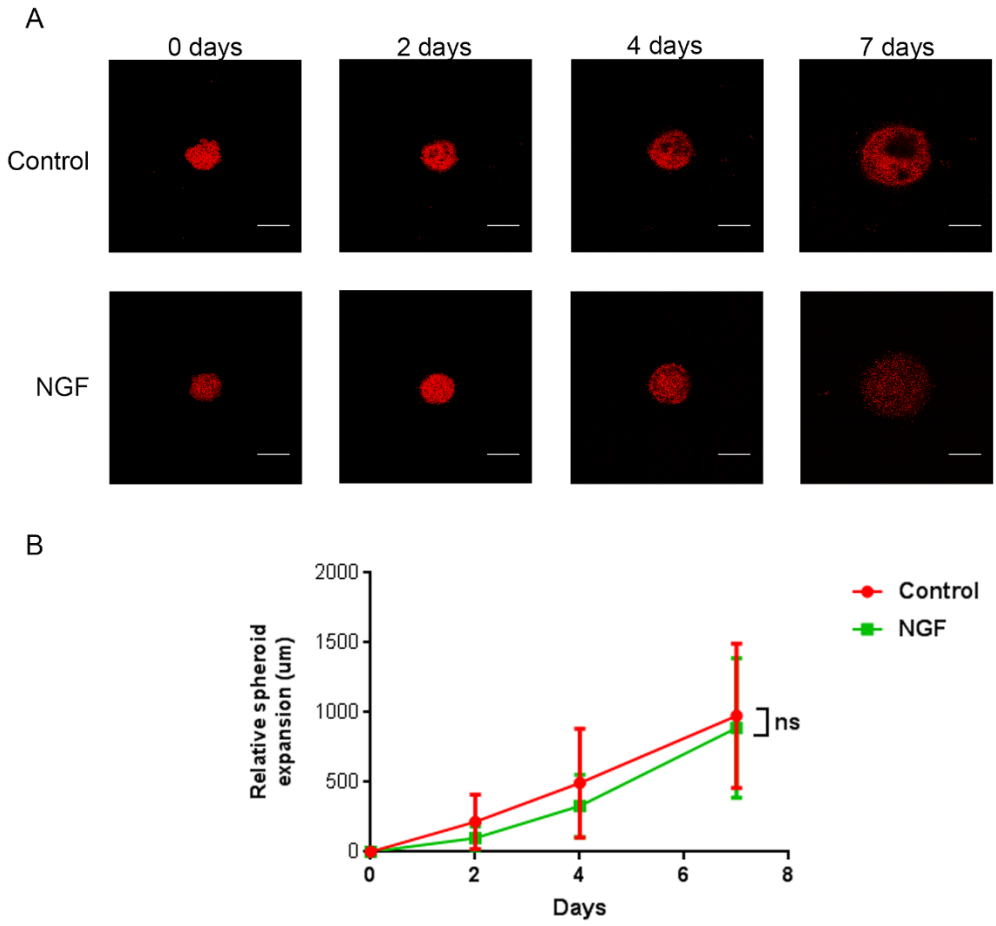


Figure 4-28: NGF does not increase the spheroid size of control untreated WM35 RFP cells
A. Representative immunofluorescence images of wild-type WM35 spheroids treated in the presence of absence (control) of 1mg/ml NGF for 3 days and incorporated into collagen gels for 0, 2, 4 and 7 days, and B. Relative spheroid size (μm) of WM35 spheroids (red line) or WM35 spheroids treated for 72 hrs with 1mg/ml NGF (green line) (relative to initial spheroid size at day 0) over 7 days. Each bar is the mean of three replicate experiments ± SD. Scale bar 100 μm

However, WM35 previously subjected to pre-treatment with 16 nM of trametinib for 9 days, and subsequent treatment with NGF demonstrated a trend (albeit not significant) for increased spheroid expansion compared trametinib-resistant cells not subjected to treatment with NGF (

Figure 4-29). Collectively these data suggest, as with melanoma subpopulations constitutively expressing CD271, that trametinib-induced drug resistant CD271 expressing melanoma subpopulations also display reduced spheroid expansion potential, in line with published literature indicating that CD271 expression is associated with a less invasive phenotype both *in vitro* and *in vivo* (Saltari *et al.*, 2016). Interestingly, exogenous NGF selectively increased proliferation of trametinib –induced drug resistant CD271 expressing melanoma cells suggesting CD271 expression alone is not adequate to promote tumour cell proliferation. Taken together, these data suggest NGF may play an integral role in the promotion of both the proliferation and invasion of trametinib-induced CD271 expressing melanoma subpopulations.

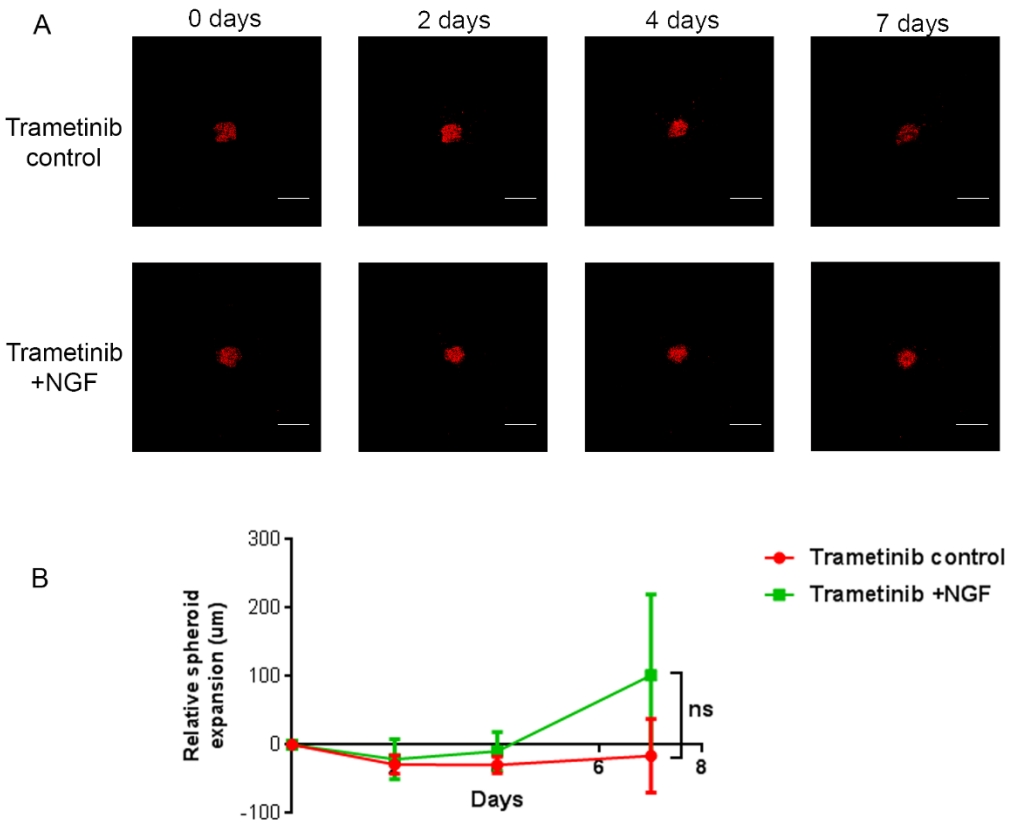


Figure 4-29: NGF does not significantly increase the spheroid expansion of trametinib-induced resistant WM35 cells

A. Representative immunofluorescence images of WM35 spheroids pre-treated with 16 nM trametinib for 9 days in the presence (Trametinib +NGF) of absence (Trametinib control) of subsequent treatment with 1mg/ml NGF for 3 days and incorporated into collagen gels for 0, 2, 4 and 7 days and B. Relative spheroid size (μm) of WM35 spheroids pre-treated for 9 days with 16 nM trametinib (blue line) or with the additional treatment for 72 hrs with 1mg/ml NGF (pink line) (relative to initial spheroid size at day 0) over 7 days. Each bar is the mean of three replicate experiments ± SD. Scale bar 100 μm

4.3 **Discussion**

4.3.1 CD271 expressing melanoma subpopulations emerge in response to BRAF and MEK inhibitors

Although the recent introduction of BRAF and MEK inhibitors have revolutionized the management of patients with metastatic melanoma (Chapman *et al.*, 2011; Long *et al.*, 2014), the development of acquired resistance evolves early following the initiation of these treatment modalities and significantly impacts on the durability of clinical response. A number of resistance mechanisms have been reported including the paradoxical hyper activation of NRAS (Poulikakos *et al.*, 2010), the activation of RTK (Nazarian *et al.*, 2010) or more recently the induction of melanoma subpopulations that are innately more resistant to targeted therapies and express stem cell surface markers such as CD271 (Ravindran Menon *et al.*, 2014). Recent literature highlights, in particular, the association of CD271 induction and development of resistance to BRAF/MEK inhibitors and standard chemotherapy temozolomide and dacarbazine (Chartrain *et al.*, 2012; Ravindran Menon *et al.*, 2014).

In line with previous observations, results from this present study demonstrate the emergence of CD271 expressing melanoma subpopulations in response to both BRAF and MEK inhibitors (Chartrain *et al.*, 2012; Ravindran Menon *et al.*, 2014). Interestingly, and again in line with published literature, long term exposure of A375 cells to trametinib resulted in the transient induction of CD271 expressing melanoma subpopulations which subsequently declined and returned to baseline (Ravindran Menon *et al.*, 2014) and therefore suggesting CD271 induction as a marker of early drug resistance that is possibly not required to sustain tumour growth once resistance is established. Data also highlighted the time dependent decrease in apoptotic cell death observed in melanoma cells in response to sustained treatment with trametinib with a concurrent induction in CD271 expression. Taken together these data further support the hypothesis that CD271 expression is associated with increased resistance to trametinib.

It is interesting and somewhat perplexing that there was a rapid initial reduction in pERK expression in BRAF mutant A375 cells following exposure to trametinib which then gradually increased in line with the emergence of CD271 expression and development of early drug resistance. Expression of pERK continued to decline in A375 cells following long term exposure to trametinib, however at these time points CD271 expression had also declined suggesting the observed further reduction in pERK expression and continued resistance to trametinib may be mediated by additional mechanisms independently to the induction of CD271.

The development of resistance to BRAF/MEK inhibition is associated with the rapid recovery of pERK expression as a result of resistant cells that escape the effects of BRAF/MEK inhibition (Paraiso *et al.*, 2010). However, it is still not clear what mediates the recovery of pERK expression and re-activation of the MAPK pathway following long term exposure to trametinib and the establishment of acquired resistance. It is therefore possible that other generic pro-survival pathways are activated to mediate trametinib-induced resistance, such as the unfolded protein response (Yan *et al.*, 2015), inhibition of anti-apoptotic proteins including BCL-2 (Mukherjee *et al.*, 2015) or activation of autophagy to sustain tumour growth independently of the MAPK pathway.

4.3.2 Trametinib-induced CD271 expressing melanoma subpopulations are associated with increased autophagic activity

There is increasing evidence in other cancer types that autophagy is crucial for the maintenance and survival of cancer stem cells (Maycotte *et al.*, 2015a). As autophagy is a principal survival mechanism harnessed by cancer cells to survive hostile micro environmental conditions and promote metabolic homeostasis, (Rabinowitz and White, 2010), it is therefore likely that autophagy contributes to the survival of melanoma cells, including drug resistant subpopulations regardless of whether such populations are a true cancer stem cell phenotype or not. Results from this present study demonstrated a clear association between trametinib-induced CD271 expressing subpopulations and activated autophagy, with data demonstrating

the concurrent induction of CD271 and autophagy (reflected by increased LC3-II and decreased p62 expression) in BRAF mutant cell lines following prolonged exposure to both the BRAF inhibitor PLX4720 and the MEK inhibitor trametinib. Studies of autophagic flux in BRAF mutant cells with dual tagged LC3-RFP-GFP additionally revealed an increase in LC3 flux in trametinib-induced CD271 expressing melanoma cells at a single cell level, further supporting the hypothesis that trametinib-resistant CD271 expressing melanoma cells harness autophagy to promote tumour cell survival.

What is still unclear is whether autophagy in this context is activated in response to targeted inhibition of MAPK signalling (Goulielmaki *et al.*, 2016) or whether emerging CD271 expressing drug resistant subpopulations specifically use autophagy as a survival mechanism. Future work could include the evaluation of the association of CD271 induction in the context of trametinib resistance with downstream signalling molecules such as RHoA/ROCK or JNK which could activate either a pro-survival, pro-apoptotic or migratory response and further tease out whether drug-induced CD271 plays a role in tumour cell migration or has a pro-survival role.

There is also increasing evidence in the literature to suggest AMPK activated by glucose starvation or ATP depletion to sustain the metabolic and energetic cellular demands in the micro environment may provide the link to the activation of autophagy (Cantó *et al.*, 2009) though inactivation of m-TORCH1 (Hardie, 2011) or direct phosphorylation of ULK1 (Kim *et al.*, 2011a). Indeed data from the present study demonstrated the significant reduction in ATP release and concurrent increase in pAMPK levels in trametinib-induced drug resistant CD271 expressing melanoma subpopulations, supporting the notion that autophagy activation in these subpopulations is mediated by a depletion of cellular ATP release that results in metabolic reprogramming via pAMPK activation. Nevertheless these observations do not preclude from the possibility that autophagy induction observed in trametinib-induced drug resistant subpopulations results directly from CD271 induction or is a generic survival mechanism induced in response to treatment with trametinib. In an attempt to address these possibilities, subsequent experiments focussed on modulating CD271 expression in melanoma cells (both wild type and in trametinib-induced drug resistant subpopulations) prior to determining the subsequent impact on autophagy. Studies of CD271 over expression in A375

and WM35 melanoma cells revealed a concurrent increase in basal autophagy, however, the cellular localisation of CD271 was lobular and sub membranous rather than being an expected diffuse membranous expression pattern and raising concerns with regard to the functionality of CD271 following overexpression and the possibility that autophagy induction may have been mediated by cellular stress and the induction of ER stress (Hoyer-Hansen and Jaattela, 2007). However, studies of ER stress gene expression in melanoma cells following over expression of CD271 did not reveal any significant association suggesting the observed autophagy induction did not arise as a consequence of cellular stress induced by transfection of CD271. Nevertheless, in contrast to published data (Lehraiki *et al.*, 2015) results from the present study also revealed there was no protective effect of CD271 expression on trametinib-induced cell death suggesting that it is possible that CD271 is not functional..

To further tease out the association between drug-induced CD271 expression and autophagy activation CD271 expression was down regulated using a stable (ShRNA) a transient (SiRNA) approach in A375 cells. Stable CD271 knockdown resulted in increased cell death, suggesting, in line with previous observations (Lehraiki *et al.*, 2015) that CD271 expression plays an integral role in melanoma cell survival. Subsequent experiments of CD271 knockdown were thus performed using a transient approach (siRNA), however, the observed, albeit non-significant, increase in autophagic activity and reduction in pAMPK expression in cells following transient knockdown of CD271, may not reflect autophagic activity in such cells and rather, may have been related to transfection-associated autophagy induction. Nevertheless, knockdown of CD271 also resulted in a significant reduction in cell viability in response to treatment with trametinib indicating that CD271 might indeed play a protective role in the response of BRAF mutant melanoma cells to BRAF/MEK inhibition. Taken together, these data suggest that CD271 knockdown impairs the ability of melanoma cells to resist the cytotoxic effects of trametinib although the association between drug-induced CD271 abrogation and autophagy is not entirely clear. To tease out if drug-induced CD271 initiates autophagy or if autophagy is induced by drug resistance resulting in increased CD271 future experiments could include the use of modern and more efficacious technologies for gene editing such as CRISPR/Cas9 (Sander and Joung, 2014) to guide transcriptional activators/repressors to the

CD271 gene promotor region, which will allow for the modulation of CD271 at the DNA level. This approach will allow for more physiological activation compared to exogenous cDNA to overexpress CD271, and repression could be controlled more accurately than RNAi. Alternative approaches would be to manipulate autophagy regulatory genes and subsequently evaluate the effect on CD271 expression or the effect of trametinib treatment on CD271 induction or tagging autophagy regulatory proteins with a fluorescent probe to allow for analysis of the direct interaction between CD271 and autophagy in response to trametinib using super-resolution microscopy.

4.3.3 Trametinib-induced CD271 expressing melanoma subpopulations are not consistently associated with the induction of other stem cell markers

The hypothesis that CD271 expressing melanoma subpopulations are a true stem cell population is a matter of ongoing debate. Recent literature in this context is controversial with some reports indicating a potential association between drug-induced CD271 expressing melanoma subpopulations and the co-expression of other stem cell markers (Redmer *et al.*, 2014a) and other reports contradicting a potential stem cell origin of the CD271 expressing cells (Cheli *et al.*, 2014a). The presence of de-differentiated cancer stem cells has been reported to be associated with more aggressive disease (Bao *et al.*, 2013; Lasorella *et al.*, 2014) via exploiting their ability to escape immunological surveillance (Bruttel and Wischhusen, 2014). It is also known that cancer stem cells harness pro-survival mechanisms such as autophagy (Lei *et al.*, 2017) and increased resistance to apoptosis (Fulda, 2013) and therefore to further tease out whether drug-induced CD271 expressing melanoma subpopulations are a true stem cells population is of importance. Results derived in chapter 3 highlighted a lack of any significant association between constitutively activated CD271 expressing melanoma cells and the co-expression of other stem cell markers including Jarid-1B (Roesch *et al.*, 2010), ABCB5 (Chartrain *et al.*, 2012) and SOX10 (Graf *et al.*, 2014a). Consistent with finding in chapter 3, results from studies of stem cell marker expression in CD271 expressing trametinib resistant CD271 expressing melanoma subpopulations also highlight a lack of any association between those cells that express CD271 and the co expression of ABCB5, Jarid-1B, Nestin or

SOX10 again suggesting such CD271 expressing drug resistant subpopulations are not a stem cell phenotype. If, however, CD271 expression is not associated with a stem cell phenotype in melanoma, it is still not certain why CD271 expressing cells emerge in drug resistance and what their constitutive role is in melanoma development and progression. Recent evidence highlights the association between BRAF therapy-induced TNFα/NF-kB pathway and CD271 induction (Lehraiki *et al.*, 2015). In the context of drug resistance, recent evidence supports the activation of the TNFα/NF-kB in response to targeted BRAF inhibition, followed by the increased expression of CD271 expressing melanoma subpopulations (Lehraiki *et al.*, 2015), indicating that there is possibly a direct link between NF-kB activation and CD271 induction in the context of drug resistance. Recent literature indicates that the NF-kB pathway is constitutively activated in many cancers (Smith *et al.*, 2014) and in melanoma, TNFα secretion by macrophages and T-cells in the tumoural micro environment leads to an alteration in the equilibrium between differentiated and de-differentiated melanoma cells and the emergence of CD271 expressing melanoma cells, and allowing CD271 positive cells to escape immune surveillance (Landsberg *et al.*, 2012). Interestingly, accumulating data also highlight the complex interplay between autophagy and NF-kB pathway. Autophagy-dependent degradation of NF-κB signaling components such as Hsp90 and IKKa and b (Djavaheri-Mergny and Codogno, 2007; Colleran *et al.*, 2011) can either terminate or activate NF-kB activation (Trocoli and Djavaheri-Mergny, 2011). Conversely, NF-κB signaling can either activate or inhibit signaling pathways that lead to the induction of autophagy by regulating the transcription of a subset of pro-autophagic-regulating genes (Djavaheri-Mergny and Codogno, 2007; Lee *et al.*, 2007a). It is therefore possible that there is a complex regulation between BRAF inhibitor-induced NF-kB pathway activation, autophagy activation and CD271 induction. However, it is not entirely clear what is the sequence of events following the emergence of resistance to BRAF/MEK inhibitors, knowledge which would potentially be of benefit to identify novel targets to overcome acquired resistance to BRAF/MEK inhibition in melanoma.

4.3.4 Exogenous NGF increases trametinib-induced CD271 expressing cell proliferation but has no effect on tumour spheroid expansion *in vitro*

Data derived from chapter 3 suggest that constitutive expression of CD271 is associated with reduced spheroid collagen expansion, supporting previous observations reporting the presence of CD271 expression is associated with reduced invasion *in vitro* and *in vivo* (Saltari *et al.*, 2016). Similarly data from the present chapter also revealed trametinib induced CD271 expressing melanoma cell populations also display reduced expansion when cultured in collagen gels *in vitro* compared to untreated control cells and collectively supporting the notion that although CD271 expression *per se* might play a role in melanoma survival (Lehraiki *et al.*, 2015) and progression (Beretti *et al.*, 2015) it may not be linked or be require to support the invasion of drug-resistant subpopulations. However, there is increasing evidence that constitutive expressing CD271 melanoma subpopulations may require additional stimuli to promote tumour invasion such as the presence of NGF (Herrmann *et al.*, 1993). NGF has been suggested to stimulate cell proliferation and survival via its interaction with TrkA and CD271 (Demont *et al.*, 2012), and in melanoma, the interaction of CD271 with NGF has been suggested to increase tumour cell invasion *in vitro* (Herrmann *et al.*, 1993). To evaluate the effect of NGF stimulation on trametinib induced CD271 expressing melanoma cells, drug resistant cells were further treated with NGF which resulted in a significant increase in cell proliferation. However, although there was some increase in spheroid size, NGF failed to significantly induce collagen spheroid expansion of trametinib induced CD271 expressing cells. Nevertheless, the observed increase in trametinib induced CD271 expressing spheroid expansion following treatment with NGF, although not significant, might indicate that *in vitro* invasion requires the presence of exogenous factors, including NGF, which *in vivo* could be derived from the tumour microenvironment. Recent reports highlight the secretion of NGF by human keratinocytes (Pincelli *et al.*, 1994; Shi *et al.*, 2013) indicating that *in vivo*, CD271 expression in melanoma cells may be driven by NGF secretion in the tumour microenvironment leading to the promotion of tumour invasion. It is still, however, not clear how NGF stimulates drug-induced CD271 cell proliferation. It is possible that CD271 requires the direct binding of NGF to the ligand to drive tumour cell invasion or requires the activation

of downstream target genes such as ESM1 (Chen *et al.*, 2016) to promote tumour invasion. However previous reports highlight the use of smaller doses of NGF up to 100 ng/ml (Fabricant *et al.*, 1977; Paraiso *et al.*, 2010) which is in contrast with the higher dose of NGF used in this study. It is likely that higher dose of NGF could signal through other pathways independent of CD271 such as TrkA (Paraiso *et al.*, 2010) and therefore the results involving NGF presented in this Thesis should be interpreted with caution. Future work to include further optimisation of exogenous NGF dosing and evaluate the effect on trametinib resistant cells is required to achieve firm conclusions. To further decipher the role of CD271 in tumour invasion and spheroid expansion in collagen gels, subsequent experiments described in chapter 5 investigated the role of CD271 inhibition using small molecule inhibitors TAT-PEP5 and Ro-08 to block CD271 expression and evaluate the effect of CD271 blockade on tumour cell spheroid expansion *in vitro*.

Collectively results derived from the present chapter highlight the emergence of CD271 expressing subpopulations following the exposure of BRAF mutant melanoma cells to BRAF or MEK specific inhibition. Data also highlight the intimate relationship between pro-survival autophagy and CD271 expression in the context of trametinib-induced melanoma resistance. However, whether pro-survival autophagy is activated as a consequence of drug-induced CD271 expression or vice versa is still not entirely clear. Findings reported in this chapter, additionally identify novel therapeutic targets and pave the way to several strategies that could potentially overcome the resistance of or re-sensitise patients with MAPK drug-resistant metastatic melanoma to the cytotoxic effects of trametinib, discussed fully in chapter 5.

4.4 **Summary**

- BRAF and MEK inhibition in BRAF mutant melanoma cells leads to induction of CD271 expressing subpopulations in melanoma

- Trametinib-induced CD271 expressing melanoma subpopulations are associated with an increase in autophagic activity

- Overexpression of CD271 is associated with increased autophagic activity but not induction of ER stress markers

- Knockdown of CD271 in BRAF mutant melanoma cell subpopulations in vitro is not associated with the co-expression of other neuroectodermal stem cell markers

- Trametinib-induced CD271 expressing melanoma subpopulations display reduced spheroid expansion and size when incorporated into collagen gels *in vitro* compared to untreated control BRAF mutant melanoma cells

- Treatment of trametinib-induced CD271 expressing melanoma subpopulations with exogenous NGF increases proliferation but does not significantly increase spheroid size *in vitro*

Chapter 5

Targeting drug resistant subpopulations in melanoma as a strategy to overcome therapeutic resistance to MAPK inhibition

Chapter 5 Targeting drug resistant subpopulations in melanoma as a strategy to overcome therapeutic resistance to MAPK inhibition

5.1 Introduction

As highlighted throughout the current thesis, the development of acquired resistance is the major limiting factor in the targeted treatment of patients with BRAF or NRAS mutant metastatic melanoma with BRAF or MEK specific inhibitors, leading to a plethora of studies focused towards novel means through which to overcome such resistance. Such approaches have included the inhibition of downstream ERK (Ishii et al., 2013), the targeting of other activated signalling pathways such as PI3K-PTEN-AKT (Sánchez-Hernández et al., 2012) or combined BRAF and MEK inhibition, including combined treatment with the BRAF inhibitor, dabrafenib, and the MEK inhibitor, trametinib; a strategy that results in prolonged disease free survival (Long et al., 2014) and following adoption by the National Institute for Health and Care Excellence (NICE) is now used as first line treatment for patients with BRAF mutant metastatic melanoma (NICE, 2016). Additionally in an attempt to delay the emergence of acquired resistance, sequential rather than combination approaches with targeted agents have been made (Kirk, 2013). However, to date none of these strategies have been able to overcome the inevitable development of acquired resistance by BRAF/NRAS mutant metastatic melanomas to BRAF and or MEK inhibitor therapy, emphasizing the acute need for novel and more effective approach.

Data from chapter 4 demonstrated the emergence of CD271 expressing subpopulations in the drug resistance of BRAF mutant melanoma cells to the BRAF and or MEK specific inhibition, paralleled by the activation of AMPK and pro-survival autophagy and thus highlighting the potential for CD271, AMPK or autophagy inhibition as a potential therapeutic strategy to prevent the survival and invasion of such drug resistant subpopulations.

Results reported in chapter 4 have also highlighted the intimate relationship between trametinib-induced CD271 expressing melanoma subpopulations and activation of pro-

survival autophagy, possibly via activation of the AMPK pathway. AMPK blockade has been shown to act synergistically with radiation therapy and reduce cell viability of colorectal cancer cells (Jin *et al.*, 2016). Similarly, targeting tumour cell metabolic reprogramming via AMPK activation in breast cancer cells has been proposed as a promising treatment strategy to overcome drug resistance (Long *et al.*, 2016). Evidence from other cancer types additionally indicates autophagy plays an important role in maintaining tumour cell homeostasis and survival, including in BRAF mutant melanoma (Armstrong *et al.*, 2011; Corazzari *et al.*, 2015) and leading to a number of pre-clinical and clinical studies of autophagy inhibition as a therapeutic approach to prevent drug resistance. Studies in chronic myeloid leukaemia demonstrate a superior anti-leukemic effect by combined treatment with tigecycline and chloroquine (Lu *et al.*, 2017). Similarly, autophagy inhibition with chloroquine has been shown to augment the effects of 5-FU (5-fluorouracil) in colorectal cancer (Li *et al.*, 2010), collectively suggesting that inhibiting autophagy in combination with other chemotherapeutic agents may present a viable means through which to re-sensitize CD271 expressing MEK inhibitor resistant melanoma cells once again to the cytotoxic effects of trametinib.

Interestingly, recent data also reports autophagy exacerbation as a strategy to promote tumour cell death (Scarlatti *et al.*, 2008), with studies in melanoma specifically showing use of the cannabinoid derivative THC (Tetrahydrocannabinol) results in autophagy induction, already increased basally by the presence of a BRAF mutation (Corazzari *et al.*, 2015) and which leads to tumour cell apoptosis (Armstrong *et al.*, 2015b) thereby suggesting this as a possible means through which to enhance cell death of drug resistant CD271 expressing melanoma subpopulations.

Given the clear association between MEK-inhibitor induced CD271 expressing melanoma subpopulations and pro-survival autophagy, the aim of the current chapter was to evaluate the potential for chemical inhibition of CD271 or the modulation of autophagy (either through inhibiting pAMPK or by chemical inhibition with chloroquine or specific autophagy inhibitors or by exacerbation with agents that promote cytotoxic autophagy) in trametinib-induced drug resistant BRAF mutant melanoma subpopulations as a strategy through which to over-come resistance to targeted MEK inhibitor therapy.

5.2 **Results**

5.2.1 Chemical Inhibition of CD271 inhibits cell viability, pAMPK and autophagy in trametinib-induced drug resistant subpopulations but has no impact on trametinib-induced inhibition of spheroid expansion *in vitro*

To test the potential for chemical inhibition of CD271 as a strategy to overcome CD271 associated resistance to MEK inhibitor therapy, two small molecule inhibitors, TAT-PEP5 (which inhibits the interaction of CD271 with Rho-GDI (Mochizuki *et al.*, 2016) and Ro 08-2750 (a small molecule which inhibits the binding of NGF to p75NTR (Niederhauser *et al.*, 2000) were used to inhibit CD271 activity in melanoma cells pre-treated with trametinib.

Dose response experiments were first performed in order to establish the optimal dose of each agent able to inhibit the cell viability of A375 or WM35 melanoma cells pre-treated for 9 days with 16 nM trametinib. Results demonstrated the dose dependent inhibition of both A375 and WM35 cell viability following treatment with either TAT-PEP5 or RO-08 for 72 hours, (

Figure 5-1 A, B), with the consistent and significant inhibition of cell viability of either cell line pre-treated with trametinib induced by 5 μM TAT-PEP5 and 10 μM RO-08 (Figure 5-2A, B) (One way ANOVA with Tukey's multiple comparison test, **P<0.01, ****P<0.0001).

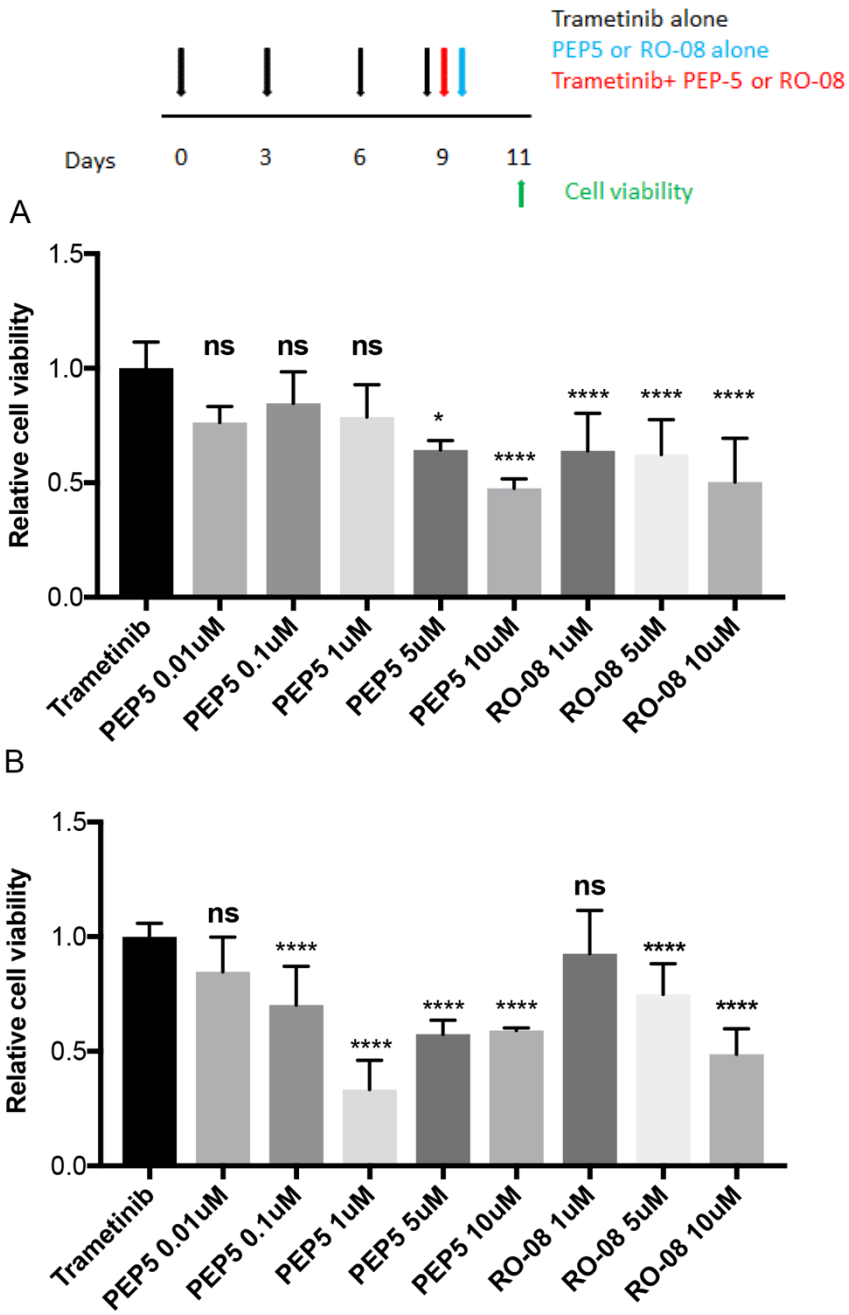


Figure 5-1:TAT-PEP5 and RO-08 induce dose dependent inhibition of cell viability of trametinib-induced drug resistant WM35 and A375 cells

Relative cell viability of WM35 (A) or A375 (B) cells, pre-treated for 9 days with 16nM trametinib, and subsequently treated for a further 72 hours with trametinib alone or with trametinib in combination with 0.01-10 μM TAT-PEP5 (PEP5) or 1-10 μM RO-08 (RO-08). Each bar is the mean +/- SD of 3 independent experiments expressed relative to control (9 day 16 nM trametinib treated), each containing 4 replicate treatment conditions. *P<0.05, ****P < 0.0001.

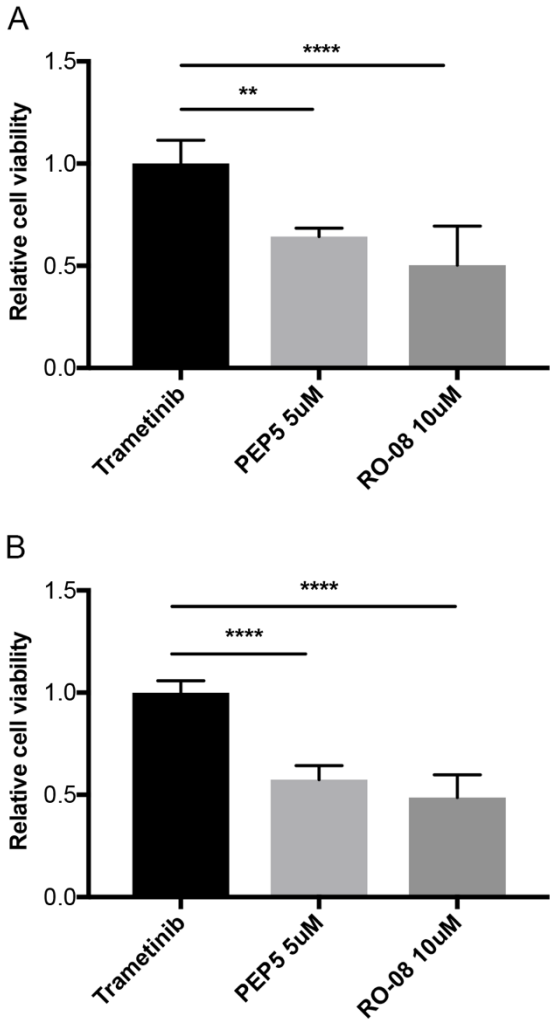


Figure 5-2: TAT-PEP5 and RO-08 significantly inhibit the cell viability of trametinib-induced drug resistant WM35 and A375 cells

Relative cell viability of WM35 (A) or A375 (B) cells pre-treated for 9 days with 16nM trametinib in the absence (trametinib) or presence of subsequent treatment for 72 hours with 5 μM TAT-PEP5 (PEP5 5 uM) or 10 μM RO-08 (RO-08 10uM). Each bar is the mean +/- SD of 3 independent experiments expressed relative to control (9 day trametinib treated WM35 or A375 cells).*P<0.05, ****P < 0.0001.

To further evaluate the effect of CD271 inhibitors on CD271 expression, autophagic activity and pAMPK expression, A375 pre-treated for 9 days with 16nM trametinib were again treated with 16 nM trametinib, 5 μM TAT-PEP5, 10 μM RO-08 or combined trametinib and PEP5/RO-08 for 72 hours prior to assessing the expression of CD271, p62, LC3 I/II or pAMPK by western blotting. Results demonstrated TAT-PEP5 and RO-08 induced the down regulation of CD271 expression which was paralleled with a reduction in autophagic activity and pAMPK expression (**Error! Reference source not found.** A) in cells pre-exposed to trametinib for 9 days. However, f

urther exposure to trametinib in the presence or absence of either CD271 inhibitor, resulted in the induction of CD271 expression to similar levels induced by the original exposure to trametinib for 9 days (Figure 5-3 A) which was also paralleled an increase in autophagic activity as well as pAMPK expression (Figure 5-3 A), likely reflecting that the CD271 inhibitors although they inhibit CD271 protein expression in drug induced CD271 expressing cells, the addition of trametinib, which induces CD271 expression, antagonizes the CD271 inhibitor function and results in increase in CD271 expression, autophagy and pAMPK levels. It is also likely that the observed reduction in CD271 expression in cells treated with the CD271 inhibitors alone is a result of the withdrawal of trametinib rather than a direct effect of the CD271 inhibitors on CD271 expression and therefore re-introduction of trametinib results in again increased expression of CD271.

Cleaved PARP expression demonstrated increased expression levels in A375 cells that have been treated with 16 nM trametinib for 9 days (Figure 5-3 A and v) which were further increased in cells that have been subsequently treated with combination of trametinib and CD271 inhibitors (Figure 5-3 A and v), albeit non-significant, indicating that the combination of trametinib with the CD271 inhibitors induces apoptotic cell death in trametinib induced CD271 expressing subpopulations.

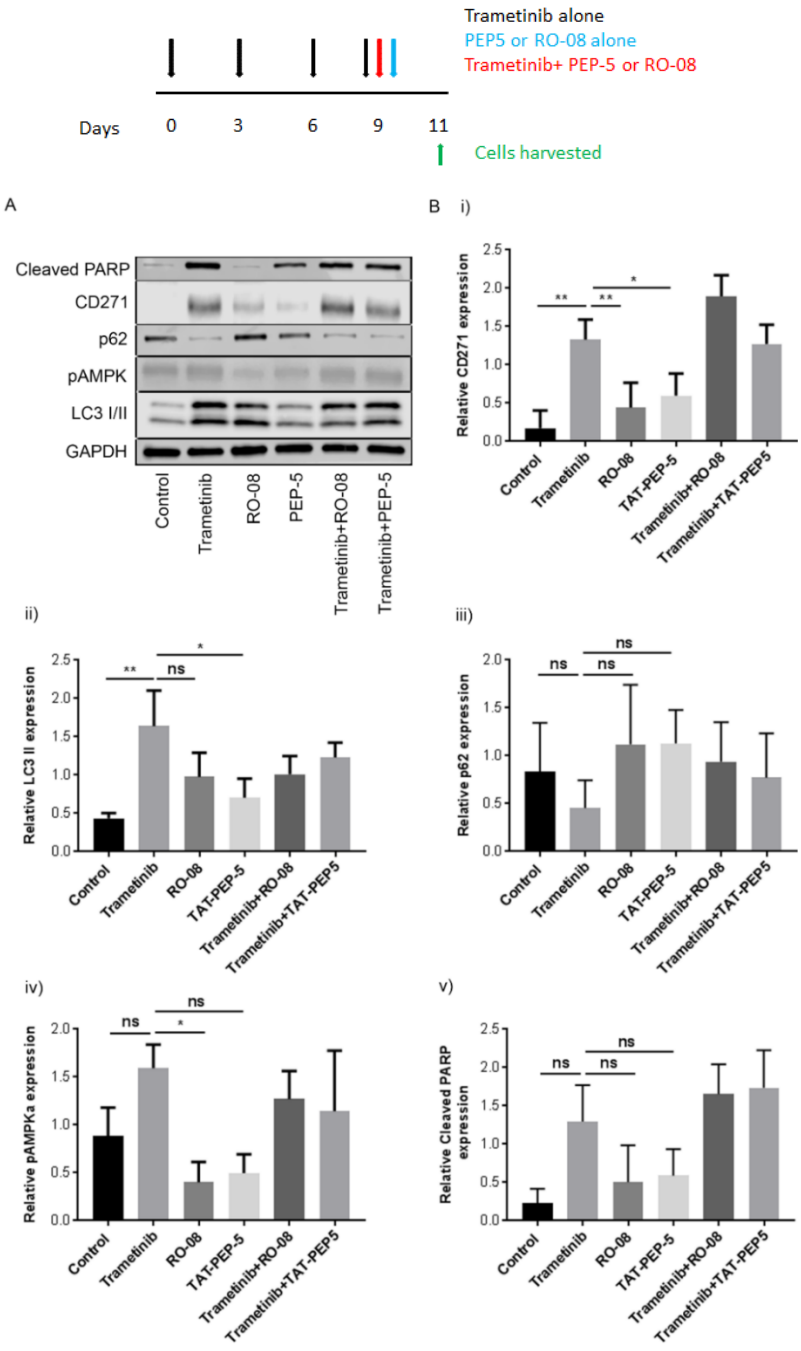


Figure 5-3: TAT-PEP5 and RO-08 inhibit MEK inhibitor-induced CD271, autophagy and pAMPK in A375 melanoma cells, but do not however prevent the induction of CD271, autophagy and pAMPK upon re-exposure to trametinib.

A.Representative immunoblots for the expression of Cleaved PARP (89 kDa), CD271 (45 kDa), pAMPK (62 kDa), p62 (62 kDa), LC3 I/II (16,18 kDa) or GAPDH loading control (38 kDa) in DMSO treated A375 cells (control) or A375 cells pre-treated for 9 days with 16 nM trametinib (trametinib) and subsequently for 72 hours with DMSO vehicle control (control), 16 nM trametinib, , 5 μM TAT-PEP5 (PEP5), 10 μM RO-08 (RO-08) or combined trametinib and TAT-PEP5 (Trametinib +PEP5) or RO-08 (Trametinib+RO-08). B. Relative CD271 (i), LC3 II (ii), p62 (iii), pAMPK (iv) or cleaved PARP (v) expression levels relative to GAPDH in 3 individual experiments. Each bar is the mean or 3 experiments ± SD (*P<0.05, **P<0.01).

To assess the impact of chemical inhibition of CD271 activity on the *in vitro* invasion of trametinib-induced drug resistant WM35 cells, WM35 H2B RFP cells pre-treated for 9 days with 16nM trametinib were seeded in collagen gels prior to further treatment for up to 7 days with either DMSO control or trametinib in the presence or absence of 5 µM TAT-PEP5 or 10 µM RO-08, and the monitoring of spheroid size over 7 days. Results demonstrated neither TAT-PEP5 or RO-08 alone exerted the capacity to prevent increase in spheroid size of wild-type control (DMSO treated) WM35 cells (Two-way ANOVA test with Sidak post hoc correlation, NS P>0.05,

Figure 5-4 A, B, Figure 5-5 A,B), while a significant reduction in spheroid size was observed in cells pre-treated for 9 days with trametinib or subsequently treated with trametinib for a further 7 days in the presence or absence of either CD271 inhibitor (

Figure 5-4 A,B, Figure 5-5 A,B) (Two-way ANOVA test with Sidak post hoc correction, **P<0.01, ***P<0.001), indicating that the CD271 inhibitors alone do not impact on spheroid size *in vitro*, whereas the cells treated with combination of trametinib and the CD271 inhibitors demonstrate a significant reduction in spheroid size which is, however, not significantly different compared to the cells treated with trametinib alone, suggesting that CD271 inhibitors do not affect the spheroid size of trametinib-induced CD271 expressing subpopulations *in vitro*.

Collectively these data indicate chemical inhibition of CD271 inhibits the viability of trametinib-induced drug resistant melanoma cells and increases their spheroid size.

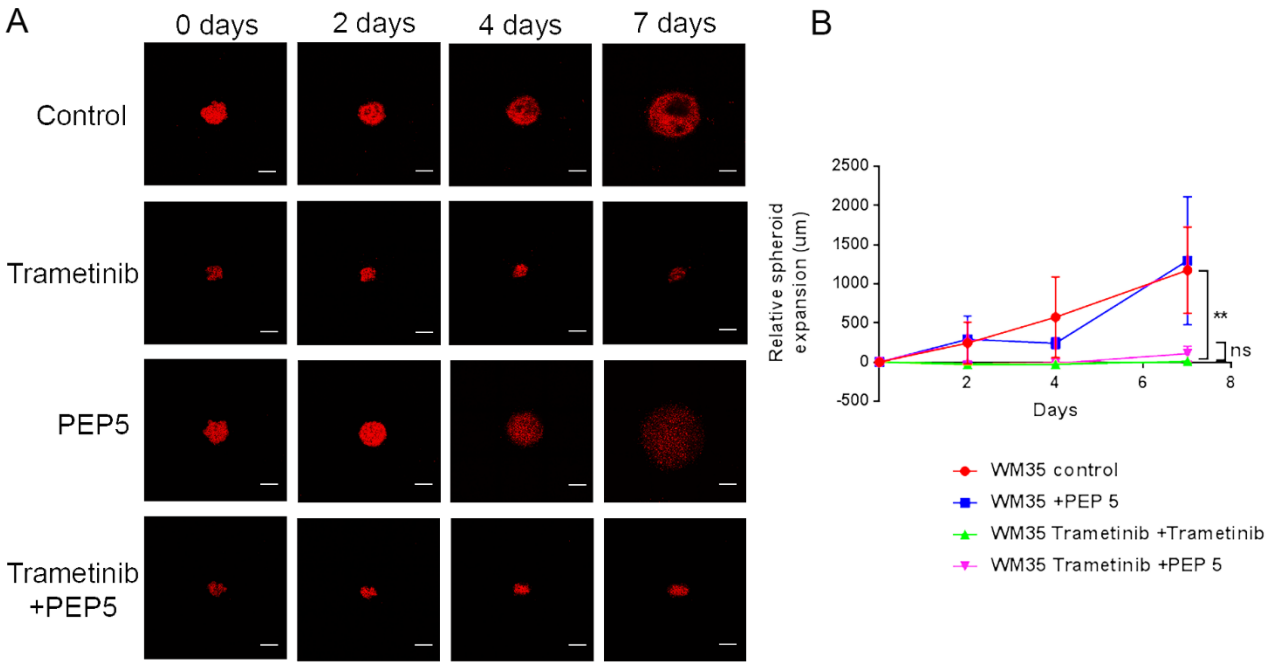


Figure 5-4: TAT-PEP5 does not prevent an increase in spheroid size of Trametinib-induced drug resistant WM35 cells in vitro

A.Representative immunofluorescence images of WM35 H2B RFP untreated control cells (control) or pre-treated with 16 nM trametinib for 9 days followed by continuation of 16 nM trametinib (trametinib), 5 μM TAT-PEP5 (PEP5) or combination of 16 nM trametinib and 5 μM TAT-PEP5 (Trametinib+PEP5) for up to 7 days in collagen gels for 0 , 2, 4 or 7 days. Scale bar 100 μm. B. Quantification of spheroid expansion demonstrated as relative size (μm) compared to initial spheroid size (0 day) **P<0.01.

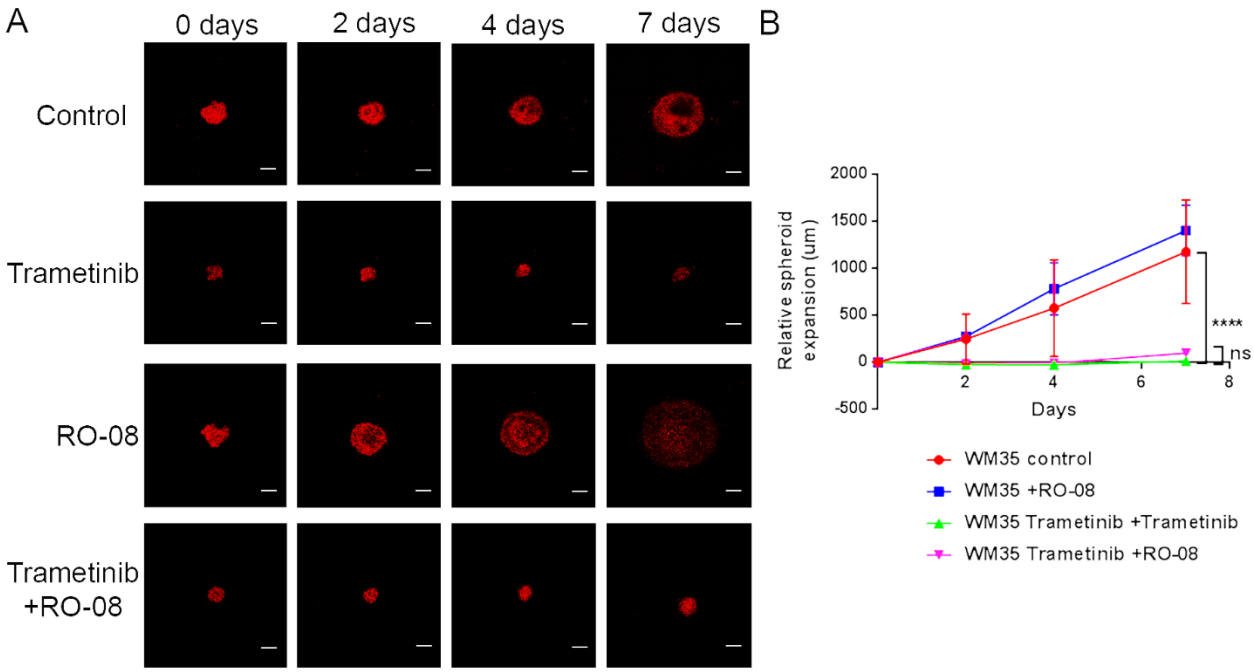


Figure 5-5 RO-08 does not prevent an increase in spheroid size of Trametinib-induced drug resistant WM35 cells in vitro

A.Representative immunofluorescence images of WM35 H2B RFP untreated control cells (control) or pre-treated with 16 nM trametinib for 9 days followed by continuation of 16 nM trametinib (trametinib), 10 μM RO-08 (RO-08) or combination of 16 nM trametinib and 10 μM RO-08 (Trametinib+RO-08) for up to 7 days in collagen gels for 0 , 2, 4 or 7 days. Scale bar 100 μm. B. Quantification of spheroid expansion demonstrated as relative spheroid size (μm) compared to initial spheroid size (0 day) **P<0.01.

5.2.2 Inhibition of pAMPK increases autophagic activity in trametinib-induced CD271 expressing melanoma subpopulations

To evaluate the potential of inhibiting pAMPK activity as a means to inhibit trametinib-induced pro-survival autophagy in melanoma cells, trametinib-induced drug resistant WM35 or A375 subpopulations were re-exposed to trametinib in the presence or absence of a potent cell permeable AMPK inhibitor (Liu *et al.*, 2014) prior to assessing the impact on autophagy activity by western blotting for p62 and LC3 I/II expression. Experiments using compound C were performed in collaboration with Dr Noel Edwards, Newcastle University and results demonstrated a reduction in pAMPK expression in both WM35 and A375 following treatment with Compound C which was paralleled with a reduction in CD271 expression (Figure 5-6 A, Figure 5-7 A). However, LC3 II expression was increased in both cell lines following treatment with compound C (Figure 5-6 A, Figure 5-7 A) with a concurrent reduction in p62 expression in WM35 cells (Figure 5-6 A) but increase in p62 expression in A375 cells (Figure 5-7 A), collectively suggesting pAMPK inhibition enhances rather than reduces autophagic activity in trametinib-induced drug resistant melanoma cell populations. The reasons for these findings are unclear but may reflect the fact that Compound C has anti-cancer effects that are independent of the AMPK pathway and therefore is not a specific AMPK inhibitor (Liu *et al.*, 2014).

The alternative approach of directly modulating autophagy either with specific inhibitors or through exacerbation with agents to harness its cytotoxic effect was therefore adopted subsequently to evaluate the potential of autophagy modulation as a strategy to re sensitize MEK inhibitor drug resistant melanoma subpopulations to the cytotoxic effects of trametinib.

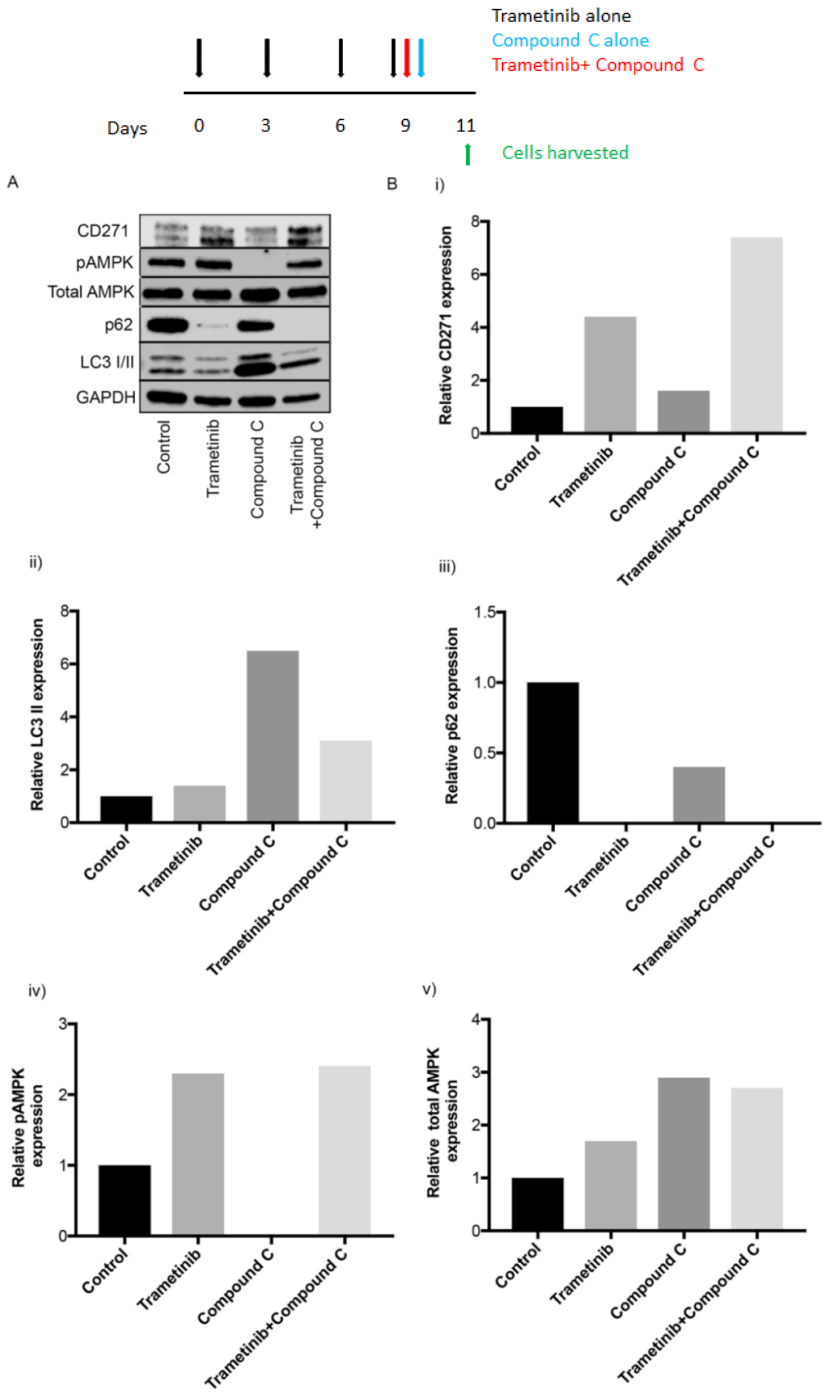


Figure 5-6: Compound C reduces pAMPK, CD271 expression but increases autophagy trametinib-induced drug resistant WM35 melanoma subpopulations.

A.Representative immunoblots for the expression of CD271 (45 kDa), pAMPK (62 kDa), total AMPK (62 kDa), p62 (62 kDa), LC3 I/II (16, 18 kDa) or GAPDH loading control (38 kDa) in WM35 cells following treatment with vehicle DMSO control (control) or WM35 cells treated with 16 nM trametinib for 7 days and subsequently treated with 16 nM trametinib (trametinib),5 µM Compound C or combination 16 nM trametinib and 5 µM Compound C (Trametinib+Compound C) for 48 hours. B.Relative CD271 (i), LC3 II (ii), p62 (iii), pAMPK (iv) or total AMPK (v) expression levels relative to GAPDH (n=1).

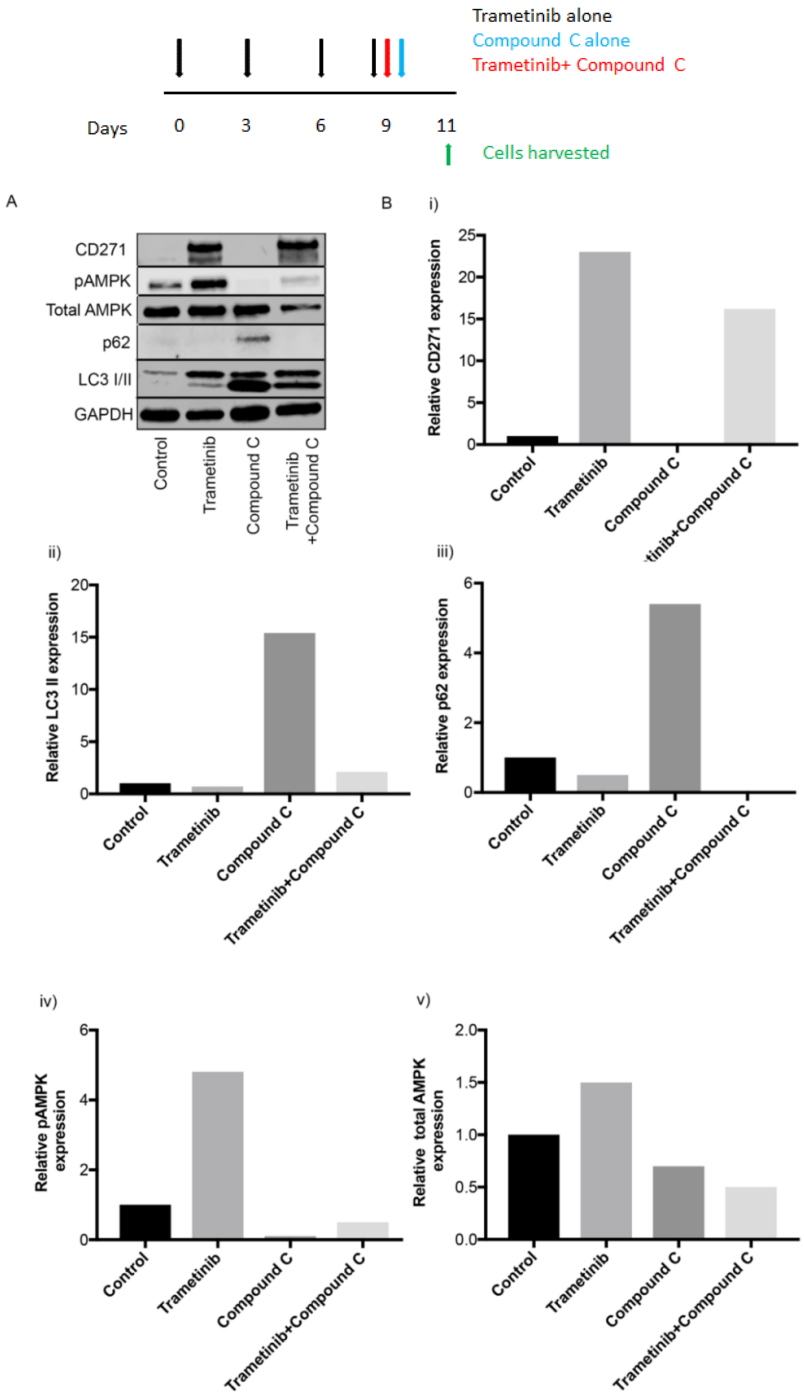


Figure 5-7: Compound C reduces pAMPK, CD271 expression but increases autophagy trametinib-induced drug resistant A375 melanoma subpopulations.

A.Representative immunoblots for the expression of CD271 (45 kDa), pAMPK (62 kDa), total AMPK (62 kDa), p62 (62 kDa), LC3 I/II (16, 18 kDa) or GAPDH loading control (38 kDa) in A375 cells following treatment with vehicle DMSO control (control) or A375 cells treated with 16 nM trametinib for 7 days and subsequently treated with 16 nM trametinib (trametinib),5 µM Compound C or combination 16 nM trametinib and 5 µM Compound C (Trametinib+Compound C) for 48 hours. B.Relative CD271 (i), LC3 II (ii), p62 (iii), pAMPK (iv) or total AMPK (v) expression levels relative to GAPDH (n=1).

5.2.3 Autophagy Inhibition re-sensitizes MEK inhibitor-induced CD271 drug resistant melanoma subpopulations to the cytotoxic effects of trametinib.

Autophagy in the context of cancer resistance can be a direct result of cytotoxic chemotherapy (Kanzawa *et al.*, 2004) or targeted therapy treatment (Goulielmaki *et al.*, 2016) but also can be harnessed by specific chemo resistant tumour cell subpopulations to escape the cytotoxic effects of these agents (Brigger *et al.*, 2015). Given the clear association between autophagy activation in trametinib-induced CD271 expressing drug-resistant subpopulations, the potential for autophagy inhibition was first evaluated *in vitro* as a strategy to re-sensitize drug resistant melanoma subpopulations to the cytotoxic effects of trametinib. To test this hypothesis autophagy was inhibited in trametinib-induced drug resistant subpopulations with the lysosomal inhibitor chloroquine and a specific inhibitor of the Vps34/Beclin 1 complex, PIK III (Ronan *et al.*, 2014).

5.2.4 Autophagy inhibition with Chloroquine reduces cell viability, colony forming potential and tumour spheroid size of prolonged trametinib-induced resistant CD271 expressing melanoma subpopulations *in vitro*.

To evaluate the potential for chloroquine to re-sensitize drug resistant melanoma cells to the cytotoxic effects of trametinib, WM35, SK-mel-28, A375 and WM266-4 cells pre-treated for 9 days with 16nM trametinib were re-exposed to 16nM trametinib for 48 hrs in the presence or absence of co-treatment with 10 μM chloroquine prior to the assessment of cell viability and colony forming potential. Analysis of the effects on cell viability revealed the combined treatment of all cell lines with trametinib and chloroquine significantly increased inhibition of cell viability of all cell lines (

Figure 5-8 A, B, C, D) (One way ANOVA with Tukey multiple comparison test, *P<0.05, **P <0.001, ****P <0.0001), resulting in significant re-sensitization of resistant cells (initially treated for 9 days with 16nM trametinib) to the cytotoxic effects of trametinib.

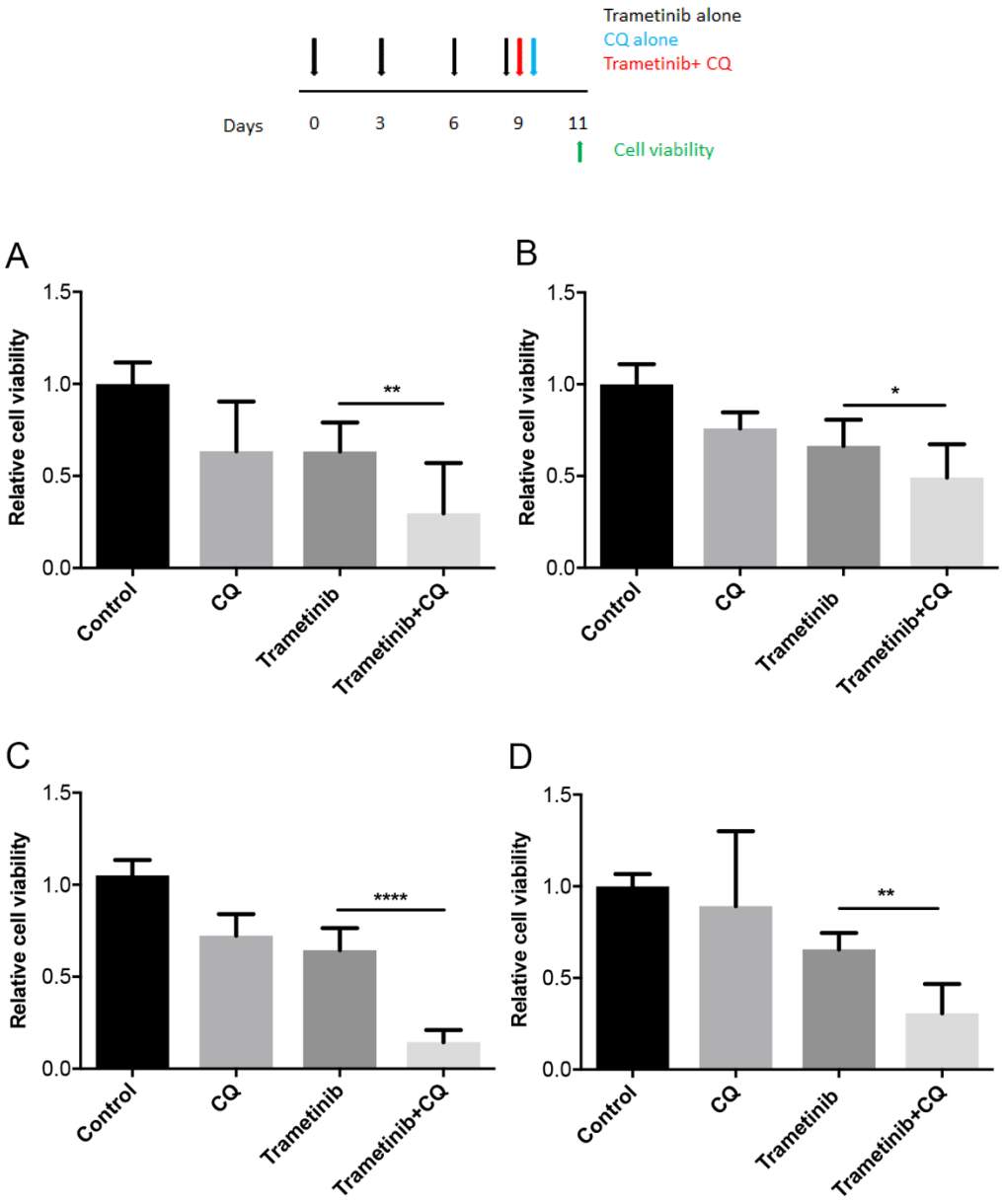


Figure 5-8: Chloroquine re-sensitizes trametinib-resistant melanoma cells to trametinib-induced inhibition of cell viability.

Relative cell viability of trametinib-resistant WM35 (A), A375 (B), SK-mel-28 (C) and WM266-4 (D) cells (initially treated for 9 days with 16 nM trametinib) following treatment for 48 hrs with DMSO vehicle control (control), 10 µM chloroquine (CQ), 16 nM trametinib (Trametinib), or combined CQ and trametinib (Trametinib+CQ). Each bar is the mean +/- SD of 3 independent experiments each containing 4 replicate treatment conditions, expressed relative to untreated control. *P<0.05, **P <0.001, ****P < 0.0001.

However, the clinically achievable chloroquine concentrations are between 25 and 50 mg/Kg (Oduola *et al.*, 1998; Ursing *et al.*, 2016) which might not be equivalent to 10 µM dose used in tissue culture experiments. To evaluate the effect of a lower, clinically achievable dose of chloroquine on the cell viability of trametinib resistant cells, identical experiments were repeated in WM35 and A375 cells using lower doses of 5 µM chloroquine, in which results again revealed the significantly increased inhibition of cell viability of both trametinib-induced resistant WM35 and A375 cells following combined treatment for a further 48 hours with 16 nM trametinib and 5 µM chloroquine (one way ANOVA with Tukey multiple comparison test, ***P < 0.001, **P <0.001, Figure 5-9 A and B).

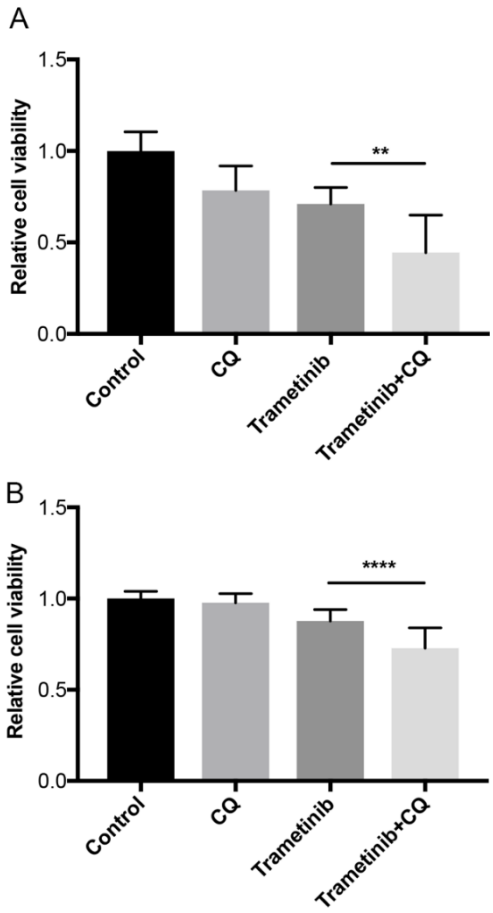


Figure 5-9: Lower dose chloroquine re-sensitizes trametinib-resistant melanoma cells to trametinib-induced inhibition of cell viability.

Relative cell viability of trametinib-resistant WM35 (A) and A375 (B) cells (treated initially for 9 days with 16nM trametinib) following treatment for 48 hrs with DMSO vehicle control, 5 µM chloroquine (CQ) ,16nM trametinib (Trametinib), or combined CQ and trametinib (Trametinib+CQ). Each bar is the mean +/- SD of 3 independent experiments each containing 4 replicate treatment conditions, expressed relative to untreated control. **P <0.001. ***P < 0.001.

To further confirm the potential for chloroquine to increase cell death of trametinib-induced drug resistant melanoma cell populations following re exposure to trametinib, colony forming assays were performed in SK-mel-28 and A375 cells. Both cell lines were initially treated with 16 nM trametinib for 9 days, followed by subsequent treatment for 48 hrs in the presence or absence of 10 µM chloroquine, 16 nM trametinib or the combination of both agents. Results demonstrated the colony forming efficiency of both SK-mel-28 (Figure 5-10 and B) and A375 cells (Figure 5-10 A and C) cells was reduced by both single agent chloroquine and trametinib but was enhanced by combined treatment with both agents (One way ANOVA with Tukey's post hoc correlation, *$P<0.05$, NS $P>0.05$, (Figure 5-10 B and C).

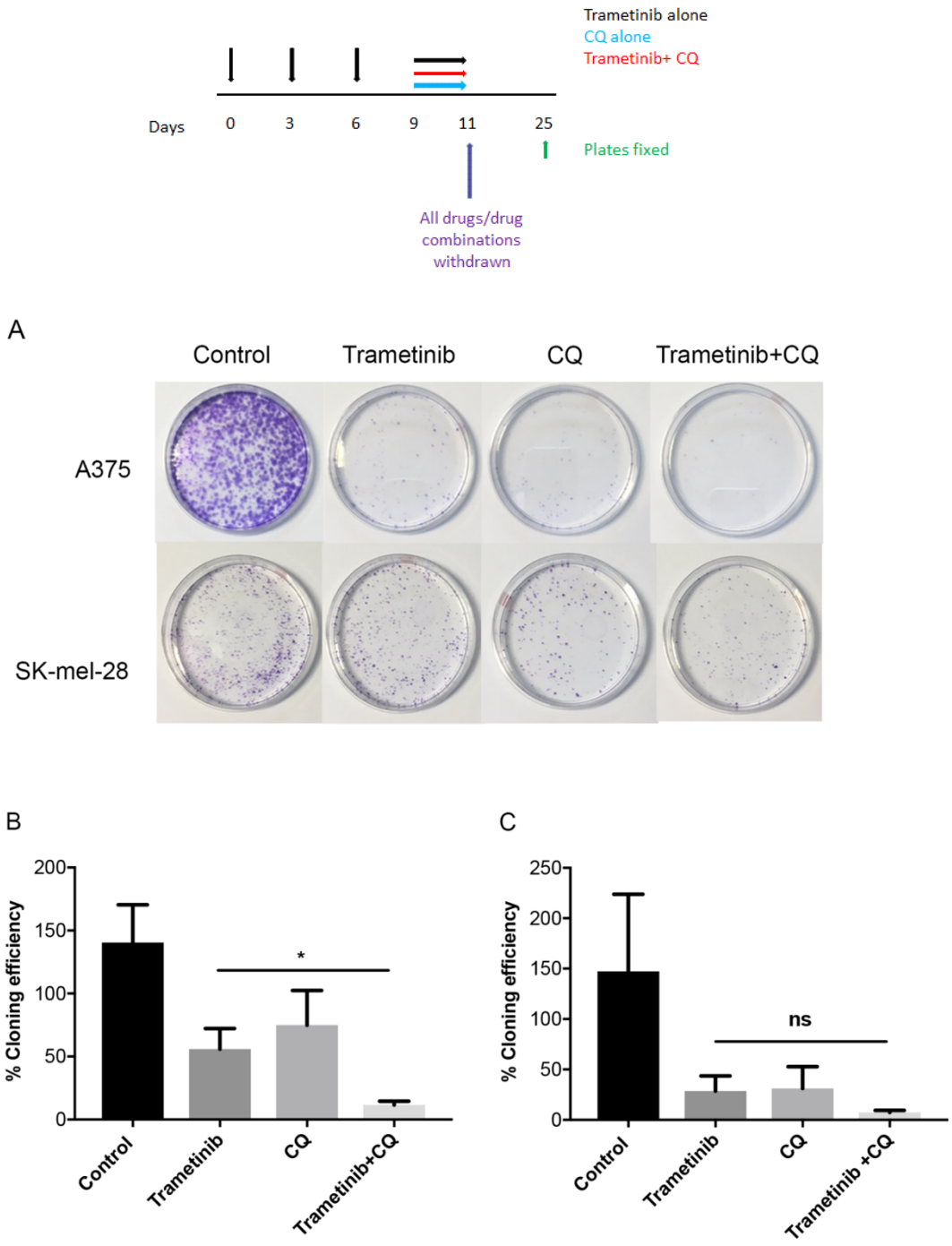


Figure 5-10: Chloroquine inhibits the colony forming capacity of trametinib-induced drug resistant A375 and SK-mel-28 cells.

A: Representative images of A375 and SK-mel-28 colonies or % cloning efficiency of A375 (B) or SK-mel-28 cells (C) untreated control (control) or following initial exposure for 9 days to 16 nM trametinib and subsequent treatment for 48 hrs in the presence of 16 nM Trametinib, 10 µM chloroquine (CQ), or combined trametinib and CQ chloroquine prior to seeding at a density of 1000 cells/petri dish and further culture for 2 weeks. Each bar is the mean of three independent replicate experiments what +/- SD compared to untreated control. *P<0.05

Collectively these data thus suggest that inhibiting trametinib-induced pro-survival autophagy in drug resistant CD271 expressing melanoma subpopulations re-sensitizes them to the cytotoxic effects of trametinib. Nevertheless to confirm the effects of chloroquine on the inhibition of trametinib-induced CD271 and autophagy as well as the induction of cell death and specifically apoptosis, additional experiments were carried out to evaluate the effects of chloroquine on trametinib-induced CD271, PARP cleavage and p62 and LC3 I/II expression. WM35 and A375 cells were initially treated in the presence of 16 nM trametinib for 9 days before subsequent treatment for a further 48 hours with either 16 nM trametinib, 10 μM chloroquine or combined trametinib and chloroquine.

Results demonstrated an increased CD271 expression which was non-significant in drug-resistant WM35 (Figure 5-11) but a significant increase in CD271 expression was observed in A375 cells (One way ANOVA with Tukey's multiple comparison test, *P<0.05, Figure 5-12 A, Bi) following further treatment with trametinib for 48 hrs, which was not, in general, altered by treatment with chloroquine or in cells treated subsequently for 48 hrs with combined chloroquine and trametinib (Figure 5-11 and Figure 5-12). Data also demonstrated trametinib-induced LC3 II expression which was further induced in the presence of chloroquine, signifying a block in autophagic flux, an observation supported by an associated increase, albeit non-significant, in p62 expression in trametinib-induced drug resistant cells treated with chloroquine and collectively further confirming the inhibition of trametinib-induced autophagy by chloroquine in both cell lines (One way ANOVA with Tukey's multiple comparison test, ***P<0.001 for LC3 II in WM35 cells (Figure 5-11 A, Bii), **P<0.01 for LC3 II in A375 cells, (Figure 5-12 A, B ii)). Furthermore, results also revealed a non-significant increase in cleaved PARP expression in trametinib-induced drug resistant populations in both cell lines following exposure to chloroquine, further confirming the potential for autophagy inhibition with chloroquine to re-sensitize cells to trametinib-induced apoptosis (Figure 5-11 and Figure 5-12).

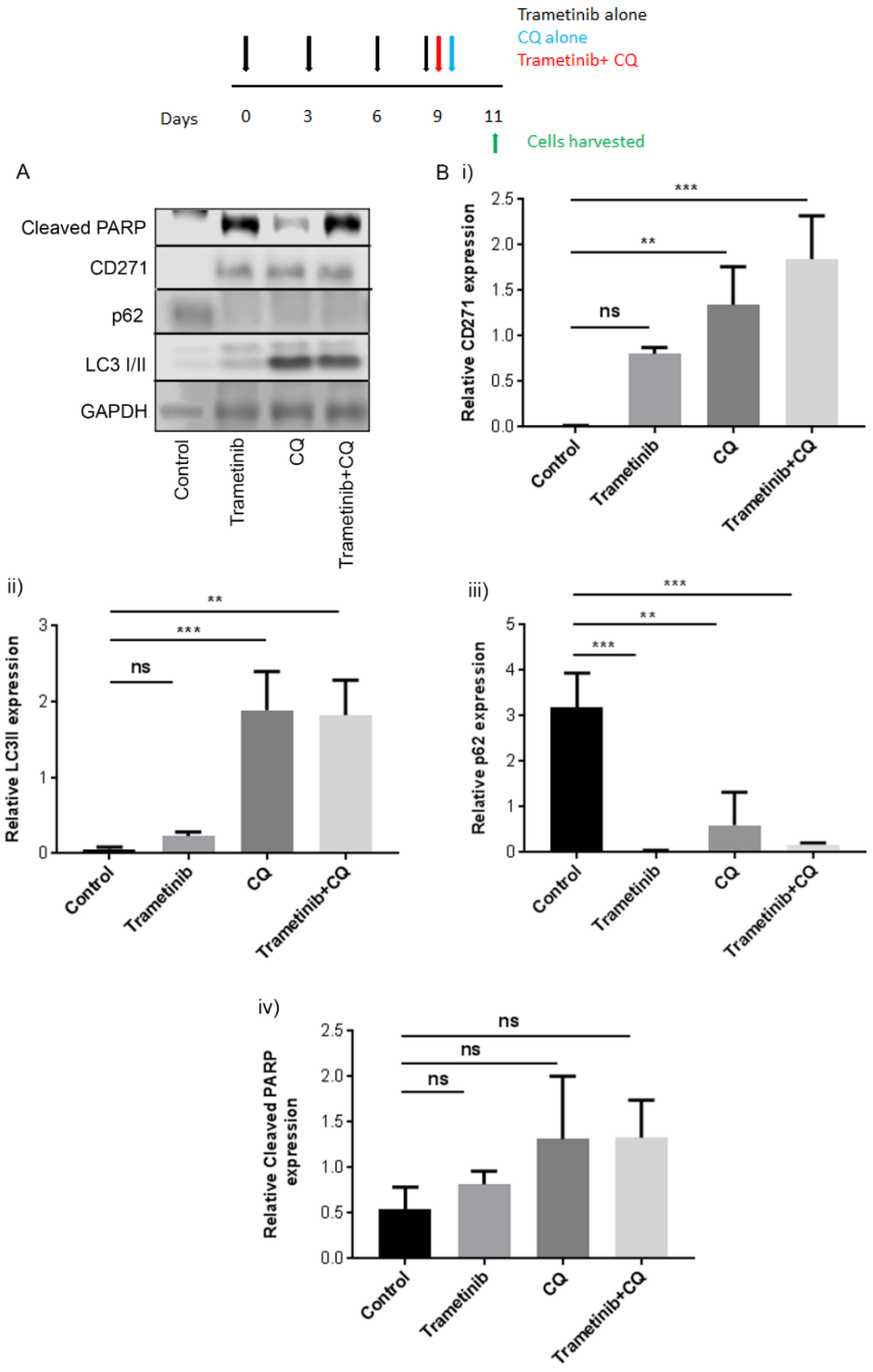


Figure 5-11 Chloroquine inhibits trametinib-induced autophagy and potentiates apoptosis of drug resistant WM35 cells

A.Representative immunoblots or B: Relative expression of CD271 (45 kDa, B i), LC3 I/II (16,18 kDa, or LC3II, B ii), p62 (62 kDa, B iii), Cleaved PARP (89 kDa, B iv), or GAPDH loading control (38 kDa) in WM35 cells following treatment with vehicle DMSO control (control) or pre-treatment with 16 nM trametinib for 9 days (trametinib) and subsequent treatment for 48 hours with 10 μM chloroquine (CQ) or combined trametinib and chloroquine (Trametinib +CQ) or for 48 hours. Each bar is the mean or 3 experiments ± SD relative to GAPDH in 3 individual experiments (**P<0.01, ***P<0.001).

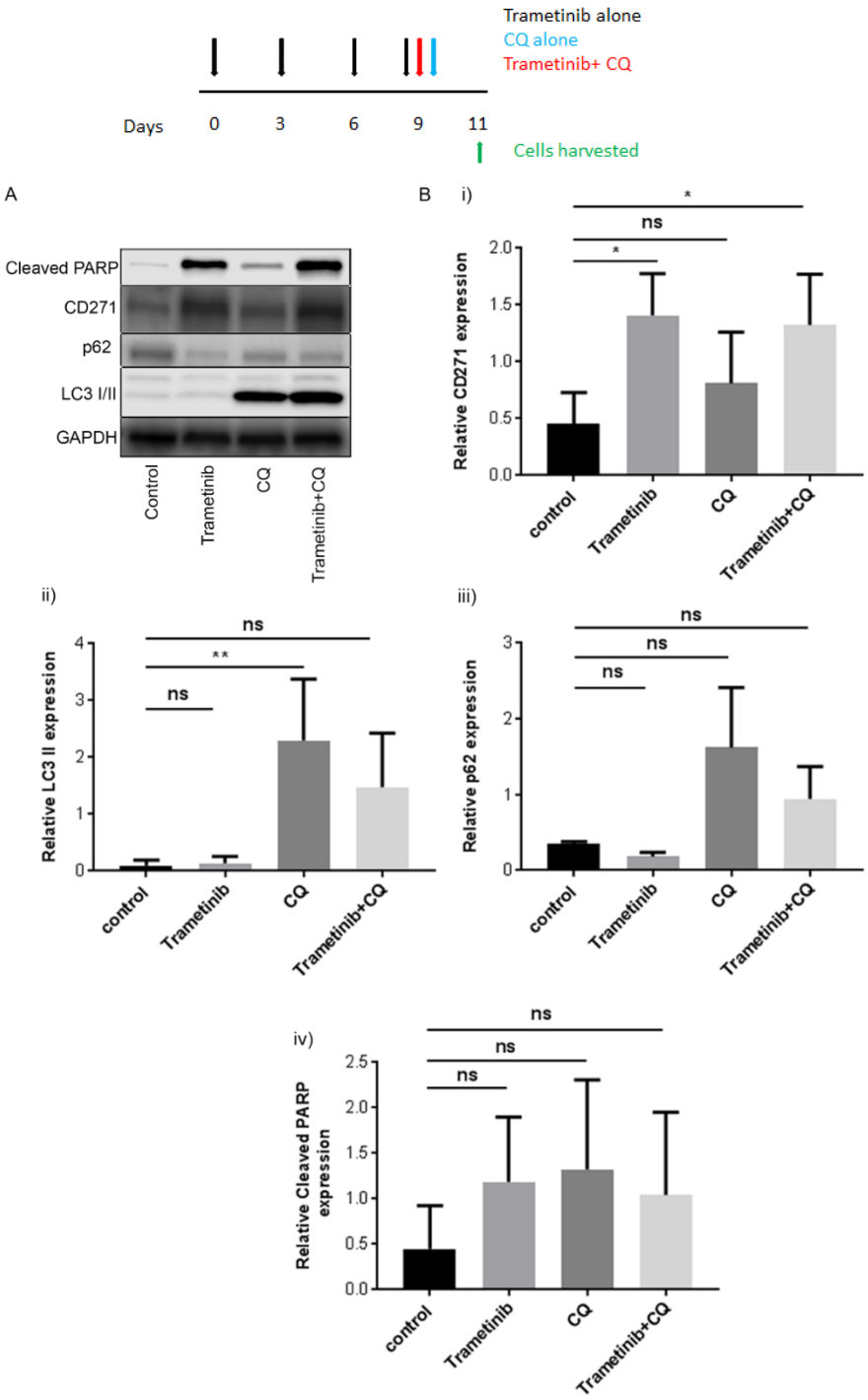


Figure 5-12: Chloroquine inhibits trametinib-induced autophagy and potentiates apoptosis of drug resistant A375 cells

A.Representative immunoblots or B: Relative expression of CD271 (45 kDa, B i), LC3 I/II (16,18 kDa, or LC3II, B ii), p62 (62 kDa, B iii), Cleaved PARP (89 kDa, B iv), or GAPDH loading control (38 kDa) in A375 cells following treatment with vehicle DMSO control (control) or pre-treatment with 16 nM trametinib for 9 days (trametinib) and subsequent treatment for 48 hours with 10 μM chloroquine (CQ) or combined trametinib and chloroquine (Trametinib +CQ) or for 48 hours. Each bar is the mean or 3 experiments ± SD relative to GAPDH in 3 individual experiments. (*P<0.05, **P<0.01).

Together, these data thus support the pro-survival role of autophagy in trametinib-induced CD271 expressing drug resistant subpopulations and the therapeutic potential for autophagy blockade to overcome trametinib-induced drug resistance of BRAF/NRAS mutant melanoma.

Finally to investigate the effects of chloroquine on the invasion of trametinib-induced CD271 expressing drug-resistant melanoma subpopulations *in vitro*, A375 cells continuously pre-treated in the presence of 16 nM trametinib for 42 days were seeded into collagen gels and subsequently further treated with either complete media control, 16 nM trametinib, 10 μM chloroquine or combined trametinib and chloroquine for up to 7 days. Results demonstrated the significant reduction in collagen invasion of cells that have been co-treated with trametinib and CQ for 7 days in collagen gels compared to the invasion of either control cells at the same time point (Two way ANOVA with Sidak post hoc correlation, ****P<0.0001) (Figure 5-13 A and B) or cells that have been continuously treated with trametinib for 7 days following incorporation in to collagen invasion assays- (Two way ANOVA with Sidak multiple comparison test (***P<0.001), (Figure 5-13 A and B).

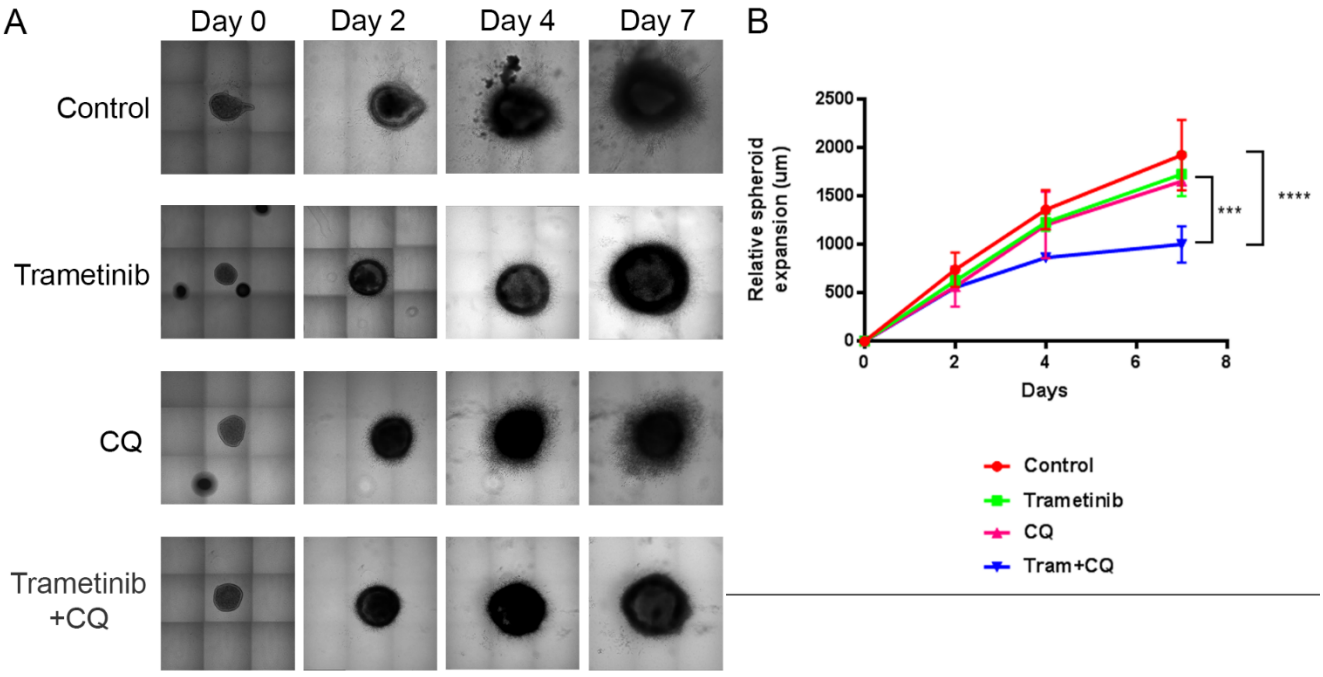


Figure 5-13: Combined treatment of trametinib-induced drug resistant A375 cells (pre exposed to trametinib for 42 days) with trametinib and chloroquine inhibits spheroid expansion in vitro

A.Representative bright field images of drug resistant A375 spheres pre-treated with 16 nM trametinib for 42 days and incorporated into collagen invasion gels prior to subsequent treatment for 0, 2, 4 or 7 days with medium control (control), 16 nM trametinib, 10 μM chloroquine (CQ) or combined trametinib and chloroquine (Trametinib+CQ). Scale bar 100 μm. B. Quantification of collagen invasion of drug resistant A375 cells following subsequent treatment for 0, 2, 4 or 7 days with control, trametinib, CQ or trametinib + CQ expressed as relative invasion (μm) compared to initial spheroid size at day 0. Each point is the mean of three replicate independent experiments +/-SD compared to control (42 day trametinib treated A375 cells), ***P<0.001, ****P<0.0001.

5.2.4.1 Vps34 inhibition reduces cell viability, but has no impact on the inhibition of invasion of prolonged trametinib-induced resistant CD271 expressing melanoma subpopulations in vitro.

To evaluate the potential for specific autophagy inhibition to re-sensitize drug resistant melanoma cells to the cytotoxic effects of trametinib, WM35 and A375 cells pre-treated for 9 days with 16 nM trametinib or prolonged exposure to 16 nM trametinib for 42 days were re-exposed to 16 nM trametinib for 24 hrs in the presence or absence of co-treatment with 5 μM PIK III (a selective inhibitor of the Vps34/Beclin-1 (Dowdle *et al.*, 2014) prior to the assessment of cell viability and tumour invasion *in vitro.*

Results demonstrated a trend for PIK II-induced inhibition of cell viability of either WM35 or A375 cell pre-treated for 9 days with 16 nM trametinib (Figure 5-14). Consistent with previous findings re exposure of 9 day resistant WM35 or A375 cells to 16 nM trametinib for 24 hours also had no effect on the reduction of cell viability (Figure 5-14). However, subsequent treatment of 9 day resistant WM35 or A375 cells with trametinib in combination with PIK III resulted in the significant inhibition of viability of both drug-resistant cell lines (One way ANOVA with Tukey's post hoc correlation, **P <0.001 for WM35 and ****P < 0.0001 for A375 cells, Figure 5-14 A, B).

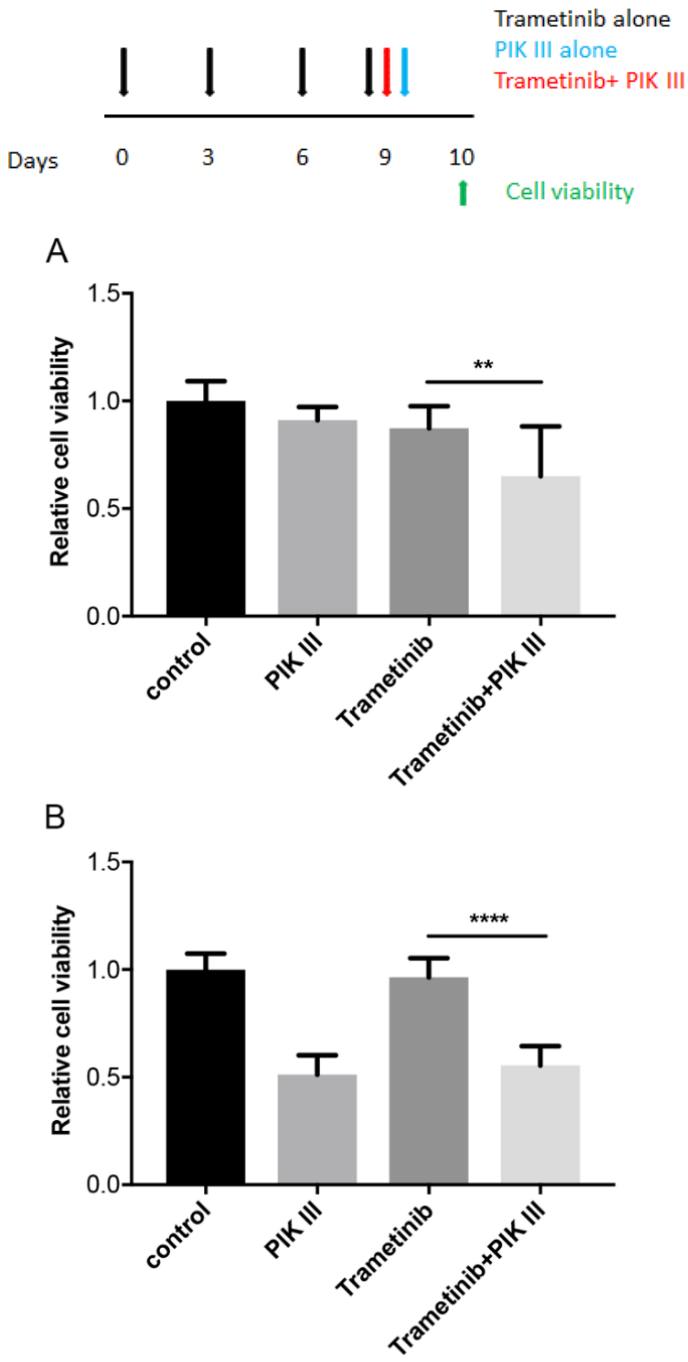


Figure 5-14: Vps34 inhibition re-sensitizes drug resistant WM35 and A375 cells to the cytotoxic effects of trametinib.

Relative cell viability of trametinib-resistant WM35 (A) and A375 (B) cells pre-treated for 9 days with 16nM trametinib and subsequently treated for 24 hrs In the presence of DMSO vehicle control, 5 µM PIK III (PIK III) ,16nM trametinib (Trametinib), or combined PIK III and trametinib (Trametinib+PIK III). Each bar is the mean +/- SD of 3 independent experiments each containing 4 technical replicates and expressed relative to untreated control**P <0.001. ****P < 0.0001.

To further evaluate the potential effect of PIK III on trametinib-induced CD271 and autophagy, as well as apoptosis, drug resistant WM35 and A375 cells (pre-treated with 16 nM trametinib for 9 days) were also treated with PIK III in the presence of absence of re-exposure to 16nM trametinib prior to assessing the expression of CD271, LC3 I/II, p62, pAMPK and cleaved PARP and by Western blotting (Figure 5-15, Figure 5-16). Results demonstrated the significant induction of CD271 expression in 9 day resistant WM35 cells in the presence of absence of PIKII (One way ANOVA with Tukey's post hoc correction, * P< 0.05, Figure 5-15 A, Bi) but which was reduced in 9 day resistant cell treated subsequently with trametinib and PIK III compared to drug resistant WM35 cells subsequently treated with trametinib alone (Figure 5-15 A, Bi). However, in drug resistant A375 cells pre-exposed to treatment for 9 days with 16 nM trametinib, CD271 was apparently induced by further treatment with PIK III and which was not reduced by combined additional treatment with PIK III and trametinib (Figure 5-16 A, Bi), perhaps reflected by the highly variable data derived by these experiments and the large error bars incurred.

Treatment of WM35 or A375 cell treated with 16 nM trametinib for 9 days also resulted in both the induction of LC3-II and inhibition of p62 expression (Figure 5-15 and Figure 5-16 A and B ii and iii), which was significant in WM35 cells (One way ANOVA with Tukey's multiple comparison test ** P< 0.01 for LC3-II and **** P< 0.0001 for p62, Figure 5-15 B ii and iii), albeit only significant in case of LC3 II expression in A375 cells (One way ANOVA with Tuckey's multiple comparison test * P< 0.05 for LC3-II and non-significant for p62,Figure 5-16 B ii and iii).

Single agent trametinib and PIK III also appeared to increase pAMPK levels in both 9 day trametinib resistant WM35 and A375 cells (although only significant in drug resistant A375 cells (One way ANOVA with Tukey's multiple comparison test, **P < 0.01, Figure 5-16 B iv). Furthermore, subsequent treatment of both 9 day trametinib resistant WM35 and A375 cells for 24 hrs with combined trametinib and PIK III resulted in further induction of pAMPK (One way ANOVA with Tukey's multiple comparison test, **P < 0.01, Figure 5-15 B iv in case of WM35 cells and **** P<0.0001, Figure 5-16 B iv in case of A375 cells) suggesting that it is possible that autophagy blockade with PIK III in combination with trametinib results in paradoxical feedback signalling further activating AMPK possibly to further induce autophagy

as a survival mechanism. Finally, analysis of PARP cleavage revealed induction in 9 day trametinib-induced drug resistant WM35 and A375 cells following further treatment for 24 hours with single agent trametinib or PIK III which was significantly enhanced by the combination of both agents (One way ANOVA with Tukey's multiple comparison test, ** $P < 0.01$ for WM35 (Figure 5-15 B v) and ****$P < 0.0001$ for A375 (Figure 5-16 B v). Collectively these data suggest that PIK III inhibits trametinib-induced CD271 and autophagy to varying degrees and combination of trametinib and PIK III significantly induces apoptosis in trametinib-induced CD271 expressing melanoma cells.

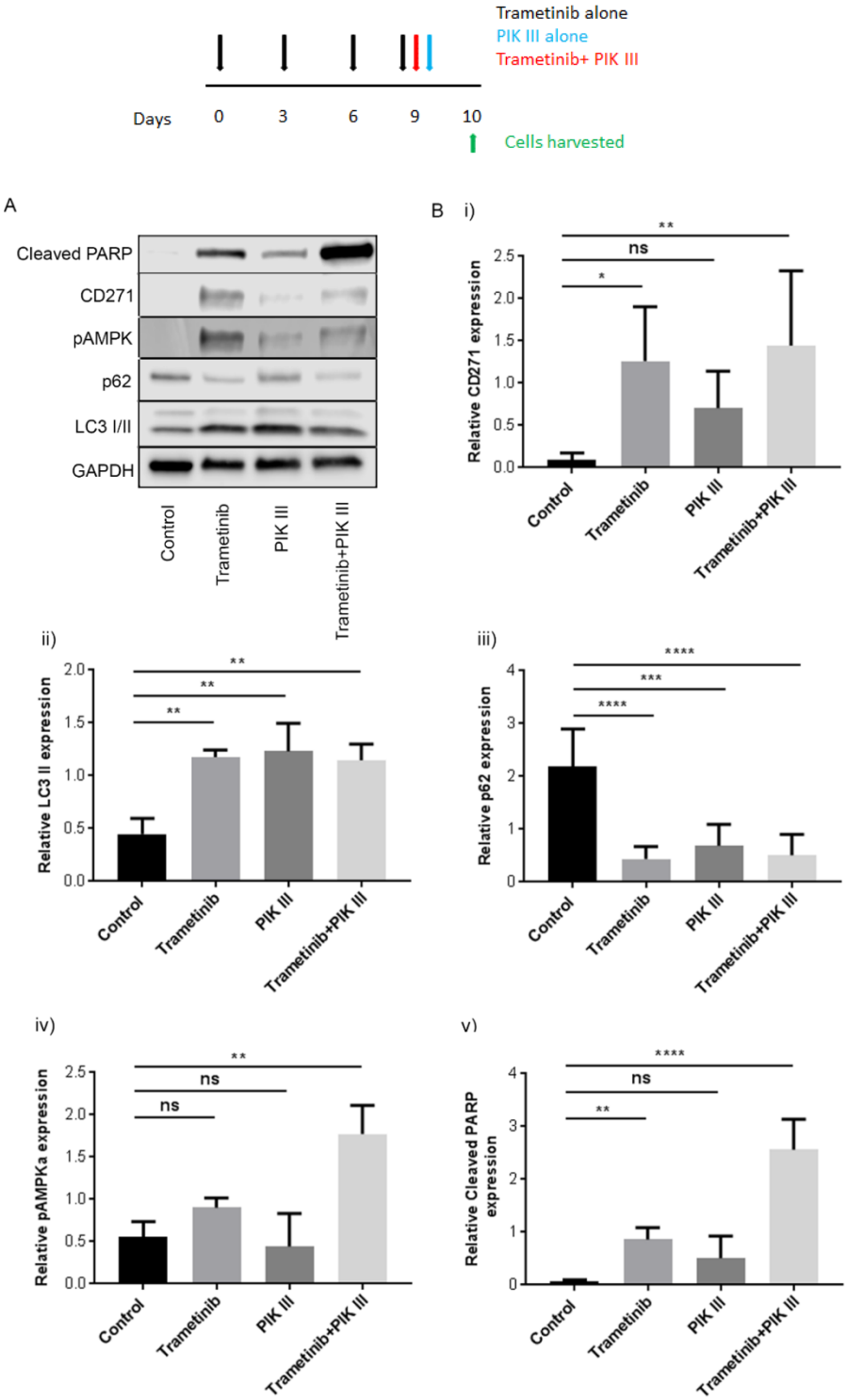


Figure 5-15 PIK III partially inhibits trametinib-induced CD271 and autophagy in WM35 cells
A.Representative immunoblots for the expression of Cleaved PARP (89 kDa), CD271 (45 kDa), p62 (62 kDa), LC3 I/II (16, 18 kDa) or GAPDH loading control (38 kDa) in WM35 cells pre-treated for 9 days with 16 nM trametinib and treated subsequently for 24 hrs with vehicle DMSO control (control), 16 nM trametinib, 5 μM PIK III (PIK III) or combined trametinib and PIK III (Trametinib +PIK III). B. Relative CD271 (i), LC3 II (ii), p62 (iii), pAMPK (iv) or Cleaved PARP (v) expression relative to GAPDH in 3 individual experiments. Each bar is the mean or 3 experiments ± SD (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001)

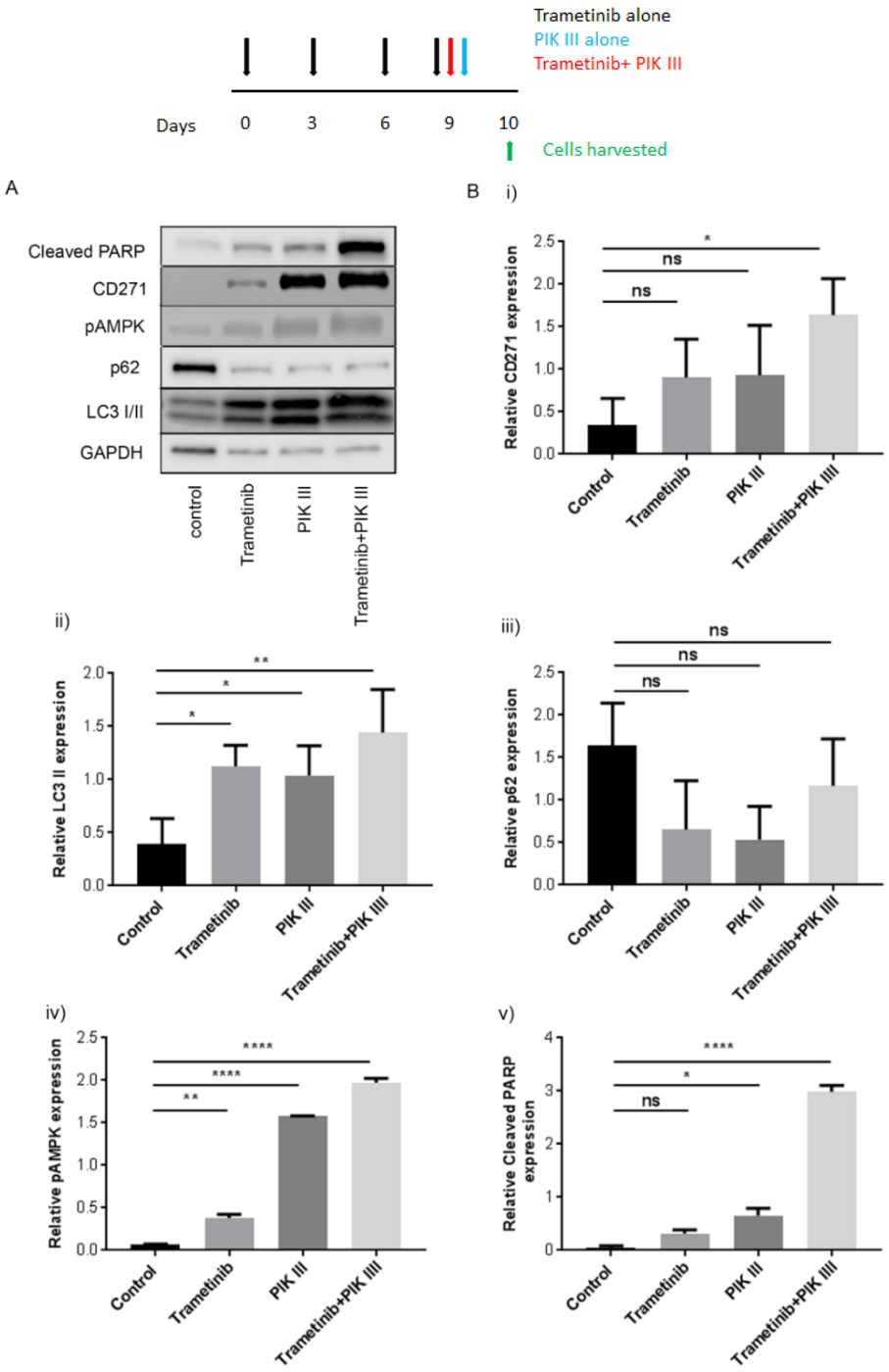


Figure 5-16: Combination of trametinib with PIK III increases CD271 expression, increases autophagic activity, pAMPK and apoptotic cell death in trametinib treated A375 cells

A.Representative immunoblots for the expression of Cleaved PARP (89 kDa), CD271 (45 kDa), p62 (62 kDa), LC3 I/II (16, 18 kDa) or GAPDH loading control (38 kDa) in A375 cells pre-treated for 9 days with 16 nM trametinib and treated subsequently for 24 hrs with vehicle DMSO control (control), 16 nM trametinib, 5 μM PIK III (PIK III) or combined trametinib and PIK III (Trametinib +PIK III). B. Relative CD271 (i), LC3 II (ii), p62 (iii), pAMPK (iv) or Cleaved PARP (v) expression relative to GAPDH in 3 individual experiments. Each bar is the mean or 3 experiments ± SD (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001)

To further investigate the effect of Vps34 inhibition on the spheroid expansion of trametinib-induced drug resistant melanoma cells *in vitro*, A375 cells subjected to long term exposure to 16 nM trametinib for 42 days were incorporated into collagen gels further treated in the presence or absence of 16 nM trametinib, 5 µM PIK III or combined of trametinib for up to an additional 7 days. In contrast to results derived from studies of long term trametinib-resistant A375 cells treated with chloroquine, however, these studies revealed no significant effect of PIK III alone or in combination with trametinib on relative spheroid size *in vitro,* (Two-way ANOVA with Sidak post hoc correlation, NS P>0.05, (Figure 5-17A, B).

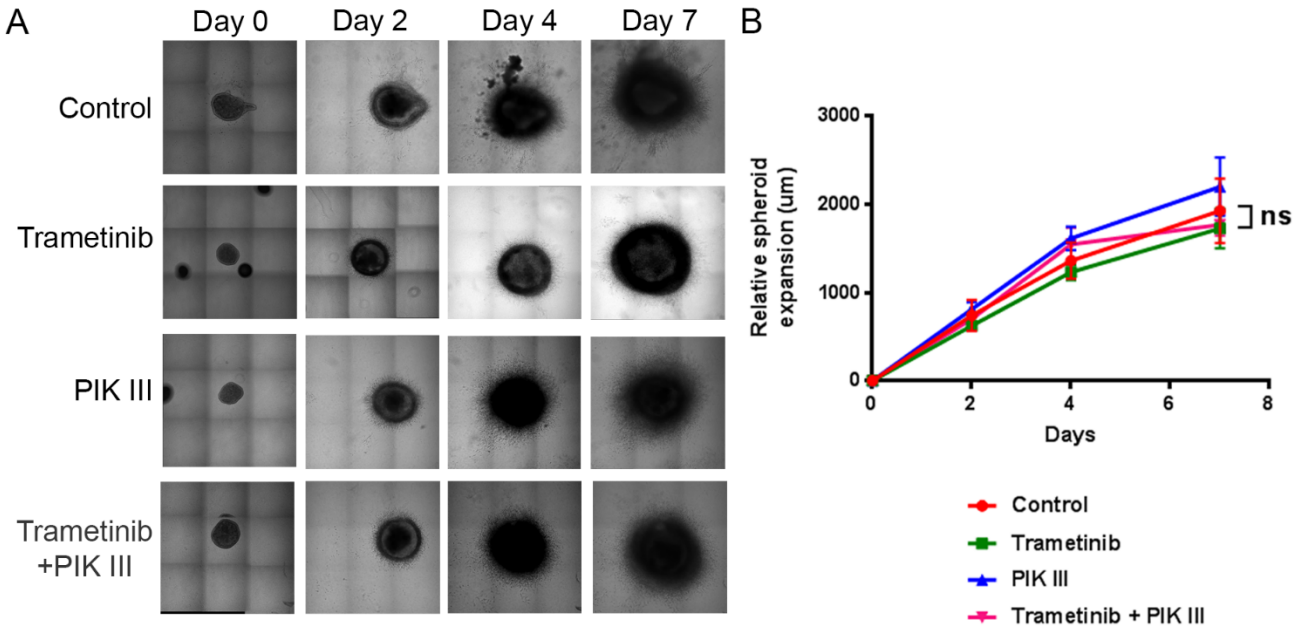


Figure 5-17 PIK III does not inhibit spheroid expansion of A375 cells subjected to prolonged trametinib-induced drug resistance

A.Representative bright field images of A375 spheres pre-treated with 16 nM trametinib for 42 days followed by incorporation into collagen gels and subsequent treatment for up to 7 days with DMSO vehicle control, 16 nM trametinib (trametinib), 5 µM PIK III (PIK III) or combined trametinib and PIK III (Trametinib+PIK III). Scale bar 100 µm. B. Relative spheroid size (in µm) of A375 cells pre-treated for 42 days with 16 nM trametinib and treated subsequently for 0, 2 , 4 or 7 days with DMSO vehicle control, 16 nM trametinib, 5 µM PIK III or combined trametinib and PIK III (Trametinib+PIK III). Each point is the mean three independent replicate experiments ± SD expressed relative to the mean initial spheroid size (0 day time point of 42 day trametinib treated A375 cells).

5.2.5 Combined MEK and Vps34 inhibition inhibits melanoma dissemination *in vivo*

5.2.5.1 Optimization of an in vivo zebrafish model of human melanoma systemic metastasis

To evaluate the invasive capacity of trametinib-induced drug resistant CD271 expressing melanoma subpopulations *in vivo* and the effect of autophagy inhibition on tumour dissemination, a zebrafish model of human metastatic melanoma was established in collaboration with Dr David Hill (Institute of Cellular Medicine, Newcastle University) and Dr Bill Chaudhry (Institute of Genetic Medicine, Newcastle University, see chapter 2). Initial experiments were executed to determine optimal cell growth and temperature conditions for zebrafish embryos inoculated with human A375 and WM35 cells.

To establish optimal temperature and environmental oxygen conditions for the growth of both human melanoma cells lines and the transparent transgenic Casper *(flk1:GFP) tagged* zebrafish embryo development, both zebrafish and human melanoma cell lines were first cultured at different temperatures and under differing oxygen content before evaluating the effects on melanoma cell viability and effect on the growth of zebrafish embryos. Results demonstrated that both hypoxia (5% O_2) and lower temperatures (28 and 33^{o}C) resulted in reduced number of both WM35 and A375 (Figure 5-18A and B) with the most profound effect seen in cells grown at 28^{o}C (Figure 5-18A, B, 28^{o}C, 5%O_2) effect which is likely due to reduced melanoma cell proliferation.

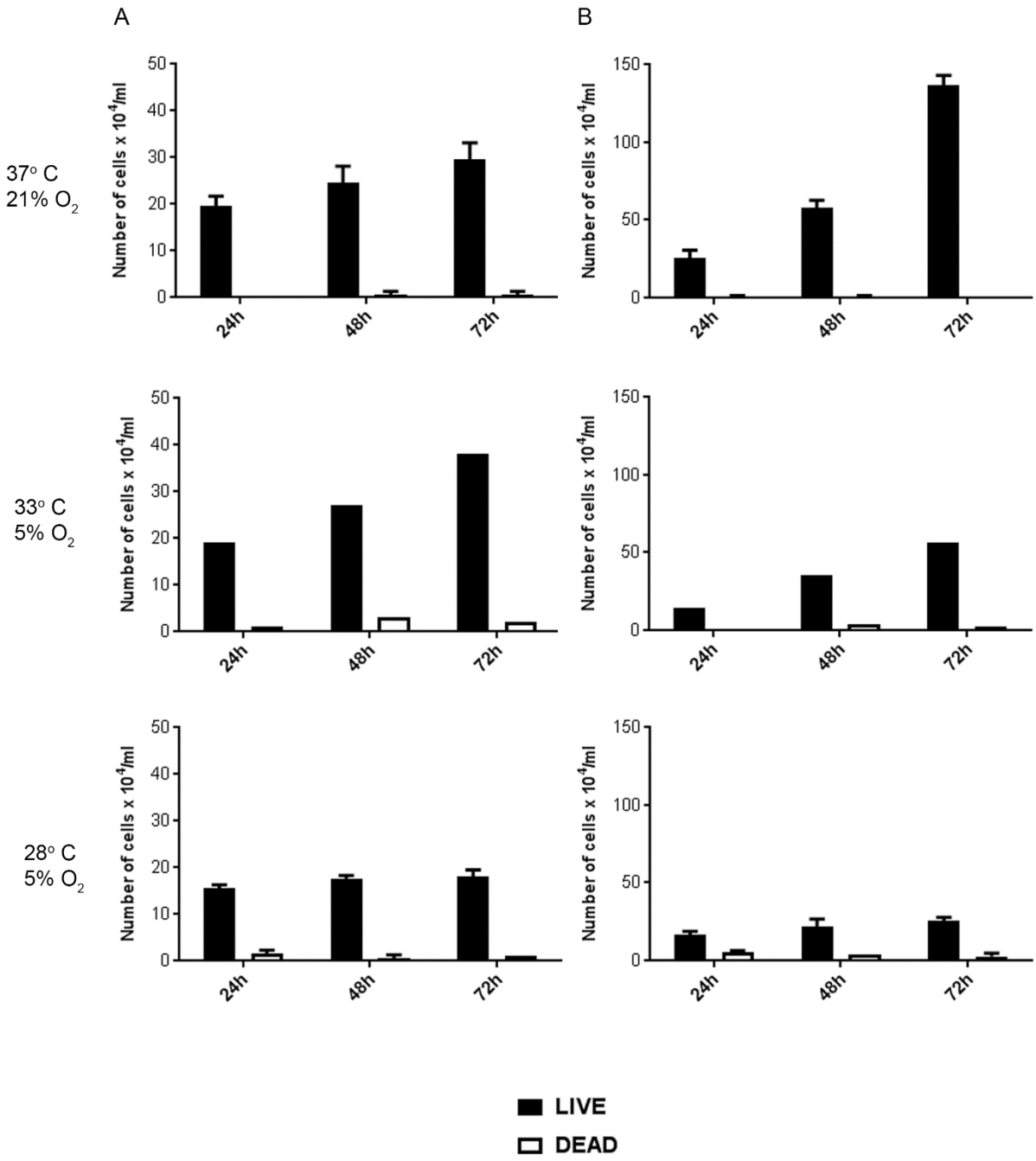


Figure 5-18: Hypoxia and low cell culture temperature reduces number of melanoma cells
Number of live (black bar) or dead (white bar) WM35 (A) or A375 (B) cells X 10^4/ml following culture for 24, 48 or 72 hrs in normal cell culture conditions (37°C, 21% O_2) or hypoxic/lower cell culture temperature conditions (28°C, 5% O_2 or 33°C, 5% O_2). Each bar is the mean ±SD of two replicate experiments in all conditions apart from both WM35 and A375 cells cultured at 33°C and 5% O_2 in which the represented bar is the result of a single experiment.

Normal conditions for the growth of the zebrafish embryos over 5 days are 28°C, in 21% O_2 and 0.04% CO_2 (Figure 5-19) but since melanoma cells proliferated more effectively at higher temperatures of 37°C (Figure 5-18) additional experiments were performed to investigate the effect of differing temperatures on the development of zebrafish embryos. Results demonstrated physiological and morphological anomalies in zebrafish embryos that were maintained at 37°C and either 5 or 10% O_2 (Figure 5-20). In particular Zebrafish embryos developed pericardial effusions when grown at higher temperatures (Figure 5-20) and hence all subsequent experiments of Zebrafish embryos inoculated with A375 or WM35 cells were performed in zebra fish maintained at 33°C in 5% O_2.

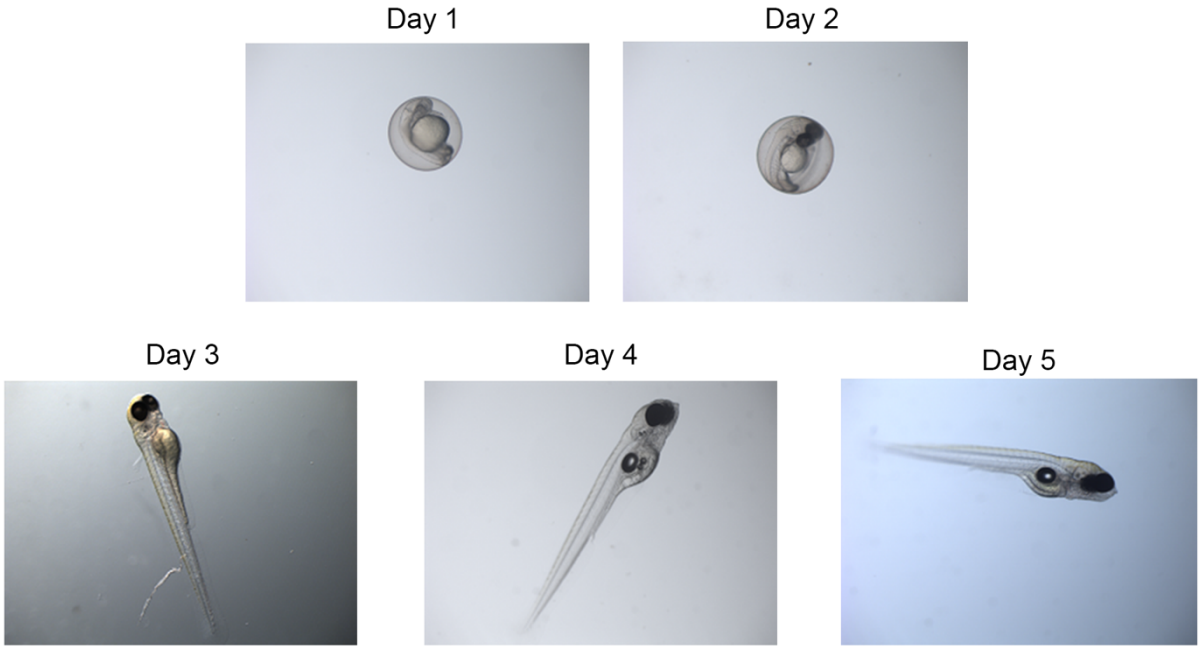


Figure 5-19: Normal development of zebrafish larvae from day 1 to day 5 post fertilization
Representative bright field images of normal development of zebrafish larvae from day 1 to day 5 post fertilization.

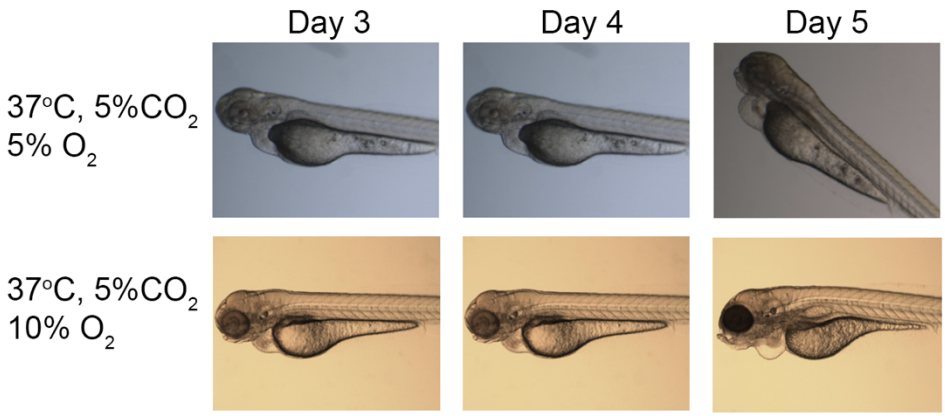


Figure 5-20: Hypoxia and high temperature leads to pericardial effusion of zebrafish embryos

Representative bright field images of day 3, 4 or 5 post fertilization zebrafish embryos following growth at 37°C and either 5 or 10% O_2 leading to pericardial effusion and abnormal zebrafish growth.

5.2.5.2 PIK III inhibits the dissemination and promotes cell death of trametinib-induced resistant A375 cells in vivo

To investigate the effect of autophagy inhibition with PIK III on the invasion of trametinib-induced resistant melanoma cells in vivo, dil (red) labelled A375 cells continually pre exposed for 42 days to 16nM trametinib were inoculated into the yolk sac of 2 day zebra fish embryos subsequently maintained in E3 media at 33^{o}C in 5% O_2 in the presence or absence of 16 nM trametinib, 5 μM PIK III or combined trametinib and PIK III for 72 hours (experiments were performed in collaboration with Dr David Hill, Institute of Cellular Medicine, Newcastle university). Real time confocal microscopy analysis revealed tumour cell dissemination in the yolk sac and tail of zebrafish inoculated with control 42 day trametinib-induced drug resistant A375 cells (Figure 5-21 A i) and ii), which was partially inhibited following further exposure to 16nM (Figure 5-21 B i) and ii)) and by the additional treatment with single agent PIK III (Figure 5-21 C i) and ii)). However, treatment of zebrafish bearing 42 day trametinib-induced dry resistant A375 cells with combined trametinib and PIK III, resulted not only in reduced tumour cell migration and invasion, but also in reduced tumour cell viability and cell death (Figure 5-21 D i) and ii), as evidenced by the leakage of RFP fluorescent dye into the yolk sac. Collectively these data indicate inhibition of autophagy by Vps34 inhibition may present a viable means through which to re-sensitize MEK inhibitor-resistant metastatic melanoma cells to the cytotoxic effects of trametinib. However, as discussed in chapter 4, long term exposure to trametinib leads to a transient induction of CD271 expressing melanoma subpopulations and A375 cells that have been exposed to trametinib for 42 days have lost the expression of CD271. Future experiments to investigate the relationship between autophagy inhibition and CD271 associated tumour cell invasion *in vivo* will focus on cells that have been treated in the presence of the MEK inhibitor for 9 days and at which time point CD271 expression peaks.

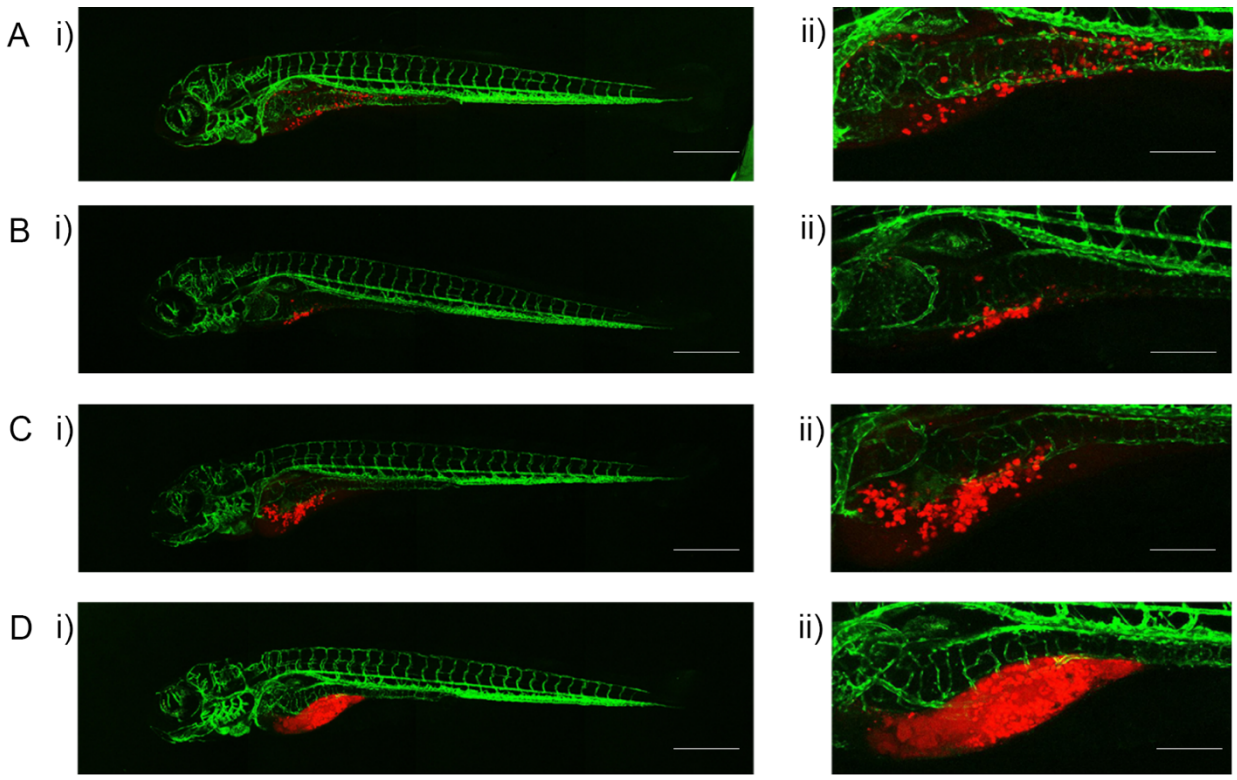


Figure 5-21: PIK III inhibits invasion and re-sensitizes drug resistant A375 cells to the cytotoxic effects of trametinib.

Representative immunofluorescence images of 5 day post fertilization zebrafish embryos injected with A375 cells pre exposed to 16 nM trametinib for 42 days (red cells, A) and subsequently maintained in the presence of 16nM trametinib (B), 5 µM PIK III alone (C) or combined trametinib and PIK III. A, B, C, Di) × 10 magnification, A, B, C, Dii) x 20 magnification. Scale bar 100 µm

5.2.6 Induction of cytotoxic autophagy with THC increases cell death of trametinib-resistant CD271 expressing melanoma subpopulations *in vitro*

Previous studies in the Lovat lab have highlighted the ability of the synthetic cannabinoid derivative ∆(9)-tetrahydrocannabinol (THC) to promote cytotoxic autophagy of metastatic melanoma cells (Armstrong *et al.*, 2015b). To evaluate the potential of this strategy as a means through which to promote autophagy-mediated death of trametinib-induced CD271 expressing drug resistant melanoma cells, A375 and WM35 cells previously treated with 16 nM trametinib for 9 days were further treated with 4.5 μM THC for 24 hours prior to evaluating the effect on cell viability, CD271 expression and the expression of LC3 I/II and p62 and cleaved PARP.

Results demonstrated THC induced the inhibition of both WM35 and A375 cells pre-treated with trametinib for 9 days (Figure 5-22). Furthermore THC also significantly inhibited the cell viability of 9 day trametinib-induced drug resistant WM35 and A375 cells subsequently re-exposed to trametinib (One way ANOVA with Tukey's multiple comparison test, *P < 0.05 in WM35 and **P<0.01 in A375 cells, (Figure 5-22 A and B).

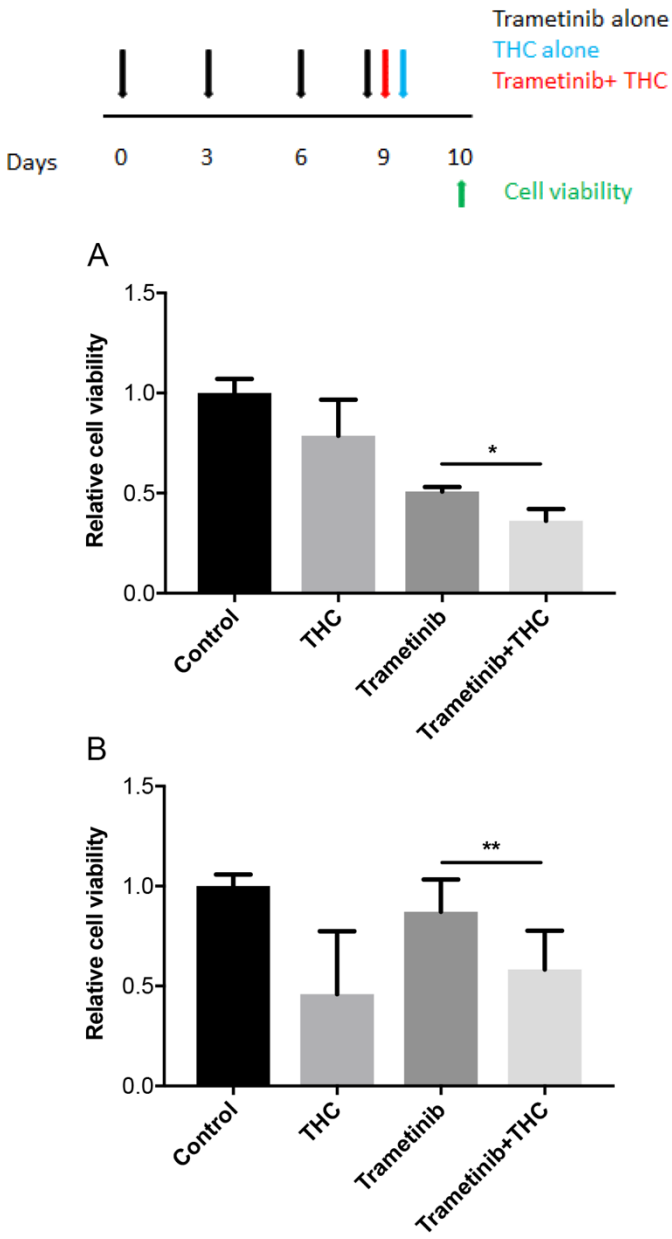


Figure 5-22: THC re-sensitizes trametinib-induced resistant WM35 and A375 cells to trametinib-induced inhibition of cell viability.

Relative cell viability of WM35 (A) and A375 (B) cells pre-treated for 9 days with 16nM trametinib and treated subsequently for 24 hrs with vehicle control (control), 4.5 µM THC (THC) ,16nM trametinib (Trametinib), or combined THC and trametinib (Trametinib+THC). Each bar is the mean +/- SD of 3 independent experiments each containing 4 technical replicates and expressed relative to control (9 day trametinib treated cells). *P <0.05. **P < 0.01.

To investigate the effect of THC on CD271 expression and to confirm THC-induced autophagy and cell death of trametinib-induced drug resistant melanoma cells, the expression of CD271, pAMPK, LC3 I/II, p62 and cleaved PARP was also determined in WM35 (Figure 5-23) and A375 (Figure 5-24) cells initially treated with 16 nM trametinib for 9 days and further treated for 24 hours with 16 nM trametinib, 4.5 μM THC or combined THC and trametinib by western blotting. Results demonstrated trametinib and THC (alone and in combination)-induced expression of CD271 which was not significant in case of WM35 cells (One way ANOVA with Tukey's multiple comparison test, Figure 5-23 A and Bi), however significant in case of A375 cells (One way ANOVA with Tukey's multiple comparison test, *P<0.05, **P<0.01), (Figure 5-24 A and Bi) which was paralleled by a significant increase in LC3 II and significant decrease in reduction in p62 expression (One way ANOVA with Tukey's multiple comparison test, **P<0.01 for LC3 II and ****P<0.0001 for p62 in WM35 cells Figure 5-23 A, B ii and iii, One way ANOVA with Tukey's multiple comparison test,*P<0.05 for LC3 II, ****P<0.0001 for p62 in A375 cells Figure 5-24 A, B ii and B iii).

Interestingly, pAMPK expression increased in both 9 day trametinib resistant WM35 and A375 cells following re treatment with trametinib (Figure 5-23 A and B iv, Figure 5-24 A and B iv) as well as in both drug resistant cell lines treated with THC, with the further induction of pAMPK by combined trametinib and THC treatment, at least in drug resistant A375 cells (one way ANOVA with Tukey's post hoc correlation, **P<0.001, Figure 5-24 B iv). Results also demonstrated the significant induction of PARP cleavage in drug resistant WM35 and A375 cells following re exposure to trametinib in the presence of THC (one way ANOVA test with Tukey's post hoc correlation, ****P<0.0001 for WM35, Figure 5-23 B v and ** P< 0.01 for A375 cells, Figure 5-24 B v).

Taken together these data indicate that exacerbation of autophagy with THC is an effective means through which to promote cytotoxic autophagy in trametinib-induced drug resistant melanoma cells.

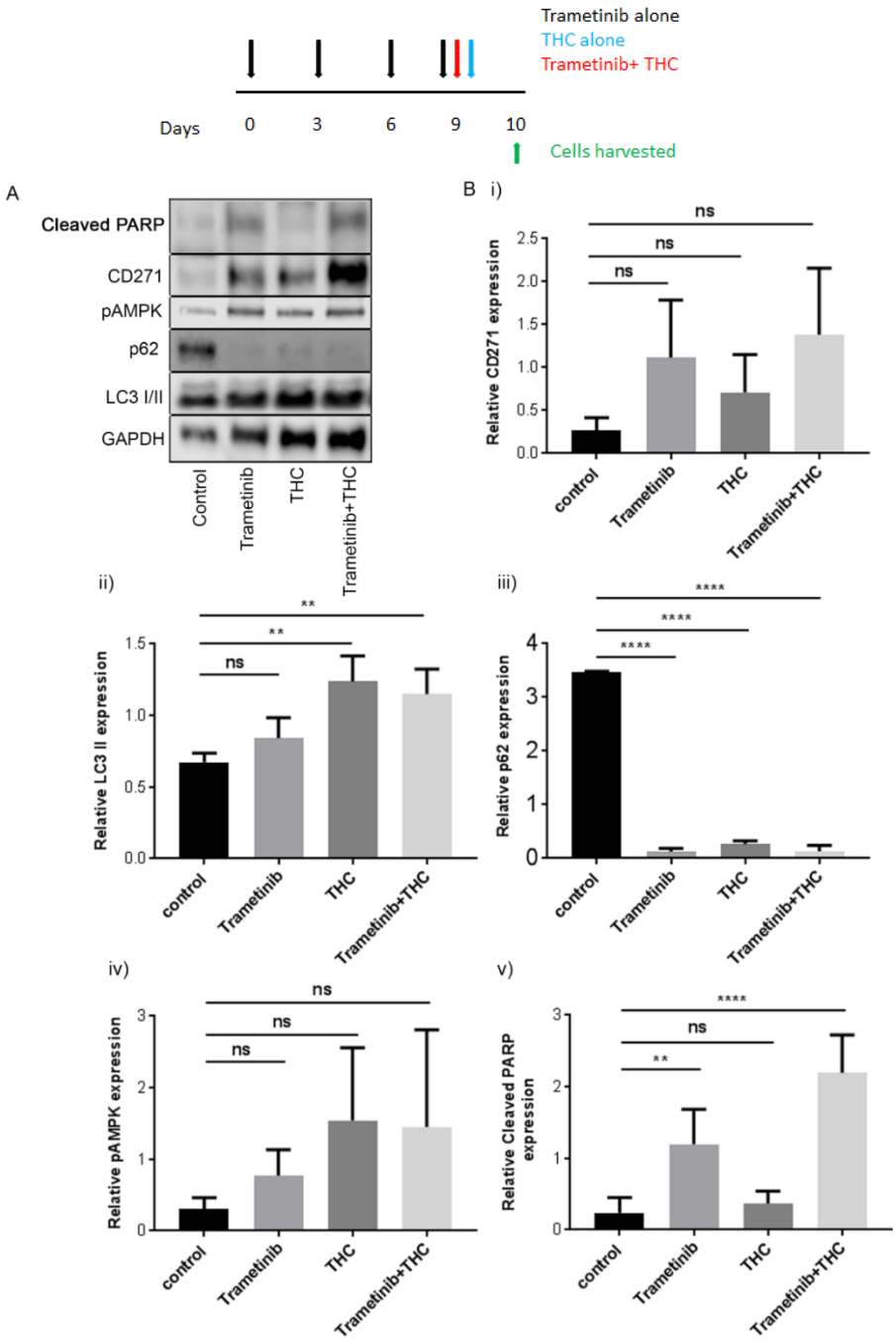


Figure 5-23: Treatment with combination of THC and trametinib increases CD271 expression, autophagy induction and apoptosis in trametinib resistant CD271 expressing WM35 cells

A.Representative immunoblots for the expression of Cleaved PARP (89 kDa), CD271 (45 kDa), pAMPK (62 kDa), p62 (62 kDa), LC3 I/II (16, 18 kDa) or GAPDH loading control (38 kDa) in WM35 cells pre-treated for 9 days with 16 nM trametinib and treated subsequently for 24 hours with vehicle DMSO control (control), 16 nM trametinib, 4.5 µM THC (THC) or combined trametinib and THC (Trametinib+THC). B. Relative CD271 (i), LC3 II (ii), p62 (iii), pAMPK (iv) or cleaved PARP (v) expression levels relative to GAPDH in 3 individual experiments. Each bar is the mean or 3 experiments ± SD (**P<0.01, ***P<0.001, ****P<0.0001).

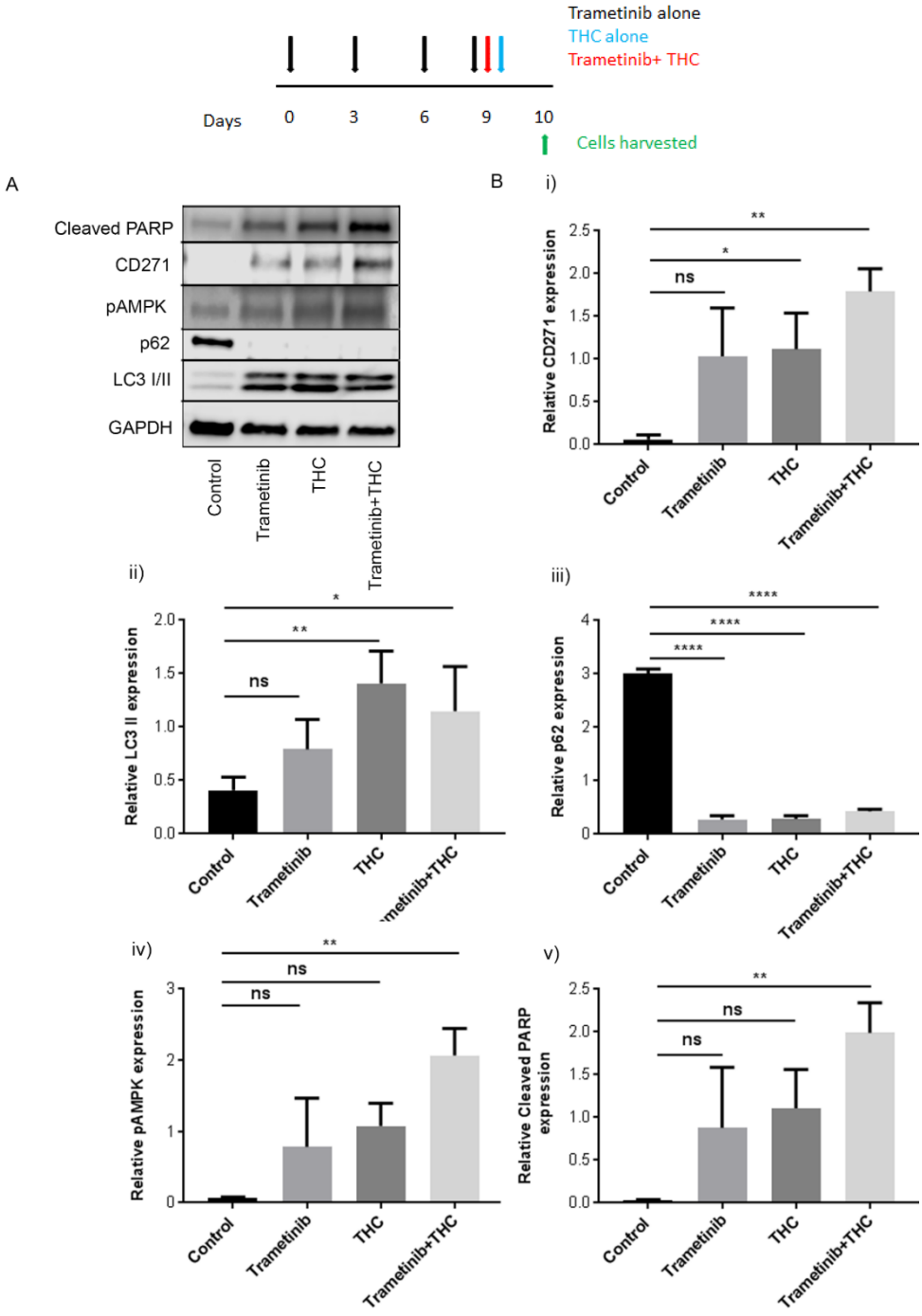


Figure 5-24: Treatment with combination of THC and trametinib increases CD271 expression, autophagy induction and apoptosis in trametinib resistant CD271 expressing A375 cells

A.Representative immunoblots for the expression of Cleaved PARP (89 kDa), CD271 (45 kDa), pAMPK (62 kDa), p62 (62 kDa), LC3 I/II (16, 18 kDa) or GAPDH loading control (38 kDa) in A375 cells pre- treated for 9 days with 16 nM trametinib and treated subsequently for 24 hours with vehicle DMSO control (control), 16 nM trametinib, 4.5 µM THC (THC) or combined trametinib and THC (Trametinib+THC). B. Relative CD271 (i), LC3 II (ii), p62 (iii), pAMPK (iv) or cleaved PARP (v) expression levels relative to GAPDH in 3 individual experiments. Each bar is the mean or 3 experiments ± SD (**P<0.01, ***P<0.001, ****P<0.0001).

To evaluate whether the combination treatment with trametinib and THC also reduced the cell viability of melanoma cells exposed to prolonged treatment with trametinib, control A375 cells or A375 cells pre-treated with trametinib for 42 days were further treated for 24 hours with either with 16 nM trametinib, 4.5 μM THC or combined trametinib and THC prior to assessing cell viability. Results demonstrated THC induced the significant reduction in cell viability of both A375 naïve and long term trametinib resistant cells (

Figure 5-25 A, B) (One way ANOVA with Tukey's post hoc correlation, ****P<0.0001).

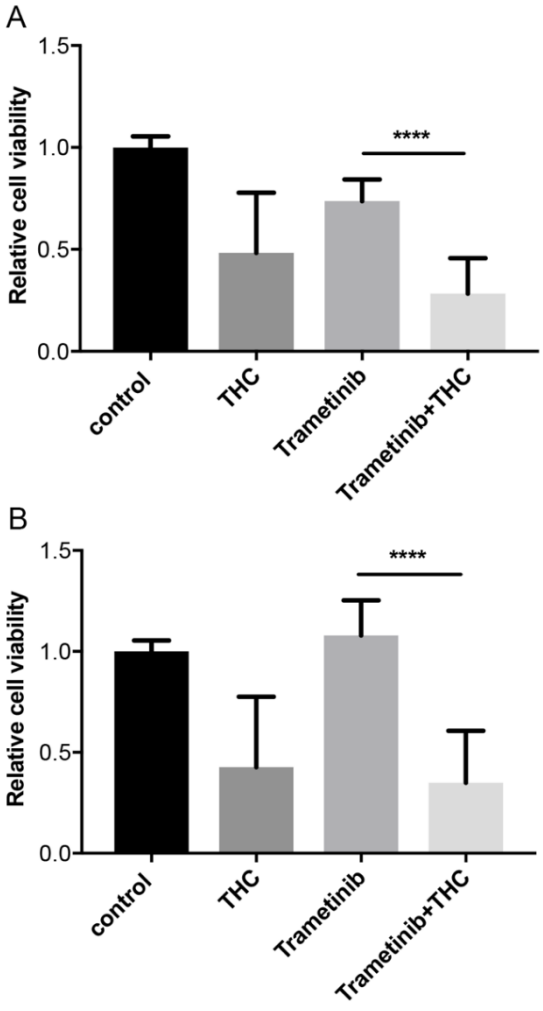


Figure 5-25: THC promotes the inhibition of cell viability of A375 cells exposed to pronged treatment with trametinib

Relative cell viability of trametinib naive A375 (A) or (B) A375 cells pre-treated for 42 days with 16 nM trametinib resistant A375 (B) cells following treatment for 24 hrs with DMSO vehicle control, 4.5 μM THC (THC) ,16 nM trametinib (Trametinib), or combined THC and trametinib (Trametinib+THC). Each bar is the mean +/- SD of 3 independent experiments each containing 4 replicate treatment conditions. ****P < 0.0001

5.3 **Discussion**

Data from the previous chapter implies drug-induced CD271 expression and specifically MEK inhibitor-induced CD271 expression appears to be intimately related to the induction of autophagy which plays a key role in the survival of such subpopulations, leading to the hypotheses tested in the present chapter that inhibition of CD271 directly, the inhibition of pAMPK or autophagy modulation may represent potential therapeutic strategies through which to overcome the resistance of BRAF/NRAS metastatic melanoma to MEK inhibitor targeted therapy with trametinib.

5.3.1 CD271 inhibition results in reduction of cell viability, inhibition of pAMPK and autophagy but increase in spheroid size *in vitro* of trametinib-induced CD271 expressing melanoma cells

The potential to target cancer stem cell populations with some success has been shown in several cancer types (Wang *et al.*, 2010) including the use of antibodies targeting the cancer stem cell surface marker CD47 in acute lymphoblastic leukaemia (Chao *et al.*, 2011) or antibodies against CD133 in glioblastoma (Wang *et al.*, 2011). However, data in the present study to not support CD271 as a true stem cell marker and coupled with the heterogeneity of melanoma, the transient nature of CD271 expression induced by drug resistance and the likely contribution of micro environmental factors to tumour cell survival, targeting CD271 in the context as a potential cancer stem cell marker may not be the best approach. Nevertheless given the recent emergence of small molecule inhibitors to CD271 (Niederhauser *et al.*, 2000; Mochizuki *et al.*, 2016) and results from the present study showing knockdown of CD271 in trametinib-induced drug resistant melanoma subpopulations re-sensitized them to the cytotoxic effects of MEK inhibition, it may be that chemical inhibition of CD271 may present a viable strategy.

Trametinib-induced CD271 expressing subpopulations appear to be associated with the development of early resistance to the MEK inhibitor trametinib. Targeting CD271 at the critical point of development of early resistance may therefore provide a viable strategy

through which to prevent the establishment of trametinib-induced resistance. Supporting this hypothesis, data revealed the treatment of early (9 days) trametinib-induced CD271 expressing drug resistant melanoma cells with specific CD271 inhibitors (TAT-PEP5 and RO-08) resulted in significant inhibition of cell viability, paralleled by the reduced expression of CD271, and pAMPK as well as autophagy.

Data showing reduced autophagic activity of drug-resistant melanoma cells in response to treatment with CD271 inhibitors suggests a link between autophagy activation and CD271 expression. However, further work will be required to determine whether there is a direct causal relationship between CD271 and autophagy as alternatively drug-resistant melanoma cells may activate autophagy independently of CD271 as a parallel pro-survival mechanism in response to metabolic stress (Degenhardt *et al.*, 2006; Karantza-Wadsworth *et al.*, 2007) or to resist apoptosis (Pattingre *et al.*, 2005). Alternatively autophagy may be activated in response to the changing tumour microenvironment, in response to hypoxia or nutrient deprivation (Ravindran Menon *et al.*, 2014; Yang *et al.*, 2015d) which occurs in solid tumours such as melanoma. Autophagy is also reportedly induced in response to treatment with targeted therapies for example in colorectal cancer following treatment with BRAF inhibitors (Goulielmaki *et al.*, 2016).

Data also revealed inhibition of CD271 resulted in the significant inhibition of spheroid expansion of trametinib-induced drug resistant subpopulations compared to the wild-type WM35 cells. As well as an increase in cell death of CD271 positive populations, it is possible that the decreased function and expression of CD271 in trametinib-induced CD271 expressing subpopulations mediated by the CD271 specific inhibitors may also have resulted in an increase in CD271 negative subpopulations that are inherently more invasive, as highlighted in chapters 3 and 4 and in line with the current literature indicating CD271 negative melanoma subpopulation exhibit a more invasive phenotype (Saltari *et al.*, 2016). It is perhaps therefore not surprising that by reducing the number of CD271 positive tumour cells, the equilibrium between CD271 positive and negative subpopulations is distorted, allowing the favoured emergence of CD271 negative melanoma cells with a more invasive phenotype.

Targeting CD271 at the point of development of early resistance may thus eliminate CD271 positive drug resistant populations but at the risk of increasing CD271 negative subpopulations and therefore contribute to rather than inhibit tumour progression. It is therefore important to evaluate the efficiency of targeting the survival mechanism these resistant subpopulations use to promote tumour survival, which is the case of trametinib-induced CD271 associated cells is likely autophagy. Evidence from other cancer types highlight the alternative approach of targeting cancer stem cell survival mechanisms such as the Wnt, Notch and Hedgehog pathway (Takebe *et al.*, 2015) and the ability of cancer stem cell populations to resist apoptosis (Signore *et al.*, 2013). Since autophagy has been shown to contribute to other cancer stem cell survival (Song *et al.*, 2013; Yang *et al.*, 2015a; Yang *et al.*, 2015c) and the current study highlights the contribution of autophagy to the survival of drug-induced CD271 expressing melanoma subpopulations, it is not surprising that autophagy blockade reduced the cell viability of CD271 expressing subpopulations. What would be of interest, however, would be to investigate the effect of concomitant autophagy and CD271 blockade in trametinib-induced CD271 cell invasion and survival.

5.3.2 AMPK inhibition in trametinib resistant BRAF mutant melanoma cells increases autophagic activity

The AMPK pathway is important in regulating metabolic homeostasis and maintaining cellular energy supply by activating pathways that generate ATP such as glucose metabolism and fatty acid oxidation (Li *et al.*, 2015b).However the direct link between AMPK activation in cancer is not fully established. Recent evidence suggests that AMPK activation is associated with a reduction in tumour growth *in vitro* mediated through activation of tumour suppressor pathways such as the Hippo pathway (Wang *et al.*, 2015) or cell cycle regulation (Lee *et al.*, 2007b). Interestingly, AMPK can also act as a tumour promoter by enhancing cellular ability to survive conditions of metabolic stress such as nutrient deprivation and hypoxia (Zaugg *et al.*, 2011) or by activating autophagy (Egan et al., 2011). AMPK is known to activate autophagy by phosphorylation of ULK 1 (Egan *et al.*, 2011; Kim *et al.*, 2011b) but also inactivation of

mTORCH1 (Wullschleger et al., 2006). In the context of trametinib-induced CD271 expressing drug resistant melanoma populations, results demonstrated a reduction in ATP release accompanied by an increase in pAMPK. Treatment of control or trametinib-induced drug resistant WM35 and A375 cells with the cell-permeable AMPK inhibitor, Compound C (Liu et al., 2014) also resulted in efficient AMPK inhibition but had no significant effect on CD271 expression. Nevertheless, despite the anticipated reduction in autophagic activity, an increase in LC3 II expression was observed. A possible explanation for this observation may be related to the nature of compound C, which is not a specific AMPK inhibitor (Liu *et al.*, 2014). Several studies have reported compound C to inhibit various biological cascades independently of AMPK inhibition such as the inhibition of the hypoxic activation of HIF-1 (Emerling *et al.*, 2007) or increasing p21 levels (Nam et al., 2008), indicating that compound C may have off target effects that could lead to the activation of autophagy. Interestingly recent data have also shown compound C mediates an AMPK-inhibition independent increase in autophagy in human glioma cells, though blockade of the Akt/mTOR pathway (Vucicevic et al., 2011) further reinforcing the complex interaction of the AMPK pathway and autophagy and highlighting the complexities of attempting to target AMPK for the therapeutic benefit of melanoma.

5.3.3 Inhibition of autophagy or the promotion of cytotoxic autophagy promotes apoptosis of trametinib-induced drug resistant CD271 expressing melanoma cells

The paradoxical activation of autophagy or its activation by many chemotherapeutics is a well-known phenomenon linked to chemo resistance (Kanzawa et al., 2004) including the resistance to targeted agents (Eritja et al., 2017) of many tumour types and its use by specific chemo resistant tumour cell subpopulations to escape the cytotoxic effects of chemotherapy (Brigger et al., 2015). Indeed studies have shown that increased autophagy in melanoma is associated with decreased survival (Ma et al., 2011), and that basal autophagy is increased in BRAF mutant melanoma cells (Corazzari et al., 2015) as well as being induced by temsirolimus

(Xie et al., 2013) and collectively suggesting the inhibition of this process may represent a viable means through which to overcome trametinib-induced drug resistance.

Inhibition of autophagy has been the subject of many studies, most frequently employing the lysosomal inhibitor chloroquine, in both clinical (Rangwala *et al.*, 2014b) and pre-clinical setting (Liang *et al.*, 2016), and has shown to act synergistically to chemotherapeutic agents to promote cell death (Schmukler *et al.*, 2014). Promising phase II clinical trial results in patients with advanced pancreatic cancer using single agent hydroxychloroquine demonstrate a possible therapeutic advantage in patients treated with hydroxychloroquine compared to the control group (Wolpin *et al.*, 2014). However, more promising early clinical trial outcomes derive from studies in which chloroquine or hydroxychloroquine are combined with standard chemotherapy or radiation therapy (Sotelo *et al.*, 2006). A clinical trial in glioblastoma has demonstrated a possible advantage in combining hydroxychloroquine to temozolomide and radiation therapy (Rosenfeld *et al.*, 2014), however, the poor tolerability of higher doses of hydroxychloroquine highlight the need for more specific autophagy inhibitors for clinical use. In melanoma, pre-clinical data combining temsirolimus with hydroxychloroquine has demonstrated that inhibition of the mTOR and autophagy pathways promotes apoptosis and could be a new therapeutic approach for the treatment of melanoma (Xie et al., 2013) and there are ongoing clinical trials in melanoma combining mTOR and autophagy inhibitors in melanoma (Rangwala *et al.*, 2014a).

The current study demonstrated that autophagy inhibition with chloroquine in trametinib-induced CD271 expressing melanoma subpopulations results in increased cell death and induction of apoptosis, evidenced by increase in cleaved PARP expression, therefore supporting the potential clinical utility of treatment with chloroquine as a means through which to prevent the induction of pro-survival autophagy and increase apoptosis of trametinib-induced CD271 expressing drug-resistant sub populations.

Nevertheless autophagy inhibition with chloroquine or hydroxychloroquine may not present the most effective clinical approach through which to inhibit pro-survival autophagy. Evidence suggests some tumours more sensitive than others (Bristol et al., 2013) and that it sensitizes normal cells to the cytotoxic effects of chemotherapeutics (Kimura *et al.*, 2013). In addition,

pharmacokinetic studies suggest clinically achievable concentrations of hydroxychloroquine may not be as effective at inhibiting autophagy *in vivo* as *in vitro* (Bristol *et al.*, 2013). Coupled with the fact that pre-clinical models do not clarify the impact on the immune system, likely important for effectiveness, and the fact that chloroquine is not a specific inhibitor of autophagy, these data have led to the investigation of more specific inhibitors of autophagy such as those against Vps34, currently in clinical trials in combination with mTOR inhibitors for patients with renal cell carcinoma (Pasquier, 2015). These novel molecules specifically inhibit autophagy at early stages, making those inhibitors a promising alternative treatment approach to chloroquine (Ronan et al., 2014)

In the present study treatment of trametinib-induced resistant melanoma subpopulations with Vps34 inhibitor PIK III, resulted in significant reduction of cell viability and increased apoptosis of such populations both *in vitro* and *in vivo*. However, although *in vitro*, chloroquine in combination with trametinib significantly reduced the spheroid size of drug resistant A375 cells pre-exposed clinically achievable concentrations of trametinib for 42 days, Vps34 inhibition with PIK III alone or in combination with trametinib did significantly affect spheroid expansion *in vitro*. Such findings may indicate that the utilised dose of PIK III may not have been high enough to diffuse through the collagen to have a significant effect on spheroid expansion *in vitro*, and suggests further dose response studies of the Vps34 inhibitor are warranted. Alternatively, the effects derived by chloroquine, may not have been specific and may have resulted from off target-autophagy independent mechanisms such as direct effect on tumour vasculature independent of autophagy (Rubinsztein *et al.*, 2012; Maes *et al.*, 2016) or by modulating immune-mediated response (Townsend *et al.*, 2012). Nevertheless, the autophagy-independent effects of chloroquine on tumour cells and tumour microenvironment remain to be fully elucidated. In contrast however, studies of Vps34 inhibition trametinib-induced drug resistant melanoma cells *in vivo* demonstrated the combined exposure of zebra fish xenografts bearing trametinib-induced drug resistant melanoma cells to PIK III and trametinib resulted in reduction in tumour cell migration away from the injection site. However, it is possible that the accumulated doses of trametinib and PIK III within the zebrafish xenografts may have been sufficient enough to inhibit autophagy and therefore inhibit tumour cell invasion, and facilitating the observed induction in cell death

of tumour cells remaining in the yolk sac. Nevertheless more complex pharmacokinetic studies are required both *in vitro* and *in vivo* to establish drug concentrations reached in the environment of both zebrafish xenografts and collagen invasion gels. However, downstream autophagy blockade with chloroquine has different effects on cancer cells when compared to upstream autophagy blockade using a Vps34/Beclin1 inhibitor such as PIK III. To further investigate the contribution of autophagy to the invasion and survival of drug induced melanoma subpopulations, blocking autophagy at the transcriptional level, to completely abrogate autophagic activity would allow a better understanding of the contribution of autophagy to the survival of such subpopulations and the relationship between autophagy and CD271 induction in response to MEK inhibitors.

Finally, since recent studies have shown melanoma cell death can be induced by harnessing the cytotoxic effects autophagy induced by the synthetic cannabinoid exacerbation THC (Armstrong *et al.*, 2015b) studies were undertaken to test the potential of this strategy to enhance apoptosis of trametinib-induced drug resistant melanoma cells. Results demonstrated THC induced the significant inhibition of trametinib-induced CD271 expressing melanoma cell viability *in vitro*, however, interestingly this effect was only induced by THC in combination with trametinib treatment and not by THC alone. Such observations may indicate that THC requires the induction of pro-survival autophagy induced by trametinib in order to exert its cytotoxic effect. Nevertheless, taken together, these data indicate that treatment of trametinib-induced CD271 expressing melanoma subpopulations with THC may present a viable strategy through which to overcome the acquired resistance of BRAF/NRAS mutant melanoma to MEK inhibition, and is a strategy that warrants further investigation *in vivo*.

5.4 **Summary**

- CD271 inhibition using small molecule CD271 inhibitors inhibits the cell viability of trametinib-induced CD271 expressing melanoma cells but increases tumour invasion *in vitro*
- Inhibition of AMPK activity results in the reduction of trametinib-induced CD271 expression but increases autophagy in drug resistant subpopulations.
- Autophagy inhibition with chloroquine re-sensitizes drug resistant CD271 expressing melanoma subpopulations to the cytotoxic effects of trametinib and inhibits spheroid size *in vitro*
- Autophagy inhibition with the Vps34 inhibitor, PIK III re-sensitizes drug resistant CD271 expressing melanoma subpopulations to the cytotoxic effects of trametinib but does not inhibit spheroid expansion *in vitro*
- Autophagy inhibition with the Vps34 inhibitor, PIK III re-sensitizes drug resistant CD271 expressing melanoma subpopulations to the cytotoxic effects of trametinib and inhibits tumour invasion and dissemination *in vivo*
- THC in combination with trametinib inhibits the cell viability of trametinib-induced CD271 expressing melanoma cells

Chapter 6

Overall Discussion

Chapter 6 Overall Discussion

Malignant melanoma is the most aggressive form of skin cancer and although associated with an excellent prognosis when diagnosed early, once metastatic disease evolves, clinical outcome remains poor (Charles et al., 2009). In the past decade however, the management of metastatic melanoma has fundamentally changed, with the introduction of novel targeted and immunotherapies. Prior to this, treatment approaches were limited to the use of chemotherapy with dacarbazine, temozolomide or various chemotherapy combinations (Bajetta et al., 2006; Pflugfelder et al., 2011), all of which only resulted in a low objective clinical response (Eggermont and Kirkwood, 2004; Quirbt et al., 2007). The recent discovery of driver BRAF mutations in cutaneous melanoma however, has unveiled a new era in the use of agents that specifically target these mutations for therapeutic benefit. Approximately 60% of all cutaneous melanomas harbour a mutation in BRAF (Ascierto et al., 2012) which leads to hyper-activation of MAPK signalling resulting in enhanced tumour cell proliferation and cell cycle progression (Davies et al., 2002; Mar et al., 2015). As a result, the inhibition of activated MAPK signalling has become an attractive therapeutic target for BRAF and NRAS mutant melanomas. The recent introduction of BRAF and more recently MEK inhibitors has indeed revolutionized the therapeutic management of patients with advanced melanoma, with the specific BRAF inhibitors targeting mutant BRAF, (vemurafenib and subsequently dabrafenib), demonstrating significantly improved patient survival with an approximate 50% response rate and a median duration of response of 6-7 months (Chapman et al., 2011; Hauschild et al., 2012). However the success of these agents is limited by the development of acquired resistance, which occurs within months of treatment initiation resulting in catastrophic clinical outcomes for patients (Gowrishankar et al., 2012).

As melanoma is a heterogeneous cancer, it is perhaps not surprising that the mechanisms mediating BRAF/MEK inhibitor-acquired resistance are varied. Paradoxical re-activation of the MAPK pathway is a well described mechanism of resistance, mediated for example by overexpression of CRAF or COT1 (Montagut *et al.*, 2008; Johannessen *et al.*, 2010a; Poulikakos *et al.*, 2011) leading to restored MAPK activity and continued tumour growth. Additionally, secondary activating mutations in NRAS and MEK also lead to re-activation of MAPK signalling

and tumour cell proliferation (Nazarian et al., 2010). Other MAPK-independent mechanisms of resistance include the overexpression of the receptor tyrosine kinases such as platelet-derived growth factor receptor-β (PDGFRβ) and IGF-1R (Villanueva et al., 2010; Shi et al., 2011) or the loss of PTEN and activated PI3K/AKT signalling (Paraiso *et al.*, 2011).

Perhaps one of the most recent and controversial mechanisms mediating the resistance of BRAF mutant melanoma to BRAF/MEK inhibitor therapy is the emergence of chemo resistant stem-like subpopulations (Roesch *et al.*, 2010; Chartrain *et al.*, 2012; Ravindran Menon *et al.*, 2014), an observation that has led to a flurry of studies investigating the role, if any, of cancer stem cells and their contribution to tumour initiation as well as chemo resistance (Boiko *et al.*, 2010a; Chen *et al.*, 2010; Boyle *et al.*, 2016). The presence and importance of cancer stem cells in melanoma remains an area of ongoing debate. Proposed stem cell markers in melanoma include CD271 (Boiko *et al.*, 2010a), Jarid-1B (Roesch *et al.*, 2010), ABCB5 (Chartrain *et al.*, 2012) and SOX10 (Zhang and Herlyn, 2014), with CD271 expressing melanoma subpopulations also described as a key stem- like subpopulation that emerges as part of drug-induced chemo-resistance to BRAF/MEK targeted therapy (Ravindran Menon *et al.*, 2014; Li *et al.*, 2015a; Redmer *et al.*, 2017). Understanding the role of constitutive and targeted therapy-induced CD271 expression in melanoma, and the mechanisms leading to its induction and the survival of CD271 expressing melanoma cells thus presents an opportunity for their targeting and as a potential means to overcome MAPK inhibitor induced drug resistance.

To this aim the present study revealed that constitutive as well as drug-induced CD271 expression is associated with an increase in autophagic activity and that autophagy blockade with either chloroquine of specific Vps34 inhibitors resulted in significant reduction in cell viability of CD271 expressing melanoma subpopulations. Alternatively treatment of drug resistant CD271 expressing subpopulations with the autophagy inducing agent THC also resulted in re-sensitization of trametinib-induced CD271 expressing melanoma cells to the cytotoxic effect of MEK inhibition, collectively supporting the concept of autophagy modulation as a strategy to overcome trametinib-induced drug resistance.

6.1 **Understanding the difference between constitutive and drug induced CD271 expression, pAMPK and autophagy in melanoma**

To understand the difference between constitutive and drug-induced expression of CD271 in melanoma, and the association with autophagy, CD271 expression was initially correlated with p62 expression in a panel of naevi and primary cutaneous melanomas of differing AJCC stage as well as with basal autophagy activity in CD271 expressing subpopulations derived from BRAF mutant melanoma cell lines. Results revealed an inverse correlation with p62 expression in AJCC stage II melanomas *in vivo* and an increase in LC3-II flux in isolated CD271 expressing melanoma cells *in vitro*.

Furthermore over expression of CD271 *in vitro* resulted in an increase in autophagic activity, collectively suggesting the constitutive expression of CD271 by melanoma cells is associated with an increase in basal autophagy. Interestingly however, despite the anticipated decrease in autophagic activity expected following knockdown of CD271, autophagic activity increased. It is possible that the observed sustained autophagic activity in this case may have reflected the only partial decrease in CD271 expression induced by a transient RNAi approach. However, experiments of stable CD271 knockdown in A375 cells resulted in cell death indicating that CD271 is important for melanoma cell proliferation and growth.

Although melanoma cells constitutively expressing CD271 are known to be present in melanoma cell lines (Saltari *et al.*, 2016) and primary melanoma tissue (Beretti *et al.*, 2015), the relevance of such expression in tumour initiation (Boiko *et al.*, 2010a; Quintana *et al.*, 2010) is controversial. Evidence also suggests that constitutive CD271 expression by primary melanomas is associated with more aggressive disease (Beretti *et al.*, 2015). CD271 expression is also well known to be regulated by micro environmental factors such as hypoxia and nutrient deprivation (Ravindran Menon *et al.*, 2014) which may explain per se the link between constitutive expression by melanomas in a hypoxic/nutrient deprived environment and the observed increase in autophagy, also induced by hypoxia and nutrient starvation and known to promote tumour cell survival in hostile micro-environmental conditions (Hu *et al.*, 2012a; Kim *et al.*, 2015). Since autophagy is also activated as a pro-survival response mechanism to counter act the cytotoxic effects of targeted therapies and chemotherapy (Lee

et al., 2015; Goulielmaki *et al.*, 2016), this may also explain the observed increase in autophagy observed in drug-induced resistant CD271 expressing melanoma, which could be independent of CD271 induction. However, the effect of CD271 on autophagic activity may be different in drug-treated cells compared to unstressed cells constitutively expressing CD271. In the context of drug-induced CD271 expression, this is a well-known phenomenon associated with the resistance of BRAF mutant melanomas to MEK inhibitor therapy (Ravindran Menon et al., 2014; Lehraiki et al., 2015), and supported by results from the present study.

However, trametinib-induced CD271 expression observed in the present studies was transient, while the associated increase in basal autophagy was sustained in drug-resistant subpopulations, indicating that CD271 expression may not be required for autophagy induction or that CD271 induction is independent of autophagy induction and further questioning whether trametinib-induced autophagy activation in the tumour microenvironment contributes to the emergence of CD271 expressing subpopulations or whether CD271 expressing subpopulations are induced independently of autophagy but harness pro-survival autophagy to survive. It is therefore possible that any increase is CD271 is a survival response mechanism to micro environmental stress and that autophagy activation enables CD271 expressing cells to survive, detach from the original tumour site and gain entry to systemic circulation. It is also possible and, again in line with results presented in this thesis, that CD271 expression is only required until the tumour has repopulated and once tumour cell proliferation is established, CD271 expression is not further required, reflected by a decline in its expression.

Furthermore, If CD271 expressing cells are induced independently of direct autophagy activation, other signalling pathways may also be mediating their induction. Recent studies have shown BRAF inhibition mediates the activation of NF-κB signalling in melanoma cells via an increase in TNFa secretion leading to the induction of CD271 (Lehraiki et al., 2015). Given the fact that NF-κB can either activate or inhibit signalling pathways that lead to the induction of autophagy and regulates the transcription of a subset of pro-autophagic regulating genes (Trocoli and Djavaheri-Mergny, 2011), it is possible that CD271 induction may result from trametinib-induced activation of NF-κB independently of autophagy activation or a complex interplay between NF-kB activation resulting in autophagy activation which then leads to

induction of CD271 (Figure 6-1). To address this possibility future experiments would need to assess the effect of trametinib treatment of melanoma cells in which CD271 expression has been abrogated on TNFa/NF-Kb pathway induction and autophagic activity or through alternative blockade of the NF-kB pathway with small molecule inhibitors prior to evaluating such effects of trametinib on CD271 induction.

What is also surprising was the lack of correlation between CD271 expression and the expression of other stem cell markers such as Nestin, Jarid-1B, ABCB5 and SOX10. In the setting of constitutive CD271 expression the present study demonstrated a lack of any correlation the expression of other stem cell markers, supporting data previously highlighting a poor overlap between CD271 and ABCB5 expression (Cheli *et al.*, 2014b). However, in the context of drug resistance, CD271 appeared to correlate with an increase, albeit non-significant, in Jarid-1B and SOX10 and a decrease in Nestin expression. What is still not clear, however, is whether these subpopulations emerge independently or whether expression overlaps in any way. Is it possible that subpopulations expressing different stem cell markers emerge in response to targeted therapies independently, contributing to the heterogeneity of melanoma and the clinical observation that combination therapy is essential to promote tumour shrinkage. Alternatively it is also possible that targeting one subpopulation might allow for the growth of subpopulations expressing other markers, and therefore contribute to continued tumour growth, and suggesting therefore that the targeting of a generic survival mechanism may be a more efficient treatment approach rather than targeting each subpopulation independently.

Whether CD271 initiates autophagy or if autophagy is induced by drug resistance resulting in increased CD271 remains enigmatic (Figure 6-1) and to further delineate this association experiments using modern technologies for gene manipulation such as CRISPR/Cas9 (Sander and Joung, 2014) to manipulate CD271 or key autophagy regulatory genes such as ULK 1, ATG5 or ATG7 are hence required and furthermore to allow for a more physiological means through which to analyse the interaction between CD271 and autophagy either in the constitutive setting or in response to trametinib. Nevertheless, data from the present study support the potential for inhibiting CD271 or autophagy as a therapeutic strategy to re-sensitize drug resistant cells to cytotoxic effects of trametinib.

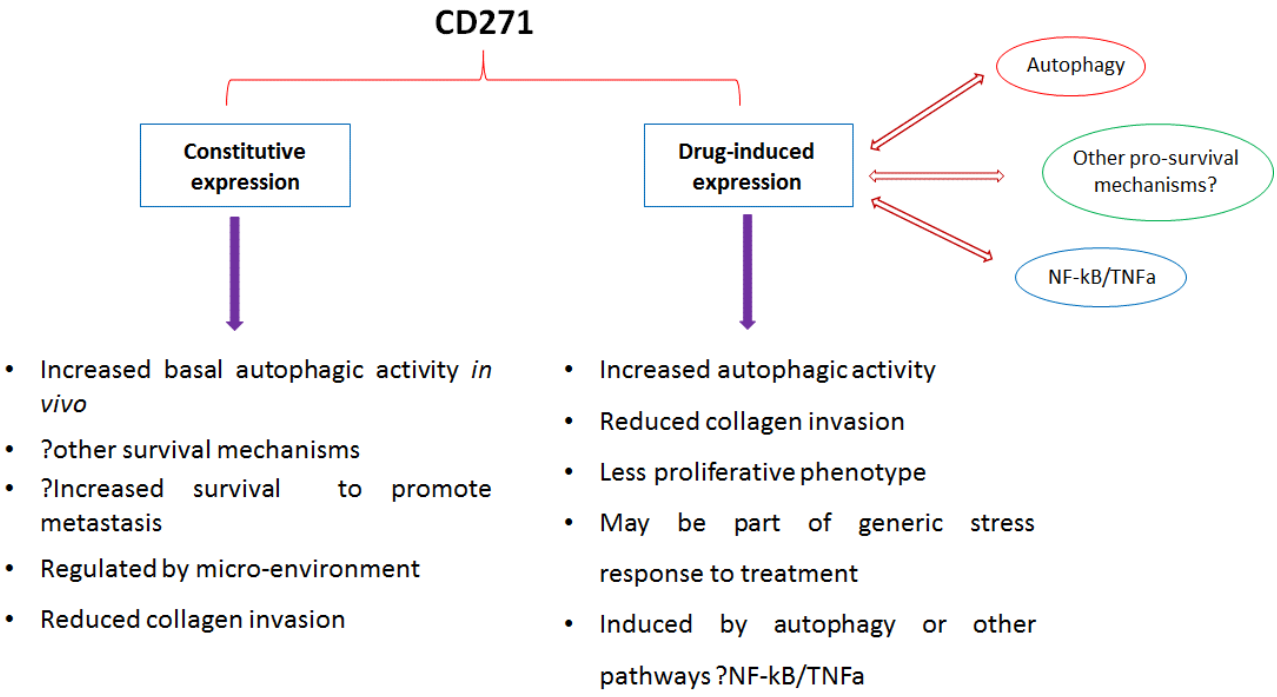


Figure 6-1: Proposed model of CD271 expression
Expression of CD271 in the constitutive and drug-induced setting. Constitutive expression is associated with increased basal autophagy in vivo and its expression is regulated by micro-environmental factors. Constitutive CD271 expression is associated with reduced collagen invasion *in vitro*. Drug-induced CD271 expression may be induced by the induction in autophagic activity, mediated by NF-kB pathway or as a result of other generic pro-survival mechanisms. Drug induced CD271 is associated with an increase in basal autophagy and a less invasive and less proliferative phenotype *in vitro.*

6.2 **The role of CD271 in tumour cell invasion and the importance of micro environmental factors**

Results presented in this thesis demonstrate that both constitutive and drug-induced CD271 expressing melanoma subpopulations is associated with reduced collagen invasion *in vitro*. It is likely that CD271 expression is regulated by micro-environmental stresses or drug induced stresses which also contributes to the upregulation of survival pathways such as autophagy. However, despite the anticipated result of CD271 expressing melanoma subpopulations being innately more invasive, and supporting recently published studies, results highlighted both the constitutive and drug-induced expression of CD271 in melanoma cells was associated with a less invasive phenotype *in vitro*, (Saltari *et al.*, 2016) and perhaps in the context of drug resistance mediated by trametinib-induced cell cycle arrest (G1 arrest) (Haass *et al.*, 2014). It is therefore possible long term exposure of melanoma cells to trametinib results in G1 arrest mediated induction of pro-survival autophagy which prevents cell invasion. However, what is still unclear is why melanoma cells constitutively expressing CD271 might exhibit a less invasive phenotype in the absence of any drug-induced stress. It is possible that melanoma cells that constitutively express CD271 cells are more sensitive to external stresses or remain in an undifferentiated state which affects cell cycle and metabolism. Such observations could also be explained by the hypothesis that CD271 positive cells have a more regulatory/drug resistant phenotype and their role in disease progression and metastasis is limited. Coupled with the observations that CD271 negative cells were more invasive and CD271 expression *in vitro* is dynamic and unstable, it is possible that CD271 positive cells can switch from a CD271 positive to a CD271 negative phenotype depending on micro environmental factors and stresses and acquire different properties based on tumour progression and migration which could be facilitated by CD271 negative cells or tumour survival facilitated by CD271 positive cells.

Taken together these data demonstrate the complex and situational association between CD271 activation and autophagic activity which indeed appears to be contextual in the setting of the constitutive or drug-induced CD271 respectively and also the dynamic expression of CD271 which appears to be regulated by the tumoural need to either promote tumour survival

or metastasis and further highlighting the different cellular properties and capabilities CD271 expressing cells exhibit in constitutive and drug-induced setting.

6.3 Targeting CD271 or pro-survival autophagy as a therapeutic strategy to overcome resistance of melanoma cells to trametinib

Despite the complexity of CD271 expression, it is clear that CD271 expressing cells are associated with early resistance to targeted therapy, which correlates with an induction in autophagic activity. The majority of therapeutic approaches for melanoma only transiently effects tumour growth and ultimately, given the heterogeneous nature of melanoma and its propensity to activate pro-survival and anti-apoptotic pathways, acquired resistance to treatment approaches inevitably occurs. Studies have highlighted the potential role for targeting autophagy in combination with chemotherapy (Li *et al.*, 2010) and targeted therapies (Goulielmaki *et al.*, 2016) to overcome resistance in of targeted therapy-associated drug resistance. However, the induction of specific cell subpopulations poses the question of whether it will be more beneficial to target the chemo resistant subpopulations themselves or the processes they harness to survive, including autophagy. Given the upregulation of tumour cell subpopulations expressing specific surface markers (Singh *et al.*, 2003; Yamashita and Wang, 2013) in response to chemotherapy and bearing in mind that in the case of many cancer types these subpopulations contribute to the development of acquired resistance, specific antibodies or small molecules have been developed and trialled for their efficiency in targeting specifically these subpopulations (Jin *et al.*, 2006; Stratford *et al.*, 2013)) for therapeutic benefit.

Inhibiting CD271 using two small molecule inhibitors, TAT-PEP5 and RO-08 (Ting-Hsien *et al.*, 2014; Mochizuki *et al.*, 2016) resulted in increased cell death of trametinib-treated melanoma cells but also in increased spheroid expansion *in vitro* suggesting this approach may not be beneficial in a clinical setting as it may increase the tumour's metastatic potential.

Furthermore, since melanoma is a very heterogeneous cancer and resistance to targeted therapies is likely associated with the emergence of a number of resistant subpopulations, it

is possible that targeting CD271 expressing subpopulations directly might allow for other, smaller subpopulations that differ in resistance profiles and might therefore not be efficiently targeted by a single therapeutic agent, to emerge and contribute to chemo resistance.

Another consideration to take into account when targeting cancer 'stem-like cells' is the potential for off-target effects, for example targeting CD271 might influence the renewal and maintenance of neural crest and neuronal tissue as CD271 is a crucial receptor for neurite cell growth and survival (Johnson *et al.*, 1986). Finally, it is still possible that targeting cancer stem like cells, in the tumour bulk might facilitate the development of resistance to cancer stem cell-directed therapies and therefore, combination therapies that involve both CSC-directed agents as well as tumour bulk-targeted regimens would be predicted to prove most effective in improving clinical treatment responses and patient outcomes. Taken together, these observations suggest that although it is possible to directly target CD271 expressing melanoma subpopulations, selecting for CD271 negative subpopulations by eliminating the CD271 positive subpopulations might increase the tumour's metastatic potential or promote the emergence of other stem cell subpopulations that do not express CD271. As a result, targeting the process these subpopulations use to survive could represent a more efficient treatment approach leading to the alternative possibility that modulation of pro-survival autophagy or AMPK, harnessed by trametinib-induced CD271 expressing melanoma cells may provide a better approach. Blockade of AMPK with compound C resulted in efficient blockade of pAMPK but did not have any significant effect on CD271 expression or autophagic activity. It should be noted however that Compound C, is not a specific AMPK inhibitor (Liu *et al.*, 2014) and since there are no specific AMPK inhibitors currently available, the benefit of AMPK inhibition is at present limited. As AMPK is only one of several upstream modulators of autophagic activity specific inhibition of autophagy regulatory proteins may therefore present a more effective therapeutic strategy.

Increasing evidence supports a pivotal role of autophagy activation in chemo resistance (Hu *et al.*, 2012b; Sui *et al.*, 2013). Manipulating autophagy, either by targeting autophagy in combination with chemotherapy (Rangwala *et al.*, 2014b; Lu *et al.*, 2017) or exacerbating autophagy (Armstrong *et al.*, 2015b) to target resistant cancer cells (Liu *et al.*, 2013a) appears to be beneficial in overcoming resistance to chemotherapy. Inhibition of autophagy with the

lysosomal inhibitor chloroquine or the specific Vps34 inhibitor PIK III as well as inducing cytotoxic autophagy with THC, all resulted in the re-sensitization of trametinib-induced resistant melanoma cells to the cytotoxic effects of MEK inhibition and hence may each provide a viable treatment approach to overcome trametinib-associated resistance (Figure 6-2). However, although autophagy inhibition with chloroquine resulted in effective re-sensitization of melanoma cells the cytotoxic effects of trametinib results from clinical trials indicate that the maximum clinically tolerated dose of chloroquine or hydroxychloroquine might not be sufficient to cause efficient autophagy blockade *in vivo* (Amaravadi *et al.*, 2011). Coupled with the fact that chloroquine is not a specific autophagy inhibitor, novel autophagy inhibitors may thus provide a more efficacious therapeutic approach. Novel inhibitors specifically targeting the Vps34/Beclin-1 complex have been developed and are currently in clinical use for patients with follicular lymphoma (Gopal *et al.*, 2014; Miller *et al.*, 2015; Pasquier, 2015). Treatment of trametinib-induced CD271 expressing melanoma cells with PIK III resulted in significant reduction in cell viability and reduction in tumour cell dissemination in a zebrafish model of metastatic melanoma *in vivo.* However, the combination of trametinib and PIK III did not demonstrate a significant effect in reducing spheroid expansion *in vitro* in a collagen gel assay which might, however, be a dose dependent effect. Nevertheless, given the safety and applicability of Vps34 inhibitors in clinical practice combined with the pre-clinical data supporting the efficiency of these specific inhibitors in targeting drug-induced CD271 expressing subpopulations, clinical trials of combination of Vps34 inhibitors with MAPK inhibition might be of clinical relevance. Finally, since previous studies in melanoma have shown THC promotes autophagy mediated apoptosis (Armstrong *et al.*, 2015b) , which coupled with the compelling data from the present study showing THC also promotes apoptosis of trametinib-induced drug resistant CD271 expressing melanoma cells and current clinical trials (Chakravarti *et al.*, 2014) in glioblastoma multiforme (Velasco *et al.*, 2016a), suggests the use of THC may also represent viable therapeutic option to overcome the resistance of BRAF/NRAS mutant melanoma to MEK inhibition.

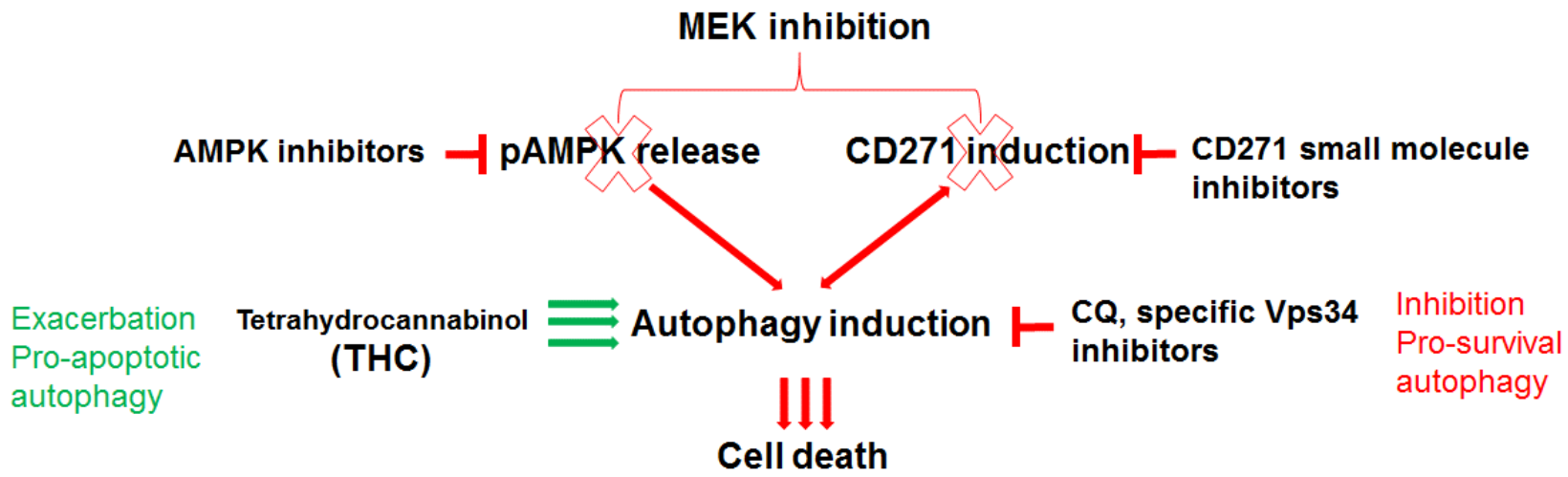


Figure 6-2: Targeting CD271 or pro-survival pathways to overcome resistance to MEK inhibition

Proposed targets to overcome CD271 mediated resistance to MEK inhibitors. MEK inhibitors can induced CD271 expressing melanoma subpopulations directly or induce AMPK release leading to autophagy induction which subsequently can lead to CD271 induction. Alternatively, MEK induced CD271 expressing melanoma cells can harness autophagy as a pro-survival mechanism. CD271 associated resistance can be targeted by either blocking the AMPK pathway, CD271 using small molecular CD271 inhibitors or by modulating autophagy either by blocking pro-survival autophagy with chloroquine or specific autophagy inhibitors or alternatively exacerbating pro-apoptotic autophagy using THC.

In conclusion, although there has been extensive research into the signalling pathways contributing to drug resistance in melanoma this work highlights the pivotal role of CD271 expressing melanoma subpopulations in trametinib-induced resistance and their association with the activation of pro-survival autophagy. Trametinib-induced CD271 expressing melanoma cells contribute to the development of acquired resistance via a complex link with activated metabolic pathways such as AMPK and autophagy. Although the link between drug induced CD271 expression melanoma subpopulations and autophagy is clear, the association between constitutive expression of CD271 *in vitro* and *in vivo* is weak and indicates that the role of CD271 might differ in a constitutive or drug-induced setting. Drug induced CD271 expressing cells only transiently emerge in response to long term treatment with trametinib therefore suggesting that CD271 might only be important initially in the development of acquired resistance and not once full resistance is established. This observation further highlights the complex regulation of CD271 and its clinical implications as targeting CD271 to overcome resistance in the clinical setting will require regular patient tissue sampling to look for the emergence of CD271 expressing cells which will be difficult outside a clinical trial setting. However, and more importantly, manipulating autophagy, the pro-survival mechanism CD271 expressing cells use to survive might be a more realistic approach. Data from the current study highlight that drug- induced CD271 expressing subpopulations harness autophagy as a pro-survival mechanism and manipulating autophagy either by blocking pro-survival autophagy or inducing cytotoxic autophagy sensitizes drug-resistant BRAF mutant melanoma cells to the cytotoxic effects of trametinib. Although it is not clear whether trametinib-induced activation of autophagy results in CD271 induction of vice versa, targeting autophagy as described above nevertheless results in increased cell death irrespective of the sequence of these events and therefore may present viable treatment option to overcome the resistance of BRAF /NRAS mutant melanomas to MEK inhibition.

Inhibition of the MAPK pathway is likely to continue to be a cornerstone of therapeutic interventions for BARF mutant cutaneous melanoma despite the introduction of immunotherapy. However, novel treatment approaches using combination of BRAF/MEK inhibition and autophagy modulation might offer a superior therapeutic benefit for BRAF mutant cutaneous melanoma, delay the development of acquired resistance and improve clinical outcomes.

Chapter 7 References

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List of published Manuscripts arising from this Thesis

Verykiou S, Edwards N, Hill D (2017)

How breakthroughs in translational research have impacted treatment strategies for melanoma

Accepted for publication at British Journal of Dermatology, *in press*

Muthiah S, Tang D, Nars B, **Verykiou S** (2017)

A new era in holistic care - bridging the gap between dermatologists and oncologists for the treatment of malignant melanoma.

Accepted for publication at British Journal of Dermatology, *in press*

List of published Abstracts arising from this Thesis

Verykiou S, Hill DS, Plummer R and Lovat PE

Modulation of autophagy reduces survival of trametinib-resistant, CD271 expressing melanoma subpopulations

Oral presentation British Society for Investigative Dermatology, Manchester, April 2017

Verykiou S, Rohman A, Bowler K, Plummer R, Lovat PE and Hill DS

Inhibition of autophagy reduces survival of trametinib-resistant, CD271 expressing melanoma subpopulations

Poster presentation Society for Melanoma Research, Boston USA, November 2016

Verykiou S, Hill DS, Plummer R and Lovat PE

Contribution of autophagy to the survival of melanoma-initiating CD271-positive subpopulations

Oral presentation at The British Society for Investigative Dermatology annual meeting, Southampton 2015

Verykiou S, Hill DS, Plummer R and Lovat PE

Contribution of autophagy to the survival of melanoma-initiating CD271-positive subpopulations

Oral presentation at The Royal Society of Medicine AbbVie Dermatology Trainee Research Prize meeting, Royal Society of Medicine, April 2015

Hill DS, **Verykiou S**, Robinson N, Armstrong J, Przyborski S, Chaudhry B, Lovat PE

Modelling melanoma metastasis using organotypic skin equivalent and zebrafish models

Pig Cell and Mel Res: 27:6- 1236

Poster presentation Society for Melanoma Research, Zurich 2014