TARGETING ADAPTATIVE MECHANISMS TO ENDOPLASMIC RETICULUM STRESS IN MELANOMA

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B. Biotechnology

Thesis submitted in fulfilment of the requirements for obtaining the degree of

DOCTOR OF PHILOSOPHY in Surgical Science

School of Medicine and Public Health
University of Newcastle

November 2013
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 this copy of my thesis, when deposited in the University Library**, being made available for loan and photocopying subject to the provisions of the Copyright Act 1968.

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I hereby certify that the work embodied in this thesis contains a published paper/s/scholarly work of which I am a joint author. I have included as part of the thesis a written statement, endorsed by each co-author, attesting to my contribution to the joint publication/s/scholarly work.

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Kwang Hong Tay
ACKNOWLEDGEMENT OF COLLABORATION

I hereby certify that the work embodied in this thesis has been done in collaboration with other researchers. I have included as part of the thesis a statement clearly outlining the extent of collaboration, with whom and under what auspices at the beginning of each research chapters.

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Kwang Hong Tay
I hereby certify that this thesis is in the form of a series of published papers of which I am a joint author. I have included as part of the thesis a written statement from each co-author, endorsed by the Faculty Assistant Dean (Research Training), attesting to my contribution to the joint publications.

Kwang Hong Tay
I dedicate this thesis to all my family members and relatives, especially my parents, Jee Tay and Kwi Ying Siow, my sibling, Kwang Yew Tay, my uncle and aunt, Khai Bon Tan and Kwee Lan Siow, and my cousin, Hong Sing Tan, who have been so supportive throughout my PhD candidature. I also dedicate this thesis to my partner Hsin-Yi Tseng who accompanies me to go through the whole study and keeps me going during depressed and stressful moments.
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Publication Arising from Work in this Thesis


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- Poster Presentation (SMR (Society for Melanoma Research) Conference 2010)

- Poster Presentation (SMR (Society for Melanoma Research) Conference 2010)

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- Poster Presentation (Melbourne Lorne Cancer Conference 2010)

- Poster Presentation (HMRI Cancer Research Program 2010)
• Poster Presentation (Melbourne Lorne Cancer Conference 2011)


• Poster Presentation (SMR (Society for Melanoma Research) Conference 2010)
• Poster Presentation (HMRI Cancer Research Program 2010)
• Poster Presentation (Melbourne Lorne Cancer Conference 2011)


• Oral Presentation (AACBS (Australian Association of Chinese Biomedical Scientist) 2011)
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• Poster Presentation (AACR ( American Association for Cancer Research) 2012)


• Poster Presentation (HMRI Cancer Research Program 2011)
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SYNOPSIS

Melanoma is a skin cancer that remains a major public health problem in Australia because of high incidence, morbidity and mortality associated with the disease. Previously, reports from our laboratory have shown that melanoma cells have largely adapted to endoplasmic reticulum (ER) stress. This adaptation is not only responsible for resistant of melanoma cells to apoptosis induced by ER stress, but also contributes to the resistance of melanoma cells to many chemotherapeutic and biological agents. Therefore, hope for a new approach in treatment of melanoma comes from identification of the mechanisms employed in induction of apoptosis by ER stress and the possible resistance mechanisms in melanoma cells against ER stress-induced apoptosis. The aim of this thesis was to further elucidate mechanisms of ER stress-induced apoptosis and interactions between ER stress pathways and other signalling pathways in melanoma, thus providing more information in identification of treatment approaches that will increase the sensitivity of melanoma to apoptosis induced by ER stress.

In chapter 3, we showed that sustained activation of the IRE1α and ATF6 pathways of the UPR is critical for survival of melanoma cells undergoing prolonged ER stress. This indicates that persistent activation of the pathways is an important adaptive mechanism to ER stress in melanoma cells, and suggest that interruption of IRE1α and ATF6 signalling may be useful in combination with drugs that induce ER stress to kill melanoma cells. Moreover, the observation that the MEK/ERK pathway is required for sustained activation of IRE1α and ATF6 in melanoma cells under ER stress reinforces the importance of inhibition of the pathway in the treatment of melanoma.

A characteristic of human melanoma is the constitutive activation of the MEK/ERK pathway due to activating mutations of key components of the pathway, in particular, BRAF. Studies in Chapter 4 extend the findings in Chapter 3 by showing that oncogenic activation of the MEK/ERK pathway is not only a mechanism of adaptation to ER stress, but also a source of chronic ER stress in melanoma cells. While activation of MEK/ERK is important for enabling increased protein synthesis by activation of eIF4E, it consequently causes ER stress. Nonetheless, MEK/ERK signalling potentiates IRE1α and ATF6 signalling of the UPR, which protects melanoma cells against ER stress.
stress-induced apoptosis. These results reveal that regulation of the UPR is another mechanism by which oncogenic activation of the MEK/ERK pathway promotes the pathogenesis of melanoma.

Although ER stress triggers apoptosis by activating Bim in various types of cells, Bim activation is suppressed in melanoma cells undergoing ER stress. In Chapter 5, it is demonstrated that ER stress reduces PP2A activity leading to increased ERK activation and subsequent phosphorylation and proteasomal degradation of the Bim protein. The increase in ERK activation is, at least in part, due to reduced dephosphorylation by PP2A, which is associated with downregulation of the PP2A catalytic subunit. These results suggest that pharmacological activation of PP2A may improve treatment results of agents that induce ER stress in melanoma cells.

Results presented in this thesis provided new insights into mechanisms involved, and suggest that targeting MEL/ERK signalling, interruption of the IRE1α and ATF6 pathways, and activations of PP2A are potentially useful strategies to improve the therapeutic efficacy of agents that inducing ER stress.
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<td>4E-BP</td>
<td>eIF4E-Binding Protein</td>
</tr>
<tr>
<td>4-OHT</td>
<td>4-hydroxytamoxifen</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP-Binding Cassette</td>
</tr>
<tr>
<td>AJCC</td>
<td>American Joint Commission on Cancer</td>
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<tr>
<td>ASK1</td>
<td>Apoptosis Signal-Regulating Kinase 1</td>
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<tr>
<td>ATF4</td>
<td>Activating Transcription Factor 4</td>
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<td>ATF6</td>
<td>Activating Transcription Factor 6</td>
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<td>BAD</td>
<td>Bcl-2-Antagonist of Cell Death</td>
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<td>BAK</td>
<td>Bcl-2 Antagonist/Killer</td>
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<td>BAX</td>
<td>Bcl-2-Associated X Protein</td>
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<td>Bcl-2</td>
<td>B Cell Lymphoma Gene 2</td>
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<td>Bcl-Xₐ</td>
<td>B-Cell Lymphoma-extra Large</td>
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<td>BID</td>
<td>BH3-Interacting-Domain Death Agonist</td>
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<td>BIK</td>
<td>Bcl-2-Interacting Killer</td>
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<td>BIM</td>
<td>Bcl-2-Interacting Mediator of Cell Death</td>
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<td>BRAF</td>
<td>Rapidly Accelerated Fibrosarcoma Isoform B</td>
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<td>bZIP</td>
<td>Basic Leucine Zipper</td>
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<td>CARD</td>
<td>Caspase Recruitment Domain</td>
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<td>CDDP</td>
<td>Cisplatin</td>
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<td>CDK</td>
<td>Cyclin Dependent Kinase</td>
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<td>CDKN2A</td>
<td>Cyclin Dependent Kinase Inhibitor 2A</td>
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<td>CHOP</td>
<td>C/EBP Homologous Protein</td>
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<td>CIP2A</td>
<td>Cancerous Inhibitor of PP2A</td>
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<td>CTLA4</td>
<td>Cytotoxic T-Lymphocyte-associated Antigen 4</td>
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<td>DED</td>
<td>Death Effector Domain</td>
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<tr>
<td>DIABLO</td>
<td>Direct Inhibitor of Apoptosis-Binding Protein with Low pI</td>
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<td>DISC</td>
<td>Death-Inducing Signalling Complex</td>
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<td>DMEM</td>
<td>Dulbecco's Modified Eagle's Medium</td>
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<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
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<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
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<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
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<td>eIF</td>
<td>Eukaryotic Initiation Factor</td>
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<td>ERAD</td>
<td>ER-Associated Degradation</td>
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<td>ERK</td>
<td>Extracellular Signal Regulated Kinase</td>
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<td>ERK</td>
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<td>FCS</td>
<td>Foetal Calf Serum</td>
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<td>FDA</td>
<td>Food and Drug Administration</td>
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<td>Acronym</td>
<td>Description</td>
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<td>GADD153</td>
<td>Growth Arrest- and DNA Damage-Inducible Gene 153</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
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<td>GRP78</td>
<td>78kDa Glucose-Regulated Protein</td>
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<td>GTP</td>
<td>Guanosine Triphosphate</td>
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<td>HEAT</td>
<td>Huntington/Elongation/A-subunit/TOR</td>
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<td>IAP</td>
<td>Inhibitors of Apoptosis Proteins</td>
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<td>IFN</td>
<td>Interferons</td>
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<td>IGF-1</td>
<td>Insulin Growth Factor-1</td>
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<td>IL</td>
<td>Interleukin</td>
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<td>IP3</td>
<td>Inositol Triphosphate</td>
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<td>Inositol-Requiring Enzyme 1</td>
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<td>JNK</td>
<td>c-Jun N-terminal Kinase</td>
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<td>LB</td>
<td>Lysogeny Broth</td>
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<td>LCMT1</td>
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<td>LDH</td>
<td>Lactic Acid Dehydrogenase</td>
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<td>Mitogen-Activating Protein Kinase</td>
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<td>MC1R</td>
<td>Melanocortin receptor 1</td>
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<td>MEK</td>
<td>Mitogen-Activated Protein Kinase Kinase</td>
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<td>MNK</td>
<td>MAP Kinase Signal-Integrating Kinase</td>
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<td>OA</td>
<td>Okadiac Acid</td>
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<td>PCR</td>
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<td>PI</td>
<td>Propidium Iodide</td>
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<td>PI3K</td>
<td>Phosphatidylinositol-3-kinase</td>
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<td>PIB5PA</td>
<td>Phosphatidylinositol 4,5-biphosphate 5-phosphate A</td>
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<td>PIP2</td>
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<td>PIP3</td>
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<td>Protein Phosphatase 2A</td>
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<td>PTEN</td>
<td>Phosphatase and Tensin Homologue Deleted on Chromosome 10</td>
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<td>PTPA</td>
<td>Phosphotyrosyl Phosphatase Activator</td>
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<td>PUMA</td>
<td>p53-Upregulated Modulator of Apoptosis</td>
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<td>RNA</td>
<td>Ribonucleic RNA</td>
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<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<td>RTK</td>
<td>Receptor Tyrosine Kinase</td>
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<td>S1P</td>
<td>Site-1 Protease</td>
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<td>SAPK</td>
<td>Stress-Activated Protein Kinase</td>
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<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulphate-Polycrylamide Gel Electrophoresis</td>
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<tr>
<td>shRNA</td>
<td>Short Hairpin RNA</td>
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<tr>
<td>siRNA</td>
<td>Small Interfering RNA</td>
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<tr>
<td>Smac</td>
<td>Second Mitochondria-Derived Activator of Caspase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
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<td>-------------</td>
</tr>
<tr>
<td>SNB</td>
<td>Sentinel Node Biopsy</td>
</tr>
<tr>
<td>SOC</td>
<td>Super-Optimal Broth with Catabolite Repression</td>
</tr>
<tr>
<td>SQ</td>
<td>Subcutaneous</td>
</tr>
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<td>TBE</td>
<td>Tris Borate EDTA</td>
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<td>TBS-T</td>
<td>Tris-Buffered Saline-Tween 20</td>
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<td>TEMED</td>
<td>N,N,N',N'-Tetramethylethylenediamine</td>
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</tr>
<tr>
<td>TM</td>
<td>Tunicamycin</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour Necrosis Factor</td>
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<td>TRAF2</td>
<td>TNF Receptor-associated Factor 2</td>
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<td>TRAIL</td>
<td>TNF-Related Apoptosis-Inducing Ligand</td>
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<td>UPRE</td>
<td>Unfolded Protein Response Promoter Element</td>
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<td>UV</td>
<td>Ultraviolet</td>
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<td>XBP-1</td>
<td>X-box-Binding Protein 1</td>
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<td>α-MSH</td>
<td>α-Melanocyte-stimulating hormone</td>
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CHAPTER ONE

GENERAL INTRODUCTION
1.1. Melanoma

1.1.1 Epidemiology

Melanoma is a malignant tumour arising from melanocytes. It occurs predominantly in skin but can also be found in any part of the body contains melanocytes, such as the eye and the bowel [1]. Melanoma is a heterogeneous disease, which suggests a highly complex aetiology. Although melanoma is less common than basal and squamous cell skin cancers, it is the most dangerous form [2]. The global incidence of melanoma is increasing constantly in the last a few decades. In particular, Australia has the highest incidence of melanoma of any country in the world, which has continued to increase over the last 10 years by 11% in females and 15% in males, with mortality rates showing no decrease in the past 36 years [3]. The life time risk of diagnosis of melanoma by the age of 85 is estimated to be 1 in 24 females and 1 in 13 males in Australia [3]. Moreover, melanoma has an excessively heavy impact on the productive years of the lives of young Australians, because it is the most common cancer in females and males aged 15-29 and 25-54 years, respectively [3]. Males were 1.6 times more likely to be diagnosed with melanoma and 2.9 times more likely to die from it, compared to females [3]. Figure 1.1 shows the incidence and mortality rates of melanoma in both females and males in the state of New South Wales (NSW) of Australia. Given that the global incidence and the death rate of melanoma are increasing rapidly and there is currently no curative treatment of melanoma once it spreads beyond its original site, research into the development of more effective therapeutics against melanoma is urgently needed.
Figure 1.1 The mortality and incidence rate of melanoma in both males and females in NSW from 1972 to 2010. Adapted from Tracey, 2010 [3].
1.1.2 Aetiology of Melanoma

1.1.2.1 UV Light in Melanoma Development

Human melanocytes are frequently exposed to ultraviolet (UV) light. Considerable epidemiological evidence and clinical studies suggest a role of sun exposure in the development of melanoma [4, 5]. The occurrence and number of melanocytic nevi are among the most important known risk factors for the development of malignant melanoma and reducing sun exposure at an earlier stage of life is effective in reducing naevus prevalence and subsequent melanoma risk [4, 5]. The damage to DNA by UV irradiation is believed to be causative of melanoma. The dark-skinned individuals appear to be more resistant to melanoma than the fair-skinned people [6]. Additionally, individuals with xeroderma pigmentosum, a recessively transmitted disorder of DNA repair in which the ability to repair DNA damage caused by UV light is deficient [7], have higher risks of melanoma [8]. Furthermore, the incidence of melanoma among Caucasians is inversely related to the latitude of residence [9], with the highest incidence being found in lower latitudes closer to the equator. Indeed, melanoma occurs most frequently after intermittent exposure to the sun and in people with frequent sunburns. Nevertheless, whereas intense, intermittent exposure causes genetic damage, chronic or low-grade exposures to UV light results in protection against DNA damage [10].

UV irradiation has multiple effects on cellular functions that might contribute to the development of melanoma. These include direct mutagenic effects on DNA; stimulating the cellular constituents of the skin to produce growth factors; reducing cutaneous immune defences; generation of reactive oxygen species of melanin that promote oxidative stresses and contribute to malignant changes; activation of signal pathways that ultimately trigger transcription of genes involved in cellular proliferation, migration, and resistance to programmed cell death; and alterations in proto-oncogene and tumour suppressor gene expression [8, 10, 11]. Besides UV irradiation from sun exposure, additional factors appear involved in the aetiology of melanoma [12].
1.1.2.2 Genetic Factors

The risk of an individual developing a melanoma is greatly increased if there is a family history of the disease [10, 13, 14]. Genetic factors that determine phenotypic characteristics such as naevi, skin colour, especially those with blond or red hair who sunburn and freckle easily have a higher risk of melanoma [6, 15]. Melanomas in blacks and Asians tend to occur at sites not exposed to the sun, such as the nail beds and sole of the foot [8, 16].

The tanning response is a defensive measure in which melanocytes synthesise melanin and transfer it to keratinocytes, where it absorbs and dissipates UV energy [8]. At the molecular level, exposure to UV light increases skin pigmentation, in part through the action of α-melanocyte-stimulating hormone (α-MSH) on its receptor, the melanocortin receptor 1 (MC1R) [17]. Binding of the hormone to the receptor stimulates intracellular signalling in melanocytes, thereby increasing the expression of enzymes involved in the production of melanin. Fair-skinned and red-haired people often carry germ-line polymorphisms in the MC1R gene that reduce the activity of the receptor [15, 17]. It is well-documented that these polymorphisms increase the risk of melanoma [18]. Thus, in fair-skinned people, the basis of increased susceptibility to melanoma is a genetic impairment in the production of melanin.

In addition to these inherited phenotypic characteristics, there is evidence for genetic predisposition to melanoma that can be traced in families [19]. Genetic studies of this heritable trait in large cohorts of melanoma-prone families ultimately led to the initial identification of cyclin-dependent kinase inhibitor 2A (CDKN2A) as the familial melanoma gene [20]. CDKN2A is located at chromosome 9p21. LOH or mutations at this locus co-segregated with melanoma susceptibility in familial melanoma kindred [21, 22]. Homozygous deletions of this locus centred on CDKN2A were also frequently observed in cancer cell lines of non-melanocytic origins [23, 24].

Activating rapidly accelerated fibrosarcoma isoform B (BRAF) mutations are the most prevalent somatic genetic event in human melanoma. Recently, a genome-wide mutation detection strategy revealed mutations in the BRAF gene in a high proportion of melanoma cell lines and primary tumours [25]. These mutations clustered in specific
regions of biochemical importance, with the predominant mutation being a single phosphomimetic substitution in the kinase activation domain (V600E), which confers constitutive activation in ~50-60% of melanomas [20, 26]. Importantly, subsequent studies have shown the presence of activating BRAF mutations in up the 82% of benign naevi [27, 28], suggesting that mutations in BRAF is an early event in melanoma development. BRAF$^{V600E}$ mutation accounts for approximately 80% of all BRAF mutations [29]. Other common BRAF mutations in melanoma, found in the same codon, are V600K (approximately 16% of mutations in melanoma) and V600D/R (3% of all mutations) [30]. Moreover, activating mutations in N-RAS, H-RAS, c-Kit, ERBB4, or the G-protein α-subunit GNAQ are also responsible in subsets of melanomas [19]. Melanomas without BRAF mutations usually carry mutations in other components of the MAPK pathway such as NRAS, KIT and GNAQ [31-33].

The phosphatidylinositol-3-kinase (PI3K) pathway is often hyperactivated in melanoma [34]. Elevated phosphorylated (activated) Akt levels appear to correlate adversely with patient survival [35]. Mechanisms leading to Akt activation can involve: (a) mutations in the upstream regulators PI3K and tensin homolog deleted on chromosome 10 (PTEN) [36, 37]; (b) overexpression of the gene due to increased copy number or activating point mutations in Akt itself [38]; (c) deletion of negative regulators such as PTEN [20, 39]; (d) altered expression of interacting proteins such as TCL1, HSP90, APPL1, and RasGAP [40, 41]. In addition, loss or reduced expression of phosphatidylinositol 4, 5-bisphosphate 5-phosphate A (PIB5PA) also contributes to activation of PI3K/Akt signalling in melanoma cells [42-44], whereas integrins and growth factors promote melanoma cell growth and survival via PI3K/Akt activation [20, 45]. Furthermore, activation of PI3K/Akt signalling has been shown to cooperate with mutant BRAF in melanomagenesis using in vivo models [46-48].

### 1.1.3 Pathobiology of Melanoma

Melanocytes have a limited capacity of proliferate in normal situations and are at risk for mutation after DNA damage. They are derived from the neural crest, migrate to the epidermis during embryogenesis and thereafter reside in the basal layer, in contact with keratinocytes forming the so-called epidermal melanin unit [8]. Generally, melanin has a photoprotective function in the skin, directly absorbing ultraviolet photons as well as
reactive oxygen species (ROS) generated by the interaction of ultraviolet photons with membrane lipids and other cellular chromophores [8, 49]. Mammalian melanocytes produce two types of melanin, the phaeomelanin and eumelanin [50, 51]. Eumelanin is a brown-black polymer that is more dominant in dark-skinned individuals. On the other hand, phaeomelanin is a cysteine-containing red-brown polymer that is the dominant melanin in fair-skinned individuals [52]. Rather than protecting the skin against UV, phaeomelanin may actually contribute to UV-induced skin damage. In contrast, eumelanin absorbs UV radiation well and this protects the skin [51, 52].

Approximately 18-35% of primary cutaneous melanomas arise from pre-existing naevi [53]. In fact, the number of both common (benign or congenital) and dysplastic naevi is the strongest risk factor for melanoma. Dysplastic naevi are distinct from common naevi and are clinically termed as atypical naevi. There are usually at least 5mm in diameter with flatness of texture, an irregular asymmetric outline, variable pigmentation, and/or indistinct borders [53]. Further progression of atypical naevi leads to in situ and radial growth phase (RGP) primary melanoma. In this phase, the lesions tend to be circular or oval but do not show the ability of rapid growth or metastasis [54]. Under vertical growth phase (VGP), primary melanomas gain the capacity to invade the dermis and have potentials for systemic dissemination. In late stages of melanoma progression, tumours may invade into the lymphatic systems and spread into regional lymph nodes and/or bloodstream (Figure 1.2) [54].

Clinical features of melanoma such as changes in shape, size and colour of naevi can be detected early in its development. Currently, the best prognostic indicator for patient survival is tumour thickness as measured by the method of Breslow [55].
Figure 1.2 Melanoma cell precursors progress via several phases toward malignant transformation. Each phase is characterised by certain histological features that are the visible manifestation of underlying genetic changes. Reproduced with permission from Miller, 2006 [11], Copyright Massachusetts Medical Society.
1.1.3.1 Survival Pathways

So far, melanoma diagnosis has been based on pathology, but recent studies demonstrated that genome-wide alterations in DNA copy number together with analysis of individual somatic mutation can be used to distinguish between the different melanoma subtypes with 70% accuracy [56, 57]. Melanoma is a complex genetic disease, the management of which will require an in-depth understanding of the biology underlying its initiation and progression. This will allow improved staging and subtype classification, and will lead to the design of better therapeutic agents and approaches.

1.1.3.1.1 Mitogen-Activated Protein Kinase (MAPK) Pathway

Mitogen-activated protein kinase (MAPK) pathways are evolutionarily conserved kinase modules that link extracellular signals to the machinery that controls fundamental cellular processes such as growth, proliferation, differentiation, migration and apoptosis [58]. MAPK pathways are comprised of a three-tier kinase module in which a MAPK is activated upon phosphorylation by a mitogen-activated protein kinase kinase (MAPKK), which in turn is activated when phosphorylated by a mitogen-activated protein kinase kinase kinase (MAPKKK) (Figure 1.3a) [58]. There are three main MAPK family members: extracellular signal regulated kinase (ERK), Jun N-terminal kinase (JNK) and p38. Deregulation of MAPK signalling pathways is a frequent event in multiple human cancers [59].

1.1.3.1.1.1 Ras/Raf/MEK/ERK Signalling

The Ras/Raf/MEK/ERK pathway is the best studied mammalian MAPK pathway and is dysregulated in approximately one-third of all human cancers [58]. ERK1/2 is activated through the Ras/Raf/MEK module [60]. Upon extracellular signal activation, cell surface receptor tyrosine kinases (RTKs) triggers guanosine triphosphate (GTP) loading of the Ras GTPase, which can then recruit Raf kinase to the plasma membrane for activation. The activated Raf phosphorylates MEK1/2, which further activates ERK1/2 by phosphorylation (Figure 1.3b) [60].
Active ERK phosphorylates numerous cytoplasmic and nuclear targets, including kinases, phosphatases, transcription factors and cytoskeletal proteins [61]. ERK signalling can, depending on the particular cell type, regulate diverse processes such as proliferation, differentiation, survival, migration, angiogenesis and chromatin remodelling [61, 62]. It has been demonstrated that ERK1/2 functions in stabilisation of e-Myc and the activation of cyclin dependent kinase (CDK) proteins [60, 63]. Moreover, it is well-established that blockade of the Raf/MEK/ERK pathway inhibits melanoma cell growth and proliferation [64, 65].
Figure 1.3 A schematic illustration of the structure of MAPK pathways. (a) General setup of MAPK pathways; (b) the Ras/Raf/MEK/ERK pathway in particular. This figure was originally published in Kolch, 2000 [66] © the Biochemical Society.
1.1.3.1.1.2 JNK Signalling

JNKs, also known as the stress-activated protein kinases (SAPKs), have been linked to controlling cell growth, differentiation, transformation and cell death [67]. The family is encoded by three genes: JNK1, JNK2 and JNK3. Alternative splicing of these genes creates a total of 10 JNK isoforms. The JNK pathway is predominantly activated by cytokines, UV radiation, growth factor deprivation, agents that interfere with DNA and protein synthesis and other cellular stress, where the activation requires dual phosphorylation on tyrosine and threonine residues at a distinctive TPY motif, a reaction is catalysed by MEK4 and MEK7 [68].

Several studies have demonstrated JNK functions as a pro-apoptotic protein [69]. The anti-apoptotic protein Bcl-2 is a direct target of JNK activated by microtubule inhibitors. Phosphorylation of JNK can abrogate the anti-apoptotic function of Bcl-2 by affecting its ability to bind to and sequester pro-apoptotic Bcl-2 protein such as BAX, thereby activating the apoptotic pathway [70]. On the other hands, evidence has been accumulating that suggests the involvement of JNK in cell survival. In mice deficient in both JNK1 and JNK2, apoptosis was enhanced in hindbrain and forebrain regions [71, 72]. Furthermore, it has been reported that JNK functions as an anti-apoptotic molecule in an isoform-specific manner in certain tumors [73]. Despite the numerous evidence pointing JNK towards a pro-apoptotic role, equal numbers of studies have reported JNK to be anti-apoptotic, which might be cell-type specific [69].

1.1.3.1.1.3 p38-MAPK Signalling

p38 are strongly activated by environmental stresses and inflammatory cytokines. p38 is required for expression of TNFα and interleukin-1 during inflammatory responses. The four vertebrate isoforms of p38: α, β, γ, and δ are characterized by the presence of the conserved Thr-Gly-Tyr (TGY) phosphorylation motif in their activation loop [74]. This motif is phosphorylated by MEK3 an MEK6, which are activated by various MAPKKKs that are induced by physical and chemical stresses [58]. In some instances p38 can also be activated by MEK4, a kinase that is better known as an activator of JNK. Similar to the JNK pathway, the involvement of p38 signalling in apoptosis is also diverse. It has been reported that p38 is involved in p53-induced apoptosis and p53-
mediated cell cycle progression [75, 76]. Nevertheless, inhibition of p38 activity enhances apoptosis in response to DNA-damaging agents such as doxorubicin and cisplatin as well as microtubule-disrupting agents such as taxol, vicristine and vinblastine [77, 78]. These results suggest that p38 pathway is required for apoptosis or survival in a cell-type specific manner.

1.1.3.1.2 The PI3K/Akt Pathway

The phosphatidylinositol-3-kinase (PI3K) signalling pathway is crucial to many aspects of cell growth and survival. It is targeted by genomic aberrations including mutation, amplification and rearrangement more frequently than any other pathway in human cancer [79]. In addition, the PI3K pathway is stimulated as a physiological consequence of many growth factors and regulators. Inappropriate activation of the PI3K pathway results in disturbance of control of cell growth and survival, which contributes to a competitive growth advantage, metastatic competence and, frequently, therapy resistance [79].

The PI3K family constitutes a large family of lipid and serine/threonine kinases [80-82]. PI3K is composed of heterodimers of an inhibitory adaptor/regulatory (p85) and a catalytic (p110) subunit. p85 binds and integrates signals from various cellular proteins, including transmembrane tyrosine kinase-linked receptors and intracellular proteins, providing an integration point for activation of p110 and downstream molecules [79]. PI3K signalling begins with the engagement of growth factors to receptor tyrosine kinases. PI3K is then recruited to plasma membrane-anchored receptors and is activated and phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3), leading to the activation of the downstream effector, Akt (Figure 1.4) [83]. Activated Akt in turn phosphorylates the downstream cellular proteins that promote cell proliferation and survival [79, 83, 84]. Two crucial amino acids that are phosphorylated and necessary for activation of Akt: one in kinase domain (threonine 308) which is phosphorylated by constitutively active phosphoinositide-dependent kinase 1 (PDK1), whereas phosphorylation of the other in the hydrophobic C-terminal domain (serine 473) by PDK2 [85, 86].
Activated Akt phosphorylates up to 100 substrates to regulate a variety of cellular functions. First, Akt signaling exerts a strong anti-apoptotic effect through phosphorylation and inhibition of key pro-apoptotic proteins, such as BAD, pro-caspase 9 and members of the Forkhead family of transcription factors leading to suppression of Bim [87, 88]. Second, Akt activates cell proliferation by inactivating the CDK inhibitor p27Kip1 and inhibiting glycogen synthase kinase 3 (GSK3)-mediated Myc and cyclin D1 inhibition [89]. Third, Akt regulates a subset of proteins involved in growth, metabolism and angiogenesis. Additionally, Akt is one of the main regulators of protein translation and ribosome biogenesis by regulating the mTORC1 protein complex. mTORC1 is composed of the protein kinase mTOR and a series of interactors. Activation of the mTORC1 pathway by Akt turns on the translational machinery to produce ribosomes and increases the protein synthesis rate [90, 91]. Therefore, PI3K/Akt pathway is an attractive target for developing the treatment for melanoma.
Figure 1.4 The PI3K/Akt signalling pathway. In the cytoplasm, PI3K (complex of p85 and catalytic p110) is activated downstream of receptor tyrosine kinases (RTKs). PI3K phosphorylates PIP2 to generate PIP3, which leads to Akt activation and enhance cell growth, proliferation, and survival. PTEN negatively regulates Akt activation through dephosphorylation of PIP3.
1.1.4 Melanoma Classification and Staging

1.1.4.1 Classification of Melanoma

Melanoma can be classified according to its site of origin, tumour thickness, ulceration, and histologic subtype. Ulceration and tumour thickness are the most potent predictors for survival of patients [92]. There are four major histogenetic subtypes of melanoma including superficial spreading, lentigo maligna, acral lentiginous and nodular [27, 93]. Superficial spreading melanoma is the most common type of melanoma that comprises approximately 80% of all primary melanomas in Caucasian populations [93]. The common sites for superficial spreading melanoma are on the trunk in males and lower extremities in females. Nodular melanomas is the second most common type of melanoma, responsible for 10-15% of all melanomas [16]. The main characteristic of nodular lesions is rapid growth [93]. Lentigo maligna melanoma represents around 10% of all melanomas. The affected patients are usually in the seventh decade of life and the face is the most common site [93]. Acral lentiginous melanomas are relatively rare, which account for approximately 2-8% of all melanomas in Caucasian populations. However, it is the most common form of melanoma in African-American, Asian and Hispanic populations with a relatively low incidence of melanoma [93, 94].

1.1.4.2 Staging and Grading of Melanoma

Melanoma can grow in different directions (radial growth and vertical growth). Staging of malignant melanoma is generally used as a tool to assess disease progression and can be determined according to the level of invasion (Clark level) and vertical thickness (Breslow depth) [55, 95]. The thickness of the melanoma is correlated to the local recurrence and risk of metastases [55].

The prognosis of a patient with a newly diagnosed cutaneous melanoma depends mainly on two factors, the thickness of the primary tumour and the presence or absence of metastasis to regional lymph nodes [10]. The Breslow’s method has proven the most reliable indicator or prognosis by measuring the depth of melanoma invasion into the body. The distance from the dermal-epidermal junction, the two layers of skin, to the
most deeply invasive cells in the body is measured in millimetres [55]. A melanoma with a measurement of less than 0.76mm is considered confined to the skin and has the best prognosis. A melanoma with invasion deeper than 1.5mm can still be confined to the skin yet has a decreased survival rate [55]. Beyond that measurement, the regional lymph nodes are involved. Once melanoma is in the lymph system, it poses a problem for spreading to other parts of the body [96].

There are a number of ways to classify the stages of melanoma. One classification system is provided by the American Joint Commission on Cancer (AJCC). It classifies melanoma into four different stages depending on distant metastases, the presence of lymph node and tumour thickness [97, 98] (Table 1.1). In Brief, Stage IA melanomas have thickness equal to or less than 1.0mm. Stage IB has melanomas equal to or less than 2.0mm or ulceration of melanomas equal to or less than 1.0mm. Stage IIA melanomas have thickness greater than 2.01mm but less than 4.0mm or equal to or less than 2.0mm if they are ulcerated. Stage IIB melanomas have thickness greater than 4.0mm or greater than 2.0mm if they are ulcerated. Stage III specifies regional lymph node involvement and includes Stage IIIA, IIIB and IIIC depending on the size of the metastases, number of lymph nodes and presence of intralymphatic metastases. Stage IV melanomas indicate distant metastases to sites beyond regional lymph nodes [97].
Table 1.1. Anatomic Stage Grouping for Cutaneous Melanoma. Modified from Blach, 2001 [97].

<table>
<thead>
<tr>
<th>Pathologic Stage</th>
<th>Thickness (mm)</th>
<th>Ulceration</th>
<th>No. + Nodes</th>
<th>Nodal Size</th>
<th>Distant Metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>IA</td>
<td>1</td>
<td>No</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>IB</td>
<td>1</td>
<td>Yes</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>1.01-2.0</td>
<td>No</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>II A</td>
<td>1.01-2.0</td>
<td>Yes</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>II B</td>
<td>2.01-4.0</td>
<td>Yes</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>II C</td>
<td>&gt; 4.0</td>
<td>No</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>III A</td>
<td>Any</td>
<td>No</td>
<td>1</td>
<td>Micro*</td>
<td>–</td>
</tr>
<tr>
<td>III B</td>
<td>Any</td>
<td>Yes</td>
<td>1</td>
<td>Micro*</td>
<td>–</td>
</tr>
<tr>
<td>III C</td>
<td>Any</td>
<td>Yes</td>
<td>1</td>
<td>Macro*</td>
<td>–</td>
</tr>
<tr>
<td>IV</td>
<td>Any</td>
<td>Any</td>
<td>Any</td>
<td>Any</td>
<td>Skin, SQ, Other Visceral</td>
</tr>
</tbody>
</table>

Abbreviation: SQ, Subcutaneous

*Micro (Micrometastasis): diagnosed after sentinel lymph node biopsy

*Macro (Macrometastasis): diagnosed clinically detectable nodal metastases confirmed pathologically.
1.1.5 Treatment of Melanoma

1.1.5.1 Surgical Treatment of Melanoma

Treatment of most solid tumours has evolved from surgery alone to multidisciplinary approaches. However, malignant melanoma remains an exception, with surgery excision remains the principle for treatment of primary lesions [92]. Early diagnosis combined with appropriate surgical therapy is currently the only curative treatment. Ideally, surgery should provide both local control of the disease and long-term survival without significant functional impairment [99]. Excision biopsy is essential for accurate diagnosis and microstaging, which is usually performed with a 2mm lateral margin and a small amount of subcutaneous fat. In order to determine the choice of further therapy and provide important prognostic information, the pathology report routinely includes the Breslow thickness (mm), presence of ulceration, mitotic index, Clark level, lateral and deep margin size (mm) and the presence of local metastasis [99, 100]. The recommended margin of excision for a primary melanoma with up to 2.0mm of thickness is 2.0cm. For a melanoma thicker than 2.0mm, a 2.0cm margin of excision is appropriate. For a melanoma in situ, the recommended margin is 0.5-1cm [99, 101, 102].

Sentinel node biopsy (SNB) is frequently done to determine whether melanoma has spread to regional lymph nodes [99]. If regional lymph nodes are positive for melanoma cells, subsequent lymphadenectomy might improve survival. Lymphadenectomy for melanoma has two goals: it may be curative, or it may simply prevent further relapse at regional sites. Both can only be achieved by meticulous and thorough removal of all involved and at-risk nodes [99]. Surgery is also the most effective approach to remove distant metastases if it is technically feasible, if risk of morbidity and mortality are low, and if the patient is likely to live long enough to accrue benefit. Completely resected single distant metastasis may occasionally be associated with long survival [99, 103].
1.1.5.2 Chemotherapy

Single agent dacarbazine or temozolomide continues to be widely used in systemic therapy of metastatic melanoma because of their fairly low toxicity and simplicity of administration [10, 104]. Dacarbazine and the analog drug temozolomide are alkylating agents that damage DNA by introducing alkyl groups to guanine bases, eventually causing cell death via apoptosis and other cell death mechanisms [105]. Dacarbazine is a standard chemotherapeutic agent for metastatic melanoma in over three decades but it has never been shown to increase overall survival in this patient population. Generally, dacarbazine is associated with a response rate of approximately 10% to 20% and a progression-free survival of approximately 3 to 6 months [105, 106]. Temozolomide, a dacarbazine analogue was not found to be significantly more efficacious compared to dacarbazine in term of overall survival and progression-free survival [105, 107].

1.1.5.3 Immunotherapy

Immunotherapy continues to be investigated intensively in both adjuvant and advanced disease setting, and attempts are being made to target the major defences that melanoma mounts against an effective immune response [10]. Immunotherapy agents include interleukin 2 (IL-2), interferons (IFNs), monoclonal antibodies against immunological checkpoints, adoptive cell therapy and melanoma vaccines [108]. Initially discovered in 1976 [109, 110], IL-2 gained Food and Drug Administration (FDA) approval for high dose intravenous use in treating metastatic melanoma in 1998 [111, 112]. However, the overall response rate is low and the majority of toxicities associated with high dose IL-2 are severe [113]. IFN α-2β is the first immunotherapeutic agent approved for adjuvant treatment of stage IIB/III melanoma by FDA. High dose IFN α-2β significantly reduces the risk of recurrence of melanoma. However, tolerability is an issue with this regimen because of fever, anorexia, fatigue, and depression [113-116].

Recently, ipilimumab, also known as Yervoy, a fully humanized monoclonal antibody (IgG1) that blocks cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) to promote anti-tumour immunity has been approved by the FDA as an immunotherapeutic drug to treat advanced melanoma [92]. CTLA4 is expressed on the surface of T-cells and is a potent negative regulator of T-cell activation. It has been demonstrated that CTLA4
blockade results in anti-tumour immunity by removing inhibitory signals in the co-stimulatory pathway that down-regulated T-cell responses [117, 118]. The most common adverse events are immune-related ones, which occurred in approximately 60% of the patients treated with Yervoy [118]. Adverse effects of administration of Yervoy such as colitis, hepatitis, rash and diarrhoea are usually readily manageable [119].

In recent years, programmed cell death 1 (PD-1) has emerged as one of the most important inhibitory molecules in the immune system [120]. The primary role of the PD-1-mediated pathway is to limit T-cell activity in the setting of an inflammatory response [121, 122]. PD-1 is expressed on activated T, B and natural killer (NK) cells, whereas its ligands, PD-L1 and PD-L2 are expressed on immune cells as well as on many non-hematopoietic cells, including various malignant tumour cells such as melanoma [123]. Importantly, PD-1 is highly expressed on circulating melanoma antigen-specific T cells [124, 125], and PD-L1 expressed on melanoma cells is able to induce immunosuppression [126]. Several PD-1- or PD-L1- blocking antibodies have been developed [127]. In a phase I study, Nivolumab, a specific anti-PD-1 antibody, revealed a response rate of 28% in patients with metastatic melanoma, with 50% of these responses lasting more than 1 year [127]. Most recently, the results of a phase I study combining PD-1 blockade in escalating doses with CTLA4 blockade were reported [128]. The response rate was promising, which is approximately 40% in patients receiving concurrent therapy. Although the incidence of adverse events was also increased, the majority of these were reversible with anti-inflammatory treatment [128].

Based on ex vivo and in vitro data, it is now established that tumour-specific T cells can be detected in peripheral blood of melanoma patients, even in the presence of progressive tumours [129]. However, such T cells often display functional impairments as a consequence of the tumour environment, which inhibits their acquisition of final effector functions [130]. Culture of these tumour-sensitized T cells in vitro with appropriate activation stimuli has been shown not only to restore normal functional properties [131], but also to confer effector activity that could potentially sustain tumour rejection upon re-infusion [132]. As a consequence of these observations, adoptive cell immunotherapy, consisting in the administration of immunologically active cellular
Chapter One

effectors, has been tested in clinical settings. Adoptive cell therapy has yielded some of the most dramatic responses among patients with metastatic melanoma. Objective response rates in highly selected patients have been stated to range between 49% and 72% [133]. However, adoptive cell therapy is complex and has several critical steps, which is labour-intensive as well as costly [133].

1.1.5.4 Target Therapy

BRAF mutations are found in approximately 50% of melanomas which leads to constitutive activation of the MEK/ERK pathway that is important for melanoma cell growth and survival [29]. Therefore, selectively targeting mutated, activated BRAF appears to be a promising strategy in the treatment of melanoma. Indeed, a novel structure guided development strategy has led to the development of PLX4032 (vemurafenib), a BRAF inhibitor designed to selectively bind the mutant form of BRAF [134]. Vemurafenib has been approved by the FDA in August 2011 for the treatment of melanoma by targeting mutated BRAF [135, 136]. Of note, another mutant BRAF inhibitor, dabrafenib, which was approved for use against melanoma by the FDA in May 2013, showed similar promising effects in the treatment of mutant BRAF melanoma, with marked inhibition of phosphorylated ERK and high clinical response rates [137].

Although vemurafenib and dabrafenib have achieved unprecedented clinical responses in the treatment of melanomas with activating BRAF mutations, complete remission is rare and a proportion of mutant BRAF melanomas are less responsive to the inhibitors [138-141]. On the other hand, durations of responses are commonly limited with most patients relapsing within 1 year [138-141]. Mechanisms that have been reported to contribute to the resistance include activating mutations of NRAS, overexpression of COT (MAP3K8), activation of receptor tyrosine kinases such as PDGFRβ and IGF1-R, mutations of MEK, and signal-switching among the RAF isoforms [142-144]. Furthermore, it has been recently shown that vemurafenib-resistant mutant BRAF melanoma cells may become drug-dependent for their continuous proliferation [145, 146].
Since all these resistance mechanisms of melanoma cells to BRAF inhibitors largely converge on activation of ERK and /or Akt, novel combinations of co-targeting mutant BRAF and MEK and co-targeting mutant BRAF and PI3K/Akt are currently in evaluation in clinical trials [147, 148]. Trametinib (GSK1120212), a MEK inhibitor, was approved by the FDA recently in the treatment of melanoma, mediates blockade of MAPK kinase (MEK), which was downstream of BRAF in the MAPK pathway. Early findings demonstrated that the combinations of BRAF inhibitors and trametinib were tolerable and showed promising clinical activity [147] where the response rate was improved from up to 76% and progression free survival was also significantly improved [147, 149].
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1.2 Endoplasmic Reticulum Stress and the Unfolded Protein Response

The endoplasmic reticulum (ER) is an organelle of a lacy network of cisternae that has essential roles in many cellular processes required for cell survival, growth and other functions. These include intracellular calcium homeostasis, protein folding and glycosylation, and lipid biosynthesis [150]. Moreover, because the ER releases calcium through ion channels in response to second messengers such as inositol triphosphate (IP3) and protein kinases, it is also regarded as a signalling organelle [151].

ER stress is a condition under which intracellular or extracellular disturbances, such as nutrient deprivation, hypoxia, alterations in glycosylation status, disturbances of calcium flux, cause accumulation and aggregation of unfolded and/or misfolded proteins in the ER lumen [151-153]. Cells undergoing ER stress respond to protect themselves by activating the unfolded protein response (UPR), which alters transcriptional, translational, and post-translational programs, resulting in up-regulation of ER chaperones, general translational attenuation, and enhanced ER-associated degradation (ERAD) of misfolded and unfolded proteins. The signalling pathways of the UPR are initiated by three ER transmembrane proteins, activating transcription factor 6 (ATF6), inositol-requiring enzyme 1 (IRE1), and double-stranded RNA-activated protein kinase-like ER kinase (PERK) [151-153]. Under unstressed conditions, the luminal domains of these sensors are occupied by ER chaperone proteins, in particular, 78kDa glucose-regulated protein (GRP78). Upon ER stress, increased binding of GRP78 with misfolded and unfolded proteins causes removal of GRP78 from ATF6, IRE1, and PERK, thus resulting in release of these proximal ER stress sensors and activation the UPR (Figure 1.5). GRP78 has therefore been termed the “master regulator” of the UPR [154, 155].

1.2.1 IRE1α

IRE1α is an unusual protein in that it acts as both a serine/threonine protein kinase and an endoribonuclease [151-153]. The latter activity processes an intron leading to the catalytic removal of a 26-base intron from the mRNA of the gene, X-box-binding
protein 1 (XBP-1) (Figure 1.5). This splicing and re-ligation results in a translational frameshift to produce the active XBP-1 protein, a basic leucine zipper (bZIP) family transcription factor that can bind to both the ER stress response element (ERSE) and the unfolded protein response promoter elements (UPRE), thus transcriptionally up-regulating a number of genes involved in the UPR [156]. In addition, IRE1α is also necessary for cleavage and post-transcriptional degradation of many other mRNAs encoding secreted proteins. This plays a part in reducing protein loading onto the ER [157].

As a protein kinase, IRE1α can form protein complexes with TNF receptor-associated factor 2 (TRAF2) and apoptosis signal-regulating kinase 1 (ASK1). This causes activation of ASK1 that in turn activates Jun N-terminal kinase (JNK) [158, 159]. Moreover, binding of IRE1α to TRAF2 leads to activation of a number of other protein kinases implicated in immunity and inflammation [159]. IRE1α can also bind to the multi-domain pro-apoptotic Bcl-2 family proteins Bax and Bak [160]. This association may cause activation of IRE1α, and thus modulating calcium flux and UPR signalling.

1.2.2 ATF6

ATF6 has two isoforms, α and β [151-153]. They are members of the bZIP transcription factor family and have conserved protein domain, but interestingly, divergent transcriptional activation domains. ATF6α is the better characterized of the two that shares many of the activities of IRE1α. Upon ER stress it is freed from the ER membrane and translocates to the Golgi compartment, where it is cleaved by the site-1 protease/ site-2 protease (S1P/S2P) serine protease to produce a transcription factor that binds to ERSE elements, thus activating genes encoding proteins involved in the UPR including XBP-1, C/EBP homologous protein (CHOP), and ER chaperone proteins such as GRP78 and GRP94 (Figure 1.5) [161, 162].

1.2.3 PERK

PERK is a serine/threonine protein kinase. Oligomerization of PERK at the ER membrane leads to its autophosphorylation and activation [151-153]. Activated PERK phosphorylates the translation initiation factor, eukaryotic initiation factor 2α (eIF2α),
leading to its inactivation and attenuation of translation. However, selective mRNAs can be preferentially translated. The best characterized of these is the transcription factor ATF4 that activates the transcription of genes including CHOP, GRP78, and GRP94 (Figure 1.5). PERK−/− cells and cells expressing eIF2α that cannot be phosphorylated are hypersensitive to ER stress-induced cell death, indicating the importance of signals initiated by PERK in protection of cells from ER stress [163].
Figure 1.5 A schematic illustration of signalling pathways of the UPR.
1.3 ER Stress and Cell Death

Although ER stress activates the UPR that is essentially a cyto-protective response, prolonged or excessive activation of the UPR can result in cell death by inducing primarily apoptosis [151-153]. In some circumstances, ER stress can also trigger autophagy that contributes to degradation of excessive proteins and protects cells from apoptosis [164], but similar to the UPR, excessive autophagy can lead to autophagic cell death [165]. Moreover, ER stress has been shown to play a role in oncogene-induced senescence [166].

Induction of apoptosis by ER stress in most cell types involves many of the same molecules that have important roles in other apoptotic cascades including Bcl-2 family proteins and caspases [167, 168]. However, how ER stress-induced apoptotic signalling is triggered may vary among different cell types and ER stress inducers.

1.3.1 Apoptosis

1.3.1.1 The Extrinsic Pathway

The extrinsic signalling pathways that initiate apoptosis involve transmembrane receptor-mediated interactions. These involve death receptors that are members of the tumour necrosis factor (TNF) receptor gene superfamily [169]. Members of the TNF receptor family share similar cysteine-rich extracellular domains and have a cytoplasmic domain of about 80 amino acids called the “death domain”, which plays a critical role in transmitting the death signal from the cell surface to the intracellular signalling pathways [170]. The ligands principally involved are TNF-α, FAS ligand (FASL) and TNF-related apoptosis-inducing ligand (TRAIL). Binding of the ligands to their receptors is believed to cause aggregation (trimerization) of receptors and results in the binding of the adapter protein Fas-associated death domain (FADD), which then associates with procaspase-8 via dimerization of the death effector domain. At this point a death-inducing signalling complex (DISC) is formed, resulting in the auto-catalytic activation of pro-caspase-8 (Figure 1.6) [171]. Once caspase-8 is activated, the execution phase of apoptosis is triggered.
Depending on the cell type, activated caspase-8 induces apoptosis by two different signalling pathways. In type I cells, large amounts of active caspase-8 directly cleave and activate procaspase-3 independently of mitochondria. In type II cells, small amounts of active caspase-8 are insufficient to activate procaspase-3 directly. Instead, caspase-8 cleaves the “BH3-only protein” Bid, generating an active fragment (tBid) that activates the mitochondrial death pathways [172] (Figure 1.6).

1.3.1.2 The Intrinsic Pathway

The intrinsic signalling pathways that initiate apoptosis involve a diverse array of non-receptor-mediated stimuli that produce intracellular signals that act directly on targets within the cell and are mitochondrial-initiated events [173]. The stimuli that initiate the intrinsic pathway cause changes in the inner mitochondrial membrane that result in an opening of the mitochondrial permeability transition pore, loss of the mitochondrial transmembrane potential and leakage of molecules such as cytochrome c, second mitochondria-derived activator of caspase /direct inhibitor of apoptosis-binding protein with low pi (Smac/DIABLO), Omi/HtrA2, AIF and endonuclease G into the cytoplasm [174, 175]. Cytochrome c binds and activates Apaf-1 as well as procaspase-9, forming an “apoptosome” [176]. The clustering of procaspase-9 in this manner leads to caspase-9 activation. Caspase-9 then activates the effector caspases-3, -6, and -7 [177]. Smac/DIABLO and Omi/HtrA2 are reported to promote apoptosis by inhibiting inhibitors of apoptosis proteins (IAP) activity (Figure 1.6) [173, 178].
Figure 1.6 Intrinsic and extrinsic pathways of apoptosis. Adapted from Tait, 2010 [179].
1.3.2 The Mitochondrial Apoptotic Pathway and ER Stress-Induced Apoptosis

Involvement of the mitochondrial apoptotic pathway, also known as the intrinsic apoptotic pathway, in ER stress-induced apoptosis has been well-documented [180, 181], although there have also been reports showing that, at least in some circumstances, ER stress can trigger apoptosis independently of mechanisms mediated by mitochondria [182].

The mitochondrial apoptotic pathway is tightly regulated by interactions between pro- and anti-apoptotic Bcl-2 family proteins [183-187]. Among them, pro-survival proteins including Bcl-2, Bcl-XL, and Mcl-1, protect mitochondrial integrity, whereas the pro-apoptotic members of the family promote the release of apoptogenic proteins such as cytochrome c, Smac/DIABLO from mitochondria [188-190]. The pro-apoptotic proteins of the Bcl-2 family can be further divided into the BH3-only proteins including PUMA, Bim, Noxa and their effectors, the multidomain proteins Bax and Bak (Figure 1.7) [186, 191-194]. Activation of BH3-only proteins are essential in induction of apoptosis, as they act as “death ligands” to activate Bax and Bak by directly binding to them, or by indirectly displacing them from anti-apoptotic Bcl-2 family members (Figure 1.8) [183, 188, 193, 194]. The interactions between anti-apoptotic proteins and BH3-only proteins are highly selective [195]. For example, while Bim and PUMA can tightly engage all the anti-apoptotic proteins, thus being particularly potent apoptosis initiator, Bad can only bind to Bcl-2 and Bcl-XL, but not Mcl-1. In addition, Bid and Bim have been reported to be the only BH3-only proteins that can directly bind to and activate Bax [196]. Anti-apoptotic proteins of the family may also constitute more than one functional class. For instance, Mcl-1 was proposed to play a unique apical role, elimination of which is required at an early stage of induction of apoptosis [184, 197].
Figure 1.7 Three groups of Bcl-2 family proteins base on their structure and the absence or presence of BH domains (BH1-BH4). Adapted from Walensky, 2006 [198].
Figure 1.8 Models for Bax/Bak activation by BH3-only proteins. (A) Direct Activation Model. BH3-only proteins can be divided into two groups: "sensitizers" or "derepressors" (e.g. Bad) that bind only to pro-survival proteins and "activators" (e.g. Bim) that can also directly engage Bax and Bak. "Sensitizers/derepressors" induce apoptosis by displacing "activators" from pro-survival proteins, which then proceed to trigger Bax/Bak activation. (B) Pro-survival Neutralization Model. Pro-survival proteins inhibit Bax and Bak, perhaps through direct interaction as has been demonstrated for Bak. BH3-only proteins induce apoptosis by neutralizing pro-survival molecules and Bax/Bak activation occurs spontaneously in the absence of pro-survival activity. Adapted from van Delft, 2006 [193].
The multidomain pro-apoptotic Bcl-2 family proteins Bax and Bak can localize to the ER membrane, where they bind to IRE1α, which has been shown to be required for activation of IRE1α and subsequent activation of XBP-1 [199]. Loss of Bax/Bak leads to, at least in some cases, impairment of adaptation to ER stress [160]. It appears therefore that Bax/Bak may have a pro-survival role in the ER stress response, presumably due to activation of the IRE1α/XBP-1 pathway. Indeed, ER-localized Bax/Bak has been suggested to act as an adaptive mechanism under conditions of mild or transient ER stress, but as pro-apoptotic molecules under prolonged or strong ER stress conditions [200, 201].

Involvement of Bax- and Bak-dependent mitochondrial apoptotic pathway strongly suggests that one or more BH3-only proteins play critical roles in ER stress-induced apoptosis. This is further supported by inhibition of ER stress-induced apoptosis with over-expression of Bcl-2 or one of its anti-apoptotic homologs [167, 168]. Indeed, ER stress can upregulate PUMA and Noxa in various types of cells [202-204]. This has been shown to be p53-dependent or –independent presumably related to different types of cells used in varying studies and ER stress inducers in question. For example, p53-dependent upregulation of PUMA and Noxa has been demonstrated in mouse embryo fibroblasts exposed to tunicamycin and thapsigargin [203], which are commonly used laboratory tools as ER stress inducers. Tunicamycin induces ER stress by inhibition of glycosylation, whereas thapsigargin, by inhibition of ER Ca²⁺ ATPases. In contrast, p53-independent activation of PUMA was observed in human osteosarcoma SAOS-2 cells, and colon cancer HCT116c cells subjected to ER stress [205].

Another BH3-only protein that plays an important role in ER stress-induced apoptosis in certain circumstances is Bim. This was initially suggested by translocation of Bim into the ER upon ER stress induction, and was further supported by the finding that ectopic expression of ER-targeted Bim induced apoptosis [191, 192, 206]. More recently, Bim was found to be transcriptionally up-regulated in diverse types of cells under stress. This is mediated by binding of heterodimers of the transcription factors CHOP and C/EBPα to an unconventional promoter within the first intron of Bim gene. In addition, ER stress triggers protein phosphatase 2A (PP2A)-mediated dephosphorylation of Bim, which prevents its ubiquitination and proteasomal degradation [206].
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The BH3-only protein BIK is predominantly located to the ER, where it forms complex with GRP78. BIK has been shown to regulate Bax- and Bak-dependent release of calcium from the ER and mitochondrion-mediated apoptosis in cells under ER stress [207, 208]. Another BH3-only protein that has been reported to be activated by ER stress is Bad, which is under physiological conditions phosphorylated and sequestered in the cytoplasm by the protein 14-3-3, but was dephosphorylated in response to several ER stress stimuli [209, 210].

Besides up-regulation or activation of pro-apoptotic Bcl-2 family proteins, down-regulation of pro-survival Bcl-2 family members in response to ER stress also contributes to apoptosis induced by ER stress. Transcriptional repression of Bcl-2 by CHOP has long been reported [211], but this is not universally observed in every cell types. Induction of ER stress may also impinge on Bcl-2 phosphorylation and stability. This has been suggested to be mediated by PP2A. Down-regulation of Mcl-1 has also been frequently observed, which is, at least in some cases, due to inhibition Mcl-1 mRNA translation [212].

1.3.3 The Caspase Cascade

Caspases belong to a conserved family of proteases that use a cysteine residue as a catalytic nucleophile to cleave their substrates specifically after aspartic acid residues. The first member of the family, caspase-1, was discovered in humans in 1992 as an important regulator of inflammation and originally called interleukin-1β-converting enzyme (ICE) [213]. Caspases share similarities in amino acid sequence, structure and substrate specificity. The caspases are synthesised as inactive pro-enzyme (zymogens) that contain three domains: an amino-terminal prodomain; a large internal domain (p20) containing a large catalytic subunits; and a carboxy-terminal small domain (p10) [214].

At least 7 of the 14 known mammalian caspases have important roles in apoptosis and generally divided into two classes: the initiator caspases, which include caspase-2, -8, -9 and -10; and the effector caspases, which include caspase-3, -6 and -7 (Figure 1.9) [215, 216]. The initiator caspases are characterized by long prodomains (>90 amino acids)
that serve as platforms for the recruitment of activating adaptor proteins. The prodomains of caspase-2 and -9 contain a caspase recruitment domain (CARD), whereas caspase-8 and -10 possess two tandem repeats of the death effector domain (DED). The effector caspases contain short prodomains (20-30 amino acids) (Figure 1.9) [217]. Caspase activation involves proteolytic cleavage at specific internal aspartate (Asp) residues, followed by association of large and small subunits to form a heterodimer, which eventually form the active heterotetrameric protease to initiate apoptosis. Each subunit contributes amino acids to form a catalytic site at opposite ends of the molecule (Figure 1.10) [217].
Figure 1.9 Apoptotic caspases in mammals. The position of the intra-chain activation cleavage (between the large and small subunits, ~p20 and ~p10, respectively) is highlighted by a black arrow, whereas other sites of cleavage are represented by grey arrows. The prodomains in initiator caspases invariably contain homotypic interaction motifs, such as the caspase-recruitment domain (CARD) and the death-effector domain (DED). Adapted from Riedl, 2004 [215].
Figure 1.10 General structure and the activation of mammalian caspases. Caspase proenzymes contain three domains: an amino-terminal prodomain; a large subunit (p20); and a carboxy-terminal small subunit (p10). Two cleavage events at aspartate (Asp) residues are required to activate caspases. The first divides the proenzyme into large and small caspase subunits, and the second removes the N-terminal prodomain. The resulting functional caspase is a tetramer of two large (p20) and two small (p10) subunits. Adapted from Clarke, 2009 [214].
1.3.4 Caspases and ER Stress-Induced Apoptosis

Activation of initiator caspases is a proximal event in induction of apoptosis by either the intrinsic (mitochondrial) or extrinsic (death receptor) apoptotic pathways. Although processing of caspase-8, -9, and -2 has been observed in cells under ER stress, which eventually leads to activation of effector caspases such as caspase-3 and -7, activation of caspase-12 in rodents and caspase-4 in human appears essential in ER stress-induced apoptosis [168, 218].

Caspase-12 is located to the ER and is selectively activated by ER stress [218]. Caspase-12 deletion inhibits apoptosis induced by a variety ER stress inducers such as tunicamycin and thapsigargin. In contrast, over-expression of caspase-12 induces apoptosis. There is large body of evidence showing that caspase-12 is activated by ER stress up-stream of effector caspases, indicating it functions as an initiator caspase. However, caspase-12 is expressed only in rodents. Its human homologue is silenced by several mutations during evolution [219]. Human caspase-4 is the closest homologue to murine caspase-12 and is at least partially located to the ER [218]. Caspase-4 has been shown to fulfill the function of caspase-12 in ER stress-induced apoptosis in some types of human cells [218, 220].

How caspase-12 and caspase-4 is activated in cells under ER stress remains to be determined. A number of biological events have been shown to be associated with activation of caspase-12 in murine cells [159, 167, 200, 221]. First, caspase-12 activation has been linked to release of calcium from ER and consequent activation of calpain. Inhibition of calpain by chemical inhibitors and genetic approaches and chelation of intracellular calcium can block caspase-12 activation induced by various ER stress inducers [221]; second, ER stress triggers recruitment of caspase-7 to the ER where it complexes with caspase-12 leading to its activation. GRP78 also exists at the complex and plays a role in inhibiting both caspase-7 and -12 [222]; and third, it has been suggested that caspase-12 is associated with TRAF2, but under ER stress recruitment of the TRAF2/caspase-12 complex to IRE1 provides a scaffold for caspase-12 activation [223]. However, if human caspase-4 is similarly activated as murine caspase-12 by ER stress remains to be clarified.
1.4 Adaptation of Melanoma Cells to ER Stress

Although excessive or prolonged UPR can result in apoptosis, most melanoma cell lines are not sensitive to apoptosis induced by pharmacological ER stress inducers, tunicamycin and thapsigargin. This suggests that melanoma cells may have adapted to ER stress conditions by development of resistance mechanisms against ER stress-induced apoptosis.

There is ample evidence showing that the UPR is activated in various solid tumours due to both intrinsic and extrinsic factors [152, 224]. Increased expression of GRP78, a commonly used indicator of activation of the UPR, has been reported in a variety of cancers. In some cases, GRP78 expression is associated with tumour growth and resistance to chemotherapy [152, 224]. Consistently, GRP78 is also expressed at varying, but commonly higher levels in melanoma cells relative to melanocytes both in vitro and in vivo [225, 226]. In addition, the levels of GRP78 correlate with progression of the disease and other markers of prognosis such as tumour thickness and mitotic rate [226]. Similarly, the active form of XBP-1 mRNA is expressed at higher levels in cultured melanoma cells [227]. Therefore, melanoma cells have adapted to ER stress, which appears to be imposed on melanoma cells at early stages of development, in that UPR has been shown to be activated at initiation stages of melanoma by the oncogenic form of HRAS (HRAS$^{G12V}$) [166].

It is conceivable that cells in a developing tumour without sufficient blood supply may undergo hypoxia, nutrient starvation and acidosis [152, 224]. In addition, mutated proteins in cancer cells may also contribute to ER stress. Another ER stress-inducing mechanism of cancer cells is increased glycolytic activity due to the Warburg effect. High levels of lactic acid dehydrogenase (LDH) are readily apparent in patients with melanoma, and the levels in sera are the single most powerful predictor of prognosis in metastatic disease [98, 228]. In support of the importance of increased glycolysis in induction of ER stress in melanoma, the levels of LDH5 are correlated with the levels of GRP78 in melanoma cells in vivo [229].
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1.5 Adaptive Mechanisms of Melanoma Cells to ER Stress

Adaptation to ER stress is believed to be an intrinsic consequence of low level activation of the UPR. However, it remains a paradox how the UPR switches between the pro-survival and pro-apoptotic signalling pathways. Nevertheless, adaptive processes converge on mechanisms that inhibit apoptosis. Because ER stress can induce apoptosis through multiple mechanisms, it is conceivable that adaptation of melanoma cells to ER stress is the consequence of multiple anti-apoptotic mechanisms.

1.5.1 Up-regulation of Mcl-1 is Essential for Survival of Melanoma Cells upon ER Stress

Mcl-1 is an anti-apoptotic Bcl-2 family protein that is of particular importance in melanoma, in that its expression increases with melanoma progression and is associated with poor prognosis [230]. Moreover, Mcl-1 is a major resistance mechanism of melanoma cells to apoptosis induced by various apoptotic stimuli [231, 232]. As a protein with a rapid turn-over rate, Mcl-1 expression is frequently regulated by post-translational mechanisms [233]. Nevertheless, an increase in the Mcl-1 protein levels often correlates with an increase in its mRNA levels, mostly due to enhanced transcription [234]. In addition, Mcl-1 can also be regulated by translation level. For example, the Mcl-1 protein levels have been shown to be greatly reduced by thapsigargin due to translational repression mediated by phosphorylation of eIF2α downstream of PERK [235, 236].

However, in contrast to down-regulation, Mcl-1 along with Bcl-2 are increased in melanoma cells in response to ER stress induced by thapsigargin or tunicamycin, while the expression levels of Bcl-XL, another pro-survival Bcl-2 family protein remain unaltered [212, 231, 234]. Although Bcl-2 was up-regulated, it does not appear to be critical for protection of melanoma cells from ER stress-induced apoptosis, in that inhibition of Bcl-2 by siRNA has only a minimal effect on sensitivity of melanoma cells to ER stress-induced apoptosis, whereas over-expressing Bcl-2 can only delay the onset of apoptosis, but does not rescue melanoma cells from apoptosis induced by ER stress when Mcl-1 is deficient. On the other hand, knocking down Mcl-1 by siRNA readily
enhances ER stress-induced apoptosis, and over-expression of Mcl-1 efficiently protects melanoma cells from apoptosis induced by tunicamycin or thapsigargin, even when Bcl-2 is inhibited. Therefore, Mcl-1, but not Bcl-2, plays a determining role in survival of melanoma cells under ER stress conditions. Indeed, while the Mcl-1 expression is correlated with melanoma progression, the expression of Bcl-2 is down-regulated during progression of melanoma [226, 230, 231, 234]. Importantly, the Mcl-1 protein levels in melanoma cells are correlated with the levels of GRP78, suggesting that the increase in Mcl-1 in melanoma maybe a consequence of activation of the UPR and an adaptive mechanism of melanoma cells to ER stress [226].

ER stress-induced apoptosis in melanoma cells is associated with activation of Bax/Bak, reduction in mitochondrial membrane potential, and the release of apoptotic proteins from mitochondrial when Mcl-1 is inhibited [236, 237]. Along with these apoptotic events, the BH3-only proteins Noxa and PUMA are increased in melanoma cells upon ER stress. However, inhibition of either Noxa or PUMA only inhibit apoptosis partially when Mcl-1 is knocked down by shRNA, suggesting that Noxa and PUMA may act together in ER stress-induced apoptosis of melanoma cells deficient in Mcl-1. It is of note that although PUMA can be antagonized by Mcl-1 and Bcl-2, Noxa can only bind to Mcl-1. This may explain, at least in part, why targeting Bcl-2 is relatively ineffective against apoptosis induced by ER stress in melanoma cells [212, 231]. Regardless, it appears that the up-regulation of Mcl-1 has a critical part in antagonizing Noxa and PUMA.

Up-regulation of the Mcl-1 protein in melanoma cells under ER stress is associated with increases in the Mcl-1 mRNA, which is efficiently inhibited by actinomycin D, a general transcription inhibitor, indicating that a transcriptional increase is involved in ER stress-induced up-regulation of Mcl-1 [231]. Analysis of the Mcl-1 promoter region identifies a binding site for the E26 transformation specific sequence (Ets)-1 that is activated by treatment with tunicamycin or thapsigargin [234]. Ets-1 is a transcription factor from the Ets family that plays roles in many biologic processes such as cell growth and survival [238, 239]. Indeed, inhibition of Ets-1 by siRNA or mutations in the Ets-1 binding site inhibits the increase of the Mcl-1 transcript by ER stress, and recapitulates the effect of Mcl-1 inhibition on sensitization of melanoma cells to
apoptosis induced by ER stress [234]. Therefore, Ets-1 is responsible for transcriptional up-regulation of Mcl-1 by ER stress in melanoma cells.

Ets-1 is transcriptionally upregulated in melanoma cells upon induction of ER stress, which is partially inhibited in melanoma cells deficient in IRE1α and ATF6, indicating that these branches of the UPR are involved in the ER stress-triggered increase in Ets-1 in melanoma cells [234]. The convergence of the IRE1α and ATF6 pathways on XBP-1 suggest that XBP-1 may be involved in upregulation of Ets-1 by the UPR. Indeed, inhibition of XBP-1 blocks ER stress-induced upregulation of Ets-1 [234]. Therefore, XBP-1 plays an important role in up-regulation of Ets-1, and subsequent upregulation of Mcl-1 in melanoma cells subjected to ER stress.

Taken together, it appears that a signalling nodule of XBP-1-Ets-1-Mcl-1 leading to transcriptional upregulation of Mcl-1 is activated in melanoma cells upon activation of the UPR, which is important for protection of melanoma cells against ER stress-induced apoptosis by antagonizing pro-apoptotic Bcl-2 family proteins, such as PUMA and Noxa that are also increased by ER stress in melanoma cells (Figure 1.11) [231, 234].
Figure 1.11 A schematic illustration of Ets-1-mediated upregulation of Mcl-1 downstream of XBP-1 in melanoma cells under ER stress. Adapted from Zhang, 2011 [240].
1.5.2 Induction of GRP78 in Adaptation of Melanoma Cells to ER Stress

As the “master regulator” of the response of cells to ER stress, GRP78 plays an important role in survival of cells under ER stress [155, 224]. Multiple mechanisms have been reported to contribute to GRP78-mediated inhibition of apoptosis in cell undergoing ER stress. These include its binding to unfolded/misfolded proteins to limit their aggregation, binding to calcium to maintain calcium homeostasis, and binding to caspase-12 and caspase-7 to inhibit their activity [155, 224]. In addition, GRP78 has been shown to promote cell proliferation and to be necessary for ER stress-induced autophagy [241]. Although the three branches of the UPR can all contribute to induction of GRP78, the ATF6 pathway plays a dominant role in regulation of GRP78 expression [242, 243].

As a typical ER luminal chaperone, GRP78 can also be expressed on the surface of various types of cancer cells including melanoma cells, in particular, when cells are under ER stress [224, 244]. Relocation of GRP78 to the cell surface appears to be mediated its C-terminal ER retention motif, as deletion of the motif alters its cell surface presentation [244]. Cell surface GRP78 interacts with a number of cell surface proteins and soluble ligands such as activated α(2)-macroglobulin and acts as an initiator of intracellular signalling pathways, thus promoting cell survival and proliferation [244, 245].

Auto-antibodies that react with GRP78 expressed on the cell surface can be detected in the sera of patients with prostate cancer, ovarian cancer and melanoma [244]. These auto-antibodies are a negative prognostic factor in prostate cancer [244]. However, if they are of prognostic value in melanoma patients has not been established. Nevertheless, binding of auto-antibodies to the cell surface GRP78 has been reported to promote tumour growth of a murine melanoma model [246]. Because its preferential expression on the cell surface of cancer cells, GRP78 has been suggested to be a tumour-associated antigen [247].
1.5.3 Adaptation to ER stress as a Resistance Mechanism of Melanoma Cells to Chemotherapeutic Drugs

A growing body of evidence shows that induction of ER stress and subsequent activation of the UPR can alter chemosensitivity of cancer cells [152, 224, 248]. On the other hand, induction of apoptosis is associated with induction of ER stress in many types of cancer cells by a number of different classes of chemotherapeutic drugs, such as the DNA-damaging agent cisplatin, the non-steroidal anti-inflammatory drug celecoxib, the proteasomal inhibitor bortezomib, and the general kinase inhibitor sorafenib, suggesting that induction of ER stress may be an important mechanism in killing of cancer cells by chemotherapeutic drugs [152, 224, 227, 248-250]. On the other hand, melanoma cells have developed multiple adaptive mechanisms that render the cells largely resistant to ER stress-induced apoptosis, suggesting that adaptation to ER stress may contribute to resistance of melanoma cells to induction of apoptosis [248]. Some adaptive mechanisms to ER stress, such as induction of GRP78 and Mcl-1, have been shown to play important roles in resistance of melanoma cells to various chemotherapeutic drugs [225, 227, 248].

1.5.3.1 GRP78 Contributes to Resistance of Melanoma Cells to DNA-damaging Agents

Although cisplatin and adriamycin are conventionally regarded as DNA-damaging agents, they can induce ER stress in melanoma cells as shown by increased expression of GRP78 and activation of XBP-1 [227]. Whether induction of ER stress plays a part in induction of apoptosis by the drugs remains to be clarified, but GRP78 protects melanoma cells against cytotoxic effects of the drugs [225]. This is mediated, at least in part, by the inhibitory effect of GRP78 on activation of caspase-4. The latter is bound to and kept inactive by GRP78 in melanoma cells [220, 225]. Inhibition of GRP78 by siRNA in melanoma cells subjected to ER stress frees caspase-4 and leads to its activation [220].

There was no correlation between the GRP78 expression levels and sensitivities of melanoma cell lines to cisplatin or adriamycin, suggesting that, besides GRP78, other mechanisms may also contribute to regulation of responses of melanoma cells to the
drugs [225]. For example, expression of ATP-binding cassette (ABC) transporters and increased DNA repair are known to contribute to resistance of cancer cells against cisplatin and adriamycin [251, 252]. In addition, activation survival signalling pathways such as the PI3K/Akt and MEK/ERK pathways is a common cause for resistance of melanoma to apoptosis [253, 254]. Regardless, targeting GRP78 may be a useful strategy in sensitizing melanoma cells to these chemotherapeutic drugs.

1.5.3.2 Melanoma Cells under ER Stress are more Resistance to Microtubule-Targeting Drugs

Unlike cisplatin and adriamycin, the microtubule-targeting drugs docetaxel and vincristine do not trigger ER stress in melanoma cells as shown by their inability to induce GRP78 and the spliced XBP-1 mRNA. However, their cytotoxicity is attenuated in melanoma cells subjected ER stress [227]. This was demonstrated by the observation that treatment with the ER stress inducer tunicamycin or thapsigargin before the addition of docetaxel or vincristine reduced the levels of apoptosis induced by the drugs. GRP78 does not appear to be involved in that sensitivity of melanoma cells to docetaxel- and vincristine-induced apoptosis cannot be enhanced by inhibition of GRP78. In contrast, activation of the PI3k/Akt pathway downstream of XBP-1-mediated signalling is critical in protection of melanoma cells against the drugs by preloaded ER stress [227].

How Akt is activated by XBP-1 signalling in melanoma cells remains to be further studied, but it was recently shown that ER stress activated Akt in a zebrafish embryonic cell line through XBP-1-mediated up-regulation of insulin growth factor-1 (IGF-1) [255]. However, IGF-1/IGF-1 receptor signalling in melanoma cells originates mainly from exogenous IGF-1 because melanoma cells express no, or minimal, IGF-1 [256, 257]. Nevertheless, it is possible that XBP-1 may activate a factor or factors similar to IGF-1 that in turn causes activation of the PI3K/Akt pathway in melanoma cells under ER stress. In any case, it appears that activation of XBP-1 signalling as a consequence of adaptation to ER stress is an important resistance mechanism of melanoma cells to the microtubule-targeting drugs docetaxel and vincristine.
1.5.4 Early Attenuation of IRE1α Signalling during Persistent ER Stress Sensitizes Cells to ER Stress-induced Cell Death

No trigger for ER stress has been identified that selectively elicits only protective responses or apoptosis. However, the duration of activation of individual arms of the UPR plays an important role in determining cell fate in response to ER stress. The IRE1 pathway is rapidly attenuated after induction of ER stress even in the presence of ER stress inducers, whereas the ATF6 branch is also attenuated, albeit with slow kinetics. In contrast, the PERK pathway of the UPR persists for considerably longer periods, and is presumably responsible for induction of apoptosis in the absence of activation of IRE1 and ATF6 (Figure 1.12) [258]. It appears that cyto-protective outputs of the initial combined activation of three arms of the UPR outweigh pro-apoptotic outputs. However, attenuation of IRE1 and ATF6 signalling create an imbalance that leads to apoptosis. Consistent with this, IRE1α - or ATF6- deficient cells demonstrate reduced survival rate in cells treated with ER stress inducers [258, 259].

In agreement with this model, deficiency in IRE1α - or ATF6- renders sensitivity of melanoma cells to ER stress-induced apoptosis. In contrast, melanoma cells deficient in PERK remain relatively resistant to apoptosis induced by ER stress [248]. It has been found that elevated levels of GRP78 along with phosphorylated PERK and eIF2α persisted for at least 36 hours in the presence tunicamycin and thapsigargin [225, 248]. This suggests that, in contrast to observations made in other cell types, IRE1 and ATF6 signalling in melanoma cells were not rapidly attenuated under prolonged ER stress. Therefore, a testable hypothesis is that perpetuation of IRE1 and/or ATF6 signalling with or without attenuation of the PERK pathway is an essential mechanism of adaptation of melanoma cells to ER stress.
Figure 1.12 Both cell survival and cell death responses can be triggered by accumulation of misfolded proteins and/or unfolded proteins (ER stress), but with different duration of 3 individual arms of the UPR. Adapted from Lin, 2007 [258].
1.5.5 Eukaryotic Initiation Factor 4E (eIF4E) in Adaptation of Melanoma Cells to ER Stress

The UPR is often constitutively activated in cancer cells, indicative of adaptation of the cells to ER stress [152, 224]. Indeed, cells in a developing solid tumour without sufficient blood supply may undergo acidosis, hypoxia, and nutrient starvation, therefore resulting in ER stress [224, 260]. Moreover, regularly increased synthesis of mutated proteins that are required to sustain malignancy of cancer cells may also directly uncouple the ER protein folding load with the ER protein folding capacity [152, 224]. The rate of mRNA translation plays an important role in regulating protein expression and is primarily controlled at the stage of mRNA translation initiation [261, 262].

1.5.5.1 Eukaryotic Initiation Factor 4E (eIF4E)

Initiation of translation of most mRNAs is mediated by the cap-dependent mechanism, whereas cap-independent initiation is involved in translation of a subset of mRNAs, especially under cellular stress conditions [261, 263, 264]. Cap-dependent translation initiation is governed by the eukaryotic initiation factor (eIF) 4F (eIF4F) complex that consists of the cap-binding protein eIF4E, the RNA helicase eIF4A, and the scaffolding protein eIF4G [261-264]. Among them, eIF4E is the rate-limiting factor that potentially influences the expression of almost every protein in cells [264-266] and the key factor for the assembly of eIF4F at the 5’ cap (Figure 1.13).

The activity of eIF4E is regulated by its availability that is controlled by the eIF4E inhibitory binding proteins (4E-BPs), in particular, 4E-BP1 [264-266]. Hypophosphorylated 4E-BP1 shares the same binding motif with eIF4G and thus competes with eIF4G for binding to and sequestering eIF4E [264-266]. In addition, phosphorylation of eIF4E at serine 209 upon binding to eIF4G by MAP kinase signal-integrating kinase 1 (MNK1) and MNK2 enhances its activity [266-268]. Importantly, overexpression of eIF4E promotes cellular transformation and tumorigenesis [269-271]. This is associated with increased translation of various mRNAs encoding cancer-promoting proteins [264, 269, 271, 272]. Indeed, eIF4E is expressed at increased levels in many types of cancers, such as those of the breast and colon [273-275], which is
often associated with disease progression and poor prognosis of patients [264, 273-275]. However, the mechanism(s) responsible for upregulation of eIF4E in cancer cells remains elusive.
Figure 1.13 mRNA recruitment by eukaryotic initiation factors during translation initiation. Adapted from Hou, 2012 [267].
1.6 Protein Phosphatase 2A (PP2A)

The serine/threonine phosphatase PP2A represents a family of holoenzyme complexes that regulate numerous intracellular signalling cascades mostly by reversing the action of kinases [276, 277]. Typically, PP2A contains a highly active core dimer composed of a catalytic subunit (PP2A-C) and a structural A subunit (PP2A-A) that recruits one of the multiple regulatory B subunits (PP2A-B) to form the PP2A heterotrimeric complex (Figure 1.14) [276, 277]. The substrate specificity of PP2A is determined by the B subunit in the heterotrimeric complex, whereas the dynamic exchange of B subunits in the complex is regulated by reverse methylation and phosphorylation of the C-terminal tail of the C subunit. PP2A activity is also regulated by a number of endogenous PP2A inhibitors such as SET and cancerous inhibitor of PP2A (CIP2A), and activators such as phosphotyrosyl phosphatase activator (PTPA) [276, 277].

1.6.1 The Catalytic Subunit (PP2A-C)

The catalytic subunit contains a large conserved domain that forms a bimetallic active site for phosphor-ester hydrolysis. It targets either serine or threonine residues of the phosphate group and under certain conditions harbours activity toward phosphorylated tyrosine [278]. PP2A catalytic activity is encoded by two distinct ubiquitously expressed genes [279], the Cα and Cβ subunits, the latter being expressed approximately 10 fold lower due to a weaker promoter [280]. Both are 35kDa in size and share 97% sequence identity.

The studies of the crystal structure of the PP2A holoenzyme determined that the highly conserved C-terminal tail (TPDYFL) of the PP2A-C subunit resides at a critical interface between the PP2A-A subunit and the B subunit B′γ [281, 282]. As such, methylation and phosphorylation patterns of the C-terminal tail of PP2A-C subunit tightly regulate the recruitment of B subunit to the core enzyme. Methylation on the carboxyl group Leu by S-adenosylmethionine-dependent leucine carboxyl methyltransferase 1 (LCMT1) was shown to be required for the binding of the PR55B family members but not for other B subunits [283]. The methylation of the C-terminal
tail can be reversed by the specific PP2A-methylesterase (PPME) thus adding another dimension to holoenzyme regulation (Figure 1.15) [283].
Figure 1.14 Schematic representation of the structural diversity of the PP2A holoenzyme complex. PP2A enzymes are heterotrimers consisting of core dimer scaffold (A) and a catalytic (C) subunit that is associated with one of the regulatory (B) subunits. Adapted from Seshacharyuku, 2013 [284].
Figure 1.15 Post transitional modification of PP2A. Phosphorylation and methylation are two major modifications that have been shown to modulate PP2A subunit associations and catalytic efficiency. Adapted from Shi, 2009 [277].
1.6.2 The Structural Subunit (PP2A-A)

PP2A-A scaffold subunit is encoded by two distinct genes, PPP2R1A and PPP2R1B, resulting in two isoforms, Aα and Aβ. Both are ubiquitously expressed and share 86% sequence similarity [284, 285]. In about 90% of the PP2A assemblies, the core and/or holoenzyme is composed of the Aα scaffold subunit. On the other hand, Aβ is found in 10% of PP2A assembly leading to differential preference of interaction with the catalytic and regulatory subunits [284].

The catalytic subunit interacts with the PP2A-A scaffold protein at four C-terminal Huntington/elongation/A-subunit/TOR (HEAT) repeats [286]. Upon the formation of the core enzyme, the scaffold protein forms a more horseshoe shape-like structure by folding in on itself [276, 277]. The bending of the structural subunit allows the catalytic subunit unobstructed access to the PP2A substrate, which is recruited to the holoenzyme by the B regulatory subunit [285].

1.6.3 The Regulatory Subunit (PP2A-B)

PP2A-B is though to be the master regulator of the PP2A holoenzyme and it is likely to act as a targeting modulator to provide temporal and spatial specificity [284, 287]. Up to the date, there are 15 genes have been identified in the human genome that encode at least 26 different alternative transcripts and splice forms representing the B subunits of the PP2A holoenzyme [279]. Around 30 PP2A holoenzyme combinations are possible without including predicted splice variants [288-290]. Even though all the different family members and isoforms of PP2A-B bind to similar recognition sequences of PP2A-A, they do not possess similar gene sequences. Regulatory subunits are multiform and are classified into four different families: B (B55/PR55), B’ (B56/PR61), B” (PR48/PR72/PR130), and B’’’ (PR93/PR110) [284].

1.7 The Role of PP2A in Tumour Suppression

PP2A was first suggested to act as a tumour suppressor based on the tumour-promoting actions of okadiac acid (OA), a PP2A inhibitor which promotes tumour growth in
mouse skin [291], stomach and liver [292] and induces genomic instability [293]. Recently, there is increasing evidence showing that PP2A has an important tumour suppressive role [279, 288, 292]. Indeed, mutations have been identified in different components of the PP2A complex, which have been linked to a variety of human cancers [148, 279, 284, 294, 295]. Particularly, a truncated form of PP2A-B56γ has been found in the mouse melanoma cell line B16 that is not able to dephosphorylate specific targets and plays a role in malignant progression [294, 295]. Although no mutations have been found in patients, the expression of the PP2A-B56γ gene is frequently reduced in human melanoma compare to naevi [296]. Similarly, PP2A-B56α has recently been shown to be expressed at lower levels in metastatic compared to primary melanomas [297]. Down-regulation of various PP2A subunits has also been reported in several other cancers [148, 279, 284].

1.7.1 PP2A Activation as an Anti-Cancer Therapy

PP2A has tumour suppressing activity by regulating cell signalling cascades in a way opposite to the activity of kinase oncogenes [279, 298]. Therefore, it suggests that pharmacological activation of PP2A activity would retrain the activity of the kinase oncogenes and could be beneficial for cancer treatment.

The first example of PP2A activator is FTY720 (Fingolimod), a synthetic myriocin analogue structurally similar to sphingosin. It is a known immunomodulator, which is approved by FDA for the treatment of patients with either multiple sclerosis or undergoing renal transplantation [148, 299, 300]. Although the precise mechanism underlying induction by FTY720 of PP2A activation is unclear, evidence suggests that, like ceramide, FTY720 has a direct effect on the PP2A heterotrimeric complexes [301, 302].

PP2A activators have been recently suggested to use in treatment of different types of human leukemia. FTY720 mediates toxic effects in cell lines representing different B-cell malignancies and primary B cells from patients with chronic lymphocytic leukemia. Expose with FTY720 of transformed B-cells results in decreased levels of phosphor-ERK1/2 and Bcl-2 independent apoptosis [303]. Furthermore, FTY720 has been proven effective in suppression of e-Kit-mediated tumourigenesis of acute myeloid leukemias.
[304]. These findings indicate that incorporation of PP2A activating drugs in current therapeutic protocols for cancers may offer a novel attractive therapeutic strategy.
1.8 Aims of Studies

As indicated above, melanoma is a major Australian health problem. However, there is currently no curative treatment once the disease spreads beyond the loco-regional site. This is because melanoma generally has low response rates to a number of different biologic agents and is largely resistant to available chemotherapy. Previous studies in our laboratory have demonstrated that melanoma cells have largely adapted to ER stress. This adaptation is not only responsible for resistance of melanoma cells to apoptosis induced by ER stress, but also contributes to the resistance of melanoma cells to varying therapeutic agents. In this PhD project, we wish to continue the ongoing studies on investigation of adaptive mechanisms of melanoma cells to ER stress. We will focus on testing two hypotheses: 1) **Activation of the IRE1α and ATF6 arms of the UPR is prolonged in melanoma cells undergoing ER stress, which plays a crucial role in protecting the cells from apoptosis induced by ER stress;** and 2) the **CHOP/Bim axis of the UPR is dysregulated and does not function to transmit apoptosis signalling.** Our overall goal is to understand mechanisms by which melanoma cells adapt to ER stress, and to identify new therapeutic targets for overcoming resistance of melanoma to treatment. The specific aims are:

1. To clarify the kinetics and duration of activation of individual arms of the UPR in melanoma cells upon ER stress;
2. To examine the mechanism(s) by which activation of the ATF6 and IRE1α arms of the UPR is sustained in melanoma cells under ER stress;
3. To confirm that the apoptosis-inducing potential of the CHOP/Bim axis of the UPR is suppressed in melanoma cells undergoing ER stress;
4. To elucidate the mechanism(s) by which the apoptosis-inducing potential of the CHOP/Bim axis is suppressed in melanoma cells under ER stress.
CHAPTER TWO

MATERIALS AND METHODS
Chapter Two

2.1 Cells Lines

The series of melanoma cells with the prefix Mel- were isolated from patients of the Newcastle and Sydney Melanoma Units. Mel-RM was from bowel. Mel-JD and Mel-CV were from lymph nodes. MM200 was isolated from primary melanoma and gifted from Dr Pope and Dr Parsons (Queensland Institute for Medical Research, QLD, Australia). ME1007 and ME4405 were also isolated from primary melanoma and kindly supplied from Dr. Parmiani (National Cancer Institute, Milan, Italy). IgR3 was established from primary melanoma and was provided by Dr. Hope (Genetics Department, University of Adelaide, SA, Australia). All melanoma cell lines were tested positive for Tyrosinase and MART-1 mRNA by RT-PCR tests. MCF-7 was breast carcinoma cell line kindly provided by Dr. Sutherland (Garvan Institute, Darlinghurst, NSW, Australia). A2058 was established from a brain metastasis and was kindly provided by Dr. Todaro (Bristol-Myers Squibb, WA, Australia). HEK293 was purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). All these cell lines were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 5% Foetal Calf Serum (FCS) (Commonwealth Serum Laboratories, VIC, Australia) at 37°C in a humidified atmosphere of 5% CO₂. Cell lines were routinely tested for mycoplasma. Melanocytes HMMn-MP and HEMn-DP and its respective medium were purchased from Clonetics (Edward Kellar, VIC, Australia). pCDH-CMV-MCS-EF1-copGFP containing Myc-tagged BRAF^{V600E} or BRAF^{wt} expressed melanocyte and their respective controls were gifted from Associate Professor Rizos (Westmead Institute for Cancer Research, University of Sydney, NSW, Australia). All melanoma cells culture were handled under sterile conditions in class II biosafety hoods.
### 2.2 Antibodies and Recombinant Proteins

Table 2.1 List of Antibodies

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Table 2.2 List of Recombinant Proteins and Other Reagents

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2.3 Apoptosis

2.3.1 BrdU Incorporation Assay

BrdU cell proliferation assays were carried out using an assay kit (Cell Signalling, Beverly, MA, USA) and according to the manufacturer’s instructions. Briefly, cells seeded at $5 \times 10^3$ per well in 96-well plates overnight before treatments desired. BrdU (10mM) was added and cells were incubated for 4 hours before BrdU assays were carried out. Absorbance was read at 450nm using a Synergy 2 multi-detection microplate reader (BioTek, Winooski, VT, USA).

2.4 Flow Cytometry

2.4.1 Propidium Iodide (PI) Assay

Apoptotic cells were determined by PI assay. Melanoma cells were adhered overnight in a 24-well plate at a density of $1 \times 10^5$ cells/well in DMEM containing 5% FCS. Cells with treatment were incubated for a desired period of time. The medium containing the dead cells was collected into a Falcon polystyrene tube while the remaining adherent cells were washed once with PBS, collected into the respective tubes and centrifuged at 200 x g for 10 minutes. Supernatants were then discarded. Hypotonic buffer (PI, 50mg/ml, in 0.1% Sodium tri-citrate and 0.1% Triton X-100) was added to the adhered cells in the 24-well plate and incubated at 37°C in a humidified atmosphere of 5% CO$_2$, for 10 minutes or until cells detached from the plate. Cells were pipetted off and collected into the respective tubes. Samples were kept in the dark at 4°C overnight before flow cytometric analysis. The PI fluorescence of individual nuclei was measured in the red fluorescence using flow cytometry (FACSscan, Becton Dickinson, Sunnyvale, CA, USA) and the data registered in a logarithmic scale. At least $1 \times 10^4$ cells of each sample were analysed. Apoptotic nuclei appeared as a broad hypodiploid DNA peak, which was easily distinguished from the narrow hyperdiploid peak of nuclei in the melanoma cells.
2.5 **Protein Analysis**

### 2.5.1 Whole Cell Protein Extraction

Melanoma cells were seeded in 6-well plated at 4 x 10^5 cells per well and were allowed to adhere to the plate and reach exponential growth for 24 hours before treatment. Cells were harvested by trypsinisation, washed with PBS, followed by lysing with NP-40 based lysis buffer (10mM Tris-HCl, pH 7.4; 140mM NaCl; 0.5mM CaCl\(_2\); 0.5mM MgCl\(_2\); 0.02% NaN\(_3\); 1% NP-40; 1x protease inhibitor cocktail) and incubated on ice for at least 30 minutes. Samples were then centrifuged at 14,000 x g for 30 minutes at 4°C. The supernatants that contained the proteins were transferred into fresh tubes. Protein concentrations were then determined by protein assay solution and read with spectrophotometer (Spectronic Genesys 8, UK).

### 2.5.2 Immunoprecipitation

Immunoprecipitation was performed following the manufacturer’s instructions. Briefly, 500µl of cell lysates were pre-cleared with 50µl of anti-mouse/ anti-rabbit IgG beads by incubating on ice for 30 minutes followed by centrifuging at 10,000 x g for 3 minutes. The supernatants were transferred into clean tubes and were incubated on ice with 5µg of primary antibodies (2µg for Mouse/ Rabbit IgG isotype control) for 1 hour. After incubation, 50µl of fresh anti-mouse/ anti-rabbit IgG beads were added and samples were incubated at 4°C on a rocker for 2 hours. The beads were washed 3 times with lysis buffer and collected by centrifugation. SDS-PAGE sample buffer (12.5mM Tris-Cl/SDS [pH 6.8]; 20% glycerol; 4% SDS; 0.001% bromophenol blue; 0.02% β-mercaptoethanol) was added to the collected beads and boiled at 100°C for 10 minutes. The samples were then centrifuged at 10,000 x g for 3 minutes before subjected to SDS-PAGE and western blot analysis.

### 2.5.3 SDS-PAGE

Proteins can be separated by using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The Mini-PROTEAN 3 electrophoresis system was
Chapter Two

purchased from Bio-Rad. 1.5mm thick of polyacrylamide gels were prepared from bis/acrylamide solution. 7.5-15% gels were used for separating 10-250 kDa proteins. Each gel consists of two layers, resolving gel (resolving gel buffer (1.5M Tris [pH 8.8]), 30% acrylamide solution, 10% SDS, 10% ammonium persulphate (APS), TEMED) and stacking gel (stacking gel buffer (800mM Tris [pH6.8]), 30% acrylamide solution, 10% SDS, 10% APS, TEMED). The resolving gel mixture was poured into the pre-setup spacer and short plates, and topped up with 70% ethanol to remove air bubbles. Ethanol does not readily mix with resolving gel mixture due to different density. Once the resolving gel layer get polymerized, the water was discarded, and stacking gel mixture was layered over the resolving gel layer. A multi-well comb was placed in the stacking gel layer before the gel polymerized. After polymerization was complete, the gel was mounted in an electrophoresis apparatus. The comb was removed and the apparatus was topped up with SDS running buffer (25mM Tris, 200mM glycine, 0.1%SDS). A total of 40-100μg of proteins were mixed with an equal volume of 2x SDS-PAGE sample buffer. The mixture was then boiled at 100ºC for 3 minutes. Samples were loaded into wells together with unstained and/ or pre-stained protein molecular weight markers and electrophoresed.

2.5.4 Western Blot

Following electrophoresis, the proteins on the polyacrylamide gels were electrophoretically transferred onto 0.45μm nitrocellulose membranes (Bio-Rad, Regents Park, NSW, Australia) in transferring buffer (20mM Tris, 150mM glycine, 20% methanol) using Mini Trans-Blot® electrophoretic transfer cell (Bio-Rad, Regents Park, NSW, Australia). Non-specific interactions on the nitrocellulose membranes were blocked with blocking buffer (5% skim milk or 5% BSA in Tris-buffered saline-Tween 20 (TBS-T)) at room temperature for 1 hour. Membranes were incubated overnight at 4ºC with primary antibodies diluted in blocking buffer. Membranes were then washed 3 times with TBS-T, each for 20 minutes. After the washing step, membranes were incubated with horseradish peroxidase-conjugated secondary antibody at room temperature for 2 hours. Labelled bands were detected with Luminata Crescendo Western HRP and images were captured on ImageReader LAS-4000 (Fujufilm Corporation, Japan).
2.5.5 Nascent Protein Synthesis Assay

Melanoma cells were seeded at 4 x 10^5 cells per well in 6-well plates and were allowed to adhere to the plate and reach exponential growth for 24 hours before treatment. Nascent protein synthesis was performed using Click-iT chemistry following manufacturer’s instructions. Briefly, plates were washed once with warm PBS followed by replacing media with methionine-free media and incubated at 37ºC for 1 hour to deplete methionine reserves. Cells were incubated with 50µM of Click-iT® HPG diluted in methionine-free media at 37ºC in a humidified atmosphere of 5% CO₂ for 2 hours. Cells were washed once with PBS, followed by fixing with 4% paraformaldehyde in PBS for 15 minutes. Cells were then collected into Falcon polystyrene tubes and permeabilised with 1% BSA/ 0.1% Saponin in PBS for 15 minutes. After washing the cells once with 3% BSA in PBS, 500µl of Click-iT® reaction cocktail were added to each sample and incubated at room temperature for 30 minutes in the dark. Cells were washed once with 3% BSA in PBS and stained with Alexa Fluor® 488 and incubated at room temperature for 30 minutes in the dark prior to flow cytometry analysis.

2.5.6 PP2A Phosphatase Activity Assay

PP2A activity was determined using the PP2A immunoprecipitation phosphatase assay kit. Briefly, whole-cell lysates were incubated with 4µg of antibody against PP2A-C and 25-40µl agarose beads for 2 hours at 4ºC with constant rocking. After three washes with TBS, 60µl of diluted phosphopeptide and 20µl of assay buffer were added and incubated for 10 minutes at 30ºC in a shaking incubator. Briefly centrifuged and transferred 25µl into each well of the microtiter plate. 100µl of Malachite Green Phosphate detection solution was added and let the colour develop for 10-15 minutes at room temperature. Fluorescence was read by a microplate reader (Synergy 2, BioTek, Winooski, VT, USA) at excitation 650nm.
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2.6 Molecular Biology Assays

2.6.1 RNA Extraction

Melanoma cells were seeded at 4 x 10^5 cells per well in 6-well plates and allowed to reach approximately 70% confluence on the day of treatment. Cells were harvested by trypsinisation and washed twice in cold PBS. Total RNA was isolated using RNeasy mini kit following the manufacturer’s instruction. Briefly, 350 µl of RLT lysis buffer was added to the cell pellets, followed by homogenizing the lysate using vortexing. One volume of 70% ethanol was mixed with the homogenized lysates. The mixture was transferred into RNeasy spin columns, centrifuged at 8,000 x g for 15 seconds, and washed once with RW1 buffer and twice with RPE buffer provided by the kit. RNAs were finally eluted with 30-50 µl of nuclease free water. RNA concentrations were determined by using Nanodrop 2000 spectrophotometer (Thermo Scientific, Scoresby, VIC, Australia).

2.6.2 Reverse Transcription

1-2 µg of RNA was reversed transcribed into cDNA using qScript™ SuperMix following the manufacturer’s instruction. Briefly, 4 µl of qScript™ SuperMix was added to the diluted RNA, making up to a volume of 20 µl. This mixture was heated to 25ºC for 5 minutes, 42ºC for 30 minutes and followed by 85ºC for 5 minutes. cDNAs were then further diluted with 30-60 µl of nuclease free water.

2.6.3 Real-Time Polymerase Chain Reaction (qPCR)

cDNA products from reverse transcription were used as templates for qPCR. cDNA amplification was performed by 7900HT Fast Real-Time PCR System (Applied Biosystems, Mulgrave, VIC, Australia) with specific gene primers. The following PCR conditions were used: Standard Fast Cycle: 95ºC for 20 seconds, 40 cycles of 95ºC for 1 second and 60ºC for 20 seconds using Fast SYBR Green mastermix. Cycle threshold (C_T) values for specific genes were normalized to the C_T value for the house-keeping gene, β-actin. The fold changes of mRNA expressed were determined by comparison
with β-actin, where the control sample was arbitrarily designated as 1, and the values of the relative abundance of mRNA of other samples were calculated accordingly.

Table 2.3 List of Primers for Real-Time PCR

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence 5′→3′</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active XBP-1</td>
<td>F*-TGC TGA GTC CGC AGC AGG TGC</td>
</tr>
<tr>
<td>β-actin</td>
<td>F*-GGC ACC CAG CAC AAT GAA G</td>
</tr>
<tr>
<td></td>
<td>R*-GCC GAT CCA CAC GGA GTA CT</td>
</tr>
<tr>
<td>BIM</td>
<td>F*-TGC AGA CAT TTT GCT TGT TCA A</td>
</tr>
<tr>
<td></td>
<td>R*-GAA CCG CTG GCT GCA TAA T</td>
</tr>
<tr>
<td>EIF4E(S209D) mutant</td>
<td>F*-CAC AGC TAC TAA GAG CGG CGA CAC CAC TAA</td>
</tr>
<tr>
<td></td>
<td>AAA TAG GTT TGT T</td>
</tr>
<tr>
<td></td>
<td>R*-CAA CAA ACC TAT TTT TAG TGG TGT CGC CGC TCT TAG TAG CTG TGT G</td>
</tr>
<tr>
<td>GRP78</td>
<td>F*-TTG CGC TGT GCT CCT GTG CT</td>
</tr>
<tr>
<td></td>
<td>R*-TTT TTG AAC ACG CCG ACG ACG CAG G</td>
</tr>
<tr>
<td>p-EIF4E</td>
<td>F*-TCA CGC AGC AGC AGT CTT GC</td>
</tr>
<tr>
<td></td>
<td>R*-AGG GGT GGT TTC CGG TTC GAC A</td>
</tr>
<tr>
<td>XBP-1</td>
<td>F*-CGG TGC GCG GTG CGT AGT CTG GA</td>
</tr>
<tr>
<td></td>
<td>R*-TGA GGG GCT GAG AGG TGC TTC CT</td>
</tr>
</tbody>
</table>

F* stands for Forward Primer  R* stands for Reverse Primer

2.6.4 Polymerase Chain Reaction (PCR) Thermal Cycling

PCR was performed according to the manufacturer’s instructions. GoTaq® Flexi DNA Polymerase was used. PCR reaction and cycling conditions for each polymerase are listed in Table 2.4 and Table 2.5 respectively.

Table 2.4 PCR Reaction for GoTaq® Flexi DNA Polymerase

<table>
<thead>
<tr>
<th>GoTaq® Flexi DNA Polymerase</th>
<th>5X Colourless GoTaq® Flexi Buffer</th>
<th>MgCl2 Solution (25mM)</th>
<th>dNTP (10mM)</th>
<th>Forward Primer (10μM)</th>
<th>Reverse Primer (10μM)</th>
<th>GoTaq® DNA Polymerase</th>
<th>DNA template (&lt;0.5μg)</th>
<th>Nuclease Free Water</th>
<th>X, variable volume</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 μl</td>
<td>3 μl</td>
<td>1 μl</td>
<td>1 μl</td>
<td>1 μl</td>
<td>0.25 μl</td>
<td>X</td>
<td>To 50 μl</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.5 PCR Cycling Conditions for GoTaq® Flexi DNA Polymerase

<table>
<thead>
<tr>
<th>Step</th>
<th>Temp</th>
<th>Time (min)</th>
<th>Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>95ºC</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95ºC</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>X</td>
<td>0.5</td>
<td>40</td>
</tr>
<tr>
<td>Extension</td>
<td>72ºC</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Final Extension</td>
<td>72ºC</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Cooling</td>
<td>95ºC</td>
<td>∞</td>
<td></td>
</tr>
</tbody>
</table>

X, variable temperature

2.6.5 Restriction Enzyme Digestion

Digestion of restriction site was performed according to the manufacturer’s protocols. Briefly, digestion reaction was prepared as listed in Table 2.6 on ice and digested at optimum temperature for 1-16 hours.

Table 2.6 Reaction for Restriction Digestion

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x Restriction Enzyme Buffer</td>
<td>2 μl</td>
</tr>
<tr>
<td>100x Acetylated BSA (10μg/μl)</td>
<td>0.2 μl</td>
</tr>
<tr>
<td>Plasmid DNA</td>
<td>X</td>
</tr>
<tr>
<td>Nuclease Free Water</td>
<td>To 19-19.5 μl</td>
</tr>
<tr>
<td>Restriction Enzyme*</td>
<td>0.5-1 μl</td>
</tr>
</tbody>
</table>

X, variable volume  *Restriction Enzyme should be added last

2.6.6 Mutagenesis

Phosphomimetic S209D (serine-to-aspartic acid) EIF4E mutant was performed by using QuickChangeII Site-Directed Mutagenesis Kit (Agilent Technologies, Mulgrave, VIC, Australia) and following the manufacturer’s protocol. Briefly, thermal cycling was performed to denature DNA template and anneal the oligonucleotide primers containing the desire mutation. PfuTurbo DNA polymerase was used to extend and incorporate the mutagenic primers resulting in nicked circular strands. The methylated, nonmutated parental DNA template was digested with DpnI. The circular, nicked dsDNA was
transformed into XL1-Blue supercompetent cells. After the transformation, the XL1-Blue supercompetent cells repair the nicks in the mutated plasmid.

2.6.7 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used for visualized PCR and digested products. A 0.75-1.5% agarose gel was used for separating 100bp-10kb DNA. Agarose gels were prepared by mixing agarose powder with 1X Tris Borate EDTA (TBE) (89mM Tris base, 89mM Boric Acid, 2mM EDTA [pH 8.0]) and heated in a microwave. Ethidium Bromide (125pg/ml) was added into the molten agarose, followed by pouring it into a casting chamber and a multi-well comb was placed in the agarose gel. After the gel was set at room temperature, it was then mounted into an electrophoresis apparatus and topped up with 1X TBE. Colourless samples were then mixed with 6X loading dye and loaded into the wells together with the DNA ladders.

2.7 Gene Transfection in Mammalian Cells

2.7.1 Transient Transfection of small interference RNA (siRNA Knockdown)

Transient transfection was performed by using Dharmacon SMARTpool reagent. Melanoma cells were seeded according to Table 2.7 and allowed to reach approximately 50% confluence on the day of transfection. Non-targeting siRNA was transfected as a negative control simultaneously with targeting siRNA constructs (Table 2.8). Cells were transfected with 50-100nM siRNA diluted in Opti-MEM medium using Lipofectamine™ 2000 according to the manufacturer’s transfection protocol. 6 hours post-transfection, transfection medium was replaced with fresh non-antibiotic DMEM with 5% FCS and incubated at 37°C in a humidified atmosphere of 5% CO₂ overnight. The cells were treated with different agents and harvested for further studies. Efficiency of RNA interference (RNAi) was measured by immunoblotting or qPCR.
Table 2.7 Cell Density in Respective Plates for Transfections

<table>
<thead>
<tr>
<th>Transfection Type</th>
<th>Type of Plate</th>
<th>Cell Density</th>
</tr>
</thead>
<tbody>
<tr>
<td>siRNA</td>
<td>96-well</td>
<td>5 x 10^3</td>
</tr>
<tr>
<td>siRNA</td>
<td>24-well</td>
<td>3.5 x 10^4</td>
</tr>
<tr>
<td>siRNA</td>
<td>6-well</td>
<td>1.68 x 10^5</td>
</tr>
<tr>
<td>DNA</td>
<td>24-well</td>
<td>1 x 10^5</td>
</tr>
<tr>
<td>DNA</td>
<td>6-well</td>
<td>4 x 10^5</td>
</tr>
</tbody>
</table>

Table 2.8 List of siRNA Constructs used for Transfection Knock Down

<table>
<thead>
<tr>
<th>siRNA constructs</th>
<th>Catalog Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHOP siGENOME SMARTpool</td>
<td>M-004819-03-0005</td>
</tr>
<tr>
<td>ATF6 siGENOME SMARTpool</td>
<td>M-009917-01-0010</td>
</tr>
<tr>
<td>EIF4E siGENOME Individual 1</td>
<td>D-003884-01-0005</td>
</tr>
<tr>
<td>EIF4E siGENOME Individual 2</td>
<td>D-003884-02-0005</td>
</tr>
<tr>
<td>ERK1 siGENOME SMARTpool</td>
<td>M-003592-03-0005</td>
</tr>
<tr>
<td>ERK2 siGENOME SMARTpool</td>
<td>M-003555-04-0005</td>
</tr>
<tr>
<td>GRP78 siGENOME Individual 1</td>
<td>D-008198-03-0005</td>
</tr>
<tr>
<td>GRP78 siGENOME Individual 2</td>
<td>D-008198-04-0005</td>
</tr>
<tr>
<td>IRE1α siGENOME SMARTpool</td>
<td>M-004951-01-0010</td>
</tr>
<tr>
<td>MEK1 siGENOME SMARTpool</td>
<td>M-003571-01-0010</td>
</tr>
<tr>
<td>XBP1 siGENOME Individual 1</td>
<td>D-009552-01-0002</td>
</tr>
<tr>
<td>XBP1 siGENOME Individual 2</td>
<td>D-009552-02-0002</td>
</tr>
<tr>
<td>non-targeting siRNA SMARTpool</td>
<td>D-001206-13-20</td>
</tr>
</tbody>
</table>

All the siRNA constructs were purchased from Dharmacon (Lafayette, CO, USA).

2.7.2 Transfection of DNA (Over-expression)

2.7.2.1 Transformation

The expression and empty vectors were transformed into DH5α™ competent cells following the manufacturer’s protocol. Briefly, plasmid vectors (Table 2.9) were gently mixed into 50μl of DH5α™ competent cells in Falcon polystyrene tubes and incubated on ice for 30 minutes. pUC19 control DNA provided by the kit was transformed into the competent cells as positive control. The mixture was heat shocked for exactly 20 seconds in a 42°C water bath and immediately placed on ice for 2 minutes. The transformed cells were topped up to 1ml with pre-warmed SOC medium and left in a shaking incubator at 37°C for exactly 1 hour at 225rpm. The cells were then plated on LB agar plates containing appropriate antibiotics and incubated overnight at 37°C.
Table 2.9 List of Plasmid Vectors used for Transfection Over-Expression

<table>
<thead>
<tr>
<th>Plasmid Vector</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCMV6-XL4-BimEL</td>
<td>Origene: Rockville, MD, USA</td>
</tr>
<tr>
<td>pCMV6-AC-CHOP</td>
<td>Origene: Rockville, MD, USA</td>
</tr>
<tr>
<td>pCMV6-AC-PP2A-C</td>
<td>Origene: Rockville, MD, USA</td>
</tr>
<tr>
<td>pCMV6-entry-XBP-1</td>
<td>Origene: Rockville, MD, USA</td>
</tr>
<tr>
<td>pcDNA3.1-GRP78</td>
<td>Supplied by Dr. Amy Lee (Keck School of Medicine of the University of Southern California, Los Angeles, CA)</td>
</tr>
</tbody>
</table>

2.7.2.2 Preparation of Plasmid DNA

A few colonies were picked from the LB agar plates, inoculated into 5ml LB medium with appropriate antibiotics and incubated in a 37°C shaker overnight. 1 ml of transformed cells was transferred into an eppendorf tubes and purified using QIAprep Spin Miniprep kit following the manufacturer’s instructions. The purified plasmid was digested with enzyme and subsequently run by electrophoresis to determine the size of the plasmid. The remaining positive bacteria containing the correct size of plasmid was mixed into 200ml LB medium containing appropriate antibiotics and incubated in a 37°C shaker overnight. Plasmid was isolated by using Hispeed Plasmid Midi Kit following the manufacturer’s instructions. DNA concentrations were determined using Nanodrop 2000 spectrophotometer.

2.7.2.3 Transfection Procedures

Melanoma cells were seeded according to Table 2.7 and allowed to reach approximately 80% confluence on the day of transfection. Cells were transfected with 2μg expression or empty vectors in Opti-MEM medium using Lipofectamine™ 2000 according to the manufacturer’s transfection protocol. Six hours post-transfection, transfection medium was replaced with fresh non-antibiotic DMEM supplemented with 5% FCS and incubated at 37°C in a humidified atmosphere of 5% CO₂ overnight. For transient transfection, the cells were treated with different agents and harvested for further studies.
2.7.2.4 Stable Transfection of short hairpin RNA (shRNA Knockdown)

Melanoma cells were seeded at $1 \times 10^4$ cells/well in 96-well plate and allowed to reach exponential growth overnight. Sigma MISSION® Lentiviral Transduction Particles for shRNA-mediated knockdown (Table 2.10) of a certain gene were applied to approximately 70% confluent cells in the presence of polybrene (4 or 8μg/ml) at MOIs of 0.5, 1 or 5 in 100μl DMEM for 16-24 hours. The culture medium was then replaced and incubated at 37ºC in a humidified atmosphere of 5% CO₂ for another 24 hours. Cells were selected with 2μg/ml puromycin for 3 days until mock-transduced controls (polybrene only) were completely dead. For each transduced melanoma cell line, up to 4 wells of cells per lentiviral clone were tested for knockdown via Western Blot and/or qPCR. Cells with the lowest level of gene and/or protein expression were expanded for experimental use.

<table>
<thead>
<tr>
<th>Lentiviral Transduction Particles</th>
<th>Catalog Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRE1α shRNA</td>
<td>SHCLNV-NM_001433</td>
</tr>
<tr>
<td>ATF6 shRNA</td>
<td>SHCLNV-NM_007348</td>
</tr>
<tr>
<td>eIF2α shRNA</td>
<td>SHCLNV-NM_032025</td>
</tr>
</tbody>
</table>

2.7.3 Inducible Expression System

A lentivirus-based inducible gene expression system was used to conditionally express Bim and CHOP in melanoma cells. Briefly, the system involved coinfection of two lentiviral particles: one encoding the inducible transcriptional activator Gal4 1-147 ER<sup>T2</sup>VP16 (GEV16) cloned into pFU-GEV16-PGK-Hygro containing a Hygromycin-B-resistance gene, and another, Bim or CHOP cDNA cloned into pF-5xUAS-SV40-puro containing a puromycin-resistance gene. Dual antibiotic selection was applied deriving a cell population carrying both GEV16 and Bim or CHOP. Application of low concentration (10nM) of 4-hydroxytamoxifen (4-OHT) drives the expression of Bim or CHOP.
CHAPTER THREE

SUSTAINED IRE1α AND ATF6 SIGNALLING IS IMPORTANT FOR SURVIVAL OF MELANOMA CELLS UNDERGOING ER STRESS
ACKNOWLEDGEMENT OF COLLABORATION

I hereby certify that the work embodied in this Chapter has been done in collaboration with other researchers.

The work in this Chapter was accepted by Cellular Signalling on 7th November 2013. I, Kwang Hong Tay, was the first author of this manuscript, and the work embodied in this chapter was performed by myself, with the exception of the following:

IRE1α, ATF6 and eIF2α stable knockdown cell lines were constructed by Amanda Croft.

The work embodied by these experiments is inextricably tied to the overall findings of the manuscript, and therefore this work will be discussed in Chapter 6 of this thesis.

____________________
Kwang Hong Tay
Sustained IRE1α and ATF6 signalling is important for survival of melanoma cells undergoing ER stress

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Key Words: ERK, Endoplasmic Reticulum Stress, UPR, Melanoma
Chapter Three

Abstract

Apoptosis triggered by endoplasmic reticulum (ER) stress is associated with rapid attenuation of the IRE1α and ATF6 pathways but persistent activation of the PERK branch of the unfolded protein response (UPR) in cells. However, melanoma cells are largely resistant to ER stress-induced apoptosis, suggesting that the kinetics and durations of activation of the UPR pathways are deregulated in melanoma cells undergoing ER stress. We show here that the IRE1α and ATF6 pathways are sustained along with PERK signalling in melanoma cells subjected to pharmacological ER stress, and that this is, at least in part, due to increased activation of the MEK/ERK pathway. In contrast to an initial increase followed by rapid reduction in activation of IRE1α and ATF6 signalling in control cells that were relatively sensitive to ER stress-induced apoptosis, activation of IRE1α and ATF6 by the pharmacological ER stress inducer tunicamycin (TM) or thapsigargin (TG) persisted in melanoma cells. On the other hand, the increase in PERK signalling lasted similarly in both types of cells. Sustained activation of IRE1α and ATF6 signalling played an important role in protecting melanoma cells from ER stress-induced apoptosis, as interruption of IRE1α or ATF6 rendered melanoma cells sensitive to apoptosis induced by TM or TG. Inhibition of MEK partially blocked IRE1α and ATF6 activation, suggesting that MEK/ERK signalling contributed to sustained activation of IRE1α and ATF6. Taken together, these results identify sustained activation of the IRE1α and ATF6 pathways of the UPR driven by the MEK/ERK pathway as an important protective mechanism against ER stress-induced apoptosis in melanoma cells.
Introduction

Several cellular stress conditions, such as nutrient deprivation, hypoxia, alterations in glycosylation status, and disturbances of calcium flux, lead to accumulation and aggregation of unfolded and/or misfolded proteins in the endoplasmic reticulum (ER) lumen and cause so-called ER stress [1-3]. The ER responds to the stress conditions by activation of a range of stress response signalling pathways to alter transcriptional and translational programs, which couples the ER protein folding load with the ER protein folding capacity and is termed the unfolded protein response (UPR) [1-3].

The UPR of mammalian cells is initiated by three ER transmembrane proteins, activating transcription factor 6 (ATF6), inositol-requiring enzyme 1 (IRE1), and double-stranded RNA-activated protein kinase-like ER kinase (PERK), which act as proximal sensors of ER stress. Under unstressed conditions, the luminal domains of these sensors are occupied by the ER chaperone glucose-regulated protein 78 (GRP78) [1-3]. Under ER stress, sequestration of GRP78 by unfolded proteins activates these sensors by inducing phosphorylation and homodimerization of IRE1 and PERK and relocalisation of ATF6 to the Golgi where it is cleaved by site 1 and site 2 proteases (S1P and S2P) leading to its activation as an transcriptional factor [1-3].

The UPR is fundamentally a cytoprotective response, but excessive or prolonged UPR can result in cell death primarily by induction of apoptosis. Although many mechanisms have been demonstrated to contribute to apoptosis induced by ER stress [4-8], the varying durations of activation of the UPR pathways may have an essential role in determining the fate of cells upon ER stress [9]. After initial activation, IRE1α, and to a
lesser extent, ATF6 signalling is rapidly attenuated even in the face of persistence of ER stress [9]. In contrast, activation of the PERK pathway sustains and plays an important role in executing ER stress-induced apoptosis [9]. It seems therefore that initial activation of the IRE1α and ATF pathways of the UPR produces anti-apoptotic outputs that outweigh pro-apoptotic outputs generated by PERK signalling in cells subjected to ER stress [9, 10]. However, attenuation of IRE1α and ATF6 signalling tilts the balance towards induction of apoptosis [9, 11]. Consistent with the role of the PERK pathway in ER stress-induced apoptosis, upregulation of the BH3-only protein BIM by PERK signalling is responsible for apoptosis induced by ER stress in diverse types of cells [12, 13].

Melanoma cells have largely adapted to ER stress and are insensitive to ER stress-induced apoptosis [14-16]. However, the potential role of deregulation of kinetics and durations of activation of the UPR pathways in resistance of melanoma cells to ER stress-induced apoptosis has not been defined. We show in this report that activation of the IRE1α and ATF6 pathways of the UPR is sustained along with PERK signalling in melanoma cells subjected to pharmacological ER stress, and that this plays an important role in protecting melanoma cells from ER stress-induced apoptosis. In addition, we demonstrate that persistent activation of IRE1α and ATF6 signalling is driven, at least in part, by activation of the MEK/ERK pathway.
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Materials and Methods

Cell lines

The human melanoma cell lines Mel-RM, Mel-RMu, Mel-CV and MM200 were obtained as described previously [17, 18]. HEK293 was purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). They were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 5% Fetal Calf Serum (FCS) (Commonwealth Serum Laboratories, Melbourne, Victoria, Australia).

Antibodies, recombinant proteins, and other reagents

The mouse monoclonal antibodies (mAbs) against p-ERK and the rabbit polyclonal antibodies (pAbs) ATF4 and IRE1α were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The rabbit pAb against ERK, eIF2α and p-eIF2α (Serine 51) were purchased from Cell Signaling Technology (Beverly, MA, USA). The rabbit pAbs for p-IRE1α (S724) was purchased from Abcam (Cambridge, MA, USA), and the mouse mAb GAPDH antibody was purchased from Ambion (Austin, TX, USA). The ATF6 mouse mAb was purchased from Imgenex (San Diego, CA, USA). U0126 was purchased from Promega (Madison, WI, USA) and was dissolved at 10mM in DMSO. The β-tubulin mAb, salicylaldehyde, tunicamycin (TM) and thapsigargin (TG) were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia).
Apoptosis

Apoptotic cells were quantified by measurement of sub-G1 DNA content using propidium iodide (PI) on a flow cytometer (Becton Dickinson, Sunnyvale, CA) as described elsewhere [19].

Western blotting

Western blot analysis was carried out as described previously [19]. Labelled bands were detected by Luminata™ Crescendo Western HRP substrate (Millipore, Billerica, MA) and images were captured with ImageReader LAS-4000 (Fujifilm Corporation, Japan).

Quantitative reverse transcription and real-time PCR (qPCR)

Total RNA was isolated using RNeasy mini kit (Qiagen, Australia) following the manufacturer’s instructions. RNA was reverse transcribed into cDNA using Superscript III (Invitrogen, USA) following the manufacturer’s instructions. qPCR was performed using the ABI Prism 7900HT sequence detection system (Applied Biosystems, Australia) with specific-gene primers: Active XBP-1 forward 5’- TGC TGA GTC CGC AGC AGG TGC -3’; Active XBP-1 reverse 5’- GCT TGG CTG ATG ACG TCC CCA C-3’; GRP78 forward 5’- TTG CGC TGT GCT CCT GTG CT -3’; GRP78 reverse 5’- TTC TTG AAC ACG CCG AGC GAG C -3’; β-actin forward, 5’-GTC ACC CAG CAC AAT GAA G-3’; β-actin reverse, 5’-GCC GAT CCA CAC GGA GTA CT-3’). The following PCR cycle was used: Standard Fast Cycle 95°C for 20 seconds, 40 cycles of 95°C for 1 second and 60°C for 20 seconds using Fast SYBR Green mastermix.
(Applied Biosystem, Australia). Cycle threshold ($C_T$) values for specific genes were normalised to the $C_T$ value for the house-keeping gene, $\beta$-actin. The fold changes of mRNA expressed were determined by comparison with $\beta$-actin, where the control sample was arbitrarily designated as 1, and the values of the relative abundance of mRNA of other samples were calculated accordingly. The specificity of the qPCR was controlled using non-template control.

**Small interference RNA**

The siRNA constructs were purchased from Dharmacon (Lafayette, CO, USA). The siRNA constructs used are: IRE1$\alpha$ siGENOME SMARTpool (M-004951-01-0010), ATF6 siGENOME SMARTpool (M-009917-01-0010) and non-targeting siRNA pool (D-001206-13-20) as control. Transfection of siRNA pools was carried out as described previously [20].

**Short hairpin RNA (shRNA) knockdown**

Melanoma cells were seeded at $1 \times 10^4$ cells/well in 96-well plate and allowed to reach exponential growth overnight. Sigma-Aldrich MISSION® Lentiviral Transduction Particles for shRNA-mediated knockdown of IRE1$\alpha$ (SHCLNV-NM_001433), ATF6 (SHCLNV-NM_007348) and eIF2$\alpha$ (SHCLNV-NM_032025) were applied to approximately 70% confluent cells in the presence of polybrene ($8\mu$g/ml) at MOIs of 0.5, 1 or 5 in 100$\mu$l DMEM for 16-24 hours. The culture medium was then replaced and incubated at 37°C in a humidified atmosphere of 5% CO$_2$ for another 24 hours. Cells were selected with 2-10 $\mu$g/ml puromycin every 3 days until mock-transduced controls
(polybren only) were completely dead. For each transduced melanoma cell line, up to 4 wells of cells per lentiviral clone were tested for knockdown via Western Blot and/or qPCR. Cells with the lowest level of gene and/or protein expression were expanded for experimental use.
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Results

Persistent activation of the IRE1α and ATF6 pathways of the UPR in melanoma cells undergoing ER stress

Rapid reduction in activation of IRE1α, and to a lesser extent, ATF6 signalling plays an important role in conferring sensitivity to ER stress-induced apoptosis in sensitive cells such as HEK293 cells [9]. We examined whether resistance of melanoma cells to apoptosis induced by ER stress is associated with deregulation of the IRE1α pathway by comparing the kinetics of XBP-1 activation between melanoma and HEK293 cells. Quantitation of the active (spliced) form of the XBP-1 mRNA by qPCR showed that XBP-1 was rapidly activated in both HEK293 cells and melanoma cells (Mel-RM and MM200 cells) (Fig. 1a & b). Consistent with previous reports, the levels of the spliced XBP-1 mRNA decreased swiftly after the initial increase in HEK293 cells (Fig. 1b) [9]. In contrast, its levels continue to increase in melanoma cells up to 36 hours after treatment (Fig. 1a). In line with these observations, the levels of phosphorylated (activated) IRE1α were reduced after initial elevation in HEK293 cells, but were continuously increased in Mel-RM and MM200 cells (Fig. 1c). Of note, the onset of ER stress-induced phosphorylation of IRE1α appeared slower in melanoma cells than HEK293 cells (Fig. 1c). The difference in the kinetics of IRE1α activation in response to ER stress was also reflected by the active XBP-1 mRNA levels in HEK293 and Mel-RM cells treated with the other pharmacological ER stress inducer TG (Fig. 1d).

We also examined the dynamics of activation of ATF6 in melanoma in comparison with HEK293 cells upon exposure to pharmacological ER stress inducers. Similar to
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Figure 1
Figure 1 - Persistent activation of the IRE1α and ATF6 pathways of the UPR in melanoma cells under ER stress. (A) Total RNA from HEK293 cells with or without treatment with TM (3μM) for indicated periods was subjected to qPCR analysis for the expression of active XBP-1 mRNA. The relative abundance of the active XBP-1 mRNA before treatment was arbitrarily designated as 1. The data shown are the mean ± SE of three individual experiments. (B) Total RNA from Mel-RM and MM200 cells with or without treatment with tunicamycin (TM) (3μM) for indicated periods was subjected to qPCR analysis for the expression of active XBP-1 mRNA. The relative abundance of the active XBP-1 mRNA in each cell line before treatment was arbitrarily designated as 1. The data shown are the mean ± SE of three individual experiments. (C) Whole cell lysates from Mel-RM, MM200 and HEK293 cells with or without treatment with TM (3μM) for indicated periods were subjected to western blot analysis of pIRE1α, IRE1α, ATF6 and β-tubulin (as loading control). The data shown are representative of three individual experiments. (D) Total RNA from Mel-RM and HEK293 cells with or without treatment with thapsigargin (TG) (1μM) for indicated periods was subjected to qPCR analysis for the expression of active XBP-1 mRNA. The relative abundance of the active XBP-1 mRNA in each cell line before treatment was arbitrarily designated as 1. The data shown are the mean ± SE of three individual experiments. (E) Total RNA from Mel-RM, MM200 (upper panel) and HEK293 (lower panel) cells with or without treatment with TM (3μM) for indicated periods was subjected to qPCR analysis for the expression of GRP78 mRNA. The relative abundance of the GRP78 mRNA in each cell line before treatment was arbitrarily designated as 1. The data shown are the mean ± SE of three individual experiments. (F) Total RNA from Mel-RM and HEK293 cells with or without treatment with TG (1μM) for indicated periods was subjected to qPCR analysis for the expression of GRP78 mRNA. The relative abundance of the GRP78 mRNA in each cell line before treatment was arbitrarily designated as 1. The data shown are the mean ± SE of three individual experiments. (G) Whole cell lysates from Mel-RM, MM200 and HEK293 cells with or without treatment with TM (3μM) for indicated periods were subjected to western blot analysis of pPERK, pelF2α, eIF2α, ATF4 and GAPDH (as loading control). The data shown are representative of three individual experiments.
phosphorylation of IRE1α, ER stress-induced cleavage (activation) of ATF6 commenced later in melanoma cells than HEK293 cells (Fig. 1c). However, the increased level of cleaved form of ATF6 was sustained up to 36 hours in Mel-RM and MM200 cells, whereas it reduced rapidly after the initial increase in HEK293 cells (Fig. 1c). By 36 hours, the level of cleaved ATF6 in HEK293 cells was comparable to that before treatment.

The expression of GRP78 was primarily regulated by IRE1α and ATF6 at the transcriptional level [21-23]. We therefore examined whether induction of the GRP78 transcript by ER stress is consistent with the kinetics of activation of these proximal UPR transducers in melanoma cells. Indeed, the GRP78 mRNA was increased shortly after exposure of Mel-RM and MM200 cells to TM, and the elevated levels persisted up to 36 hours (Fig. 1e). In contrast, it declined after an initial increase in HEK293 cells, consistent with previous reports (Fig. 1e) [9]. The different dynamics of ER stress-induced GRP78 transcription was also observed in Mel-RM and HEK293 cells treated with TG (Fig. 1f). Taken together, these results indicate that, in contrast to their attenuation in HEK293 cells, activation of the IRE1α and ATF6 pathways of the UPR is largely sustained in melanoma cells undergoing unmitigated ER stress.

**Activation of the PERK pathway is sustained in melanoma cells undergoing ER stress**

Since the PERK branch of the UPR plays an important role in executing ER stress-induced apoptosis, it remained possible that resistance of melanoma cells to ER stress-induced apoptosis was due to attenuation of PERK signalling in the cells undergoing ER
stress. To test this, we examined the kinetics of activation of the PERK pathway by monitoring accumulation of phosphorylated (activated) PERK and its downstream events, phosphorylation of eIF2α and upregulation of ATF4. Consistent with the previous finding that activation of PERK signalling persists in cells under prolonged ER stress, the increases in phosphorylation of PERK and eIF2α and upregulation of ATF4 lasted up to 36 hours after treatment in both HEK293 and melanoma cells (Fig. 1g). Thus, similar to its persistent activation in HEK293 cells, activation of PERK signalling in melanoma cells with prolonged ER stress is also sustained.

**Persistent activation of the IRE1α and ATF6 pathways is critical for survival of melanoma cells undergoing ER stress**

Having established that activation of the IRE1α and ATF6 pathways is sustained in melanoma cells with unmitigated ER stress, we examined whether this plays a role in protecting melanoma cells from ER stress-induced apoptosis by taking advantage of Mel-RM and MM200 cells with IRE1α or ATF6 stably knockdown by lentiviral transduction of IRE1α or ATF6 shRNA (Fig. 2a) [24]. Both Mel-RM and MM200 cells with IRE1α or ATF6 knocked down displayed marked increase in apoptosis in response to treatment with TM or TG in comparison with those transduced with the control shRNA (Fig. 2b), pointing to a critical role of activation of these pathways in protection of melanoma cells from ER stress. The role of persistence in activation of the IRE1α /XBP-1 axis of the UPR in survival of melanoma cells undergoing ER stress was further confirmed by use of salicylaldehyde that inhibits the IRE1α endonuclease activity thus blocking generation of XBP-1s [25, 26]. As shown in Fig.2c & d, the addition of
salicylaldehyde after induction of ER stress by TM caused increase in apoptosis that was associated with diminution of the cleaved form of XBP-1 mRNA.
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Figure 2
Figure 2 – Persistent activation of the IRE1α and ATF6 pathways is critical for survival of melanoma cells undergoing ER stress. (A) Mel-RM and MM200 melanoma cells were transduced with the control or IRE1α short hairpin RNA (shRNA) or ATF6 shRNA. Whole cell lysates were subjected to western blot analysis of IRE1α (upper panel), ATF6 (lower panel) and GAPDH (as a loading control). The data shown are representative of three individual experiments. (B) Mel-RM and MM200 melanoma cells transduced with control or IRE1α shRNA or ATF6 shRNA were treated with TM (3μM) or TG (1μM) for 48 hours. Cells were subjected to quantitation of apoptotic cell death by measurement of sub-G1 DNA content. The data shown are the mean ± SE of three individual experiments. *p<0.05; two-tailed student’s t test. (C) Mel-RM and MM200 melanoma cells treated with salicylaldehyde (60μM) with or without TM (3μM) pretreatment for 24 hours were subjected to quantitation of apoptotic cell death by measurement of sub-G1 DNA content. The data shown are the mean ± SE of three individual experiments. *p<0.05; two-tailed student’s t test. (D) Total RNA from Mel-RM and MM200 melanoma cells treated with salicylaldehyde (60μM) with or without TM (3μM) pretreatment for 24 hours were subjected to qPCR analysis for the expression of active XBP-1 mRNA. The relative abundance of the active XBP-1 mRNA in each cell line before treatment was arbitrarily designated as 1. The data shown are the mean ± SE of three individual experiments. *p<0.05; two-tailed student’s t test.
PERK signalling kills melanoma cells when the IRE1α or ATF6 pathways is blocked

Although we have previously shown that the PERK pathway is deregulated in melanoma cells, which contributes to resistance of the cells to ER stress-induced apoptosis [13], it is likely that when IRE1α and/or ATF6 pathways were attenuated, pro-apoptosis signalling arising from the PERK pathway may become sufficient to kill melanoma cells undergoing ER stress. To test this, we stably knocked down eIF2α in Mel-RM and MM200 by lentiviral transduction of eIF2α shRNA (Fig. 3a). The addition of salicylaldehyde after induction of ER stress by TM induced apoptosis in Mel-RM and MM200 transduced with the control shRNA, but not in those with eIF2α stably knocked down (Fig. 3b & c), confirming the role of the PERK pathway in ER stress-induced apoptosis in melanoma cells with IRE1α/XBP-1 inhibited. Consistently, while ER stress did not significantly induce apoptosis in Mel-RM and MM200 cells with eIF2α and IRE1α or ATF6 co-knocked down, it killed Mel-RM and MM200 cells carrying the control shRNA with IRE1α or ATF6 knocked down (Fig. 3d & e). Thus, even though the PERK pathway of the UPR is deregulated in melanoma cells, it can induce apoptosis should activation of IRE1α and/or ATF6 signalling be interrupted.
Figure 3
Figure 3 – PERK signalling kills melanoma cells when the IRE1α and ATF6 pathways is blocked. (A) Mel-RM and MM200 melanoma cells were transduced with the control or eIF2α shRNA. Whole cell lysates were subjected to western blot analysis of eIF2α and GAPDH (as a loading control). The data shown are representative of three individual experiments. (B) Control or eIF2α shRNA transduced Mel-RM and MM200 melanoma cells were treated with salicylaldehyde (60μM) with or without TM (3μM) pretreatment for 24 hours. Cells were subjected to quantitation of apoptotic cell death by measurement of sub-G1 DNA content. The data shown are the mean ± SE of three individual experiments. *p<0.05; two-tailed student’s t test. (C) Control or eIF2α shRNA transduced Mel-RM and MM200 melanoma cells were treated with salicylaldehyde (60μM) with or without TM (3μM) pretreatment for 24 hours. Total RNA were subjected to qPCR analysis for the expression of active XBP-1 mRNA. The relative abundance of the active XBP-1 mRNA in each cell line before treatment was arbitrarily designated as 1. The data shown are the mean ± SE of three individual experiments. (D) eIF2α shRNA transduced Mel-RM and MM200 cells were transfected with IRE1α small interference RNA (siRNA), ATF6 siRNA or control siRNA. Whole cell lysates were subjected to western blot analysis of pIRE1α, IRE1α, ATF6 and GAPDH (as a loading control). The data shown are representative of three individual experiments. (E) eIF2α shRNA transduced Mel-RM and MM200 cells were transfected with IRE1α siRNA, ATF6 siRNA or control siRNA with or without TM (3μM) treatment. Cells were subjected to quantitation of apoptotic cell death by measurement of sub-G1 DNA content. The data shown are the mean ± SE of three individual experiments. *p<0.05; two-tailed student’s t test.
Activation of the MEK/ERK pathway is necessary for persistent activation of IRE1α and ATF6 signalling in melanoma cells undergoing ER stress

A characteristic of human melanoma is constitutive activation of the MEK/ERK pathway [27, 28], which plays an important role in adaptation of melanoma cells to ER stress [29-33]. To examine whether activation of the pathway contribute to persistence in activation of the IRE1α and ATF6 pathways of the UPR in melanoma cells undergoing ER stress, we compared the kinetics of activation of IRE1α and ATF6 pathways in Mel-RM and MM200 cells with or without treatment with the MEK inhibitor U0126. In keeping with previous results [22], U0126 sensitized melanoma cells to ER stress-induced apoptosis (Fig. 4a). This was associated with attenuation of ER stress-induced IRE1α phosphorylation, cleavage of XBP-1, cleavage of ATF6, and induction of GRP78 mRNA (Fig. 4b-e). In line with this, concurrent knockdown of ERK1/2 with siRNA similarly reduced the levels of XBP-1s in Mel-RM and MM200 cells upon ER stress (Fig. 4f & g).
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Figure 4
Figure 4 – Activation of the MEK/ERK pathway is necessary for persistent activation of IRE1α and ATF6 signalling in melanoma cells undergoing ER stress. (A) Mel-RM and MM200 cells treated with TM (3μM) or TM plus U0126 (20μM) for indicated periods were subjected to quantitation of apoptotic cell death by measurement of sub-G1 DNA content. The data shown are the mean ± SE of three individual experiments. (B) Whole cell lysates from Mel-RM and MM200 cells treated with TM (3μM) or TM plus U0126 (20μM) for indicated periods were subjected to western blot analysis of pIRE1α, IRE1α, ATF6 and β-tubulin (as a loading control). The data shown are representative of three individual experiments. (C) Total RNA from Mel-RM and MM200 cells treated with TM (3μM) or TM plus U0126 (20μM) for indicated periods were subjected to qPCR analysis for the expression of GRP78 mRNA. The relative abundance of the GRP78 mRNA in each cell line before treatment was arbitrarily designated as 1. The data shown are the mean ± SE of three individual qPCR analyses. *p<0.05; two-tailed student’s t test. (D) Total RNA from Mel-RM and MM200 cells treated with TM (3μM) or TM plus U0126 (20μM) for indicated periods were subjected to qPCR analysis for the expression of XBP-1s mRNA. The relative abundance of the active XBP-1 mRNA in each cell line before treatment was arbitrarily designated as 1. The data shown are the mean ± SE of three individual qPCR analyses. *p<0.05; two-tailed student’s t test. (E) Whole cell lysates from Mel-RM and MM200 cells treated with TM (3μM) or TM plus U0126 (20μM) for indicated periods were subjected to western blot analysis of pERK1/2, ERK1/2, and GAPDH (as a loading control). The data shown are representative of three individual experiments. (F) Total RNA from Mel-RM and MM200 cells transfected with control or ERK1 siRNA plus ERK2 siRNA were treated with TM (3μM) for indicated periods were subjected to qPCR analysis for the expression of XBP-1s mRNA. The relative abundance of active XBP-1 mRNA in each cell lines transfected with the control siRNA and before treatment was arbitrarily designated as 1. The data shown are the mean ± SE of three individual qPCR analyses. *p<0.05; two-tailed student’s t test. (G) Whole cell lysates from Mel-RM and MM200 cells transfected with control or ERK1/2 siRNA were treated with TM (3μM) for indicated periods were subjected to western blot analysis of pERK1/2, ERK1/2, and GAPDH (as a loading control). The data shown are representative of three individual experiments.
**Discussion**

In this report, we have provided evidence that, in contrast to their attenuation with prolonged ER stress in other types of cells, the IRE1α and ATF6 pathways of the UPR persist in melanoma cells undergoing ER stress, which plays an important role in resistance of melanoma cells to ER stress-induced apoptosis. When IRE1α or ATF6 signalling is interrupted, ER stress induces apoptosis through the PERK pathway, although its potential to induce apoptosis is known to be weakened in melanoma cells [13]. In addition, our results show that sustained activation of IRE1α and ATF6 signalling in melanoma cells undergoing ER stress is, at least in part, due to activation of the MEK/ERK pathway.

Although the UPR is fundamentally a cyto-protective response, excessive and/or prolonged ER stress kills cells predominantly by induction of apoptosis [4, 5, 32, 34]. This switch from the cyto-protective to pro-apoptotic output of the UPR has been shown in various types of cells to be caused by attenuation of the IRE1α, and to a lesser extent, ATF6, pathways, whereas the PERK branch of the UPR sustains with unmitigated ER stress and is responsible for ER stress-induced apoptosis [9, 11]. However, melanoma cells have largely adapted to ER stress and are not sensitive to apoptosis induced by pharmacological ER stress inducers [14, 15, 22, 35, 36]. Although a number of mechanisms have been shown to contribute to survival of melanoma cells under ER stress [15, 22, 37], we found in this study that resistance of melanoma cells to ER stress-induced apoptosis was associated with persistent activation of the IRE1α and ATF6 pathways along with PERK signalling. This was demonstrated by sustained presence of phosphorylated IRE1α and cleaved ATF6, activation of XBP-1, and induction of...
GRP78. In contrast, activation of IRE1α and ATF6 signalling decreased rapidly after initial elevation in HEK293 cells that were known to be sensitive to ER stress-induced apoptosis [9], suggesting that sustained activation of the IRE1α and ATF6 pathways by ER stress is specific to melanoma cells.

The IRE1α and ATF6 pathways of the UPR play predominantly pro-survival roles in cells undergoing ER stress [9, 11, 38]. Consistent with this, our results showed that knockdown of IRE1α or ATF6 by siRNA sensitized melanoma cells to ER stress-induced apoptosis. Importantly, selective disruption of the IRE1α/XBP-1 axis of the UPR by the specific inhibitor salicylaldehyde after its activation induced apoptosis of melanoma cells, indicating the critical role of continuation in activation of IRE1α signalling in survival of melanoma cells undergoing prolonged ER stress. Indeed, IRE1α signalling is known to mediate activation of the PI3K/Akt pathway and is also responsible for upregulation of Mcl-1 that are critical for survival of melanoma cells upon ER stress [24]. ATF6 transcriptionally regulates GRP78 along with XBP-1 that also play important roles in resistance of melanoma cells to ER stress-induced apoptosis [16, 30].

Activation of Bim downstream of PERK signalling is responsible for induction of apoptosis by ER stress in diverse types of cells [12, 13]. However, Bim is rapidly reduced after initial upregulation by increased activation of ERK as a consequence of reduction in the activity of PP2A, a protein phosphatase that can dephosphorylate (inactivate) ERK, in melanoma cells submitted to ER stress, indicating that the apoptosis inducing potential of the PERK pathway in ER stress-induced apoptosis of melanoma cells is compromised [13]. Nevertheless, when IRE1α or ATF6 was knocked
down or IRE1α signalling was disrupted by salicylaldehyde, ER stress induced apoptosis in melanoma cells through the PERK pathway, as it failed to induce apoptosis in melanoma cells with co-knockdown of eIF2α, a key downstream target of PERK [11]. These results emphasize the importance of cooperation between sustained activation of IRE1α and ATF6 pathways and compromised PERK signalling in protection of melanoma cells from ER stress-induced apoptosis. However, should IRE1α and/or ATF6 signalling be attenuated, even the reduced pro-apoptosis output resulting from the PERK pathway is able to trigger apoptosis in melanoma cells.

An important finding of this study is that the activity of MEK/ERK signalling is required for sustained activation of the IRE1α and ATF6 pathway in melanoma cells undergoing ER stress. This was demonstrated by attenuation of ER stress-induced IRE1α phosphorylation, cleavage of XBP-1, cleavage of ATF6, and induction of GRP78 mRNA in melanoma cells treated with the MEK inhibitor U0126 or with ERK1/2 knocked down by siRNA. Consistent with this, MEK/ERK signalling is known to be required for maximal upregulation of GRP78 in melanoma cells undergoing ER stress [22, 39, 40]. How the MEK/ERK pathway interacts with IRE1α and ATF6 signalling in melanoma cells submitted pharmacological ER stress requires further investigation. Nevertheless, it is known that constitutive activation of the MEK/ERK pathway that was found in the vast majority of melanomas triggers chronic ER stress as manifested by activation of the IRE1α and ATF6 pathway by enhancing nascent protein synthesis [25]. On the other hand, acute ER stress induces further activation of MEK/ERK that plays an important role in protection of melanoma cells from ER stress-induced apoptosis [13, 22, 25].
Conclusion

Our results showing that sustained activation of the IRE1α and ATF6 pathways of the UPR is critical for survival of melanoma cells undergoing prolonged ER stress indicate that persistent activation of the pathways is an important adaptive mechanism to ER stress in melanoma cells, and suggest that interruption of IRE1α and ATF6 signalling may be useful in combination with drugs that induce ER stress to kill melanoma cells. Furthermore, the observation that the MEK/ERK pathway is required for sustained activation of IRE1α and ATF6 in melanoma cells under ER stress reinforces the importance of inhibition of the pathway in the treatment of melanoma.
References


CHAPTER FOUR

ONCOGENIC ACTIVATION OF MEK/ERK PRIMES MELANOMA CELLS FOR ADAPTATION TO ENDOPLASMIC RETICULUM STRESS
ACKNOWLEDGEMENT OF COLLABORATION

I hereby certify that the work embodied in this Chapter has been done in collaboration with other researchers.

The work in this Chapter was accepted by Journal of Investigative Dermatology on 7th July 2013. I, Kwang Hong Tay, was the co-first author of this manuscript, and the work embodied in this chapter was performed by myself, with the exception of the following:

Figure 1, Figure 2 and Figure 4 were performed by myself and Amanda Croft.
Figure 6 was performed by myself with assistance from Hsin-Yi Tseng.
Supplementary Figure 3, 8 and 9 were performed by Amanda Croft.
Western Blotting was performed by myself with assistance from Hsin-Yi Tseng.

The work embodied by these experiments is inextricably tied to the overall findings of the manuscript, and therefore this work will be discussed in Chapter 6 of this thesis.

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Kwang Hong Tay
Chapter Four

Oncogenic Activation of MEK/ERK Primes Melanoma Cells for Adaptation to Endoplasmic Reticulum Stress

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Cancer cells commonly undergo chronic endoplasmic reticulum (ER) stress, to which the cells have to adapt for survival and proliferation. We report here that in melanoma cells intrinsic activation of the ER stress response/unfolded protein response (UPR) is, at least in part, caused by increased outputs of protein synthesis driven by oncogenic activation of mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK/ERK) and promotes proliferation and protects against apoptosis induced by acute ER stress. Inhibition of oncogenic BRAFV600E or MEK-attenuated activation of inositol-requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6) signaling of the UPR in melanoma cells. This was associated with decreased phosphorylation of eukaryotic translation initiation factor 4E (eIF4E) and nascent protein synthesis and was recapitulated by knockdown of eIF4E. In line with this, introduction of BRAFV600E into melanocytes led to increases in eIF4E phosphorylation and protein production and triggered activation of the UPR. Similar to knockdown of glucose-regulated protein 78 (GRP78), inhibition of XBP1 decelerated melanoma cell proliferation and enhanced apoptosis induced by the pharmacological ER stress inducers tunicamycin and thapsigargin. Collectively, these results reveal that potentiation of adaptation to chronic ER stress is another mechanism by which oncogenic activation of the MEK/ERK pathway promotes the pathogenesis of melanoma.

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INTRODUCTION

The endoplasmic reticulum (ER) responds to ER stress that is characterized by accumulation and aggregation of unfolded and/or misfolded proteins in the ER lumen by activation of a range of signaling pathways to alter transcriptional and translational programs (Harding et al., 2002; Schroder and Kaufman, 2005; Walter and Ron, 2011; Wang and Kaufman, 2012). This couples the ER protein folding load with the ER protein folding capacity and is termed the ER stress response or unfolded protein response (UPR) (Harding et al., 2002; Schroder and Kaufman, 2005; Walter and Ron, 2011; Wang and Kaufman, 2012). The UPR of mammalian cells is initiated by three ER transmembrane proteins, activating transcription factor 6 (ATF6), inositol-requiring enzyme 1 (IRE1), and double-stranded RNA-activated protein kinase-like ER kinase (PERK) (Harding et al., 2002; Schroder and Kaufman, 2005; Walter and Ron, 2011; Wang and Kaufman, 2012), which are anchored by the ER chaperon glucose-regulated protein 78 (GRP78) at their luminal domains, but upon ER stress, sequestration of GRP78 by unfolded proteins causes the activation through inducing phosphorylation and homodimerization of IRE1 and PERK and relocation of ATFE6 to the Golgi where it is cleaved and activated as a transcriptional factor (Harding et al., 2002; Schroder and Kaufman, 2005; Walter and Ron, 2011; Wang and Kaufman, 2012). The UPR is fundamentally a cytoprotective response, but excessive or prolonged UPR can trigger cell death predominantly by induction of apoptosis (Xu et al., 2005; Boyce and Yuan, 2006). This is associated with attenuation of IRE1 and ATF6 activities, whereas PERK signaling that is essential for activation of pro-apoptotic proteins such as CHOP and Bim is maintained (Puthalakath et al., 2007; Tabas and Ron, 2011; Zhong et al., 2011). The UPR is often constitutively activated in cancer cells, indicative of adaptation to ER stress (Ma and Hendershot, 2004; Lee, 2007; Jiang et al., 2009b, 2009c; Wang et al., 2010). Indeed, cells in a developing solid cancer may undergo hypoxia, nutrient starvation, and acidosis, thus resulting in ER stress (Ma and Hendershot, 2004; Lee, 2007; Wang et al., 2010).
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2010). Moreover, increased synthesis of proteins that are often in mutated forms required to sustain malignancy of cancer cells may also directly uncouple the ER protein folding load with the ER protein folding capacity (Ma and Hendershot, 2004; Lee, 2007).

The rate of protein synthesis is primarily controlled at the stage of mRNA translation initiation (Billigines and Stokoe, 2007; Silvera et al., 2010; Bitterman and Polunovsky, 2012; Grzmił and Hemmings, 2012). Initiation of translation of most mRNAs is mediated by the cap-dependent mechanism, which is governed by the eukaryotic initiation factor 4F (eIF-4F) complex that consists of the cap-binding protein eIF-4E, the RNA helicase eIF-4A, and the scaffolding protein eIF-4G (Billigines and Stokoe, 2007; Silvera et al., 2010; Bitterman and Polunovsky, 2012; Grzmił and Hemmings, 2012). Among them, eIF-4E is the rate-limiting factor (Sonenberg and Hinnebusch, 2009; Jackson et al., 2010; Silvera et al., 2010). Although the activity of eIF-4E is negatively regulated by eIF-4E binding proteins (eIF-4BP), it is enhanced by phosphorylation at serine 209 by mTOR kinase signal-integrating kinase 1 (mSINT1) and mSINT2 upon binding to eIF-4G (Phillips and Blaydes, 2005; Sonenberg and Hinnebusch, 2009; Jackson et al., 2010; Silvera et al., 2010; Hou et al., 2012).

A characteristic of human melanoma is constitutive activation of the MEK/ERK (mitogen-activated protein kinase kinase/extracellular signal-regulated kinase) pathway (Davies et al., 2002; Platt et al., 2008). This stems primarily from oncogenic mutations of BRAF, with the most common mutation being a glutamic acid for valine substitution at position 600 (BRAF<sup>V600E</sup>) (Davies et al., 2002; Platt et al., 2008). Mutations in N-RAS, H-RAS, c-Kit, ERBB4, or the G-protein α-subunit GNAQ are also responsible in subsets of melanomas (Flaherty et al., 2010). Activation of MEK/ERK is critical for melanoma cell survival under acute, pharmacological ER stress (Jiang et al., 2007; Hersey and Zhang, 2008), but its potential role in adaptation of melanoma cells to chronic, constitutive ER stress remains to be determined. It is known that MEK/ERK signaling promotes protein synthesis (Billigines and Stokoe, 2007; Silvera et al., 2010; Bitterman and Polunovsky, 2012; Grzmił and Hemmings, 2012), which may contribute to increased ER protein folding load in cells with MEK/ERK constitutively activated. However, it was recently reported that the RAB11FIP2 FKBP12-induced ER stress, implicating that activation of MEK/ERK may alleviate ER stress in BRAF<sup>V600E</sup> melanoma cells (Beck et al., 2013).

In this study, we have examined the potential interaction between the constitutively activated MEK/ERK pathway and the UPR in melanoma cells. We show here that MEK/ERK signaling is necessary and sufficient for intrinsic activation of the UPR as a consequence of ER stress triggered by enhanced protein synthesis, which in turn promotes proliferation and protects against apoptosis induced by acute ER stress in melanoma cells. These results indicate that potentiation of adaptation to chronic ER stress is another mechanism by which activation of the MEK/ERK pathway promotes the pathogenesis of melanoma.

RESULTS AND DISCUSSION

The mutant BRAF inhibitor PLX4720 attenuates activation of the IRE1 and ATF6 branches of the UPR in BRAF<sup>V600E</sup> melanoma cells

Post studies have shown that activation of MEK/ERK is required for induction of high levels of UPR and contributes to resistance of melanoma cells to acute, pharmacological ER stress (Jiang et al., 2007; Hersey and Zhang, 2008). To study whether activation of MEK/ERK similarly contributes to chronic, constitutive activation of the UPR in melanoma cells, we tested the effect of the mutant BRAF inhibitor PLX4720 on the expression of the active (spliced) form of XBP1 (XBP1s), mRNA and GRP78, two commonly used indicators of activation of the IRE1 and ATF6 branches of the UPR (Ma and Hendershot, 2004; Lee, 2007), in melanoma cell lines. Strikingly, while XBP1s and GRP78 were downregulated in Mel-RMu and MM200 cells harboring BRAF<sup>V600E</sup>, they were both increased in Mel-RM cells carrying wild-type BRAF, by PLX4720 (figure 1a-d). Moreover, PLX4720 triggered decreases in phosphorylated (activated) IRE1α and cleaved (activated) ATF6 in Mel-RMu and MM200 cells (figure 1e and f), but caused, albeit moderately, increases in activated IRE1α and ATF6 in Mel-RM cells (Supplementary Figure S1a online). These contrasting effects of PLX4720 were associated with its different impacts on activation of ERK, which was, as anticipated, inhibited in BRAF<sup>V600E</sup>, but enhanced in wild-type BRAF, melanoma cells (Supplementary Figure S1b online) (Katzavasilis et al., 2010). PLX4720 neither reduced the levels of phosphorylated eIF-2α and ATF4, downstream targets of PERK signaling, in Mel-RMu and MM200 cells, nor did it increase their expression in Mel-RM cells, suggesting that it preferentially impinges on activation of IRE1 and ATF6 signaling (Supplementary Figure S2 online).

The inhibitory effect of PLX4720 on activation of IRE1 and ATF6 signaling was confirmed in another 4 BRAF<sup>V600E</sup> cell lines as shown by downregulation of XPB1s and GRP78 (Supplementary Figure S3 online).

In contrast to inhibition of IRE1α and ATF6 signaling by PLX4720, the mutant BRAF inhibitor vemurafenib, a close relative of PLX4720 (Tsi et al., 2008), has recently been reported to induce ER stress in BRAF<sup>V600E</sup> melanoma cells (Beck et al., 2013). To clarify this paradox, we treated Mel-RMu, MM200, and Mel-RM cells with vemurafenib. Indeed, vemurafenib upregulated XBP1s and GRP78 in Mel-RMu and MM200 cells, but strikingly, also caused upregulation of XPB1s and GRP78 in Mel-RM cells (Supplementary Figure S4a-d online). Nonetheless, vemurafenib inhibited ERK activation in Mel-RMu and MM200 cells and enhanced activation of ERK in Mel-RM cells, recapitulating the different effects of PLX4720 on ERK activation in BRAF<sup>V600E</sup> and wild-type BRAF melanoma cells (Supplementary Figure S4e and f online).

These results suggest that induction of ER stress by vemurafenib may be dissociated with its effects on activation of MEK/ERK signaling.

Inhibition of MEK or knockdown of ERK1/2 blocks IRE1 and ATF6 signaling in BRAF<sup>V600E</sup> and wild-type BRAF melanoma cells

To confirm that the contrasting effects of PLX4720 on activation of IRE1 and ATF6 signaling are due to its different effects
on activation of MEK/ERK in BRAF\textsuperscript{V600E} and wild-type BRAF melanoma cells, we treated Mel-RMu and MM200 (BRAF\textsuperscript{V600E}) and Mel-RM (wild-type BRAF) cells with the MEK inhibitor U0126. Inhibition of MEK downregulated XBP1s and GRP78 not only in BRAF\textsuperscript{V600E} but also in wild-type BRAF melanoma cells (Figure 2a and b and Supplementary Figure S5 online). In line with this, concurrent knockdown of ERK1/2 with small interfering RNA (siRNA) reduced the levels of XBP1s and GRP78 in both the Mel-RMu and Mel-RM cells (Figure 2c–e).

We also examined the effect of the other mutant BRAF inhibitor dabrafenib on XBP1s and GRP78 in melanoma cells. Dabrafenib downregulated XBP1s and GRP78 and inhibited ERK activation in MM200 but upregulated XBP1s and GRP78 and enhanced ERK activation in Mel-RM cells, recapitulating the contrasting effects of PLX4720 on activation of IRE1 and ATF6 signaling in BRAF\textsuperscript{V600E} and wild-type BRAF melanoma cells (Supplementary Figures S6a–c and S7a–c online). In contrast, the BRAF inhibitor CEP32496 that inhibits both BRAF\textsuperscript{V600E} and wild-type BRAF reduced XBP1s and GRP78 and inhibited ERK activation in both the MM200 and Mel-RM cells (Supplementary Figures S6a–c and S7a–c online) (James et al., 2012). Taken together, the above results suggest that constitutive activation of MEK/ERK signaling triggers ER stress in melanoma cells and that induction of ER stress by PLX4720 and dabrafenib in wild-type BRAF melanoma cells is associated with enhanced activation of ERK rather than caused by off-target effects of the inhibitors.

**Activation of MEK/ERK signaling is critical for sustaining de novo protein production in melanoma cells**

Activation of the MEK/ERK pathway promotes protein synthesis that is often enhanced in cancer cells and may represent an underlying mechanism of chronic ER stress by uncoupling the ER protein folding load with the ER protein folding capacity (De Benedetti and Graff, 2004; Silvera et al., 2010). Indeed, the magnitude of nascent protein production was significantly higher in melanoma cells than melanocytes (Figure 3a), which was, however, reduced by U0126 in both the BRAF\textsuperscript{V600E} (Mel-RMu and MM200) and wild-type BRAF (Mel-RM) melanoma cells (Figure 3b). Similarly, PLX4720 reduced protein synthesis in Mel-RMu and MM200 cells but decreased the synthesis in Mel-RM cells (Figure 3c). Thus, constitutive activation of MEK/ERK has an important role in maintaining high levels of protein production in melanoma cells. Notably, neither U0126 nor PLX4720 inhibits protein synthesis in melanocytes (Figure 3b and c).

MEK/ERK-mediated activation of protein synthesis involves phosphorylation of the translation initiation eIF-4E (Phillips and Blaydes, 2006; Hou et al., 2012). Consistent with this, the basal levels of phosphorylated eIF-4E were generally higher in melanoma cells than melanocytes (Supplementary Figure S8 online), which were rapidly reduced by PLX4720 in BRAF\textsuperscript{V600E}, and by U0126 in both the BRAF\textsuperscript{V600E} and wild-type BRAF, melanoma cells (Figure 3d–g), suggesting that the increased protein production in melanoma cells is coupled with phosphorylation of eIF-4E mediated by MEK/ERK.
MEK/ERK signaling triggers chronic ER stress through increased protein production in melanoma cells

To examine whether increased protein synthesis mediated by the MEK/ERK pathway causes ER stress, we treated MM200 and Mel-RM cells with the small molecule 4EGI-1, an inhibitor of elf4E (Moeke et al., 2007; Fan et al., 2010). 4EGI-1 at a concentration (10 μM) that did not induce noticeable cell death caused partial, but significant, inhibition of protein synthesis (Figure 4a). This was associated with down-regulation of XBP1s and GRP78 (Figure 4b and c), recapitulating, at least in part, the effects of inhibition of MEK/ERK on protein synthesis and activation of the UPR (Figures 1–3). In addition, 4EGI-1 abolished enhancement in protein synthesis and upregulation of XBP1s and GRP78 triggered by PLX4720 in Mel-RM cells (Supplementary Figure S5a–c online). These results suggest that MEK/ERK-induced activation of the UPR is mediated by elf4E.

We confirmed the role of enhanced protein synthesis in MEK/ERK-induced ER stress by knocking down elf4E with siRNA (Figure 4d), which significantly inhibited de novo protein synthesis and reduced XBP1s and GRP78 transcript expression in MM200 and Mel-RM cells (Figure 4e and f, and Supplementary Figure S5a online), in line with the role of elf4E and increased protein synthesis in induction of ER stress. In support of this, introduction of a construct expressing the phosphomimetic S209D (serine-to-aspartic acid) elf4E mutant into Mel-RM cells caused an increase in nascent protein synthesis and GRP78 and XBP1s expression and abolished reduction in protein synthesis and inhibition of XBP1s and GRP78 induced by U0126 (Figure 4g–i and Supplementary Figure S10b online).

Oncogenic BRAF activates elf4E and the UPR in melanocytes

To further confirm that activation of MEK/ERK signaling induces ER stress in melanocytic cells, we infected HEM-1435 human melanocytes with a lentiviral construct expressing BRAFV600E. Enforced expression of BRAFV600E caused activation of ERK, which was associated with induction of phosphorylation of elf4E (Figure 5a and b), increased protein synthesis, albeit moderately, and activation of IRE1 and ATF6 signaling of the UPR (Figure 5c–e). In contrast, overexpression of wild-type BRAF did not increase ERK activation, consistent
with previous reports (Scurr et al., 2010), nor did it cause activation of IRE1 and ATF6 signaling (Supplementary Figure S11 online). Intriguingly, the expression levels of phosphorylated eIF2α and ATF4 were also increased moderately by ectopic expression of BRAFV600E in melanocytes (Figure 5a). Regardless, these data not only confirm the role of activation of MEK/ERK in causing chronic ER stress but also suggest that occurrence of ER stress is an early event in the pathogenesis of melanoma. Indeed, BRAFV600E is found in the majority of neo- 
(Pollock et al., 2003; Michaloglou et al., 2005; Taube et al., 2009), and the UPR is activated at early stages of melanoma initiation by HRASG12V (Denoyelle et al., 2006). Interestingly, oncogenic BRAF induces senescence in melanocytes (Dhomen et al., 2009; Scurr et al., 2010), but once the senescence barrier is overcome, it drives melanomagenesis (Dhomen et al., 2009; Scurre, 2010). By analogy, induction of ER stress by oncogenic activation of MEK/ERK signaling in melanocytic cells may also set a barrier for melanoma initiation. It is conceivable that only those cells that can survive chronic ER stress triggered by MEK/ERK may acquire malignant phenotypes.

**XBP1 and GRP78 promote proliferation of melanoma cells**

We examined the functional consequence of constitutive activation of IRE1 and ATF6 signaling in melanoma cells. Although siRNA knockdown of XBP1 or GRP78 did not trigger noticeable cell death, it inhibited proliferation in MM200 and Mel-RM cells (Figure 6a and b, and Supplementary Figure S12 online). However, knockdown of XBP1 or GRP78 did not impact on inhibition of
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Figure 4. Activation of mitogen-activated protein kinase kinase/extracellular signal–regulated kinase (MEK/ERK) triggers endoplasmic reticulum stress in melanoma cells. a) Click-IT protein synthesis assay showing magnitude of caspase protein synthesis in cells treated with or without 4EG-1 (10 µM) for 12 h (n = 3), mean ± SEM; Student’s t-test; *P < 0.05. b, c) Quantitative PCR (qPCR) analysis of XBP1s (b) and glucose-regulated protein 78 (GRP78) (c) mRNA expression after 4EG-1 (10 µM) treatment (n = 3), mean ± SEM; Student’s t-test; *P < 0.05. d, g) Western blot analysis for total lysates transduced with eukaryotic initiation factor 4E (eIF4E) small interfering RNA (siRNA) (d) and phosphomimetic S209D eIF4E mutant constructs (g) (n = 3). e, f) qPCR analysis showing XBP1s (e) and GRP78 (f) mRNA expression after eIF4E knockdown (n = 3), mean ± SEM; Student’s t-test; *P < 0.05. h, i) qPCR analysis showing XBP1s (h) and GRP78 (i) mRNA expression in eIF4E-S209D transduced cells with or without U0126 (20 µM) (n = 3), mean ± SEM; Student’s t-test; *P < 0.05. MESO, dimethyl sulfoxide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Proliferation caused by PLX4720 in MM200 cells or by U0126 in MM200 and Mel-RM cells (Figure 6c and d), in line with downregulation of XBP1 and GRP78 by inhibition of MEK/ERK signaling (Figures 1a–d and 2a–c). These results suggest that XBP1 and GRP78 activated by MEK/ERK signaling primarily affect proliferation of melanoma cells under steady-state conditions. Notably, both PLX4720 and U0126 primarily exert inhibitory effects on cell proliferation at the concentrations (3 and 20 µM, respectively) used in this study, although they can induce cell death when used at higher concentrations in a proportion of melanoma cell lines (Wang et al., 2007; Jiang et al., 2011). The effect of XBP1 on melanoma cell proliferation was also shown by treatment of MM200 and Mel-RM cells with salicylaldehyde that inhibits the IRE1 endonuclease activity, thus blocking generation of XBP1s (Figure 6c) (Volkmann et al., 2011). Intriguingly, overexpression of XBP1 or GRP78 did not protect sensitive melanoma cells from PLX4720- or U0126-induced inhibition of proliferation (Supplementary Figures S13 and S14 online), suggesting that XBP1 or GRP78 at levels above those driven by MEK/ERK signaling does not provide further proliferative advantage to melanoma cells under steady-state conditions.

Activation of IRE1/XBP1 signaling contributes to survival of melanoma cells undergoing acute ER stress

Induction of ER stress that potentially leads to cell death by MEK/ERK signaling seems paradoxical, as its activation has been well established to be protective against cellular stress in melanoma cells (Hersey et al., 2006; Jiang et al., 2007). Nevertheless, MEK/ERK-mediated induction of GRP78 has an important role in protecting melanoma cells from apoptosis induced by pharmacological ER stress (Jiang et al., 2007). Similarly, IRE1/XBP1 signaling also contributed to survival of melanoma cells undergoing pharmacological ER stress, in that treatment with salicylaldehyde or siRNA knockdown of XBP1 enhanced apoptosis of MM200 and Mel-RM cells in response to the ER stress inducers tunicamycin or thapsigargin (Supplementary Figures S15a–c online). Therefore, chronic activation of IRE1 and ATF6 signaling not only promotes

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melanoma cell proliferation but also protects melanoma cells from apoptosis undergoing acute ER stress, which conceivably has a role in resistance of melanoma cells to therapeutic agents that induce ER stress (Jiang et al., 2009a).

As downregulation of protein phosphatase 2 (PP2A) activity is an important protective mechanism against pharmacological ER stress in melanoma cells (Tay et al., 2012), we examined whether constitutive activation of MEK/ERK signaling that causes chronic ER stress has a role in regulation of PP2A activity. PLX4720 did not impinge on the phosphatase activity of PP2A in MM200 cells (Supplementary Figure S16a online), indicating that relief of melanoma cells from MEK/ERK-triggered chronic ER stress is unable to enhance PP2A activity conceivably due to attenuation of feedback regulation of PP2A by ERK (Garcia et al., 2002; Letourneau et al., 2006). Nevertheless, it reduced PP2A activity in Mel-RM cells (Supplementary Figure S16b online),

**Figure 5.** Oncogenic BRAF activates eukaryotic initiation factor 4E (eIF4E) and the unfolded protein response in melanocytes. (a) Whole-cell lysates of HEMI-455 melanocytes transduced with pCDH-empty vector or pCDH-BRAFv600E-myc were subjected to western blot analysis (n = 3). (b) Quantitation of p-eIF4E as shown in a by normalizing to total eIF4E (n = 3, mean ± SEM; Student's t-test; *P < 0.05). (c) Reverse transcription-PCR analysis showing that transfection of HEMI-455 with either pCDH-empty vector or pCDH-BRAFv600E-myc alters the expression of XBP1s mRNA (n = 3). (d) Click-iT protein synthesis assay showing magnitude of nascent protein synthesis in HEMI-455 transduced with either pCDH-empty vector or pCDH-BRAFv600E-myc (n = 3). (e) Quantitative PCR analysis showing changes of glucose-regulated protein 78 (GRP78) mRNA in HEMI-455 transduced with either pCDH-empty vector or pCDH-BRAFv600E-myc (n = 3). (f) Akt, activating transcription factor 6; DSSO, dimethyl sulfoxide; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

**Figure 6.** XBP1 and glucose-regulated protein 78 (GRP78) promote proliferation of melanoma cells. (a) (Left) Whole-cell lysates from cells transfected with the control or GRP78 small interfering RNA (siRNA) were subjected to western blot analysis (n = 3). (Right) Analysis of bromodeoxyuridine (BrdU) incorporation in cells transfected with the control or GRP78 siRNA. (b) (Left) Quantitative PCR (qPCR) analysis validating XBP1s knockdown efficiency. (Right) Analysis of BrdU incorporation in cells transfected with the control or XBP1s siRNA. (c, d) Analysis of BrdU incorporation in (c) MM200 and (d) Mel-RM cells transfected with the control, GRP78 (left) or XBP1s (right) siRNA followed by PLX4720 (3 μM) or U0126 (20 μM) treatment for 48 h (n = 3, mean ± SEM; Student’s t-test; *P < 0.05). (e) (Left) qPCR analysis of XBP1s and (right) analysis of BrdU incorporation in cells treated with or without sodium selenite (60 μM), DSSO, dimethyl sulfoxide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
consistent with downregulation of PP2A activity by induction of ER stress (Tay et al., 2012). It seems likely that while acute ER stress downregulates PP2A activity, constitutive activation of MEK/ERK has a role in keeping PP2A activity in check at a minimal level. PP2A is known to have a tumor-suppressive role in many types of cancer (Janssens et al., 2005; Kalev and Sablina, 2011).

Introduction of exogenous oncocogenic BRAF has been reported to inhibit melanoma cell growth by inducing autophagy (Maddodi et al., 2010). However, the mechanisms involved remain unknown. Our results suggest that this may be associated with induction of ER stress by activation of MEK/ERK signaling, in that ER stress is known to trigger autophagy, which is nevertheless believed to have a protective role in cells undergoing ER stress (Li et al., 2008). As melanoma cells carrying endogenous oncogenic BRAF are additive to its signaling for survival and growth (Hoeflich et al., 2006), it is unlikely that autophagy, if any, resulting from constitutive chronic ER stress is pro-proliferative in BRAFV600E melanoma cell growth.

Although we have clearly demonstrated that constitutive activation of MEK/ERK signaling is not only a mechanism of adaptation to ER stress but also a source of chronic ER stress in melanoma cells, it remains puzzling how MEK/ERK signaling spares the PERK branch, the main pathway that mediates ER stress-induced apoptosis (Xu et al., 2005; Boyce and Yuan, 2006; Puthalakath et al., 2007). It is believed that there is no ER stress inducer that can selectively elicit any particular signaling pathway of the UPR (Lin et al., 2007; Walter and Ron, 2011). Indeed, activation of MEK/ERK signaling by ectopic expression of BRAFV600E resulted in activation of all the three branches of the UPR in melanocytes. As IRE1 and ATF6 activities are progressively attenuated, whereas PERK signaling is maintained and thus triggers apoptosis in many types of cells undergoing prolonged ER stress (Lin et al., 2007), it is possible that activation of MEK/ERK exerts a role in sustaining IRE1 and ATF6 activities, rather than selectively activating the pathways in melanoma cells. Also, while induction of ER stress by MEK/ERK signaling is resolved, other elements such as increased glycolysis and cellular acidosis are still able to trigger ER stress in melanoma cells (Ma and Hendrix, 2004; Liu et al., 2009; Zhuang et al., 2010). Under such conditions, IRE1 and ATF6 activities are attenuated with diminishing of MEK/ERK signaling, whereas persistent ER stress continues driving activation of the PERK pathway.

Another paradox that needs to be clarified is how vemurafenib induces ER stress in both the BRAFV600E and wild-type melanoma cells, although our results indicate that the ability of vemurafenib to induce ER stress in melanoma cells may be independent of its effect on activation of MEK/ERK signaling. This is of particular importance, as it suggests that the therapeutic efficacy of this clinically available BRAF inhibitor may be determined not only by its inhibitory effect on MEK/ERK but also by its ability to induce ER stress. Further studies using animal models and melanoma samples from patients before and after treatment with the inhibitor are clearly warranted.

MATERIALS AND METHODS

Cell lines

Human melanoma cell lines Mel-RM4, MeWo-300, Mel-CV, IgRo2, A2058, and S4Mel-28 (BRAFV600E); and Mel-RM, Mel-JD, ME4505, and ME1007 (BRAFV600T) and human melanocyte lines HEMm-AP and HEMm-AP were obtained and cultured as described previously (Jiang et al., 2010; Ye et al., 2013).

Antibodies and other reagents

Antibodies and reagents used are listed in Supplementary Table S1 and S2 online.

Apoptosis

Quantification of apoptotic cells was carried out by measurement of sub-G1 DNA content as described elsewhere (Jiang et al., 2011).

Western blot analysis

Western blot analysis was carried out as described previously (Jiang et al., 2011). Labeled bands were detected by Luminata Crescendo Western HRP substrate (Millipore, Billerica, MA). Images were captured, and the intensity of the bands was quantitated with ImageReader LAS-4000 (FujiFilm, Tokyo, Japan).

Detection of spliced XBP1 mRNA (XBP1s)

Detection of XBP1s was carried out as previously described (Jiang et al., 2008).

Lentiviral gene transduction and DNA constructs

The lentiviral vector pCDH-CMV-MCS-EF1-copGFP containing Myc-tagged BRAFV600E or BRAFV600T was used to produce lentivirus and to transduce human melanocytes as described previously (Haferkamp et al., 2009). Overexpression of BRAFV600E and BRAFV600T was confirmed by western blot using Myc tag and BRAF antibody, respectively.

siRNA knockdown

Transfection of siRNA siGENOME SMARTpools (Supplementary Table S1 online) was carried out as described previously (Jiang et al., 2011).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at https://www.nature.com/jid

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Supplementary Figure 1: a, PLX4720 increases activated IRE1α and ATF6 in Mel-RM (wild-type BRAF) melanoma cells. Whole cell lysates from Mel-RM with or without PLX4720 (3μM) treatment for indicated periods were subjected to Western blot analysis of phosphorylated IRE1α (pIRE1α), IRE1α, ATF6 and GAPDH (as a loading control). b, Differential effects of PLX4720 on ERK activation in Mel-RMu and MM200 (BRAFV600E) and Mel-RM (wild-type BRAF) melanoma cells. Whole cell lysates from Mel-RMu, MM200, and Mel-RM cells with or without PLX4720 (3μM) treatment for indicated periods were subjected to Western blot analysis of phosphorylated ERK (pERK), ERK, and GAPDH (as a loading control). The data shown are representative of three individual experiments.
Supplementary Figure 2: PLX4720 does not reduce activation of the PERK branch of the UPR. Whole cell lysates from Mel-RMu and MM200 with or without treatment with PLX4720 (3μM) for indicated periods were subjected to Western blot analysis of phosphorylated eIF2α (p-eIF2α), eIF2α (a) or ATF4 (b) and GAPDH (as a loading control). The data shown are representative of three individual experiments.
**Supplementary Figure 3**: Inhibition of mutant BRAF downregulates XBP1s and GRP78 in BRAF\(^{V_{600E}}\) melanoma cells.  

**a.** RT-PCR products of XBP-1 mRNA and β-actin mRNA (as a control) from total RNA extracts of Mel-CV, IgR3, A2058, and Sk-Mel-28 cells with or without treatment with PLX4720 (3μM) for indicated periods were digested with Apa-LI for 90 min followed by electrophoresis. The longer fragment derived from the active form of XBP1 mRNA and two shorter bands derived from the inactive form are indicated. The data shown are representative of three individual experiments.  

**b.** Total RNA from Mel-CV, IgR3, A2058, and Sk-Mel-28 cells with or without PLX4720 (3μM) treatment for 24 hours was subjected to qPCR analysis for the expression of GRP78 mRNA. The relative abundance of the GRP78 mRNA in each cell line before treatment was arbitrarily designated as 1. The data shown are the mean ± SEM of three individual experiments. *p<0.05; two-tailed student's t test.
Supplementary Figure 4: The differential effects of vemurafenib (PLX4032) and PLX4720 in BRAF<sup>V600E</sup> melanoma cells. **a** and **b**, Total RNA from MM200 and Mel-RMu cells treated with or without PLX4720 (3 μM) or PLX4032 (6 μM) for 16 hours was subjected to qPCR analysis for the expression of XBP1s (a) or GRP78 (b) mRNA. The relative abundance of the XBP1s (a) or GRP78 (b) mRNA before treatment was arbitrarily designated as 1. The data shown are the mean ± SEM of three individual experiments. *p<0.05, two-tailed student’s t test. **c** and **d**, Total RNA from Mel-RM cells with or without PLX4032 (6 μM) treatment for 16 hours was subjected to qPCR analysis for the expression of XBP1s (c) or GRP78 (d) mRNA. The relative abundance of the XBP1s (c) or GRP78 (d) mRNA before treatment was arbitrarily designated as 1. The data shown are the mean ± SEM of three individual experiments. *p<0.05, two-tailed student’s t test. **e**, Whole cell lysates from Mel-RMu and MM200 cells with or without PLX4032 (6 μM) treatment for indicated periods were subjected to Western blot analysis of phosphorylated ERK (pERK), ERK and GAPDH (as a loading control). **f**, Whole cell lysates from Mel-RM cells with or without PLX4032 (6 μM) treatment for indicated periods were subjected to Western blot analysis of phosphorylated ERK (pERK), ERK and GAPDH (as a loading control). The data shown are representative of three individual experiments.
Supplementary Figure 5: U0126 inhibits ERK activation in both BRAF^{V600E} and wild-type BRAF melanoma cells. Whole cell lysates from Mel-RMu, MM200, and Mel-RM cells with or without treatment with U0126 (20μM) for indicated periods were subjected to Western blot analysis of phosphorylated ERK (pERK), ERK, and GAPDH (as a loading control). The data shown are representative of three individual experiments.
Supplementary Figure 6: Inhibition of the mutant BRAF by CEP32496 and Dabrafenib in MM200 cells reduced XBP1 and GRP78 mRNA. a and b, MM200 cells treated with the indicated inhibitors, CEP32496 (10μM) and Dabrafenib (10nM), for 24 hours were subjected to qPCR analysis of the expression of XBP1 (a) and GRP78 (b) mRNA. The relative abundance of the XBP1 mRNA cells before treatment was arbitrarily designated as 1. The data shown are the mean ± SEM of three individual experiments. *p<0.05; two-tailed student's t test. c, Whole cell lysates from MM200 cells with or without indicated treatment for 6 hours were subjected to Western blot analysis of phosphorylated ERK (pERK) and ERK. The data shown are representative of three individual experiments.
Supplementary Figure 7: The effects of the mutant BRAF inhibitor, Dabrafenib, and the BRAF inhibitor, CEP32496, on the expressions of XBP1s and GRP78 in Mel-RM (wild-type BRAF) melanoma cells. a and b, Mel-RM cells treated with the indicated inhibitors, CEP32496 (10μM) and Dabrafenib (10nM), for 24 hours were subjected to qPCR analysis of the expression of XBP1 (a) and GRP78 (b) mRNA. The relative abundance of the XBP1 mRNA cells before treatment was arbitrarily designated as 1. The data shown are the mean ± SEM of three individual experiments. *p<0.05; two-tailed student’s t test. c, Whole cell lysates from Mel-RM cells with or without indicated treatment for 6 hours were subjected to Western blot analysis of phosphorylated ERK (pERK) and ERK. The data shown are representative of three individual experiments.
Supplementary Figure 8: eIF4E is expressed and phosphorylated at higher levels in melanoma cells than melanocytes. a, Whole cell lysates from the melanocyte lines and melanoma cell lines as indicated were subjected to Western blot analysis of phosphorylated eIF4E (p-eIF4E), eIF4E, and GAPDH (as a loading control). The data shown are representative of three individual experiments. b, Quantitation of levels of p-eIF4E in cells as shown in (a) that were normalized to those of total eIF4E. The data shown are the mean ± SEM of three individual experiments. *p<0.05; two-tailed student's t test.
Supplementary Figure 9: 4EGI-1 abolished enhancement in protein synthesis and upregulation of XBP1s and GRP78 triggered by PLX4720 in Mel-RM cells. a, Mel-RM cells treated with PLX4720 (3μM) for 24 hours with or without pretreatment with 4EGI-1 (10μM) for 1 hour were subjected to measurement of protein synthesis by the Click-iT® protein synthesis assay kit. The relative magnitude of nascent protein synthesis in cells before treatment was arbitrarily designated as 1. The data shown are the mean ± SEM of three individual experiments. b, Mel-RM cells treated with PLX4720 (3μM) for 24 hours with or without pretreatment with 4EGI-1 (10μM) for 1 hour were subjected to qPCR analysis of the expression of XBP1s mRNA. The relative abundance of the XBP1 mRNA cells before treatment was arbitrarily designated as 1. The data shown are the mean ± SEM of three individual experiments. c, Mel-RM cells treated with PLX4720 (3μM) for 24 hours with or without pretreatment with 4EGI-1 (10μM) for 1 hour were subjected to qPCR analysis of the expression of GRP78 mRNA. The relative abundance of the XBP1 mRNA cells before treatment was arbitrarily designated as 1. The data shown are the mean ± SEM of three individual experiments. *p<0.05; two-tailed student’s t test.
Supplementary Figure 10: a, Knockdown of eIF4E (as shown in Figure 4d) inhibits protein synthesis in melanoma cells. MM200 and Mel-RM cells were transfected with the control or eIF4E siRNA. Twenty-four hours later, cells were subjected to measurement of protein synthesis by the Click-it® protein synthesis assay kit. The relative magnitude of nascent protein synthesis in each cell line transfected with the control siRNA was arbitrarily designated as 1. The data shown are the mean ± SEM of three individual experiments. *p<0.05; two-tailed student’s t test. b, Expression of the phosphomimetic S209D eIF4E (as shown in Figure 4G) reverses inhibition of protein synthesis by the MEK inhibitor U0126. Mel-RM cells were transfected empty vector-myc or phosphomimetic S209D eIF4E mutant constructs. Twenty-four hours later, cells were treated with U0126 (20μM) for 12 hours before subjecting to measurement of protein synthesis by the Click-it® protein synthesis assay kit. The relative magnitude of nascent protein synthesis in cells transfected with empty vector-myc was arbitrarily designated as 1. The data shown are the mean ± SEM of three individual experiments.
Supplementary Figure 11: Overexpression of wild-type BRAF did not increase ERK activation nor did it cause activation in IRE1 and ATF6 signaling. HEM1455 human melanocytes were transduced with either pCDH-GFP-empty vector alone or pCDH-GFP-BRAF wild-type. Forty-eight hours later, a. Whole cell lysates were subjected to Western blot analysis of BRAF, phosphorylated ERK (p-ERK), ERK, phosphorylated eIF4E (p-eIF4E), eIF4E, phosphorylated eIF2α (p-eIF2α), eIF2α, ATF4 and GAPDH (as a loading control). The data shown are representative of three Western blot analyses; b and c. Total RNA was subjected to qPCR analysis of XBP1s (b) or GRP78 (c) expression. The relative abundance of the XBP1s (b) or GRP78 (c) mRNA cells before treatment was arbitrarily designated as 1. The data shown are the mean ± SEM of three individual experiments.
Supplementary Figure 12: Inhibition of GRP78 and XBP1 by siRNA does not induce significant cell death. **a**, MM200 and Mel-RM cells were transfected with either control or GRP78 siRNA. Forty-eight hours later, cells were subjected to quantitation of cell death by measurement of sub-G1 DNA content. **b**, MM200 and Mel-RM cells were transfected with either control or GRP78 siRNA. Forty-eight hours later, cells were subjected to quantitation of cell death by measurement of sub-G1 DNA content. The data shown are the mean ± SEM of three individual experiments.
**Supplementary Figure 13**: Overexpression of XBP1 did not protect MM200 from PLX4720 or U0126-induced inhibition of proliferation nor did it protect Mel-RM from U0126-induced inhibition of proliferation. MM200 and Mel-RM cells were transfected with either pCMV6-entry or pCMV6-entry-XBP1 vector. Twenty-four hours later, **a**, Whole cell lysates were subjected to Western blot analysis of the myc-tag and GAPDH (as a loading control). The data shown are representative of three Western blot analyses; **b** and **c**, MM200 and Mel-RM cells were further treated with PLX4720 (3µM) or U0126 (20µM) as indicated for 48 hours and subjected to BrdU incorporation analysis. The data shown are the mean ± SEM of three individual experiments.
Supplementary Figure 14: Overexpression of GRP78 did not protect MM200 from PLX4720- or U0126-induced inhibition of proliferation nor did it protect Mel-RM from U0126-induced inhibition of proliferation. MM200 and Mel-RM cells were transfected with either pcDNA3.1 or pcDNA3.1-GRP78 vector. Twenty-four hours later, a, Whole cell lysates were subjected to Western blot analysis of GRP78 and GAPDH (as a loading control). The data shown are representative of three Western blot analyses; b and c, MM200 and Mel-RM cells were further treated with PLX4720 (3μM) or U0126 (20μM) as indicated for 48 hours and subjected to BrdU incorporation analysis. The data shown are the mean ± SEM of three individual experiments.
Supplementary Figure 15: Inhibition of XBP1 enhanced apoptosis of melanoma cells in response to the ER stress inducers tunicamycin (TM) or thapsigargin (TG). a, MM200 and Mel-RM cells treated with TM (3μM) or TG (1μM) for 48 hours with or without salicylaldehyde (60μM) pretreatment for 4 hours were subjected to quantitation of apoptotic cell death by measurement of sub-G1 DNA content. The data shown are the mean ± SEM of three individual experiments. b, MM200 and Mel-RM cells were transfected with either control siRNA or XBP1 siRNA. Twenty-four hours later, whole cell lysates were subjected to Western blot analysis of XBP1 and GAPDH (as a loading control). The data shown are representative of three Western blot analyses. c, MM200 and Mel-RM cells were transfected with either control siRNA or XBP1 siRNA. Twenty-four hours later, cells were treated with TM (3μM) or TG (1μM) for 48 hours with or without salicylaldehyde (60μM) pretreatment for 4 hours followed by quantitation of apoptotic cell death by measurement of sub-G1 DNA content. The data shown are the mean ± SEM of three individual experiments.
Supplementary Figure 16: The relationship between chronic ER stress caused by constitutive activation of MEK/ERK signaling and PP2A activity. a, MM200 and b, Mel-RM cells treated with or without PLX4720 (3µM) treatment for indicated periods were subjected to PP2A activity assay. The data shown are the mean ± SEM of three individual experiments. *p<0.05; two-tailed student’s t test.
CHAPTER FIVE

SUPPRESSION OF PP2A IS CRITICAL FOR PROTECTION OF MELANOMA CELLS UPON ENDOPLASMIC RETICULUM STRESS
ACKNOWLEDGEMENT OF COLLABORATION

I hereby certify that the work embodied in this Chapter has been done in collaboration with other researchers.

The work in this Chapter was accepted by Cell Death & Disease on 24th May 2012. I, Kwang Hong Tay, was the first author of this manuscript, and the work embodied in this chapter was performed by myself, with the exception of the following:

Inducible cell lines were constructed by Rick Francis Throne.
Western Blotting was performed by myself with assistance from Hsin-Yi Tseng.

The work embodied by these experiments is inextricably tied to the overall findings of the manuscript, and therefore this work will be discussed in Chapter 6 of this thesis.

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Kwang Hong Tay
Suppression of PP2A is critical for protection of melanoma cells upon endoplasmic reticulum stress

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Endoplasmic reticulum (ER) stress triggers apoptosis by activating Bim in diverse types of cells, which involves dephosphorylation of BimEL by protein phosphatase 2A (PP2A). However, melanoma cells are largely resistant to ER stress-induced apoptosis, suggesting that Bim activation is suppressed in melanoma cells undergoing ER stress. We show here that ER stress reduces PP2A activity leading to increased ERK activation and subsequent phosphorylation and proteasomal degradation of BimEL. Despite sustained upregulation of Bim at the transcriputional level, the BimEL protein expression was downregulated after an initial increase in melanoma cells subjected to pharmacological ER stress. This was mediated by increased activity of ERK, whereas the phosphatase activity of PP2A was reduced by ER stress in melanoma cells. The increase in ERK activation was, at least in part, due to reduced dephosphorylation by PP2A, which was associated with downregulation of the PP2A catalytic C subunit. Notably, instead of direct dephosphorylation of BimEL, PP2A inhibited its phosphorylation indirectly through dephosphorylation of ERK in melanoma cells. Taken together, these results identify downregulation of PP2A activity as an important protective mechanism of melanoma cells against ER stress-induced apoptosis.

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A number of cellular stress conditions, such as nutrient deprivation, hypoxia, alterations in glycosylation status, and disturbances of calcium flux, trigger accumulation and aggregation of unfolded and/or misfolded proteins in the endoplasmic reticulum (ER) lumen leading to so-called ER stress.1,2 The ER responds to the stress conditions by activation of a range of signalling pathways to either transcriptional and translational programs, which couples the ER protein folding load with the ER protein folding capacity and is termed the ER stress response or the unfolded protein response (UPR).1,3 The UPR is fundamentally a cytoprotective response, but excessive or prolonged UPR resulting from irrevocable ER stress can cause apoptosis.1,4 This involves many of the same molecules that have important roles in other apoptotic cascades.3-5 Among them, the BH3-only protein Bim appears of particular importance because it mediates ER stress-induced apoptosis in diverse types of cells.5

Activation of Bim in cells undergoing ER stress is regulated by both transcriptional and post-translational mechanisms.6 Transcriptional upregulation of Bim by ER stress is mediated by the transcription factor CHOP and its cofactor C/EBPβ that form heterodimers, which activate a non-conventional promoter within the first intron of the bim gene.6 At the post-translational level, ER stress triggers protein phosphatase 2A (PP2A)-mediated dephosphorylation of Bim, in particular, the most abundant Bim isomorph, BimEL, which prevents its ubiquitination and proteasomal degradation, thus leading to its increase in expression.6 BimEL is known to subject to phosphorylation by the MEK/ERK pathway that targets it for degradation.7,8 It can also be phosphorylated by JNK resulting in its dissociation from the dynein light chain of the microtubule and induction of apoptosis.7

PP2A represents a family of serine/threonine phosphatases that regulate numerous intracellular signaling cascades.9,10 Typically, PP2A contains a highly active core dimer composed of a catalytic C subunit (PP2A-C) and a structural A subunit (PP2A-A) that recruits one of multiple regulatory B subunits (PP2A-B) to form the PP2A hetrotrimeric complex.9,10 The substrate specificity of PP2A is determined by the B subunit in the complex, whereas the dynamic exchange of B subunits in the complex is regulated by reversible methylation and phosphorylation of the C-terminal tail of PP2A-C.9,10 Noticeably, there is increasing evidence showing that PP2A has an important tumor-suppressive role, and various PP2A subunits has also been reported to be downregulated in a number of cancers including melanoma.11,12,13 Most cultured human melanoma cell lines are not sensitive to apoptosis induced by pharmacological ER stress inducers,14,15 suggesting that melanoma cells have largely

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Keywords: ER stress; PP2A; MEK/ERK; Bim; melanoma

Abbreviations: ER, endoplasmic reticulum; OA, oleic acid; 4-OH, 4-hydroxytestosterone; PP2A, protein phosphatase 2A; PP2A-A, PP2A catalytic A subunit; PP2A-B, PP2A regulatory B subunit; PP2A-C, PP2A catalytic C subunit; TGF, transforming growth factor; TRM, taxol-resistant melanoma; UPR, unfolded protein response

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adapted to ER stress conditions. In support, the UPR is constitutively activated in melanoma cells in vivo and in vitro.\textsuperscript{10,17} Although the adaptive mechanism(s) developed by melanoma cells are not fully understood, we have previously found that upregulation of the anti-apoptotic protein McI-1 is critical for survival of melanoma cells upon ER stress.\textsuperscript{14} When McI-1 is inhibited, ER stress induces apoptosis by activation of the BH3-only proteins Noxa and PUMA.\textsuperscript{14} In contrast, Bim does not have a role in ER stress-induced apoptosis of melanoma cells deficient in McI-1.\textsuperscript{14} However, the mechanism by which Bim is inhibited in melanoma cells undergoing ER stress remains undefined.

We show in this report that the Binm\textsubscript{ERL} protein expression is rapidly downregulated after an initial increase, despite sustained upregulation of the Binm transcript, in melanoma cells submitted to ER stress, and that the decrease in Binm\textsubscript{ERL} is due to reduction in PPIA activity leading to increased activation of ERK and subsequent phosphorylation and proteasomal degradation of the protein. In addition, we demonstrate that, instead of directly dephosphorylating Binm, PPIA reduces Binm phosphorylation indirectly by dephosphorylation of ERK in melanoma cells.

Results

ER stress does not induce sustained upregulation of Binm\textsubscript{ERL} in melanoma cells. ER stress triggers apoptosis through activation of Binm in diverse types of cells,\textsuperscript{5} but treatment with the ER stress inducer tunicamycin (TM) induced only a moderate, transient increase in the Binm\textsubscript{ERL} protein expression, which was followed by a decrease in Mel-RM cells (Figure 1a). By 36h after treatment, the Binm\textsubscript{ERL} protein level was even lower than that in untreated cells (Figure 1a). The decrease in the Binm\textsubscript{ERL} expression at relatively late stages (24 and 36h) after exposure to TM was also observed in another 3 melanoma cell lines (Figure 1b). In contrast, TM triggered sustained upregulation of Binm in MCF-7 breast cancer cells (Figure 1a).\textsuperscript{5} Consistent with previous reports,\textsuperscript{6,14,15} melanoma cells were relatively resistant (<20% apoptotic cells at 48h after treatment), whereas MCF-7 cells were sensitive to TM-induced apoptosis (Figure 1c).

Downregulation of the Binm\textsubscript{ERL} protein after its initial increase by ER stress in melanoma cells was further confirmed with another ER stress inducer thapsigargin (TG) (Figure 1d). The ER stress-induced changes in the Binm\textsubscript{ERL} expression in melanoma cells appeared specific because, as reported before,\textsuperscript{14} ER stress triggered sustained upregulation of the related Bcl-2 family proteins, McI-1 and PUMA, and caused no change in the expression of Bcl and Bcl-X\textsubscript{L} in melanoma cells (Supplementary Figure 1). Induction of ER stress by TM and TG was corroborated by upregulation of GRP78, CHOP, and the active form of the XBP1 mRNA (Figure 1e and Supplementary Figure 2).

Suppression of Binm has an important role in resistance of melanoma cells to ER stress-induced apoptosis. Having established that ER stress does not induce sustained upregulation of Binm\textsubscript{ERL} in melanoma cells, we examined whether this has a role in protecting melanoma cells from ER stress-induced apoptosis. To this end, we used a lentivirus-based inducible gene expression system to establish a Mel-RM sub-line (Mel-RM-Bim) to overexpress Binm\textsubscript{ERL} conditionally in response to 4-hydroxytestosterone (4-OHT) (Figure 2a).\textsuperscript{17,18} Induction of Binm\textsubscript{ERL} by 4-OHT triggered apoptosis in Mel-RM-Bim cells (Figure 2b), which was markedly enhanced by cotreatment with TM (Figures 2a and b), suggesting that melanoma cells are sensitive to ER stress-induced apoptosis provided Binm is expressed at relatively high levels. The effect of overexpression of Binm\textsubscript{ERL} on sensitivity of melanoma cells to TM-induced apoptosis was confirmed in another two melanoma lines transiently transfected with a construct expressing Binm\textsubscript{ERL} (Figures 2c and d).

ER stress induces transcriptional upregulation of Binm in melanoma cells. Like in other cell types,\textsuperscript{6} ER stress induced sustained upregulation of the Binm transcript in Mel-RM cells (Figure 3a). This upregulation was due to a transcriptional increase mediated by the transcription factor CHOP,\textsuperscript{17} as small interference RNA (siRNA) knockdown of CHOP markedly inhibited the increase in Binm mRNA levels, partially recapitulating the effect of the transcription inhibitor actinomycin D (Figures 3b and c). Upregulation of Binm mRNA by ER stress was confirmed in another three melanoma cell lines (Supplementary Figure 3).

To examine whether disassociation between the Binm protein expression and its transcriptional upregulation is specific to melanoma cells under ER stress, we established a Mel-RM sub-line (Mel-RM-CHOP) that expressed CHOP conditionally in response to 4-OHT (Figure 3d). Induction of CHOP resulted in persistent increases in Binm at both the mRNA and protein levels and caused apoptosis (Figures 3d and e), suggesting that downregulation of the Binm protein after its initial upregulation despite its sustained transcriptional increase mediated by CHOP is specific to melanoma cells undergoing ER stress.

The decrease in Binm in melanoma cells undergoing ER stress is due to proteasomal degradation mediated by MEK/ERK signaling. Treatment with the proteasome inhibitor MG132 inhibited downregulation of the Binm\textsubscript{ERL} protein in Mel-RM cells exposed to TM (Figure 4a), indicating that its decrease in melanoma cells under ER stress is associated with proteasomal degradation. In support, protein half-life analysis showed that the Binm\textsubscript{ERL} turnover rate was more rapid in melanoma cells treated with TM compared with those without exposure to TM (Figure 4b), in contrast to the increased Binm\textsubscript{ERL} half-life time in MCF-7 cells undergoing ER stress (Supplementary Figure 4).\textsuperscript{16} Similarly, the Binm\textsubscript{ERL} protein underwent a retarded mobility shift (slower migration in SDS-PAGE), consistent with increased phosphorylation of Binm\textsubscript{ERL},\textsuperscript{7,8,9} in melanoma cells during ER stress (Figures 1a, b and d), whereas it underwent an opposite pattern of mobility shift (faster migration in SDS-PAGE) (Figure 1a), consistent with its dephosphorylation,\textsuperscript{6} in MCF-7 cells. The increase in Binm\textsubscript{ERL} phosphorylation in melanoma cells undergoing ER stress was confirmed using an antibody that specifically recognizes Binm\textsubscript{ERL} phosphorylated at Ser69 (Figures 4c and d). Consistently, although there was an
increase in ubiquitination of Bim in melanoma cells after treatment with TM, Bim ubiquitination was reduced in MCF-7 cells undergoing ER stress as reported by others (Figure 4e). As MEK/ERK activity can phosphorylate Bim EL, leading to its ubiquitination and proteasomal degradation, we examined whether this is involved in Bim EL phosphorylation in melanoma cells upon ER stress. Treatment with the MEK inhibitor U0126 upregulated Bim EL in melanoma cells exposed to TM, which was associated with reduction in its phosphorylation (Figure 4f), indicating that MEK/ERK signaling has an important role in phosphorylation of Bim EL and downregulation of its expression. Notably, phosphorylation of ERK was increased, albeit moderately, by induction of ER stress (Figure 4f and Supplementary Figure 5). The effect of MEK/ERK signaling on phosphorylation of Bim in melanoma cells under ER stress was further confirmed by knockdown of MEK1 (Figure 4g). Moreover, the effect was similarly observed in additional two melanoma lines treated with U0126 plus TM (Supplementary Figure 6).

ER stress reduces the phosphatase activity of PP2A in melanoma cells. The PP2A is responsible for dephosphorylation of Bim and contributes to its upregulation in many
types of cells undergoing ER stress,\textsuperscript{8} indeed, treatment of Mel-RM cells with the pharmacological PP2A activator, FTY720 (fingolimod),\textsuperscript{9} resulted in upregulation of Bim\textsubscript{EL} in melanoma cells undergoing ER stress (Figure 5a and Supplementary Figure 7), whereas treatment with okadaic acid (OA) at a concentration that specifically inhibits PP2A led to increased phosphorylation of Bim\textsubscript{EL} (Figures 5b and c),\textsuperscript{9} indicating that PP2A has the similar regulatory effect on the Bim\textsubscript{EL} expression in melanoma cells as in other cell types.\textsuperscript{8} Nevertheless, phosphorylation of Bim was increased in melanoma cells undergoing ER stress (Figures 1a, b, d and 4c), suggesting that PP2A activity is repressed by ER stress in melanoma cells. This was confirmed by reduction in the phosphatase activity of PP2A in melanoma cells after treatment with TM, which was in contrast to the moderate increase in PP2A activity in MCF-7 cells induced by the same treatment (Figure 5d).

We monitored the expression of the catalytic C subunit of PP2A in Mel-RM in comparison with MCF-7 cells undergoing ER stress. Strikingly, PP2A-C was progressively downregulated in Mel-RM cells, whereas it was markedly increased in MCF-7 cells, after treatment with TM (Figure 5e). Downregulation of PP2A-C was also observed in another two melanoma lines (Figure 5e). The role of downregulation of PP2A-C in reduction of PP2A activity in melanoma cells under ER stress was confirmed by transfection of a PP2A-C expressing construct into Mel-RM cells, which showed that overexpression of PP2A-C caused an increase in PP2A activity and upregulation of Bim\textsubscript{EL} in cells with or without treatment with TM, and enhanced ER stress-induced apoptosis (Figures 5f and g, and Supplementary Figure 8).

**Dethosphorylation of ERK is necessary for PP2A-mediated inhibition of Bim\textsubscript{EL} phosphorylation in melanoma cells.** Interestingly, treatment with FTY720 or overexpression of PP2A-C downregulated ERK phosphorylation, whereas exposure to OA caused an increase in the phosphorylated ERK (pERK) levels (Figures 5a, b and f), suggesting that PP2A has a role in dephosphorylating ERK in melanoma cells. In contrast, neither FTY720 nor OA caused noticeable changes in the ERK phosphorylation status in MCF-7 cells (Figures 5a and b). These results raised a possibility that dephosphorylation of ERK may have an indirect role in PP2A-mediated inhibition of Bim\textsubscript{EL} phosphorylation in melanoma cells,\textsuperscript{6,8} even though it is known that PP2A directly targets Bim\textsubscript{EL} for dephosphorylation in MCF-7 cells and other types of cells.\textsuperscript{6}

Although the role of MEK/ERK signaling in phosphorylation of Bim\textsubscript{EL} in melanoma cells under ER stress had been established by inhibition of MEK with U0126 and siRNA
PP2A is physically associated with ERK but not Bim in melanoma cells. PP2A exerts its phosphatase effect by physical interaction with its substrates.19,21 We therefore examined the potential physical association of PP2A with ERK and Bim in melanoma cells in comparison with MCF-7 cells. Strikingly, although Bim in MCF-7 cells was readily co-precipitated with PP2A-C in protein extracts from MCF-7 cells under ER stress, no Bim in melanoma cells was detected in PP2A-C precipitates from Mel-RM cells either with or without treatment with OA. In contrast, little ERK was co-precipitated with PP2A in MCF-7 cells, but it was readily co-precipitated with PP2A in Mel-RM cells with or without undergoing ER stress (Figure 6d). These results further suggest that, in contrast to dephosphorylating Bim directly, PP2A inhibits phosphorylation of Bim indirectly through dephosphorylating ERK in melanoma cells.

Discussion
Past studies have shown that upregulation of the anti-apoptotic protein Mcl-1 is critical for survival of melanoma...
Suppression of PPI2a in melanoma under ER stress

(H1 Tay et al)

Chapter Five

Figure 4  Downregulation of the Bim<sub>EL</sub> protein in melanoma cells undergoing ER stress is mediated by MEK/ERK signaling. (a) The proteasome inhibitor MG132 reverses downregulation of the Bim<sub>EL</sub> in melanoma cells under ER stress. Mel-RM cells with or without pretreatment with the proteasome inhibitor MG132 (10 μM) for 1 h were treated with TM (3 μM) for 24 h. Thirty microgram of total protein of whole-cell lysates were then subjected to western blot analysis of Bim and GAPDH (as a loading control). The data shown are representative of three individual experiments. (b) ER stress accelerates the turnover rate of Bim<sub>EL</sub> in melanoma cells. Upper panel: a Mel-RM cell line treated with the proteasome synthesis inhibitor cycloheximide (CHX) (10 μg/ml) with or without the addition of TM (3 μM) for indicated periods. Thirty microgram of total protein of whole-cell lysates were then subjected to western blot analysis of Bim and GAPDH (as a loading control). The data shown are representative of three individual experiments. Lower panel: quantitative expression levels of Bim<sub>EL</sub> as shown in the upper panel that were normalized to GAPDH. Quantitation of each band was determined using ImageJ software. Mel-RM and MCF-7 (upper panel) and Igf3, Sk-Me-2b, and MM200 (lower panel) cell lines were treated with TM (3 μM) for indicated periods. Thirty microgram of total protein of whole-cell lysates were then subjected to western blot analysis using an antibody that specifically recognizes Bim<sub>EL</sub> phosphorylated at Ser99. Western blot analysis of GAPDH was included as a loading control. The data are representative of three individual experiments. (c) TM induces phosphorylation of Bim<sub>EL</sub> in melanoma cells. Mel-RM and MCF-7 cells were treated with TG (1 μM) for indicated periods. Thirty microgram of total protein of whole-cell lysates were then subjected to western blot analysis using an antibody that specifically recognizes Bim<sub>EL</sub> phosphorylated at Ser99. Western blot analysis of GAPDH was included as a loading control. The data are representative of three individual experiments. (d) TG induces phosphorylation of Bim<sub>EL</sub> in melanoma cells. Mel-RM and MM200 cells were treated with TG (1 μM) for indicated periods. Thirty microgram of total protein of whole-cell lysates were then subjected to western blot analysis using an antibody that specifically recognizes Bim<sub>EL</sub> phosphorylated at Ser99. Western blot analysis of GAPDH was included as a loading control. The data shown are representative of three individual experiments. (e) TM increases an increase in ubiquitination of Bim<sub>EL</sub> in Mel-RM melanoma cells, but a decrease in MCF-7 breast cancer cells. Whole-cell lysates from Mel-RM melanoma cells and MCF-7 breast cancer cells with or without treatment with TM (3 μM) for 36 h were subjected to immunoprecipitation using an antibody against Bim. Thirty microgram of total protein of the resulting precipitates were subjected to SDS-PAGE and probed with an antibody against ubiquitin and an antibody against Bim. The data shown are representative of three individual experiments. (f) The MEK inhibitor U0126 inhibits phosphorylation of Bim<sub>EL</sub> and increases its expression in melanoma cells undergoing ER stress. Thirty microgram of total protein of whole-cell lysates from Mel-RM cells treated with U0126 (20 μM), TM (3 μM), or U0126 plus TM for 24 h were subjected to western blot analysis of Bim, pERK1/2, and GAPDH (as a loading control). The data shown are representative of three individual experiments. (g) Knockdown of MEK1 by siRNA inhibits phosphorylation of Bim<sub>EL</sub> and increases its expression in melanoma cells undergoing ER stress. Mel-RM cells were transfected with the control or MEK1 siRNA. Twenty-four hours later, cells were treated with TM (2 μM) for a further 24 h. Thirty microgram of total protein of whole-cell lysates were then subjected to western blot analysis of Bim, MEK1, pERK1/2, and GAPDH (as a loading control). The data shown are representative of three individual western blot analyses.
Figure 5  Downregulation of the BimEL protein in melanoma cells under ER stress is associated with reduction in PP2A activity that is, at least in part, due to downregulation of the PP2A catalytic C subunit (PP2A-C). (a) Pharmacological activation of PP2A reverses downregulation of BimEL by ER stress in melanoma cells. Left panel: 30 μg of total protein of whole-cell lysates from Mel-RM melanoma cells or MCF-7 breast cancer cells treated with the pharmacological PP2A activator FTY720 (2.5 μM), TM (3 μM), or FTY720 plus TM for 24 h were subjected to western blot analysis of Bim, pERK, ERK, and GAPDH (as a loading control). The data shown are representative of three individual experiments. Right panel: Mel-RM cells were treated with FTY720 (2.5 μM), TM (3 μM), or FTY720 plus TM for 48 h before apoptosis was measured by the PI method using flow cytometry. The data shown are the mean ± S.E. of three individual experiments. (b) Inhibition of PP2A further promotes phosphorylation of BimEL and increases phosphorylation of ERK in Mel-RM cells under ER stress. Thirty microgram of total protein of whole-cell lysates from Mel-RM melanoma cells or MCF-7 breast cancer cells treated with the OA (50 nM), TM (3 μM), or OA plus TM for 24 h were subjected to western blot analysis of Bim, pERK, ERK, and GAPDH (as a loading control). The data shown are representative of three individual experiments. (c) Inhibition of PP2A further promotes phosphorylation of BimEL in SK-Mel- 28, 41B, and MM200 melanoma cells. Thirty microgram of total protein of whole-cell lysates from SK-Mel-28, 41B, and MM200 cells treated with the OA (50 nM), TM (3 μM), or OA plus TM for 24 h were subjected to western blot analysis of Bim and GAPDH (as a loading control). The data shown are representative of three individual experiments. (d) ER stress reduces PP2A activity in Mel-RM melanoma cells, but increases PP2A activity in MCF-7 breast cancer cells. Mel-RM cells and MCF-7 cells were treated with TM (3 μM) for indicated periods before the phosphatase activity of PP2A was quantified using a PP2A-C immunoprecipitation phosphatase assay kit. The PP2A phosphatase activity in cells without treatment was arbitrarily designated as 1. The data shown are the mean ± S.E. of three individual experiments. (e) ER stress downregulates PP2A-C in melanoma cells but upregulates its expression in MCF-7 breast cancer cells. Upper panel: Thirty microgram of total protein of whole-cell lysates from Mel-RM melanoma cells or MCF-7 breast cancer cells treated with TM (3 μM) for indicated periods were subjected to western blot analysis of PP2A-C and GAPDH (as a loading control). The data shown are representative of three individual experiments. Lower panel: Thirty microgram of total protein of whole-cell lysates from 41B and MM200 melanoma cells treated with TM (3 μM) for 24 h were subjected to western blot analysis of PP2A-C and GAPDH (as a loading control). The data shown are representative of three individual experiments. (f) Overexpression of PP2A-C upregulates BimEL and decreases ERK phosphorylation in melanoma cells. Mel-RM cells were transiently transfected with vector alone or pCMV encoding PP2A-C. Twenty-four hours later, cells were treated with TM (3 μM) or TG (1 μM) for a further 24 h. Thirty microgram of total protein of whole-cell lysates were then subjected to western blot analysis of Bim, PP2A-C, pERK, ERK, and GAPDH (as a loading control). The data shown are representative of three individual western blot analyses. (g) Overexpression of PP2A-C causes increased PP2A activity in melanoma cells with or without undergoing ER stress. Mel-RM cells were transiently transfected with vector alone or pCMV encoding PP2A-C. Twenty-four hours later, cells were treated with TM (3 μM) for a further 24 h. The phosphatase activity of PP2A was then quantitated using a PP2A-C immunoprecipitation phosphatase assay kit. The PP2A phosphatase activity in cells transfected with vector alone without TM treatment was arbitrarily designated as 1. The data shown are the mean ± S.E. of three individual experiments.
cells upon ER stress. When McI-1 is inhibited, ER stress induces apoptosis of melanoma cells by activation of the BH3-only proteins Noxa and PUMA, whereas Bim, which is responsible for induction of apoptosis by ER stress in many other cell types, is not involved. Nevertheless, Bim has an important role in killing of melanoma cells by other apoptotic stimuli such as histone deacetylase inhibitors and inhibitors of the RAF/MEK/ERK pathway. These results collectively suggest that Bim is suppressed in melanoma cells under ER stress. In this study, we identified reduction in PP2A activity as an important mechanism to keep Bim levels in check by enhancing its post-translational degradation in melanoma cells subjected to pharmacological ER stress inducers.

We found that, in agreement with observations in a number of other cell types including MCF-7 cells, the Bim transcript was increased in melanoma cells by ER stress through the transcription factor CHOP. However, in contrast to the sustained increase in the Bim mRNA, the Bim\textsubscript{P} protein was downregulated after an initial increase in melanoma cells upon ER stress. This downregulation was important in protection of melanoma cells, as ER stress induced markedly increased apoptosis when Bim was overexpressed. These results, along with our previous finding that PUMA and Noxa that are upregulated in melanoma cells upon ER stress are inhibited by McI-1, demonstrated that melanoma cells have developed multiple mechanisms to maintain the balance between pro- and anti-apoptotic Bcl-2 family proteins to survive ER stress.

Downregulation of Bim at the protein level despite the sustained increase in its transcript mediated by CHOP is specific to melanoma cells under ER stress, as overexpression of CHOP resulted in upregulation of the Bim mRNA as well as the Bim\textsubscript{P} protein. It appears therefore that in response to ER stress, melanoma cells activate a powerful post-translational mechanism(s) leading to robust suppression of the Bim\textsubscript{P} protein expression. Indeed, pro-apoptotic degradation of the Bim\textsubscript{P} protein was accelerated in melanoma cells treated with TM. This was in sharp contrast to the observation in MCF-7 cells in which the half-life time of the protein was prolonged upon ER stress. Consistently, although phosphorylation and ubiquitination of Bim\textsubscript{P} were reduced in MCF-7 cells, these post-translational modifications of the protein were enhanced in melanoma cells under ER stress.

Bim\textsubscript{P} can be phosphorylated by protein kinases such as ERK and JNK and dephosphorylated by the PP2A. The latter is of particular importance in cells under ER stress as PP2A-mediated dephosphorylation of Bim has an essential role in upregulation of Bim in many types of cells undergoing ER stress. Strikingly, while ERK activation was increased and had an essential role in phosphorylation of Bim\textsubscript{P} in
melanoma cells subjected to ER stress, the phosphatase activity of PP2A was reduced by ER stress in melanoma cells. This suggests that the increased phosphorylation and subsequent proteasomal degradation of BimEL in melanoma cells under ER stress is caused by the predominant phosphorylating effect of ERK that overrides the dephosphorylating effect of PP2A on the protein. We did not observe a role of JNK in phosphorylation of Bim by ER stress in melanoma cells (data not shown), but whether other protein kinases such as PKA are involved remains to be defined.25,27

ER stress-triggered reduction in PP2A activity seems to be highly specific to melanoma cells in that PP2A activity is increased in many other cell types by ER stress.6,8,20 In the search for the mechanism(s) responsible for the differential regulation of PP2A by ER stress, we found that PP2A-C was progressively decreased in melanoma cells, but markedly increased in MCF-7 cells, after treatment with TM. Introduction of a PP2A-C-expressing construct increased PP2A activity not only in healthy melanoma cells, but also in those subjected to ER stress, indicating that downregulation of PP2A-C is responsible, at least in part, for suppression of PP2A activity in melanoma cells. Similar to the pharmacological PP2A activator, overexpression of PP2A-C reversed downregulation of BimEL in melanoma cells under ER stress, confirming the importance of reduction in PP2A activity in ER stress-induced suppression of Bim in melanoma cells.

A question remaining unaddressed is how PP2A-C is selectively downregulated by ER stress in melanoma cells. Similarly, it is unclear whether additional mechanisms such as possible alterations in the expression of other PP2A subunits are involved in regulating PP2A activity in cells undergoing ER stress. Mutations have been identified in different components of the PP2A complex, which have been linked to a variety of human cancers.11-13 In particular, a truncated form of PP2A-B55α has been found in the mouse melanoma cell line B16 that is not able to dephosphorylate specific targets and has a role in malignant progression.14-16 Although no mutations have been found in patients, the expression of the PP2A-B55α gene is frequently reduced in human melanoma compared to normal skin.16,17 Similarly, PP2A-B55α has recently been shown to be expressed at lower levels in metastatic compared to primary melanomas.27,28 Regardless, our results clearly demonstrated that reduction in PP2A activity associated with downregulation of PP2A-C is an important mechanism triggered by ER stress in melanoma cells to suppress the BimEL expression. Notably, despite the reduction in its activity, PP2A retained part of its dephosphorylating effect on Bim, in that treatment with OA caused an increase in BimEL phosphorylation in melanoma cells treated with TM. Nevertheless, this residual effect was apparently not adequate to supersede the phosphorylating effect of ERK on BimEL to accumulate the protein to such a level required for efficient induction of apoptosis.

Activation of the MEK/ERK pathway has been well documented to protect cells from ER stress-induced apoptosis.29,30 However, how ER stress triggers activation of the pathway remains undefined. We found that the increase in ERK activation in melanoma cells under ER stress was closely associated reduction in PP2A activity, which otherwise directly targets ERK for dephosphorylation. This was demonstrated by (1) activation of PP2A by FTY720 or overexpression of PP2A-C caused downregulation of pERK in melanoma cells with or without subjected to ER stress; (2) inhibition of PP2A by OA upregulated ERK phosphorylation, in particular, in melanoma cells treated with TM; and (3) ERK was readily co-prefectivated with PP2A-C in melanoma cells with or without subjected to ER stress. In contrast, we did not observe any noticeable effect of PP2A on ERK activation in MCF-7 cells. On the other hand, although PP2A directly targets Bim for dephosphorylation in MCF-7 and a number of other types of cells undergoing ER stress,6,8 it did not appear to directly act on Bim in melanoma cells with or without subject to ER stress. Instead, the inhibitory effect of PP2A on Bim phosphorylation was mediated indirectly by its dephosphorylating effect on ERK. This was suggested by reduced phosphorylation of BimEL mediated by OA when ERK was inhibited, and was further supported by the lack of physical association between BimEL and PP2A in melanoma cells. It is known that the C subunit in the PP2A heterotrimeric complex dictates the substrate specificity of PP2A, and that the dynamic exchange of PP2A-B in the complex is regulated by reversible methylation and phosphorylation of the C-terminal tail of the C subunit, involving the PP2A specific methyltransferase PME1 and methyltransferase, LCN1.9-12 It is conceivable that the differential effects of PP2A on ERK and Bim are associated with cell type- and context-dependent recruitment of different B subunits into the PP2A heterotrimeric complex, but the mechanism responsible for this remains to be defined.13,15-19,24

In conclusion, we have shown in this study that reduction in PP2A activity is an important mechanism responsible for activation of ERK, downregulation of BimEL, and resistance to apoptosis in melanoma cells undergoing ER stress. This reduction is, at least in part, due to downregulation of PP2A-C. Moreover, we have demonstrated that the inhibitory effect of PP2A on BimEL phosphorylation is predominantly mediated by its dephosphorylating effect on ERK (Supplementary Figure 9). These results suggest that pharmacological activation of PP2A may improve treatment results of agents that reduce ER stress in melanoma cells such as cisplatin and sorafenib.30,31 In addition, the dephosphorylating effect of PP2A on ERK in melanoma cells may also have important implications in improving efficacy of targeting the RAF/MEK/ERK pathway, which is a promising strategy in the treatment of metastatic melanoma.29,32

Materials and Methods

Cell lines. The human melanoma cell lines Mel-RM, MM200, IgR1, and SK-Mel-28 and the human breast cancer cell line MCF-7 were described previously.18,32 They were cultured in Dulbecco's modified Eagle medium supplemented with 5% fetal calf serum (Commonwealth Serum Laboratories, Melbourne, Vic., Australia).

Antibodies, recombinant proteins, and other reagents. The rabbit polyclonal antibodies (pAb) against Bim were supplied by Imgenex (San Diego, CA, USA); the mouse monoclonal antibodies (mAb) against CHOP, pERK, Mcl-1, and Beclin-1, and the rabbit pAb against GRP78 were from Santa Cruz Biototechnology (Santa Cruz, CA, USA); the rabbit pAbs against phosphorylated Erk, Bim, and PI3K and the mouse mAbs against Bim and Bcl-2 were from Cell Signaling Technology (Beverly, MA, USA); the mouse mAb against GAPDH was from Ambion (Austin, TX, USA). The restriction enzyme AplII was purchased from New England Biolabs (Ipswich, MA, USA). The PP2A inhibitor OA,
Adonomin D, TM, and TG were purchased from Sigma-Aldrich (St. Louis, MO, USA). The PKA activator FTY720 and M1G22 were from Caymen Chemicals (Ann Arbor, MI, USA). The MEK inhibitor U0126 was from Promega (Madison, WI, USA). Cycloheximide was purchased from CalBiochem (La Jolla, CA, USA).

Apopotaxis analysis. Apoptotic cells were quantified by measurement of sub-G1 DNA content using propidium iodide (PI) on a flow cytometer (Beckton Dickinson, Sunnyvale, CA, USA) as described elsewhere.

Western blot analysis. Western blot analysis was carried out as described previously. Labeled bands were detected by Luminata Crescendo Western HRP substrate (Millipore, Billerica, MA, USA) and images were captured and the intensity of the bands was quantitated with ImageJReader LAS-4001 (FujiFilm Corporation, Tokyo, Japan).

Immunoprecipitation. Immunoprecipitation experiments were carried out as described previously.

PP2A phosphatase activity assay. PP2A activity was determined using the PP2A immunoprecipitation phosphatase assay kit (Millipore). Briefly, whole cell lysates in 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1% NP40 were incubated for 2 h at 4 °C with 4 μg of an antibody against PP2A-C and protein A-agarose. After three washes, immunoprecipitates were used in a phosphatase reaction according to the manufacturer's instructions. As an internal control, the specificity of the reaction was assessed by inhibiting PP2A activity with 1 mM OA prior to reaction. The percentage of phosphatase activity was determined by dividing the free phosphate of the last cells by that of the untreated cells.

Establishment of melanoma cell lines carrying an inducible Bim or CHOP expression system. A lentivirus-based inducible gene expression system described previously was used to express Bim and CHOP conditionally in melanoma cells. Briefly, the system involves confection of two lentiviral particles: one encoding the inducible transactivation factor G1-s (I-TAC) ER\(^{1+}\) (VE616) doped into FU2-VE616-PKG-Hygro containing a byronycin-B resistance gene and another, Bim or CHOP DNA doped into p8-SWAS-SV40-puro containing a puromycin-resistance gene. Dual antibiotic selection was applied deriving a well population carrying both VE616 and Bim or CHOP. Application of low medium concentrations of (–OHT) drives the expression of Bim or CHOP. The melanoma cell line MoM/RM was used to establish cultures carrying inducible excogenous Bim (MoM/Bim) or CHOP (MoM/CHOP).

Plasmid vectors and transfection. The pCMV/1-κB-Luc (Sigma, St. Louis, MO), pCMV/AC-CHOP (Shea2004), pCMV/AC-PPPA-C (SBC24101) were purchased from Origene (Rockville, MD, USA). Cells were transfected with 2 μg plasmid as well as the empty vector in Opti-MEM medium (Invitrogen, Carlsbad, CA, USA) with Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol.

Quantitative reverse transcription and real-time PCR. Total RNA was isolated using RNAasy mini kit (Qiagen, Donovar, VIC, Australia) following the manufacturer's instructions. RNA was reverse transcribed into cDNA using Superscript III (Invitrogen) following the manufacturer's instructions. Quantitative PCR (qPCR) was performed using the ABI Prism 7900 sequence detection system (Applied Biosystems, Mulgrave, VIC, Australia) with specific-gene primers: Bim forward, 5'-TGCAGACTTCTGCTGTTCA-3'; Bim reverse, 5'-GAAGCTGAGGTCGTTATAGTT-3'; jn-jn forward, 5'-GGCACCACGACAGAATGAG-3'; jn-reverse, 5'-GCTGATCCACGACAGAATGAG-3'. The PCR conditions were used: standard fast cycle for 95 °C for 10 s, 40 cycles of 95 °C for 1 s and 60 °C for 20 s using Fast SYBR Green mastermix (Applied Biosystems). Cycle threshold (C\(_T\)) values for specific genes were normalized to the C\(_T\) value for the housekeeping gene, jn-jn. The fold changes of mRNA expression were determined by comparison with jn-jn, where the control sample was arbitrarily designated as 1, and the values of the relative abundance of mRNA of other samples were calculated accordingly. The specificity of the qPCR was controlled using non-template control.

siRNA knockdown. The siRNA constructs used were obtained as the sGENOME SMARTpool reagents (Dharmacon, Lafayette, CO, USA). The siRNA constructs used were: MK1-siGENOME SMARTpool (M-003571-01-0010), EMA-siGENOME SMARTpool (M-000352-03-0010), ERK1-siGENOME SMARTpool (M-000305-04-0010), CHOP-siGENOME SMARTpool (M-004810-05-0010), and non-targeting siRNA pool (D-001000-10-20) as control. Transfection of siRNA pools was carried out as described previously.

Detection of XBP1 mRNA splicing. Reverse transcription-PCR (RT-PCR) products of XBP1 mRNA were obtained from total RNA extracted using primers 5'-CGCTGTCGGCGGTGTGAGTCTGA-3' (sense) and 5'-TGGGG CGCGCTGAGGCTGCTCCT-3' (antisense). As a 26-bp fragment containing an ApeIl site is spliced on activation of XBP1 mRNA, the RT-PCR products were digested with ApeIl to distinguish the active spliced form from the inactive unspliced form. Subsequent electrophoresis revealed the inactive form as two closed fragments and the active form as a non-framed fragment.

Conflict of Interest

The authors declare no conflict of interest.

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Suppression of PPI2A in melanoma under ER stress

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**Supplementary Figure 1:** ER stress induces sustained upregulation of McI-1 and PUMA, and does not induce notable changes in the expression of Bid and Bcl-X<sub>L</sub>. Thirty microgram of total protein of whole cell lysates from Mel-RM cells treated with TM (3μM) for indicated periods were subjected to Western blot analysis of McI-1, PUMA, Bid, Bcl-X<sub>L</sub>, and GAPDH (as a loading control). The data shown are representative of three individual experiments.
Supplementary Figure 2: Induction of ER stress by TG in Mel-RM and MM200 melanoma cells and MCF-7 breast cancer cells. Mel-RM, MM200, and MCF-7 cells were treated with TG (1μM) for 12 hours. For examining the expression of GRP78 and CHOP, Thirty microgram of total protein of whole cell lysates were subjected to Western blot analysis of GRP78, CHOP and GAPDH (as a loading control). The arrowhead points to a non-specific band generated with the antibody against CHOP. For examining activation of XBP-1, RT-PCR products of XBP-1 mRNA and b-actin mRNA (as a control) from total RNA extracts were digested with Apa-LI for 90 min followed by electrophoresis. The longer fragment derived from the active form of XBPI mRNA and two shorter bands derived from the inactive form are indicated. The data shown are representative of three individual experiments.
Supplementary Figure 3: ER stress induces upregulation of the Bim transcript in melanoma cells. Sk-Mel-28, IgR3, and MM200 melanoma cells were treated with TM (3μM) for indicated periods. Total RNA was then isolated and subjected to qPCR analysis for the Bim mRNA expression. The relative abundance of the Bim mRNA in each cell line before treatment was arbitrarily designated as 1. The data shown are the mean ± SE of three individual experiments.
Supplementary Figure 4: ER stress prolongs half-life time of \( \text{Bim}_{\text{EL}} \) in MCF-7 breast cancer cells. A, MCF-7 cells were treated with the protein synthesis inhibitor cycloheximide (CHX) (10\( \mu \text{g/ml} \)) with or without the addition of TM (3\( \mu \text{M} \)) for indicated periods. Thirty microgram of total protein of whole cell lysates were then subjected to Western blot analysis of Bim and GAPDH (as a loading control). The data shown are representative of three individual experiments. B, Quantitative expression levels of \( \text{Bim}_{\text{EL}} \) as shown the upper panel that were normalized to GAPDH. Quantitation of each band was carried out with ImageReader LAS-4000. The data shown are representative of three individual experiments.
Supplementary Figure 5: ER stress upregulates activation of ERK in melanoma cells. Thirty microgram of total protein of whole cell lysates from Mel-RM cells treated with TM (3μM) for indicated periods were subjected to Western blot analysis of phosphorylated ERK (pERK) and ERK. The data shown are representative of three individual experiments.
**Supplementary Figure 6:** The MEK inhibitor U0126 inhibits ER stress-induced phosphorylation of Bim_{EL} in melanoma cells. Thirty microgram of total protein of whole cell lysates from Mel-RM cells treated with U0126 (20μM), TM (3μM), or U0126 plus TM for 24 hours were subjected to Western blot analysis of Bim, pERK, ERK, and GAPDH (as a loading control). The data shown are representative of three individual experiments.
Supplementary Figure 7: The PP2A activator FTY720 upregulates BimEL in melanoma cells under ER stress. Sk-Mel-28, IgR3, and MM200 melanoma cells were treated with TM (3μM) with or without the addition of FTY720 (2.5μM) for 24 hours. Thirty microgram of total protein of whole cell lysates were subjected to Western blot analysis of Bim and GAPDH (as a loading control). The data shown are representative of three individual experiments.
Supplementary Figure 8: Overexpression of PP2A-C sensitizes melanoma cells to ER stress-induced apoptosis. Mel-RM cells were transiently transfected with vector alone or cDNA encoding PP2A-C. Twenty-four hours later, cells were treated with TM (3μM) for a further 48 hours. Apoptosis was measured by the propidium iodide method using flow cytometry. The data shown are the mean ± SE of three individual experiments.
Supplementary Figure 9: A schematic illustration of regulation of phosphorylation of ERK and Bim<sub>E</sub> by PP2A in melanoma cells.
CHAPTER SIX

GENERAL DISCUSSION
Chapter Six

General Discussion

Melanoma is a major Australian health problem. It is the third most common cancer in men and women, and has a disproportionately heavy impact on the productive years of the lives of young Australian, because it is the most common cancer in males aged 25-54 years and in females aged 15-29. Although early detection and surgical excision of primary melanoma commonly result in cure, there is currently no curative treatment once the disease has spread beyond the primary site [253, 254]. This is closely related to resistance of melanoma cells to available chemotherapeutic, targeted therapeutic drugs, and biological reagents. Inappropriate activation of survival-signalling pathways and deregulation of apoptotic pathways, either as consequences of genetic and/or epigenetical alterations or resulting from environmental stimulations plays a central role in resistance of melanoma cells to treatment [253, 254].

The importance of the UPR in cancer development and progression has inspired a great deal of interest in exploring therapeutic potentials of targeting critical components of the response. Solid cancer cells commonly undergo ER stress as a consequence of hypoxia, nutrient deprivation, DNA damage, metabolic and oxidative stress, and display constitutive activation of the UPR. In contrast, the UPR is rarely activated in most normal cells [224, 260, 305]. This discrepancy may offer an opportunity for targeting the UPR in the treatment of cancer. However, most melanoma cell lines and fresh melanoma isolates are insensitive to apoptosis induced by ER stress [248, 306]. This suggests that melanoma cells may have adapted to ER stress conditions. In support, melanoma cells constitutively express increased levels of GRP78 and the active form of XBP-1 mRNA, two commonly used indicators of activation of UPR [225]. Indeed, past studies have identified a number of mechanisms that contribute to adaptation of melanoma cells to pharmacological ER stress. These include upregulation of the anti-apoptotic Bcl-2 family protein Mcl-1, induction of GRP78 and XBP-1, activation of the MEK/ERK and PI3K/Akt pathways, and expression of the apoptosis repressor with caspase recruitment domain (ARC) protein [220, 225, 227, 231]. Nevertheless, better understanding of adaptive mechanisms to ER stress is clearly required for development of approaches to render melanoma cells sensitive to ER stress-induced apoptosis. Therefore, the overall aim of this PhD project was to further elucidate mechanisms responsible for adaptation of melanoma cells to ER stress.
Chapter Six

The results embedded in this thesis have shown that, 1) in contrast to rapid attenuation after initial activation in other types of cells, activation of the IRE1α and ATF6 signalling pathways of the UPR is sustained in melanoma cells undergoing persistent ER stress, which plays a critical role in protection of melanomas from ER stress-induced apoptosis; 2) activation of the MEK/ERK pathway is required for sustained activation of IRE1α(ATF6) signalling; 3) potentiation of adaptation to chronic ER stress is another mechanism by which oncogenic activation of the MEK/ERK pathway promotes the pathogenesis of melanoma; 4) suppression of Bim that is responsible for ER stress-induced apoptosis in diverse type of cells by the protein phosphatase PP2A also plays a role in resistance of melanoma cells to ER stress-induced apoptosis.

Although the UPR is fundamentally a cyto-protective response, excessive and/or prolonged UPR can result in cell death predominantly by induction of apoptosis [152, 167, 168, 307]. This switch from the cyto-protective to pro-apoptotic output of the UPR has been shown in various types of cells to be caused by attenuation of the IRE1α, and to a lesser extent, ATF6, signalling [258, 259]. However, results presented in Chapter 3 have shown that, in contrast to observation made in other types of cells, the IRE1α and ATF6 pathways of the UPR persist in melanoma cells undergoing ER stress, which plays an important role in resistance of melanoma cells to ER stress-induced apoptosis. When IRE1α and ATF6 signalling is interrupted, ER stress induces apoptosis through the PERK pathway.

The IRE1α and ATF6 pathways of the UPR play predominantly pro-survival roles in cells upon ER stress [180, 258, 259]. Consistent with this, knockdown of IRE1α or ATF6 pathways with siRNA or interruption of IRE1α pathway with a specific inhibitor induced apoptosis in melanoma cells, indicating that the continuation in activation of IRE1α signalling plays a critical role in survival of melanoma cells undergoing prolonged ER stress. Indeed, activation of PI3K/Akt pathway is known to be mediated by IRE1α signalling, which is also responsible for upregulation of Mcl-1 that are critical for survival of melanoma cells upon ER stress [225, 234]. Furthermore, ATF6 transcriptionally regulates GRP78 along with XBP-1 that also play important roles in resistance of melanoma cells to ER stress-induced apoptosis [225, 227].
Chapter Six

An important finding in Chapter 3 is that the activity of MEK/ERK signalling is required for sustained activation of the IRE1α and ATF6 pathway in melanoma cells undergoing ER stress. This was demonstrated by attenuation of ER stress-induced activation of the IRE1α and ATF6 signalling in melanoma cells treated with MEK inhibitor U0126 or with ERK1/2 knocked down by siRNA. How the MEK/ERK pathway interacts with IRE1α and ATF6 signalling in melanoma cells subjected to pharmacological ER stress requires further investigation. Nonetheless, it is known that acute ER stress induces further activation of MEK/ERK that plays an important role in protection of melanoma cells from ER stress-induced apoptosis [64, 308].

A characteristic of human melanoma is constitutive activation of the MEK/ERK pathways [253, 309-311]. Results described in Chapter 4 of this thesis have provided evidence that oncogenic activation of the MEK/ERK pathway is not only a mechanism of adaptation to ER stress, but also a source of chronic ER stress in melanoma cells. This was demonstrated by suppression of activation of the IRE1α and ATF6 branches by inhibition of MEK/ERK signalling with various strategies, including inhibition of MEK or knockdown of ERK1/2 in wild type BRAF melanoma cells and by inhibition of mutant BRAF or MEK in BRAFV600E melanoma cells. In addition, while PLX4720 activated ERK in wild-type BRAF melanoma cells, consistent with previous reports [312], it enhanced activation of IRE1α and ATF6 signalling.

Activation of MEK/ERK pathway promotes protein synthesis that is often required by cancer cells to sustain their malignant characteristics such as enhanced survival and proliferation [261-264]. Indeed, the magnitude of nascent protein production was remarkably higher in melanoma compare to melanocytes, which might uncouple the ER protein folding load with ER protein folding capacity, thus causing ER stress. It has been reported that MEK/ERK-mediated activation of protein synthesis involves phosphorylation of the rate-limiting factor of cap-dependent translation initiation eIF4E [267, 268]. Consistent with this, the basal level of phosphorylated eIF4E were generally higher in melanoma cells compared to melanocytes, which appeared to be mediated by MEK/ERK signalling. Therefore, it seems that enhancement in nascent protein synthesis mediated by eIF4E is the major mechanism by which activation of MEK/ERK pathway triggers ER stress in melanoma cells. This was consolidated by the finding that
knockdown of eIF4E reduced XBP-1s and GRP78 even in melanoma cells with induced activation of MEK/ERK signalling.

The finding that ectopic expression of BRAF\textsuperscript{V600E} causes phosphorylation of eIF4E, increases in nascent protein synthesis, and activation of the UPR indicative of induction of ER stress is of particular importance, in that this not only confirms the role of activation of the MEK/ERK pathway in triggering ER stress in melanocytic cells, but also suggests that occurrence of ER stress is an early event in the pathogenesis melanoma, as activating mutations in BRAF is also found in the majority of nevi [28, 313, 314]. Activating mutations in BRAF induce oncogenic senescence in melanocytes. However, once the senescence barrier is overcome, activated BRAF drives melanomagenesis [315, 316]. By analogy, induction of ER stress by oncogenic activation of the MEK/ERK pathway in melanocytes may also set a barrier for melanoma initiation. It is conceivable that only those cells that can survive chronic ER stress triggered by MEK/ERK may acquire malignant phenotypes.

GRP78 is known to play an important role in protecting melanoma cells from ER stress-induced apoptosis [225, 253]. Our finding demonstrated that IRE1\textalpha/XBP-1 signalling similarly contributes to survival of melanoma cells undergoing ER stress. Given the essential role of activation of the MEK/ERK pathway in enhancing protein production and maintaining IRE1\textalpha and ATF6 activities, it appears that induction of ER stress by MEK/ERK signalling provides melanoma cells with multifaceted mechanisms to drive their malignancy. Targeting GRP78 is in development for the treatment of cancer [224]. Selective inhibitors of IRE1\textalpha/XBP-1 have also been recently shown to be a promising therapeutic option in cancers with chronic ER stress [317, 318]. Combinations of these strategies with inhibition of MEK/ERK pathway may further inhibit pro-survival outputs of the UPR and enhance therapeutic efficacy in the treatment of melanoma.

Previous studies have shown that upregulation of pro-survival protein Mcl-1 plays a critical role for survival of melanoma upon ER stress [231]. Another two BH3-only proteins, Noxa and PUMA were also upregulated and responsible for ER stress-induced apoptosis in melanoma cells when Mcl-1 was inhibited. However, Bim, which is responsible for induction of apoptosis by ER stress in diverse type of cells, is not involved [206]. Nonetheless, Bim plays a crucial role in killing of melanoma cells by
other apoptotic stimuli such as RAF/MEK/ERK pathway inhibitors and histone deacetylase inhibitors [135, 232, 319]. These results collectively suggest that Bim is suppressed in melanoma cells under ER stress.

ER stress activates Bim through two pathways, involving PP2A-mediated dephosphorylation, which prevents its ubiquitination and proteasomal degradation and CHOP-C/EBPα-mediated direct transcriptional induction [206]. Indeed, results in Chapter 5 demonstrated that ER stress increased the Bim transcript in melanoma cells through upregulation of the transcription factor CHOP. However, in contrast to the sustained increase in Bim mRNA, the BimEL protein was downregulated after an initial upregulation in melanoma cell upon ER stress. This reduction of Bim was critical in protection of melanoma cells, as overexpression of Bim sensitized melanoma cells to ER stress-induced apoptosis. These results, along with our previous finding that Noxa and PUMA that are upregulated in melanoma cells upon ER stress are neutralized by Mcl-1 [231], demonstrated that melanoma cells have developed numerous mechanisms to sustain the balance between pro- and anti-apoptotic Bcl-2 family proteins to survive ER stress [220, 231, 248, 320].

Overexpression of CHOP resulted in upregulation of Bim mRNA as well as BimEL protein in melanoma suggested that the downregulation of Bim at the protein level despite the sustained increase in its transcript mediated by CHOP is specific to melanoma cells under ER stress. It appears that melanoma cells activate an influential post-transcriptional mechanism(s) leading to strong downregulation of BimEL protein in response to ER stress. Indeed, proteasomal degradation of the BimEL protein was accelerated in melanoma cells subjected to ER stress.

Bim is phosphorylated on multiple sites by protein kinases such as ERK, JNK and p38 isoforms, whereas it can be dephosphorylated by PP2A [321, 322]. The latter is of particular importance in cells under ER stress as PP2A-mediated dephosphorylation of Bim has an essential role in upregulation of Bim in diverse type of cells undergoing ER stress [206]. Remarkably, while ER stress increased ERK activation in melanoma cells and played a major role in phosphorylation of BimEL, the phosphatase activity of PP2A was reduced by ER stress in melanoma cells. This demonstrates that the increased phosphorylation and subsequent proteasomal degradation of Bim proteins in melanoma
cells under ER stress is caused by the predominant phosphorylating effect of ERK that overrides the dephosphorylating effect of PP2A on the protein.

ER stress-triggered suppression of PP2A activity seems to be highly specific in melanoma cells whereas PP2A activity is increased in many other cell types by ER stress [206, 323, 324]. Our finding shows that PP2A-C plays a critical role in suppression of PP2A activity in melanoma cells under ER stress as PP2A-C was progressively decreased in melanoma cell subjected to ER stress. Moreover, pharmacological PP2A activator and overexpression of PP2A-C reversed suppression of Bim$_{EL}$ in melanoma cells under ER stress, demonstrating the importance of reduction of PP2A activity in ER stress-induced suppression of Bim in melanoma cells. Additionally, we found that the reduction in PP2A activity was closely associated with the upregulation of ERK activation in melanoma cells under ER stress, where PP2A may directly targets ERK for dephosphorylation. This was demonstrated by 1) activation of PP2A by FTY720 or overexpression of PP2A-C caused reduction of activated ERK in melanoma cells with or without subjected to ER stress; 2) inhibition of PP2A by OA increased phosphorylation of ERK, in particular, in melanoma cells treated with ER stress inducer; and 3) ERK was readily co-precipitated with PP2A-C in melanoma cells with or without subjected to ER stress. Moreover, PP2A did not appear to directly act on Bim in melanoma cells with or without ER stress. Alternatively, the inhibitory effect of PP2A on Bim phosphorylation was mediated indirectly by its dephosphorylating effect on ERK. This was supported by reduced phosphorylation of Bim$_{EL}$ mediated by OA when ERK was inhibited. In addition, it was further supported by the lack of physical association between Bim$_{EL}$ and PP2A in melanoma cells. Taken together, we have shown in this Chapter 5 that reduction in PP2A activity is an important mechanism responsible for activation of ERK, downregulation of Bim, and resistance to apoptosis in melanoma cells undergoing ER stress.

It is clear that much additional work will be required for a complete understanding of adaptive mechanisms to ER stress in melanoma cells. Nevertheless, results presented in this thesis have provided new insights into mechanisms involved, and suggest that targeting MEK/ERK signalling, interruption of the IRE1$\alpha$ and ATF6 pathways, and activation of PP2A are potentially useful strategies to improve the therapeutic efficacy
of agents that inducing ER stress such as cisplatin and sorafenib in the treatment of melanoma [225, 227].
Future Directions

Although this thesis has provided new insights into adaptive mechanisms to ER stress in melanoma cells, a number of important issues remain unaddressed. Results described in Chapter 4 have clearly demonstrated that inhibition of oncogenic BRAF by PLX4720 reduces activation of the IRE1α and ATF6 branches of the UPR. However, a recent study showed that the clinically available RAF inhibitor vemurafenib, which is a close relative of PLX4720, induced ER stress in BRAFV600E melanoma cells [325]. Although this has been verified in Chapter 4, it appeared that, unlike PLX4720 that induces ER stress in wild-type, but attenuates activation of the UPR in mutant BRAF melanoma cells, vemurafenib induces ER stress in both wild-type and mutant BRAF melanoma cells, which is apparently disassociated with its effects on activation of MEK/ERK signalling. Nevertheless, how does this inhibitor induces ER stress while MEK/ERK signalling is inhibited needs further investigation. Regardless, this finding is of particular importance as it suggested that the therapeutic efficacy of this clinically available BRAF inhibitor may be determined not only by its inhibitory effect on MEK/ERK but also by its ability to induce ER stress. Further studies using animal models and melanoma samples from patients before and after treatment with the inhibitor are clearly warranted.

Although evidence provided in Chapter 5 has pointed to that PP2A indirectly dephosphorylates Bim by suppressing ERK activation in melanoma upon ER stress, the mechanism responsible for this remains to be defined. However, our results revealed that PP2A is physically associated with ERK, suggesting that PP2A may directly dephosphorylate ERK, thus leading to reduced phosphorylation of Bim. It is known that the B subunit in the PP2A heterotrimeric complex dictates the substrate specificity of PP2A [276, 277]. Indeed, past studies have shown that the PP2A-B56 subunits, PP2A-B'56β and PP2A-B'56γ directly dephosphorylate ERK [279, 297]. Nonetheless, whether these PP2A B subunits are involved in dephosphorylation of ERK in melanoma cells undergoing ER stress remains a subject of ongoing studies.

An additional potential adaptive mechanism to ER stress in melanoma cells is induction of autophagy. ER stress has been shown to induce autophagy that plays a role in protection against apoptosis in a number of other types of cells [326, 327]. However,
whether autophagy is involved in survival of melanoma cells undergoing ER stress has not been examined. Studies on a separate project carried by my colleagues and I have suggested that receptor-interacting serine/threonine-protein kinase 1 (RIPK1), which is emerging as a key regulator of survival, apoptosis and programmed necrosis in cells upon stimulation with death ligands or DNA-damaging drugs, protects melanoma cells from ER stress-induced apoptosis through activation of autophagy. This work is ongoing. We expect to demonstrate that RIPK1-mediated autophagy is another mechanism responsible for survival of human melanoma cells undergoing ER stress, which will strengthen the validity of therapeutic strategies targeting RIPK1 and autophagy in combination with ER stress inducers in melanoma.
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Suppression of PP2A is critical for protection of melanoma cells upon endoplasmic reticulum stress.

Oncogenic Activation of MEK/ERK Primes Melanoma Cells for Adaptation to Endoplasmic Reticulum Stress.

Sustained IRE1α and ATF6 Signalling is Important for Survival of Melanoma Cells Undergoing ER Stress.

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Date: 08/11/2013

Kwang Hong Tay
Date: 22/10/2013

Prof. Robert Callister
Date:
I, Peter Hersey, attest that Research Higher Degree candidate Kwang Hong Tay contributed to the 1) conception and design of the research, 2) collection, analysis and interpretation of research data and 3) drafting and revision of significant parts of the work to contribute to the interpretation of the publications entitled:

Suppression of PP2A is critical for protection of melanoma cells upon endoplasmic reticulum stress.

Oncogenic Activation of MEK/ERK Primes Melanoma Cells for Adaptation to Endoplasmic Reticulum Stress.

Prof. Peter Hersey
Date: 29/10/2013

Kwang Hong Tay
Date: 22/10/2013

Prof. Robert Callister
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I, Chen Chen Jiang, attest that Research Higher Degree candidate Kwang Hong Tay contributed to the 1) conception and design of the research, 2) collection, analysis and interpretation of research data and 3) drafting and revision of significant parts of the work to contribute to the interpretation of the publications entitled:

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Sustained IRE1α and ATF6 Signalling is Important for Survival of Melanoma Cells Undergoing ER Stress.

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Prof. Robert Callister
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Sustained IRE1α and ATF6 Signalling is Important for Survival of Melanoma Cells Undergoing ER Stress.

Dr. Hsin-Yi Tseng
Date: 08/11/2013

Kwang Hong Tay
Date: 22/10/2013

Prof. Robert Callister
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I, Lei Jin, attest that Research Higher Degree candidate Kwang Hong Tay contributed to the 1) conception and design of the research, 2) collection, analysis and interpretation of research data and 3) drafting and revision of significant parts of the work to contribute to the interpretation of the publications entitled:

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Oncogenic Activation of MEK/ERK Primes Melanoma Cells for Adaptation to Endoplasmic Reticulum Stress.

Sustained IRE1α and ATF6 Signalling is Important for Survival of Melanoma Cells Undergoing ER Stress.

Dr. Lei Jin
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Suppression of PP2A is critical for protection of melanoma cells upon endoplasmic reticulum stress.

Oncogenic Activation of MEK/ERK Primes Melanoma Cells for Adaptation to Endoplasmic Reticulum Stress.

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Date: 22/10/2013

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Oncogenic Activation of MEK/ERK Primes Melanoma Cells for Adaptation to Endoplasmic Reticulum Stress.

Sustained IRE1α and ATF6 Signalling is Important for Survival of Melanoma Cells Undergoing ER Stress.

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Oncogenic Activation of MEK/ERK Primes Melanoma Cells for Adaptation to Endoplasmic Reticulum Stress.

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Kwang Hong Tay
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Suppression of PP2A is critical for protection of melanoma cells upon endoplasmic reticulum stress.

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Date: 22/10/2013

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Date: 22/10/2013

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Suppression of PP2A is critical for protection of melanoma cells upon endoplasmic reticulum stress.

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Oncogenic Activation of MEK/ERK Primes Melanoma Cells for Adaptation to Endoplasmic Reticulum Stress.

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Date: 22/10/2013

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I, Yan Ye, attest that Research Higher Degree candidate Kwang Hong Tay contributed to the 1) conception and design of the research, 2) collection, analysis and interpretation of research data and 3) drafting and revision of significant parts of the work to contribute to the interpretation of the publications entitled:

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Date: 23/10/2013

Kwang Hong Tay  
Date: 22/10/2013

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I, Qi Luan, attest that Research Higher Degree candidate Kwang Hong Tay contributed to the 1) conception and design of the research, 2) collection, analysis and interpretation of research data and 3) drafting and revision of significant parts of the work to contribute to the interpretation of the publications entitled:

Sustained IRE1α and ATF6 Signalling is Important for Survival of Melanoma Cells Undergoing ER Stress.
Cellular Signalling. 2013. (Accepted on 7th November)

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Date: 22/10/2013

Prof. Robert Callister
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I, Suzanah Boyd, attest that Research Higher Degree candidate Kwang Hong Tay contributed to the 1) conception and design of the research, 2) collection, analysis and interpretation of research data and 3) drafting and revision of significant parts of the work to contribute to the interpretation of the publications entitled:

Oncogenic Activation of MEK/ERK Primes Melanoma Cells for Adaptation to Endoplasmic Reticulum Stress.

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Kwang Hong Tay
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