DEFECIANCY OF *Puma* AND *Noxa* FOR MELANOMAGENESIS IN A MOUSE MODEL OF MELANOMA

PHANG SU LING

UNIVERSITI SAINS MALAYSIA

2017

DEFECIANCY OF *Puma* AND *Noxa* FOR MELANOMAGENESIS IN A MOUSE MODEL OF MELANOMA

by

PHANG SU LING

Thesis is submitted in fulfilment of the requirements
for the Degree of
Master of Science

July 2017

ACKNOWLEDGEMENT

Dear Dr Nethia, for being a teacher and for the opportunity to learn and grow, I thank you. It is true when they say actions speak louder than words as I thank you for your dedication, the invaluable lesson of keeping integrity at work, and for showing us the value of hard work – all of which you have thought us through your actions and principals. And for the countless sleepless nights you've invested in this, thank you.

A thank you note to everyone who has been with me through this project; my parents, Bong, BFFs and friends at home and most importantly lab members of Lab 207, Ben, Vani, Prabu, and Fairus for their many thoughtful words, guidance and encouragements, and for playing a huge role in the completion of this thesis and project.

Past and present lab members of Lab 308, 414, 418 and staff members from different parts of USM, especially from the Animal Research Facility, AMDI and School of Biological Sciences, as well as the long list of many colleagues turned friends, I owe this to each and every one of you. To all the friends I have met and made out of this project, thank you for walking with me through this journey, you decorated my life.

I give all the glory to God.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

β-met	2-mercaptoethanol β-mecapto
ACS	acetyl-CoA synthetase
AIF	Apoptosis-inducing factor
AMDI	Advanced Medical and Dental Institute
APAF-1	Apoptosis peptidase activating factor-1
ARF	Alternative reading frame
BAD	BCL-2-associated death promoter
BAK	BCL-2 antagonist killer
BAX	BCL-2-associated X protein
BCL-2	B-cell lymphoma 2
BCL-w	B-cell lymphoma-2 like protein
BCL-XL	BCL-2-related gene, long isoform
BFL-1	BCL-2-related gene A1
BH3	BCL-2 homology domain 3
BID	BCL-2-interacting domain death agonist
BIK	BCL-2 interacting killer
BIM	BCL-2 interacting mediator of cell death
BIM	BCL-2-interacting mediator protein
BMF	BCL-2 modifying factor
BOK	Bcl-2 related ovarian killer
BSA	Bovine Serum Albumin
$CaCl_2$	Calcium chloride
CDK4	Cyclin-dependant Kinase 4
CDKN2A	Cyclin-dependant Kinase Inhibitor 2A

cDNA	Complementary DNA
CO_2	Carbon dioxide
COT	Cancer Osaka Thyroid
CSD	Chronically sun-damaged
CTAB	Hexadecyltrimethylammonium bromide
DAPI	4',6-diamidino-2-phenylindol
ddH_2O	Double disuntiled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
DR	Death receptor
DTIC	Dacarbazine
EDTA	Ethylenediaminetetraacetic acid
endoG	Endonuclease G
ER	Endoplasmic reticulum
FADD	FAS-associated death domain protein
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FGF	Fibroblast Growth Factor
FVB/NJ	Friend Virus B NIH Jackson
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GEMM	Genetically-modified mouse models
HCl	Hydrochloric
HGF	Hepatocyte Growth Factor
HMB45	Human black melanoma 45

HRAS	Harvey Ras
HRK	Harakiri protein
IGF-1R	insulin-like growth factor 1
IHC	Immunohistochemistry
MAPK	Mitogen-activated protein kinase
MCL-1	Myeloid cell leukemia 1
MDM2	Murine double minute-2
MEK	Mitogen-activated protein kinase kinase
$MgCl_2$	Magnesium chloride
MOMP	Mitochondrial outer membrane permeabilisation
Na_2HPO_4	Disodium phosphate
Nalp3	NACHT, LRR and PYD domains-containing protein 3
NaOH	Sodium hydroxide
NF1	Neurofibrometosis
NOXA/Pmaip	Phorbal-12-myristate-13-acetate-induced protein-1
OMM	Outer mitochondrial membrane
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PD	Programme cell death
PDGFRβ	Platelet-derived growth factor beta-recepto
PGK	Phosphoglycerate kinase
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
pRB	Retinoblastoma protein
PTEN	Phosphatase and tensin homolog
PUMA	p53-up-regulated modulator of apoptosis

RGP	Radial growth phase
RIPK3	Receptor-interacting serine/threonine-protein kinase 3
RNA	Ribonucleic acid
RTK	Receptor tyrosine kinases
RT-PCR	Real time PCR
SCF	Stem Cell Factor
SEER	Surveillance, Epidemiology and End Results
SEM	Standard error of the mean
tBID	Truncated BID
TM	Transmembrane
TMZ	Temozolomide,
TNF	Tumour necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
T-Vec	Talimogene laherparepvec
Tyr	Tyrosinase
UV	Ultraviolet
VDAC	Voltage-dependant anion channel
VGP	Vertical growth phase

KEKURANGAN *Puma* DAN *Noxa* BAGI MELANOMAGENESIS DALAM MODEL MENCIT MELANOMA

ABSTRAK

Kegagalan sel melanoma untuk menjalani apoptosis (sejenis kematian sel terancang) sebagai respon terhadap rawatan konvensional seperti dadah sitotoksik, dipercayai merupakan sebab utama melanoma menunjukkan rintangan terhadap terapi. Kajian bagi menentukan sumbangan laluan apoptosis intrinsik biasanya diterhad dengan penggunaan model kultur sel in vitro dan xenograf. Model mencit melanoma, Cdkn2a -/-, Tyr-HRASG12V, telah digunakan dalam projek ini bagi mangatasi masaalah tersebut. Model Cdkn2a -/-, Tyr-HRASG12V dikacukkan dengan baka Noxa -/- atau Puma -/- untuk menghasilkan Cdkn2a -/-, Tyr-HRASG12V, Noxa -/- dan Cdkn2a -/-, Tyr-HRASG12V, Puma -/- masing masing. Generasi baka ini membolehkan kami menyiasat kepentingan laluan apoptosis intrinsik bagi genesis melanoma. Protein terhad BH3 Puma dan Noxa telah dipilih sebagai protein relevan untuk dieksploitasikan dalam laluan apoptosis atas bukti dari kajian lain yang melaporkan penglibatan mereka dalam biologi melanoma dan respon rawatan. Di sini, kami melaporkan bahawa tumor dari semua ketiga-tiga kohort disahkan sebagai melanoma melalui pengggunaan stain petanda standard emas HMB45, S100 and MelanA. Kehilangan Puma memecut melanomagenesis berbanding dengan kohort kawalan tetapi hanya secara marginal. Sebaliknya, kehilangan Noxa mengurangkan insiden melanoma, menunda genesis melanoma dan mengurangkan bilangan melanoma berbanding dengan baka kawalan dan Puma -/-. Tambahan pula, sebaik

sahaja melanoma tumbuh dalam mencit Noxa -/-, melanoma membesar dengan kadar yang lebih perlahan berbanding dengan melanoma dalam kohort kawalan dan kohort Puma -/-. Walaupun melanoma sering tumbuh di bahagian telinga, bilangan melanoma di bahagian telinga kohort Noxa -/-, ternyata lebih kurang berbanding dengan dua kohort yang lain. Pertumbuhan tumor intrakranial dalam kohort Puma -/- adalah di luar jangkaan kami. Ini menunjukkan bahawa ketiadaan Puma boleh menyumbang kepada pertumbuhan tumor yang jarang ini. Kami mengemukakan dua hipotesis sebagai kemungkinan bagi kelewatan genesis melanoma dalam kohort Noxa -/- (1) pampasan lampau oleh protein terhad BH3 yang mempunyai potensi yang lebih tinggi (e.g. Puma, Bim atau Bid) ataupun (2) kesan bersepadu antara dua protein terhad BH3.

DEFECIANCY OF *Puma* AND *Noxa* FOR MELANOMAGENESIS IN A MOUSE MODEL OF MELANOMA

ABSTRACT

It is commonly believed without sufficient evidence that the impairment of the ability to undergo apoptosis (a mode of programmed cell death) in response to conventional treatment such as cytotoxic drugs, gained melanoma its notorious resistance to therapy. Studies to determine the contribution of the intrinsic apoptosis pathway for melanomagenesis are often deterred by utilisation of *in vitro* cell culture models and xenograft models. An established mouse model of melanoma, the *Cdkn2a -/-*, Tyr-*HRAS*^{G12V}, was utilised in this study to obviate limitations posted by the usage of *in vitro* and xenograft models. The *Cdkn2a -/-*, Tyr-*HRAS*^{G12V} was crossed with either a *Puma -/-* or *Noxa -/-* line yielding in *Cdkn2a-/-*, Tyr-*HRAS*^{G12V}, *Noxa -/-* or *Cdkn2a -/-*, Tyr-*HRAS*^{G12V}, *Puma -/-* strains respectively. Generation of these strains enabled us to investigate the significance of the intrinsic apoptosis pathway for melanomagenesis. The BH3-only proteins *Puma* and *Noxa* were chosen as relevant proteins to be exploited in the apoptosis pathway as there are evidence from other studies reporting on their involvement in melanoma biology and treatment response. Here, we report that tumours from all three cohorts were stained for gold standard markers HMB45, S100 and MelanA and were confirmed as melanoma. Loss of *Puma* only marginally accelerated melanomagenesis compared to the control cohort. On the other hand, loss of *Noxa* decreased melanoma incidence, delayed melanomagenesis and decreased number of melanomas compared to the controls and *Puma -/-* cohorts. Upon establishment, melanomas in the *Noxa -/-* mice, grew

relatively slower compared to melanomas in the control and *Puma* -/- cohorts. Although melanoma frequently established on ears, the *Noxa* -/- cohort developed significantly less ear melanomas compared to the other two cohorts. Unexpectedly, the *Puma* -/- cohort developed intracranial tumours which indicates that absence of *Puma* could contribute to the onset of these infrequent tumours. We hypothesise two possibilities for the delay in melanomagenesis in the *Noxa* -/- cohort (1) overcompensation by more potent BH3-only proteins (e.g. Puma, Bim or Bid) or (2) a concerted effect between two BH3-only proteins.

CHAPTER 1

INTRODUCTION

Melanoma is a type of skin cancer which despite being low in occurrence, is high in its contribution to death tolls due to its nature of being resistant to therapy and high metastasising capacity (Surveillance, Epidemiology and End Results (SEER), 2015). Melanoma can be divided into two subtypes namely the chronically sun-damaged (CSD) melanoma and non-CSD melanoma. CSD melanoma is usually caused by mutation due to overexposure to UV radiation while the non-CSD melanoma is commonly hereditary (reviewed by Shain and Bastian, 2016). The stages of the progression of melanoma are characterised by various genetic mutations, among which, mutation in the *BRAF* and *CDKN2A* genes being the few most common (Tucker et al., 1997, Ciarletta et al., 2011).

Number of reported cases of melanoma has been on the rise and has increased by 200% since 1973 (Reed et al., 2012) with Australia and New Zealand being the two countries with the highest record of melanoma patients in the world (Australian Institute of Health and Welfare (AIHW), 2014). Melanoma in many nations affects the younger population at large and, is most common among those aged 15 to 49 (Cancer Registration Statistics, England, 2013). Melanoma, albeit being scarce, accounts for 0.4% of the total number of male cancer patients and 0.3% of the total

number of female cancer patients in the year 2007, in Malaysia (Omar and Ibrahim Tamin, 2011).

Efforts to fight melanoma has also been increased in parallel to the increasing number of melanoma patients. A number of drugs have been approved by the U.S. Food and Drug Administration (FDA) for melanoma treatment thus far but the effort is not spared from further complication with the involvement of mutation in multiple pathways and the diversity of tumours. Melanoma cells are believed to be resistant to anti-cancer drugs due to their resistance to apoptosis, which is commonly assumed to be mediated by tumour suppressor protein p53 (reviewed by Brown and Attardi, 2005). Hence, cells that are resistant to apoptosis due to mutations in p53 are expected to be recalcitrant to drugs (reviewed by Brown and Attardi, 2005). However, mutation of p53 is rare in melanoma (Soengas and Lowe, 2003). Why then are melanoma still insusceptible to drugs? With these apposing notions, the role of apoptosis in melanomagenesis is questionable. Thus, an appropriate model is needed to address this notion.

The 2D, 3D and xenograft models, despite their many advantages, falls short in several ways in mimicking human melanoma. The 2D models is deprived of the natural environment for cell growth while the 3D model lacks the impairment of the immune system which are of vital importance for tumour development (Beaumont et al., 2014). The xenograft models which require transplantation of cells, subject cells to strong selection for defective apoptosis (Beaumont et al., 2014). Disadvantages in the rest of the models will be overcome in this project by using the *Cdkn2a-/-*, Tyr-*HRAS*^{G12V} mouse model of melanoma. The *Cdkn2a-/-*, Tyr-*HRAS*^{G12V} which mimics common mutations in human melanoma will be crossed with either a *Puma* or *Noxa* deleted mice line, to abrogate the apoptosis pathway. Results of the crossing will be

the generation of *Cdkn2a-/-*, Tyr-*HRAS*^{G12V}, *Noxa -/-* and *Cdkn2a -/-*, Tyr-*HRAS*^{G12V}, *Puma -/-* strain respectively. The pro-apoptotic protein genes *Noxa* (phorbol-12-myristate-13-acetate-induced protein 1) and *Puma* (p53 up-regulated modulator of apoptosis) were selected as the representative genes from the apoptosis pathway to be manipulated due to their common association in melanoma biology and treatment response.

The predisposed genetically modified mouse model will provide us an avenue to assess the degree of involvement of defective intrinsic apoptosis pathway for melanomagenesis through detailed observation of the consequences of the earlier mentioned genetic modifications. We hypothesised that loss of the pro-apoptotic protein genes *Noxa* or *Puma* will to lead to faster appearance, more rapid and invasive growth of melanoma in the mouse model.

The rationale of this work stemmed from the preliminary data that were obtained from Centenary Institute, Sydney, Australia. Preliminary data showed that rather than accelerating melanomagenesis, loss of *Noxa* delayed the onset of melanoma, decreased melanoma penetrance and melanoma burden. Given the number of mice in the cohort was low, the effect of loss of *Noxa* on melanomagenesis might be a statistical fluke. Hence, significantly larger experimental groups would be required to attain better statistical significance and draw a more confident conclusion and this is what is aimed to be achieved in this project. We generated 32 *Noxa -/-* mice and 21 mice *Puma -/-* mice. In total, we had 44 mice in the *Noxa -/-* cohort (32 mice generated in AMDI + 12 mice generated in Sydney) and 39 mice in the *Puma -/-* cohort (21 mice generated in AMDI + 18 mice generated in Sydney). Unfortunately, we could not generate more *Cdkn -/-* mice as the *Cdkn -/-* line was lost most probably due to error in breeding in Australia before

the mice were imported to AMDI, USM, Penang. Therefore, data showed for the *Cdkn -/-* cohort in this chapter, were data generated in Sydney, Australia as none was bred in Malaysia.

Preliminary study of gene expression, particularly the expression of selected pro- and anti-apoptotic genes in mice tumours will be conducted. Cell lines will also be derived from mice tumours for the use in other probable future work.

1.1 Objectives

Objective 1: *To investigate the effect of loss of pro-apoptotic genes, Puma and Noxa for melanomagenesis in a predisposed mouse model of melanoma*.

Objective 2: *To investigate changes in levels of the other pro and anti–apoptotic proteins in the intrinsic apoptosis pathway when either Noxa or Puma is manipulated.*

CHAPTER 2

LITERATURE REVIEW

Incidences of skin cancers have been on the rise over the past decade. Approximately 76,380 cases of melanoma have been diagnosed in the year 2016 (American Cancer Society, Cancer Facts and Figures, 2016). While accounting for only less than 2% of all skin cancers, melanoma is responsible for a large majority of skin cancer deaths due to its resistance to therapy and highly metastatic nature (Surveillance, Epidemiology and End Results (SEER), 2015). In order to understand melanoma, we shall first get to know our skin.

2.1 Getting acquainted with our skin – The Three Prong Fork

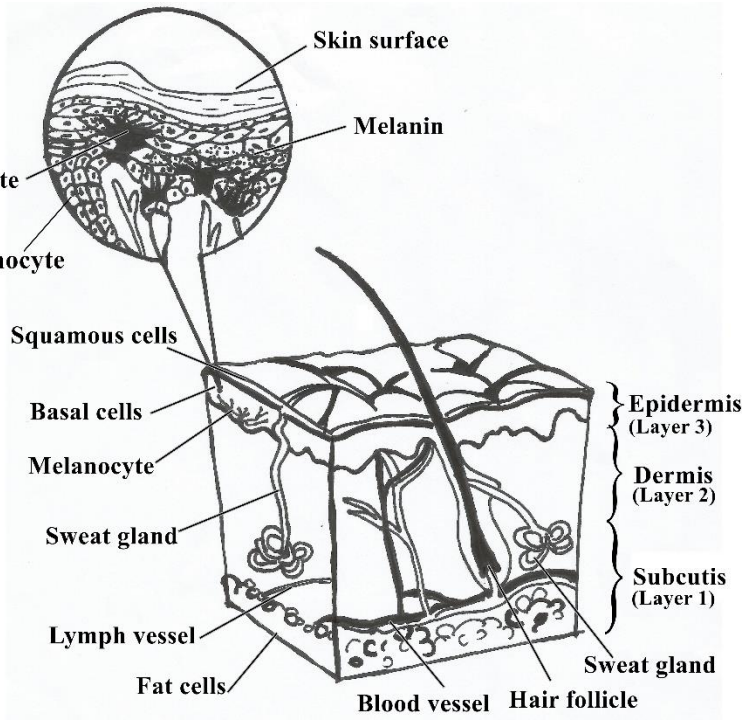


Figure 2.1 Cross section of the skin. The skin can be divided into three major layers namely, the subcutis (layer 1), dermis (layer 2) and epidermis layer (layer 3). The subcutis layer binds the skin to adjacent organs and contains fat tissue for energy storage. The dermis consists of mostly connective tissues and is important for thermal and nutrient regulation due to the rich network of blood and lymphatic vessels. The outer most layer is the epidermis which contains the pigment melanin, produced by melanocytes which gives rise to the colour of our skin. Melanin protects living cells from DNA damage or mutations caused by UV radiation. Melanocytes, keratinocytes and the pigment melanin are shown in the magnification bubble.

Think of the skin as a fork made up of three prongs, each with their individual important roles but nonetheless, interconnected. The first prong, the subcutis or subcutaneous layer, is the inner most layer of the skin which binds the skin to the adjacent organs through loose connective tissues (Mescher, 2013) (Figure 2.1). This layer contains adipocytes that stores energy (Mescher, 2013).

The second prong, the dermis consists of two sub-layers – the papillary layer and the reticular layer consists of mostly connective tissue (McGrath and Uitto 2010, Mescher, 2013). The dermis region supports the epidermis layer and connects it to the subcutaneous tissue (Kolarsick et al., 2011) (Figure 2.1). This layer of the skin plays an important role in nutritive and thermoregulatory functions due to the rich network of blood and lymphatic vessels (Mescher, 2013).

The third and final prong is the outer most layer of the skin called epidermis (Figure 2.1). The epidermis layer is further divided into several subcutaneous layers (Mescher, 2013). The deepest layer, named stratum basale contains melanocytes which are the melanin producing cells (reviewed by Shain and Bastian, 2016) (Figure 2.1 – see magnification bubble). Melanin, a pigment which gives rise to skin colour is contained in a cytoplasmic organelle called melanosome and transported to the most superficial layer of the epidermis called the stratum corneum (Mescher, 2013). At the stratum corneum, melanosomes accumulate within keratinocytes.

Keratinocytes are matured basal cells which act as protective barrier of living cells against environmental threats such as virus, bacteria, fungi and water loss as well as UV radiation (reviewed by Shain and Bastian, 2016). Melanin within the keratinocytes absorb and scatter UV radiation as harmless heat to protect living cells from DNA damage or mutation (Mescher, 2013). Having said that, exposure to UV radiation remains the major risk factor for development of melanoma. Exposure to UV weakens the immune system and aids in the formation of DNA damaging reactive oxygen species (ROS). Other risk factors which contribute to melanoma include family history of melanoma, previous diagnosis of melanoma, immunosuppression and multiple benign (>100) or dysplastic nevi (>6) (Rigel and Carucci, 2000, Miller and Mihm, 2006, Ibrahim and Haluska, 2009).

2.2 Introduction to melanoma

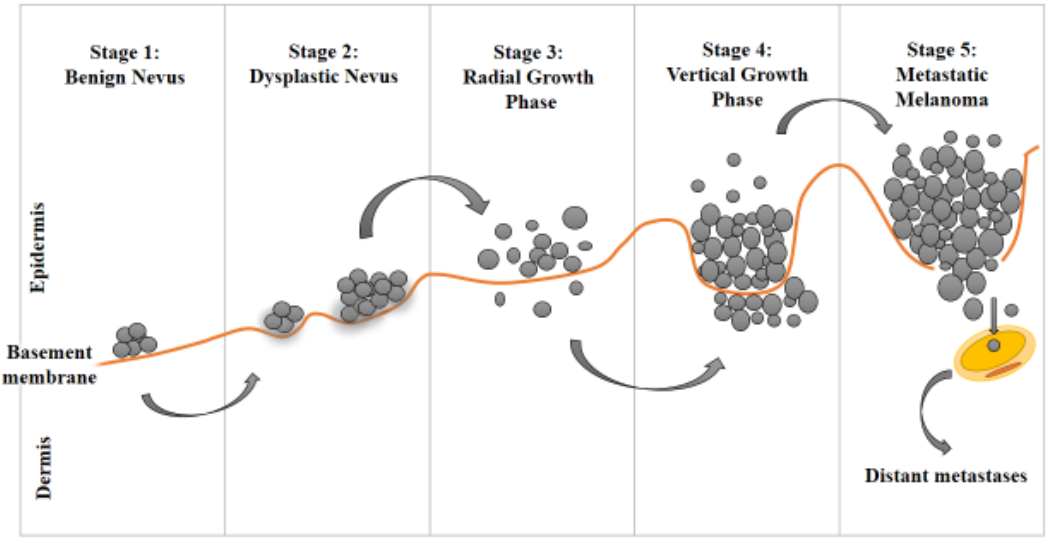
Melanoma originates in the melanocytes and is the deadliest skin cancer as it highly metastatic and therapy resistant. Melanoma can be divided into two subtypes namely the chronically sun-damaged (CSD) melanoma and non-CSD melanoma (reviewed by Shain and Bastian, 2016). The CSD melanoma are highly associated to mutation and damage done by UV radiation through primarily high levels of sun exposure and thus occupies areas such as the head and neck which are most exposed to the sun in comparison to other parts of the body (reviewed by Shain and Bastian, 2016). CSD melanoma are usually diagnosed in patients over the age of 50 (Bastian, 2014). On the other hand, the non-CSD melanoma are commonly hereditary. The presence of epidermal nevus which are commonly known as moles or birthmarks, often increases the risk of melanoma in young patients, usually in the first decade of human life (reviewed by Criscito et al., 2016, Shain and Bastian, 2016). Mutations in

CSD melanoma are usually of the *NRAS* and *BRAF*^{nonV600E} while mutation in non-CSD melanoma are commonly of the *BRAF*^{V600E} (Curtin et al., 2005). However, mutation which causes melanoma is not only confined to the genes mentioned above, but may affect many other proteins in pathways which controls cell proliferation such as NF1 (Krauthammer et al., 2015) and cell cycle control such as the CDKN2A which encodes tumour suppressor proteins p16^{INK4A} and p14^{ARF} (Fargnoli et al., 1998). The *CDKN2A* and *CDK4* located on chromosome 9p21 and 12q14 respectively are strongly associated with family history of melanoma (Meyle and Guldberg, 2009). The C*DKN2A* gene locus will be further elaborated in section 2.6.

The major potential cause of CSD melanoma is sun exposure, in the form of UVA and UVB (Gandini et al., 2005). Penetration of UVB is enhanced by the increasing depletion of the ozone layer leading to a higher risk for UV-induced carcinogenesis (Fabo, 2005). The risks of melanoma is also on the rise with the rising popularity of sunbedding for aesthetic purposes (The International Agency for Research on Cancer Working Group on artificial ultraviolet and skin, 2007). Non-CSD melanoma usually caused by other endogenous risk factors of skin cancer such as number of melanocytic nevi, phototype, skin and eye colour, presence of dysplastic nevi, and personal history or hereditary of skin cancer (Fagundo et al., 2011).

The evolution of melanoma occurs in five stages based on the Clark model (Wallace et al., 1984). An illustration of the stepwise progression of melanoma is as shown in Figure 2.2. The first stage, the benign melanocyte nevi is the commonly acquired mole caused by the proliferation of normal melanocytes. The second stage of the progression, the dysplastic nevus, is caused by *BRAF* mutation in pre-existing nevus or at a new location. This stage is characterised by the formation of random

and asymmetric lesions of multiple pigmentation with irregular border (Tucker et al., 1997). Dysplastic nevus which experience genetic mutation, for instance, a silenced *CDKN2A* tumour suppressor gene with an activated MAPK pathway will progress to the radial growth phase (RGP). Cells at this phase will acquire the ability to proliferate intra-epidermally, but have no ability to metastasise and is confined to the epidermis (Ciarletta et al., 2011). With the loss of E-cadherin, the progression enters the vertical growth phase (VGP) where cells acquire the ability to invade the dermis and subcutaneous tissue. The final stage of the progression is metastatic melanoma that can spread to other areas of the body, usually first to lymph nodes then to skin, subcutaneous soft tissue, lungs, liver and the brain (Ibrahim and Haluska, 2009).



Adapted from: McMaster Pathophysiology Review

Figure 2.2 Stepwise evolution of melanoma based on the Clark model. Stage 1: Benign nevus, Stage 2: Dysplastic nevus; Stage 3: RGP; Stage 4: VGP; Stage 5: Metastatic melanoma. Once melanoma cells are independent from the control of keratinocytes, they breach the basement membrane and are able to interact with fibroblasts, lymphatic and vascular endothelial cells, which allow them to eventually invade to other areas of the body.

2.3 Melanoma around the world

Incidences of melanoma is definitely of no stranger to the society and have caused deaths in a large part of the world both in the northern and southern region of the globe, especially within the Caucasian populations. New Zealand and Australia are the two countries with the highest number of melanoma in the world (Australian Institute of Health and Welfare (AIHW), 2014). It was estimated that one person becomes a victim of melanoma in every six hours in Australia specifically in Queensland (Australian Institute of Health and Welfare (AIHW), 2014). A ratio of 71 out of 10,000 people in the year 2009 to 2013 was diagnosed with melanoma in Queensland alone, exceeding all the other number of cases reported worldwide (Australian Institute of Health and Welfare (AIHW), 2014).

A recent study reported on the projection of future melanoma incidences in six populations (US whites and populations of the United Kingdom, Sweden, Norway, Australia and New Zealand) (Whiteman et al., 2016). Melanoma incidences in the US whites and the populations of the UK, Sweden and Norway have always been in the rise and are projected to continue increasing until 2022. Melanoma burden in Australia has been declining at -0.7% each year since 2005 but is still on the rise in New Zealand although it is estimated to decline soon (Whiteman et al., 2016). Nonetheless, new melanoma cases are projected to increase in all six populations because of the longer age expectancy (Whiteman et al., 2016). Moving to South America, in Brazil, 1,073 (543 male patient, 530 female patient) cases of melanoma was reported in 2015 (Vazquez et al., 2015).

Unlike the aforementioned countries, melanoma is rare in Malaysia. According to the National Cancer Registry Report, 2007, a total of 61 cases of melanoma was reported (Omar and Ibrahim Tamin, 2011). Among the 61 cases

reported, 34 were males - 11 Malay, 10 Chinese and 2 Indians. Out of the total of 27 female melanoma patients, 14 of them were Malays, 11 were Chinese and one Indian (Omar and Ibrahim Tamin, 2011). In a study conducted at the University Malaya Medical Centre from 1998 to 2008, 32 cases of cutaneous melanoma were recorded within the ten years of the study among which, the largest group of 19 people were Chinese patients, followed by 10 Malays and 3 Indians (Pailoor et al., 2012). According to a news report from the Star newspaper in February 2015, data from the Dermatology Clinic, Hospital Kuala Lumpur from year 2006 to 2014, revealed that melanoma is uncommon in Malaysia as it only accounted for 5.4% of the patients in the Dermatology Clinic of Hospital Kuala Lumpur.

2.4 Therapies for melanoma

Survival rate of melanoma falls drastically once it enters the metastatic stage (Coventry et al., 2015). Melanoma among all skin cancers, has possibly the highest proficiency to spread to other parts of the body and is resistant to many therapies (Wu and Singh, 2011, Braeuer et al., 2014). Treatment methods of melanoma is usually given based on the stage of melanoma. Some treatment methods include surgery, immunotherapy, radiation therapy, chemotherapy and targeted therapies.

Melanoma at the early stage is usually treated by surgery. However, adjuvant therapy with drugs is needed when melanoma has metastasised to other parts of the body. Dacarbazine (DTIC) was among the first drug to be developed for the treatment of metastatic melanoma in 1975. However, treatment of melanoma went into to its dark age for the next 20 years as no other drug was developed until the year 1995. A better understanding of the role of the immune system in melanomagenesis, brought to the development of three drugs namely Intron A,

Proleukin and Sylatron for treatment of metastatic melanoma. From then onwards, development of drugs for treatment of melanoma took flight with more understanding of proteins and signalling pathway mutations (e.g. *BRAF*) involved in melanomagenesis (eg. Zelboraf, Mekinis, Tafinlar). Over the years, a number of drugs have been approved by the U.S. Food and Drug Administration (FDA) for melanoma treatment to be used as single agent or in combination.

As many cancer researchers are embracing the personalised medicine approach for cancer treatments and care, existing cancer treatment drugs have been exploited for their potential in this treatment approach. Personalised medicine utilises understanding of the unique genetic and clinical information of each individual patient at a molecular level in order to optimise the strategy used for their treatment and health care (Chan and Ginsburg, 2011, Foroutan B, 2015).

In personalised medicine, drugs are given to patients according to the type of genetic alteration in the various pathways involved in melanomagenesis among which the common ones are the MAPK (mitogen-activated protein kinases) and PI3K/AKT (phosphatidylinositol-4,5-bisphosphate 3-kinase/ protein kinase B) signalling pathways. The MAPK pathway allows the transfer of signals from the receptor on the surface of a cell to the cell's DNA through a chain of proteins, while the PI3K/AKT pathway regulates cell cycle in response to extracellular signals (reviewed by Sun et al., 2015, Maryu et al., 2016). Regulation of the pathway often involve cross-talking with other signalling pathways such as the MAPK pathway (Mendoza et al., 2011). Both these pathways are important in signaling apoptosis, cell proliferation and growth (reviewed by Chang et al., 2003, Sun et al., 2015, Maryu et al., 2016). As such, mutation in either or both of these pathways result in

development of cancer (reviewed by Pappalordo et al., 2016). The MAPK pathway is further elaborated in section 2.5 reflecting its focus in this project.

Drugs such as vemurafenib (Chapman et al., 2011) and trametinib (Infante et al., 2012) which are specific inhibitors of BRAF and the MAPK pathway respectively, have been widely used in the field of targeted therapies in personalised medicine with remarkable responses. Imatinib has been used for treatment of melanoma patients with C-KIT gene (activated KIT transmits signal via the MAPK pathway (reviewed by Zhang and Liu, 2002) alterations (Hodi et al., 2008). Despite the great early outcome, tumours treated with these inhibitors become irresponsive to their specific inhibitors due to additional alterations in the MAPK pathway namely mutations in neuroblastoma RAS viral oncogene homolog (NRAS) (Nazarian et al., 2010) and mitogen-activated protein kinase (MEK) (Wagle et al., 2011), amplifications or slice variants of BRAF (Whittaker et al., 2010, Poulikakos et al., 2011, Shi et al., 2012) or CRAF (Montagut et al., 2008) (Figure 2.3). Other resistance factors include loss of neurofibromatosis (NF1) (negative regulator of the ras signal transduction pathway) (Whittaker et al., 2013, Maertens et al., 2013), increased expression of Cancer Osaka Thyroid (COT) (a MAPK antagonist) (Johannessen et al., 2010), upregulation of receptor tyrosine kinases namely the beta-type platelet-derived growth factor receptor (PDGFRβ) (Nazarian et al., 2010) and insulin-like growth factor 1 (IGF-1R) (Villanueva et al., 2010) which activates the PI3K/AKT pathway or loss of tumour suppressor protein, phosphatase and tensin homolog (PTEN) (PTEN inhibits the activation of the PI3K/AKT pathway) (Paraiso et al., 2011). In order to overcome resistance, current treatment strategies aim at treating melanoma cells with combination of MAPK and PI3K pathway inhibitors.

Besides targeting the proliferative pathways (e.g. MAPK and/or PI3K/AKT pathway(s)), the BCL-2 family proteins, the critical regulators of the intrinsic apoptosis pathway, have also gained attention as attractive therapeutic targets for melanoma treatment. The interest to target these proteins mounted with the development of small molecule inhibitors such as ABT-737 and now ABT-263 (Navitoclax, AbbVie) which specifically target these proteins. The BCL-2 family proteins became ideal targets for treatment, given the perception that melanoma cells are exceptionally resistant to apoptosis. Based on this perception, substantial amount of time and cost were invested in developing antisense BCL-2 and BH3 mimetics to target melanoma cells. Although much time and effort were invested in developing drugs to target the BCL-2 proteins, there is surprisingly very little effort focused on understanding the contribution of the intrinsic apoptosis pathway for melanoma biology and whether it affects drug resistance. The broad aim of the project is to understand the contribution of the intrinsic apoptosis pathway for melanoma biology and drug resistance. This project particularly focuses on comprehending the involvement of the intrinsic apoptosis pathway for melanomagenesis. These points will be further expanded in the following sections, which will first introduce the MAPK pathway, *CDKN2A* gene locus and BCL-2 family members. The last two sections will discuss the missing link between the intrinsic apoptosis pathway and melanomagenesis, and the advantages of using genetically engineered mouse models in our study.

2.5 The Mitogen Activated Protein Kinase (MAPK) Pathway

The MAPK pathway (Figure 2.3) is involved in a variety of fundamental cellular processes and is frequently abrogated in melanoma. The pathway is activated

by a number of growth factors including Stem Cell Factor (SCF), Fibroblast Growth Factor (FGF) and Hepatocyte Growth Factor (HGF) (Bohm et al., 1995) which stimulate membrane-bound receptor tyrosine kinases (RTKs). Activation of RTK, phosphorylates RAS. There are three isoforms of RAS: HRAS, NRAS and KRAS (Castellano and Santos, 2011). Activated RAS phosphorylates RAF which is a serine/threonine kinase (Figure 2.3). Similar to RAS, RAF has three isoforms: ARAF, BRAF and RAF1 (also known as CRAF) (reviewed by An et al., 2015). Phosphorylated RAF in turn phosphorylates another serine/threonine kinase, MEK (exists as two isoforms: MEK1 and MEK2). Activated MEK phosphorylates ERK which exists as two isoforms ERK1 and ERK2 (reviewed by Buscà et al., 2016) (Figure 2.3). ERK1 and 2 induce genes that are involved in cell proliferation and protects cells from being apoptosed (Sekulic et al., 2008, Haass et al., 2008).

Constitutive activation of the *HRAS* transgene in the melanocyte lineage was required for the manifestation of spontaneous melanoma in the *Cdkn2a -/-* mouse model. Requirement for the constitutive activation of the *HRAS* transgene, reiterates the importance of the MAPK pathway for melanoma establishment. The constitutive activation of the *HRAS* transgene recapitulates the activation of the MAPK pathway in human melanomas as would normally occur by mutations in *NRAS* and *BRAF*.

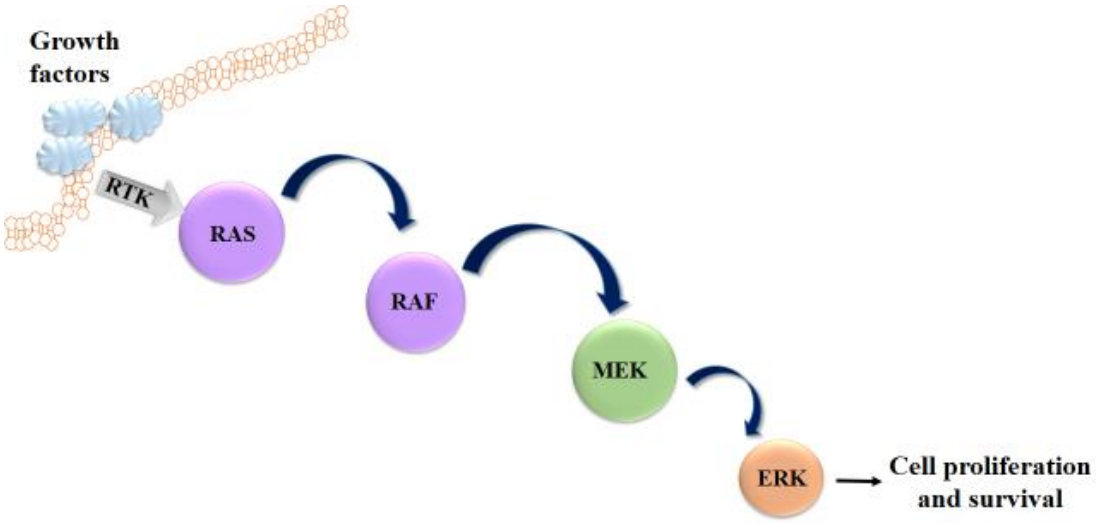
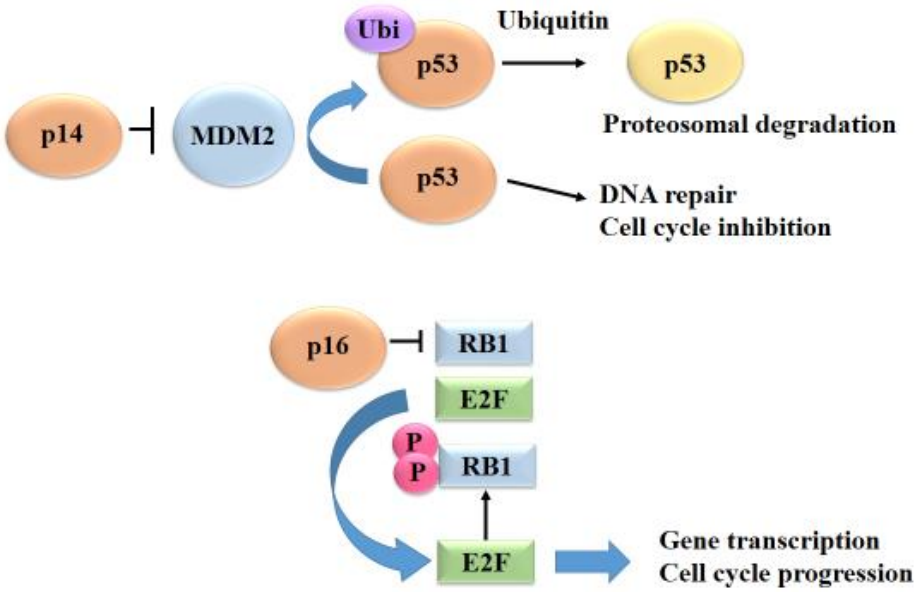


Figure 2.3 The Mitogen Activated Protein Kinase (MAPK) Pathway. The pathway is activated by various growth factors which stimulate membrane bound receptor tyrosine kinases (RTKs). Activation of RTK, phosphorylates RAS. Phosphorylated RAS phosphorylates MEK which in turn phosphorylates ERK.

2.6 The *CDKN2A* gene locus

The *CDKN2A* (Cyclin-Dependent Kinase Inhibitor 2A) gene encodes two distinct tumour suppressor proteins with overlapping reading frames $p16^{INK4A}$ (cyclin dependent kinase inhibitor 2A) and $p14^{ARF}$ (alternative reading frame) (Ouelle et al., 1995). $P19^{ARF}$ is the mouse homolog of human $p14^{ARF}$ (Fecher et al., 2007). Mutation in the *CDKN2A* gene locus which is common in familial melanoma can occur in either or both of the $p16^{INK4A}$ or $p14^{ARF}$ gene causing deregulation of cell cycle progression (reviewed by Zhao et al., 2016). The $p16^{INK4A}$ tumour suppressor protein inhibits the phosphorylation of the Retinoblastoma protein (pRb). PRb in its hypophosphorylated form, inhibits the E2F transcription factor and represses induction of genes required for cells to enter the S phase from the G1 cell cycle phase (reviewed by Zhao et al., 2016) (Figure 2.4). $P16^{INK4A}$ loss leads to hyperphosphorylation of the pRb protein which in turn liberates E2F. E2F then induces genes which are required for the unscheduled entry of cells into S phase

from G1 (reviewed by Zhao et al., 2016) (Figure 2.4). The ARF tumour suppressor protein stabilises p53 by inhibiting MDM2 (human homolog HDM2). P53 senses cell stress signals such as DNA damage caused by chemotherapeutic drugs and activate the apoptosis pathway (Figure 2.7). In the absence of ARF, MDM2 targets p53 for degradation through the ubiquitin-proteasome pathway (reviewed by Pant and Lozano, 2014) (Figure 2.4).



Adapted from: Griewank et al., 2014

Figure 2.4 The *CDKN2A* gene locus. Products of the *CDKN2A gene* locus, $p16^{INK4A}$ and $p14^{ARF}$ negatively regulate the pRb (retinoblastoma protein) and p53 pathway respectively. Loss of the *CDKN2A* locus leads to uncontrolled cell proliferation and evasion from apoptosis.

The mouse model of melanoma utilised in this study was established based on known history of mutation in the *CDKN2A* locus which is often found in familial melanoma (Hussussian et al., 1994, Kamb et al., 1994). In order to investigate the roles of $p16^{Ink4a}$ and $p19^{Arf}$ in melanoma, mice models harbouring deletions in either genes were established (Chin et al., 1997). Unfortunately the *$p16^{Ink4a}$* null mice

developed melanoma with very low penetrance (Chin et al., 1997) and the *p19*^{Arf} null mice developed zero melanomas (Chin et al., 1997). With the realisation that loss of either *p16*^{Ink4a} or *p19*^{Arf} is insufficient for melanomagenesis, the *HRAS* transgene, active in the melanocyte lineage was crossed to the initial models and this time, the established models developed spontaneous melanomas. The latency of melanoma formation was however, slow with median latencies of 40 weeks (Chin et al., 1997). The model was then improved by deleting both *p16*^{Ink4a} and *p19*^{Arf} with constitutive activation of the *HRAS* transgene (Chin et al., 1997, Chin et al., 1999). The improved model developed spontaneous melanoma with high frequency at a median latency of 22 weeks thus confirming the importance of both p16^{Ink4a} and p19^{Arf} for melanoma suppression.

2.7 The BCL-2 family members

The BCL-2 family proteins are the key regulators of the intrinsic apoptotic pathway (reviewed by Delbridge et al., 2016). The members of the family share one or more of the BCL-2 homology (BH) domains namely, BH1, BH2, BH3 and BH4 which give the members the ability to interact with one another (reviewed by Delbridge et al., 2016). The BCL-2 family members can be classified into two groups: anti-apoptotic proteins and pro-apoptotic proteins. The pro-apoptotic proteins can be further divided into the pro-apoptotic effector proteins, and the BH3-only pro-apoptotic proteins (Figure 2.5). The BH3-only proteins, only share the BH3 domain with the rest of the sub-groups (Figure 2.5). The transmembrane (TM) domain which is present in all proteins with the exception of BFL-1, BAD, BID, BMF and PUMA, facilitates association of the proteins with the outer mitochondrial membrane (OMM) (reviewed by Delbridge et al., 2016) (Figure 2.5).

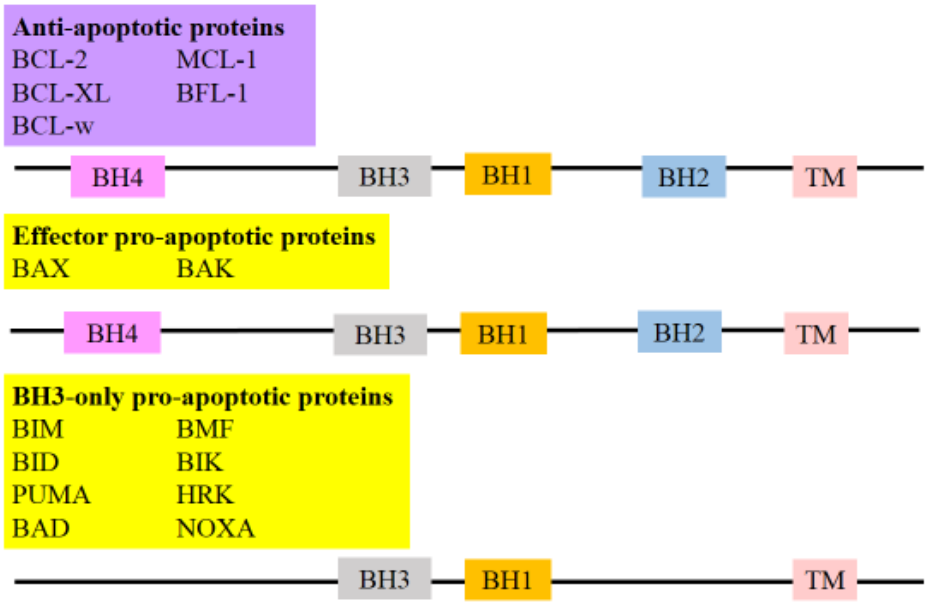


Figure 2.5 The BCL-2 family members and their BH domains. BCL-2 family members are divided into two groups: the anti-apoptotic proteins and the pro-apoptotic proteins (effector proteins and BH3-only proteins). The anti-apoptotic and effector pro-apoptotic proteins contain all four BH domains while BH3-only pro-apoptotic proteins only share the BH3 domain with the other proteins. The transmembrane (TM) domain which is usually present in all members except for BFL-1, BAD, BID, BMF and PUMA, facilitates association of the proteins with the outer mitochondrial membrane (OMM).

The anti-apoptotic members are BCL-2 (B cell CLL/lymphoma-2), BCL-XL (BCL-2-related gene, long isoform), BCL-w (B-cell lymphoma-2 like protein), MCL-1 (myeloid cell leukaemia 1) and BFL-1/A1 (BCL-2-related gene A1) (reviewed by Delbridge et al., 2016). They have all the four BCL-2 homology domains (BH1 to 4) and inhibit cell death by binding to the BH3-only proteins or to activated BAK and BAX (reviewed by Llambi et al., 2011) (Figure 2.7).

The pro-apoptotic effector proteins consist of BAX (BCL-2-associated X protein), BAK (BCL-2 antagonist killer) and BOK (Bcl-2 related ovarian killer) (reviewed by Delbridge et al., 2016). BAX and BAK are ubiquitously expressed while the rest of the BCL-2 family members are tissue specific and is only expressed when stimulated (reviewed by Delbridge et al., 2016). BAX is a monomeric protein

in the cytosol that only travels to the mitochondria upon inactivation of the anti-apoptotic proteins by the BH3-only proteins (Fröhlich et al., 2014). Activated BAX inserts into the OMM and oligomerises with BAK to induce MOMP (Gillies and Kuwana, 2014). The induction of MOMP by BAK and BAX can occur through either the formation of pore or ion channels by BAK and BAX (Lucken-Ardjomande and Martinou, 2005, Kinnally and Antonsson, 2007) or, through the unity of BAX and BAK with pre-existing ion channel(s) such as the voltage-dependant anion channel (VDAC) (Shimizu et al., 1999, Cheng et al., 2003). Expression of Bok is only found to be restricted to ovarian granulosa cells and several reproductive tissues characterized by hormonally regulated cyclic cell turnover (Hsu et al., 1997). Unlike BAX and BAK, BOK is not sensitive to antagonist effects of anti-apoptotic proteins and induces MOMP independently of other BCL-2 family members (reviewed by Llambi et al., 2016). These suggests that BOK is regulated hormonally and thus is only expressed in reproductive organs.

The BH3-only pro-apoptotic proteins only share the BH3 domain with the other members, hence their name. The members are: BAD (BCL-2-associated death promoter), BIK (BCL-2 interacting killer), Hrk (Harakiri), NOXA (Phorbol-12-myristate-13-acetate-induced protein 1), PUMA (p53-up-regulated modulator of apoptosis), BID (BH3 interacting-domain death agonist), BIM (BCL-2-interacting mediator) and BMF (BCL-2 modifying factor) (reviewed by Kiraz et al., 2016).

The BH3 domain is an amphipathic α-helix that serves as a binding motif for interaction with a hydrophobic groove on either multidomain anti- or pro-apoptotic BCL-2 family members (Shamas-Din et al., 2013). BID, BIM and PUMA are non-selective and has binding motif that allows them to bind and inhibit all of the anti-apoptotic proteins. BAD binds and inhibits BCL-2, BCL-XL and BCL-w. BIK and

Hrk bind to BCL-XL, BCL-w and BFL/A1. The binding motif on NOXA can only fit to the groove of MCL-1 and BFL1/A1 thus, making NOXA's inhibition specific to MCL-1 and BFL1/A1 only (reviewed by Besbes et al., 2015) (Figure 2.6).

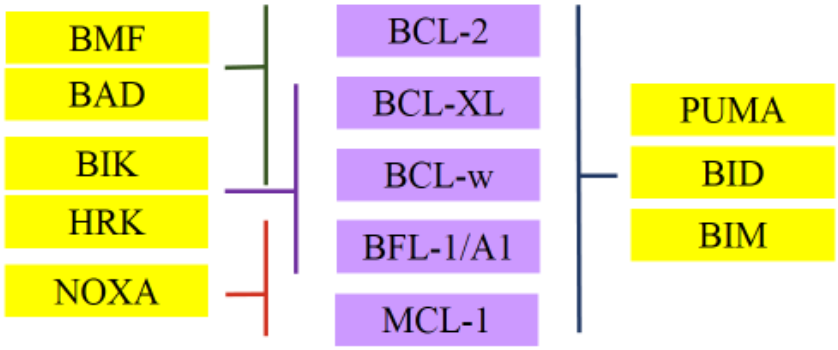


Figure 2.6 Selective inhibition of the anti-apoptotic proteins by some members of the BH3-only proteins. PUMA, BID and BIM inhibit all anti-apoptotic proteins. HRK and BIK inhibit BCL-XL, BCL-w and BFL-1/A1. BAD and BMF inhibit BCL-2, BCL-XL and BCL-w. NOXA specifically inhibits MCL-1 and BFL-1/A1. Yellow boxes: BH3-only pro-apoptotic proteins; purple boxes: anti-apoptotic proteins, solid lines: direct inhibition and different line colours indicate specific inhibition of the anti-apoptotic proteins by the BH3-only pro-apoptotic proteins.

2.8 Apoptosis Pathways

Apoptosis is a mode of programmed cell death. The pathway is important in the maintenance of normal cell turnover and proper function of the immune system, embryo development, hormone-dependant atrophy, and chemical induced cell death (reviewed by Onal et al., 2016). Abrogation of the pathway can lead to cancer manifestation or degenerative diseases (Tait and Green, 2010). Cells engage the apoptosis pathway following various stress signals, such as DNA damage or endoplasmic reticulum stress caused by chemotherapeutic agents, activation of oncogenes, growth-factor deprivation and ultraviolet radiation (reviewed by Mohana-Kumaran et al., 2014). Apoptosis can be initiated through the intrinsic apoptosis pathway (the mitochondrial pathway) or extrinsic apoptosis pathway (death receptor

pathway). The extrinsic pathway will be briefly introduced and the intrinsic pathway and its associated proteins will be discussed at a greater length, reflecting the focus of this study.

2.8.1 The Extrinsic Apoptosis Pathway

Activation of the extrinsic pathway begins when death receptors (DR) located at the plasma membrane are activated by their respective ligands namely FASL, TNF and TRAIL. Members of the DR includes the tumour necrosis factor (TNF), related apoptosis-inducing ligand (TRAIL) receptor 1 (other names: DR4 and TNFRSF10A), TRIAL2 (other names: DR5 and TNRSF10B), FAS (other names: CD95 and APO1) and TNF receptor I (other name: TNFRSF1A) (reviewed by Ichim and Tait, 2016). The death receptors contain a cystolic "death domain" which enables them to interact with other cystolic proteins. Cystolic FADD (FAS-associated death domain), caspases-8 and -10 are recruited by the receptors once the ligands bind to their respective death receptor (Figure 2.7). Activated caspase-8 in turn, activates executioner caspases-3 and -7 which initiate the proteolytic mechanism of apoptosis in cells (reviewed by Ichim and Tait, 2016) (Figure 2.7). The extrinsic pathway crosstalks with the intrinsic pathway through the actions of caspase-8. Caspase-8 truncates and activates BH3-only protein BID to its truncated form, tBID (Li et al., 1998, Luo et al., 1998). The pathway thereafter, follows the intrinsic pathway (Figure 2.7).

2.8.2 The Intrinsic Apoptosis Pathway

The intrinsic apoptosis pathway can be triggered by various extracellular and intracellular stresses such as DNA damage caused by chemotherapeutic agents,

growth factor deprivation, ultra violet radiation and oncogene activation (reviewed by Mohana-Kumaran et al., 2014). For example, damage in the DNA caused by chemotherapeutic agents lead to p53 accumulation in the cells. Accumulation of p53 activate the BH3-only proteins. Induction of the BH3-only proteins lead to inhibition of the anti-apoptotic proteins (Figure 2.7). As a result, BAX and BAK oligomerise and induce mitochondrial outer membrane permeabilisation (MOMP) which leads to the release of cytochrome c (see section 2.7 on the possible mechanisms how BAX and BAK oligomerise and induce MOMP). Cytochrome c together with APAF-1, form apoptosome and activate initiator caspase-9 (Figure2.7). Activation of caspase-9 in turn activates a series of executioner caspases-3, -6 and -7 and finally, activation of apoptosis in cells (Kiraz et al., 2016) (Figure 2.7).

Caspases are a family of genes important for maintaining homeostasis through regulating cell death and inflammation (Enari et al., 1998). Caspases-3 and -7 cleave a large set of substrates, for example, resulting in the characteristic morphological and biochemical characteristics of apoptosis such as nuclear condensation, phosphatidylserine exposure, and genomic DNA fragmentation (Lamkanfi and Kanneganti, 2010). For example, caspase-3 cleaves Inhibitor of Caspase Activated DNase (ICAD), causing CAD to become activated. CAD cleaves DNA and degradation of nucleus DNA results in apoptosis (Enari et al., 1998).

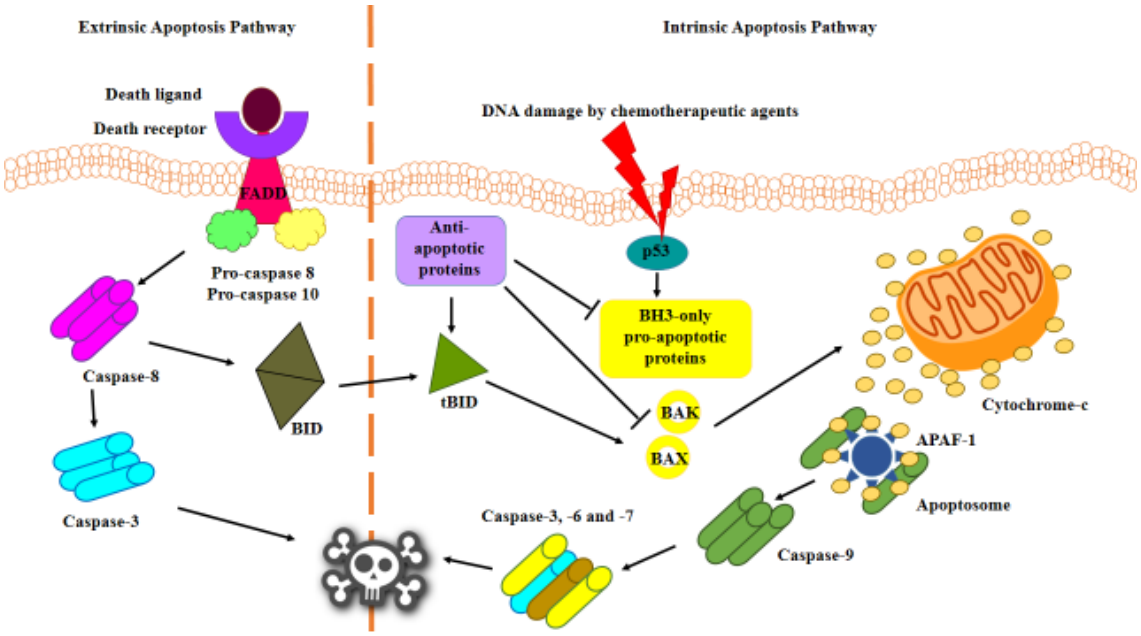


Figure 2.7 The Apoptosis Pathways. The intrinsic pathway can be induced by the accumulation of p53 as a response to various cellular stresses. The accumulation of p53 protein activate the BH3-only pro-apoptotic proteins which in turn inhibit the anti-apoptotic proteins leading to BAX/BAK oligomerisation. The oligomerisation of effector proteins BAX/BAK induces MOMP resulting in cytochrome-c release to the cytoplasm. Cytochrome-c together with APAF-1 form the apoptosome and activate caspase-9. Activation of caspase-9, in turn activates executioner caspases-3, -6 and -7 which consequences in apoptosis. The extrinsic apoptosis pathway is activated by death ligands and their individual death receptors which leads to the recruitment of FADD as well as caspases-8 and -10. Activated caspases-8 and -10 activate executioner caspases-3, -6, and -7 and subsequently, activation of apoptosis. The intrinsic and extrinsic pathway crosstalk through the actions of caspase-8. Caspase-8 activates BH3-only protein, BID to its truncated form, tBID. Truncated BID activates BAX and BAK directly which leads to the activation of the intrinsic pathway. Solid lines: direct inhibition or activation, yellow boxes: BH3-only pro-apoptotic proteins, purple box: anti-apoptotic proteins, and skull: activation of apoptosis in the cell.

2.9 Melanomagenesis and the Intrinsic Apoptosis Pathway – The Missing Link

It has been widely accepted without much evidence that treatment of cancer cells with chemotherapeutic drugs, induce apoptosis mediated by tumour suppressor protein p53. Hence, it is believed that cells that are resistant to apoptosis due to inactivation or loss of p53 will be recalcitrant to drugs (reviewed by Brown and