Expression of the uncharacterised isoform, BCL2β, in melanoma

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Statement of Originality

The thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to the final version of my thesis being made available worldwide when deposited in the University’s Digital Repository, subject to the provisions of the Copyright Act 1968.

Signed,

Chloe Warren
Acknowledgements and Dedications

I’d like to thank my wonderful and supportive supervisor, Dr Nikola Bowden, without whom I would have mistaken my first stumble for what it felt like at the time (i.e. a bear trap), and abandoned this journey pretty much immediately. You are the greatest mentor I could have hoped for and I will never forget your kindness, compassion, patience and wisdom.

I’d like to thank my fiancée for taking care of me and reminding me to take care of myself whenever I would listen.

I’d like to thank my parents for supporting me whenever the time zones would allow and sometimes when they wouldn’t.

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Collinson for being a patient, well organised and generally delightful lab manager/ PhD student tolerator.

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<th>MEANING</th>
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<tr>
<td>ALM</td>
<td>acral lentiginous melanoma</td>
</tr>
<tr>
<td>AMO</td>
<td>antisense morpholino oligonucleotides</td>
</tr>
<tr>
<td>BER</td>
<td>base excision repair</td>
</tr>
<tr>
<td>BH</td>
<td>BCL2 homology</td>
</tr>
<tr>
<td>BRAF V600E</td>
<td>T to A substitution at codon 600 in the BRAF gene</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CLL</td>
<td>chronic lymphoid leukaemia</td>
</tr>
<tr>
<td>CPD</td>
<td>cyclopyrimidine dimers</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DSB</td>
<td>double strand break</td>
</tr>
<tr>
<td>dsDNA</td>
<td>double stranded DNA</td>
</tr>
<tr>
<td>DTIC</td>
<td>dacarbazine</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ESI</td>
<td>electron spray ionisation</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
</tr>
<tr>
<td>IP</td>
<td>immunoprecipitation</td>
</tr>
<tr>
<td>LMM</td>
<td>Lentigo maligna melanoma</td>
</tr>
<tr>
<td>LSB</td>
<td>Antibody LS-B11858 (Life Span Bioscience)</td>
</tr>
<tr>
<td>m/z</td>
<td>mass/charge</td>
</tr>
<tr>
<td>MMR</td>
<td>mismatch repair</td>
</tr>
<tr>
<td>MOM</td>
<td>mitochondrial outer membrane</td>
</tr>
<tr>
<td>MOMP</td>
<td>mitochondrial outer membrane permeability</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MRM</td>
<td>multiple reaction monitoring</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
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<td>nucleotide excision repair</td>
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<tr>
<td>NHEJ</td>
<td>non-homologous end joining</td>
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<tr>
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<td>non-Hodgkin lymphoma</td>
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<td>NMM</td>
<td>nodular malignant melanoma</td>
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<tr>
<td>O/N</td>
<td>overnight</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>PI</td>
<td>propidium iodide</td>
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<td>phosphatidylserine</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative PCR</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
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<tr>
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<td>superficial spreading melanoma</td>
</tr>
<tr>
<td>TF</td>
<td>transcription factor</td>
</tr>
<tr>
<td>TGA</td>
<td>Australian Therapeutic Goods Administration</td>
</tr>
<tr>
<td>TIL</td>
<td>tumour infiltrating lymphocytes</td>
</tr>
<tr>
<td>TM</td>
<td>transmembrane</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
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<td>well plate</td>
</tr>
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<tr>
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<td>GENE NAME</td>
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<td>APE1</td>
<td>AP endonuclease 1</td>
</tr>
<tr>
<td>BAK</td>
<td>BCL2 antagonist killer</td>
</tr>
<tr>
<td>BAX</td>
<td>BCL2 associated X protein</td>
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<td>FADD</td>
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<tr>
<td>FAS</td>
<td>Fas cell surface death receptor</td>
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<td>HD-IL2</td>
<td>high dose interleukin-2</td>
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<td>mitogen activated protein kinase kinase</td>
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<td>VDAC</td>
<td>voltage dependent ion channel</td>
</tr>
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<td>XP</td>
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Abstract

There are two known isoforms of the anti-apoptotic protein BCL2. While BCL2α is well characterised and known predominantly for its role in apoptosis, BCL2β has not yet been described. We sought to confirm the presence of this isoform in melanoma and characterise its role in the apoptotic response.

We were able to verify expression of the rare isoform at the protein level in cell lines using multiple reaction monitoring tandem mass spectrometry. We also examined the role of the isoforms in apoptosis and melanin synthesis by a) monitoring gene response to stress at the mRNA level and b) using siRNAs targetted to each individual transcript. We treated cells with a variety of stressors and then monitored their apoptotic response using flow cytometry. We also quantified melanin production in response to UVB.

The BCL2α response to stress (i.e. downregulation prior to apoptosis) was matched across melanocyte and melanoma cell lines. However, BCL2β response was varied across the melanoma cell lines, but matched that of BCL2α in melanocytes. Knock-down of both isoforms (individually) resulted in increased apoptosis in melanoma cell lines.

We quantified mRNA of both BCL2 isoforms in our cohort of 189 FFPE melanoma tumours using qPCR. We also quantified total BCL2 protein expression in this same cohort using immunohistochemistry.

Interestingly, expression of the BCL2β isoform in tumours was significantly associated with increased overall survival (686.4 weeks, 95% CI 462.5-910.3). BCL2β expression was also elevated in metastatic tissue compared with
primary. This pattern was actually seen in the reverse at the protein level; total BCL2 protein was elevated in primary compared with metastatic.

Analysis of SNP rs3943258 revealed that possession of the mutant T allele in melanoma patients corresponded with increased $BCL2\beta$ expression. This contrasts with previous observations from a different study on healthy controls.

Our current understanding of the role of $BCL2\beta$ is based on the concept that it lacks the C-terminal transmembrane domain, and is thus incapable of localising to target organelles. It is generally assumed that the isoform is of null function. However, these observations have been based based on studies of non-representative synthetic versions of $BCL2\beta$. This is the first time the naturally transcribed version of the rare isoform has been studied, and the first time its role has been investigated since the 1980s. We have demonstrated herein that $BCL2\beta$ performs an anti-apoptotic role within the cell, and that its regulation may be disrupted in melanoma.
1 Chapter One: The BCL2 family and their isoforms in the context of melanoma

1.1 The biology of melanoma

Melanoma occurs as the consequence of the oncogenic transformation of melanocytes, the skin cells responsible for skin pigmentation and ultraviolet (UV) tanning response. Melanocytes are found in the basal epidermis of the skin, as well as the lining of respiratory, gastrointestinal, mucosal and meningeal surfaces (Cichorek, Wachulska et al. 2013); it is possible for primary melanomas to develop in each of these regions, although cutaneous melanoma (i.e. of the skin) is the most commonly diagnosed (90%) (Chang, Karnell et al. 1998). For a summary of the subtypes of melanoma, see Table 1.1-1.
Table 1.1-1 Subtypes of melanoma.

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Prevalence</th>
<th>Appearance/ Histological features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superficial spreading melanoma (SSM)</td>
<td>70% of all cutaneous melanoma.</td>
<td>Flat, multi-shaded, irregular borders. Arises from pre-existing lesion.</td>
</tr>
<tr>
<td>Nodular malignant melanoma (NMM)</td>
<td>10-15% of melanoma incidence.</td>
<td>Pigmented/ non-pigmented, thick.</td>
</tr>
<tr>
<td>Lentigo maligna melanoma (LMM)</td>
<td>4-10% of melanoma incidence.</td>
<td>Brown, large, flat. Often seen on sun-damaged skin.</td>
</tr>
<tr>
<td>Acral lentiginous melanoma (ALM)</td>
<td>3-5% of Caucasian melanomas, 30-50% of dark skinned melanomas.</td>
<td>Variable in colour. Found on palms, soles of feet, nail beds.</td>
</tr>
</tbody>
</table>

In response to intense UV irradiation, melanocytes will initiate the tanning response. This culminates in the generation of melanosomes, which physically shield the nuclei of skin cells. This protective response requires melanocytes to be partially resistant to UV, unlike other skin cells which may undergo apoptosis or stall the cell cycle in response to this same stimulus (Sermadiras, Dumas et al. 1997, McGill, Horstmann et al. 2002, Cichorek, Wachulska et al. 2013). In addition, melanin synthesis itself is an oxidative process (i.e. it produces reactive oxygen species, ROS), and thus melanocytes have an increased tolerance for oxidative stress (Denat, Kadekaro et al. 2014). Melanocytes are therefore intrinsically enriched for survival advantage, which confers their aggressive nature if
they undergo the oncogenic transformation to melanoma (Soengas and Lowe 2003). Indeed, although melanoma accounts for just 3% of skin cancers, it is responsible for 75% of skin cancer related deaths (Tracey, Kerr et al. 2008).

If diagnosed before metastasis has occurred, approximately 90% of melanoma patients achieve 5-year survival (Siegel, DeSantis et al. 2012), as surgical excision of the primary melanoma is sufficient to prevent metastatic disease and poor outcomes. However, if the diagnosis is made once the primary has already metastasized, just 15% of patients will survive for more than 5 years (Siegel, DeSantis et al. 2012). These poor survival rates are of particular concern when we consider that incidence is on the increase in Australia; the number of invasive melanomas removed per year increased by 325% between 1985 and 2009 (Czarnecki 2014).
1.2 Melanoma risk factors

There are several risk factors for the development of melanoma. These include the autosomal recessive disorder Xeroderma pigmentosum, XP (which displays a 2000 fold increase in melanoma risk (Bradford, Goldstein et al. 2011)), and genetic predisposition associated with mutations/polymorphisms in CDKN2A (cyclin-dependent kinase inhibitor 2A) and MC1R (melanocortin 1 receptor). In addition to these known genetic risk factors, there are several phenotypic and environmental risk factors, most notably UV exposure (see Table 1.2-1).

Table 1.2-1 Summary of risk factors associated with melanoma.

<table>
<thead>
<tr>
<th>Genetic</th>
<th>Risk</th>
<th>Detail</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDKN2A</td>
<td>Familial melanoma accounts for 8-12% of melanoma incidence; of these, 33-40% harbour a CDKN2A mutation (Swick and Maize 2012, Wangari-Talbot and Chen 2012).</td>
<td>CDKN2A encodes two distinct proteins via alternative splicing mechanisms; p16 (cyclin-dependent kinase inhibitor 2A, multiple tumour suppressor 1) and p14Arf (alternate reading frame tumour suppressor p14), both of which are tumour suppressors (Tsao, Chin et al. 2012).</td>
</tr>
<tr>
<td>MC1R</td>
<td>MC1R is a major regulator of melanocyte function, and is essential for tanning response and UV tolerance. MC1R variants are associated with red hair, pale skin, high tendency to burn and difficulty tanning (Lin and Fisher 2007, Fukunaga-Kalabis and Herlyn 2012).</td>
<td>Approximately 65% of melanoma patients harbour MC1R polymorphisms. There is no single genetic pattern of MC1R polymorphisms which is associated with melanoma development, although recent evidence suggests that different combinations of these polymorphisms are associated with specific epidemiologic, phenotypic and histological features of the disease (Peña-Vilabelda, García-Casado et al. 2014).</td>
</tr>
</tbody>
</table>
**XP**

XP is a rare autosomal disease, with a typical prevalence of 1 in 10,000,000. XP can manifest in 7 complementation groups, each caused by a mutation in a different XP gene, through XPA to XPG (Knoch, Kamenisch et al. 2012).

All XP variants are hyper sensitive to UV light due to their characteristic ineffective DNA repair pathways. Each complementation group has a slight variation in risk to both melanoma skin cancers and non-melanoma skin cancers (Nouspikel 2009).

<table>
<thead>
<tr>
<th>Phenotypic/ Genetic</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fitzpatrick Scale I</strong></td>
<td>Individuals with a phototype ranking of 1 on the Fitzpatrick Scale have up to double the risk of developing cutaneous melanoma than darker skinned individuals (Cancer Council Australia, Australian Cancer Network et al. 2008).</td>
</tr>
<tr>
<td>Highly pigmented skin is more resistant to melanoma as the increased concentration of pigment improves the capacity for nuclear shielding (Lin and Fisher 2007).</td>
<td></td>
</tr>
<tr>
<td><strong>Number of moles</strong></td>
<td>Individuals with &gt;100 nevi have a 7x higher risk of developing melanoma than those with &lt;15 (Cancer Council Australia, Australian Cancer Network et al. 2008).</td>
</tr>
<tr>
<td>Both SSM and LMM can develop from previously benign nevi (Hong, Robert C. Bast et al. 2010).</td>
<td></td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td>In general, risk of melanoma increases with age (Upendra and Jane 2013).</td>
</tr>
<tr>
<td>Due to the accumulation of DNA damage throughout life (partially attributable to UV exposure), elderly people are at a higher risk of developing melanoma (Cancer Council Australia, Australian Cancer Network et al. 2008).</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Environmental</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>UV exposure</strong></td>
<td>It is estimated that 90% of cutaneous melanoma cases are caused by UV exposure (Armstrong and Kricker 1993).</td>
</tr>
<tr>
<td>UV exposure directly causes the production of 6-4 photoproducts, CPDs and bulky adducts, and indirectly causes 8-oxo-2'deoxyguanosine (via oxidative damage) (Diderich, Alanazi et al. 2011).</td>
<td></td>
</tr>
</tbody>
</table>
1.3 Melanoma therapies

1.3.1 Traditional chemotherapy

Prior to the advent of immunotherapy (see Section 1.3.3), the most common therapies for advanced metastatic melanoma were fotemustine (an alkylating agent), dacarbazine (DTIC) and temozolomide (both of which are platinum based). A 2016 review of the therapeutic landscape for melanoma revealed that these drugs elicited a complete response in just 2% of patients and a partial response in 5% (Middleton, Dalle et al. 2016). Despite this, all three of these drugs have been approved as a first-line therapy for melanoma at some point over the last 30 years (Davey, van der Westhuizen et al. 2016).

The failure of DNA damaging agents to treat melanoma has led to theories which place melanoma’s insufficient DNA damage detection and repair systems at the core of explanations for its aggressive tendencies (Bradbury and Middleton 2004). In particular, perturbations in the NER (nucleotide excision repair) pathway, which is responsible for the removal of UV induced DNA damage, is a defining feature of melanoma (Tomescu, Kavanagh et al. 2001, Bowden, Ashton et al. 2010, Bowden, Ashton et al. 2013, Li, Yin et al. 2013, Murray, Maltby et al. 2015, Budden, Davey et al. 2016).

1.3.2 Molecular targeted therapies

A major shift in the past 10 years has led to an increase in the application of a personalised medicine perspective in the approach to melanoma research and treatment.
The observation that *BRAF* (B-Raf proto-oncogene, serine/threonine kinase), a tyrosine kinase, is frequently mutated in melanoma has been made in multiple cell lines and clinical cohorts since 2002 (Davies, Bignell et al. 2002, Gorden, Osman et al. 2003, Curtin, Fridlyand et al. 2005, Luke and Hodi 2012). Up to 66% of cutaneous melanomas harbour a *BRAF* mutation, and 90% of these mutations are a T to A substitution at codon 600; known as *BRAF* V600E (Swick and Maize 2012). The *BRAFV600E* mutation causes hyperactivation of the *MEK* (mitogen activated protein kinase kinase) signalling pathway, which upregulates cell proliferation, invasion, survival and angiogenesis (Thomas 2006). Vemurafenib (Plexxacon/ Genentech) was the first drug to be developed and used successfully to extend survival in melanoma patients with the *BRAFV600E* mutation (Flaherty, Puzanov et al. 2010). Although early phase clinical trials looked promising (patients with the mutation had a 69% response rate) (Flaherty, Puzanov et al. 2010), 50% of patients succumbed to disease progression within 7 months of starting treatment due to the development of drug resistance (Rizos, Menzies et al. 2014). Tactics in personalised medicine tend to now be focussed on combinatorial therapies, application of immunotherapy and investigations into the mechanisms of drug resistance.

1.3.3 Immunotherapy

In order to metastasise and thrive, cancers of all types need to be successful in evading the body’s immune system. While the idea of reprogramming or manipulating tumours such that they become
vulnerable to the immune system is not a new concept, marked success for this approach in the clinic has only really been seen within the last five years and the advent of PD1 (programmed death 1) based therapies (see Section 1.3.3.3).

1.3.3.1 Cytokine therapy

The interleukin 2 (IL2) cytokine induces proliferation of lymphokine activated killer (LAK) cells which detect and destroy tumour cells (Papa, Mule et al. 1986). IL2 therapy has been tested on a number of different cancers, including melanoma.

A study in the 1990s saw a total of 7% of patients responding for up to 91+ months, but with substantial adverse side-effects (Rosenberg, Yang et al. 1994). Dosage is restricted for this reason. Although success of the drug has been examined in a number of combination trials in more recent years, response was still demonstrated to be no better than any of the previous standards of care (Elias, Zapas et al. 2005) (Pretto, Elia et al. 2014).

1.3.3.2 Vaccines

Although cancer vaccines can fall into a wide range of categories (protein, whole cell, DNA or carbohydrate based), the most promising vaccine in the context of melanoma is a DNA based vaccine, derived from the herpes simplex virus (Rizos, Menzies et al. 2014).

Although early studies on the vaccine looked promising, with 26% of patients showing response to the drug (Senzer, Kaufman et al. 2009), the
following larger trials did not show any significant difference in survival between patients randomised to the T-VEC treatment and those receiving the current standard of care (Andtbacka, Ross et al. 2016).

**1.3.3.3 PD1 based therapies**

In 2011, Yervoy/ Ipilimumab (Bristol Myers-Squibb) was approved by the TGA (Australian Therapeutic Goods Administration). The drug is a CTLA4 (cytotoxic T-lymphocyte associated protein 4) inhibitor (see Figure 1.3.3.3-1). Cytotoxic T-lymphocytes can recognise and kill tumour cells and can be inhibited from doing so via activation of CTLA4 (Schadendorf, Hodi et al. 2015). Blockage of this signalling pathway allows the lymphocytes to attack and destroy cancer cells (Mukherjee, Schwan et al. 2015). The drug has been widely successful in extending the life of metastatic melanoma patients. In a trial of 1861 patients, 21% of the participants on Ipilimumab survived for over 10 years (Figure 1.3.3.3-2) (Schadendorf, Hodi et al. 2015).
Figure 1.3.3.3-1 Summary of immune checkpoint therapies in melanoma.

Immune and tumour cells express immune check-point proteins which can signal their status as foreign/abnormal (and therefore threatening) or non-threatening. These signals can activate or deactivate the immune system and therefore be manipulated therapeutically to prompt an immune response to cancer. Image taken from (Drake, Lipson et al. 2014)
Figure 1.3.3.3-2 Overall survival of patients treated with Ipilimumab.

Overall survival of 1,861 metastatic melanoma patients treated with Ipilimumab (pooled from 10 prospective trials and two retrospective). Median overall survival was 11.4 months. Data and graph taken from (Schadendorf, Hodi et al. 2015).

Pembrolizumab, (Merck, Sharp & Dohme) and Nivolumab (Bristol Myers-Sqibb) have also seen marked success in the clinic (Robert, Schachter et al. 2015). These drugs target the PD1 (programmed death 1) protein. PD1 is typically expressed on the surface of T-cells and functions to down-regulate the immune response. Blocking the receptor therefore stimulates immune activity and promotes tumour-killing (see Figure 1.3.3.3-1)(Drake, Lipson et al. 2014). In trials where Pembrolizumab/Nivolumab were administered to patients whose melanoma was unresponsive to Ipilimumab or targetted therapy, 31-40% of patients responded (Ivashko and Kolesar 2016).
Interestingly, it has been observed that patient tumour regression is markedly improved when proliferating T cells localise within the tumour (Tumeh, Harview et al. 2014).

Unfortunately, only 10-15% of melanoma patients respond to Ipilimumab (Hodi, O'Day et al. 2010) and the relapse rate for Pembrolizumab is as yet unknown. Research is currently underway as to how immune response can be triggered in non-responding patients.

1.3.4 The future of melanoma therapy

One of the approaches for increasing the responsiveness of melanoma to treatment is to directly target the cancer cells’ apoptotic machinery (Figure 1.3.4-1). Cellular regulation mechanisms are complex, thus there are many ways that a pharmacological treatment can be side-stepped due to the redundant nature of these pathways. Manipulation of cancer cells’ apoptotic decision making machinery would arguably provide fewer opportunities for the development of resistance, as, once entered into, apoptosis is a committal process. In this vein, we can remove the opportunities for cancerous cells to evolve resistance to treatments which may target molecules or pathways several stages upstream of cell fate decision making (Anvekar, Asciolla et al. 2011).
Figure 1.3.4-1 Targetting cellular apoptotic machinery.

Targetting apoptotic machinery is potentially a more effective strategy to cancer therapy as the apoptotic machinery (highlighted in the two rectangles) is downstream of a number of other pathways (highlighted in the ovals). Image taken from (Mukherjee, Schwan et al. 2015)
1.4 Apoptosis

Apoptosis is the mechanism whereby a cell undergoes programmed cell death in response to stress or as part of normal development (Alberts 2008). During apoptosis, the dying cell and its microenvironment work to minimise the effect of cell death on the rest of the tissue/organism (Kerr, Wyllie et al. 1972, Ellis and Horvitz 1986). It has long been appreciated that perturbations in apoptosis play a central role in cancer development (Hanahan and Weinberg 2011).

There are two major pathways which can lead to apoptosis; intrinsic and extrinsic. The extrinsic pathway is triggered by extracellular signalling, whereas the intrinsic pathway is triggered when the mitochondrial integrity becomes compromised as a result of intracellular stress signalling pathways (Alberts 2008). Both mechanisms of apoptosis share the ultimate goal of activating effector caspases which in turn cause the morphological changes which characterise apoptosis, such as chromosome condensation and breakdown of the cytoskeleton (Kerr, Wyllie et al. 1972). For an overview of extrinsic and intrinsic apoptosis, see Figure 1.4-1.
Figure 1.4-1 Regulation of intrinsic and extrinsic apoptosis.

Extrinsic apoptosis is triggered by the binding of the extracellular signalling molecule, FAS (Fas cell surface death receptor), to FADD (Fas associated via death domain) on the cell surface. This activates pro-caspase 8, which cleaves the effector caspase-3; this causes apoptosis. The extrinsic pathway is also capable of triggering the intrinsic pathway via BID (BH3 interacting-domain death agonist). Intrinsic apoptosis is regulated by the permeability of the outer mitochondrial membrane. This is controlled by the BCL2 (B-cell lymphoma 2) family of proteins. In turn, the BCL2 family is regulated by numerous cell signalling pathways which convey cellular stress levels. Leakage of mitochondrial compounds, such as cytochrome c, into the cytoplasm activates pro-caspases, which can then cleave and activate effector caspases (image adapted from (Pope 2002)).
1.5 The BCL2 Family

Following the initial discovery of the membrane-bound anti-apoptotic protein BCL2 in the 1980s (Tsujimoto, Finger et al. 1984, Tsujimoto, Cossman et al. 1985, Tsujimoto 1989), a number of homologous proteins have been identified. These proteins are collectively known as the BCL2 family as they all contain BCL2 homology (BH) domains and are involved in the regulation of apoptosis. They are commonly localised to the mitochondria.

Traditionally, the proteins are categorised into one of three sub-families: anti-apoptotic, BH3-only and executioner proteins. Subfamily categorization is based on BH and TM (transmembrane) domain and anti-/pro-apoptotic function status, as well as the proteins’ capacity to form pores in a phospholipid membrane (see Table 1.5-1) (Yin, Oltvai et al. 1994, Petros, Medek et al. 2001). It should be noted that the “BH3-only” proteins contain more BH domains than just BH3 – the BCL2 family classification system will be addressed in more detail in Section 1.5.2.
### Table 1.5-1 BCL2 subfamilies and members.

<table>
<thead>
<tr>
<th>Primary structure</th>
<th>BH- domain status</th>
<th>Role</th>
<th>Subfamily name</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BCL2</strong></td>
<td>BH4, BH3, BH1, BH2, TM</td>
<td>Anti-apoptotic</td>
<td>Multidomain “Anti-apoptotic”</td>
</tr>
<tr>
<td><strong>BCLXL</strong></td>
<td>BH4, BH3, BH1, BH2, TM</td>
<td>Anti-apoptotic</td>
<td></td>
</tr>
<tr>
<td><strong>MCL1</strong></td>
<td>BH3, BH1, BH2, TM</td>
<td>Anti-apoptotic</td>
<td></td>
</tr>
<tr>
<td><strong>BCLW</strong></td>
<td>BH4, BH3, BH1, BH2, TM</td>
<td>Anti-apoptotic</td>
<td></td>
</tr>
<tr>
<td><strong>BFL1/A1</strong></td>
<td>BH3, BH1, BH2</td>
<td>Executioners</td>
<td></td>
</tr>
<tr>
<td><strong>BCL2L12</strong></td>
<td>BH2</td>
<td>Anti-apoptotic</td>
<td></td>
</tr>
<tr>
<td><strong>BAX</strong></td>
<td>BH4, BH3, BH1, BH2, TM</td>
<td>Pro-apoptotic</td>
<td></td>
</tr>
</tbody>
</table>

- BH: Bcl-2 homology domain
- TM: Transmembrane domain

**Primary Structure Diagrams:**
- **BCL2:** BH4, BH3, BH1, BH2, TM
- **BCLXL:** BH4, BH3, BH1, BH2, TM
- **MCL1:** BH3, BH1, BH2, TM
- **BCLW:** BH4, BH3, BH1, BH2, TM
- **BFL1/A1:** BH3, BH1, BH2
- **BCL2L12:** BH2
- **BAX:** BH4, BH3, BH1, BH2, TM
Of the BCL2 family, the executioners are the gatekeepers to apoptosis. Whereas BAK (BCL2 antagonist killer) is constitutently localised to the mitochondrial outer membrane (MOM), BAX (BCL2 associated X protein) relocates here from the cytosol after activation (Wolter, Hsu et al. 1997). Once within the MOM and active, the executioners come together to form pores and trigger mitochondrial outer membrane permeability (MOMP) and therefore apoptosis (see Figure 1.5-1) (Martinou and Green 2001, Nechushtan, Smith et al. 2001, Chipuk, Kuwana et al. 2004).
Figure 1.5-1 BCL2 family members regulate MOMP.

BAX and BAK (represented by ovals) activation is regulated by the anti-apoptotic and BH3-only proteins. The release of cytochrome C triggers a cascade of cellular signalling which concludes with apoptosis. Image taken from (Gimenez-Cassina and Danial 2015).

Although there are many hypotheses regarding the precise roles of the BCL2 family members, it is generally agreed that in a state of cell survival, the BH3-only (i.e. pro-apoptotic) and anti-apoptotic members maintain the cell above an apoptosis commitment threshold by fulfilling a specific balance which holds the executioners in their inactivated state (Certo, Del Gaizo Moore et al. 2006). The balance of anti-apoptotic and BH3-only proteins can be altered by a number of signalling pathways, and can result in the cell’s commitment to apoptosis once the executioners are active (Igney and Krammer 2002) (van Delft and Huang 2006). Each of the BH3-only or anti-apoptotic proteins has a distinct pattern of activation,
localisation and responses to specific death/survival stimuli. All of these can vary across cell type, stress type and developmental stage; the nature of the BCL2 family is extensively complex.

1.5.1 Theories of BAX/BAK activation

There is some dispute over how exact regulation of BAX/BAK activation occurs. Significant points of controversy include; the hierarchy of anti-apoptotic and BH3-only proteins regarding BAX/BAK activation, BAX/BAK inhibition mechanisms (i.e. protein sequestering, oligomerisation blocking, active site occupation) and the specific role of the mitochondrial membrane (Table 1.5.1-1).
Table 1.5.1-1 Theories of BAX/BAK activation.
Executioners are depicted by ovals, BH3-only proteins by circles and anti-apoptotic proteins by semi-circles.

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Diagram</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct Activation</td>
<td>The executioners are directly activated by the binding of BH3-only proteins. The availability of these BH3-only proteins for executioner binding can be controlled by anti-apoptotic proteins. Neither the mitochondrial matrix nor the MOM is necessary for this interaction to be facilitated. (Kuwana, Mackey et al. 2002) (Vela, Gonzalo et al. 2013)</td>
<td><img src="image1.png" alt="Diagram" /></td>
</tr>
<tr>
<td>Indirect Derepressor</td>
<td>Although some BH3 proteins are capable of binding to BAX, apoptosis is not dependent on these interactions. Instead, BH3-only proteins interact with anti-apoptotic proteins, sequestering them and thus preventing their inhibition of BAX. (Willis, Fletcher et al. 2007)</td>
<td><img src="image2.png" alt="Diagram" /></td>
</tr>
<tr>
<td>Indirect Activation</td>
<td>BH3-only proteins can directly activate executioners.</td>
<td><img src="image3.png" alt="Diagram" /></td>
</tr>
<tr>
<td>Direct Derepressor</td>
<td>Alternatively, anti-apoptotic proteins can block the BH3 proteins from directly activating the executioners.</td>
<td><img src="image4.png" alt="Diagram" /></td>
</tr>
<tr>
<td>Indirect Derepressor</td>
<td>Anti-apoptotic proteins can block executioner activation; or they can be sequestered by BH3-only proteins.</td>
<td><img src="image5.png" alt="Diagram" /></td>
</tr>
<tr>
<td>Unified</td>
<td>Sequestering anti-apoptotic proteins allows executioners to become active:</td>
<td></td>
</tr>
<tr>
<td>---------</td>
<td>---------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td></td>
<td><img src="image1.png" alt="Diagram" /> Mode One: anti-apoptotic proteins can block executioners.</td>
<td></td>
</tr>
<tr>
<td></td>
<td><img src="image2.png" alt="Diagram" /> Mode Two: anti-apoptotic proteins can block BH3-only proteins.</td>
<td></td>
</tr>
<tr>
<td>Two Class</td>
<td>There are two modes of pro-survival activity, wherein the anti-apoptotic proteins have different binding properties. The prevailing mode within a cell can differ depending on cell and stress type. During Mode One, anti-apoptotic proteins bind BH3-only members. During Mode Two, they bind the executioners. Mode Two is less efficient in suppressing apoptosis than Mode One. (Llambi, Moldoveanu et al. 2011)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><img src="image3.png" alt="Diagram" /> Activators (a subtype of BH3-only proteins) can bind to and directly activate executioners.</td>
<td></td>
</tr>
<tr>
<td></td>
<td><img src="image4.png" alt="Diagram" /> Sensitisers (a subtype of BH3-only proteins) can bind to anti-apoptotic proteins, which stops the anti-apoptotic proteins from sequestering activators.</td>
<td></td>
</tr>
<tr>
<td>Two Class</td>
<td>The two class model suggests that the BH3-only subfamily can be further subdivided into sensitizers and activators. Whereas activators are capable of directly binding and activating BAX, sensitizers will bind BCL2. Because BCL2 can bind to and sequester activators, this sensitiser binding acts to liberate activators in a pro-apoptotic manner. (Letai, Bassik et al. 2002)</td>
<td></td>
</tr>
</tbody>
</table>
This theory emphasizes the significance of the mitochondrial membrane. BAX and BAK have multiple conformational states which occur in dynamic equilibrium. This equilibrium can be shifted by binding of BCL2 family members or other mitochondrial localised proteins, as well as by interactions with the mitochondrial membranes. It is only when BAX/BAK adopts a specific conformation that permeabilisation of the membrane occurs. (Leber, Lin et al. 2007)

The mitochondrial membrane is vital for all of the interactions of the BCL2 family.

1.5.2 BH and TM domains

The BH domains facilitate the family members’ interactions with each other and can indicate pro- or anti-apoptotic function (Adams and Cory 1998).

The BH1 and BH2 domains are the most highly conserved of the BH domains, and are found across the BCL2 family (Aouacheria, Rech de Laval et al. 2013). Interestingly, although the topology of the BH1-BH2 binding cleft is nearly identical across the anti-apoptotic subfamily, each member has variable specificity for pro-apoptotic binding partners (Kvansakul and Hinds 2013). The key differences in the binding cleft structure across members are described here (Lee, Dewson et al. 2014).

The BH3 domain is the best characterised of these domains; it is present in almost all pro-apoptotic members and may or may not be present in
anti-apoptotic members (Christos and Andreas 2012, Aouacheria, Rech de Laval et al. 2013). BH3 has four key hydrophobic residues that form an amphipathic helix. This helix is capable of binding a hydrophobic groove; most commonly formed by the BH1 and BH2 domains of other BCL2 family proteins (Kawatani, Uchi et al. 2003, Gurudutta, Verma et al. 2005). The domain plays a key role in the BH3-only proteins as it facilitates their interaction with both pro- and anti-apoptotic members (Aouacheria, Rech de Laval et al. 2013). It has been shown that it is necessary for interactions with and the activation of executioners (Willis, Chen et al. 2005, Willis, Fletcher et al. 2007). In some cases, interactions with anti-apoptotic members are pro-apoptotic: the anti-apoptotic protein is rendered unable to bind and block activation of executioners. In other cases, this interaction is anti-apoptotic: the BH3-only protein is rendered incapable of binding to and activating executioners (Table 1.5.1-1) (Willis, Fletcher et al. 2007, Llambi, Moldoveanu et al. 2011). It is difficult to generalise the effect of these interactions as it is dependent on individual family members.

Finally, the BH4 domain is the least well defined of the BH domains. Initial studies suggested that it is essential for anti-apoptotic function and that it is only present in anti-apoptotic BCL2 members (David, Jerry et al. 1998). However, ongoing redefinition of the BH4 sequence signature has seen this theory overturned as BH4 domains have been identified across the diversity of the BCL2 family (Aouacheria, Rech de Laval et al. 2013). The BH4 domain can be responsible for interactions with non-BCL2
family members, such as calcineurin (Shibasaki, Kondo et al. 1997), IP3R (inositol 1,4,5-triphosphate receptor, type 3) (Rong, Bultynck et al. 2009) and VDAC (voltage dependent ion channel) (Shimizu, Konishi et al. 2000).

The presence or absence of a TM domain can also determine BCL2 family function. Some of the interactions within the family are only facilitated when respective members are or embedded within the outer mitochondrial membrane, which can require the presence of a TM domain (Kale, Liu et al. 2012).

1.5.3 Alternative roles for anti-apoptotic BCL2 members

The similarity of BCL2 family members is somewhat perplexing; although some of the proteins have opposite functions, i.e. are pro- or anti-apoptotic, the exact differences in structure which account for these opposing functions are unknown. This mystery of the structure-function relationships within the BCL2 family is emphasised when we consider that both pre- and post-translational modifications (namely splicing and cleavage) can actually reverse, alter or abolish protein function (Boise, Gonzalez-Garcia et al. 1993) (Kirsch, Doseff et al. 1999). These multiple roles of anti-apoptotic family members are summarised in Table 1.5.3-1.
### Table 1.5-3-1 Roles within the anti-apoptotic BCL2 subfamily.

<table>
<thead>
<tr>
<th>Name of gene/Role of isoform</th>
<th>Gene splice control mechanism/ Name of isoform/ Primary structure</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BCL2</strong></td>
<td>Unknown</td>
</tr>
<tr>
<td>Anti-apoptotic</td>
<td>BCL2α</td>
</tr>
<tr>
<td>Unknown</td>
<td>BCL2β</td>
</tr>
<tr>
<td><strong>BCLX/ BCL2L1 (BCL2-like 1)</strong></td>
<td>There are several regulatory regions within $BCLX$ mRNA, and these can be broken down into four main regions (B2, B3, B1, and SB1). Research has also highlighted the importance of two ceramide responsive elements, as well as the internal ribosomal entry site (Massiello, Salas et al. 2004, Massiello, Roesser et al. 2006, Durie, Hatzoglou et al. 2013).</td>
</tr>
<tr>
<td>Anti-apoptotic</td>
<td>BCLXL (long)</td>
</tr>
<tr>
<td><strong>Pro-apoptotic; inhibits BCL2 from enhancing survival, generally expressed in cells with high turnover rate (Boise, Gonzalez-Garcia et al. 1993), can sensitise cells to chemotherapy agents (Sumantran, Ealovega et al. 1995).</strong></td>
<td><strong>BCLXs (short)</strong></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>MCL1 (myeloid leukaemia sequence 1)</strong></td>
<td><strong>MCL splicing is thought to be closely linked to cell cycle control (Moore, Wang et al. 2010, Gao and Koide 2013).</strong></td>
</tr>
<tr>
<td>Anti-apoptotic; expression increases when cells are exposed to cell-survival inducing tumourogenic compounds, expressed in viable cells (Bingle, Craig et al. 2000).</td>
<td><strong>MCL1L (long)</strong></td>
</tr>
<tr>
<td>Pro-apoptotic; transfection into epithelial and HeLa cells induces apoptosis (Bingle, Craig et al. 2000).</td>
<td><strong>MCL1s (short)</strong></td>
</tr>
<tr>
<td><strong>BCLW/ BCL2L2 (BCL2-like 2)</strong></td>
<td><strong>Unknown.</strong></td>
</tr>
<tr>
<td>Anti-apoptotic: increased expression confers resistance to multiple cytotoxic insults (Gibson, Holmgreen et al. 1996).</td>
<td><strong>BCLW (Isoform 1)</strong></td>
</tr>
<tr>
<td>Unknown.</td>
<td><strong>BCLW (Isoform 3)</strong></td>
</tr>
<tr>
<td><strong>BFL1/A1 (BCL2 related protein A1)</strong></td>
<td>Unknown.</td>
</tr>
</tbody>
</table>
Anti-apoptotic: Confers survival advantage in monocytes (Noble, Wickremasinghe et al. 1999), noted correlation between expression and stomach cancer development (Choi, Park et al. 1995).

Anti-apoptotic: The protein is localised to the nucleus and has anti-apoptotic activity (Ko, Lee et al. 2003).
1.6 The BCL2 gene

Many of the anti-apoptotic subfamily members and their respective isoforms and splicing mechanisms have been characterised, but BCL2β, an isoform of the first member of the BCL2 family to be discovered, is yet to be fully investigated. The BCL2 gene is capable of producing two proteins: BCL2α (commonly referred to as BCL2) and BCL2β.

The BCL2 gene was initially identified due to its role in B-cell lymphoma. A t(14:18) chromosomal translocation in the disease causes enhancement of BCL2α transcription, which confers a survival advantage to the cancerous cells as the protein is anti-apoptotic (Tsujimoto, Finger et al. 1984, Tsujimoto, Cossman et al. 1985, Tsujimoto 1989). The BCL2α protein is comprised of six α-helices with a hydrophobic groove at the surface, which is responsible for its binding to pro-apoptotic proteins. The BCL2 gene is comprised of three exons. The first two exons encode the four BH domains and the final exon encodes the TM domain that anchors the protein to intracellular membranes (Seto, Jaeger et al. 1988, Chen-Levy, Nourse et al. 1989). The two isoforms of BCL2, BCL2α and BCL2β, are depicted in Figure 1.6-1).
Figure 1.6-1 Primary protein structures of BCL2α and BCL2β.

This figure is based on experiments on BCL2 and the highly homologous BCLXL. It illustrates the similarities between the isoforms. BH1, 2 and 3 are required for heterodimerisation with BCL2 family members (Oltvai, Milliman et al. 1993, Yin, Oltvai et al. 1994, Muchmore, Sattler et al. 1996). Channels are formed by α-helices 5 and 6 (Schendel, Xie et al. 1997). Phosphorylation by MAPK8 (mitogen activated kinase 8) at specific residues between BH4 and 3 can modify binding to Beclin-1 (Wei, Pattingre et al. 2008). Caspase 3 cleavage at aa34-34 can destroy protein function (Kirsch, Doseff et al. 1999). The two proteins are identical until residue 196, wherein they differ by i) BCL2β lacking a transmembrane domain and ii) BCL2β having a specific C-terminal 9aa sequence (Tsujimoto and Croce 1986). Adapted from (Belka and Budach 2002).
1.6.1 Roles of BCL2α

Although it was for its role in apoptosis that BCL2α was originally discovered, the isoform is now known to be involved with numerous other pathways, such as DNA repair, endoplasmic reticulum (ER) stability/ Ca2+ levels and autophagy (Liu, Naumovski et al. 1997, Pattingre, Tassa et al. 2005, White, Li et al. 2005). These different roles of BCL2α are summarised below in Table 1.6.1-1.
<table>
<thead>
<tr>
<th>Cellular process</th>
<th>Description of feature</th>
<th>Role of BCL2/ BCL2α</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autophagy</td>
<td>Autophagy is a survival mechanism resorted to during starvation, wherein intracellular contents can be recycled for nutritional value.</td>
<td>BCL2α is capable of inhibiting autophagy via its interaction with Beclin-1, although only when localised at the ER membrane (Pattingre, Tassa et al. 2005).</td>
</tr>
<tr>
<td>Apoptosis via p53 (protein 53)</td>
<td>p53 is a major tumour suppressor.</td>
<td>BCL2α can prevent p53 from up-regulating pro-apoptotic genes. Interestingly, p53 can also negatively regulate BCL2α itself (Beham, Marin et al. 1997) (Bharatham, Chi et al. 2011).</td>
</tr>
<tr>
<td>Transcription factor (TF) control</td>
<td>TFs regulate gene expression.</td>
<td>BCL2 can regulate NFKb, AP1, CRE and NFAT by blocking them from entering the nucleus (Massaad, Portier et al. 2004).</td>
</tr>
<tr>
<td>T cell development</td>
<td>Adaptive immune cells which mature in the thymus.</td>
<td>Levels of BCL2 expression fluctuate according to the stage of T cell maturity (Gratiot-Deans, Ding et al. 1993, Veis, Sentman et al. 1993)</td>
</tr>
<tr>
<td>Regulation of Ca2+ at the ER</td>
<td>The ER is the central storage centre for Ca2+, a major cellular signalling molecule.</td>
<td>BCL2 is capable of modulating the activity of IP3R (a Ca2+ channel) (White, Li et al. 2005).</td>
</tr>
<tr>
<td>NER</td>
<td>NER repairs bulky, helix distorting DNA damage induced by UV irradiation.</td>
<td>Overexpression of BCL2 attenuates CPD removal and the stalling of DNA replication following exposure to UV light (Liu, Naumovski et al. 1997).</td>
</tr>
<tr>
<td>Break Excision Repair (BER)</td>
<td>BER occurs throughout the cell cycle to repair non-helix distorting lesions, such as mismatched or damaged single bases.</td>
<td>Overexpression of BCL2 down regulates BER via APE1 (AP endonuclease 1) blockage (Kuo, Shiah et al. 1999, Jin, May et al. 2006, Zhao, Gao et al. 2008).</td>
</tr>
<tr>
<td>Mismatch Repair (MMR)</td>
<td>MMR repairs bases which have been mis-incorporated during DNA replication and recombination.</td>
<td>BCL2 can inhibit MMR via its direct interaction with MSH2 (MutS protein homolog 2) (Youn, Cho et al. 2005) (Hou, Gao et al. 2007).</td>
</tr>
<tr>
<td>Double strand break (DSB) repair, non-</td>
<td>NHJE is a mechanism of DSB that re-joins short DNA overhangs</td>
<td>Cells with higher expression of BCL2 have lower rates of EJ and vice versa. This is thought to be</td>
</tr>
</tbody>
</table>
**homologous end joining (NHEJ)**

on the ends of broken DNA.

due to the ability of BCL2 to interact with KU proteins (which form a molecular scaffold for the DSB repair machinery) (Wang, Gao et al. 2008, Kumar, Kari et al. 2010). BCL2 can also regulate DSB via its interaction with BRCA1 (Laulier, Barascu et al. 2011).

**ds and ssDNA repair via PARP1**

PARP1 is required for ssDNA and dsDNA (double strand DNA) break repair.

BCL2 can relocate to sites on the chromatin where it can directly interact with and inhibit PARP1. This interaction can be disrupted by BH3-only proteins (and BH3 mimetic drugs) (Dutta, Day et al. 2012)

**Melanin synthesis**

Melanin synthesis (by melanocytes) is triggered by UV exposure. The pigment acts as a protective shield to the nucleus to reduce DNA damage.

Melanin synthesis has oxidative by-products; to prevent spontaneous apoptosis, BCL2 expression is increased in the local areas (and melanin synthesis is restricted to specialised compartments known as melanosomes) (Sermadiras, Dumas et al. 1997, Cichorek, Wachulska et al. 2013).

BCL2 is a downstream target of MITF (Microphthalmia-associated transcription factor), a major transcription factor for melanin synthesis enzymes (McGill, Horstmann et al. 2002).

### 1.6.2 Current evidence for a role of BCL2β

Despite the accumulation of evidence for the many roles of BCLα, there has been very little investigation into the role of BCL2β.

Functional protein studies on BCL2 tend to focus on the wildtype BCL2α.

Where the BCL2β isoform is addressed, it is assumed that a recombinant
version of a C-terminal truncated BCL2α shares the same function as BCL2β, as both lack the TM domain (Tsujimoto and Croce 1986).

Investigations which have assessed the significance of the TM domain on the capacity for BCL2 to: regulate apoptosis and p53 and to interact with BAX and BRCA1 all concluded that the domain was vital for the efficiency of BCL2 in these roles (Tanaka, Saito et al. 1993) (Froesch, Aime-Sempe et al. 1999) (Peng, Lapolla et al. 2009) (Laulier, Barascu et al. 2011). In contrast, some investigations that have concluded that the TM domain is not essential for function. These focused on the separate steps of apoptosis activation and/or apoptosis across different cell types (Borner, Martinou et al. 1994, Kawatani, Uchi et al. 2003).

Despite these inconsistencies in the literature, it is important to note that C-terminal truncated BCL2α does not accurately represent BCL2β, due to the isoform’s specific 9aa C-terminal sequence (see Figure 1.6-1).
1.7 Evolving disruption of the traditional research perspectives of the BCL2 family

1.7.1 Transmembrane domain functionality

The assertion that the TM domain is vital for a BCL2 family member’s function is easily disproved when we look to the other family members and their isoforms. Several of these lack the domain and yet still maintain their functionality in the cell (see Table 1.5-3-1). Interestingly, truncation of the C-terminal domain of MCL1 does not destroy its capacity to locate to specific sub-cellular compartments, it just modifies it (Perciavalle, Stewart et al. 2012). Indeed, the TM membrane is not necessary for the trafficking of a protein to an organelle. Recent functional studies on BCLXL concluded that the N-terminal amino acids 1-28 were sufficient for targeting to the mitochondria; not the C-terminus (McNally, Soane et al. 2013).

1.7.2 BH domain status

BH domain status is no longer considered predictive or indicative of protein function (Del Gaizo Moore and Letai 2013). Several other important sequences which can contribute to apoptotic activity have now been identified (Ko, Choi et al. 2007). The significance of the BH3 domain is being called into question (Kale, Liu et al. 2012) and pore forming activity is no longer considered to be exclusive to executioners (Peng, Lapolla et al. 2009, Landeta, Valero et al. 2014). Apoptotic function of BCL2 family members can be post translationally modified (Kutuk and Letai 2008).
1.7.3 Roles outside of apoptosis regulation

Many of the traditional roles of the BCL2 family are currently being revised. It is now well established that family members have roles outside of apoptosis. Discovery of such roles and indeed of the inherent diversity of BCL2 family function has led to two key hypotheses. Firstly, that the proteins serve out their non-apoptotic “day jobs” and will only revert to their apoptotic role following a call to arms i.e. cellular stress (Hetz and Glimcher 2008). Alternatively, it can be argued that the BCL2 family serves to monitor cellular status and thus inform apoptotic decision making (Laulier and Lopez 2012). Regardless of these hypotheses, it is clear that the traditional view of the BCL2 family as one locked into the regulation of MOMP is outdated.

1.7.4 Multiple isoforms with different functions

Adding another level of complexity is the fact that all of the BCL2 family members have multiple isoforms, most of which have alternate roles (Akgul, Moulding et al. 2004). Of particular interest are MCL1 and BCLX; both genes encode two proteins with opposing functions. While MCL1L and BCLXL are anti-apoptotic, MCL1s and BCLXs are pro-apoptotic (Boise, Gonzalez-Garcia et al. 1993, Bingle, Craig et al. 2000). This phenomena of isoform role diversity has long been appreciated, though its relevance to the BCL2 family and the development of future BCL2 targetting therapeutics is not widely addressed.
1.8 Targetting the BCL2 family

When we consider that many of the anti-apoptotic BCL2 family members were initially discovered in a cancer background, and also that the family has a key role in apoptotic regulation, it is unsurprising that attempts have been made to manipulate the anti-apoptotic BCL2 subfamily in a therapeutic context. These attempts include retrovirus systems (Kagawa, Pearson et al. 2000), the alteration of localisation apparatus (Bartholomeusz, Talpaz et al. 2007), activity blocking antibodies (Piche, Grim et al. 1998), RNAi (Agarwala, Keilholz et al. 2009) and miRNAs (Schultz, Lorenz et al. 2008). However, the most successful method of BCL2 family targetting is via BH3 mimetic molecules.

1.8.1 BH3 mimetics

Initial proof of concept experiments that highlighted the potential of BH3 mimetics showed that small molecules which bound to the hydrophobic groove of BCLXL could block its anti-apoptotic function (Wang, Liu et al. 2000). Since then, many different BH3 mimetics have been developed with the intention to lower the apoptotic threshold within the cell. These molecules are summarised in Table 1.8.1-1.
<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Detail for development</th>
<th>Targets</th>
<th>Stage Clinical Trials</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ABT-737</strong></td>
<td>AbbVie: Lead compound. Mimics BH3 domain of BAD. (Oltersdorf, Elmore et al. 2005)</td>
<td>BCL2, BCLXL, BCLW</td>
<td>Phase I/II</td>
</tr>
<tr>
<td><strong>Navitoclax/ ABT-263</strong></td>
<td>AbbVie: based on ABT-737 but has longer half-life and is orally available (Tse, Shoemaker et al. 2008). Currently in clinical trials.</td>
<td>BCL2, BCLXL, BCLW</td>
<td>Phase I/II</td>
</tr>
<tr>
<td><strong>ABT-199/ venetoclax</strong></td>
<td>AbbVie/ Genentech: Derivative of Navitoclax.</td>
<td>BCL2</td>
<td>Approved for use in chronic lymphocytic leukaemia in patients with specific c17 mutation.</td>
</tr>
<tr>
<td><strong>Gossypol/ BL-193/ AT-101</strong></td>
<td>Naturally occurring compound isolated from cotton seeds (Oliver, Miranda et al. 2005).</td>
<td>BCL2, BCLXL, BCLW, MCL1</td>
<td>Phase I/II</td>
</tr>
<tr>
<td><strong>Apogossypol</strong></td>
<td>Derivative of gossypol.</td>
<td>BCL2, BCLXL, BCL2, MCL1</td>
<td>Phase I/II</td>
</tr>
<tr>
<td><strong>Apogossypol/ ApoG2</strong></td>
<td>Ascenta. Derivative of gossypol: reduced toxicity and non-specific binding (Hu, Zhu et al. 2008).</td>
<td>BCL2, BCLXL, MCL1</td>
<td>Phase I/II</td>
</tr>
<tr>
<td><strong>Sabutoclax/ BI-97C1/ ONT-701</strong></td>
<td>Oncothyreon, Inc: Derivative of gossypol (Wei, Stebbins et al. 2010).</td>
<td>BCL2, BCLXL, BCLW, MCL1, Bfl-1</td>
<td>Phase I/II</td>
</tr>
<tr>
<td><strong>BI-97D6</strong></td>
<td>Derivative of gossypol (Wei, Stebbins et al. 2011).</td>
<td>BCL2, BCLXL, MCL1, Bfl-1</td>
<td>Preclinical</td>
</tr>
<tr>
<td><strong>TW-37</strong></td>
<td>Derivative of gossypol: has higher affinity to targets</td>
<td>BCL2, BCLXL</td>
<td>Phase I/II</td>
</tr>
<tr>
<td>Compound</td>
<td>Description</td>
<td>Molecules Involved</td>
<td>Stage</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>S1</td>
<td>Organic non-DNA intercalative compound (Zhang, Jin et al. 2007)</td>
<td>BCL2, BCLXL, MCL1</td>
<td>Preclinical</td>
</tr>
<tr>
<td>BH3-M6</td>
<td>Synthetic terphenyl scaffold which mimics BH3 α-helix (Kazi, Sun et al. 2011)</td>
<td>BCL2, BCLXL, MCL1</td>
<td>Preclinical</td>
</tr>
<tr>
<td>Maritoclax</td>
<td>Isolated from Streptomyces.</td>
<td>MCL1</td>
<td>Preclinical</td>
</tr>
<tr>
<td>MIM1</td>
<td>Result of screening for MCL1 binding activity (Kritzer 2012).</td>
<td>MCL1</td>
<td>Preclinical</td>
</tr>
<tr>
<td>BAM7</td>
<td>Direct activator of BAX (Gavathiotis, Reyna et al. 2012).</td>
<td>-</td>
<td>Preclinical</td>
</tr>
<tr>
<td>Obatoclax/GX15-070</td>
<td>Derivative of collection of BH3 mimetics developed by Cephlon (Parikh, Kantarjian et al. 2010).</td>
<td>BCL2, BCLXL, BCLW, MCL1</td>
<td>Phase I/Phase III in combination</td>
</tr>
<tr>
<td>HA14-1</td>
<td>Result of in silico screening (Wang, Liu et al. 2000).</td>
<td>BCL2, BCLXL</td>
<td>Preclinical</td>
</tr>
<tr>
<td>A-1155463</td>
<td>Discovered through NMR screening (Tao, Hasvold et al. 2014).</td>
<td>BCLXL</td>
<td>Preclinical</td>
</tr>
<tr>
<td>A-1210477</td>
<td>Derived from indole-2-carboxylic acid core (Bruncko, Wang et al. 2015, LeVerson, Zhang et al. 2015).</td>
<td>MCL1</td>
<td>Preclinical</td>
</tr>
</tbody>
</table>

Though a number of these BH3 mimetics have shown promising results in preclinical cancer models, only Navitoclax and ABT-199 (a derivative of Navitoclax) have made it through to clinical trials. These molecules...
have shown the greatest efficacy in haematological cancers. Navitoclax has been trialled in both chronic lymphoid leukaemia (CLL) (Roberts, Seymour et al. 2012) and non-Hodgkin lymphoma (NHL) (Wilson, O’Connor et al. 2010), but dosage escalation studies were limited by thrombocytopenia, thought to be a result of targetting BCLXL (Zhang, Nimmer et al. 2007). In contrast, ABT-199, which has reduced binding to BCLXL and BCLW compared to Navitoclax, does not induce platelet deficiency (Souers, Levenson et al. 2013). ABT-199 is currently in clinical trials for CLL, NHL and multiple myeloma (Anderson, Huang et al. 2014, Cang, Iragavarapu et al. 2015).

As for the other BH3 mimetics, at least 20 clinical trials for gossypol (the first naturally occurring BH3 mimetic) alone have been conducted, yet none of these therapies have been approved for cancer treatment (Vela and Marzo 2015).

The limited success of this group of molecules is attributed to high toxicity due to off target affects, as well as the co-incidental induction of autophagy, which can protect cancerous cells against apoptosis (Malik, Orhon et al. 2011) (Rahmani, Aust et al. 2012). These unpredicted effects have driven the search for and optimisation of BH3-memetic response prediction methods. More recently, investigations have highlighted the importance of looking beyond the profiling of anti-apoptotic subfamily members (Certo, Del Gaizo Moore et al. 2006, Yamaguchi, Janssen et al. 2011, Renault, Elkholi et al. 2014). This includes looking at pro-apoptotic members, the specific interactions between BCL2 family
members and the conformational changes of the proteins that occur throughout these interactions.

Repeated studies have highlighted the significance of low MCL1 expression conferring sensitivity to BH3 mimetics in cell lines (Yamaguchi and Perkins 2011) (Suryani, Carol et al. 2014). Unsurprisingly therefore, a major shortcoming of BH3 mimetics is their failure to effectively target MCL1L in the cases where tumour growth is MCL1L dependent. It is considered that MCL1L is the most potent of the BCL2 family as it has a particularly high affinity with pro-apoptotic members (Willis, Chen et al. 2005). Silencing of MCL1 has been found to elicit a pro-apoptotic response in a number of models (Senft, Berking et al. 2012, Wang, Wu et al. 2014). Due to this potency and high turnover however, targetting MCL1L therapeutically has proved difficult (Czabotar, Lessene et al. 2014). Although a number of molecules with the potential for binding MCL1L have been developed, none of them have had sufficient binding affinity to be functionally effective at a clinically feasible dose (Leverson, Zhang et al. 2015). The BH3 mimetic A-1210477, developed in 2015, is the first molecule proposed to act directly on MCL1L to promote apoptosis in cell lines (Leverson, Zhang et al. 2015) (Besbes and Billard 2015).

As with the many approaches to chemotherapy, combination therapy is thought to hold the most promising potential for BH3 mimetics. For example, a study on the longevity and mutational effects of targetted therapy revealed that resistance to BRAF inhibitors in melanoma cell
lines can be partially rescued with administration of the BH3 mimetic, ABT-737 (Perna, Karreth et al. 2015). Several studies have also highlighted the success of combining BH3 mimetics with earlier immunotherapy trials (Begley, Vo et al. 2009, Karlsson, Lindqvist et al. 2013).

1.8.2 Splicing

As described in Table 1.5.3-1, splicing can modify BCL2 family member function. Manipulation of gene splicing in favour of their pro-apoptotic transcripts (i.e. $BCLX$ in favour of $BCLX_s$ and $MCL1$ in favour of $MCL1_s$) holds the most therapeutic potential. However, targetting splicing in a clinical context has only been realised and explored recently due to a number of technological difficulties (Juliano 2016).

Introduction of oligonucleotides which effectively alter splicing of $BCLX$ from $BCLXL$ to $BCLX_s$ in cells in culture and mice with melanoma xenografts has been shown to grant in anti-tumour benefits (Bauman, Li et al. 2010). In addition, antisense morpholino oligonucleotides (AMOs) have been designed to knock down exon 2 in the pre-mRNA of $MCL1$, thereby increasing the expression of $MCL1_s$ compared to that of $MCL1L$ (Shieh, Liu et al. 2009).

Another way to manipulate splicing is by targetting SAP155 (spliceosome associated factor 155), a splicing factor which acts on $MCL1$ and $BCLX$. Inhibition of this protein by meamycin B has been used to switch splicing in favour of $MCL1_s$ in cell culture. Interestingly, the combination of
meaymycin B with ABT-737 also induces apoptosis (Gao and Koide 2013). The activity of SAP155 has also been successfully blocked using an anti SAP155-antibody. Like the aforementioned study, manipulation of the protein’s activity primed the cell for response to apoptosis inducing treatment (Massiello, Roesser et al. 2006).
1.9 BCL2 and melanoma

The involvement of the BCL2 family in cell death has led to investigation in the context of multiple cancers. BCL2 is of particular interest in melanoma due to its part in melanin synthesis and melanocyte development (McGill, Horstmann et al. 2002) (Table 1.6.1-1). Research regarding the role of BCL2 in melanoma is reviewed in Table 1.9-1.

Table 1.9-1. Evidence for the role of BCL2 in melanoma development and progression. (IHC=immunohistochemistry)

<table>
<thead>
<tr>
<th>Study reference</th>
<th>Cohort numbers</th>
<th>Methods</th>
<th>Conclusions</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Grover and Wilson 1996)</td>
<td>Tissue samples: 42 metastatic</td>
<td>Protein analysis, Flow cytometry analysis of cells which had been immunostained using unspecified BCL2 antibody</td>
<td>Patients whose tumours expressed BCL2 (30% of the cohort) protein had significantly shorter survival than those whose tumours did not.</td>
</tr>
<tr>
<td></td>
<td>Tissue samples and cell lines:</td>
<td>Protein analysis, Western Blotting using unspecified BCL2 antibody</td>
<td>BCL2 was expressed in the majority of metastatic tissue and in the majority of melanocytic cell lines. There was no difference in BCL2 protein expression between metastatic tissue and melanocytes.</td>
</tr>
<tr>
<td></td>
<td>12 metastatic tumour tissue samples, 5 melanocyte cell lines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Tang, Tron et al. 1998)</td>
<td>Tissue samples: Western Blotting: 10 metastatic</td>
<td>Protein analysis, Western Blotting and IHC using unspecified BCL2 antibody</td>
<td>Overall levels of BCL2 protein expression were lower in metastatic tissue compared to primary.</td>
</tr>
</tbody>
</table>
(Leiter, Schmid et al. 2000)

| Tissue samples: | RNA and protein analysis | RNA: A significantly higher number of metastatic tissue samples compared to primaries expressed BCL2α transcript. This difference was not extensive enough to predictably discriminate between tissue types.
| q-PCR: 16 normal, 15 nevi, 13 primary, 16 metastatic | q-PCR targeting BCL2α transcript, IHC using unspecified BCL2 antibody |
| IHC: 5 normal, 10 nevi, 9 primary, 29 metastatic | Protein: No significant difference in BCL2 expression between tissue types. |

(Sviatoha, Rundgren et al. 2002)

| Tissue samples: | Protein analysis | Expression of BCL2 was positively correlated with survival. |
| 90 metastatic | IHC using unspecified BCL2 antibody |

(Utikal, Leiter et al. 2002)

| Tissue samples: | RNA and protein analysis | RNA: BCL2 was expressed in more metastatic tissue samples (90%) than primary tissue samples (50%). Protein: BCL2 was expressed in more metastatic tissue samples (71%) than primary tissue samples (38%). |
| q-PCR: 4 normal, 20 nevi, 20 primary, 15 surgical margins, 30 metastatic | q-PCR targeting both BCL2 transcripts, and IHC using unspecified BCL2 antibody |
| IHC: 7 normal, 26 nevi, 7 surgical margins, 50 primary, 34 metastatic |

(Gradilone, Gazzaniga et al. 2003)

| Tissue samples: | RNA and protein analysis | There was no evidence of there being a relationship between BCL2 protein expression and the progression to metastatic disease. |
| 36 metastatic: 36/36 used for RNA studies, 17/36 used for protein studies | q-PCR targeting BCL2α transcript only, IHC using unspecified BCL2 antibody |

(Zhang and Rosdahl 2006)

| Tissue samples: | Protein analysis | Total BCL2 consistently higher (across all samples) in metastatic compared to primary tissue. |
| Seven (7) matched primary and metastatic samples | IHC using BCL2 antibody predicted to recognise both BCL2 isoforms |
### (Fecker, Geilen et al. 2006)

<table>
<thead>
<tr>
<th>Tissue samples:</th>
<th>Protein analysis</th>
<th>There was no evidence of there being a relationship between BCL2 protein expression and the progression to metastatic disease.</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 primary tissue samples: of which 12 patients has survived the 10 year follow up period and 12 had developed metastases and died.</td>
<td>IHC using BCL2 antibody predicted to recognise both BCL2 isoforms</td>
<td></td>
</tr>
</tbody>
</table>

### (Zhuang, Lee et al. 2007)

<table>
<thead>
<tr>
<th>Tissue samples:</th>
<th>Protein analysis</th>
<th>BCL2 protein expressed in 100% of thin primaries and 90% of thick primaries. This is in comparison to 63% of subcutaneous and 35% of lymph node metastatic tissue.</th>
</tr>
</thead>
<tbody>
<tr>
<td>18 thin primaries (&lt;1.0mm), 24 thick primaries (&gt;1.0mm), 38 metastatic: 21 subcutaneous, 17 lymph node</td>
<td>IHC using BCL2 antibody predicted to recognise both BCL2 isoforms</td>
<td></td>
</tr>
</tbody>
</table>

### (Tas, Duranyildiz et al. 2008)

<table>
<thead>
<tr>
<th>Patient serum:</th>
<th>Protein analysis</th>
<th>Serum levels of circulating BCL2 in patients was significantly higher than in healthy controls. Serum BCL2 was also elevated in stages III and IV compared to earlier stages of disease.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum taken from 51 patients with metastatic melanoma and 18 healthy controls</td>
<td>ELISA using unspecified BCL2 antibody.</td>
<td></td>
</tr>
</tbody>
</table>

While some studies show that BCL2 expression is higher in metastatic disease than primary (Leiter, Schmid et al. 2000, Utikal, Leiter et al. 2002, Zhang and Rosdahl 2006), other studies demonstrate the opposite (Tang, Tron et al. 1998, Zhuang, Lee et al. 2007). Similarly, there is no agreement in the literature as to whether expression of BCL2 is beneficial or detrimental in terms of survival (Grover and Wilson 1996, Sviatoha, Rundgren et al. 2002). These inconsistent patterns of expression in melanoma tissue are most likely due to the highly heterogeneous nature and plastic tendencies of the disease (Hartman and Czyz 2013). In
addition, while this table demonstrates that there has been research into the role of BCL2 in melanoma, none of the studies have made a distinction between the BCL2 isoforms. These discrepancies in the literature could be influenced by this oversight.
1.10 Project Rationale

Investigations into improvement of therapies for melanoma, which up until the past decade were limited to platinum based therapies, are ongoing. Although targeted therapies and immunotherapies do hold promise, there is still a subset of patients which are not eligible for these therapies or have developed resistance.

As discussed, the BCL2 family is involved in the regulation of apoptosis and therefore plays a vital role in protecting the body against cancer. Targetting the apoptotic pathway directly is a valid option for improving or developing new chemotherapies.

The anti-apoptotic BCL2 subfamily is of particular interest as most of its composite members have a variety of splice variants with an array of different roles within the cell. Most of these splice variants have been extensively characterised; except for BCL2β.

The *BCL2* gene is especially relevant to melanoma cancers as it is involved with melanin regulation and melanocyte development. Studies on the role of *BCL2* in melanoma have been inconclusive and, in some ways, contradictory. This may be due to the heterogeneity of melanoma tissue, but also due to the tendency for researchers to ignore *BCL2* splice variants when investigating protein as well as mRNA.

It is with this rationale in mind that we intend to investigate the roles of both BCL2 isoforms in the context of melanoma.
1.11 Hypotheses & Aims

We broke down our hypotheses and aims into separate chapters, the structure of which are outlined as follows:

1.11.1 Chapter Two: Confirmation of expression of the BCL2 isoforms

Major hypothesis:
- Both isoforms of BCL2 are present at both the mRNA and protein levels in melanoma lines.

Key aims:
- Refine methodologies for the quantification of both BCL2 isoforms at the i) mRNA and ii) protein level for further application in these investigations.
- Assess expression of both BCL2 isoforms at the i) mRNA and ii) protein level within melanoma cell lines.
1.11.2 Chapter Three: \textit{BCL2} isoform expression in melanoma

Hypotheses:
- \textit{BCL2} isoforms have different associations with melanoma clinical parameters and TILs.
- \textit{BCL2} expression is elevated in metastatic melanoma tumour tissue in comparison to primary tissue.
- SNP rs3943258 is associated with \textit{BCL2} isoform mRNA expression.

Key aims:
- Confirm mRNA and protein expression of \textit{BCL2} isoforms across our cohort of melanoma tissue.
- Interrogate the relationship between the SNP rs3943258 \textit{BCL2} polymorphism and expression of the \textit{BCL2} isoforms within the cohort.

1.11.3 Chapter Four: Functional analysis of the \textit{BCL2} isoforms

Hypotheses:
- The two \textit{BCL2} isoforms have different roles in the apoptotic response.
- p53 is capable of regulating both isoforms in response to different stimuli.
- Melanin synthesis will differ between the knockdown (KD) conditions of the two \textit{BCL2} isoforms.

Key aims:
- Measure \textit{BCL2} isoform transcript expression throughout apoptosis induction.
- Knock down both \textit{BCL2} isoforms separately prior to the induction of apoptosis and melanin synthesis.
Chapter Two: Confirmation of expression of \textit{BCL2} isoforms

2.1 Introduction

As addressed in Section 0, the \textit{BCL2} gene has two isoforms which are rarely studied separately. The purpose of this chapter is to confirm the expression of the isoforms at both the mRNA and protein level.

Initial interest in the two isoforms was piqued following analysis of an investigative gene expression microarray experiment (Figure 2.4.1-1). Within this preliminary dataset, differential expression of the two isoforms was observed and marked the beginnings of these investigations.

2.1.1 Quantifying mRNA expression

Real-time PCR (quantitative PCR; qPCR) was used to confirm the expression of the isoforms; for more detail on the discrepancies and differences between gene expression microarray assays and qPCR, see Section 2.5.1.

qPCR is a PCR based method of mRNA quantification; it is dependent on the annealing of primers or probes to their targetted cDNA sequence. TaqMan Gene Expression Assays (often referred to as simply, ‘probes’) contain forward and reverse primers, as well as a gene specific probe labelled with a reporter fluorescent dye and a quencher. The primers bind their target sequence at the 3’ and 5’ ends, and the probe binds in the middle. As the elongation phase occurs, the probe is displaced and cleaved, causing a detectable fluorescent signal. As the cycles of PCR
continue, the number of targetted cDNA strands grows exponentially. By dividing the fluorescence of the reporter dye by the fluorescence of a passive reference dye (i.e. background), we generate a value for \( \Delta Rn \). Plotting \( \Delta Rn \) against cycle number generates an amplification plot. The \( \Delta Rn \) value at the threshold crossover (i.e. wherein the curve enters the exponential phase) gives us the Ct value. Comparing the Ct value of a gene of interest to that of a steady state housekeeping gene gives us the relative gene expression (Nolan, Hands et al. 2006).

Single stranded conventional primers can also be used in conjunction with a fluorescent polymerase (i.e. SYBR Green) as an alternative to TaqMan assays. Under these conditions, the formation of a double stranded complex causes the fluorescent signal which is quantified by the qPCR machine and used to calculate relative expression. Primers are marginally less efficient and specific than TaqMan Assays. This is because the fluorescent signal is associated with the formation of any kind of double strand, as opposed to only that associated with the binding of targetted primers with their mRNA sequence. Primer dimer formation (wherein primers bind to themselves) can occur and interfere with the read-out of the result. For this reason, it is important to analyse the amplification curve in conjunction with a melt curve, wherein primer dimers can be visualised (Rodriguez, Rodriguez et al. 2015).
2.1.2 Quantifying protein expression

2.1.2.1 Western blotting and immunoprecipitation

Traditionally, western blotting is used to detect the presence of a protein within a biological sample. This method takes advantage of an individual protein’s specific i) size and charge and ii) unique surface structure.

Proteins within the sample are first separated on an acrylamide gel according to their size and relative charge. The gel is transferred to a nitrocellulose blot which can be probed with labelled antibodies targetted to the protein of interest (Renart, Reiser et al. 1979).

Because of the upper limit on the loading volume within acrylamide gel wells, samples wherein the target protein is only lowly expressed have to be enriched for the target protein prior to western blotting analysis. This can be done using immunoprecipitation (IP), wherein antibodies can be used to pull-down the protein of interest from a sample and thus increase its concentration prior to western blot analysis (Bonifacino, Dell'Angelica et al. 2001).

For rarely studied or new proteins, expression typically has to be confirmed following western blot analysis. This is achieved by analysing the sample using mass spectrometry (MS).

2.1.2.2 Mass spectrometry

There are many different types of MS which can be used to analyse the protein components of a biological sample (Mehmood, Allison et al. 2015).
The first step of MS is fragmentation of proteins into peptide fragments via addition of cleavage enzymes. These peptides are then separated according to size via liquid chromatography. A high voltage is applied to create an aerosol of ionised peptides: this process is known as electrospray ionisation (ESI). The ionised peptide fragments can be analysed downstream based on their mass/charge (m/z) ratio (Mehmood, Allison et al. 2015).

2.1.2.2.1 Ion trap mass spectrometry

This method, where used for confirming the approximate size and presence of a specific protein, is dependent on immunoprecipitation (IP) and therefore antibodies.

Ion trap MS uses electric fields directed via a cylindrical ring electrode to scatter ionised peptide fragments (see Figure 2.1.2.2.1-1). The machine’s detector records the fragments’ m/z values such that their precursor proteins can be identified. This is done by matching the m/z values are matched to their specific peptide fragment fingerprint in a database of known m/z ratios (Mehmood, Allison et al. 2015).
Figure 2.1.2.2.1-1 An ion trap. The ions can be maintained stably within the device or their trajectories can be disrupted (such that they are deflected to the detector) via application of a voltage to the ring electrode. Adapted from (Glish and Vachet 2003).

This form of MS is appropriate for identifying multiple proteins within a complex mixture, though it is not ideal for detecting small quantities of protein. In addition, the method is limited by the database used for protein identification – if it does not contain the proteins of interest then they will not be detected.

2.1.2.2.2 Multiple reaction monitoring mass spectrometry

Multiple reaction monitoring (MRM) MS is performed using a triple quadrupole instrument (Figure 2.1.2.2.2-2). The apparatus scans a very specific mass range, which is in contrast to full scanning MS techniques, such as that described previously (in Section 2.1.2.2.1). Prior to running the experiment, signature peptides for the proteins of interest are selected in silico by performing a theoretical trypsin digest. It must be
ensured that the peptides are unique to the proteins of interest. This can be achieved by performing a protein BLAST (Basic Local Alignment Search Tool) (Karlin and Altschul 1990, Karlin and Altschul 1993, Altschul, Madden et al. 1997, Shevchenko, Sunyaev et al. 2001). These peptide fragments (also called the precursor ions) of interest are selected in the first quadrupole (based on their m/z value), which behaves as a filter. The precursor ions are further fragmented via collision induced dissociation in the second quadrupole. The predefined transition pairs (i.e. a precursor ion and its respective fragment peptide ions) of interest are filtered in the third quadrupole. This step must be optimised such that the fragment ions with optimal signal intensity are selected. The instrument detector monitors the transitions and generates a plot of retention time vs signal intensity for each ion (Liebler and Zimmerman 2013). This plot can be used to relatively quantify the protein of interest, by measuring the area under the curve and comparing this value to the total volume of protein loaded onto the machine. In order to absolutely quantify the protein, known quantities of synthetic heavy labelled versions of the signature peptides can be run alongside the sample. This method is preferred as it is not dependent on antibodies and its lower limit for detection is up to 100x lower than that of alternative MS methods (Lange, Picotti et al. 2008).
Figure 2.1.2.2.2-1 Schematic diagram of a triple quadrupole mass spectrometer.

Samples are fed into the triple quadrupole instruments via ESI processing. Precursor ions or ‘signature ions’ are selected in the first quadrupole. Further fragmentation occurs in the second quadrupole and fragment ions or ‘transition pairs’ are selected in the final quadrupole. Image taken from (Lange, Picotti et al. 2008).
2.2 Hypotheses & Aims

Although many of the members of the BCL2 family have well described isoforms, the isoforms of BCL2 itself are rarely acknowledged (see Section 1.5). Despite the presence of the BCL2 splice variants in various NCBI databases, only BCL2α has been characterised. This is in stark contrast to BCL2β, which has not been investigated since its initial discovery in the 1980s (Tsujimoto and Croce 1986).

We hypothesise that:

- Both isoforms of BCL2 are present at both the mRNA and protein levels in melanoma cell lines.

Within this next chapter we aim to:

- To refine methodologies for the quantification of both BCL2 isoforms at the i) mRNA and ii) protein level for further application in these investigations.

- To assess expression of both BCL2 isoforms at the i) mRNA and ii) protein level within melanoma cell lines.
2.3 Methods

2.3.1 Cell culture

Melanoma (MM200, SkMel28, Me4405 and MelRM) and melanocyte (HeMn; human epidermal neonatal melanocytes, medium pigmented; Cascade Biologics) cell lines were used in this study. MM200 was isolated from a primary melanoma (Thomas and Hersey 1998). SkMel28 was isolated from a metastatic melanoma tumour and has been identified as p53 mutant – its derivation is described elsewhere (Avery-Kiejda, Zhang et al. 2008, Bowden, Ashton et al. 2013). Me4405 is derived from a primary melanoma and has been reported to lack p53 expression (Avery-Kiejda, Zhang et al. 2008). MelRM was isolated from a fresh surgical biopsy of metastatic melanoma in the bowel of a patient attending the Sydney/Newcastle Melanoma Units (Franco, Zhang et al. 2001). The melanoma cell lines were maintained in high-glucose Dulbecco’s modified Eagle’s medium (DMEM) (HyClone) supplemented with 10% fetal bovine serum (FBS) (Interpath), a 5% penicillin/streptomycin solution (BioWhittaker) and 5% sodium bicarbonate (HyClone) at 37°C and 5% CO₂. The melanocytes were maintained in Medium 254 supplemented with HMGS (Human melanocyte growth supplement) (ThermoFisher) at 37°C and 5% CO₂.

To remove cells from flasks or wells, the cells were rinsed with PBS (phosphate buffered saline) prior to a 5 minute incubation (37°C, 5% CO₂) with 5ml 2x 0.5% Trypsin-EDTA dissociation reagent (Gibco). Appropriate cell culture media was added to neutralise the trypsin and...
prevent cell rupture. In order to maintain cell lines, pelleted cells were sub-cultivated at a ratio between 1:2 and 1:8. Throughout prolonged periods wherein cells were not necessary for experimentation, cells were frozen in DMEM with 10% DMSO and stored in liquid nitrogen.

2.3.2 Microarrays

All cell lines under interrogation were treated with 10µg/ml cisplatin and harvested before treatment and at 6 and 24h after treatment for gene expression analysis (Bowden, Ashton et al. 2013).

RNA extracted from the cell lines was amplified, biotinylated and hybridised to Whole Genome Gene Expression Human Ref8 V3 BeadChip (Illumina). The chips were scanned using a Bead Array Reader (Illumina). The transcript expression results were cubic spline normalised using BeadStudio 2.0 software (Illumina), and the remaining analyses was performed using GeneSpring GX 11.0. To account for bias or skewing of expression results, all the gene expression profiles and each individual gene were normalised to the median resulting in two way normalisation. This data is available for public access in the Gene Expression Omnibus repository under accession number GSE47980. For details regarding the probe sequences for $BCL2\alpha$ and $\beta$, see Figure 2.3.2-1.
Figure 2.3.2-1 Location of BCL2α and BCL2β microarray probes mapped to the mRNA transcripts.

Nucleotide BLAST result of the a) BCL2α probe 4150201 and b) BCL2β probe 3870474 shows the locations of the probe/primers relative to the BCL2 transcripts, c) where the shaded circle indicates BCL2α and the non-shaded circle indicates BCL2β. (probe references according to Illumina HumanRef-8 v3.0) (Karlin and Altschul 1990, Karlin and Altschul 1993, Altschul, Madden et al. 1997)
2.3.3 RNA extraction from cell lines

Cell pellets were resuspended in 1 ml Trizol (ThermoFisher) and lysed by passage through a 19-gauge syringe. Lysates were incubated for a minimum of 5 minutes before 200 μl chloroform was added to each one. Samples were then shaken vigorously for 15 seconds and incubated for a further 3 minutes at room temperature (RT). Phase separation was induced by centrifugation at 10,000 RPM, 4°C, 15 minutes.

For the melanoma cell samples, the uppermost (aqueous) layer was removed and combined with 500 μl isopropanol and incubated at 10 minutes, RT. Samples were centrifuged at 10,000 RPM, 4°C, 5 minutes. RNA pellets were washed with 1 ml 75% ethanol (8000 RPM, 4°C, 5 minutes) and the remaining pellet was resuspended in 20 μl RNase free water.

Melanocyte samples were processed using RNeasy kits (QIAGEN), as per manufacturer’s instructions.

*RNA clean-up* In order to minimise melanin contamination (melanin is a PCR inhibitor) (Eckhart, Bach et al. 2000), clean-up was performed on the melanocyte RNA samples using One-Step PCR inhibitor removal kits (Zymo). The final step of this process was to clean the column with 100 μl RNase free water – to increase the final RNA concentration, samples were partially dehydrated by vacuum centrifuge.

Samples were processed for reverse transcription immediately or stored at -80 °C.
**RNA quantification** Final RNA concentration was determined using the Qubit RNA BR or HS Assay Kit (ThermoFisher), a fluorometric quantitation method.

### 2.3.4 Reverse transcription

A final volume of 1μg RNA was reverse transcribed using High Capacity cDNA Reverse Transcription Kits (Applied Biosystems) as per manufacturers’ instructions (see Appendix 7.1).

### 2.3.5 qPCR

All qPCRs were performed on the ViiA7 (Applied Biosystems). Test genes (*BCL2α, BCL2β*) were normalised to housekeeping genes (*GAPDH, 18S RNA, β-ACTIN*) to quantify relative expression. qPCRs requiring custom made primers were carried out using SYBR Green Dye (Applied Biosystems), whereas reactions using TaqMan probes (see Table 2.3.5-1) were carried out with TaqMan Fast Advanced Master Mix (Applied Biosystems). For TaqMan qPCR experimental details, see Appendix 7.2.

Biological and technical replicates were carried out in triplicate. Quality control was set at standard error <0.33 for relative expression measured using TaqMan probes and standard error <1 for relative expression measured using primers. A threshold for Ct value was set at 40 cycles – where the Ct value was above this threshold, expression was considered to be null.
Table 2.3.5-1 Details of TaqMan probes and primers.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers/ TaqMan Probe</th>
<th>TaqMan Probe ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>TaqMan</td>
<td>4326317E</td>
</tr>
<tr>
<td>18S RNA</td>
<td>TaqMan</td>
<td>4319413E</td>
</tr>
<tr>
<td>β-ACTIN</td>
<td>TaqMan</td>
<td>4326215E</td>
</tr>
<tr>
<td>BCL2α</td>
<td>TaqMan (Section 2.3.6)</td>
<td>Hs00608023_m1</td>
</tr>
<tr>
<td>BCL2β</td>
<td>Primers</td>
<td>NA</td>
</tr>
</tbody>
</table>

2.3.6 Primer design and optimisation

Specific BCL2β TaqMan probes are not yet available. Primers were therefore designed as specified in Table 2.3.6-1 and Figure 2.3.6-1. To ensure optimal primer efficiency and PCR conditions, a standard curve was generated using an identical cDNA source to perform a 5-fold serial dilution (Figure 2.4.2-3). Optimal qPCR conditions were achieved when the slope was >-3 and R² >0.95 (Figure 2.4.2-1). For SYBR Green qPCR experimental details, see Appendix 7.3.
Table 2.3.6-1 Sequence information for BCL2β primers.

<table>
<thead>
<tr>
<th>Forward/Reverse</th>
<th>Sequence (5’-&gt;3’)</th>
<th>GC%</th>
<th>Tm (°C)</th>
<th>Length (bp)</th>
<th>Self-3’ complementarity</th>
<th>Self-5’ complementarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>TTGGTGATGTGA</td>
<td>55</td>
<td>59.96</td>
<td>20</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>R</td>
<td>TTTATTCGCCCAGTCCACA</td>
<td>50</td>
<td>60.32</td>
<td>20</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>
Figure 2.3.6-1 Location of $BCL2\alpha$ probe and $BCL2\beta$ primers mapped to the mRNA transcripts.

Nucleotide BLAST result of the a) TaqMan $BCL2\alpha$ probe and b) the primers designed to target $BCL2\beta$ shows the locations of the probe/primers relative to the $BCL2$ transcripts, where the shaded circle indicates $BCL2\alpha$ and the non-shaded circle indicates $BCL2\beta$. (Karlin and Altschul 1990, Karlin and Altschul 1993, Altschul, Madden et al. 1997)
2.3.7 Antibodies

At the time of these investigations, no antibodies specific to BCL2β were available. However, the AbCam ab692 antibody targets a region which is present in both BCL2 isoforms: see Figure 2.3.7-1.
Figure 2.3.7-1 Location of the synthetic peptide sequence used to raise the AbCam antibody mapped to the BCL2 isoforms.

Protein BLAST result of the synthetic peptide sequence used to raise the AbCam antibody showing a hit for a) BCL2α and b) BCL2β. Figure c) shows the locations of the AbCam antibody targeting region relative to the primary sequences of the BCL2 isoforms (Karlin and Altschul 1990, Karlin and Altschul 1993, Altschul, Madden et al. 1997).
2.3.8 Western blotting

**Cell harvest:** Cells were grown to confluency (approximately 5x10^6 cells/sample) prior to harvesting (see Section 2.3.1). Cell pellets were washed 2x in ice cold PBS prior to immediate processing or freezing at -80°C.

**Protein processing with RIPA:** Cell pellets were thawed on ice prior to adding approximately 4x volume of chilled RIPA lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% Na deoxycholate, 0.1% SDS + protease inhibitor). Cells were then lysed mechanically using a 23-gauge needle and syringe. Samples were kept on ice, vortexed every 15 minutes for 45 minutes and centrifuged for 5 minutes, 12,000RPM, 4°C. The supernatant protein extract was stored at -80°C or immediately processed.

**Protein processing with NucBuster Protein Extraction Kit (Merck Millipore):** The samples were processed as per the manufacturer’s instructions to generate cytoplasmic and nuclear protein fractions.

**Protein quantification:** Protein was quantified using the colorimetric Pierce BCA Protein Kit (ThermoFisher) and the Epoch microplate spectrophotometer (Biotek).

**Sample preparation for gel loading (non-immunoprecipitation samples):**
Prior to loading protein samples in the gel (12%) (see Table 2.3.8-1 and Table 2.3.8-2 for gel components) or 20% Mini-Protean TGX Gel (BioRad), 20-50ug protein was mixed with an equal volume of 2x sample loading buffer (10% glycerol, 5% B-mercaptoethanol, 3% SDS, 0.06M
Tris-HCl pH 6.8, 0.1% Bromophenol Blue) and boiled at 100°C for 5 minutes. Samples were run between 100-150V.

**Table 2.3.8-1 Components of western blot resolving gel.**

<table>
<thead>
<tr>
<th>Volume (ml)</th>
<th>12%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 gel</td>
</tr>
<tr>
<td>H₂O</td>
<td>2.034</td>
</tr>
<tr>
<td>30% Acrylamide</td>
<td>2.40</td>
</tr>
<tr>
<td>Resolving Gel Buffer</td>
<td>1.50</td>
</tr>
<tr>
<td>10% APS</td>
<td>0.060</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.006</td>
</tr>
</tbody>
</table>
Table 2.3.8-2 Components of western blot stacking buffer gel.

<table>
<thead>
<tr>
<th></th>
<th>1x 1mm gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>H$_2$O</td>
<td>1.815</td>
</tr>
<tr>
<td>Stacking Gel Buffer</td>
<td>0.750</td>
</tr>
<tr>
<td>30% Acrylamide</td>
<td>0.400</td>
</tr>
<tr>
<td>10% APS*</td>
<td>0.030</td>
</tr>
<tr>
<td>TEMED*</td>
<td>0.003</td>
</tr>
</tbody>
</table>

**Transfer:** Blots were transferred to nitrocellulose at 110V for 70 minutes or using the TransBlot Turbo system (BioRad). Transfer of protein was checked by Ponceau staining.

**Blotting:** Blots were placed in Odyssey brand blocking buffer for 1hr RT, and incubated with appropriate primary antibody/s diluted in PBS (BCL2, ab692 AbCam, 1:500; GAPDH, EPR6256 AbCam, 1:2500) overnight, 4°C. Blots were washed 3x in PBS-T (PBS-Tween) and incubated with the secondary antibody prior to blot development.

**Development using fluorescence:** Blots were incubated with appropriate secondary antibodies (1:50,000 goat-anti-mouse 800 or 1:50,000 700 donkey-anti-rabbit, diluted in PBS) for 1h RT. Blots were washed 3x in PBS-T prior to scanning on Odyssey imaging system (LI-COR).

**Development using chemiluminescence:** Blots were incubated with appropriate HRP-conjugated secondary antibodies diluted in PBS.
(1:15,000 goat-anti-mouse or 1:200 goat-anti-rabbit) for 1h RT. Blots were washed 3x in PBS-T. For signal boost, Vectastain ABC kit (Vector Laboratories) reagent was used in conjunction with SuperSignal West Femto Maximum Sensitivity Substrate (ThermoFisher).

2.3.9 Direct immunoprecipitation with CoIP and RIPA buffers

*Cell harvest:* See Section 2.3.8. Each cell pellet constituted a minimum of 10^7 cells, harvested from a T150 culture flask.

*Protein processing with CoIP and RIPA buffer:* Each cell pellet was mixed thoroughly with approximately 1ml CoIP buffer (20 mM Imidazole, 2 mM EDTA, 2 mM EGTA, 0.1% Triton X-100, pH 7 + protease inhibitor) per 10^7 cells and left to incubate at 4°C for a minimum of 1h whilst rotating. The samples were then centrifuged at maximum speed for 15 minutes at 4°C and the supernatant (cytoplasmic protein) was removed and kept on ice for downstream processing. RIPA buffer (4x volume cell pellet) was then used to resuspend the cell pellet. Samples were vortexed, sonicated then centrifuged at maximum speed for 10 minutes. The supernatant (nuclear protein) was removed and kept on ice for downstream processing. Protein was quantified as described previously (Section 2.3.8).

Magnetic beads (PureProteome Protein A/G Mix, Merck Millipore) were washed 3x in sterile PBS-T. A pre-cleared control cytoplasmic protein sample was prepared by adding 100μg cytoplasmic protein to 50μl beads and incubating at 4°C for a minimum of 30 minutes whilst rotating. The beads were stored at -20°C.
All samples were incubated with 10μg antibody (BCL2 antibody ab692, AbCam; concentration 0.2μg/μl, total volume per IP 50μl) at 4°C for a minimum of 2h whilst rotating.

Protein/antibody preparations were combined with 50μl washed beads and incubated at 4°C O/N (overnight) whilst rotating.

Total lysates were removed from all beads and kept on ice for downstream processing. The beads (including those from the pre-clearing) were washed 3x in PBS-T prior to incubating with 60μl low pH buffer for 10 minutes whilst rotating. The low pH elute was boiled with sample loading buffer (with B-mercapoethanol) for 5 minutes. Elution of the beads was completed by boiling them with sample loading buffer (with B-mercapoethanol) for 5 minutes. The following controls were also prepared with sample loading buffer and run on a 20% Mini-Protean TGX Gel for silver staining: 0.5g antibody, 5μl washed beads, total lysate.

IP samples were assessed by western blot, as described in Section 2.3.8.

2.3.10 Indirect immunoprecipitation

Magnetic beads were washed 3x in sterile PBS-T. The beads were then blocked in 2x volume PBS-BSA (10% BSA) (Bovine serum albumin), and incubated at 4°C for a minimum of 2h whilst rotating. The beads were washed 3x in PBS-BSA, while control non-blocked beads were washed 3x in PBS-T. All beads were resuspended in 50μl PBS-BSA.
A total of 500μg protein was added to 5μg antibody (BCL2 antibody ab692, AbCam; concentration 0.2μg/μl, total volume per IP 25μl) and incubated at 4°C for a minimum of 2h whilst rotating.

The beads were combined with the protein/antibody mix and incubated at 4°C for a minimum of 2h whilst rotating.

Supernatant was removed from beads (this sample is herein named Super 1) before they were washed 3x in RIPA (with protease inhibitor). Super 1 was prepared for gel loading by boiling 10μl with 10μl 2x sample loading buffer (see Section 2.3.8, Western blotting methods). The beads were then boiled for 5 minutes with 30μl 2x sample loading buffer without B-mercapoethanol. This supernatant (Super 2) was also prepared for gel loading by boiling in 1.5μl sample loading buffer (with B-mercapoethanol) for 5 minutes. The beads were finally boiled in 30μl sample loading buffer (with B-mercapoethanol) to generate Super3. Samples were run on a 20% Mini-Protean TGX Gel for western blotting.

Indirect IP samples were assessed by Western blot as described previously (See Section 2.3.8).

2.3.11 Ion trap mass spectrometry

Silver staining, sample processing, machine loading and output analysis were performed by the University of Newcastle Analytical and Biomolecular Research Facility. The mass spectrometer model was the Amazon 3D Ion Trap with ETD/ PTR (Bruker).
2.3.12 Tandem mass spectrometry, multiple reaction monitoring

**Sample preparation** Cells were grown to confluence in a T-150 cell culture flask prior to harvesting (see Section 2.3.1). Approximately $10^6$ cells were used for downstream processing. The cells were lysed in 100μl ice cold 0.1M Na2CO3, pH 11 with protease and phosphatase inhibitors. Samples were sonicated 3x for 10 seconds on ice to dissolve the pellet, then left rotating at 4°C for 30 minutes. The phosphatase inhibitor cocktail, Phosphostop (Roche) was added to each sample (100μl 12M Urea + Phosphostop). The proteins were then reduced and alkylated using 20mM DDT and 40nM iodacetamide. Proteins were fragmented using 20ug Trypsin and Lys-C (an alternative to chromotrypsin), per 1mg sample and left for 3h, RT. Samples were then diluted 10x in 50mM TEAB, pH 7.8 and run in duplicate.

Reverse phase chromatography was carried out on the samples using Oasis separation pack short cartridge columns (Waters). Protein was quantified using the BCA Protein Kit and the samples were loaded onto the mass spectrometer.

**Bioinformatics preparation** Prior to running the samples on the triple quadrupole instrument, three peptides were selected to quantify the two proteins of interest. The peptides were identified using the ExPASy PeptideCutter tool (http://web.expasy.org/peptide_cutter/) and selecting fragments in regions specific to each protein of interest (see Figure 2.3.12-1) (Wilkins, Gasteiger et al. 1999).
Figure 2.3.12-1 Specific BCL2 isoform peptide fragments mapped to the isoform primary sequences.

ExPasy PeptideCutter was used to identify a) Peptides 1, b) 2 and c) 3. The abbreviations, ‘Tryps’ refers to trypsin and ‘Ch_hi’ to chymotrypsin. In d) the location of the peptide fragments and mapped to their respective protein primary structure (Wilkins, Gasteiger et al. 1999).
Transition selection Once unique peptides had been identified in silico, they were filtered for in the first quadrupole of the mass spectrometer (see Figure 2.1..2.2.1-1). The final output from the machine was filtered such that unsuitable fragment ions (i.e. non-specific or with poor intensity) were eliminated from the analysis. The remaining filtered fragment ions were used to calculate the relative expression of the peptide i.e. protein of interest.

Bioinformatics analysis Skyline software (MacLean, Tomazela et al. 2010) was used to analyse the MS output.

2.3.13 Statistics

In order to investigate statistical differences between mRNA across the cell lines, the Mann Whitney test was used. The Mann Whitney test is a non-parametric t-test; the sample size for each time point was relatively small (n=9; biological repeats=3, technical repeats=3) and was therefore unlikely to follow standard distribution.

Statistical analyses were performed in GraphPad Prism.
2.4 Results

2.4.1 Quantification of BCL2 isoforms by gene expression microarray

BCL2 isoform transcript expression was investigated in melanoma and melanocyte cell lines following cisplatin treatment across a time series using gene expression microarrays. Relative expression was normalised to global gene expression.
Figure 2.4.1-1 Response of BCL2 isoforms (mRNA) to cisplatin exposure in melanoma and melanocyte cell lines.

Melanoma (MM200, MelRM, SkMel28 and Me4405) and melanocyte (HeMn) lines were treated with 10µg/ml cisplatin. Relative BCL2α and BCL2β mRNA expression was monitored at 0, 6 and 24h following exposure. Relative expression was measured in duplicate and normalised to global gene expression as measured by transcriptomic gene expression microarray.
Both isoforms were detected in melanoma and melanocyte cell lines before and after cisplatin treatment. While $BCL2\alpha$ was generally downregulated in response to cisplatin treatment (in melanocytes and melanoma cell lines: Mel-RM, MM200, Sk-Mel28), $BCL2\beta$ had a varied response across the cell lines. While the transcript was unresponsive in some of the cell lines (Me4405 and SkMel28), it was actually upregulated in melanocytes and Mel-RM, but downregulated in MM200 (see Figure 2.4.1-1).

The observations that the two $BCL2$ isoforms responded differently to stress informed our hypotheses for further studies (see Chapter 4).

2.4.2 qPCR - primer optimisation

Primers were designed to detect $BCL2\beta$ mRNA as TaqMan probes were not available. The primers are designed to target the isoform specific 128aa sequence at the C-terminus of the $BCL2\beta$ mRNA.

Every PCR product has a specific melting temperature which is dependent on its length and GC content. Steadily increasing the temperature of a completed PCR reaction will cause the PCR product to dissociate at its specific melting temperature – this melting temperature can be determined by monitoring fluorescence (i.e. SYBR Green). Because SYBR Green fluoresces in the presence of dsDNA, the fluorescence of a reaction will drop as dsDNA becomes single stranded. By plotting the derivative of fluorescence vs temperature, we can visualise how many PCR products are present in a reaction. An overly pronounced ‘shoulder’ on the plot indicates that there is a substantial
amount of primer dimer (i.e. self-annealing PCR products) (Rodriguez, Rodriguez et al. 2015).

Figure 2.4.2-1 Dissociation curve of BCL2β qPCR product.

This melt curve was produced by plotting the 2°C ramp from 60°C to 95°C, following a qPCR on positive and negative cDNA controls.

The melt curve for the BCL2β primers (Figure 2.4.2-1) had one unique peak at 86°C for each of the positive control samples and there was no amplification of the negative control.

In order to test the efficiency of the primers, we performed qPCR on a serial dilution of DNA standards to plot a standard curve. If we propose a model wherein the efficiency of a reaction is 100%, the Ct values would be 3.3 cycles apart in a 10- fold serial dilution (there is a 2-fold change for each change in CT). Therefore, on a logarithmic scale, the gradient of the plot (y) will be -0.33, and the correlation coefficient (R²) will be 1 (as, in this model, the data would perfectly fit line of regression) (Figure 2.4.2-2) Realistically however, we accept an R² value of >0.95 and a slope
between -3 - -3.5 (Nolan, Hands et al. 2006, Rodriguez, Rodriguez et al. 2015).

Figure 2.4.2-2 Theoretical plot of a standard curve with 100% primer efficiency (y=-3.33x+z, R²=1).

$y = -3.33x + 23.33$

$R^2 = 1$
Figure 2.4.2-3 Standard curve for the BCL2β primers ($y=-3.2x+z$, $R=0.95$).

This plot was generated by performing qPCRs (in triplicate) using the BCL2β primers on a five-fold serial dilution of cDNA. The BCL2β forward primer was at a concentration of 40nM and the reverse primer at 20nM.

The gradient of the slope of Ct vs quantity for the BCL2β primers was -3.2 and the correlation coefficient ($R^2$) was 0.95 (Figure 2.4.2-3). These metrics meet the standard required for quality control, as described previously.
2.4.3 Confirmation of *BCL2* isoform mRNA expression

![Graph showing relative mRNA expression of BCL2 isoforms](image)

**Figure 2.4.3-1 Relative mRNA expression of the BCL2 isoforms in melanoma cell lines.**

Relative mRNA expression was quantified by qPCR in triplicate. Expression was normalised to the geometric mean of the housekeeping genes, *18s*, *GAPDH* and *B-actin*.

*BCL2β* transcript expression was semi-quantified using qPCR for melanoma cell lines. Both isoforms were detected in the three melanoma cell lines MM200, SkMel28 and MelRM. *BCL2α* was expressed higher...
than $BCL2\beta$ across all cell lines (Mann Whitney, MM200 $p<0.01$, SkMel28 $p<0.01$, MelRM $p=0.0081$) (Figure 2.4.3-1).

2.4.4 Confirmation of BCL2 isoform protein expression

Once expression of the isoforms at the mRNA level had been confirmed, the focus of the investigations turned to confirmation of both isoforms at the protein level.

2.4.4.1 Western blotting, immunoprecipitation and ion-trap mass spectrometry

![Western blot analysis of four melanoma cell line lysates.](image)

Cell lysates were probed with GAPDH and BCL2 antibodies. A total of 15μg protein (extracted using RIPA buffer) for each sample was loaded onto a 12% gel, which was run at 150V for 1h and transferred to nitrocellulose at 100v for 1h.
Figure 2.4.4.1-2 Western blot analysis of four melanoma and one melanocyte cell line lysates.

Cell lysates were probed with BCL2 antibody. A total of 20μg protein (extracted using RIPA buffer) for each sample was loaded onto a 12% gel, which was run at 150V for 1.5h and transferred to nitrocellulose at 100v for 1h.

BCL2 protein was detected by western blot in the same three melanoma cell lines as demonstrated previously by qPCR (Figure 2.4.3-1), as well as the melanocyte cell line, HeMn (Figure 2.4.4.1-2). It was not expressed in Me4405. The GAPDH control demonstrated even loading of the samples; SkMel28 had the highest BCL2 expression (Figure 2.4.4.1-1, Figure 2.4.4.1-2).
Figure 2.4.4.1-3 Silver stained immunoprecipitated melanocyte lysate, enriched for BCL2.

The HeMn lysate was enriched for BCL2 via direct IP of protein extracted using CoIP (yielding cytoplasmic protein) and RIPA (yielding nuclear protein) buffers. A total of 1000μg of protein was processed via direct IP and 30μl protein per sample was loaded onto a 12% gel. The gel which was run at 150V for 1.5h then transferred to nitrocellulose at 100v for 1h. Arrows indicate sections of gel which were cut out and processed via ion trap MS.

After confirmation of the presence of BCL2 protein by western blot, we endeavoured to enrich for the isoforms using IP. To maximise the separation of the proteins, the elution conditions were introduced in-step such that proteins with different binding affinities could be visualised separately. Protein was eluted off the beads in low pH conditions in both the nuclear and cytoplasmic lysates – these protein bands are approximately 25 and 20kDa in size (see Figure 2.4.4.1-3). These bands were negative for any BCL2 protein as well as any of the
known BCL2α binding partners when assessed by MS. For MS results of the individual gel plugs, see Appendix 7.3.

Figure 2.4.4.1-4 Western blot analysis of four melanoma and one melanocyte cell line lysates.

Cell lysates were probed with BCL2 antibody. A total of 20μg protein (processed using NucBuster protein extraction kit) for each sample was loaded onto a 12% gel, which was run at 150V for 1.5h and transferred using the FastTransfer system. Solid rectangles indicate a band at ~26kDa, dashed rectangles indicate two bands at ~26 and ~22kDa.
After the negative MS results, western blots were used to determine if either BLC2 isoform was able to be quantified after IP by a second method. Both BCL2 isoforms were expressed in the nuclear fraction of HeMn, SkMel28, MM200 and MelRM. There was only one isoform (BCL2α) expressed in Me4405 and, like the other cell lines, it was only expressed in the nuclear fraction (Figure 2.4.4.1-4).
Cell lysates were enriched for BCL2 via indirect iP of protein extracted using the NucBuster protein extraction kit (only the nuclear fraction was analysed). A total of 500μg protein was processed where 20μl protein for each sample was loaded onto a 12% gel. The gel which was run at 150V for 1.5h then transferred to nitrocellulose at 100v for 1h. Total lysate refers to protein lysate before IP. Super 1 was the first elution (poor protein-antibody associations) and Super 2 was the final elution (strong protein-antibody associations). Solid rectangles indicate a band at ~26kDa, dashed rectangles indicate two bands at ~26 and ~22kDa.
Both isoforms were present in Super 1 of MelRM. The total lysate and Super 1 of MelRM and SkMel28 were positive for two bands of different weights (i.e. BCL2α and BCL2β). HeMn was positive for the heavier band (i.e. BCL2α) in both total lysate and super 1. MM200 and Me4405 total lysates were also positive for BCL2α (Figure 2.4.4.1-5). The MM200, MelRM and SkMel28 samples were selected for a silver stain prior to ion trap MS analysis.

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**Figure 2.4.4.1-6 Silver stained immunoprecipitated melanoma cell lysates, enriched for BCL2.**

Cell lysates were enriched for BCL2 via indirect IP of protein extracted using the NucBuster protein extraction kit (only the nuclear fraction was analysed). These samples are identical to those in Figure 2.4.4.1-5 although 50μl of protein was loaded onto this gel. Super 1 was the first elution (poor protein-antibody associations) and Super 2 was the final elution (strong protein-antibody associations). Arrows indicate sections of gel which were cut out and processed via ion trap MS.
The MM200 samples were all negative for protein. MelRM and SkMel28 Super 2 stained positive for protein around the 25kDa mark (Figure 2.4.4.1-6). Upon analysis via MS, these bands were negative for any BCL2 protein as well as any of the known BCL2α binding partners. For results of the individual gel plugs, see Appendix 7.4.

2.4.4.2 Multiple reaction monitoring mass spectrometry

To quantify the BCL2 isoforms in a non-antibody dependant manner, MRM MS was utilised. The following graphs serve as an example of the transitions which were selected for the analysis due to their specificity and intensity (Figure 2.4.4.2-1). Transitions such as these were selected for each sample.
Figure 2.4.4.2-1 Filtered transitions for MM200 protein sample.

Depicted are a) Peptide 1 (aligns with both isoforms), b) Peptide 2 (unique to BCL2α) and c) Peptide 3 (unique to BCL2β) (see Figure 2.3.12-1 for alignment).

In order to calculate relative protein expression, the area under the graph for each transition was calculated and averaged out between experimental duplicates. The quantity of BCL2 isoform in each sample
was normalised relative to protein concentration in the original sample (Figure 2.4.4.2-2). Both BCL2 isoforms were detected and reliably quantified in the MM200, SkMel28 and MelRM melanoma cell lines.

Figure 2.4.4.2-2 Relative protein expression of the BCL2 isoforms in melanoma cell lines.

Protein expression was calculated by quantifying the area under the graph of retention vs intensity for transition pairs specific to each isoform. ‘Total’ refers to a transition pair which mapped to both isoforms (see Figure 2.3.12-1). Expression is normalised to the concentration of protein in each sample – expression was measured in duplicate.

The results of this study clearly confirmed that both BCL2 isoforms were present at the mRNA and protein level in three of the melanoma cell lines.
2.5 Discussion

In this chapter, we hypothesised that both $BCL2$ isoforms would be present at the mRNA and protein levels in our melanoma cell lines. In setting out to prove this hypothesis, we also sought to refine methods to quantify this expression (see Section 0).

2.5.1 Microarray data

It was clear that the transcript variants were both present and responded differently to cisplatin treatment across the cell lines (Figure 2.4.1-1). These preliminary data informed the decision to further investigate the role of the $BCL2$ isoforms in melanoma. Furthermore, treatment of the cell lines with cisplatin was repeated as part of this project (see Chapter 4).

2.5.2 Primer optimisation

Primer efficiency can be compromised by non-specific primer design, non-optimal primer concentrations, PCR inhibitors and contaminants. In order to verify that our primers were i) efficient and ii) specific, we performed a melt curve analysis and standard curve respectively (Figure 2.4.2-1, Figure 2.4.2-3)(Wong and Medrano 2005).

We determined that the optimal concentration for the $BCL2\beta$ primers was 40nM and 20nM for the forward and reverse primers respectively (see Figure 2.4.2-3). The gradient of the slope of a standard curve using these concentrations was -3.2 and the correlation coefficient ($R^2$) was 0.95; these values correspond to QC standards as outlined in Section 2.4.2 (Wong and Medrano 2005).
In addition, the melt curve for the \textit{BCL2}β primers demonstrated that they were amplifying a single specific product, for which the melting temperature is approximately 86°C (Figure 2.4.2-1).

In establishing these protocols, we had succeeded with our aim to develop a method to quantify mRNA expression of both \textit{BCL2} isoforms.

\textbf{2.5.3 Confirmation of \textit{BCL2} isoform mRNA expression}

These data confirm our hypothesis that both \textit{BCL2} isoforms are expressed at the mRNA level in each of the melanoma cell lines (Figure 2.4.3-1). \textit{BCL2}β mRNA is present at significantly lower levels than its counterpart, \textit{BCL2}α.

\textbf{2.5.4 Confirmation of \textit{BCL2} isoform protein expression}

\textit{2.5.4.1 Western blotting, immunoprecipitation and ion-trap mass spectrometry}

The initial western blot using the AbCam antibody confirmed the presence of \textit{BCL2} in three of the four melanoma cell lines (Figure 2.4.4.1-1). Although it was hypothesised that the antibody would bind both \textit{BCL2} isoforms, the gel was not run for long enough (i.e. 1h) to distinguish the two proteins at 22kDa and 26.2kDa. It was determined that the gel should be run for 90 minutes to observe the separate proteins.

In order to eliminate background (which was high in the previous blot, Figure 2.4.4.1-1), no GAPDH control was loaded for the next experiment. However, despite reduction in background and longer gel running time, the two isoforms were either indistinguishable from each other or only
one of them was expressed in these samples (Figure 2.4.4.1-2). Alternatively, the band below the 21kDa marker could be BCL2β. Current records from NCBI suggest that the protein is 22kDa, although it has not been studied in vivo. The protein may be smaller/larger than current records show due to presence/absence of post translational modifications.

To further optimise detection of the BCL2β isoform, IP of HeMn lysate was performed to enrich the experimental protein sample for BCL2 (Figure 2.4.4.1-3). HeMn was selected as it had been observed to have the highest expression of $BCL2\beta$ at the mRNA level (Figure 2.4.3-1). Due to uncertainty regarding the molecular weight of the proteins, the IP samples were analysed using ion trap MS. However, this analysis revealed that none of the samples were positive for either of the BCL2 isoforms (see Appendix 7.3).

It is important to note that the IgG (immunoglobulin G) light chain of antibodies is approximately 25kDa, and therefore it would appear on the gel in the same region as BCL2 isoforms (i.e. it would co-elute). This is demonstrated when we look at the antibody only lane—there is a lot of protein around the 25-20kDa mark (Figure 2.4.4.1-3).

Previous western blotting methods used to visualise the BCL2 isoforms were developed using a fluorescence based method, and the protein had been repeatedly extracted using the same cell lysis methods (i.e. RIPA buffer). To optimise the protocol, the next set of western blots were developed using chemiluminescence, and the protein lysate was
generated using the NucBuster protocol. One of the benefits of the
NucBuster protocol is the ability to separate cellular fractions. If a protein
is subcellular, it will be enriched in the subcellular lysate fraction as
opposed to a whole cell lysate (i.e. lysate generated via RIPA based lysis
methods). In addition, NucBuster is a commercially available selection of
buffers and so results are more consistent. Finally, considering we are as
yet unaware of BCL2β function and cellular location, this approach is
optimal for visualisation of the isoform.

The western blot data confirmed that BCL2 protein was present in HeMn,
MelRM, MM200 and SkMel28 (Figure 2.4.4.1-4). Interestingly, Me4405
has positive results for BCL2 expression in this particular blot, despite
previous blots suggesting it is absent (Figure 2.4.4.1-1, Figure 2.4.4.1-2).
This may be due to the alternative sample processing methods. All BCL2
bands were present in the nuclear fraction as opposed to the cytoplasmic
fraction. Double banding (suggesting successful separation of both
isoforms) is evident for HeMn, MelRM, MM200 and SkMel28 (Figure
2.4.4.1-4).

Following the success of this blot, the same methods for protein
extraction and development were used for IP, with the intention to enrich
the sample with BCL2 and confirm the expression of the isoforms using
ion trap MS.

On the next blot, double banding suggesting the presence of both BCL2
isoforms is evident in Super 1 of MelRM. Supers 1 and 2 of MelRM,
MM200 and SkMel28 also show bands of separate weights; the BCL2
isoforms may have eluted off under different conditions (Figure 2.4.4.1-5). MM200, MelRM and SkMel28 lysates were selected to run on a silver stain to analyse with ion trap MS (Figure 2.4.4.1-6).

The silver stain shows there is no protein present in the MM200 supernatants, suggesting the protein degraded in these samples. The samples with positive staining in the appropriate weight range are the Super 1 for SkMel28 and MelRM (Figure 2.4.4.1-6). However, these protein bands appear to be a different molecular weight than those visible on the corresponding western blot, suggesting that we are again seeing co-elution of short chain IgG. Indeed, once the samples were analysed using MS, there were no positive hits for BCL2 isoforms no any of their known binding proteins (see Appendix 7.4).

Because the mass of our proteins of interest are so similar to IgG, the use of antibodies complicated the process of protein confirmation. For this reason, MRM MS was used in the following attempts to verify protein expression.

2.5.4.2  Multiple reaction monitoring mass spectrometry

Analysis of the melanoma protein lysates with MRM MS confirmed our hypothesis that both isoforms are expressed across the cell lines (Figure 2.4.4.2-2). The standard error for each cell line was larger than expected due to duplicate samples and relative expression calculations. Further experimentation would therefore require the inclusion of more replicates as well as the use of spiked-in peptides (see Section 2.1.2.2.2) to absolutely quantify the protein.
In addition, due to the nature of the protein extraction methods used, wherein total protein was run on the mass spectrometer, as opposed to cellular fractions, we are still unclear about the sub-cellular location of the isoform. Further investigation of sub cellular fractions would thus be informative.

In establishing this protocol, we had succeeded with our aim to develop a method to quantify protein expression of both BCL2 isoforms.

2.5.4.2.1 Limitations of multiple reaction monitoring mass spectrometry

Unfortunately, these techniques were not able to be used for the remaining investigations described in this thesis. MRM MS is an expensive and highly involved technique requiring specialised expertise. We were unable to repeat MRM MS at every stage of experimentation. In particular, the analysis of paraffin-embedded tissue is yet to be optimised for MRM MS, which comprises our central clinical cohort for this study.
2.6 Conclusion

At the outset of this chapter, we set out to establish a methodology whereby we could test our hypothesis that both BCL2 isoforms are expressed at the mRNA and protein levels across our cell lines.

These investigations proved our hypothesis correct and we also developed a means to further assess BCL2 isoform expression in other biological contexts.

It is interesting to observe that while the difference between BCL2α and BCL2β mRNA expression within the same cell line was drastic (Figure 2.4.3-1); the difference between expression at the protein level was far less pronounced.

As well as the assumptions about BCL2β (see Section 1.6.2) which have likely led to the isoform being left by the research wayside, low mRNA levels may have contributed to this.

NOTE: Unfortunately, HeMn protein samples were unavailable at the time of MRM MS experimentation and so we were unable to confirm protein expression in this cell line. However, throughout the duration of our investigations described in Chapter Four, HeMn had higher BCL2β mRNA expression than any of the other cell lines.
Chapter Three: \textit{BCL2} isoform expression in melanoma

3.1 Introduction

The \textit{BCL2} protein is involved in the regulation of apoptosis, melanin production and melanocyte development (McGill, Horstmann et al. 2002). It is for this reason that it has been extensively studied in the context of melanoma. Although there have been many studies on the role of \textit{BCL2} in melanoma development and prognosis (see Section 0), none of these studies discriminate between \textit{BCL2α} and \textit{BCL2β}.

Not only is the existence of \textit{BCL2β} not often acknowledged, it is commonly assumed that the isoform has no function (see Section 1.6.2). Our preliminary studies (see Figure 2.4.1-1) suggest otherwise.

Using methods developed in Chapter 2, we quantified \textit{BCL2} isoform mRNA using qPCR in a large cohort of melanoma tissue samples. We looked to examine the regulation of \textit{BCL2} gene splicing by studying SNPs which have previously been shown to be associated with isoform expression (Gullem, Amat et al. 2015).

We also utilised an IHC (immunohistochemistry) methodology for the examination of \textit{BCL2} isoform proteins for which antibodies are unavailable. By performing these experiments in the context of a melanoma tissue cohort with extensive clinical parameters, we were able to investigate the relationship of \textit{BCL2} with melanoma.
Considering the use of immunotherapy in modern melanoma treatment, the role of immune cells in patient treatment is increasingly important (see Section 1.3.3). In particular, the scale of tumour infiltrating lymphocytes (TILs) has been shown to be indicative of patient response to treatment (Tumeh, Harview et al. 2014). It is for this reason that we have retrospectively scored our cohort for TILs.

We are also interested in TILs as it has long been established that \textit{BCL2} plays a central role in T cell development and survival (Gratiot-Deans, Ding et al. 1993, Veis, Sentman et al. 1993, Charo, Finkelstein et al. 2005). While the anti-apoptotic role of \textit{BCL2}α contributes to T cell stability in this context, the role of \textit{BCL2}β (if any) is unknown.
3.2 Hypotheses & Aims

In previous chapters (Section 2.5), we have established that the two BCL2 isoforms are differentially expressed between melanocytes and melanoma cell types. We have also outlined that, although there are some discrepancies regarding the role of BCL2 in melanoma, most investigations have demonstrated that BCL2 expression is higher in metastatic melanoma than primary (Section 0) (Leiter, Schmid et al. 2000, Utikal, Leiter et al. 2002, Zhang and Rosdahl 2006). In addition, other researchers have found a correlation between SNP rs3943258 and BCL2 isoform expression (Guillem, Amat et al. 2015).

We therefore hypothesise:

- BCL2 isoforms have different associations with melanoma progression/ survival and TILs.
- BCL2 expression is elevated in metastatic melanoma tumour tissue in comparison to primary tissue.
- SNP rs3943258 is associated with BCL2 isoform mRNA expression.

Within this next chapter we aim to:

- Confirm mRNA and protein expression of BCL2 isoforms across our cohort of melanoma tissue.
- Interrogate the relationship between the SNP rs3943258 BCL2 polymorphism and expression of the BCL2 isoforms within the cohort.
- Investigate the relationship between BCL2 i) mRNA ii) protein iii) SNP rs3943258 and iv) TILS with the clinical parameters of the cohort (including survival data).
3.3 Methods

3.3.1 Tumour cohort

196 FFPE (formalin fixed, paraffin-embedded) melanoma samples were collected for diagnostic purposes at the Hunter Area Pathology Service, NSW, Australia between 2004 and 2009. 189 tumours were identified with sufficient tissue (> 2mm width and length). All cases utilised had stage 2 or greater disease as either metastatic (lymph node biopsy) or primary melanomas greater than 2mm were used. The Hunter New England Area Health Service Human Ethics Committee approved the study: approval number 08/08/20/5.17. Clinical parameters for the dataset can be viewed in Table 3.3.1-1.
Table 3.3.1-1 Summary of the clinical parameters for the melanoma tumour cohort.

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3.3.2 Microarrays

These microarray data were used for gene expression analysis for other studies conducted in our lab (Bowden, Ashton et al. 2010, Bowden, Ashton et al. 2013, Budden and Bowden 2013).

RNA extracted from the tumours was amplified, biotinylated and hybridised to Whole Genome Gene Expression Human Ref8 V3 BeadChips (Illumina, USA). The BeadChips were scanned using a Bead Array Reader (Illumina USA). The transcript expression results were cubic spline normalised using BeadStudio 2.0 software (Illumina, USA), and the remaining analyses was performed using GeneSpring GX 11.0. To account for bias or skewing of expression results, all the gene expression profiles for each individual probe were normalised to the median resulting in two way normalisation. This data is available for public access in the Gene Expression Omnibus repository under accession number GSE47980.

3.3.3 Deparaffinisation and rehydration of FFPE samples for DNA/RNA extraction

The FFPE tumour cores were deparaffinised by washing twice in xylene for 30 minutes at 55°C, twice in 100% ethanol for 5 minutes at 55°C, twice in 70% ethanol for 5 minutes at 55°C and twice in water for 5-10 minutes at RT.
3.3.4 RNA extraction from FFPE

A 2mm punch biopsy was taken through the tumour block and de-paraffinised using xylene (See Section 3.3.3). RNA was extracted using Life Technologies RNA extraction kit as per manufacturer’s instructions.

For RNA clean-up and quantification steps, see Section 2.3.3.

3.3.5 Reverse transcription

A final amount of 500μg RNA was reverse transcribed using High Capacity cDNA Reverse Transcription Kits as per manufacturers’ instructions (see Appendix 7.1).

3.3.6 qPCR

Relative expression was normalised to GAPDH, as it was the least variable of the three housekeeping transcripts used for this study (GAPDH, 18s, β-actin). GAPDH and BCL2 expression were measured twice in triplicate using TaqMan Gene Expression Assays, whereas BCL2β expression was measured using custom designed primers and SYBR Green (see Section 2.3.5). For thermal cycling conditions and reaction volume components, see Appendices 7.2 and 7.3. As an FFPE RNA quality control measure, wherever measurement of GAPDH expression failed in triplicate or standard error was >1, the sample was excluded from the analyses. A threshold for Ct value was set at 40 cycles – where the Ct value was above this threshold, expression was considered to be null.
Quality control measures and troubleshooting steps for qPCR are described in Chapter Two.

3.3.7 Immunohistochemistry

Sections (4μm) of FFPE tissue were cut in duplicate and mounted onto coated DAKO flex IHV full face microscopy slides alongside sections of control tissue from three different sources (bowel, tonsil and skin) of an identical thickness. Following rehydration, the tissue was emerged in 10% bleach O/N to remove melanin prior to dehydration and staining.

Antigen retrieval (CC1 antigen retrieval buffer, pH 9) and tissue staining was performed using the Discovery Ventana Ultra Immunohistochemistry system.

Antibody ab692 (herein abbreviated to AbCam) was used at a final concentration of 1/50 and LS-B11858 (herein abbreviated to LSB) at 1/4000.

At the time of thesis submission, the ab692 AbCam antibody had been used to investigate BCL2 protein expression in 26 published studies, according to the manufacturer’s website (http://www.abcam.com/bcl-2-antibody-100d5-ab692-references.html). The LSB antibody has not yet been used in any published literature but it is the only antibody which is predicted to target solely BCL2α, based on the sequence of the peptide used to raise it.
The AbCam and LSB antibodies were used in conjunction with each other such that overlap of the differentially targeted regions could be assessed to determine BCL2 isoform expression, as described in Figure 3.3.7-1.

Tissue was scored for intensity of staining as described in Figure 3.3.7-2, as well as for distribution of staining pattern, as described in Table 3.3.7-1. These scores were analysed separately.

Tissue scoring was performed by the candidate and their primary supervisor. The supervisor has been trained by a pathologist and was deemed capable of scoring by the aforementioned pathologist. Both researchers were blinded to the origin and clinical parameters of each slide.

IHC counting software was not used in this study. Other members of this research group have compared pathologist scoring to IHC automated software and concluded that IHC automated software is not as reliable (not yet published).
Figure 3.3.7-1 Location of BCL2 antibody targetting regions respective to primary structure of BCL2 isoforms.

Protein BLAST result of the synthetic peptide sequence used to raise AbCam, showing a hit for a) BCL2α and b) BCL2β. Figure c) shows the locations of the antibody targetting region for AbCam and LSB relative to the primary sequences of the BCL2 isoforms. Theoretically, tissues staining for LSB and AbCam are positive for BCL2α, whereas tissues staining for AbCam are positive for BCL2α and BCL2β. (Karlin and Altschul 1990, Karlin and Altschul 1993, Altschul, Madden et al. 1997)
<table>
<thead>
<tr>
<th>Percentage of stained cells</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-25</td>
<td>1</td>
</tr>
<tr>
<td>26-50</td>
<td>2</td>
</tr>
<tr>
<td>51-75</td>
<td>3</td>
</tr>
<tr>
<td>76-95</td>
<td>4</td>
</tr>
<tr>
<td>&gt;96</td>
<td>5</td>
</tr>
</tbody>
</table>
Figure 3.3.7-2 Scoring system for IHC staining.

All images are at 20x magnification. Tumours were scored for staining as indicated in labelled images; 0 (negative), 1 (weak), 2 (weak>strong), 3 (strong>weak) and 4 (strong).
3.3.8 DNA extraction

Following steps described in Section 3.3.3, the samples were lysed using the Levy lysis method (see Table 3.3.8-1) overnight at 55°C on a shaker.

**Table 3.3.8-1 Levy lysis reaction volume components.**

<table>
<thead>
<tr>
<th></th>
<th>Volume/sample (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Levy lysis buffer (components)</td>
<td>371</td>
</tr>
<tr>
<td>10% Tween-20</td>
<td>20</td>
</tr>
<tr>
<td>Proteinase K (added immediately prior to use)</td>
<td>9</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>400</strong></td>
</tr>
</tbody>
</table>

Samples were pulse vortexed before incubation at 100°C for 15 minutes prior to adding 8 µl 0.05M EDTA. The samples were then centrifuged at 13000 RPM for 5 minutes.

DNA was precipitated by adding 1/10 volume 3M Na Acetate (pH 5.2) and 2x volume ice cold 100% ethanol. Samples were incubated at 70°C for a minimum of 30 minutes then centrifuged at maximum speed at 4°C for 30 minutes. The supernatant was discarded and replaced with 95% ethanol prior to centrifuging at maximum speed at 4°C once more. Ethanol was removed from the pellets which were left to air dry, prior to resuspension in TE for storage at -20°C-4°C or immediate downstream processing.
Melanin (a PCR inhibitor) (Eckhart, Bach et al. 2000) was removed from the DNA samples by running them through One-Step PCR inhibitor removal kits. Final DNA concentration was determined using the Qubit DNA BR or HS Assay Kit (ThermoFisher), a fluorometric quantitation method.

3.3.9 SNP assays

Genotyping was performed using the Viia7. Between 1-10ng genomic DNA was added to the TaqMan Genotyping Master Mix and specific TaqMan probe (rs3943258) to generate a total reaction volume of 10μl (see Table 3.3.9-1). For thermal cycling conditions, see Table 3.3.9-2. For SNP assay sequence and fluorescence information, see Table 3.3.9-3.

Table 3.3.9-1 Genotyping assay reaction volume components.

<table>
<thead>
<tr>
<th></th>
<th>10μl Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>TaqMan Genotyping</td>
<td></td>
</tr>
<tr>
<td>Master Mix (2X)</td>
<td>5</td>
</tr>
<tr>
<td>TaqMan genotyping</td>
<td></td>
</tr>
<tr>
<td>assay mix (20X)</td>
<td>0.5</td>
</tr>
<tr>
<td>Genomic DNA (1-10ng)</td>
<td>4.5</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
</tr>
</tbody>
</table>
Table 3.3.9-2 Thermal cycling conditions for SNP assays.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Temperature (°C)</th>
<th>Time (seconds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hold</td>
<td>60</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>95</td>
<td>20</td>
</tr>
<tr>
<td>PCR (40 cycles)</td>
<td>95</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>20</td>
</tr>
<tr>
<td>Post-read</td>
<td>60</td>
<td>30</td>
</tr>
</tbody>
</table>

Table 3.3.9-3 SNP sequence and fluorescence information.

<table>
<thead>
<tr>
<th>SNP ID</th>
<th>Sequence [allele 1/allele 2]</th>
<th>Location in BCL2 gene transcript</th>
<th>Probe fluorescent signal allele 1/allele 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs394325 8</td>
<td>GGCTGTCCCTGAGCCC CCAAGAAAA[C/T]GGAG CGAGGTAACCTAGAGGCA GTG</td>
<td>Second intron</td>
<td>VIC/FAM</td>
</tr>
</tbody>
</table>

DNA extracted from FFPE tissue can degrade over time, therefore genomic DNA from cell lines was run on each plate of genomic DNA from FFPE tissue for quality control purposes.

3.3.10 Tumour infiltrating lymphocytes

Tumours were scored visually according to the system as described in Table 3.3.10-1 and Figure 3.3.10-1. This TIL scoring method is described in more detail in (Schalper, Velcheti et al. 2014) and (Klintrup, Makinen et al. 2005).
Tissue scoring was performed by the candidate and their primary supervisor. The supervisor has been trained by a pathologist and was deemed capable of scoring by the aforementioned pathologist. Both researchers were blinded to the origin and clinical parameters of each slide.

**Table 3.3.10-1 Scoring system for TILs.**

<table>
<thead>
<tr>
<th>Intensity</th>
<th>Distribution</th>
<th>Grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild</td>
<td>Focal</td>
<td>1</td>
</tr>
<tr>
<td>Moderate</td>
<td>Focal</td>
<td>1</td>
</tr>
<tr>
<td>Mild</td>
<td>Multifocal</td>
<td>1</td>
</tr>
<tr>
<td>Marked</td>
<td>Focal</td>
<td>2</td>
</tr>
<tr>
<td>Moderate</td>
<td>Multifocal</td>
<td>2</td>
</tr>
<tr>
<td>Marked</td>
<td>Multifocal</td>
<td>2</td>
</tr>
<tr>
<td>Mild</td>
<td>Diffuse</td>
<td>2</td>
</tr>
<tr>
<td>Moderate</td>
<td>Diffuse</td>
<td>3</td>
</tr>
<tr>
<td>Marked</td>
<td>Diffuse</td>
<td>3</td>
</tr>
</tbody>
</table>
Figure 3.3.10-1 Scoring system for TILs.
All images are at 20x magnification. 1 = Mild & focal; moderate & focal; or mild & multifocal. 2 = Marked & focal; moderate & multifocal; marked & multifocal or mild & diffuse. 3 = Moderate & diffuse or marked & diffuse TILs.
3.3.11 Statistics

In order to investigate correlation between: gene expression quantification methods, expression of the two $BCL2$ isoforms, expression of mRNA and protein and mRNA/ protein and non-discrete clinical parameters, the Spearman’s rank test was used. The test was chosen due to its robustness against outliers.

Comparison of survival curves was conducted using the non-parametric log-rank Mantel Cox test, which was selected due to the censored nature of the survival data.

The Mann Whitney test was used to compare mRNA/ protein expression levels between categories (e.g. tumour tissue type, genotype) as these values did not adhere to standard distribution.

Statistical analyses were performed in GraphPad Prism.

Where there were missing data in the cohort (see Table 3.4.1-1), analyses were performed on the remaining data. Imputation would be an inappropriate means to compensate for the missing data due to the size of the cohort (133 samples with available mRNA, 108 samples with available protein and 131 samples with available DNA) and the amount of missing data. Imputation in this context would arguably bias the results (Sterne, White et al. 2009).
3.4 Results

3.4.1 Confirmation of mRNA microarray expression data using qPCR

![Graph comparing relative expression of BCL2α and BCL2β mRNA](image)

Figure 3.4.1-1 Comparison of relative expression of a) BCL2α and b) BCL2β mRNA as quantified by two different methods.

Gene expression of the two BCL2 isoforms was measured across the melanoma tumour cohort (n=133), using microarray gene expression and qPCR. Microarray gene expression was measured in duplicate and qPCR in triplicate.

The BCL2α gene expression data acquired by microarray analysis was positively correlated with that acquired by qPCR (Spearman, r=0.35, p=0.0002). However, the two groups of gene expression data for BCL2β expression were not correlated (p=0.77) (Figure 3.4.1-1). qPCR is a more quantitative method than gene expression microarrays – whereas the
microarrays were merely a preliminary experiment, the qPCR dataset can be used to assess mRNA expression with increased accuracy and reliability.

3.4.2 Summary of mRNA, protein and DNA methods used to analyse the melanoma clinical cohort

The quality of mRNA and DNA in the FFPE samples was variable, and so not all samples reached the QC standards required for experimental analyses. Similarly, some of the IHC slides were unable to be scored. This was due to failed control tissue, melanin contamination or insufficient tissue quantity.
Table 3.4.1-1 Final numbers of tumour samples for which data were analysed following QC steps.

<table>
<thead>
<tr>
<th></th>
<th>AbCam</th>
<th>LSB</th>
<th>AbCam &amp; LSB</th>
<th>BCL2α mRNA</th>
<th>BCL2β mRNA</th>
<th>BCL2 mRNA</th>
<th>SNP 3943258</th>
</tr>
</thead>
<tbody>
<tr>
<td>AbCam</td>
<td>121</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LSB</td>
<td>108</td>
<td>130</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BCL2α mRNA</td>
<td>86</td>
<td>92</td>
<td>75</td>
<td>133</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BCL2β mRNA</td>
<td>84</td>
<td>90</td>
<td>75</td>
<td>133</td>
<td>133</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BCL2 mRNA</td>
<td>84</td>
<td>90</td>
<td>75</td>
<td>133</td>
<td>133</td>
<td>133</td>
<td></td>
</tr>
<tr>
<td>SNP 3943258</td>
<td>91</td>
<td>94</td>
<td>78</td>
<td>79</td>
<td>79</td>
<td>79</td>
<td>131</td>
</tr>
</tbody>
</table>

Final numbers of available samples were reduced for some parameters in comparison to the original cohort (Table 3.3.3-1, Table 3.4.1-1).
3.4.3 mRNA expression across the melanoma cohort

Figure 3.4.3-1 Comparison of mRNA expression of the two $BCL2$ isoforms across the melanoma cohort.

Gene expression of the two $BCL2$ isoforms was measured across the melanoma tumour cohort using qPCR (n=133). qPCR was performed in triplicate.

There was a positive correlation between $BCL2\alpha$ and $BCL2\beta$ mRNA across the cohort (Spearman, n=133, r=0.3, p=0.0004) (Figure 3.4.3-1).
3.4.4 *BCL2β* expression is associated with improved survival in melanoma patients

![Kaplan-Meier survival plot comparing melanomas positive and negative for BCL2β expression.](image)

**Figure 3.4.4-1** Kaplain-Meier survival plot comparing melanomas positive and negative for *BCL2β* expression.

Expression of *BCL2β* mRNA was quantified in 116 melanoma tumours (for which survival data was available) by qPCR (*BCL2β* present, n=53, *BCL2β* absent, n=63). Patients whose tumours expressed *BCL2β* had significantly longer survival than those whose did not (p<0.05). qPCR was performed in triplicate.

Survival for patients with *BCL2β* expressing melanomas was significantly longer (686.4 weeks, 95% CI 462.5-910.3) than those with non-*BCL2β* expressing melanomas (310.1 weeks, 95% CI 166.5-453.8) (log-rank Mantel Cox, p=0.043*) (Figure 3.4.4-1).

Correlation analysis of *BCL2α* and β in the context of the clinical parameters revealed that *BCL2β* correlated to Overall Survival
(Spearman, r=0.2, p=0.03*). \(BCL2\beta\) expression also correlated with Time to Stage IV (Spearman, r=0.3, p=0.003**), a confounding factor for survival time.

3.4.5 \(BCL2\beta\) expression is increased in metastatic melanoma tissue compared to primary

![Graph showing relative expression of BCL2β mRNA in melanoma tissue](image)

**Figure 3.4.5-1 Relative expression of \(BCL2\beta\) mRNA in the melanoma tumour cohort according to tissue type.**

Expression of the \(BCL2\beta\) isoform was quantified by qPCR in 91 melanoma tumours (27 primary and 64 metastatic). Expression of \(BCL2\beta\) was significantly higher in metastatic vs primary tissue (p<0.05). qPCR was performed in triplicate.
Primary/ metastatic tissue data was available for a total of 137 tumours (45 primary and 92 metastatic). qPCR was performed in triplicate.

Expression of \textit{BCL2β} was significantly higher in metastatic vs primary tissue (Mann Whitney, n=91, p=0.015) (Figure 3.4.5-1). Interestingly, there was no significant difference in survival time between primary tissue and metastatic tissue, indicating the better overall survival associated with presence of \textit{BCL2β} is independent of disease stage (Figure 3.4.5-2).
3.4.6 BCL2 protein expression across the cohort

Table 3.4.6-1 Positive and negatively stained tumour sections for the two BCL2 antibodies, AbCam and LSB.

<table>
<thead>
<tr>
<th></th>
<th>AbCam</th>
<th>LSB</th>
<th>AbCam/LSB</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ve</td>
<td>68</td>
<td>60</td>
<td>42</td>
</tr>
<tr>
<td>-ve</td>
<td>40</td>
<td>48</td>
<td>22</td>
</tr>
<tr>
<td>+ve/-ve</td>
<td></td>
<td></td>
<td>26</td>
</tr>
<tr>
<td>-ve/+ve</td>
<td></td>
<td></td>
<td>18</td>
</tr>
</tbody>
</table>

According to our hypothesis regarding antibody targeting, 26 tumours stained positively for BCL2β protein, as these stained negatively for LSB and positively for AbCam (i.e. total BCL2 positive, BCL2α negative, therefore BCL2β positive). Of these 26 tumours, nine were also positive for BCL2β mRNA transcripts. However, 18 tumours stained positively for LSB and negatively for AbCam, which should not occur as both antibodies target BCL2α, i.e. if LSB is positive AbCam should be positive too (Table 3.4.6-1). These false results confirmed IHC is not accurate enough to investigate specific isoforms. Therefore, we conducted further analysis of the two antibodies rather than of BCL2α and BCL2β.
**Figure 3.4.6-1** Immunohistochemistry results of the melanoma cohort stained by two BCL2 antibodies.

BCL2 expression at the protein level was categorically quantified using the AbCam (n=121) and LSB (n=130) antibodies. Scores were designated as follows: 0= negative, 1=weak, 2=weak>strong, 3=weak<strong, 4=strong.
3.4.7 BCL2 protein expression is not associated with survival in melanoma

Figure 3.4.7-1 Kaplan-Meier survival plot of melanomas stained using the AbCam and LSB antibodies.

BCL2 expression at the protein level was categorically quantified using the AbCam (n=109: positive=68, negative=41) and LSB (n=118: positive=49, negative=69) antibodies. There was no significant difference between survival of patients based on the BCL2 staining of their tumour tissue (p>0.05).

We compared the survival times for patients with positive and negatively stained melanoma tissue for two BCL2 antibodies. There was no significant difference in survival across the differentially stained tumour tissue (Figure 3.4.7-1).
3.4.8  BCL2 protein expression is increased in primary melanoma tissue compared to metastatic

Figure 3.4.8-1 Immunohistochemistry results of the melanoma cohort stained by the AbCam antibody, separated by tissue type.

Expression of BCL2 protein was quantified in 91 melanoma tumours (25 primary and 66 metastatic) by IHC. Metastatic tissue had lower BCL2 protein expression than primary tissue (p<0.05).

Metastatic tissue had lower BCL2 protein expression than primary tissue (Mann Whitney, n=91, p=0.0323)(Figure 3.4.8-1). There were statistically more null/ weakly stained metastatic tissue than strongly stained metastatic tissue (Mann Whitney, staining intensity of 0/1 vs 2/3/4, n=91, p<0.0001).
3.4.9 BCL2 protein expression does not correlate with mRNA expression

![Graphs showing correlation](image)

Figure 3.4.9-1 Correlation of BCL2 isoform protein and mRNA expression across the melanoma cohort.

*BCL2* isoform expression was quantified by qPCR (mRNA) and IHC using two different antibodies, AbCam and LSB (protein). Shown are non-significant correlations for a) BCL2α to AbCam (n=86), b) BCL2β to AbCam (n=85), c) BCL2α to LSB (n=92) and d) BCL2β to LSB (n=91). For IHC staging, see Figure 3.3.7-2. qPCR was performed in triplicate.
Protein expression determined by categorical scoring of IHC did not correlate with mRNA expression for the two BCL2 isoforms (Figure 3.4.9-1).

3.4.10 Population distribution and Hardy-Weinberg equilibrium analysis of SNP rs3943258

![Figure 3.4.10-1 Distribution of SNP rs3943258 alleles across the cohort of melanoma patients.](image)

Genotyping of SNP rs3943258 was determined by SNP assay in 131 melanoma tissue samples. Genotyping was performed in duplicate.

This distribution of the alleles within this population is in Hardy Weinberg equilibrium ($\chi^2=0.79$, $p=0.38$, 1 degree of freedom)(Figure 3.4.10-1). This principle states that the genetic variation in a population will remain constant across generations in the absence of disrupting factors, such as
natural selection, genetic drift and gene flow. We can therefore interpret that genetic variation at this locus (rs3943258) will be maintained across generations and that these allelic frequencies will remain constant.
3.4.11 SNP rs3943258 genotype is associated with \textit{BCL2}\textbeta{} expression.

![Figure 3.4.11-1](image)

**Figure 3.4.11-1** Expression of the \textit{BCL2} isoforms at mRNA level according to SNP rs3943258 genotype.

a) ratio of \textit{BCL2}\textalpha{}: \textit{BCL2}\textbeta{} expression, b) \textit{BCL2}\textalpha{} expression and c) \textit{BCL2}\textbeta{} expression, categorised according to their SNP rs3943258 genotype; CC (n=24), TT (n=14) and CT (n=41). The T allele conferred significantly higher expression of \textit{BCL2}\textbeta{} (p<0.05). qPCR was performed in triplicate and genotyping was performed in duplicate.
Figure 3.4.11-2 Expression of BCL2 protein according to SNP rs3943258 genotype.

Shown are staining intensity values for the a) AbCam antibody and b) LSB antibody, categorised according to their rs3943258 genotype; CC (n=30), TT (n=54) and CT (n=51). Genotyping was performed in duplicate.

Neither the ratio of $BCL2\alpha: BCL2\beta$ mRNA expression nor $BCL2\alpha$ mRNA expression was not associated with or influenced by SNP rs3943258.
genotype. However, the T allele conferred significantly higher expression of \( BCL2\beta \) (Mann Whitney, presence vs absence, \( p=0.0317^* \), Mann Whitney, TT vs CC, \( p=0.02^* \)) (Figure 3.4.11-). BCL2 protein levels were not associated with SNP rs3943258 genotype (Figure 3.4.11-2).

3.4.12 TIL scores are not associated with melanoma survival

![Kaplan-Meier survival plot comparing patients according to the TIL score of their melanoma tissue.](image)

**Figure 3.4.12-1** Kaplan-Meier survival plot comparing patients according to the TIL score of their melanoma tissue.

TILs were scored as described in Figure 3.3.10-1 in a total of 109 melanoma tumours. Of the tumours, 44 were scored as “1”, 38 as “2” and 44 as “3”.

There was no difference in survival according to TIL score Figure 3.4.12-1). Separating the patients according to tissue type (primary vs metastatic) also showed no difference in survival according to TIL score. There was also no significant difference in survival for patients whose
TILs were positive or negative for BCL2 (using neither AbCam nor LSB staining) (Log-rank Mantel-Cox $p>0.05$).

3.4.13 BCL2 expression is not indicative of TIL infiltration and vice versa

There was no association between BCL2 staining status of TILs and TIL density, distribution or score (Spearman Correlation, $p>0.05$). There was no difference in BCL2 staining between tissue with high and low TIL scores (Mann Whitney, $p>0.05$).

There was also no association between $BCL2$ isoform mRNA expression levels (neither $BCL2\alpha$ nor $BCL2\beta$) and TIL density, distribution or score (Spearman Correlation, $p>0.05$). Similarly to the protein level, there was no difference in mRNA expression levels between tissue with high and low TIL scores (Mann Whitney, $p>0.05$).
3.5 Discussion

In this chapter, we hypothesised that BCL2 is elevated in metastatic melanoma tumour tissue in comparison to primary tissue, that the BCL2 isoforms have different associations with melanoma progression and that SNP rs3943258 is associated with BCL2 isoform mRNA expression (see Section 3.2).

3.5.1 Confirmation of mRNA microarray expression data using qPCR

Analysis of preliminary data (gene expression microarray data) revealed that the two BCL2 isoforms were differentially expressed in melanoma cell lines in response to cytotoxic stimuli (see Figure 2.4.1-1).

Microarray technology can be used to investigate numerous aspects of molecular biology, such as gene expression, methylation and siRNA quantification. Its application in gene expression analysis can be problematic due to individual probes’ tendency to generate false positives/ negatives (Jaluria, Konstantopoulos et al. 2007). For this reason, its usage is generally limited to ontological studies wherein generalisations can be made about groups and patterns of gene expression, as opposed to quantifying expression of individual genes. Gene expression microarray data can also be used to generate hypotheses regarding large datasets.

In order to compensate for the poor reliability of individual probes, microarray chips typically have multiple probes per gene, targetted to different regions of a gene transcript. One of the shortcomings of this is
that some chips do not allow for analysis of individual isoform expression as there are often limited isoform-specific sequences to target.

In Figure 3.4.1-1, results for gene expression for the two BCL2 isoforms as quantified by microarray and RT-PCT are compared. The two gene expression datasets do not strongly correlate. Although the qPCR results for BCL2α are positively correlated with the microarray data, the correlation is not strong. Despite the shortcomings of analysing individual transcripts from microarray data, the BCL2 isoforms were clearly expressed differentially in some of the melanoma cohort.

Interestingly, the two datasets for BCL2β expression are not at all correlated. qPCR is a more reliable method of cDNA quantification as normalisation is less complex and the method is more sensitive. It is for these reasons that gene expression was quantified using qPCR in these studies.

3.5.2 Summary of mRNA, protein and DNA methods used to analyse the melanoma clinical cohort

All tissue samples were fixed in formalin immediately post-surgical removal for long term preservation and room temperature storage and handling. However, preserving tissue by formalin fixing is not optimal for maintaining DNA and RNA quality over extended time periods (Hedegaard, Thorsen et al. 2014). There has also been some research to suggest that FFPE antigens can change over time, which affects their detection via IHC, or indeed any antibody based analytical method (Combs, Han et al. 2016).

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Due to this sample degradation, we were unable to obtain mRNA and DNA data for all of the tissue blocks in our cohort. In addition, for some samples there was no melanoma tissue left to investigate at the protein level once the blocks had been punched for mRNA and DNA studies. High melanin content also reduced the final numbers of samples available for analysis (see Table 3.4.1-1).

3.5.3 BCL2 mRNA expression

We were able to fulfil our aim of confirming mRNA expression of the BCL2 isoforms across our cohort of melanoma tissue.

Analysis of the BCL2 isoform mRNA expression results revealed that, while BCL2α was not correlated with any of the clinical parameters, BCL2β expression was associated with improved survival (Figure 3.4.4-1). This confirms our hypothesis that BCL2 isoforms have different associations with melanoma survival.

Previous studies of the relevance of BCL2 and melanoma clinical parameters have been contradictory, with some finding no correlation with survival, while others report that low BCL2 expression is associated with poor prognosis, or in fact the opposite (for a summary of the research, see Section 0). The data as presented in Section 3.4.4 reflects the general consensus that there is a positive correlation between BCL2 and survival (Sviatoha, Rundgren et al. 2002, Zhuang, Lee et al. 2007). However, these data are unique in that the distinction has been made between the transcript variants, and the survival advantage is seen in patients with elevated BCL2β (as opposed to total BCL2). This may
explain the paradox in previous studies regarding total \( BCL2 \); that the presence of an anti-apoptotic protein actually confers prognostic advantage in patients. It should be considered that \( BCL2\beta \) has an alternative role to \( BCL2\alpha \). Indeed, when we look at other anti-apoptotic BCL2 subfamily isoforms, the result of a missing TM domain can switch the proteins’ role from anti- to pro- apoptotic (Bingle, Craig et al. 2000). The fact that \( BCL2\beta \) expression confers a survival advantage may not necessarily mean that \( BCL2\beta \) has a pro-apoptotic function. It may be regulating other oncogenes, or be a downstream effector of a different pathway contributing to the observed improved survival. It should be noted that overexpression of any one of the anti-apoptotic family members does not necessarily imply that a cell will be resistant to apoptosis. As mentioned, there is redundancy across the BCL2 family and the effect of individual family members can vary across cell types (See Section 1.5).

Another factor to consider regarding the longer survival in relation to \( BCL2\beta \) expression is the role of \( BCL2 \) in melanocyte function. \( BCL2 \) is a transcription target for MITF and is necessary for the prevention of spontaneous apoptosis as a result of the oxidative by-products of melanin synthesis (Sermadiras, Dumas et al. 1997, McGill, Horstmann et al. 2002, Cichorek, Wachulska et al. 2013). \( BCL2/ BCL2\beta \) may give a survival advantage where it is overexpressed in melanoma tissue, as expression of the protein/s is actually a “melanocyte-like” feature. In cases where melanoma cells have maintained this characteristic of the progenitor
melanocyte cell, the cancerous cells are likely to have undergone fewer mutations than those which have lost this ‘melanocyte-like’ feature. Tumour cells which retain $BCL2\beta$ would logically be less aggressive than others which lack this isoform expression.

As well as being positively associated with improved survival, $BCL2\beta$ mRNA was also enriched in metastatic tissue compared to primary tissue (see Figure 3.4.3-1). This is in agreement with one of our initial hypotheses outlined at the start of this Chapter in Section 3.2.

It should be noted that while patients diagnosed with primary melanoma have significantly overall survival than those diagnosed with metastatic (Siegel, DeSantis et al. 2012), our cohort is not representative of this pattern. Instead, there is no significant difference between survival based on tissue type (see Figure 3.4.5-2). This is because the primary tissue within this cohort were advanced primary lesions with > 2mm width and length - the smaller lesions did not contain enough DNA/ RNA/ protein to be analysed. The average thickness of primary samples in our cohort was 4.9mm, or Breslow Stage V. Breslow thickness has a strong negative association with survival (Breslow 1970). Therefore, survival for all of the thick primary melanomas in our study was similar to metastatic disease. So while it may appear that the two findings relating to $BCL2\beta$ and i) survival and ii) tissue type are conflicting, they are not in the context of this cohort.

This observation wherein $BCL2\beta$ expression was increased in metastatic tissue supports the findings of Utikal et al. and Zhang et al. with the
obvious exception that our results are \textit{BCL2}\textsubscript{β} specific (Utikal, Leiter et al. 2002, Zhang and Rosdahl 2006). Both of these studies extrapolated their findings to the protein level, whereas our protein data does not correlate with our mRNA data.

3.5.4 BCL2 protein expression

We also sought to confirm expression of the BCL2 isoforms at the protein level in accordance with our aims outlined in Section 3.2.

With reference to Section 2.5, while MRM MS is the gold standard for protein analysis, we were unable to refine these methods for FFPE tissue within the time limitations of this study. Instead we used IHC. Although we were unable to analyse the BCL2 isoforms separately by using this method, we were able to analyse total BCL2.

We hypothesised that by combining the staining results for two differentially targetted BCL2 antibodies – one to both isoforms (AbCam) and one to BCL2\textsubscript{α} only (LSB) – we would be able to determine BCL2\textsubscript{β} protein expression. Theoretically, tumours which stained positive for AbCam and negative for LSB would be positive for BCL2\textsubscript{β} (Figure 3.3.7-1).

However, there were a number of tumours which stained positive for LSB and negative for AbCam, which indicates that LSB is not specific to BCL2\textsubscript{α} and therefore we are unable to determine the differences between the two isoforms. We were therefore unable to test our hypothesis that the BCL2 isoforms have different associations with
melanoma survival and TILs at the protein level. We were also unable to assess cellular localisation of the isoforms.

The IHC results for the BCL2 antibodies did not correlate with \textit{BCL2} isoform mRNA expression (Figure 3.4.9-1). Similarly, unlike \textit{BCL2}\textsuperscript{β} expression, protein expression was not correlated with survival (Figure 3.4.7-1). This lack of continuity between the mRNA and protein results is unsurprising, as relative mRNA levels are rarely indicative of protein levels (Vogel and Marcotte 2012, Payne 2015). The method of scoring IHC is categorical and only semi-quantitative which may also effect correlation with mRNA.

Although expression of \textit{BCL2}\textsuperscript{β} mRNA was increased in metastatic compared to primary tissue, the expression of total BCL2 protein was increased in primary compared to metastatic tissue (Figure 3.4.8-1). This finding was also seen by Zhang et al. when examining total BCL2. They saw that 100\% of thin primary melanomas express BCL2 but only 63\% of subcutaneous metastases and 35\% of lymph node metastases (Zhuang, Lee et al. 2007).

Other studies have linked this elevated expression of BCL2 in primary compared to metastatic tissue to decreased expression of the BCL2 transcription factors MITF and AP-2 signalling ((Zhuang, Lee et al. 2007)). It would be interesting to analyse the effects of these transcription factors on the transcription of the individual BCL2 isoforms.
3.5.5 SNP rs3943258 and BCL2

A previous study of BCL2 polymorphisms in chronic myeloid leukaemia revealed an association between rs3943258 genotype and expression of the BCL2 isoforms in the blood of healthy controls (Guillem, Amat et al. 2015). Donors homozygous for the rs3943258 C allele had an increased ratio of BCL2β/BCL2α expression in the blood compared to those harbouring the T allele. We sought to test our hypothesis that rs3943258 was associated with BCL2 isoform mRNA expression in melanoma tissue.

Our analysis showed that there was no correlation between the SNP allele and neither the ratio of the isoforms nor the expression of BCL2α mRNA. However, there was a positive association between the presence of the T allele and expression of BCL2β in the tumour tissue (Figure 3.4.11-1). This observation was somewhat inverse to that made in the Guillem paper; they saw increased expression of BCL2β (relative to BCL2α) was associated with presence of the C allele, where we saw that increased expression of BCL2β was associated with presence of the T allele. This contradiction could be because their cohort was healthy, whereas ours consisted of melanoma patients. It may be that the splicing factor/ regulatory factor associating this SNP with BCL2β expression is perturbed in the context of cancer. Alternatively, our observation had a stronger statistical power due to an increased sample size; we may be seeing the true association.
The rs3943258 SNP is present in the second intron of BCL2, namely the intron which is differentially spliced to generate the two isoforms. It is possible that the SNP is part of an intronic splice regulatory element (SRE), such as that found in the ABCB11 gene (Davit-Spraul, Oliveira et al. 2014). Consensus sequences within SREs are highly variable and can form binding sites for a number of splicing regulatory proteins (Voelker, Erkelenz et al. 2012). SREs within other members of the BCL2 family have been well characterised; in particular, there is an abundance of literature on the splicing regulation of the BCLX gene. Four main regulatory regions exist within its mRNA (B2, B3, B1 and SB1), and these have been further divided into a number of regions; some of which are found within intronic sequences (Massiello, Salas et al. 2004, Garneau, Revil et al. 2005, Massiello, Roesser et al. 2006, Revil, Pelletier et al. 2009). Clearly, there is potential for intronic SREs to affect splicing within the BCL2 gene and further investigation is required.

3.5.6 BCL2 expression in TILs

In accordance with our aims outlined in Section 3.2, we set out to investigate the relationship between TILs and BCL2 expression.

We verified that BCL2 expression (at the protein and mRNA level) was not associated with TIL scoring and vice versa. While some heterogeneity of tumour tissue samples is unavoidable, these analyses have demonstrated as far as is possible that the observations regarding BCL2 in this context are indicative of tumour tissue and not the TILs within (Section 3.4.13).
3.5.7 TIL scoring

We also set out to confirm whether levels of TILs had a relationship with overall survival.

As addressed in Section 1.3.3, the success of immunotherapeutic approaches to melanoma has demonstrated the significance of the role of host immune response in determining prognosis. In previous studies TILs have been associated with longer term survival (Tumeh, Harview et al. 2014). In this cohort however, TIL scoring was not correlated with overall survival.

This could be due to the staining method we used; we did not use any TIL specific markers or IHC counting software; TILs were visualised manually according to size and shape. Although both methods are commonly used in the laboratory context i.e. manual and automated, there is ongoing discrepancy as to which is most specific (Rizzardi, Johnson et al. 2012).

In addition, in contrast to Tumeh’s cohort, the melanoma cohort used in this study was extensively sun damaged, which is typical of Australasian melanomas compared to those collected in the Northern Hemisphere (Banan, Marvi et al. 2016).
3.6 Conclusion

Within this chapter, we set out to investigate whether $BCL2$ isoform expression was associated with the clinical parameters of melanoma or with the possession of the rs3943258 SNP. We also examined the significance of TILs in the context of melanoma survival as well as $BCL2$ isoform expression.

Unfortunately, due to the lack of specific antibodies, we were unable to examine the protein isoforms separately in regards to clinical parameters, TILs, SNPs and cellular localisation. In addition, while every effort was made to limit contamination of samples by non-cancerous tissue (i.e. normal skin, connective tissue), the heterogeneous nature of melanoma may have caused some inconsistencies in the results. The ideal way to deal with these inconsistencies would be to perform multidimensional single cell-analysis, which was beyond the scope of the study.

We were able confirm our initial hypotheses that the two isoforms have different associations with melanoma clinical parameters, $BCL2$ expression is elevated in metastatic compared to primary tissue and that the rs3943258 SNP is associated with $BCL2$ expression levels in melanoma tissue.

Prior to these investigations, the role of $BCL2\beta$ in melanoma had not been considered.

We hereby add to the current body of literature by providing evidence that $BCL2\beta$ is actually associated with a number of melanoma parameters. It
is fascinating that none of these associations were made with \textit{BCL2}\textsubscript{α} alone, and this suggests that previous hypotheses regarding the role of \textit{BCL2} and melanoma may have been due to the contribution of \textit{BCL2}\textsubscript{β} to total \textit{BCL2} as opposed to the more frequently researched \textit{BCL2}\textsubscript{α}.

Specifically, we observed that \textit{BCL2}\textsubscript{β} mRNA is positively associated with i) melanoma survival and ii) metastatic tissue (compared with primary).

Although the exact explanation for this is unclear, we suggest that more research be conducted into the splicing control of the \textit{BCL2} gene to obtain clues about the role of the two isoforms.

This research into splicing regulation is particularly significant when we consider that i) we have also seen that presence of a T allele at the rs3943258 SNP location is associated with increased \textit{BCL2}\textsubscript{β} expression and ii) the expression of mRNA and protein was not correlated.
Chapter Four: Functional analysis of the \textit{BCL2} isoforms

4.1 Introduction

In this study, to investigate the roles of \textit{BCL2} isoforms in the apoptotic response, melanoma and melanocyte cells were treated with cisplatin and UVB. Cisplatin and UVB exposure typically trigger apoptosis due to the induction of irreparable DNA damage (Huddart, Titley et al. 1995, Shindo and Hashimoto 1998).

Cisplatin is a platinum based chemotherapy drug which introduces DNA crosslinks that interfere with the DNA replication pathway (Hashimoto, Anai et al. 2016). Exposure to UVB can induce cyclobutane pyrimidine dimers (CPDs) and 6,4 photoproducts (Budden and Bowden 2013). These types of DNA damage trigger NER; if the DNA can be repaired then the cell progresses through the cell cycle (Budden and Bowden 2013). Alternatively, intrinsic apoptosis is triggered via ATM, ERK or p53 signalling (Nowsheen and Yang 2012). It is important to note that triggering apoptosis in this context is dependent on the cells capability to detect DNA damage (which occurs via the NER as well as MMR)(Bradbury and Middleton 2004). This capacity is often attenuated in melanoma cells (Bowden, Ashton et al. 2010, Murray, Maltby et al. 2015, Budden, Davey et al. 2016).

As discussed in Section 1.5, BCL2 signalling can be integral to triggering apoptosis depending on stressor and cell type.
4.1.1 BCL2 and p53

Studies in fibroblasts have revealed that the UV induced apoptotic switch is dependent on p53 mediated BCL2 downregulation (Knezevic, Zhang et al. 2007). Conversely, similar studies in Chinese hamster ovary (CHO) and HeLa cells have concluded that UV-mediated apoptosis is BCL2 dependent but p53 independent (Isoherranen, Sauroja et al. 1999, Dunkern, Fritz et al. 2001). Clearly, there are multiple pathways linking UV induced DNA damage to apoptosis and the role of p53 in decision making is still under debate.

The p53 binding region in BCL2 is within the flexible loop domain; a region which is identical between the two isoforms (Deng, Gao et al. 2006). It is therefore likely that p53 can interact with both BCL2 proteins.

4.1.2 BCL2 and cisplatin resistance

The concept of priming cells with BH3 mimetics to increase their vulnerability to cisplatin induced cell death has been investigated in detail. Usage of the BH3 mimetic gossypol has been seen to reduce cisplatin resistance in head and neck cancer cells (Bauer, Trask et al. 2005).

Increased BCL2 expression is associated with cisplatin resistance in a number of cancers, including oropharyngeal squamous cell carcinomas, glioma, acute myeloid leukaemia and non-Hodgkin’s lymphoma (Thomas, Quinn et al. 2013).

Further research into the role of BCL2 and cisplatin is therefore warranted.
4.1.3 *BCL2* and melanin

4.2 Hypotheses & Aims

Despite the abundance of literature describing the relationship between BCL2 and apoptosis, the existence of different BCL2 isoforms is rarely acknowledged or investigated (See Section 0).

We therefore hypothesise:

- The two BCL2 isoforms have different roles in the apoptotic response.
- p53 is capable of regulating both isoforms in response to different stimuli.
- Melanin synthesis will differ between the KD conditions of the two BCL2 isoforms.

We aim to:

- Measure BCL2 isoform transcript expression throughout apoptosis induction.
- Knock down both BCL2 isoforms separately prior to the induction of apoptosis and melanin synthesis.
4.3 Methods

4.3.1 Cell culture

See Section 2.3.1, Cell Culture methods

4.3.2 Cell proliferation

Cells were seeded at $1 \times 10^5$/well in a 24 well plate (WP), and harvested for counting 24 and 72h after seeding. Cells were counted indirectly by using the CellTitre-Glo Luminescent Cell Viability Assay (Promega), according to manufacturer's instructions. In this assay, luminescence is produced as a by-product of the reaction between luciferin and cellular ATP; luminescence is proportional to the number of cells in each well. Plates were read on the Cytation 3 Cell Imaging Reader (BioTek).

4.3.3 Flow cytometry

Cells were harvested and washed 2x in PBS. For each time point, $10^5$ cells (in triplicate) were resuspended in 500ul Binding Buffer and stained with 5ul Annexin PE and 5ul 7-AAD PerPCy5 (BD Pharminogen Annexin Kit). Samples were gently vortexed for ~5 seconds and incubated for 15 minutes in the dark at RT before analysing each one in triplicate on the flow cytometer. Early apoptotic, dead and alive cells were designated as such using a flow cytometer (BD FACSCanto II) (Figure 4.4.2-1). Flow cytometry data was analysed using FlowJo software and statistics were performed using GraphPad Prism.

4.3.4 RNA processing

See Section 2.3.3 for extraction, 2.3.4 for reverse transcription, and 2.3.5 for qPCRs.
4.3.5 Cell treatment

Cells were treated with 10μg/ml cisplatin (Sigma Aldrich). The dosage of 10μg/ml of cisplatin was selected as it has been previously shown to induce apoptosis in melanocyte and melanoma cells (10-60% apoptotic cells 48h after treatment) (Bowen, Hanks et al. 2003, Avery-Kiejda, Bowden et al. 2011).

For UVB exposure, cells were treated with 650J/m² using the UVB Grobel Irradiation Chamber (Dr. Gröbel UV-Elektronik GmbH, Germany). A cell survival curve was generated using a range of UVB dosages to demonstrate that 650J/m² UVB kills at least 50% of melanocyte cell lines 24h following treatment (Budden, Davey et al. 2016). Cells were washed 1x in PBS before they were irradiated with 650J/m² UVB in 1ml PBS. The PBS was then replaced with cell culture media.

For non-transfection experiments, cells were seeded into 6WP (3mls media) at a concentration of 2x10⁵ cells/ml and left to grow for 24h prior to treatment. Cells were harvested for mRNA and cell function assays 24h post-seeding (i.e. baseline controls) as well as 12, 24 and 48h after treatment.

4.3.6 siRNA transfection

Melanoma cells were seeded at 50% confluency and melanocytes at 80% to optimise plate coverage for cell harvest, and to keep cell numbers consistent for repetition of transfections for multiple experimental purposes.
The cells were reverse transfected with siRNA—the transfection reagents were added before the cells were allowed to become adherent following seeding.

Per ml of media, 50μl Opti-MEM (Thermo Fisher), 2.5μl RNAi max lipofectamine (Thermo Fisher) and 1μl 50μM siRNA (Dharmacon) (final concentration 50nM) was added and left for 5 minutes to allow formation of liposomes. Cells were then seeded into wells (in appropriate antibiotic free media: see Section 2.3.1) and left for a minimum of 24h (and a maximum of 72h) to allow KD to occur. Cells were grown in 6WP (total 3mls media/ well) for RNA extractions and in 24WP (total 1ml media/ well) for flow-cytometry assays.

siRNAs were custom designed to target each isoform (Dharmacon, USA) (see Appendix 7.5). Non-targetting siRNA #4 (Dharmacon, USA) was used as a control.

4.3.7 Melanin quantification

Melanin was quantified indirectly by measuring absorbance at 475nm of cellular lysates (Mustapha, Bzeouich et al. 2015).

A standard curve of melanin concentrations was generated by measuring the absorbance at 475nm using the Epoch microplate spectrophotometer of a series of known concentrations of melanin (Sigma-Aldrich) (Figure 4.3.7-1).
Figure 4.3.7-1 Standard curve of absorbance of melanin standards at 475nm.

Melanocytes were seeded at ~80% (1.5x10^5/well) confluency in a 24WP as they were appropriately reverse transfected with siRNA (see Section 4.3.6). The cells were treated with UVB 24h post transfection (see Section 4.3.3) and harvested 72h post transfection. Cell pellets were resuspended in PBS and counted; 1x10^5 cells were used for melanin extraction. Pelleted cells were solubilized in 1ml 1N NaOH for 2h at 80°C while mixing. Samples were then centrifuged at 1200g for 10 minutes at RT, and supernatants were transferred to fresh tubes for absorbance at 475nm to be measured and plotted against the standard curve.

4.3.8 Statistical analysis

To investigate statistical differences between mRNA expression between time points (for the same transcript, within the same cell line), the Mann

\[
y = 0.0046x - 0.0011 \\
R^2 = 0.9992
\]
Whitney test was used. The same test was used to investigate differences between percentages of apoptotic cells and melanin concentration between time points. The Mann Whitney test is a non-parametric t-test; the sample size for each time point was relatively small (n=9; biological repeats=3, technical repeats=3) and was therefore unlikely to follow standard distribution.

Statistical analyses were performed in GraphPad Prism.
4.4 Results

4.4.1 Proliferation of cell lines

The rate of apoptosis following major stress is partially dependent on the speed of cell cycle, as apoptosis is triggered once the cell has gone through its cell cycle checkpoints (Alberts 2008). In addition, the total percentage of apoptotic cells in any given population will be lower if there is a high rate of cell turnover. For this reason, the rate of cell division of the melanoma cell lines was measured to best contextualise apoptosis.
Figure 4.4.1-1 Cell proliferation of three melanoma cell lines over 72h.

Relative luminescent units (RLU) were proportional to ATP and therefore the number of cells per plate. a) RLU was calculated by subtracting background from signal luminescence, b) fold change at 72h. Luminescence was measured in triplicate.
Proliferation was quantified as fold change increase at 72h using the CellTitre Glo assay as described in Section 4.3.2 (Figure 4.4.1-1). MM200 was the fastest growing cell line (fold change = 3), followed by MelRM (fold change = 1.7) and SkMel28 (fold change=1.5) respectively.

4.4.2 Establishing a method to discriminate alive, dead and early apoptotic cells

![Figure 4.4.2-1 Using flow cytometry to distinguish alive, dead and early apoptotic cells.](image)

The two fluorescent stains are monitored via the PerCP/7AAD channels and the PE-A channel. Depicted are MM200 melanoma cells a) 24h and b) 48h following cisplatin treatment.

Following cytotoxic treatment, cells move in an anti-clockwise direction through the quadrants of the fluorescence plot, starting from negatively stained PE-A (Annexin) and 7AAD/PerCP (Propidium Iodide, PI) (Figure 4.4.2-1).

PI is an intercalating agent whose specific fluorescent excitation energy and emission is dependent on its nucleic acid binding state. Nucleic acids
are only available for binding once the cell membrane has been fully ruptured (i.e. after apoptosis) and so PI staining is indicative of cell death (Vermes, Haanen et al. 1995).

Annexin V can bind phosphatidylserine (PS), which is located on the intercellular surface of the membrane in healthy cells. When the cellular membrane is compromised (i.e. during early apoptosis), PS is instead displayed on the extracellular surface. Cells which are dead or undergoing early apoptosis will therefore stain positively for Annexin V (Vermes, Haanen et al. 1995).

Cells which are positive for both PerCP/7AAD (i.e. PI) and PE-A (Annexin i.e. PS) are dead; those which are negative for both stains are alive, and those which are PerCP positive / PE-A negative are undergoing early apoptosis. Thus we were able to designate cells as dead, alive or early apoptotic by staining them for PI and PS (Figure 4.4.2-1)(Vermes, Haanen et al. 1995).
4.4.3 Investigation of the role of the BCL2 isoforms in the apoptotic response by monitoring transcription expression following cytotoxic treatment

4.4.3.1 Cisplatin

Figure 4.4.3.1-1 Apoptotic response to cisplatin in melanoma and melanocyte cells.

Melanocyte (HeMn) and melanoma (MelRM, MM200 and SkMel28) cell lines were treated with 10µg/ml cisplatin. Percentage (%) of early apoptotic cells was monitored at 0, 12, 24 and 48h following exposure. Asterisks (*) indicate significant difference (Mann Whitney) to % early apoptotic cells at previous time point (p<0.01**, p<0.05*). Flow measurements were made in triplicate.

Cisplatin induced apoptosis in all cell lines. Interestingly, all cell lines besides MelRM had the highest percentage of apoptotic cells at 48h. MelRM had the highest percentage of apoptotic cells at 12h (Figure 4.4.3.1-1). The remaining cells actually begin to recover and at 48h there were more alive cells than dead cells (data not shown).
The least cisplatin-sensitive cell line was the melanocytes. The maximum fold change in percentage of early apoptotic cells observed in this cell line was 2.3, whereas the average maximum fold change for the other cell lines was 8.2 (Mann Whitney, p<0.0001). In addition, the maximum percentage of early apoptotic melanocytes was 1.42%, compared to an average of 23.7% for the melanoma lines (Mann Whitney, p<0.0001) (Figure 4.4.3.1-1).
Figure 4.4.3.1-2 Response of \textit{BCL2} isoforms (mRNA) to cisplatin in melanoma and melanocyte cell lines.

a) b) Melanoma and c) d) melanocyte lines were treated with 10µg/ml cisplatin. Relative \textit{BCL2}\textit{α} and \textit{BCL2}\textit{β} mRNA expression was monitored at 0, 12, 24 and 48h following exposure. mRNA was normalised to GAPDH. Asterisks (*) indicate significant difference (Mann Whitney) to expression at previous time point (p<0.01**, p<0.05*). qPCR was performed in triplicate.
Interestingly, both isoforms of BCL2 shared the same response to cisplatin treatment in melanocytes (Figure 4.4.3.1-2). Specifically, expression decreased slightly at 12h (Mann Whitney, BCL2α p<0.0001, fold change from 0h 0.1, BCL2β p=0.0004, fold change 0.2 from 0h), increased at 24h (Mann Whitney, BCL2α p=0.0004, fold change from 12h 7.9, BCL2β p<0.0001, fold change from 12h 3.7) and increased again at 48h (Mann Whitney, BCL2α p=0.0004, fold change from 24h 77.0, BCL2β p=0.0004 fold change from 24h 40.4). In contrast, the response to cisplatin treatment varied between the isoforms in each of the melanoma cell lines studied (Figure 4.4.3.1-2).

In MelRM, where BCL2α had decreased expression until 48h (Mann Whitney, fold change from 0h 0.15, p<0.0001, fold change from 12h (0) p<0.0001, fold change from 24, (0), p<0.0001), BCL2β did not respond until 24h (Mann Whitney, p>0.0001), wherein it decreased to 0. It is interesting to note however that neither isoform was expressed in this cell line at 24h (Figure 4.4.3.1-2).

In MM200, BCL2β expression was not induced at any point throughout the 48h experimental period whereas BCL2α was induced at 48h (Mann Whitney, p=0.0004, fold change from 24h 57.9) (Figure 4.4.3.1-2).

In SkMel28, BCL2α was unresponsive to cisplatin treatment, whereas BCL2β was responsive. BCL2β was induced at 12 and 24h (Mann Whitney, p<0.0001, fold change from 0h 4.8, p<0.0001, fold change from 12h 3.3), and expression decreased slightly at 48h (Mann Whitney, p<0.0001, fold change from 24h 0.3) (Figure 4.4.3.1-2).
Figure 4.4.3.2-1 Apoptotic response to UVB in melanoma and melanocyte cell lines.

Melanocyte (HeMn) and melanoma (MM200, MelRM and SkMel28) cell lines were treated with 650kJ/m² UVB. Percentage (%) of early apoptotic cells was monitored at 0, 12, 24 and 48h following exposure. Asterisks (*) indicate significant difference (Mann Whitney) to % early apoptotic cells at previous time point (p<0.01**, p<0.05*). Flow measurements were made in triplicate.

UVB also induced apoptosis in all cell lines, particularly the melanocyte cell line HeMn. These cells had the highest peak percentage (mean 77.8%) of early apoptotic cells compared to the other cell lines (Mann Whitney, mean 30.2%; p<0.0001). All melanoma cells followed a very similar apoptotic response in that early apoptosis was induced at 12h (MelRM, p<0.0001, fold change from 0h 2.8; MM200, p<0.0001, fold change from 0h, 2.5; SkMel p=0.0002, fold change from 0h 2.1) and the percentage of early apoptotic cells continued to increase at each time
point thereafter. Apoptosis was not induced in melanocytes until 24h. Although there was a significant difference in early apoptosis between 0 and 12h, the fold change was only 1.1 (Mann Whitney, p<0.0001, fold change from 12h 5.3) (Figure 4.4.3.2-1).
Figure 4.4.3.2-2 Response of BCL2 isoforms (mRNA) to UVB exposure in melanoma and melanocyte cell lines.

A selection of a) b) melanoma and c) d) melanocyte lines were treated with 650kJ/m² UVB. Relative \( BCL2\alpha \) and \( BCL2\beta \) mRNA expression was monitored at 0, 12, 24 and 48h following exposure. mRNA was normalised to \( GAPDH \). Asterisks (*) indicate significant difference (Mann Whitney) to expression at previous time point (p<0.01**, p<0.05*). qPCR was performed in triplicate.

In melanocytes, \( BCL2\alpha \) expression decreased significantly at 12h (p<0.0001, fold change from 0h -71.5) and increased significantly at 48h (p=0.002, fold change from 24h +14.5). Although \( BCL2\beta \) expression also
increased significantly at 12h (p<0.0001, fold change from 0h +29),
expression remained unchanged at 24h and 48h (p=0.24, p=0.69)
(Figure 4.4.3.2-2).

In MelRM, \( BCL2\alpha \) and \( BCL2\beta \) expression decreased in response to UVB
12h after exposure (\( BCL2\alpha \), p<0.0001, fold change from 0h -3; p=0.0082,
fold change from 0h -3.75). Expression of \( BCL2\alpha \) then increased for the
remainder of the time course (24h p<0.026 fold change from 12h 2.13,
48h p<0.0001 fold change from 24, 14.0). \( BCL2\beta \) increased at 24h
(p<0.0001, fold change from 12h 18.2) and remained steady at 48h
(p=0.98) (Figure 4.4.3.2-2).

In MM200, both isoforms were downregulated in response to UVB
(\( BCL2\alpha \), p=0.0004, fold change from 0h 15.1; \( BCL2\beta \), p<0.0001, fold
change from 0h 9.2). However, \( BCL2\alpha \) was upregulated at 48h
(p=0.0091, fold change from 24h 2.8), but expression of \( BCL2\beta \) remained
unchanged after 12h (24h p=0.5, 48h=0.7) (Figure 4.4.3.2-2).

In SkMel28, \( BCL2\alpha \) and \( BCL2\beta \) were both downregulated throughout the
time course in response to UVB. \( BCL2\alpha \) expression decreased at 12h
(p<0.0001, fold change from 0h, 19.5) and 48h (p=0.0053, fold change
from 24h 1.6). \( BCL2\beta \) was not expressed at 12h, 24h or 48h after UVB
exposure (Figure 4.4.3.2-2).

In summary, the \( BCL2 \) isoforms did not respond to UVB exposure in the
same way in any of the cell lines.
4.4.4 Investigation of the role of the BCL2 isoforms in the apoptotic response in BCL2 knock-down cells

![Graph showing relative mRNA expression of BCL2 isoforms](image)

**Figure 4.4.4-1 Relative expression of BCL2 isoforms in MM200 melanoma cells following siRNA mediated KD.**

Cells were transfected with either scrambled control siRNA or siRNA targeted to the BCL2 isoform of interest. Relative mRNA expression was monitored 72h following transfection and normalised to housekeeping genes. Asterisks (*) indicate significant difference (Mann Whitney) to expression in control conditions (i.e. scrambled siRNA), (p<0.01**, p<0.05*). qPCR was performed in triplicate.
Figure 4.4.4-2 Relative expression of BCL2 isoforms in HeMn melanocytes following siRNA mediated knock-down.

Cells were transfected with either scrambled control siRNA or siRNA targeted to the BCL2 isoform of interest. Relative mRNA expression was monitored 72h following transfection and normalised to housekeeping genes. Asterisks (*) indicate significant difference (Mann Whitney) to expression in control conditions (i.e. scrambled siRNA), (p<0.01**, p<0.05*). qPCR was performed in triplicate.

Transfection of both cell lines under experimentation (MM200 and HeMn) with BCL2 isoform-targetting siRNA was successful in significantly decreasing mRNA expression at 72h following transfection. Specifically, BCL2α expression was reduced by 44% in MM200 and 72% in HeMn (Mann Whitney, p<0.0001, p=0.002). BCL2β expression was reduced by 42% in MM200 and 82% in HeMn (Mann Whitney, p=0.038, p=0.043) (Figure 4.4.4-1, Figure 4.4.4-2).
4.4.4.1 **Effect of knocking down BCL2 isoforms in MM200**

![Graphs showing apoptotic response of siRNA transfected MM200 melanoma cells to a) cisplatin and b) UVB.](image)

**Figure 4.4.4.1-1 Apoptotic response of siRNA transfected MM200 melanoma cells to a) cisplatin and b) UVB.**

Cells were transfected with either scrambled control siRNA or siRNA targetted to the BCL2 isoform of interest and treated with a) 10mg/ml cisplatin or b) 650J/m² UVB. Cellular response was monitored 72h following transfection/ 48h following cytotoxic treatment. Asterisks (*) indicate significant difference (Mann Whitney) to % early apoptotic cells in control conditions (i.e. scrambled siRNA) point (p<0.01**, p<0.05*). Flow measurements were made in triplicate.

Knocking down BCL2a expression in MM200 resulted in an increased death response to cisplatin in comparison to the control cells (Mann Whitney, scrambled vs targetting siRNA, dead cells, p=0.02*). However, the percentage of apoptotic cells was similar between the control and the BCL2a KD cells (KD) (Mann Whitney, scrambled vs targetting siRNA, early apoptotic cells, p>0.999) (Figure 4.4.1-1 a)).
Alternatively, knocking down $BCL2B$ expression induced an increased percentage of early apoptotic cells in comparison to the non-targetting siRNA controls (Mann Whitney, scrambled vs targeting siRNA, apoptotic cells $p=0.0079^{**}$) and a decreased percentage of early apoptotic cells in comparison to the non-targetting siRNA controls (scrambled vs targeting siRNA, dead cells $p=0.0079^{**}$) (Figure 4.4.1-1 a).

Knocking down $BCL2\alpha$ and $BCL2\beta$ had a similar effect on cellular response to UVB exposure. Specifically, knocking down the isoforms induced an increased number of early apoptotic and dead cells in comparison to the control cells (Mann Whitney, scrambled vs $BCL2\alpha$ targeting siRNA, dead cells $0.002^{**}$, scrambled vs $BCL2\alpha$ targeting siRNA, apoptotic cells $p=0.002^{**}$, scrambled vs $BCL2\beta$ targeting siRNA, dead cells $p=0.002^{**}$, scrambled vs $BCL2\beta$ targeting siRNA, apoptotic cells $p=0.01^{*}$) (Figure 4.4.1-1 b).
4.4.4.2 Effect of knocking down BCL2 isoforms in melanocytes

Figure 4.4.4.2-1 Apoptotic response of siRNA transfected melanocytes (HeMn) to a) cisplatin and b) UVB.

Cells were transfected with either scrambled control siRNA or siRNA targeted to the BCL2 isoform of interest and treated with a) 10mg/ml cisplatin or b) 650J/m² UVB. Cellular response was monitored 72h following transfection and 48h following cytotoxic treatment. Asterisks (*) indicate significant difference (Mann Whitney) between % early apoptotic cells in BCL2α KD and BCL2β KD cells, (p<0.01**, p<0.05*). Flow measurements were made in triplicate.

There was no significant difference between cellular response to UVB or cisplatin between BCL2 isoform KD and control melanocyte cells. However, there was a significant difference in response when the two BCL2 isoform KDs were compared to each other. Knocking down BCL2α resulted in an increased number of apoptotic and dead cells compared to when BCL2β was knocked down (Mann Whitney, BCL2α targetting
siRNA vs BCL2β targeting siRNA, apoptotic cells, cisplatin, p=0.001**, BCL2α targeting siRNA vs BCL2β targeting siRNA, apoptotic cells, UVB, p=0.001**) (Figure 4.4.2-1).

4.4.5 Investigation of the roles of the BCL2 isoforms in melanin synthesis

![Graph showing melanin concentration of siRNA transfected melanocytes (HeMn) lysates following treatment with UVB.](image)

**Figure 4.4.5-1 Melanin concentration of siRNA transfected melanocytes (HeMn) lysates following treatment with UVB.**

Cells were transfected with either scrambled control siRNA or siRNA targeted to the BCL2 isoform of interest and treated with 650J/m² UVB. Melanin concentration was measured 72h following transfection and 48h following UVB exposure. Melanin concentration was measured in duplicate.

Knocking down the BCL2 isoforms had no significant effect on melanin production in response to UVB exposure (Mann Whitney, scrambled vs BCL2α p=0.15, scrambled vs BCL2α p=0.95, BCL2α vs BCL2β p=0.95) (Figure 4.4.5-1).
4.5  Discussion

In this chapter we sought to investigate the role of the BCL2 isoforms in apoptotic response and melanin synthesis.

We hypothesised that the two BCL2 isoforms would have different roles within the cell (specifically within apoptosis and melanin synthesis) and that they would be regulated via different pathways.

4.5.1  Investigation of the role of the BCL2 isoforms in the apoptotic response by monitoring transcript expression following cytotoxic treatment

In agreement with our initial hypothesis, as outlined in Section 4.2, the BCL2 isoforms responded differently to apoptosis induction depending on i) cell line and ii) stress stimuli.

The variation between cell lines was to be expected, as behaviour of the BCL2 family varies depending on the genetic and epigenetic characteristics of the cell type (Salucci, Burattini et al. 2012).

Both cytotoxic treatments used in our experiments induce DNA crosslinks which are typically repaired by NER (Budden and Bowden 2013). For this reason, UV irradiation and cisplatin treatment are often used interchangeably when analysing DNA repair mechanisms. However, in this study and others, differences have been observed between cellular response to the two treatments which cannot be explained by differences in dosages alone (Smith, Ford et al. 2000). For example, co-stimulation of cells with IL-1 alongside UVB treatment induces TNFα activation, although this is not seen when the UVB
irradiation is replaced with cisplatin (Strozyk, Poppelmann et al. 2006). In our study, MelRM reached a maximum rate of approximately 30% early apoptotic cells 48h after treatment with UVB and 12h after treatment with cisplatin (Figure 4.4.3.1-1, Figure 4.4.3.2-1). Comparisons of these maximum response times to the other cell lines show their responses are dissimilar. This demonstrates that the two treatments are capable of activating different cellular pathways and therefore eliciting a different cell response. This is why it is important to use a variety of cellular stressors when examining apoptotic response.

Response to the stressors was inconsistent across the cell lines (see Figure 4.4.3.1-1, Figure 4.4.3.2-1). This is likely due to each cell line having inherent sensitivity – in future studies it may be useful to use a separate dose for each cell line, based on each one’s IC50.

Intriguingly, in the context of SkMel28 and UVB treatment, both \textit{BCL2} isoforms responded in an anti-apoptotic manner in that they were both downregulated as apoptosis is induced (Figure 4.4.3.2-1, Figure 4.4.3.2-2). However, in that same cell line, where \textit{BCL2α} was unresponsive to cisplatin treatment, \textit{BCL2β}'s response was erratic (showing no correlation with cell survival) (Figure 4.4.3.1-1, Figure 4.4.3.1-2). This again suggests that the mechanism of regulation is different between the two stimuli. This particular cell line is p53 mutant, which supports the theory that p53 can regulate \textit{BCL2} activity by direct protein binding but also via transcriptional regulation (Wu, Mehew et al. 2001, Tomita, Marchenko et al. 2006). Interestingly, although SkMel28 is p53 mutant,
the cells still responded to intensive DNA damage by undergoing cell death. As mentioned in Section 4.1.1, the role of p53 in intrinsic apoptosis pathways can vary according to cell type. These data support our hypothesis that p53 is capable of regulating both $BCL2$ isoforms.

Within the MM200 cell line, $BCL2\beta$ was unresponsive to cisplatin treatment where $BCL2\alpha$ was upregulated (Figure 4.4.3.1-2). This is unusual as $BCL2\alpha$ is typically downregulated when death is induced (Walensky 2006). Alternatively, as predicted, $BCL2\alpha$ was downregulated in response to UVB, as was $BCL2\beta$ (Figure 4.4.3.2-2). This also indicates there is a different mechanism regulating $BCL2$ isoform response depending on the stimuli.

MelRM is one of the fastest growing of the four cell lines (Figure 4.4.1-1). Unsurprisingly therefore, it was the fastest to respond to cisplatin (i.e. by undergoing apoptosis) (Figure 4.4.3.1-1). Cells with a high proliferative rate are generally most sensitive to chemotherapeutic treatment as the damage is detected by the checkpoints during the cell cycle, at which point apoptosis is triggered (Li, Sanki et al. 2006). Interestingly, the cells which survived the treatment actually started to multiply again at 48h post treatment. As this population of cells recovered, $BCL2\alpha$ and $BCL2\beta$ were upregulated, supporting the hypothesis that both isoforms are anti-apoptotic (Figure 4.4.3.1-1, Figure 4.4.3.1-2). Intriguingly however, both isoforms were also upregulated in response to UVB exposure, despite there being no recovery within the cell population (Figure 4.4.3.1-1, Figure 4.4.3.2-2). It is unclear therefore whether the upregulation of the
BCL2 isoforms in MelRM is a universal response to cytotoxic stress or whether its anti-apoptotic activity is only induced by cisplatin in MelRM.

The isoforms shared a similar response in melanocytes for both stimuli (Figure 4.4.3.1-2, Figure 4.4.3.2-2). These findings are important as the genetic background of the melanocytes is far more stable than that of melanoma cells (Bertolotto 2013). Specifically, these responses indicate that the both isoforms are anti-apoptotic. Although cisplatin did induce early apoptosis in melanocytes, the percentage of cells undergoing early apoptosis never went above 1.4%, which was likely due to the slow cell cycle of melanocytes compared to melanoma cells (Figure 4.4.3.1-1). Both BCL2 isoforms were upregulated overall in response to cisplatin, which implies that they may have an anti-apoptotic role (Figure 4.4.3.1-2). On the other hand, UVB induced apoptosis in melanocytes; the treatment induced downregulation of both isoforms, implying once more that their role is anti-apoptotic (Figure 4.4.3.2-1, Figure 4.4.3.2-2). Although this characteristic has been previously confirmed in BCL2α, current hypotheses regarding the role of BCL2β are inconsistent.

The variability in responses of the isoforms to apoptotic stimuli in the melanoma cells indicates that the traditional pathways may have been disrupted by DNA mutations which have occurred during the oncogenic transformation of the cells. Indeed, melanoma cells typically have a high mutation load due to UV mutagenesis (Hodis, Watson et al. 2012).
4.5.2 Investigation of the role of the *BCL2* isoforms by monitoring cellular response to cytotoxic treatment in *BCL2* knock-down cells

Although there were three melanoma cell lines used in this study, one of the lines (MM200) had an optimal reduction in expression of the two *BCL2* isoforms following transfection via the same experimental conditions as HeMn (Figure 4.4.4-1, Figure 4.4.4-2). HeMn is a control cell line and so we were restricted to using the same transfection conditions which were optimal for these cells on all the other cell lines.

It is not possible to directly compare the results from the apoptosis assays performed on the non-transfected cells and the apoptosis assays on the transfected cells. This is because the transfection reagents affect the cells’ metabolism and so require a separate control group than non-transfection cell experiments (i.e. non-targetting or ‘scrambled’ siRNA)(Bauer, Kristensen et al. 2006).

Similarly to previous experiments within this chapter, these transfection experiments also supported our hypothesis that the two *BCL2* isoforms have different roles in the apoptotic response.

Although the transfected melanoma cells (MM200) shared a similar response to UVB treatment, the response to cisplatin was different (Figure 4.4.4.1-1). Where knocking down *BCL2α* significantly increased the number of dead cells, knocking down *BCL2β* significantly increased the number of apoptotic cells (Figure 4.4.4.1-1). This supports the well-established theory that *BCL2α* is anti-apoptotic and also suggests a similar role for *BCL2β*. It does appear that *BCL2β* may not have quite as
potent an effect as BCL2α on affecting the cells’ death status – the majority of the responsive BCL2β KD cells are only just entering apoptosis at 48h whereas a similar percentage of BCL2α KD cells were already dead by this point. It is interesting that the percentage of dead cells was actually lower in the BCL2β KD cells than the control cells – this is likely due to a higher percentage of cells being in the early apoptotic phase (Figure 4.4.4.1-1).

Although both isoforms exhibited a response to UVB and cisplatin exposure (Figure 4.4.3.1-2, Figure 4.4.3.2-2), knocking down the BCL2 isoforms did not affect cellular response to these stressors in melanocytes (Figure 4.4.4.2-1). This is interesting as previous reports have demonstrated that melanocytes are dependent on BCL2α expression for survival (McGill, Horstmann et al. 2002). We would therefore anticipate an increased sensitivity to apoptosis in melanocytes with reduced BCL2α expression. Our data demonstrates that there must be a threshold of BCL2α (and perhaps BCL2β) upon which melanocytes are dependent on for survival. In this context, the siRNA mediated KD did not decrease BCL2 isoform expression far enough to go beyond this threshold (BCL2α expression was reduced by 72% and BCL2β by 42%) (Figure 4.4.4-2).
4.5.3 Investigation of the role of the *BCL2* isoforms in the apoptotic response by quantifying melanin synthesis following cytotoxic treatment

Contrary to our hypothesis outlined in Section 4.2, knocking down the *BCL2* isoforms had no significant effect on melanin production. We looked for this effect at 48h post UVB exposure, which is the latest time point used throughout these experiments (Figure 4.4.5-1)

This suggests that BCL2 is not involved in the melanin synthesis pathway, despite it acting downstream of the major melanin synthesis regulator, MITF (Hornyak, Jiang et al. 2009).
4.6 Conclusion

At the outset of this chapter, we set out to investigate the roles of both BCL2 isoforms in apoptosis and in melanoma synthesis.

We have confirmed that $BCL2\beta$ has a functional role within the cell. Although it is difficult to define this role in the current context, wherein results are conflicting between cell lines, these data are not unusual in that the role of the BCL2 family is notoriously variable depending on stressor and cell type (Igney and Krammer 2002, van Delft and Huang 2006).

As far as this data can demonstrate however, it appears that $BCL2\beta$ has anti-apoptotic activity and that it is regulated via different means than $BCL2\alpha$. Our hypothesis that p53 can regulate both isoforms was proved to be true and thus the role of p53 requires further investigation in this context.

In order to gain an improved insight on the role of the BCL2 isoforms in apoptosis, it would be conducive to conduct different apoptosis assays. For example, the CaspaseGlo assay is based on caspase 3/7 activation. By performing this assay, we could assess what percentage of the apoptosis is occurring via intrinsic vs extrinsic pathways. Western blotting for the various proteins involved in the apoptosis cascade (e.g. Bid, cytochrome c, various caspases) would also give insight as to which stage of apoptosis BCL2 isoforms may regulate.
We have also demonstrated that melanocyte dependency on $BCL2\alpha$ for survival is only effective up to a certain threshold of expression.

Although we hypothesised that the $BCL2$ isoforms would have a role in melanocyte synthesis, these investigations proved this to be untrue, at least in the context of short term response to UVB exposure.
5 Chapter Five: Discussion

Expression of the BCL2β isoform has been investigated only very rarely (Guillem, Amat et al. 2015) since its discovery in the 1980s (Tsujimoto, Finger et al. 1984, Bakhshi, Jensen et al. 1985). Characterisation studies on the protein have thus far been limited to cloned versions of the gene which are actually inaccurate to the naturally occurring sequence (see Figure 1.6-1) (Tanaka, Saito et al. 1993) (Froesch, Aime-Sempe et al. 1999) (Peng, Lapolla et al. 2009) (Laulier, Barascu et al. 2011). We are among the first to investigate the role of BCL2β as a naturally occurring isoform, and the very first to describe it in the context of melanoma.

In Chapter Two, we were able to confirm our hypothesis that both BCL2 isoforms were present at the mRNA and protein levels in a group of melanoma cell lines (Figure 2.4.3-1, Figure 2.4.4.2-2). This was achieved by assessing and optimising several methods to ensure the two isoforms were quantified separately and accurately. mRNA quantification by qPCR using specific probes and primer sets is the gold standard for specific transcript quantification and we confirmed both BCL2α and β were accurately assessed using this method. For isoform specific protein expression, we tested several methods and the lack of antibody specificity led to the MRM MS being the most accurate and quantitative assay for determining protein isoform expression levels. As mentioned, although the use of MRM confirmed expression of our protein of interest, it would be inappropriate to use routinely due to cost, time and availability of machinery and expertise. Although MRM MS methods can been used
to quantify protein within FFPE tissue (Kennedy, Whiteaker et al. 2016), this method has not been optimised within the University of Newcastle Analytical and Biomolecular Research Facility. It would of course be desirable to streamline these methods so that we could analyse protein expression in our melanoma cohort as well as our selection of cell lines.

In Chapter Three, we analysed expression of the two BCL2 isoforms in the context of melanoma. We were again able to prove our hypothesis that the isoforms would have different associations with melanoma clinical parameters and tissue type.

Previous findings regarding the role of the BCL2 gene in the disease have been contradictory (see Section 0 for a literature summary). Indeed, our own findings were contradictory; while BCL2β mRNA was elevated in metastatic tissue, total BCL2 protein was elevated in primary (Figure 3.4.5-1, Figure 3.4.8-1). These findings may explain why there is disparity in the field of BCL2 in melanoma – most investigations do not account for the separate isoforms. We have also observed that expression of BCL2β in melanoma tissue corresponds with extended survival (Figure 3.4.4-1). We hypothesise that maintenance of BCL2β expression throughout the oncogenic transition from melanocyte to melanoma may indicate a lower mutation rate and therefore decreased disease aggression. This requires further investigation.

Finally, in Chapter Four, we confirmed our hypothesis that the two BCL2 isoforms have different roles in the apoptotic response, and that p53 is capable of regulating both isoforms.
Significantly, we demonstrated a functional role for BCL2β in the cell; it responded to different stimuli and expression varied between the genetic backgrounds of the cell lines being analysed. Evidence indicates that the isoform has an anti-apoptotic role. Based on these findings, we can also hypothesise that the role of p53 in regulating the two isoforms differs.

We understand that these data appear counter intuitive – that BCL2β confers a survival advantage whilst also appearing to be anti-apoptotic. We suggest that it is not the anti-apoptotic role which confers this advantage. As previously outlined, it may be that BCL2β is characteristic of melanocytes and therefore a ‘marker’ of melanomas which have a lower mutation rate.

Throughout the conduction of these investigations, the biggest hindrance was the lack of a BCL2β antibody. With a specific antibody such as this, we could analyse protein expression routinely using western blots and IHC, instead of having to rely on MRM MS. We could also label the antibody in order to investigate cellular localisation of the protein.

In order to discover more about this rare isoform, it would also be desirable to develop an expression vector (containing the true BCL2β sequence) to transfec cells. We could perform functional cellular assays, such as those conducted in this thesis, and observe the consequence of BCL2β protein expression. Ideally, studies of over-expression and knock-down should be performed in parallel. Unfortunately the time constraints and difficulty with the isoform sequence similarity resulted in only the knockdown studies being completed.
It would also be informative to sequence the BCL2 gene in the cell lines as well as the clinical cohort. We observed that possession of the T allele at rs3943258 correlated with increased BCL2β expression – there may be other SNPs or mutation patterns which influence BCL2 gene splicing. On that note, it would be informative to compare our melanoma cohort to an age matched healthy one, as our SNP results contrasted with a study on healthy controls (Guillem, Amat et al. 2015).

As described in Chapter One (Section 1.5), the exact mechanism of action for the BCL2 family is still ambiguous. In order to truly understand the role of BCL2β, it would be necessary to investigate it in the context of the other BCL2 family members. In particular, the experimental process known as BH3 profiling, as developed by the Letai lab (Certo, Del Gaizo Moore et al. 2006) can be used to investigate patterns of interaction within the BCL2 family. Using this method, we could assess mitochondrial sensitivity to panels of BCL2 proteins (i.e. including BCL2β), as opposed to examining the effect of each protein individually. As described in Table 1.5.1-1, there are many theories of BAX/BAK activation (i.e. apoptosis commitment) but each theory consists of interactions between multiple BCL2 family proteins.

Although it was not described in these studies, the qPCRs on Me4405 samples yielded unusual results. These experiments were performed under the same conditions as the other qPCRs, yet standard deviation between technical and biological replicates was consistently high for BCL2α and BCL2β. In addition, BCL2 protein in this particular sample
would disappear and re-appear in western blots depending on extraction conditions. Preliminary methylation analyses on the melanoma cell lines used in these studies revealed that the three lines, MelRM, MM200 and SkMel28 had a similar methylation profile whereas Me4405 did not. Interestingly, Me4405 has been described as being p53 null (Avery-Kiejda, Zhang et al. 2008). It is unclear whether these unusual Me4405 BCL2 data are a result of methylation, mutations or p53; this requires further investigation.

In conclusion, these investigations have raised a number of questions regarding the role of the BCL2 isoforms in melanoma but also in general cellular function. However, we can confirm that the isoforms both exist at the mRNA and protein level, that they are differentially regulated and that their roles within the cell are different. We can also confirm that BCL2β expression correlates with increased survival in melanoma.

6 References


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for missing data in epidemiological and clinical research: potential and pitfalls." BMJ 338: b2393.


(VEGF), matrix metalloproteinase-3 (MMP-3), and BCL-2 in malignant melanoma." Med Oncol 25(4): 431-436.


7 Appendix

7.1 Reverse transcriptions

Table 7.1-1 Thermal cycling conditions for reverse transcription reactions.

<table>
<thead>
<tr>
<th>Step</th>
<th>Incubation</th>
<th>RT reaction</th>
<th>RT inactivation</th>
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<tbody>
<tr>
<td>Time (min)</td>
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<td>5</td>
</tr>
<tr>
<td>Temperature (°C)</td>
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<td>37</td>
<td>85</td>
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Table 7.1-2 Reaction volumes for reverse transcription reactions.

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<th>Reagent</th>
<th>Volume (μl)</th>
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</tr>
<tr>
<td>dNTP mix</td>
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<td></td>
</tr>
<tr>
<td>Random primers</td>
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<td></td>
</tr>
<tr>
<td>Reverse transcriptase</td>
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<td></td>
</tr>
<tr>
<td>RNase inhibitor</td>
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</tr>
<tr>
<td>Water</td>
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### 7.2 TaqMan qPCRs

#### Table 7.2-1 Thermal cycling conditions for TaqMan qPCRs.

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<th>Temperature (°C)</th>
<th>Time (seconds)</th>
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<tr>
<td>Hold</td>
<td>95</td>
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<tr>
<td>PCR (40 cycles)</td>
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#### Table 7.2-2 Final reaction volume for TaqMan qPCRs.

<table>
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<th>Reagent</th>
<th>Volume (μl)/ sample</th>
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<tr>
<td>TaqMan</td>
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</tr>
<tr>
<td>Probe</td>
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<tr>
<td>Water</td>
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<tr>
<td>cDNA sample</td>
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<tr>
<td>Total</td>
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7.3 SYBR Green qPCRs

Table 7.3-1 Thermal cycling conditions for SYBR Green qPCRs.

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<th>Time (seconds)</th>
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<tr>
<td>Hold</td>
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</tr>
<tr>
<td></td>
<td>95</td>
<td>600</td>
</tr>
<tr>
<td>PCR (40 cycles)</td>
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<tr>
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Table 7.3-1 Final reaction volume for SYBR Green qPCRs.

<table>
<thead>
<tr>
<th>Reagent</th>
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</tr>
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<td>cDNA sample</td>
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### MS analysis of individual gel plugs

**Table 7.4-1 Ion trap MS analysis of direct immunoprecipitation gel plugs**

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<th>pI</th>
<th>Score</th>
<th>#Pept.</th>
<th>SC [%]</th>
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<td>113</td>
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<td>G3P_HUMAN</td>
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<td>9.3</td>
<td>542.6</td>
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<td>161.6</td>
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<td>IF2A_HUMAN</td>
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<td>4.9</td>
<td>134.9</td>
<td>3</td>
<td>11.1</td>
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7.5 Dhharmacon custom siRNAs

*BCL2α* sequence including overhangs:

5'AAGAAGUGACAUUCAGCUU3

*BCL2β* sequence including overhangs:

5'ACUUGUGAUGUGAGUCGUU3'