From Department Of Oncology-Pathology
Karolinska Institutet, Stockholm, Sweden

Hitting the right targets: A study of molecular mediators of sensitivity to novel potential targeted therapies in cutaneous malignant melanoma

Ishani Das
ঈশানি দাস
इशानी दास

Karolinska Institutet

Stockholm 2020

Published by Karolinska Institutet.
Printed by Eprint AB 2020

ISBN 978-91-7831-766-0

Hitting the right targets: A study of molecular mediators of sensitivity to novel potential targeted therapies in cutaneous malignant melanoma
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Ishani Das

Principal Supervisor:
Suzanne Egyházi Brage, PhD
Karolinska Institutet
Department of Oncology-Pathology

Co-supervisor(s):
Rainer Tuominen, PhD
Karolinska Institutet
Department of Oncology-Pathology

Johan Hansson, MD, Professor Emeritus
Karolinska Institutet
Department of Oncology-Pathology

Ulrika Warpman Berglund, PhD
Karolinska Institutet
Department of Oncology-Pathology

Opponent:
Andrew E. Aplin, Professor
Thomas Jefferson University, Philadelphia, USA
Department of Cancer Biology

Examination Board:
Nico Dantuma, Professor
Karolinska Institutet
Department of Cellular and Molecular Biology

Gustav Ullenhag, MD, Associate Professor
Uppsala University
Department of Immunology, Genetics and Pathology

Leonard Girnita, MD, Associate Professor
Karolinska Institutet
Department of Oncology-Pathology

To my family

'Winners….are not those who never fail, but those who never quit'

- APJ Abdul Kalam

ABSTRACT

Cutaneous malignant melanoma (CMM) is the deadliest form of skin cancer with a 5-year overall survival (OS) rate of less than 10% among patients diagnosed with disseminated disease. During the past decade the emergence and use of novel targeted therapy and immunotherapy has significantly increased the 5-year OS rate (34%). There are some CMM patients who harbor primary resistance to current therapeutic regimes and hence never respond to treatment, whereas a major subset of the patients with objective response ultimately develops a refractory disease due to acquired resistance. Moreover, clinical biomarkers that can be used to find patient subgroups who are most likely to benefit from a particular therapy remains elusive.

In paper I, we found that the combination treatment of ERBB family inhibitor afatinib and MET/ALK inhibitor crizotinib was cytotoxic to CMM cells independent of *BRAF/NRAS* mutation status. The observations were validated both *in vitro* and *in vivo*.

In paper II, we explored the molecular mechanisms behind the combination treatment effect of afatinib and crizotinib. We found that the combination treatment downregulated IRS-1, RPS6KB1 and RPS6 protein expression. PMEL/Melanoma gp-100 and PI3K-p85 were upregulated in cells with induced resistance to the combination. We also showed that the resistance to the combination was reversible after a drug holiday.

In paper III, we found that the efficacy of MTH1 inhibitor TH1579 is independent of *BRAF/NRAS* mutation status. TH1579 abrogated cell growth both *in vitro* and *in vivo*. We showed that AXL and CAV-1 play a role in mediating sensitivity to TH1579. Moreover, combination treatment of BRAF inhibitors with TH1579 further potentiated cell death in *BRAF* mutant CMM cells, including BRAF inhibitor resistant CMM cells.

In paper IV, we showed that co-expression of MTH1 and PMS2 mRNA, key players in the DNA damage response pathway, is associated with shorter progression free survival after immunotherapy in CMM. We also found that these proteins were increased in refractory tumors. Finally, we showed that co-silencing of MTH1 and PMS2 further induced apoptosis compared to silencing of either gene alone in patient-derived short-term CMM cell cultures.

Overall this thesis highlights potential novel targeted therapeutic strategies for patients with CMM and suggests possible alternate molecular markers that can be targeted to overcome resistance mechanisms that currently pose as a serious clinical challenge in the systemic treatment of CMM.

LIST OF SCIENTIFIC PUBLICATIONS

I. Combining ERBB family and MET inhibitors is an effective therapeutic strategy in cutaneous malignant melanoma independent of *BRAF/NRAS* mutation status
Ishani Das, Margareta Wilhelm, Veronica Höiom, Rodolfo Franco Marquez, Fernanda Costa Svedman, Johan Hansson, Rainer Tuominen, Suzanne Egyházi Brage
Cell Death Dis **10,** 663 (2019)

II. Downregulation of the insulin/MTOR signaling pathway by afatinib and crizotinib combination treatment confers broad cytotoxic effects in cutaneous malignant melanoma

Ishani Das, Huiqin Chen, Gianluca Maddalo, Rainer Tuominen, Vito Rebecca, Meenhard Herlyn, Johan Hansson, Michael Davies, Suzanne Egyházi Brage
Manuscript

III. AXL and CAV-1 play a role for MTH1 inhibitor TH1579 sensitivity in cutaneous malignant melanoma
Ishani Das, Helge Gad, Lars Bräutigam, Linda Pudelko, Rainer Tuominen, Veronica Höiom, Ingrid Almlöf, Varshni Rajagopal, Johan Hansson, Thomas Helleday, Suzanne Egyházi Brage, Ulrika Warpman Berglund
Cell Death Differ 2020, Jan 9 [Epub ahead of print]

IV. Co-expression of MTH1 and PMS2 is associated with shorter progression-free survival after immunotherapy in cutaneous malignant melanoma
Ishani Das, Rainer Tuominen, Thomas Helleday, Johan Hansson, Ulrika Warpman Berglund, Suzanne Egyházi Brage
Submitted

LIST OF SCIENTIFIC MANUSCRIPTS NOT INCLUDED IN THIS THESIS

I. Silencing of CEBPB-AS1 modulates CEBPB expression and re-sensitizes BRAF inhibitor resistant melanoma cells to vemurafenib

Linda Vidarsdottir, Rita Fernandes, Vasilios Zachariadis, **Ishani Das,** Elin Edsbäcker, Ingibjorg Sigvaldadottir, Alireza Azimi, Johan Hansson, Dan Grandér, Suzanne Egyházi Brage, Katja Pokrovskaja Tamm

(Manuscript under revision)

II. Pro-senescence therapy - a new strategy to overcome drug resistance and enhance immunorecognition of malignant melanoma cells

Fan Zhang, **Ishani Das**, Sofi Eriksson, Jeroen Melief, Madhurendra Singh, Marina Stantic, Alireza Azimi, Michelle Da Silva Liberio, Jacob Goodwin, Rainer Tuominen, Veronica Höiom, Fredrik Jerhammar, Suzanne Egyhazi Brage, Johan Hansson, Rolf Kiessling, Galina Selivanova, Margareta Wilhelm, Klas Wiman, and Lars-Gunnar Larsson

(***co-first author, Manuscript in preparation***)

III. Genes involved in DNA replication, chromatin remodeling and cell cycle as potential biomarkers for therapy outcome to immune therapy in patients with metastatic cutaneous malignant melanoma

Fernanda Costa Svedman, **Ishani Das**, Rainer Tuominen, E.D. Ramqvist, Johan Hansson, Veronica Höiom, Suzanne Egyházi Brage

(Manuscript in preparation)

IV. Melanoma cells are sensitized to targeted therapies by inhibition of autophagy

Alireza Azimi, **Ishani Das,** Muyi Yang, Pedram Kharaziha, Theocharis Panaretakis, Johan Hansson, Rainer Tuominen, Suzanne Egyházi Brage

(***co-first author, Manuscript in preparation***)

CONTENTS

LIST OF ABBREVIATIONS

8-oxo-dG	8-oxo-2’-deoxyguanosine
AJCC	American Joint Committee on Cancer
APC	Antigen presenting cell
ARAF	A-Raf Proto-Oncogene, Serine/ Threonine Kinase
Bcl	B-cell lymphoma
BER	Base excision repair
BRAF	B-Raf Proto-Oncogene, Serine/ Threonine Kinase
BRAFi	BRAF inhibitor
CAF	Cancer Associated Fibroblast
CAV-1	Caveolin-1
CD	Cluster of Differentiation
CDK	Cyclin-dependent kinase
CDKN2A	Cyclin-dependent kinase inhibitor 2A
cDNA	Complementary DNA
CMM	Cutaneous malignant melanoma
CR	Complete responder
CRAF	RAF1 Proto-Oncogene, Serine/ Threonine Kinase
CSD	Chronically sun damaged
ctDNA	Circulating tumor DNA
CTLA-4	Cytotoxic- T-lymphocyte associated protein 4
DFS	Disease free survival
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSB	Double strand break
EGFR	Epidermal growth factor receptor
EPHA2	Ephrin receptor A2
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence activated cell sorting
FGFR	Fibroblast growth factor receptor

FNA	Fine needle aspirate
GTP	Guanosine-5’-triphosphase
HGF	Hepatocyte growth factor
HR	Homologous recombination
ICI	Immune checkpoint inhibitor
IF	Immunofluorescence
IFN-γ	Interferon-gamma
IGF1R	Insulin-like growth factor 1 receptor
IHC	Immunohistochemistry
IRS	Insulin receptor substrate
JAK	Janus kinase
LDH	Lactate dehydrogenase
MAPK	Mitogen activated protein kinase
MAPKi	Mitogen activated protein kinase inhibitor
MEK/ MAP2K	Mitogen activated protein kinase kinase
MITF	Microphthalmia-associated transcription factor
MMR	Mismatch repair
mRNA	Messenger RNA
MSI	Microsatellite instability
MTH1	MutT homolog 1
MTH1i	MutT homolog 1 inhibitor
mTOR	Mammalian target of rapamycin
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
OIS	Oncogene induced senescence
OS	Overall survival
PD	Progressive disease
PD-1	Programmed cell death protein 1
PD-L1	Programmed death-ligand 1
PFS	Progression free survival

PI3K	Phosphoinositide- 3-kinase
PMEL	Premelanosome protein
PMS2	PMS1 protein homolog 2
PR	Partial responder
PTEN	Phosphatase and tensin homolog
RB	Retinoblastoma
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPPA	Reverse Phase Protein Array
RPS6	Ribosomal protein S6
RPS6KB1	Ribosomal protein S6 kinase beta-1
RPTOR	Regulatory Associated Protein of mTOR Complex 1
RTK	Receptor tyrosine kinase
SD	Stable disease
STAT	Signal transducer and activator of transcription
TCR	T cell receptor
TGFβ	Transforming growth factor beta
TMA	Tissue microarray
TMB	Tumor mutation burden
TP53	Tumor protein 53
Treg	Regulatory T cell
UV	Ultraviolet
WT	Wildtype

1. **BACKGROUND**

Skin is the largest organ in the body and is involved in many vital functions like protecting the human body against excessive water loss, from physical, chemical or biological assailants, in regulating our body temperature. Additionally, it also serves as a metabolic sink for storage of energy in the hypodermis. The skin microenvironment is comprised of a dynamic complex of several cellular constituents, one of them being melanocytes- the pigment producing cells in our body that protect us from harmful UV-radiation (Nestle, Di Meglio et al. 2009). Transformation and uncontrolled growth of melanocytes can give rise to malignant skin neoplasms. Cutaneous malignant melanoma (CMM) is the most aggressive form of skin cancer responsible for approximately 500 deaths annually in Sweden (Swedish melanoma registry). Globally, around 300,000 new cases with 61,000 deaths due to CMM were reported in 2018 (Pasquali, Hadjinicolaou et al. 2018). Although primary CMM can be cured with surgery, treatment of the disseminated disease is associated with poor prognosis due to intrinsic and acquired drug resistance (Somasundaram and Herlyn 2012). One of the most difficult therapeutic challenges in CMM is to overcome resistance to systemic therapies and thereby avoid disease progression. In the past 7 years several prospective randomized clinical Phase III trials have tested novel substances targeting the Mitogen Activated Protein Kinase (MAPK) pathway, potent mutant BRAF (around 50% CMM patients carry BRAF mutation) and MEK inhibitors in combination which have improved the clinical outcome with a median progression-free survival (PFS) of 21% at 4 years and 19% at 5 years. This combination has therefore been approved as first line treatment for CMM patients harboring unresectable or metastatic tumor with a *BRAF* V600E or V600K mutation (Robert, Grob et al. 2019). However, a majority relapse due to development of acquired drug resistance (Johnson and Sosman 2013). In addition, immunotherapy with check-point inhibitors has given long-term therapeutic efficacy but only for a subset of the CMM patients (Hegde, Karanikas et al. 2016, Kozar, Margue et al. 2019), thus making it essential to identify novel therapy regimes for CMM independent of *BRAF* and *NRAS* mutations status.

1.1 Skin: The multitasking organ

The skin forms a first major mechanical, dynamic, and physical barrier between our body and environmental insults. Through the production of melanin (a dark colored pigment), the skin shields us from harmful ultraviolet (UV) radiations from the sun. Skin naturally presents

itself as a model organ of choice to study the response of organisms to local and systemic injuries owing to its easy accessibility (Nestle, Di Meglio et al. 2009). The structure of the skin is a major contributing factor that helps it to perform these multiple roles. Histologically the skin can be divided into three layers- epidermis, dermis and hypodermis.

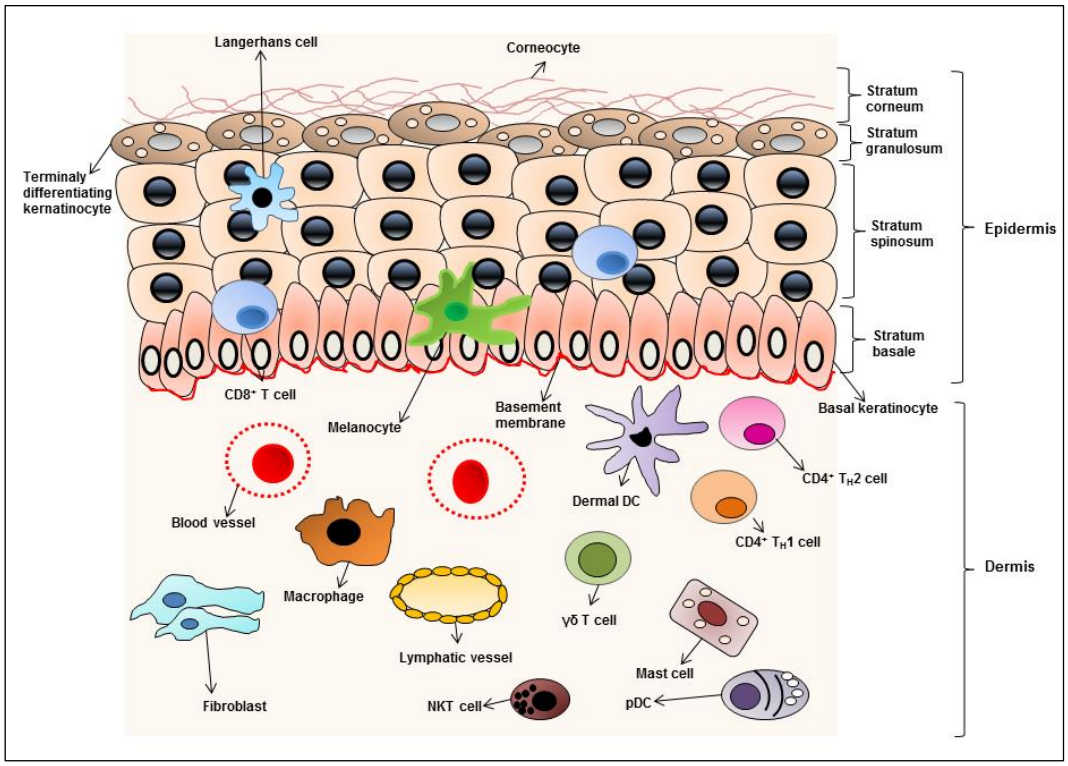

Figure 1: Skin anatomy and effector cells. Adapted from (Nestle, Di Meglio et al. 2009)

1.1.1 Epidermis

The epidermis is ectodermally derived and constitutes the outermost layer of the skin. It is a non-vascularized squamous epithelial layer mostly comprising of keratinocytes and is stratified into four sub layers: Stratum corneum, stratum granulosum, stratum spinosum and stratum basale (Chamcheu, Roy et al. 2019). The epidermis is also home to other cell types like melanocytes, Langerhans cells and T-lymphocytes (*Figure 1*). Merkel cells which are oval shaped mechanoreceptors which aid us to discern shapes and textures and also light and touch, can also be found in the epidermis (Di Meglio, Perera et al. 2011).

1.1.2 Dermis

The next layer of the skin is the dermis. It is mesodermally derived, enriched in collagen and connective tissue. The collagen matrix acts as a physiological barrier and a structural framework for blood and lymphatic vessels. The dermis is also embedded by a complex milieu of different cell types like T cells, natural killer cells, mast cells, fibroblasts, macrophages and dermal dendritic cells (*Figure 1*).

1.1.3 Hypodermis

The underlying layer of sebaceous tissue beneath the dermis is the hypodermis. It mainly constitutes of a meshwork of adipose tissue and blood vessels. The primary function of this layer is to store fat and maintain body temperature by heat insulation (Chamcheu, Roy et al. 2019).

1.2. Pathophysiology of melanoma

Malignant transformation of cells producing pigment (melanocytes), occurring primarily in the skin, but also in other parts of the body like mucosal membranes of the head and neck area, GI tract, genital organs and eyes, is the cause of malignant melanoma. UV radiation is the major environmental risk factor for developing CMM. Exposure to UV radiation can cause photocarcinogenesis mainly attributed to formation of mutations at localization of incorrectly repaired DNA photoproducts, most commonly thymine dimers. Other factors that enhance the risk of developing CMM are specific genetic aberrations, age, male sex, phenotypic appearance (hair color, skin type), number of nevi (moles) and presence of abnormal dysplastic nevi (Schadendorf, Fisher et al. 2015). Recent studies have identified several pathways that are involved in the process of melanoma-genesis; the transformation of normal melanocytes into melanoma cells (*Figure 2*). CMM onset and progression can be broadly defined into four major steps: (1) Transformation from a benign nevus with limited growth to a premalignant dysplastic nevus with aberrant proliferation; (2) Radial growth phase (horizontal growth and epidermal spreading; (3) Vertical growth phase (invasion vertically through basement membrane; (4) Metastasis (dispersal of melanoma cells to lymph nodes and other tissues) (Miller and Mihm 2006).

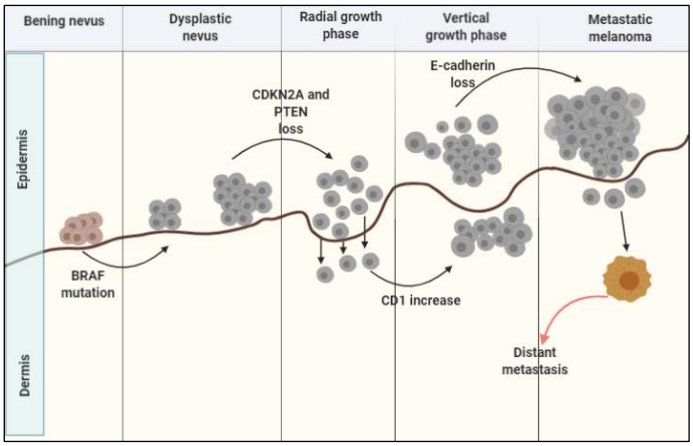

Figure 2: Molecular changes associated with CMM onset and progression. Adapted from (Miller and Mihm 2006)

CMM can be broadly classified as chronically sun damaged (CSD) versus non-chronically sun damaged (non-CSD) melanoma depending on the extent of skin damage induced by the sun at the site of origin (Shain and Bastian 2016).

High levels of reactive oxygen species (ROS) is known to cause DNA damage. Since both melanocytes and melanoma cells have elevated ROS, previous works suggest CMM to be to some extent a ROS driven tumor (Meyskens, Farmer et al. 2007, Wittgen and van Kempen 2007, Das, Gad et al. 2020). Melanin, the pigment, has been identified as a melanocyte-specific source of ROS and is synthesized, stored and transported in the melanosome organelle. Pheomelanin, one out of two forms of melanin, is known to produce ROS after exposure to UV irradiation. High levels of pheomelanin can thereby indirectly contribute to mutagenesis (*Figure 3*) (Shain and Bastian 2016). Elevated levels of ROS is a double-edged sword as, on one hand, high ROS levels may promote cell proliferation, metastasis and survival (Ishikawa, Takenaga et al. 2008), but on the other hand, elevated ROS levels may also cause DNA damage, inhibit tumor cell proliferation and induce cell death or cell growth arrest (Ramsey and Sharpless 2006, Das, Gad et al. 2020).

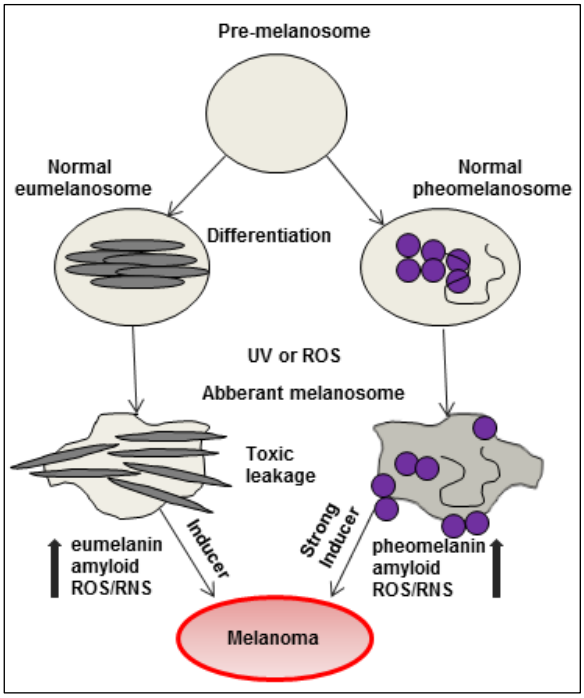

Figure 3: UV/ROS induced abberant structural changes in pheomelanosome and eumelanosome leading to development of melanoma. Adapted from (Liu-Smith, Poe et al. 2015)

1.3 DNA damage response

Genomic instability has been featured as a cancer hallmark (Hanahan and Weinberg 2011). It is one of the most pervasive cancer characteristics which stems from a combination of DNA damage, deficits in cancer repair mechanisms, and an inability for cancer cells to repair these defects before the DNA is passed onto the daughter cells. DNA damage is thereby known to cause replication stress (Lans, Hoeijmakers et al. 2019). To faithfully maintain genomic stability, cells possess complex mechanisms which are broadly referred to as DNA damage response pathways (Lord and Ashworth 2012). Solar UV radiations are known to play a major part in DNA damage induction. They are mainly responsible for the induction of pyrimidine dimers which contain deamination prone cytosine, and if left unrepaired can cause cytosine residues to be replaced by thymidine. Ionizing radiations can also cause single strand or double strand breaks (SSB or DSB) in the DNA backbone which may give rise to mutations and cause structural rearrangements within the genome. Moreover, mutations developed after UV exposure caused by error prone DNA polymerases can be mediated by ROS. In order to maintain genomic stability, melanoma cells are armored to handle the deleterious effects of ROS in different ways. One of the ways is through up-regulation of ROS metabolizing enzymes including superoxide dismutases, SODs. Tumor mutation burden (TMB) which is a readout of the number of mutations within the tumor genome has been shown to be a promising biomarker for predicting response to immune checkpoint inhibitors (ICI) (Wu, Xu et al. 2019). Moreover, reports have shown that melanoma or non-small cell lung cancer (NSCLC) patients with high TMB respond better to ICI than those with low TMB (Snyder, Makarov et al. 2014, Rizvi, Hellmann et al. 2015). Thus, genomic instability therefore paves avenues for therapeutic interventions for cancer treatment. Furthermore, a recent recommendation by the European Society of Medical Oncology (ESMO) highlights that microsatellite instability (MSI) in cancers deficient in mismatch repair (MMR) proteins, together with tumor burden and PD-1/PD-L1 expression can be collectively useful as predictive biomarkers for immunotherapy (Luchini, Bibeau et al. 2019).

1.3.1 DNA damage response pathway

Cells are equipped with a wide armory of DNA repair mechanisms to combat insults from external and endogenous agents that cause DNA damage. These DNA damage response pathways coordinate to recognize the DNA damage indicate the existence of the damage and finally mediate the repair. There exists five major DNA repair mechanisms: Base excision repair (BER), mismatch repair (MMR), nucleotide excision repair (NER), homologous recombination (HR) and non-homologous end joining (NHEJ) (Dexheimer 2013) (*Figure 4*).

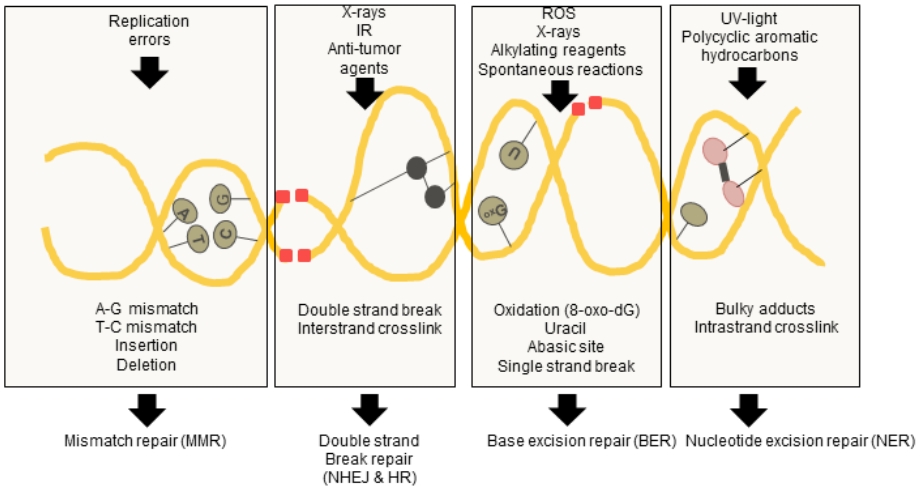

Figure 4: DNA damage and repair mechanisms. Adapted from (Dexheimer 2013).

1.3.2 PMS1 protein homolog 2 (PMS2)

PMS2 which is an important DNA repair protein in the MMR pathway dimerizes with MLH1. Patients carrying heterozygous PMS2 variants have been associated with increased risk of developing colorectal and endometrial cancers (Ten Broeke, van der Klift et al. 2018). Studies have shown that mutations in PMS2 promoter can be found in around 7.5% of CMM cases (101/1,348), which were associated with more than 5-fold higher tumor mutational burden when compared to tumors with wild-type PMS2 (Chalmers, Huang et al. 2016). Loss of PMS2 protein in primary CMM has previously been shown to be associated with higher Clark´s levels, but had limited prognostic significance (Korabiowska, Brinck et al. 2000). There has also been another study which has shown loss of PMS2 protein expression in CMM vs benign nevi (Alvino, Passarelli et al. 2014).

1.3.3 MutT Homolog 1 (MTH1)

Maintaining the genomic integrity by having optimal levels of ROS becomes an integral function of the tumor cells, which is why they often up-regulate several DNA repair mechanisms, one of them being MutT Homolog 1 (MTH1).

Although tumor cells have increased MTH1expression, it is not essential for normal cells. MTH1 is a member of the Nudix hydrolase that sanitizes the dNTP pool by hydrolyzing harmful oxidative stress induced 8-oxo-dG, thus preventing its incorporation into DNA (Nakabeppu 2014, Nakabeppu, Ohta et al. 2017). MTH1 protein has also been suggested to have a role during mitosis where it binds to tubulin, thereby ensuring correct spindle assembly, tubulin polymerization, mitosis progression and ablation of ROS generated during mitosis. In a recent study, it has been shown that MTH1 inhibitors (MTH1i) have a dual mechanism whereby it blocks mitosis, induce ROS generation and promote incorporation of

harmful 8-oxo-dG during mitotic phase (Warpman Berglund, Sanjiv et al. 2016). MTH1i mediates depolymerization of tubulin and causes cell growth to stall (cell cycle arrest) (Gad, Mortusewicz et al. 2019, Das, Gad et al. 2020). MTH1 has also been shown to cooperate with DNA glycosylases to promote survival of leukemia cells that are deficit in DNA repair enzymes (Eshtad, Mavajian et al. 2016). Previously published studies indicate that MTH1 is up-regulated in different carcinomas including colorectal cancer, glioblastoma and CMM (Koketsu, Watanabe et al. 2004, Pudelko, Rouhi et al. 2017, Wang, Liu et al. 2017). MTH1 has also been suggested to play an important role in RAS driven tumors (Giribaldi, Munoz et al. 2015, Patel, Burton et al. 2015). A recent study showed that melanoma cells are protected by Skp2/MTH1 from induced oxidative stress upon MAPK pathway activation (Wang, Liu et al. 2017). It was also demonstrated that inhibition of MTH1 made melanoma cells more susceptible to apoptosis and that the expression levels of MTH1 was determined by Skp2 which is an S-phase associated protein kinase 2 (Wang, Liu et al. 2017).

1.4 Protein phosphorylation

Protein phosphorylation regulates a major portion of cellular life, abnormal levels of which can cause onset of disease. It is one of the most common post translational modifications (PTM). Proteins can be phosphorylated on serine (86.4%), threonine (11.8%) or tyrosine (1.8%) residues (Roskoski 2012). Protein phosphorylation is a vital process that regulates multitude of cellular processes like protein synthesis, cell growth and development, signal transduction, cell division and aging. These events occur via the activation and deactivation modulated by specific kinases and phosphatases (Schwartz and Murray 2011, Ardito, Giuliani et al. 2017).

1.4.1 Kinases

The protein kinase family carries out cellular phosphorylation events. Kinases are activated by phosphorylation which leads to a downstream cascade activation. About one-third of all proteins can be modified by kinase activity, thus regulating major pathways within the cell (Ardito, Giuliani et al. 2017). These modifications can alter the activity and localization of proteins. Protein kinases including receptor tyrosine kinase are being studied extensively since they have been implicated to be one of the most frequently mutated gene families associated with cancer pathogenesis (McDonell, Kernohan et al. 2015).

1.5 Common aberrations in CMM

1.5.1 The involvement of MAPK and PI3K pathways in melanoma development

RAS/RAF/MEK/ERK (MAPK) pathway is frequently deregulated in a number of different cancer types including CMM. As with RAS family GTPases, mutant RAF proteins can also

play a role as drivers of human disease states like cancer. This pathway controls several major cellular processes including cell proliferation, survival and differentiation. Alterations in the MAPK pathway are commonly associated with CMM. Based on the tumor genetic profiles, patients can be divided into three subclasses. Around 50-60% of CMM patients have primary tumors harboring an activating mutation in the *BRAF* gene (majority have *BRAF* V600E/K). *BRAF* V600E mutations are most common in melanomas found in intermittently sun exposed skin, whereas skin high UV exposure results in primary tumors with activating *NRAS* mutation (*NRAS*Q61R, *NRAS*Q61K, *NRAS*Q61L, *NRAS*G12D) (~30% of CMM patients), and *BRAF* non V600E mutations. The *BRAF* and *NRAS* mutations are to a high degree mutually exclusive. The third group of CMM patients has primary tumors that are WT for both *BRAF* and *NRAS*. It has been shown that *BRAF* V600E mutations, an activating mutation, dramatically increase the kinase activity (Holderfield, Deuker et al. 2014). Additionally, there have also been some mutations reported in *BRAF* which causes a reduction in kinase activity (lesser than WT protein) and these tumors eventually rely on C-RAF for the transforming abilities (Freeman, Ritt et al. 2013). During normal states, RAF dimerization cause RAF kinase activation and subsequently activates MEK. However, in the event of a disease onset, MEK/ERK activation is triggered by (1) mutant RAF proteins harboring partial or completely impaired kinase activity, (2) RTKs or Ras GTPases that are activated due to a mutation, (3) homodimerization of V600E- *BRAF* splice variants that are also known to contribute to resistance to BRAF inhibitors (BRAFi) or (4) treatment with ATP-competitive RAF inhibitors in the presence of constitutively activated RAS (*Figure 5*) (Freeman, Ritt et al. 2013).

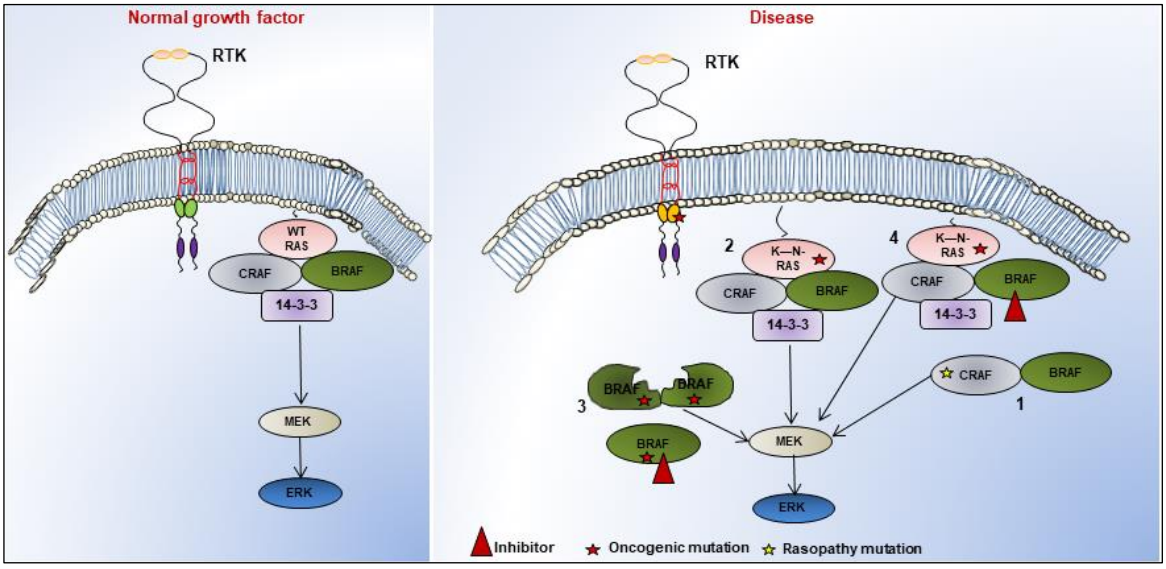

Figure 5: RAF dimerization in cell signaling. Adapted from (Freeman, Ritt et al. 2013).

Driver oncogenes like *BRAF* and *NRAS* have also been found in nevi. Mutation in these oncogenes is associated with oncogene induced senescence (OIS) but this function is inactivated in melanoma cells. For example, it has been shown in human diploid fibroblasts, senescence like growth arrest is induced in both CDKN2A and RAS-RAF-MEK pathway (Brookes, Rowe et al. 2002). However, it has also been shown that *CDKN2A* deficiency in a *BRAF*V600E knock in mouse model reduced melanocyte senescence. Additional tumor suppressor altering changes like deletion in lipid phosphatase and tensin homolog (*PTEN*) and mutation in *TP53* are associated with a rapid progression of melanocytic lesions (Peeper 2011). *PTEN* deletion has been suggested to be a bypass mechanism for OIS during melanogenesis (Wagner and Gil 2018). Moreover, accumulation of P53 and CDKN2A has also been shown to contribute towards RAS mediated premature senescence induction (Serrano, Lin et al. 1997).According to the cbioportal database for CMM cases, mutations and putative copy number alterations in *CDKN2A*, *TP53* and *PTEN* comprise of 43%, 17% and 15% respectively.

CDKN2A is a common melanoma susceptibility gene encoding two different tumor suppressor proteins $p16^{INK4A}$ and $p14^{ARF}$. The $p16^{INK4A}$ protein is an important gatekeeper of the G1/S check point, while the $p14^{ARF}$ protein stabilizes p53. $p16^{INK4A}$ binds to cyclin D1 and acts as a competitive inhibitor of CDK4. This complex keeps the tumor suppressor Retinoblastoma protein (RB) active and prevents cell cycle progression (Curtin, Fridlyand et al. 2005). Frequency of genetic and epigenetic alterations affecting $p16^{INK4A}$ only is around 20%, whereas frequency of mutations affecting both $p16^{INK4A}$ and $p14^{ARF}$ is around 40% (Rizos, Darmanian et al. 2001, McWilliams, Wieben et al. 2011). In a study to investigate the loss of $p16^{INK4A}$, it has been shown that *NRAS* mutated melanoma have a high frequency of *CDKN2A* promoter methylation and in around 30% of the cases, protein expression was lost during progression from primary to metastatic tumor (Jonsson, Tuominen et al. 2010).

Characterization of the whole genome of large patient cohorts with advanced melanoma has led to identification of other genetic alterations including inactivating mutations in *NF1*. *NF1* is a negative regulator of RAS signaling pathway and patients with *NF1* mutations have been categorized as another subset of CMM (Vogelstein, Papadopoulos et al. 2013). According to cbioportal, the proportion of melanoma tumors having genetic alterations in NF1 is around 13%.

Similar to the RAS/RAF pathway, the PI3K/AKT cascade also regulates several vital cellular processes like cell growth, cell proliferation, cell cycle and cell death and is aberrantly expressed in many different tumor types. Some seminal studies have also highlighted that the PI3K/AKT pathway has multifaceted roles in melanoma pathogenesis, heterogeneity and resistance mechanisms. PI3K comprises of a dimer containing catalytic (p110) and regulatory (p85) subunits that can be activated by multiple signals, for example by RTKs, RAS/RAF pathway, physical cell-cell interactions (*Figure 6*). Once activated, the PI3K phosphorylates plasma membrane phosphatidylinositols at 3'-OH. This ensues activation of proteins containing domains with pleckstrin homology (PH) (including AKT) and attracts them to the cell membrane. AKT has three isoforms (AKT 1/2/3). The catalytic activity of AKT is then turned on by phosphorylation at two conserved residue, Thr 308 (PDK1) and S473 (mTORC2). In turn, AKT phosphorylates and thereby activates a number of downstream effector proteins (including mTOR) which lead to a concomitant signaling cascade regulating several key cellular processes as mentioned before (Kwong and Davies 2013). PTEN which is a negative regulator of the pathway is mutated or deleted in 7.3% primary and 15.2 % metastatic CMM tumors (Agosto-Arroyo, Rosa et al. 2017). PTEN inactivates the PI3K/AKT pathway by converting phosphatidylinositol (3,4,5)- triphosphate (PIP_3) back to phosphatidylinositol (4,5)- biphosphate (PIP_2) (Dasari and Messersmith 2010). Deletions in *PTEN* often, but not exclusively, co-occur with *BRAF* mutations. PTEN loss is correlated to shorter overall survival (OS) and elevated invasive disease capacity in CMM patients with tumors harboring *BRAF* V600E mutation (Bucheit, Chen et al. 2014).

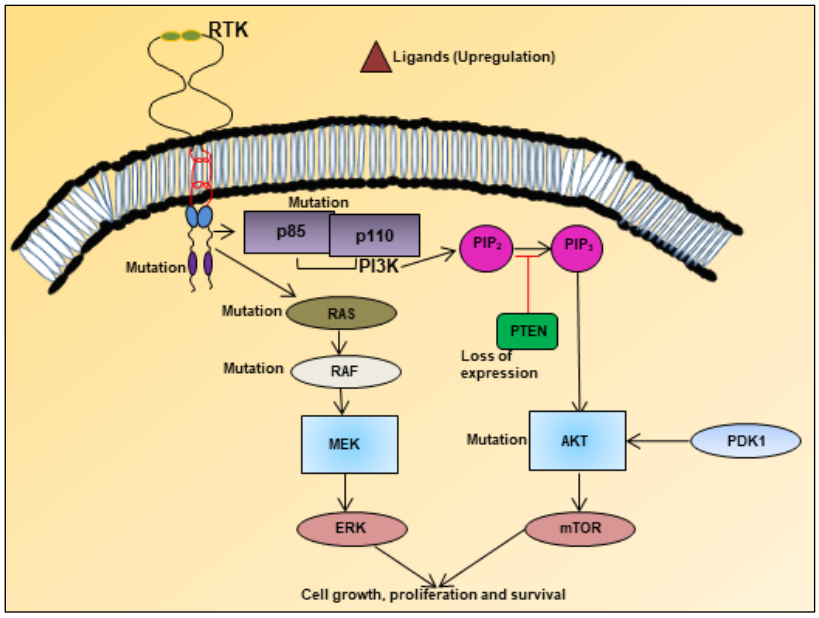

Figure 6: Schematic figure of MAPK/ERK and PI3K/AKT pathway activation. Adapted from (Dasari and Messersmith 2010).

1.5.2 Receptor tyrosine kinases: crosstalk and role in CMM

Receptor tyrosine kinases (RTKs) belong to a family of ligand-binding cell surface receptors that control different cellular functions. Currently there are 58 identified RTKs belonging to 20 subfamilies, all harbouring the same basic structure comprising of an extracellular ligand binding domain, connected via a single pass transmembrane domain to an intracellular portion containing the main kinase active domain. In a normal state, canonical RTK activity is triggered by binding of their specific ligand resulting in receptor dimerization and conformational changes. Another subset of RTKs exists as oligomers which are then activated by ligand binding. Conformational changes as a result of ligand binding cause autophosphorylation of tyrosine residues, thus activating the RTKs (McDonell, Kernohan et al. 2015).

Interactions between RTKs and PTPs result in aberrant growth and proliferation signalling that benefits the melanoma cell proliferation (*Figure 7*). RTK MET has been shown to be associated with melanoma survival, proliferation and invasion (Adachi, Sakai et al. 2016, Czyz 2018). Alterations in the expression levels of MET have been associated with changes in both growth and metastatic phenotypes. MET has been shown to play a pivotal role in melanoma metastasis to the lungs. It has been seen that inhibition of IGF1R signalling which led to the concomitant decrease in ERK signalling was able to induce cell death in melanoma cells with *BRAF*V600E mutation (Scurr, Pupo et al. 2010). Moreover, it has also been shown that AXL expression within the tumor microenvironment can contribute towards immunosuppressive and protumorigenic phenotypes in many cancer types including CMM (Rankin and Giaccia 2016). Data from the Oncomine database also demonstrated up to 5 fold increase in the mRNA expression of ERBB3 in melanoma when compared to normal skin (Easty, Gray et al. 2011), and has also been shown to contribute to metastatic transformation of melanoma cells (Tiwary, Preziosi et al. 2014). Crosstalk between different RTKs has also been studied in other cancer types where EGFR and ERBB2 act as key central hubs. ERBB3 has been shown to promote cell survival by heterodimerizing with ERBB2, causing induction of signalling predominantly via the PI3K-AKT pathway (Zhang, Wong et al. 2013). It has been reported in a study on non-small cell lung cancer (NSCLC) that induction in the expression levels of EGFR is correlated with increased levels of IGF1R (Morgillo, Woo et al. 2006). To highlight the importance of receptor cross talk across different cancer types, a study has shown that in presence of HGF ligand, EGFR can bind to several cytoplasmic and transmembrane proteins thus possibly empowering cancer cells to utilize a broader range of

growth factors from the tumor microenvironment and ultimately leading to acquired resistance to targeted therapy (Gusenbauer, Vlaicu et al. 2013).

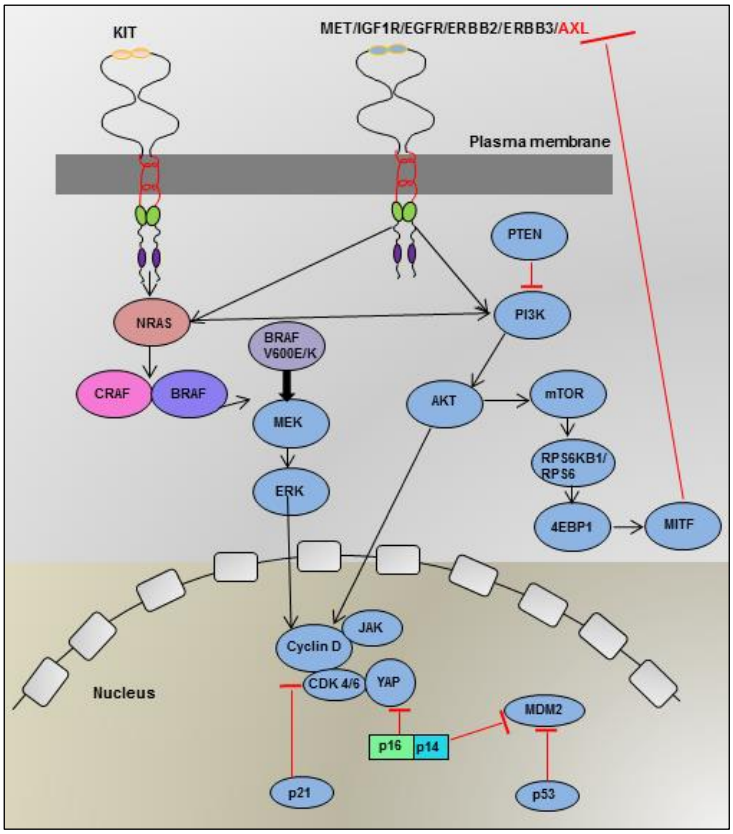

Figure 7: Key molecular signaling pathways related to melanoma tumorigenesis. Adapted from (Luke, Flaherty et al. 2017).

1.6 Systemic treatment in CMM

1.6.1 Chemotherapeutics and targeted therapies

Prior to 2011, standard of care for CMM patients with metastatic disease was mainly the chemotherapeutic agents dacarbazine or temozolomide, radiation therapy, with no discernable improvement in survival rates. However, the treatment landscape for CMM has been revolutionized since 2011 with the introduction of a number of systemic agents like BRAF inhibitors for patients with *BRAF* mutation and MEK inhibitors for blocking signaling via MAPK pathway. Monotherapy using BRAF or MEK inhibitor alone have resulted in clinical responses with limited duration in the *BRAF* mutant CMM patients, due to development of therapy resistance by reactivation of the MAPK pathway ultimately resulting in tumor recurrence (Villanueva, Vultur et al. 2011). A clinical trial designed to determine 5-year survival rates of dabrafenib (BRAFi) plus trametinib (MEKi) in a cohort of 563 CMM patients with either unresectable or metastatic melanoma showed that PFS for combination

therapy was only 19% whereas OS was 34% at 5 years (Robert, Grob et al. 2019). Since an increased PFS and OS was observed with combination treatment as compared to single treatment, the current standard of targeted treatment employs the use of combination therapy.

1.6.2 Immune checkpoint inhibitor therapy against CTLA-4 and PD-1

Emergence of immunomodulatory drugs like anti-PD1 (programmed cell death 1) drugs (nivolumab, pembrolizumab) and anti-CTLA4 (cytotoxic T-lymphocyte associated protein 4) drugs (ipilimumab) has transformed the landscape of melanoma treatment (Lugowska, Teterycz et al. 2018). The basic principle of immunotherapy accounts is blocking the interaction between the immune system of the patient and the surface molecules of the cancer cells. Immunotherapy aims to boost the anti-tumor immune responses in patients and thereby impede tumor growth and increase OS. Important players responsible for mediating immune responses against cancer are cytotoxic T-cells ($CD8^+$). This requires activation of $CD8^+$ T cells which is generally mediated by a two-step process: First, T cell receptor (TCR) mediated activation is triggered by a specific antigen on an antigen presenting cell (APC); second, by interaction between CD80/86 on APC and CD28 on the surface of the lymphocyte (Lugowska, Teterycz et al. 2018) (*Figure 8*). Proportion of $CD8^+$ tumor infiltrating lymphocytes (TILs) have been associated with response to immunotherapy and has also been reported to increase during treatment in responders but in non-responders. Furthermore, $CD8^+$ T cell localization at the tumor invasive margin has been attributed to increased immune response (Riaz, Havel et al. 2017).

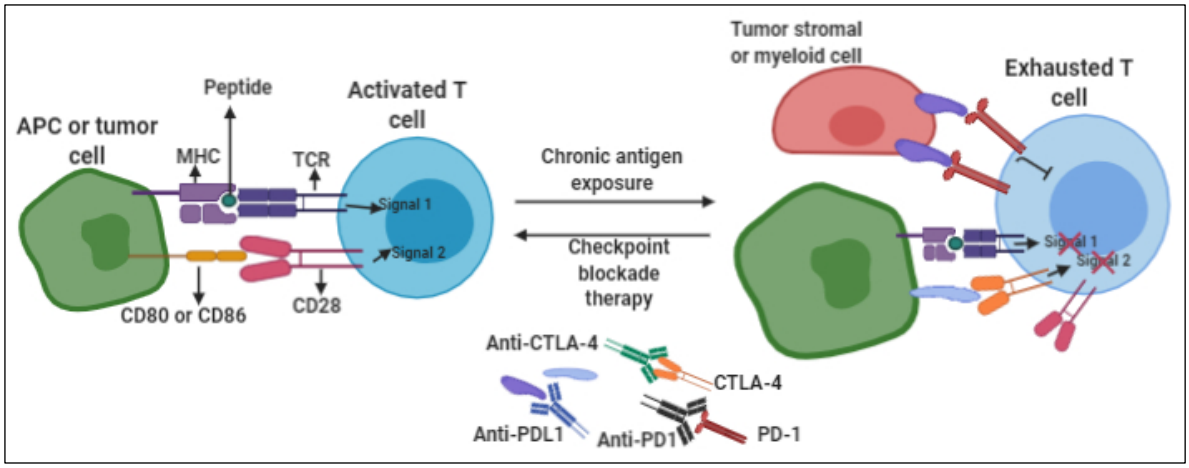

Figure 8: Immune checkpoint blockade. Adapted from (Havel, Chowell et al. 2019)

CTLA-4 is a member of the immunoglobulin superfamily and is exclusively expressed on T-cells. It is located mainly on intracellular vesicles and under healthy conditions, is only briefly expressed during activation before it is rapidly endocytosed. However, under disease conditions, CTLA-4 inhibits T-cell function and causes immunosuppression by binding to its

co-stimulatory molecule CD28 and thus quenching CD28 mediated signaling during antigen presentation. Removal of T-cell stimulatory receptors CD80 and CD86 from the cellular surface of APCs may also be orchestrated by CTLA-4, thus reducing their availability for other T-cells expressing CD28. CTLA-4 has been shown to hamper immune responses against cancer cells. Blockade of CTLA-4 by anti-CTLA4 antibody abrogates its inhibitory effects, increases T-cell cytokine production which elevates T-cell expansion and tumor cell infiltration (Domingues, Lopes et al. 2018, Seidel, Otsuka et al. 2018).

Analogous to CD28, the cell surface receptor PD-1 is expressed on effector T cells, binds to its ligands PD-L1 and PD-L2 and ablates adaptive immune responses by inhibiting immune signaling. Studies have shown that by blocking TCR/CD28 mediated signaling and IL-2 feedback loop, PD-1 dampens pro-survival signal, cell cycle progression and reduces cytokine production. Thus, blocking PD-1 using monoclonal antibodies inhibits binding of PD-1 to its ligands (Domingues, Lopes et al. 2018).

A number of recent studies have shown that these checkpoint inhibitors have dramatically improved CMM patient survival. However, a strong difference was observed in number of durable responses (PFS) between CTLA-4 and PD-1 inhibitors. In a phase III clinical trial study when comparing anti-PD1 treatment (nivolumab) with anti-CTLA4 (ipilimumab) treatment, anti-PD1 was more effective than anti-CTLA4 (6.9 months vs 2.8 months median PFS). Recent data shows that 5 year OS rate in clinical trials for PD-1 inhibitor alone is 35-40%. The study also shows that 4 year OS rate for nivolumab and ipilimumab in combination was more than 40% (Weiss, Wolchok et al. 2019). Furthermore, another report indicates OS at 5 years in advanced treatment naïve CMM patients administered with ipilimumab and nivolumab in combination to be around 52% versus 44% for nivolumab and 26% for ipilimumab alone (Larkin, Chiarion-Sileni et al. 2019) .

1.6.3 Targeted therapy and immunotherapy in combination

Preclinical studies have shown that combining targeted therapy and immunotherapy alleviates anti-tumor immunity and could therefore be a good treatment strategy for CMM patients (Cooper, Juneja et al. 2014, Hu-Lieskovan, Mok et al. 2015, Deken, Gadiot et al. 2016). Clinical trials designed to combine BRAF, MEK inhibitors together with immunotherapy seem to have some underlying benefits in terms of improved response rates, but with increased toxicity (Ribas, Puzanov et al. 2015). A clinical trial designed to combine ipilimumab (CTLA-4) with vemurafenib was terminated at a phase 1 trial due to patients developing dose limiting toxicities. Studies of combination therapy with BRAF/MEK inhibitors and PD-1/PD-L1 antibodies are ongoing (Simeone, Grimaldi et al. 2017, Clark,

Singh et al. 2018, Mooradian, Reuben et al. 2018). The response rates for these studies are variable depending on the sequence of drug administration. For example, a study where patients have first received targeted therapy after immunotherapy had a PFS of 5.6 months and an OS of 19.6 months, whereas those who progressed on targeted therapy and received anti-CTLA4 after had a much poorer response rate with PFS of 2.7 months and OS of 5 months (Pelster and Amaria 2019). Together with targeted therapy and immunotherapy, cytotoxic chemotherapy, surgical resection and radiotherapy will continue to be treatment options for CMM patients.

1.7 Mechanisms of therapy resistance

1.7.1 Resistance to chemotherapy

Treatment using chemotherapy has been ineffective for patients with metastatic melanoma, primarily due to development of intrinsic drug resistance. Both intrinsic and acquired resistance can be attributed to several factors, some of which are as follows: reduction in intracellular accumulation of chemotherapy drugs due to increased activity of drug efflux systems; diminished availability of free drugs to bind to intracellular targets due to inactivation of the drugs by altered enzyme activation; increased DNA repair activity thus impairing chemotherapy mediated cytotoxic effects; modulating the apoptotic pathway to increase pro-survival signals (Kalal, Upadhya et al. 2017). Other factors include accumulation of MITF, premelanosome gp100 (gp100/PMEL), MLANA (MART1) and melanosome trafficking related protein RAB27a (Chen, Leapman et al. 2009, Hertzman Johansson, Azimi et al. 2013).

1.7.2 Resistance to targeted therapy

Resistance evolved to targeted therapies have been described in tumors progressing on BRAF + MEK inhibitors, with alterations in *BRAF* frequently found among these resistant tumors. Multiple factors giving rise to resistance to BRAF and MEK inhibitors have been described. Amplification of mutant *BRAF* has been shown to be one of the resistance mechanisms. Dimerization of aberrantly spliced *BRAF* V600E isoforms have also been shown to contribute to BRAF inhibitor mediated resistance in 13-30% of CMM tumors (Rizos, Menzies et al. 2014, Krepler, Xiao et al. 2016). Excessive BRAF dimerization and enhanced association to MEK has been elucidated to be partially responsible for the resistance onset (Vido, Le et al. 2018).

There have been several alternative mechanisms that have been associated with resistance to MAPK inhibitors in CMM. Overexpression of *COT*, downstream mutations in *MEK1*, *AKT1*, PI3K, loss of PTEN and secondary activating mutations in *NRAS* have been demonstrated to be underlying resistance mechanisms (Eroglu and Ribas 2016),(Rizos, Menzies et al. 2014). Activation of alternate escape pathways like *PI3K-AKT* or the *YAP* pathway have also led to acquired therapy resistance. Tumors can escape cell death by increasing the expression of anti-apoptotic proteins. Higher Bcl-xL expression has been shown to decrease apoptosis via the *YAP* pathway (Frederick, Salas Fragomeni et al. 2014). A similar increase in the levels of anti-apoptotic protein Bcl2A1 was observed in *BRAF* mutant melanomas subjected to treatment with BRAF-MEK inhibitors, but MITF expression was restored. Bcl2A1 expression levels were associated with poor clinical response to BRAF inhibitors in CMM patients (Haq, Yokoyama et al. 2013). De-regulation of MITF expression has been associated with development of resistance towards targeted therapies (Kozar, Margue et al. 2019). In tumors with low MITF expression, reactivation of pathways like the *PI3K-AKT* have been associated with overexpression of RTKs MET, EGFR, ERBB3, AXL, EPHA2, PDFGβ, IGF1R and FGFR. (Chan, Singh et al. 2017, Luke, Flaherty et al. 2017) (*Figure 7*).

A study on metastatic melanoma further corroborated the importance of RTK signalling for development of drug resistance to MAPK inhibitors in CMM. This study demonstrated an inverse correlation between MITF and activated EGFR signalling, which led to vemurafenib resistance in patients with recurrent CMM (Ji, Erin Chen et al. 2015). This was observed consistently in both patient samples and melanoma cell lines. EGFR driven reactivation of MAPK pathway has also been observed in *BRAF* mutant colorectal cancers, and this has been proposed to contribute towards resistance to BRAF inhibitors (Corcoran, Ebi et al. 2012). Similarly, high EGFR expression was found in about 40% of the *BRAF* mutant tumors from patients who develop resistance towards BRAF or MEK inhibitors. This was associated with the suppression of SOX-10 which led to upregulation of TGFβ and ultimately increased expression of both EGFR and PDGFβ (Sun, Wang et al. 2014). Plausibly, this was associated with downregulated MITF, as SOX-10 is an upstream regulator of MITF. Increased levels of ERBB3 have been seen with a decrease in the expression levels of MITF, thus leading to hyper-activation of PI3K-AKT pathway and drug resistance (Alver, Lavelle et al. 2016).

Alterations in the immune compartment of CMM patients administered with MAPKi have also been shown to play a part in mediating resistance to targeted therapy. For example, it has been shown that patients progressing on targeted therapy have lower T-cell infiltration and subdued expression of melanoma differentiation antigens (MDA) in the tumor. Also,

samples collected from CMM patients during treatment on targeted therapy have shown higher PD-1 expression as compared to samples collected at progression in T-cells present in the tumor microenvironment (Pelster and Amaria 2019). Another study has also highlighted that prolonged treatment with BRAFi alone or combination of BRAFi +MEKi lead to lower CD8+ T cells activity (Pieper, Zaremba et al. 2018).

1.7.3 Resistance to immunotherapy

CMM tumors are generally considered as 'hot tumors' since they are composed of infiltrating T cells in abundance and also express tumor antigens, making them detectable to the immune system which creates a favorable microenvironment with regard to response to ICI (Lawrence, Stojanov et al. 2013, Maleki Vareki 2018). However, there are subsets of patients with 'cold tumors' who are non-responsive to ICI (Rivera Vargas and Apetoh 2019). Additionally, although patients initially respond to ICI, in many cases patients become resistant to therapy and eventually relapse.

Some factors contributing to innate resistance are constitutive expression of PD-L1 ligands on cancer cells caused by either amplification of the *PD-L1* gene or aberrant activation of oncogenic pathways like c-Jun/STAT3 in CMM. Some mechanisms for acquired resistance involve inflammatory factors secreted into the tumor microenvironment during therapy. There have been several studies reporting the role of cytokine mediated over-expression of PD-L1 while others have

highlighted the role of JAK/STAT and NFKB pathways as mediators of PD-L1 upregulation (Chen, Crabill et al. 2019). Some recent studies have proposed a few resistance mechanisms to immunotherapy. For instance, it has been shown that ICIs mediate selection pressure by enriching clones of tumor cells that can escape immune recognition and deletion through novel pathways. A recent study showed that cells taken from a PD-1 inhibitor refractory CMM patient had acquired mutations which made them less vulnerable to T-cell mediating elimination by IFN-γ response (Zaretsky, Garcia-Diaz et al. 2016). Another study showed that treatment with CTLA-4 inhibitor caused upregulation of another inhibitory receptor on tumor infiltrating immune cells, in this case V-domain Ig suppressor of T cell activation (VISTA) (Gao, Ward et al. 2017). Another study has highlighted a novel mechanism of resistance to PD-1 inhibitors where it has been shown that administered anti-PD1 inhibitors in vivo were removed by tumor associated macrophages (TAMs) (Arlauckas, Garris et al. 2017). Another study published by Trujillo *et al* in 2019 highlighted an association of β-catenin and *PTEN* loss to secondary resistance to immunotherapy in CMM (Trujillo, Luke et al. 2019).

1.8 Biomarkers in CMM

Biomarkers are generally classified as being prognostic or predictive (*Figure 9*) (Tarhini and Kudchadkar 2018). A prognostic biomarker by definition can provide an overall insight into the disease outcome of a patient, whereas a predictive biomarker gives an idea about the likelihood of therapeutic response of patient's disease to treatment. The overarching aim by using biomarkers is to improve treatment outcome by using personalized medicine. Lactate dehydrogenase (LDH) levels and S100-B levels have been used routinely as prognostic markers to test for occurrence and recurrence of CMM. LDH has been included as a determinant in staging of patients with distant metastasis in the American Joint Committee on Cancer (AJCC) melanoma staging system. Elevated LDH levels are also indicative of shorter PFS and decreased response rates to therapy (Diem, Kasenda et al. 2016). Mostly *BRAF* mutation is the biomarker used for determining choice of therapy in CMM. Additionally *NRAS* mutation status has also been used as a biomarker (LoRusso, Schalper et al. 2019). In a phase III study, they have also found that mutation and deletion in *CDKN2A* was significantly associated with poor PFS and OS in patients treated with dabrafenib+ trametinib (inhibitors of MAPK signaling) (Flaherty, Davies et al. 2016). Furthermore, the coBRIM trial studying the effects of vemurafenib single treatment vs vemurafenib and cobimetinib in combination identified two potential biomarkers where immune response gene clusters were associated with complete response and keratinization genes were associated with resistance (Yan, Robert et al. 2016).

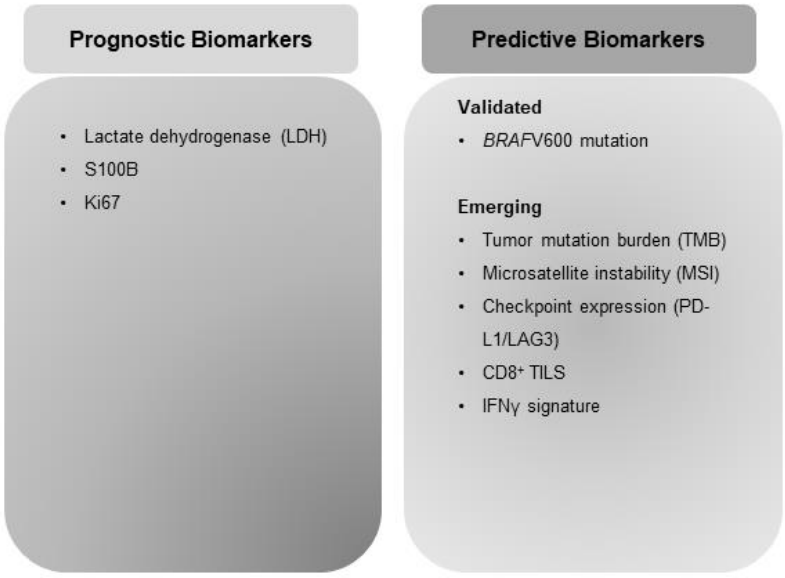

Figure 9: Prognostic and predictive biomarkers in CMM. Adapted from (Tarhini and Kudchadkar 2018)

Several parameters have been suggested as predictive biomarkers for checkpoint inhibiting therapies, such as high PD-L1 expression (in anti-PD1 therapy) specific human leukocyte antigen typing, overexpression of interferon-γ-inducible genes, presence of tumor infiltrating lymphocytes ($CD4^+/CD8^+$), expression of lymphocyte-activation gene 3 (LAG3), tumor mutational load, positivity for neoantigen expression, and the individual microbiome (Buchbinder and Flaherty 2016, Seidel, Otsuka et al. 2018).High microsatellite instability (MSI) and deficiency in DNA mismatch repair have also been validated as biomarkers of immunotherapy response in a number of different cancer types associated with poor prognosis after chemotherapy in colorectal cancer patients (Boyiadzis, Kirkwood et al. 2018). Circulating tumor DNA (CtDNA) has also been shown to be an attractive biomarker for ICI since a recent study has shown that CMM patients treated with anti-PD-1(alone or in combination with anti-CTLA4) had higher therapy response rate when they were negative for ctDNA either before or after treatment (Lee, Long et al. 2017). One more factor shown to be indicative of increased response to immunotherapy is increased expression of TGFβ in serum of CMM patients administered with anti-PD1 (Nonomura, Otsuka et al. 2016). Elevated Th1 and CTLA-4 gene expression levels are also considered good prognostic factors since they were found in responders to anti-PDL1 in many solid malignancies including CMM (Herbst, Baas et al. 2016).

1.9 Targeting RTKs in melanoma

The addition of RTK and AXL inhibitors could help to circumvent the resistance developed in *BRAF/NRAS* mutant melanomas with low MITF (Muller, Krijgsman et al. 2014). MET has been correlated to resistance to BRAF inhibitors (Adachi, Sakai et al. 2016). HGF, the ligand for MET, secreted from stromal cells in the tumor microenvironment has been shown to promote drug resistance (Beuret, Flori et al. 2007). A recent study indicated that selective inhibition of MET or PI3K isoforms may overcome the resistance to MEK inhibitors in metastatic uveal melanoma cells (Cheng, Chua et al. 2017), possibly implying a similar potential in CMM therapy. Personalized preclinical trial studies have suggested the use of MET inhibitors as second line therapies with encorafenib in double combination or encorafenib and binimetinib in triple combination for BRAF inhibitor resistant melanomas (Krepler, Xiao et al. 2016).

The RTK EPHA2 has also been associated with MAPKi drug resistance. A recent study elucidates that silencing *FLI* or *CD13* leads to de-phosphorylation of EHPA2 causing arrest in proliferation or apoptosis in melanoma cells (Azimi, Tuominen et al. 2017). RTKs

belonging to the ERBB and insulin family have also been reported to be associated with emergence of drug resistance. For example, inhibition of signaling via EGFR or SRC family kinases has been shown to revert BRAFi resistance in CMM (Girotti, Pedersen et al. 2013). Co-targeting the ERBB pathway and MAPK pathway has been suggested to enhance clinical efficacy and prolong therapeutic response of MAPKi (Abel, Basile et al. 2013). Furthermore, therapeutic destruction of the insulin receptor substrates like IRS-1 and IRS-2 have been shown to downregulate signaling mediated by IGF1R which prevented resistance acquisition to BRAFi in CMM (Reuveni, Flashner-Abramson et al. 2013).

1.10 Melanoma disease models

Over the past decades, both murine models of melanoma as well as zebrafish models have been developed to better understand the complex tapestry of the disease including melanoma etiology, molecular and cellular mechanisms behind its pathogenesis, tumor microenvironment and resistance mechanisms. These studies have led to the development of therapies targeting several molecules that are currently being used in the clinics to treat CMM patients (*Figure 9*). Some of the molecular targets are also used as biomarkers for better patient stratification during treatment. Different kinds of murine models are currently available and each one presents its own unique advantage and disadvantage depending on the research question to be answered. For example, murine models like cell-line xenograft models and patient derived xenograft models are two types which are able to mimic the complex and heterogeneous character of the disease, thereby providing us with the unique opportunity to better predict human tumor responses to therapeutic drugs (Saleh 2018). Additionally, these models are fairly simple to establish. Both genetically altered zebrafish and mice models are also currently being actively used in the field of melanoma for screening and selection of drugs for the development of personalized medicine for patients (*Figure 10*). Zebrafish which is the only vertebrate model which can be used as a powerful platform for large scale drug screens has been shown to efficiently recapitulate human diseases (Barriuso, Nagaraju et al. 2015). It has emerged as a robust animal model for pre-clinical testing of active drug compounds or drug regimes, thus providing an alternative platform instead of using mice models. Moreover, an orthotopic zebrafish model for glioblastoma has been described that can be used for high throughput screening of drugs to identify and profile novel therapies, a method which is very difficult to perform on murine models (Pudelko, Edwards et al. 2018). In this approach, thousands of zebrafish embryos can be transplanted with the cancer cells of interest, and these can then be robustly screened for drugs.

Even though zebrafish models are useful as an initial screen and understanding of the effect of the drug tested, murine models are eventually needed to obtain more in-depth understanding of the pharmacokinetic and pharmacodynamic properties of the drug. Particularly patient derived xenograft (PDX) models, so called "avatars" (personalized model for one patient's cancer generated by engrafting tumor tissue from a patient into a mouse), are proposed to be a predictive method for success in the clinic. (Lunardi and Pandolfi 2015).Additionally, transgenic tumor models have also paved the way for drug development. Both model organisms represent a wide variety of use in cancer research including transplantation assays, single-cell functional assays, *in vivo* imaging studies and transgenesis (Barriuso, Nagaraju et al. 2015).

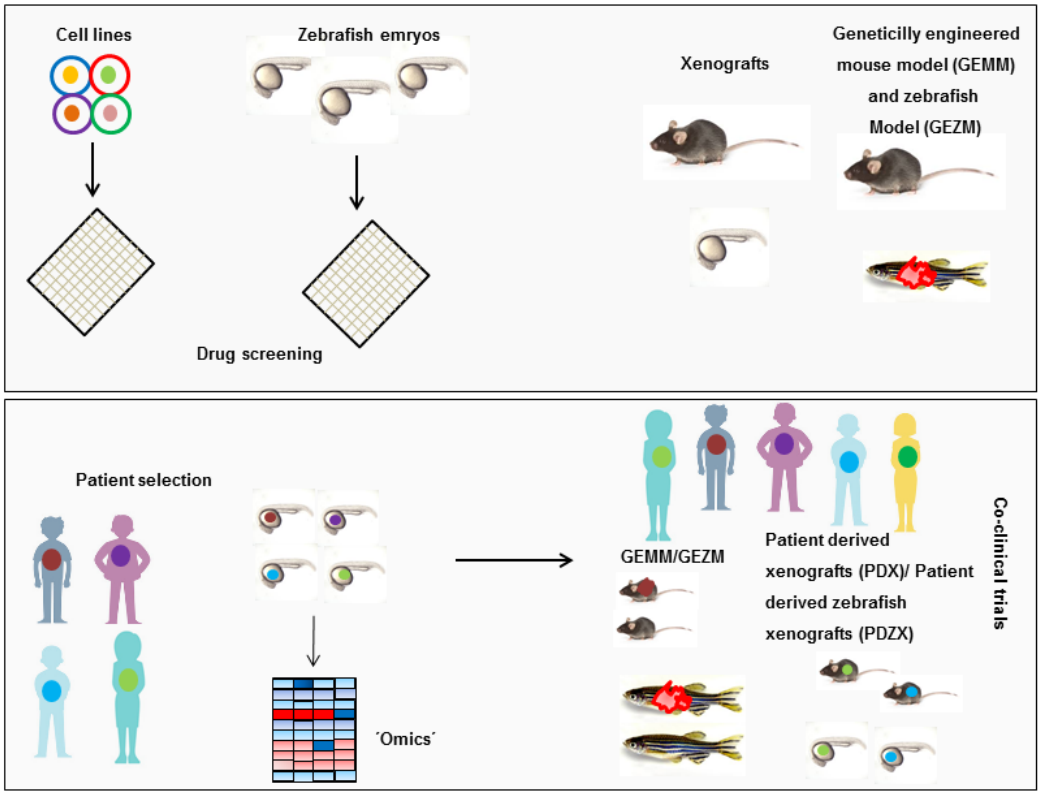

Figure 10: Animal models in clinical studies. Adapted from (Barriuso, Nagaraju et al. 2015)

Although both murine models and zebrafish models are currently being used extensively for research, each model has its limitations. For example, intradermally injected xenograft mouse models of melanoma can cause rapid tumor formation resulting in ulceration of mouse skin and thereby early termination of an experiment. Tail vein injection of cancer cells to mimic a 'metastasis model' although frequently used does not represent the patient scenario since this model skips the initial steps of metastasis from the primary tumor into blood or lymph vessels. Since established cell lines are significantly different from the originating cells,

xenograft models are often poor in predicting clinical outcome (Beaumont, Mohana-Kumaran et al. 2013). Zebrafish as a model organism also has certain drawbacks. The genome of the fish has many duplicate genes making genetic manipulation difficult. However, this is currently being overcome with the development of clustered regularly interspaced short palindromic repeats (CRISPR) technology. It is difficult to give water insoluble drugs to zebrafish due to inherent toxicity of other carrier solvents (Zhao, Huang et al. 2015). Other challenges also include possible precipitation of drugs and drug permeability (Letrado, de Miguel et al. 2018). Furthermore, zebrafish are raised in water at 33ºC which is substantially different from the human body temperature and the temperature at which cells are cultured (37ºC). This could cause the cancer cells to not grow well and give rise to tumors. Currently zebrafish xenograft studies are mostly performed using zebrafish larvae which do not have an adaptive immune system and hence the role of the immune system in these studies cannot be fully determined (Yan, Yang et al. 2019).

2. AIMS

In this thesis we aimed to find novel potential targeted therapies that could be beneficial for treatment of CMM independent of *BRAF/NRAS* mutational status.

The objectives of this research were:

- **Paper I:** To investigate whether the combination of afatinib (ERBB family inhibitor) and crizotinib (MET/ALK inhibitor) could be a potential therapeutic strategy for CMM independent of *BRAF/NRAS* mutation status
- **Paper II:** To explore the molecular mechanisms behind the broad cytotoxic effects of afatinib and crizotinib in combination in CMM
- **Paper III:** To investigate the cytotoxic potential of MTH1 inhibitor TH1579 (Karonudib) alone or in combination with MAPK targeting drugs in CMM
- **Paper IV:** To elucidate the association between PMS2 and MTH1 expression to therapy response and stage of disease in CMM

3. MATERIAL AND METHODS

Clinical samples

Patient material was collected at Karolinska University Hospital as FFPE (from pathology archives) or fine needle aspirates (FNA) (for establishing short term cultures) or as core biopsies from CMM patients with stage III/IV disease. Most of these samples were obtained either prior to onset of targeted therapy by MAPKi or checkpoint inhibitors, or collected during treatment/ at progression. All clinical material was collected after informed consent from patient. The studies have been approved by regional ethics committee in Stockholm, Sweden and has been conducted according to the Declaration of Helsinki's principles.

Tissue Microarray (TMA) containing 50 clinical sample cores in duplicates was purchased from US Biomax (Me1002a) (https://www.biomax.us/tissue-arrays/Melanoma/ME1002a). This array also contained normal skin tissue.

Tissue Microarray (TMA) containing 65 clinical samples in duplicates from CMM patients with advanced stage melanoma (Stage III/IV) were procured as gifts from Prof. Meenhard Herlyn, The Wistar Institute, Philadelphia,USA. The collection of these samples have been approved by the Institutional Review Board (IRB) and the study has been conducted according to the Declaration of Helsinki's principles.

Xenograft murine model

A375 (*BRAF* V600E) melanoma cells ($3.6x10^6$) and growth factor reduced matrigel matrix (VWR) were mixed in a 1:1 ratio and subcutaneously injected in the flank of 6-week-old CB-17/Icr-*Prkdc*$^{scid/scid}$ females (Janvier). Once tumors reached palpable size, the treatment was commenced. Measurements for tumor size were conducted by using calipers thrice weekly and tumor volume was calculated using the formula vol= (D x d^2) x 0.52, where d is the smallest diameter and D is the largest diameter. The weight of the animal was also noted at these points. In the animal model, the following dosing regime was followed. Afatinib was used at 20mg/kg, crizotinib at 15mg/kg and the combination treatment was afatinib and crizotinib (20mg/kg + 15mg/kg). The animals were treated daily using oral gavage for 5 consecutive days, followed by two days without drug administration and this schedule was repeated one more time. The animals were sacrificed 14 days after start of treatment. All animal experiments were approved by Stockholm's Ethical Committee of Animal Research and performed in accordance to Karolinska Institutet guidelines.

Zebrafish embryo transplantation, treatment and luciferase measurement

All cells were processed one hour prior to transplantation into zebrafish embryos (Pudelko, Edwards et al. 2018). Briefly, immediately prior to transplantation, highly concentrated suspension of cells was filled into non-filament micro-capillaries (World Precision Instruments) and approximately 100 cells were injected into the blastula of zebrafish embryos at 2 h post fertilization (hpf) after which they were put in E3 medium and incubated at 33°C. The next day, successfully transplanted embryos were screened for, dechorionized using Pronase (Sigma) and distributed into 6-well plates (25 embryos/well) containing E3 media with HEPES. MTH1 inhibitor TH1579 (dissolved in DMSO to 10 mM) was added directly to the medium to a final concentration of 20 μM or 40 μM. For the control, DMSO was used. For combination experiments, the embryos were instead divided into four groups (DMSO control, vemurafenib (10μM), TH1579 (20μM) or combination). During treatment, embryos were incubated at 33°C until individual tumor size detection by luminescence measurement (Hidex Sense). Randomization was performed at the onset of the treatment without any blinding.

Cell culture, treatments and transfections

Melanoma cell lines A375 and SkMEl2 were purchased from American Type Culture Collection (ATCC). A375VR4 (vemurafenib) and A375PR1 (PLX4720) are resistant sublines of A375 (Azimi, Tuominen et al. 2017). ESTDAB cell lines were obtained from European Searchable Tumor Line Database and Cell Bank (ESTDAB). Pimary short term cultures were established from patient FNA samples as described previously(Das, Wilhelm et al. 2019). Patient derived cell lines ANRU and KADA were obtained as gifts from Prof. Rolf Kiessling, Karolinska Institutet, Sweden whereas deidentified patient derived lines 1205-Lu, 1346 and 3918 were obatined fom Prof. Meenhard Herlyn, The Wistar Institute, USA. Melanoma cancer associated fibroblasts (CAF) were obtained as a gift from Prof.Erik Sahai, Francis Crick Institute, UK. All *BRAF* mutant cells were cultured in MEM (Themo Scientific, Sweden) with supplements, whereas all *NRAS* mutant and *BRAF/NRAS* WT cells were cultured in RPMI-1640 (Themo Scientific, Sweden) with supplements. Patient derived cells were cultured in RPMI-1640, whereas CAF cells were cultured in DMEM (Themo Scientific, Sweden) with 1% ITS (Themo Scientific, Sweden) and supplements. All cell lines were cultured in the aforementioned media except when experimental conditions required cell lines to be cultured in another media. In such a case, cells were condionally adapted to new culture media before start of experiments. Flourescently labeled cells were generated

for both *in vitro* and *in vivo* experiments by transfecting CMM cell lines with lentiviral plasmids followed by antibiotic selection for 1 week. H2B cells for time lapse experiments were also generated by lentiviral transfection followed by 7 days of antibiotic selection. Cells overexpressing MTH1 or transfected with shMTH1 were also established in a similar manner (Das, Gad et al. 2020). All transfections were performed using Lipofectamine 2000 (Sigma-Aldrich Chemie Gmbh, Munich, Germany) according to manufacturer's protocol. All cell lines were tested with LookOut Mycoplasma PCR detection kit (Sigma Aldrich, Stockholm, Sweden) and confirmed to be mycoplasma free.

Afatinib resistant, crizotinib resistant and combination resistant cell lines used in paper IV are sublines of A375. These cell lines were created as induced resistant lines, where A375 cells were plated in 6 well plates and treated with IC50 concentrations of afatinib, crizotinib or the combination. Dead cells were washed away, media was replaced with fresh media containing drugs and cells were subjected to constant drug pressure until resistant cell clones that were resistant to IC50 values of the drugs emerged. These clones were expanded for 2 months while keeping them under constant drug treatment.

All drugs used in this thesis was dissolved in DMSO. For the *in vitro* treatment studies in paper I and II, for most experiments unless otherwise stated, afatinib (Selleckchem) was used at 2µM, crizotinib (Selleckchem) at 2µM and the combination at 2µM+2µM. DMSO (corresponding to the % of DMSO in the combination treatment) was used as control for all experiments. For paper III vemurafenib (Selleckchem) was used at 0.1µM- 0.5µM, dabrafenib (Selleckchem) at 10nM and trametinib at 3nM (Selleckchem). For most experiments in paper III and IV, MTH1 inhibitor TH1579 (developed by the Helleday group) was used at 0.9µM.

Whole genome sequencing

Allprep universal kit was used to extract DNA from cell lines. The DNA quantification was done using NanoDrop 2000 instrument. 100 ng was subjected to whole genome sequencing (WGS) using library build-up with the Nextera DNA library prep, Illumina platform and in-house developed post-read filtering (Science for Life Laboratory, Stockholm, Sweden). Partek Flow software and DNA-Seq Toolkit for Partek Flow was used to map resulting reads, call variants and filter for variants in the coding regions and excluding indels.

2D, 3D MTS assay and drug synergy

Cell viability assay in both 2D and 3D (spheroid generated by hanging drop method) formats were performed. Briefly, 3000-4000 cells per well were plated overnight for 2D and 10,000 cells per well were plated until spheres formed and matured (3D). They were then treated with drugs for 72 h and for 2D assay, cell viablity was read out as absorbance (490nM). For the 2D assay, MTS solution (Promega, Madison, WI, USA) was used and for 3D assay MTS solution CellTiter 3D (Promega, Madison, WI, USA) was used. Cell lysis was done and luminescence was used as a read out.

For drug synergy assays, 800-1000 cells/well was dispensed in 384-well plates using a D300 digital dispenser (Hewlett-Packard, Tecan Trading AG, Switzerland). Drugs were dispensed in wells and cells were exposed for drugs for 72 h after which flourescence was measured. Drug synergy plots were created using FIMM Synergy Finder (https://synergyfinder.fimm.fi). For all experiments (Tecan Spark 10M, Tecan Trading AG, Switzerland) plate reader was used.

RNA extraction

AllPrep DNA/RNA/miRNA kit (Qiagen, Hilden, Germany) was used to extract RNA from cells. Agilent Bioanalyzer 2100 instrument (Agilent Technologies Inc., Santa Clara, CA, USA) was used to measure RNA quantity and quality.

Real-Time PCR

A 20 µl reaction using standard reagents from Invitrogen with SuperScript III reverse transcriptase (Calsbad, CA, USA) was prepared to convert the extracted RNA into cDNA which was further diluted and subjected to semi-quantitative real-time PCR reaction in a Bio-Rad CFX instrument (Hercules, CA, USA). CFX Manager software was used to analyze the results.

Extraction of data from targeted sequencing using Ion AmpliSeq ™

Targeted sequencing of fine needle aspirate or core biopsy RNA from metastases and RNA from cell lines was previously performed using the Ion AmpliSeq Transcriptome Human Gene Expression Kit for RefSeq genes ((Thermo Fisher Scientific, Waltham, MA. USA) as described in (Azimi, Tuominen et al. 2017).

Cell lysis for immunoblotting, RPPA and pRTK array

RIPA lysis buffer containing phosphatase and protease inhibitors was used to lyse cells as previously described in (Das, Wilhelm et al. 2019). For RPPA, lysates from cell lines and ~50mg tumor tissue from xenografts was homogenized and processed in lysis buffer as per recommendation by the MD Anderson Cancer Center Functional Proteomics Core (MDACC) Facility. Standard protocols were used to denature protein lysates, followed by protein measurement estimation using BCA reagent (Thermo Fischer Scientific, Germany). Appropriate amounts of protein were used for western blot analysis, pRTK array (ARY001B) or for RPPA analysis conducted at the MDACC Functional Proteomics Core Facility. The data was analysed as previously described in (Davies, Stemke-Hale et al. 2009, Gopal, Deng et al. 2010).

Immunoblotting and pRTK array

40-50µg of denatured protein was loaded on 4-12% NuPage Bis-Tris gel (Thermo Fisher Scientific, Waltham, MA, USA). After completion of run, the proteins were transferred to PVDF membrane (GE Healthcare). Once the transfer process was complete, membranes were blocked with 5% milk or 5% BSA for 1h followed by overnight incubation in 4˚C with primary antibody. The following day, membranes were washed thrice with TBS-T and incubated with secondary antibody for another 1.5 h. Membranes were washed again and developed with ECL reagent (Thermo Scientific, Waltham, MA, USA) using Image Quant LAS 4000 (GE Healthcare Europe GmbH, Freiburg, Germany). For pRTK array, 150-200µg of protein was incubated with the appropriate array membranes (previously spotted with antibodies) overnight at 4˚C. The following day, similar steps were followed as mentioned above. Anti-HRP conjugated antibody as provided with the array kit was used as secondary antibody instead.

Immunohistochemistry and immunocytochemistry

Immunohistochemistry and immunocytochemistry were performed as per manufacturer's protocol (Cell Signaling Technologies and Dako). Briefly paraffin embedded tissues sections or spheres were de-paraffinized and rehydrated. Antigen retrieval by either citrate buffer, proteinase K or EDTA was performed on sections followed by blocking in 3% hydrogen peroxide for 10 minutes and then in 2.5% horse serum for 30 minutes. Slides were incubated overnight with primary antibody against EGFR (1:50, Dako), ERBB3 (1:250, Cell Signaling Technologies), MET (1:300, Cell Signaling Technologies), Ki67 (1:400, Cell Signaling Technologies) or p-H2AX (1:200, Cell Signaling Technologies). Next day, sections stained

with ERBB3 and MET were incubated with rabbit signal stain boost (Vectastain art. 8114, Histolab Products AB, Stockholm, Sweden). For the sections stained with EGFR, ABC staining kit (Thermo Fischer Scientific, 32052) was used. DAB staining followed by counterstaining with haematoxylin was performed for all stainings followed by dehydration step. Slides were then mounted using Pertex (Histolab Products AB, Stockholm, Sweden) and imaged using Olympus Provis microscope.

Independent evaluation of all slides was performed by three observers. The intensity (negative, low (1+), moderate (2+) or strong (3+)) and proportion of ERBB3, EGFR, MET, Ki67 and p-H2AX positive tumor cells was evaluated.

Immunofluorescence

Zebrafish embryos were processed to make cryo sections as previously described (Inoue and Wittbrodt 2011) and where then placed on glass slides. Slides were equilibrated with 3 washes, 5 min each using 1X PBS-Tween 20. After this, they were blocked for 1 h at room temperature with 10 % goat serum (ab 7481) or donkey serum (ab 7475) in 1X PBS-Tween 20. The slides were incubated overnight at 4 ˚C with either anti-CAV1 (1:400) or anti-AXL (1:100). Next day, the slides were washed with low shaking (50 rpm). They were incubated with secondary antibody (1:500) for 2 h, washed with 1X PBS-Tween 20 for 3 times, 5 min each. The slides were then mounted using DAPI fluoroshield (F6057) and imaged by AxioImager M2 (Zeiss).

FFPE clinical and xenograft samples were deparaffinized and dehydrated in ethanol as per manufacturer's protocol. Antigen retrieval was performed using citrate buffer (pH 6.0) followed by blocking with H_2O_2 and serum. Sections were incubated overnight with mTOR (1:75, Cell Signaling Technologies), MET (1:200, R & D Biosystems), RPS6KB1 (1:100, Sigma- Aldrich), pRPS6KB1 (1:100, Thermo Scientific), pRPS6 and RPS6 (1:200, Cell Signaling Technologies), ACSS1 (1:150, Santa Cruz Biotechnology), MTH1 (1:100, Novus Biologics) or PMS2 (1:100, BD Biosciences). The following day, sections were washed, incubated with secondary antibodies (1:200, rabbit Alexa Flor 488 or mouse Alexa Flor 594, Cell Signaling Technologies) for 2 h, and mounted with DAPI (Sigma Aldrich), visualized and imaged using AxioImager M2 (Zeiss).

In-situ Proximity Ligation Assay (isPLA)

isPLA experiments were performed by the PLA proteomics facility, Science for Life laboratory, Sweden. Briefly Duolink in situ PLA Sigma Aldrich (according to manufacturer's protocol) were used to perform isPLA. Briefly, 0.5% Triton X-100 was used to permeabilize cells followed by TBST (TBS +0.05% Tween-20) wash and overnight blocking. Cells were incubated overnight with primary antibody and the following day, cells were stained with phalloidin (1:40) for 15 min at room temperature and mounted in media containing DAPI. Cells were then imaged using fluorescence microscope (Zeiss) and subsequently the PLA signal was measured using cell profiler software.

Co-immunoprecipitation assay

Cells plated at 85 % confluency were allowed to grow overnight. The following day, protein extraction was performed using these cells on ice using cell lysis buffer (cell signaling, #9803) with protease and phosphatase inhibitors and protein amount was quantified using BCA as previously described. Pre-washed magnetic beads (Thermo Scientific, #88802) and primary antibody (concentration used as per manufacturer's recommendation) were mixed with 300µg of protein and incubated overnight at 4 ˚C. Next day, unbound antibody from beads were washed away, beads were then boiled with 4X SDS (Thermo Scientific, # NP0007) to release the bound fraction and thereafter were the beads spun down. Supernatant was collected and analyzed using western blot.

***In vitro* kinase assay**

Test compounds were nano-dispensed in 384-well, white, low volume, non-binding plates (Costar #3824). The AXL kinase reaction (V3961, Promega) was performed according to manufacturer's protocol. Luminescence was recorded in Hidex Sense reader.

CETSA- western blot

CETSA (Cellular Thermal Shift Assay (CETSA) was performed as previously described in (Warpman Berglund, Sanjiv et al. 2016). Briefly, cells were plated to 70 % confluency. The next day, cells were treated with DMSO or TH1579 (0.9 µM) for 2 h at 37 ˚C. They were then trypsinized and resuspended in media. Around 80 µl of cell suspension was added to a

PCR tube per heating condition and treated for 3 min (Veriti 96 well thermal cycler, AB). Samples were then lysed in RIPA buffer and analyzed by western blot.

Cell confluency

In 96 well plates approximately 2-3x 10^4 CMM cells were seeded and cultured with or without CAF conditioned media. Cell confluency was measured at 0 h, 6 h, 24 h and 48 using Tecan Spark 10M (Tecan Trading AG, Switzerland). For another experimental setup, CMM cells fluorescently labeled with eGFP were grown with or without unlabeled CAF cells for 48 h or 14 days and treated with 1µM afatinib, 1µM crizotinib or the 1µM +1µM combination. eGFP signal was measured as a readout for CMM cell growth at similar time points after treatment as mentioned above. All readings in this experimental condition were normalized to DMSO.

Cell Lysis for proteomic analysis

Harvested cell pellets were resuspended in lysis buffer supplemented with phosphatase inhibitor (Roche Applied Science)). Cells were sonicated and centrifuged and protein concentration was determined as mentioned before using BCA reagent kit (Pierce). The protein lysates were reduced, enzymatically digested and the proteome and phosphoproteome were analysed using a label free method.

Phosphoproteomics analysis

200 µg of tryptic digest per sample underwent TiO_2 phosphopeptide enrichment step as previously described (Azimi, Caramuta et al. 2018); and analysed by LC-MS/MS using a Fusion OrbiTrap (Thermo Fisher, Germany). The mass spectrometer was connected to a Dionex UHPLC system (Thermo Fisher Scientific, Germany).

LC-MS/MS analyses

The proteome and phosphoproteome of each sample were analyzed on a Fusion Orbitrap (Thermo Fisher, Germany) as previously described in (Azimi, Caramuta et al. 2018).

Proteomic and phosphoproteomic data analyses

The raw data were analyzed using MaxQuant 1.5.3.30 (Cox and Mann 2008) and Andromeda (Cox, Neuhauser et al. 2011) was used to search the MS/MS data against the UniProt *Homo*

sapiens database (containing canonical and isoforms_42259 entries downloaded on 15th January 2018) complemented with a list of common contaminants, and concatenated with the reversed version of all sequences as previously described in (Azimi, Caramuta et al. 2018). Data analysis was executed using Perseus (1.6.1.2). Only total protein/phosphopeptides with expression levels >1.5-fold changes ($p<0.05$) were considered for further analyses.

Cell cycle analysis

100,000 cells/well were plated overnight in 12 well plates, followed by treatment with either DMSO or 0.9µM TH1579 for 24h. The cells were trypsinized, collected and fixed using 4% buffered formaldehyde at room temperature, followed by fixation in 95% ethanol for 1 h, and rehydrated in distilled water for 1 h. Cells were treated with subtilisin Carlsberg solution containing protease (Sigma), stained with DAPI and analyzed using a LSRII flow cytometer (Becton Dickinson). DAPI fluorescence was measured above 435 nm. For histogram analysis, the ModFit program for cell cycle analysis (Verity software house) was used. The number of nuclei/histogram was 10 000.

ROS measurement

50000 cells/well were plated overnight in 12 well plates. For ROS measurements, cells were treated with DMSO, TH1579 (0.5µM or 0.9µM) or vemurafenib (0.45µM) or the combination for 3 h, trypsinzed, stained with CM-H2DCF (Life Technologies, C8627) and analyzed by FACS as per manufacturer's protocol.

Modified Comet assay

Approximately 150,000-200,000 cells/well was seeded in 6-well plates followed by treatment with TH1579 or DMSO for 24 h on the next day. The modified comet assay was performed as earlier described (Gad et al. 2013). Briefly, cells were trypsinized, washed and resuspended in 1 x PBS at a concentration of approximately 1 million cells/ml. The cell suspension was mixed with low melting agarose, followed by the addition of the mixture in agarose coated slides. The slides were incubated in lysis buffer followed by three washes with enzyme buffer. OGG1 enzyme was added and slides were incubated for 45 min at 37°C. After this, alkaline denaturation with alkali buffer was carried out in an electrophoresis chamber for 20 min. Electrophoresis was run at 25 V and 300 mA in the same buffer for 30

min. The slides were later neutralized with neutralizing buffer for 45 min. The slides were stained with 1x SYBR gold dye immediately prior to imaging. 100 comets were counted using Comet IV software.

Time-lapse microscopy

In a 96 well black plate with transparent bottom 700-1200 cells/well were plated either in co-culture or single, treated with DMSO or vemurafenib (0.4µM) or trametinib (3nM) or TH1579 (0.45µM, 0.9µM, 1.8µM) and incubated in 95 % humidified atmosphere with 5 % CO_2 at 37°C. The plates were imaged between days 0-4 at intervals of 24 h with 10X using time lapse microscopy (ImageXpress MicroXL (Molecular Devises)). Cell profiler analyst software was used to quantify images.

Flow Cytometry

About 50000 cells/well were plated in 12 well plates overnight. The next day, cells were treated for 48 h or 72 h, followed by trypsinization and collection. Pellets were washed once with 1X PBS, the cells were resuspended in FACS buffer and then stained for 10 minutes in the dark on ice with 2% Annexin V and 2% PI solution (Sigma-Aldrich Chemie Gmbh, Munich, Germany). After staining, an additional 200 µL FACS incubation buffer was added and analysis was performed using Novocyte 3000 and Novoexpress software (ACEA Biosciences, San Diego, CA, USA) to determine induction of apoptosis and necrosis.

Scratch Assay

In 3.5cm dishes, cells were plated at 90% confluency. With the help of a 100µL filter tip, a wound was created. Following this, cells were washed gently once with PBS and were exposed to DMSO, afatinib or crizotinib alone or in combination for 5 days. Cells were washed and the gap filling/wound healing was documented using picture documentation Nikon Eclipse TS-100 microscope after 0 h, 6 h, 24 h, 48 h and 120 h treatment. Analysis of the gap filling/wound healing was performed using ImageJ software.

Transwell migration assay

Approximately 3-5 X 10^4 cells were plated on inserts (CLS3422-48EA) containing media supplemented with 2% FBS and drugs or DMSO. The lower well was filled with 750µL of media containing 7.5% FBS (used as an attractant) and cells were allowed to migrate for 16-24 h. The wells were then washed with PBS, fixed in 4% formaldehyde for 5 min followed

by 20 min in methanol. Cells were stained using crystal violet (Sigma), and imaged using Olympus Provis microscope. The crystal violet was dissolved in methanol and absorbance was measured at 540 nm using Tecan Spark 10M plate reader instrument. The readout was the number of migrated cells.

Colony Formation Assay

200-500 cells/well were plated in 6 well plates overnight ensued by treatment with either single or combination treatment for 5 days. After this media was replaced with regular media without drugs and colonies were allowed to mature for an additional 7 days, with the media being replaced every 3rd day. After this, colonies were fixed for 20 min using 4% buffered formaldehyde followed by staining with 0.05% crystal violet solution. The colonies were washed twice with 1X PBS to remove the extra stain, scanned using Epson scanner V370. To estimate amount of colony formation, crystal violet was dissolved in 100% methanol. In a 96 well plate, the crystal violet was diluted in 1:10 and absorbance was measured at 540 nm using Tecan Spark 10M plate reader instrument.

3D Invasion Assay

Each well of an 8-chamber slide was coated with ten µL of 100% Matrigel (Sigma-Aldrich Chemie Gmbh, Munich, Germany). A cell suspension containing 15000 cells/mL and 4% Matrigel was added on top of the first coating and spheres were allowed to mature for 6 days. The media was replaced with media containing DMSO, afatinib or crizotinib alone or in combination for 72 h after which wells containing spheres were washed, fixed in 4% formaldehyde followed by washing and permeabilization with Triton X-100 for 15 min. Slides were blocked with 5% horse serum for 1 h and incubated for 1.5 h with Texas Red X Phalloidin (Thermo Fisher Scientific, Waltham, MA, USA). Slides were washed thrice with 1X PBS, mounted with Fluoroshield containing DAPI (Sigma-Aldrich Chemie Gmbh, Munich, Germany) and imaged using Zeiss AxioImager M2 microscope.

Transmission Electron Microscopy

Transmission electron microscopy (TEM) was performed using standard procedures of glutaraldehyde fixation and cell scraping prior to embedding for TEM. With a focus on the cell periphery, to study and compare presence of autophagosomes in cells treated with afatinib (4 µM, 6 h), crizotinib (4 µM, 6 h), combination of both and vehicle control (DMSO).

4. RESULTS AND DISCUSSION

4.1 Paper I: Combining ERBB family and MET inhibitors is an effective therapeutic strategy in cutaneous malignant melanoma independent of *BRAF/NRAS* mutation status

Current clinically used MAPK targeting therapies for patients with disseminated CMM improve survival, but attaining a long-term clinical response is often challenging since in most cases, patients develop resistance to ongoing treatment and hence the disease relapses. A number of studies have highlighted the role of several RTKs in mediating resistance towards MAPKi. In this study, we investigated the therapeutic potential of combining MET and ERBB family inhibitors in CMM.

4.1.1 Results: In this study, we show that MET and ERBB3 is highly expressed in patients with metastatic CMM (*Paper I, Figure 1a*) (Cerami, Gao et al. 2012, Gao, Aksoy et al. 2013). Moreover, transcriptional profiling of matched cases (sampled before treatment and at progression) from two patients treated with MAPKi showed an increased mRNA expression of MET and EGFR/ERBB3 at relapse. Moreover, silencing of MET in BRAFi resistant A375VR4 cells caused an upregulation of EGFR, thus indicating RTK crosstalk (*Paper I, Figure 1b-f*). We therefore assessed the potency of afatinib and crizotinib as single treatment and in combination using cell viability as a readout. Using a panel of ten CMM cell lines (2D assay) and thirteen CMM cell lines (3D spheroid assays) with different *BRAF/NRAS* mutation status, we show that the combination treatment induced a significant ($p<0.01$) loss in cell viability as compared to DMSO or single treatments across all cell lines tested (*Figure 11, Paper I, Figure 2, Supplementary Figure S4-S5*). Dose response curves indicated that this combination treatment effect was synergistic (*Paper I, Supplementary Figure S3*).

For further functional analysis, we chose five cell lines (A375, A375VR4, SkMel2, ESTDAB102 and ESTDAB105). To assess the effects on cell proliferation and cell death, we performed colony formation and FACS assays. We were able to show that the combination treatment abrogated cell proliferation ($p<0.001$) and induced cell death via apoptosis ($p<0.05$) (*Paper I,Figure 3*) when compared to DMSO or single treatments. We validated our findings using spheroids created from CMM cells where we treated the spheres for 72 h with DMSO, single or combination treatments and stained them with Ki67 and p-H2AX to evaluate the effect on proliferation and DNA damage induction (*Paper I, Figure 4a-b, Supplementary Figure S6*). Combination treatment was able to reduce proliferation

marker Ki67 and induce DNA damage marker p-H2AX more robustly when compared to single treatments or DMSO.

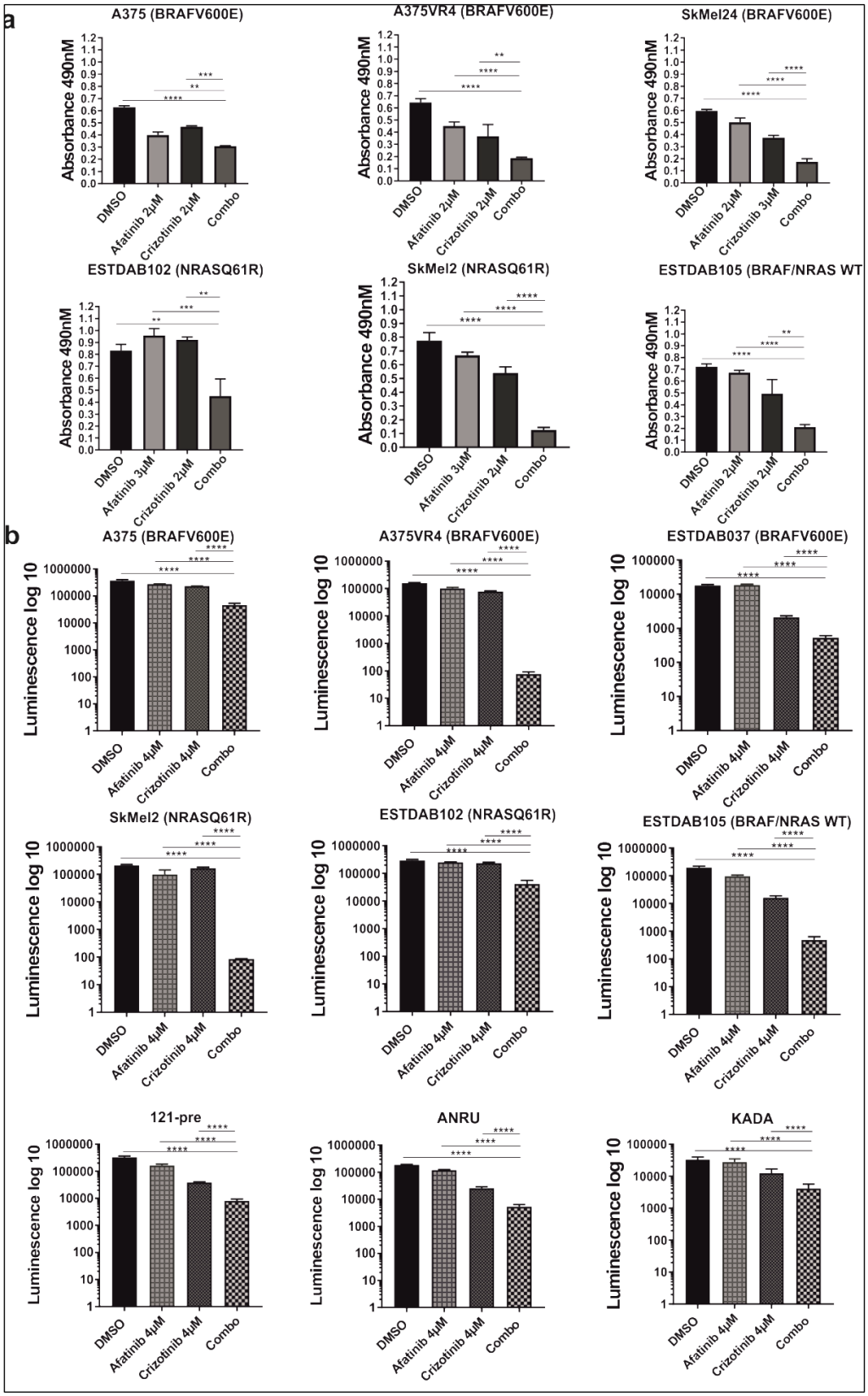

Figure 11: (a) 2D MTS assay showing single and combination treatment effects on cell viability in CMM cell lines. (b) 3D MTS assay showing single and combination treatment effects on cell viability for spheroid models generated from established and short-term patient derived cell lines.(Das, Wilhelm et al. 2019)

Furthermore, using a spheroid model, we were able to show that the combination treatment was able to significantly reduce invasive capacity of CMM cells over the single treatments and control (*Paper I, Figure 4e*). To test the effects on migration, we demonstrated using both a scratch assay and a transwell migration assay that the combination treatment significantly ($p<0.01$) attenuated CMM migratory capacity (*Paper I, Figure 4f-g, Supplementary Figure S8*). Using an *in vivo* A375 xenograft model, we showed that the combination treatment significantly caused tumors to grow at a slower rate whilst causing a significant ($p<0.01$) induction of p-H2AX and a reduction in Ki67 (*Figure 12, Paper I, Figure 5, Supplementary Figure S9*). These observations were more pronounced for the combination treatment compared to the other treatment arms.

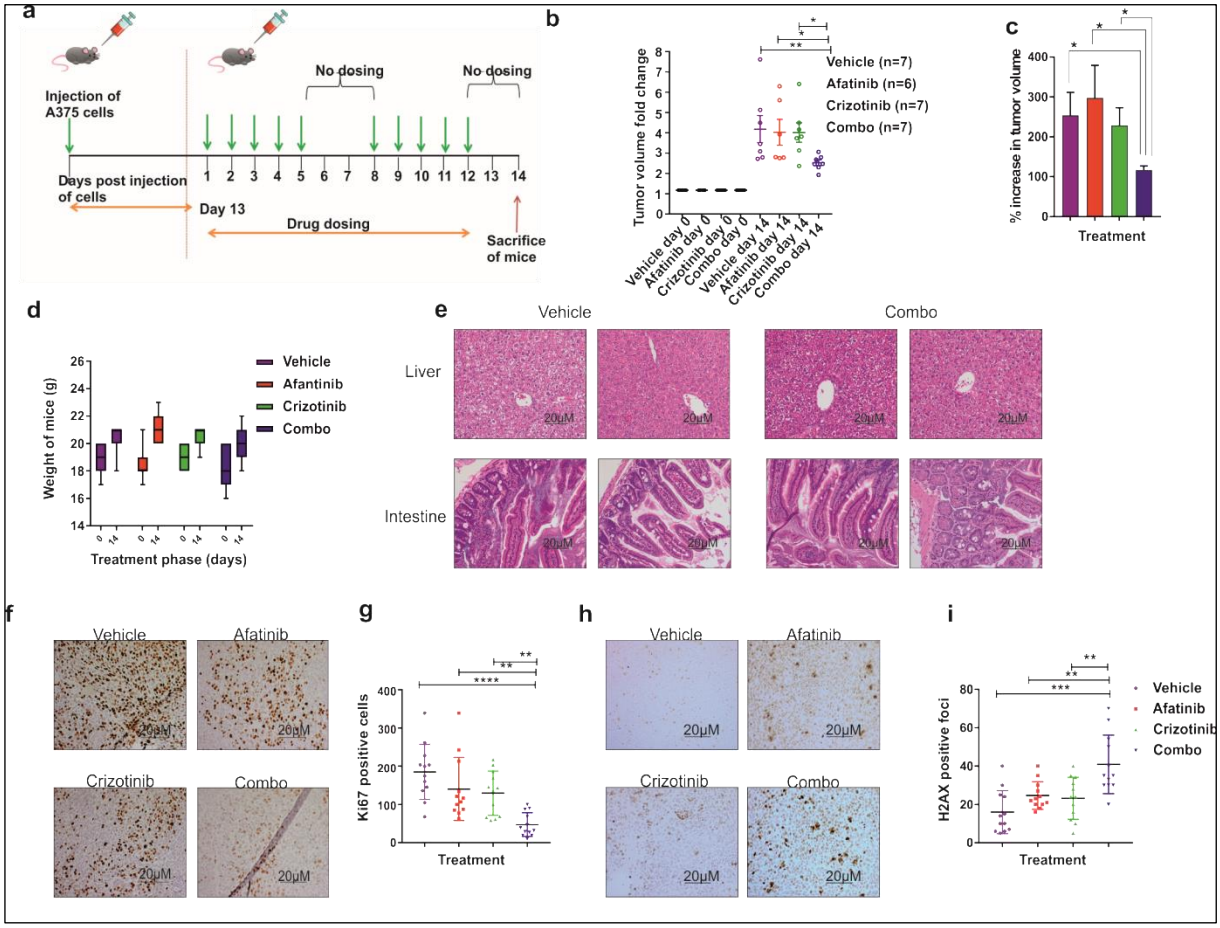

Figure 12: (a) Experimental design. (b-c) Tumor fold change and % increase in tumor volume after treatment. (d). Weight of animal recorded prior to start of treatment and at the end of treatment. (f-g) Tumor tissues showing Ki67 staining as images and quantification of Ki67 positive foci. (h-i) Tumor tissues showing H2AX staining as images and quantification of H2AX positive foci.(Das, Wilhelm et al. 2019)

Lastly, we showed that dynamic molecular rewiring occurred when comparing culturing cells in 2D or as spheroids, here reflected by the changes in protein expression patterns of different RTKs (ERBB3, AXL, MET) and their downstream effectors (AKT) (*Paper I,Figure 6b-c*).

4.1.2 Discussion: As per our knowledge, this is the first study on CMM demonstrating the broad cytotoxic effects of combining afatinib and crizotinib, clinically approved drugs for lung cancer treatment. Here we show that in *BRAF* mutant inhibitor sensitive cells and *NRAS* mutant cells, loss of WEE1 expression may contribute towards the combination mediated effects, whereas in *BRAF* mutant, inhibitor resistant cells and *BRAF/NRAS* WT cells, the combination effects may be attributed to loss of IGF1R expression (*Figure 13, Paper I, Figure 6k*).

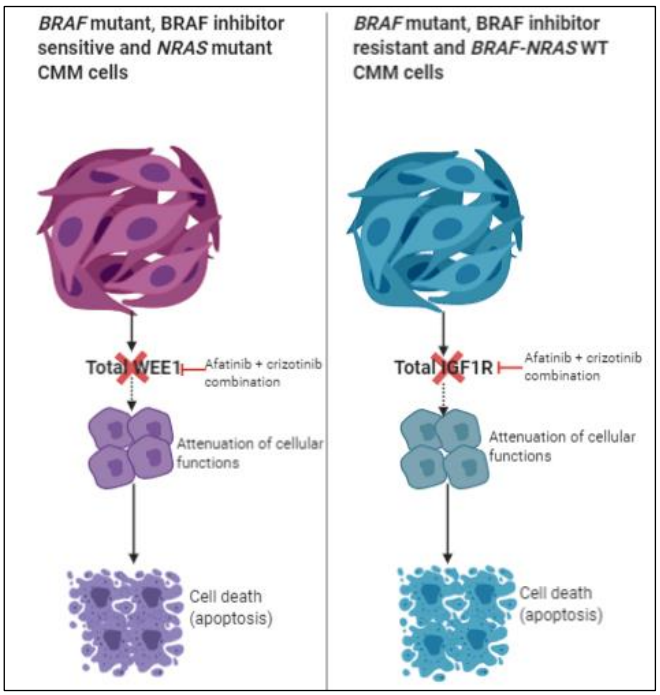

Figure 13: Schematic of proposed mechanism of action of afatinib and crizotinib combination therapy

Several studies have shown that in cancer, crosstalk between RTKs and other signaling pathways play an improtant contribution towards cancer intitiation, progression and drug resistance (Yamaguchi, Chang et al. 2014, Chan, Singh et al. 2017) . In this study we found evidence of crosstalk between MET and EGFR since downregulation of MET caused an upregulation of EGFR and AKT signaling in A375VR4 cells (*Paper I, Figure 1f*). It has also been shown that a crosstalk between ERBB3 and MET regulates invadopodia formation in melanoma cells (Revach, Sandler et al. 2019). Therefore, in this study, we employed the use

of a combination strategy to inhibit both ERBB family and MET concomitantly. One of the common resistance mechanisms towards EGFR/ERBB2 inhibitors is upregulation or activation of other RTKs, for example in non-small cell lung cancer (NSCLC), upregulation of AXL and amplification of MET and ERBB2 have been shown to contribute towards EGFR inhbitor resistance (Zhang, Lee et al. 2012). Moreover, in colon cancer, amplification of MET, ERBB2 and IGF1R has been shown to cause resistance to the EGFR inhibitor cetuximab (Bean, Brennan et al. 2007). The broad phenotypic effects of afatinib and crizotinib combination therapy can be attributed to the inhibiton of multiple targets, thus preventing plausible upregulation through RTK mediated crosstalk.

The importance of WEE1 and IGF1R in contributing towards melanoma pathogenesis has also been described before. A recent study suggests that inhibiton of WEE1 contributed towards potent inhibiton of AKT3 which has been shown to be an important therapeutic target in CMM, but AKT3 does not have much effect when targeted alone (Kuzu, Gowda et al. 2018). Additionally, high expression of WEE1 has also been associated with poor disease free survival (DFS) in CMM (Magnussen, Holm et al. 2012) which is in accordance with our observation in this study where we have also shown a trend that CMM patients (n=17) with high WEE1 expression have lower PFS after immunotherapy (*Paper I, Figure 6g*). Suppression of IGF1R has also been shown to abrogate melanoma growth and induce apoptosis in melanoma cells (Xin, Lei et al. 2018). In this study, we also observed that IGF1R is downregulated in *BRAF* mutant inhibitor resistant cells and in *BRAF/NRAS* WT cells after combination treatment (*Figure 13*, *Paper I, Figure 6k*). Furthermore, we found that patients with high IGF1R expression have a shorter PFS after immunotherapy, although not significant (*Paper I, Figure 6g*). To our knowledge no previous studies have reported a correlation between WEE1 and IGF1R mRNA expression in CMM tumors ($p<0.001$, $r=0.74$).

In summary we show that the combination treatment of afatinib and crizotinib is highly efficacious compared to single treatment, plausibly due to the concurrent inhibition of multiple RTKs preventing upregulation of alternate salvage pathways sue to receptor crosstalk. *In vitro*, the afatinib and crizotinib combination treatment ablated cell proliferation, cell viability, invasion, migration and induced apoptosis. Treating a A375 xenografted mouse model with the afatinib and crizotinib combination treatment abrogated tumor growth rate as compared to vehicle or single treatments, thus indicating that this combination is effective *in vivo* and also did not cause any severe toxicities at the tested concentrations. Lastly we showed that WEE1 and IGF1R, two non-canonical targets of the drugs are inhibited with the combination treatment and to a lesser extent with single treatments. Our study suggests that

IGF1R and WEE1 are potential targets of afatinib and crizotinib combination. Ongoing clinical trials using WEE1 inhibitor MK-1775 are being conducted for solid tumors to evaluate the clinical efficacy of WEE1 inhibition (Kuzu, Gowda et al. 2018). Targeting IGF1R in phase I/ II trials led to SD in only 38% of patients with recurrent platinum sensitive ovarian tumors. Therefore it is imperative to identify predictive biomarkers for IGF1R mediated therapies to select subset of patients who could benefit from such therapies (Werner, Sarfstein et al. 2019).

4.2 Paper II: Downregulation of the insulin/MTOR signaling pathway by afatinib and crizotinib combination treatment confers broad cytotoxic effects in cutaneous malignant melanoma

Targeted therapies have often been deemed as promiscuous because they have several off-target effects. The main known targets for afatinib are RTKs EGFR, ERBB2, ERBB4 and those for crizotinib are MET/ALK/ROS1. However, based on the results from paper I, we postulate that there are additional key molecular targets beyond these and in addition to WEE1 and IGF1R that could play a role in mediating sensitivity to the afatinib and crizotinib combination therapy. Identification of these novel targets could be of clinical benefit.

4.2.1 Results: In this study, we sought to elucidate molecular mechanisms behind the broad cytotoxic effects of afatinib and crizotinib in combination. Herein, we employed three different approaches: Phosphoproteome and whole proteome based profiling using mass-spectophotometry (MS), Reverse Phase Protein Array (RPPA) and pRTK array to determine changes occuring in CMM cells and xenograft tissues (from model used in Paper I) at the protein level upon treatment with single or combination treatment. For the MS analysis, we treated A375 (*BRAF* mutant) and SkMEl2 (*NRAS* mutant) for 3 h to capture the early and thereby most likely direct drug targets. Our results indicated that DNA damage / ribosome processing/ mTOR and Insulin signaling pathways were significantly deregulated ($p<0.05$, linear fold change=1.5). Total LAMTOR 5, pRPTOR (S859) and pRB1 (S795) for both cell lines while pIRS-2 (S391,560,577,679, 915), total LAMTOR 1 and pCDC25B (K334, S375) specific for A375 and pRPS6 (S235,236,240) for SkMel2 stood out as candidate targets that were significantly de-regulated (*Paper II, Figure 1b*). RPPA analysis was performed where A375, A375VR4, SkMel2, ESTDAB102 and ESTDAB105 were treated with drugs for 3 h (to capture early effects) and 24 h (to capture those proteins that were differentially expressed when subjected to cellular stress). In accordance with our analysis by MS, we found that PI3K/AKT and mTOR pathways were significantly deregulated. We also showed that the combination treatment mediated downregulation of p-RPS6 in all five cell lines (*Paper II, Figure 1d-e, Supplementary figure S4*). IRS-2 in the insulin signalling was among the top down-regulated candidates in A375 phosphoproteomic analysis when comparing DMSO vs combo. However, since this protein was absent in the RPPA panel, we checked for another insulin receptor substrate, IRS-1, instead and observed a slight overall decrease of total IRS-1 in protein expression at 24 h post combination treatment in SkMel2 and A375VR4 (*Paper II, Figure 1e*). Pathway analysis performed on results obtained from a similar conducted

RPPA analysis using tumor tissue lysates from A375 xenograft mouse model further corroborated the significant downregulation of insulin and PI3K/AKT pathways with the combination treatment (*Paper II, Figure 2a-b*). Due to high FDR rates for the *in vivo* RPPA results, we used immunoflourescence to validate these data. Our results indicated that a greater loss of IRS-1 nuclear signal in the combination arm compared to single treatments or vehicle. We also confirmed our *in vitro* observations of the combination treatment mediated loss of pRPS6KB1, total RPS6KB1 and pRPS6. *In vitro* validations using CMM cell lines treated for 3 h further corroborated our results (*Paper II, Figure 2c-e, Supplementary Figure S5*). Notably we also showed that induced resistance towards the combination could be reversed by a 'drug holiday' (in our study, the holiday phase was for 2 weeks). RPPA analysis performed on drug sensitive A375 and resistant A375 sublines also showed that PMEL/ gp100 (significant) and PI3K-p85 (not significant) was upregulated in combination resistant sublines compared to afatinib resistant,crizotinib resistant or drug sensitive sublines (*Paper II, Figure 3, Supplementary Figure S6*). We showed that growth of CMM cells cultured in CAF conditioned media caused upregulation of p-AKT in CMM cells which could partly attribute to the increase in cell growth observed in only the vemurafenib resistant cell line A375VR4. However, no altered protein expression was observed in IRS-1, RPS6KB1 and RPS6 under a similar setting (*Paper II, Figure 4, Supplementary figure S9*). Moreover, when analyzing tumor samples from CMM patients with stage I-IV disease, we observed differential staining and localization patterns of IRS-1, RPS6KB1 and RPS6. Our results indicated that IRS-1 was predominantly localized in the nucleus in Stage III/IV (73%) CMM compared to Stage I/II (28%). We also observed that RPS6KB1 expression was strong in Stage III/IV (53%) disease compared to Stage I/II (4%) (*Paper II, Figure 5*). In addition, we analyzed data from TCGA and found that patients with *NRAS* mutant CMM had higher IRS-1 compared to patients with *BRAF* mutant CMM (*Figure 12, Paper II, Figure 6a*). Analysis of gene expression data from previously published studies indicated that high IRS-1 was associated with poorer OS for CMM patients on immunotherapy (*Figure 14, Paper II, Figure 6b*). Finally, we demonstrated that IRS-1 and RPS6KB1 was commonly heterogeneously expressed in the CMM tumors. Additionally we observed a differential pattern of protein cellular localization on comparing treatment naïve patients to those who were previous treated (*Figure 14, Paper II, Figure 6c*).

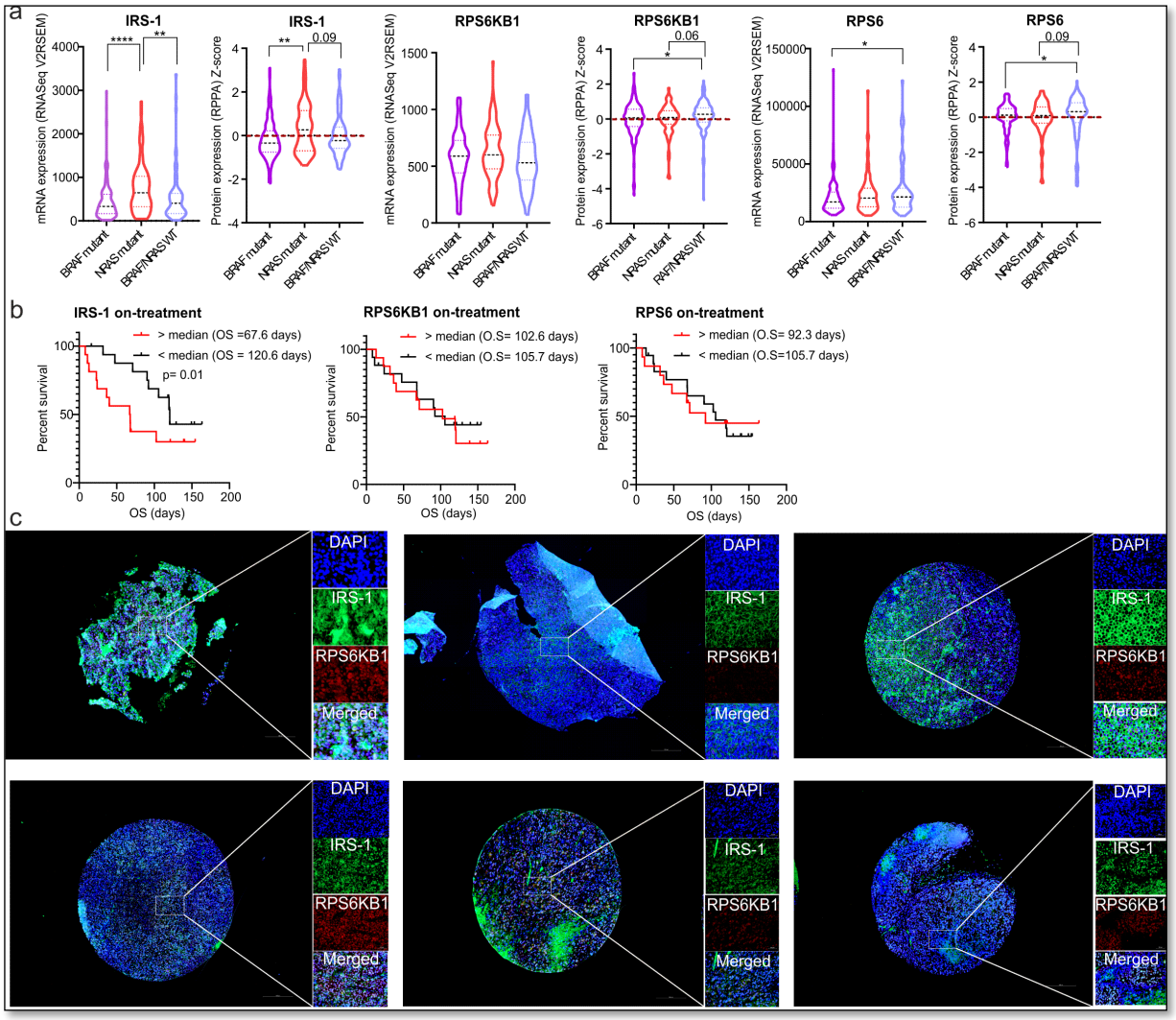

Figure 14: (a) Analysis of TCGA data showing that IRS-1 expression is higher in NRAS mutant CMM compared to BRAF mutant CMM at both mRNA and protein levels. (b) High IRS-1 is associated with shorter OS (Riaz, Havel et al. 2017). (c) IRS-1 and RPS6KB1 is heterogenously expressed in CMM clinical samples

4.2.2 Discussion: This exploratory study focused on identifying molecular targets that contribute to the broad cytotoxic response of afatinib and crizotinib combination therapy. By multiple proteomic approaches, we found that the combination of afatinib and crizotinib primarily de-regulated the expression of proteins belonging to the PI3K/AKT, mTOR, Insulin signaling, and DNA repair pathways. Herein we identified IRS-1 (known substrate molecule for IR/IGF1R), pAKT, pRPS6KB1 and pRPS6 as potential key targets contributing towards the combination treatment mediated effects. Moreover, we suggest that upregulation of PMEL/gp100 and PI3K-p85 might be important factors in mediating resistance to this novel combination treatment (*Figure 15, Paper II, Figure 6d*).

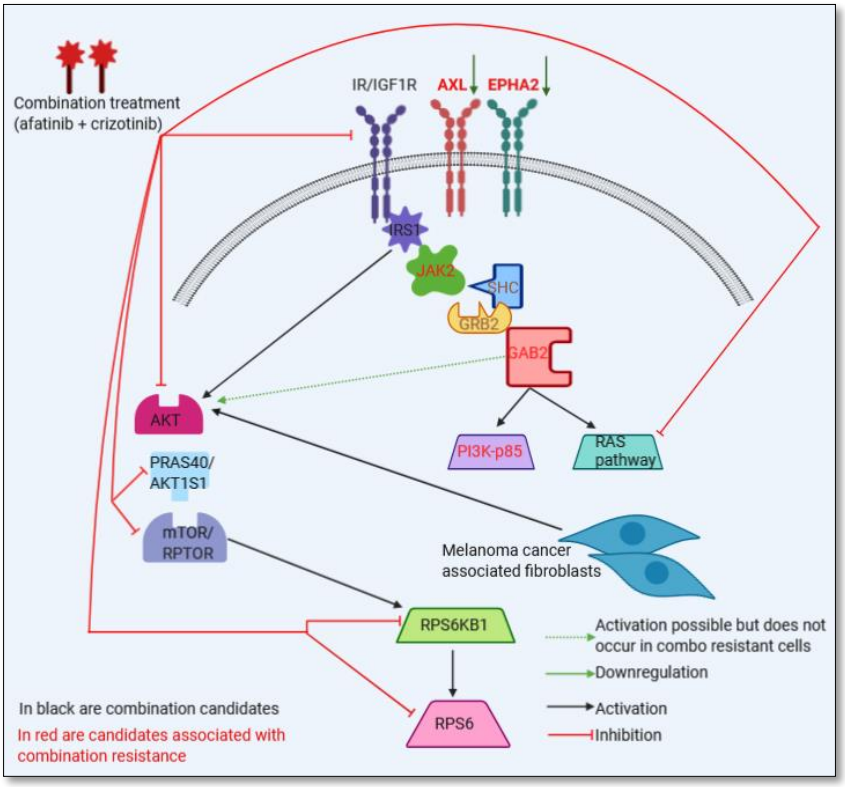

Figure 15: Schematic diagram of the proposed updated mechanism of action of afatinib and crizotinib combination

IRS-1 has been previously shown to be upregulated in vemurafenib resistant CMM cells derived from patients and it has been reported that therapeutic destruction of the insulin receptor (IR) evoked death in melanoma cells. This concept was further validated *in vivo* where the authors presented that treating a mouse model xenografted with A375 cells using the compounds targeting IR/IGF1R/IRS abrogated tumor growth (Reuveni, Flashner-Abramson et al. 2013). Herein, we showed that the afatinib and crizotinib combination treatment downregulate IRS-1 signal in CMM cell lines (3/7) and *in vivo* (*Paper II, Figure 2c-e, Supplementary Figure S4-S5*). mTOR pathway has been studied extensively and several reports have highlighted the role of mTOR in melanoma pathogenesis. mTOR activation has previously been reported in a majority of melanoma clinical samples (Karbowniczek, Spittle et al. 2008). Moreover, it has been shown that everolimus, a mTOR inhibitor reduces melanoma cell invasiveness (Ciolczyk-Wierzbicka, Gil et al. 2020). Previous publications have also suggested combining MAPK and mTOR inhibitors as an effective way to inhibit CMM growth, invasion and induce cell death (Lasithiotakis, Sinnberg et al. 2008). Moreover it has also been shown that cross resistance to BRAF and MEK inhibitors in CMM can be overcome by targeting AKT/mTOR pathway (Atefi, von Euw et al. 2011). RPS6KB1 and RPS6, other candidates identified in our study, are key players of the mTOR pathway. Abberant phosphorylation of RPS6 has been shown to confer resistance to MAPKi in *BRAF*

mutant CMM (Gao, Wang et al. 2019). Furthermore, RPS6KB1 hyperphosphorylation has been associated with poor prognosis in NSCLC patients (Chen, Yang et al. 2017). Our reults were also in accordance with these observations as we showed that RPS6KB1 and RPS6 expression was higher in stage III/IV tumors compared to stage I/II (*Paper II, Figure 5*). Using immunoblotting, RPPA and immunoflourscence, we were able to show that both activated and total forms of RPS6KB1 and RPS6 were downregulated upon treatment with the combination of afatinib and crizotinib.

When analyzing tumor samples (n=65) from patients with advanced melanoma (Stage III/IV),we have also observed that patients who were treated with radiation, chemotherapy, targeted therapy (MAPKi and RTK inhibitors) or checkpoint inhibitors before taking the biopsy had lower nuclear IRS-1 signal than patients who were treatment naïve before biopsy was taken (33% vs 50%). This suggests that treatments plausibly downregulate nuclear signal which could be therapeutically relevant. In our study, we also notably see that the combination is able to reduce nuclear IRS-1 signal.

Importantly, we also show that induced resistance towards the combination is reversible by stopping treatment for a short duration. Our results suggest PMEL/gp-100 and PI3K-p85 as mediators of resistance to the combination as both were found upregulated in afatinib and crizotinib combination resistant cells (*Paper II, Figure 3*). PMEL has been previously shown to be overexpressed in malignant cells and has been used as a therapeutic target for refractory tumors (Park, Talukder et al. 2017). gp-100 peptide vaccine is also under clinical trials as monotherapy or in combination with other immunotherapies as a possible treatment option for CMM (Domingues, Lopes et al. 2018). Upregulated expression of p85 and p110-alpha subunits of PI3K have been previously described in melanoma and therefore PI3K has been considered as a good therapeutic target (Aziz, Davies et al. 2009).

In summary we show that downregulation of IRS-1, RPS6KB1 and RPS6 could plausibly contribute to the additive effects of the combination treatment. Moreover, we show that the combination treatment downregulates AKT signaling which may further contribute towards the cytotoxic effects of this combination therapy. We highlight PMEL/gp-100 and PI3K-p85 as associated with induced resistance towards the combination treatment which we have shown is reversible (*Figure 15, Paper II, Figure 6d*).

4.3 Paper III: AXL and CAV-1 play a role for MTH1 inhibitor TH1579 sensitivity in cutaneous malignant melanoma

ROS is generated as by-products in different cellular compartments as a result of various enzymatic reactions. ROS can have deleterious effects on DNA as it can induce DNA damage and therefore compromise genomic stability. To counteract this process, cells are armoured with several defense mechanisms collectively forming the DNA repair system. One player integral to the DNA repair process is MTH1 which hydrolyzes harmful oxidative stress induced 8-oxo-dGTP, thus sanitizing the DNA pool. Several studies have shown that MTH1 is often upregulated in different cancer types, thus making it a plausible therapeutic target of choice.

4.3.1 Results: In this study we report that CMM cells are sensitive to the MTH1 inhbitor TH1579 independent of *BRAF/NRAS* mutation status, here shown as a loss in cell viability by drug treatment for 72 h in 2D (*Paper III, Figure 1B*) and 3D (*Paper III, Figure 1C*). Moreover, by using an orthotopic zebrafish embryo model, we also showed that the cytotoxic effects of TH1579 were valid *in vivo.* Staining of Ki67 in tissues obtained from these treated embryos indicated a loss in proliferation after treatment (*Paper III, Figure 1F-H*). To recapitulate molecular intra and inter tumor heterogeneity, which is often seen in CMM with presence of resistant clones which under drug pressure have a growth advantage, we mimiced this in an *in vitro* model. We co-cultured one *BRAF* mutant BRAFi sensitive cell line and the BRAFi resistant subline with WT cells in one set and two *NRAS* mutant cell lines with a WT cell line as another set and analyzed cell death and cell viability loss. We showed that in such a setting, TH1579 was most potent at killing CMM cells as compared to the other drugs (BRAFi for *BRAF* mutant cells and MEKi for *NRAS* mutant and *BRAF/ NRAS* WT cells) that are clinically used for CMM. We also used 3D spheroid co-culture model to validate our findings. Moreover, using time-lapse microscopy, we showed that TH1579 caused G2/M cell cycle arrest in *BRAF* mutant CMM cells (*Figure 16, Paper III, Figure 2, Supplementary figure S2*). Moreover cell cycle analysis experiments also showed that in *BRAF/NRAS* WT CMM cells ESTDAB 105 a G2/M cell cycle arrest was induced after TH1579 treatment. Concomitant with these observations, we also saw reduced colony formation in ESTDAB105 upon treatment with 0.9µM TH1579 (*Paper III, Supplementary figure S3*).

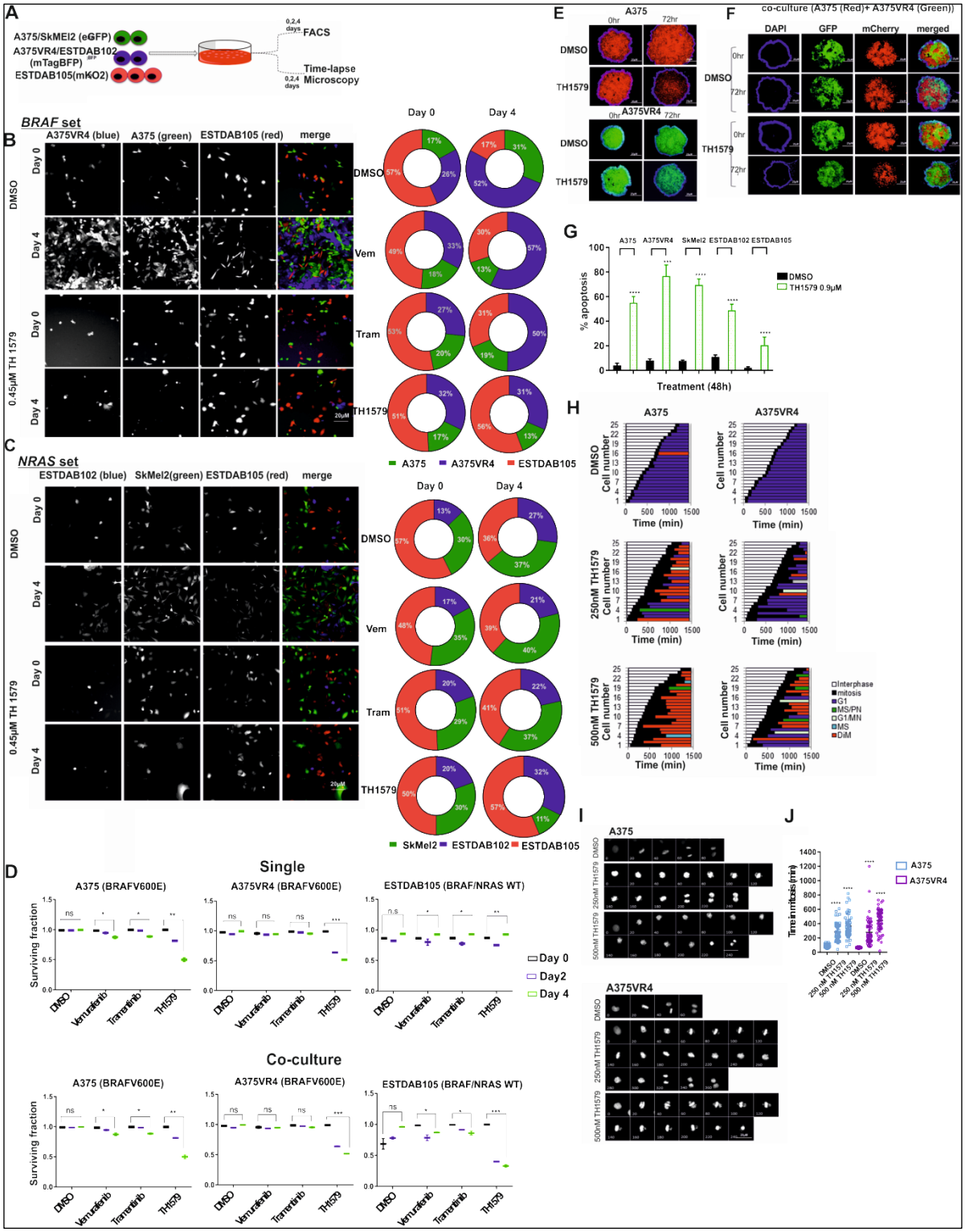

Figure 16: (A) Experimental set up for co-culture. (B,C) Representative images and quantification of viable cells from BRAF set and NRAS set after treatment at indicated time points using time lapse imaging. (D) Results from single and co-culture expreements after treatment using FACS. (E,F) Representative images of spheroids after single or co-culture and treatment with TH1579. (G) FACS results for CMM cells as single cultures treated with 0.9μM TH1579 for 48 h. (H,I,J). Representative images and quantification of time lapse imaging showing mitotic lag in TH1579 treated cells when compared to DMSO.(Das, Gad et al. 2020)

Next we demonstrated that TH1579 treatment induced ROS in CMM cells and that this ROS induction correlated significantly with percentage of apoptotic cells ($p<0.05$, $r=0.95$). Using immunoblotting and a modified comet assay, we were able to observe that the TH1579 treatment induced DNA damage (*Paper III, Figure 3, Supplementary figure S3*). To deduce the importance of MTH1 in TH1579 mediated cytotoxicity, by using cell lines transfected with a doxycycline inducable shMTH1 plasmid, we showed that in all cell lines tested except in the A375 vemurafenib resistant subline A375VR4, silencing of MTH1 alone significantly caused a reduction in cell proliferation ($p<0.01$) (here assessed by loss of colonies formed) and elevated cell death. Although shMTH1 did not cause any obvious cytotoxic effects on A375VR4, we were able to observe induction of DNA damage marker p-H2AX upon knocking down MTH1. Downregulating MTH1 in A375VR4 did not alter time spent during mitosis. (*Paper III, Figure 4*). To elucidate the molecular mechanisms behind TH1579 mediated effects, we performed immunoblotting after treatment with TH1579 to look at the changes in protein expression of key RTKs and downstream effectors previously associated with MAPKi resistance. Immunoblotting results indicated that TH1579 treatment reduced AXL, IGF1R, EPHA2 and pAKT protein levels. We also observed that cells with high AXL mRNA were more sensitive to TH1579 (*Paper III, Figure 5C*). Therefore, we over-expressed AXL in CMM cells with low endogenous AXL levels and found that we triggered an increase in sensitivity to TH1579 in these cells (*Paper III, Figure 5D-E*). To find a mechanistic explanation as to why A375VR4 was not responding the same way to MTH1 silencing as the other CMM cells, we also looked at alternative possibilities. A previous study on lung cancer reported that increased interaction of CAV-1 and MTH1 by mutant *K-RAS* resulted in inhibition in MTH1 activity (Volonte, Vyas et al. 2018). Previous work from our lab has shown that A375VR4 has higher expression of CAV-1. To deduce if CAV-1 mediates lack of sensitivity upon MTH1 knockdown in A375VR4 we checked if TH1579 had any effects on CAV-1 protein levels. We demonstrated that TH1579 downregulated CAV-1 and we also found that CAV-1 and MTH1 existed in close proximity to each other in A375VR4 cells, shown by isPLA (*Paper III, Figure 5K-L*). We couldn't however determine any physical interaction between CAV-1 and MTH1 (*Paper III, Supplementary figure S8*). We showed that TH1579 downregulated CAV-1 signal in A375VR4 (*Paper III, Figure 5M*) and AXL in both A375VR4 and SkMel2 *in vivo* (*Paper III, Supplementary figure S5*). Lastly, we observed that combination treatment of TH1579 together with BRAFi further invoked loss of cell viability compared to single treatment in BRAF mutant cells, including BRAFi resistant cells (*Figure 17, Paper III, Figure 6A, Supplementary figure S10*). We further validated the combination effects using TH1579 and vemurafenib and showed that the

combination treatment significantly triggered higher cell death and ROS induction in *BRAF* mutant cells than single agents (*Figure 17, Paper III, Figure 6B-C*). We also confirmed that this combination effect was true *in vivo*. Lastly, we showed that in A375VR4, the combination treatment effectively downregulated both CAV-1 and pAKT expression (*Paper III, Figure 6F-G*).

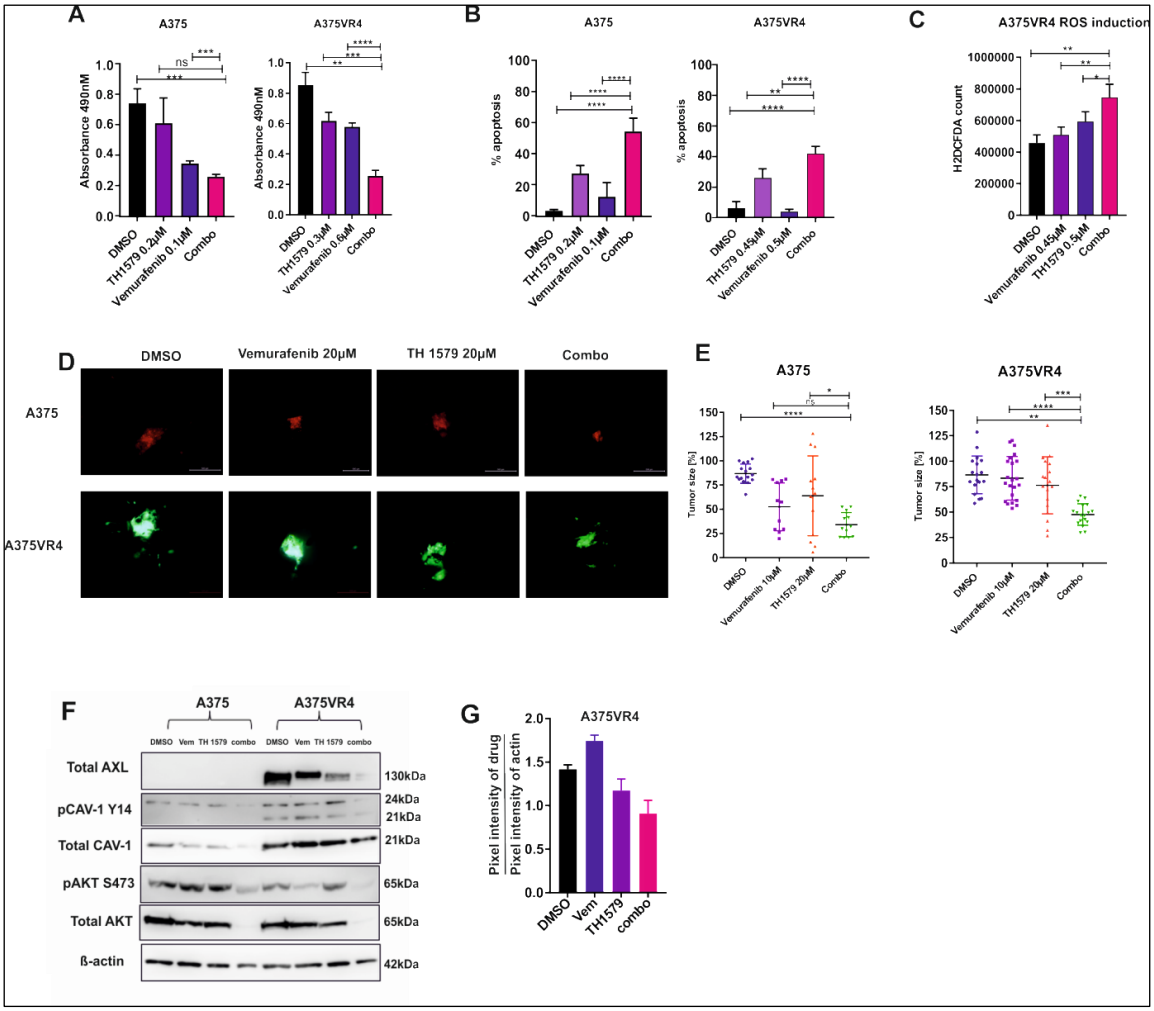

Figure 17: (A) Cell viability assay showing A375 and A375VR4 cells treated with single and combination drugs for 72 h. (B) Apoptosis assay showing effects on cell death in A375 and A375VR4 cells treated with single and combination drugs for 48 h. (C) Assay showing ROS induction in A375V4 with single and combination drugs after 3 h. (D-E) Representative image and quantification showing effects of tumor size in vivo after single and combination treatments for 72 h. (F-G) Immunoblotting showing effects on AXL, CAV-1 and AKT after drug treatment for 48 h.(Das, Gad et al. 2020)

4.3.2 Discussion: Several studies have emphasized on ROS being a critical mediator in the etiology of human diseases. Mutations induced as a result of oxidative damage to cellular DNA by ROS have been shown to contribute towards initiation and progression of cancer (Malins, Polissar et al. 1996, Behrend, Henderson et al. 2003, Waris and Ahsan 2006). Studies have linked the role of ROS in several steps during melanoma onset and progression (Wittgen and van Kempen 2007), thereby implying that targeting ROS in melanoma could

be therapeutically beneficial. To escape the deleterious effects of ROS, cancer cells often overactivate DNA repair mechanisms that promote mitosis to avoid oxidative DNA damage (Gad, Koolmeister et al. 2014, Gad, Mortusewicz et al. 2019). For example, overexpression of DNA repair protein MTH1 has been observed in many tumor types (Kennedy, Cueto et al. 1998, Pudelko, Rouhi et al. 2017, Wang, Liu et al. 2017).

In this study, we have investigated the applicability of MTH1 inhibitor TH1579 as a novel therapeutic potential in CMM. Since MTH1 has been shown to promote mitosis to evade DNA damage (Gad, Mortusewicz et al. 2019), we investigated and found that inducing mitotic arrest was one of the mechanisms by which the inhibitor TH1579 worked in our cell line panel. A study by Wang *et al* showed that ROS levels present in melanoma cells dictated their response towards TH588- an analog of TH1579 (Wang, Jin et al. 2016). As indicated in a previous study TH588 was also used to demonstrate antitumor effects in neuroendocrine cells (Aristizabal Prada, Orth et al. 2017). Herein, we showed that TH1579 treatment induced DNA damage, loss of proliferation and viability and induced apoptosis (*Paper III, Figure 1-3*) in CMM cells. Since the direct role of MTH1 in cancer therapy has been debated where some studies have highlighted MTH1 to be an important target in cancer (Koketsu, Watanabe et al. 2004, Gad, Koolmeister et al. 2014, Brautigam, Pudelko et al. 2016, Warpman Berglund, Sanjiv et al. 2016, Aristizabal Prada, Orth et al. 2017, Pudelko, Rouhi et al. 2017, Wang, Liu et al. 2017, Einarsdottir, Karlsson et al. 2018, Zhou, Ma et al. 2019), whereas other studies have shown inhibition of MTH1 to be non-toxic to cancer cells (Takagi, Setoyama et al. 2012, Kawamura, Kawatani et al. 2016), we knocked down MTH1 using shRNA to evaluate the cytotoxic effects. Our results showed that except for *BRAF* mutant vemurafenib resistant cell line A375VR4, all other cell lines investigated showed loss of proliferative cells and induced cell death upon MTH1 knockdown (*Paper III, Figure 4*). In this study, we also found that AXL and CAV-1 play a role in mediating sensitivity towards TH1579. The role of AXL and CAV-1 in cancer development and therapy resistance has been well established (Zhang, Lee et al. 2012, Muller, Krijgsman et al. 2014, Wang, Wang et al. 2015, Balaji, Vijayaraghavan et al. 2017, Ketteler and Klein 2018). Therefore, downregulating AXL and CAV-1 expression may be one of the plausible mechanisms of action behind TH1579 mediated cytotoxic effects (*Paper III, Figure 5, Supplementary figure S5 and S8*). Several recently reported studies have shown that combining different drugs with MTH1 inhibitor leads to a more effective amelioration of cancer cells (Einarsdottir, Karlsson et al. 2018, Ikejiri, Honma et al. 2018). We have also shown that combining BRAFi with TH1579 resulted in further sensitization compared with using either inhibitor alone in *BRAF mutant* CMM cells (*Paper III, Figure 6, Supplementary figure S10*).

In summary we show that TH1579 is cytotoxic for all melanoma cells independent of *BRAF/NRAS* mutation status. TH1579 treatment induces ROS in CMM cells. We also show that cells with BRAFi resistant cell line A375VR4 expressing high AXL and CAV-1 as well *NRAS* mutant cell line SkMEl2 expressing high AXL levels are sensitive to TH1579. Overexpression of AXL in AXL low cells further sensitizes them to TH1579 treatment (*Figure 18*, *Paper III, Figure 7*). Furthermore, AXL upregulation which has been associated with BRAFi resistance was downregulated by MTH1 alone and in combination treatment with BRAFi.

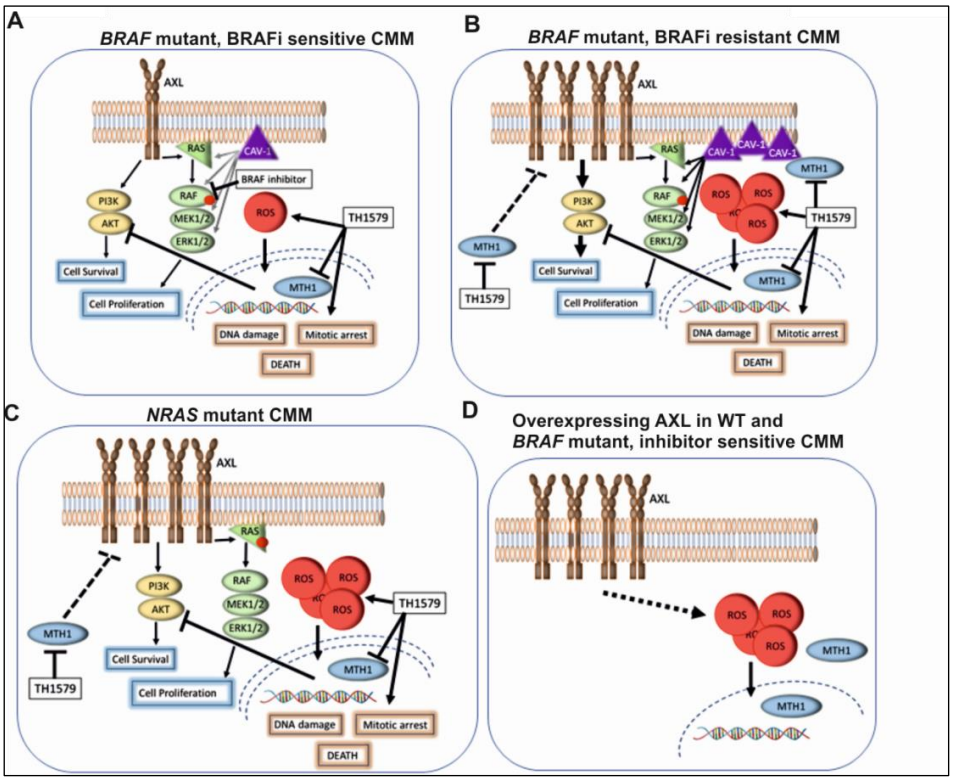

Figure 18: (A-D). Proposed mechanism of action of MTH1 inhibitor TH1579 in CMM. (Das, Gad et al. 2020)

4.4 Paper IV: Co-expression of MTH1 and PMS2 is associated with shorter progression-free survival after immunotherapy in cutaneous malignant melanoma

Genomic instability is a known hallmark of cancer and it has been shown previously that mutation burden in tumors can be an important predictive biomarker for response to immune checkpoint inhibitors (ICI). Patients with high tumor mutational burden (TMB) are predicted to respond better to ICI. Melanoma has high TMB and is considered immunologically 'hot' as the tumors generally comprise of an abundance of tumor infiltrating lymphocytes. However, many CMM patients do not respond to ICI.

4.4.1 Results: In this study we have analyzed data from TCGA and have shown that patients who express high mRNA levels of MTH1 and PMS2 have significantly ($p<0.05$) shorter disease-free survival (DFS) and OS (*Paper IV, Figure 1b*). Moreover, we found that PMS2 and MTH1 protein was co-expressed in patients with stage III/IV disease in comparison to those with stage I/II disease or normal skin (*Paper IV, Fig 1c-d*). We also observed that in stage III/IV tumors, the distribution of MTH1 localization was more common (~74%) in the nucleus as compared to Stage I/II tumors (~32%) (*Paper IV, Supplementary figure S1*). Furthermore, we have highlighted that co-expression of MTH1 and PMS2 is associated with a worse PFS in CMM patients who have been treated with ICI. However, when sub-categorizing these patients based on response to therapy using data from a previously published study, we were only able to see that median mRNA expression of MTH1 was higher (not significant) in patients with progressive disease (PD) as compared to partial responders (PR) or complete responders (CR) (*Figure 19, Paper IV, Figure 2*).

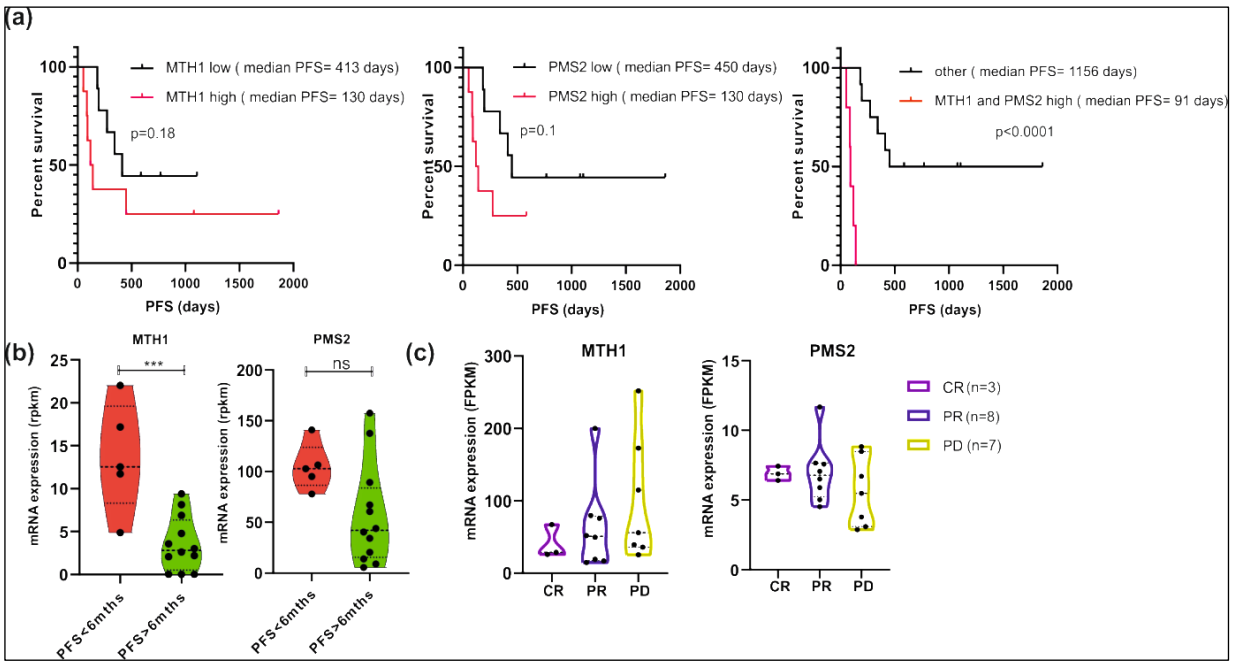

Figure 19: (a) Kaplan Meier curves showing PFS of CMM patients treated with immunotherapy with MTH1 high or low, PMS2 high or low or MTH1 and PMS2 high or low. (b) Differences in mRNA expression levels of MTH1 and PMS2 in the same cohort of patients but grouped as those with PFS < 6 months or > 6 months. (c) Difference in MTH1 and PMS2 mRNA levels in patients grouped as CR, PR or PD (dataset used from (Hugo, Zaretsky et al. 2016))

Next we showed that patients who had been treated with targeted or immunotherapy at relapse co-expressed MTH1 and PMS2 protein and also had elevated expression of the two proteins (*Paper IV, Figure 3*). Finally, we treated a selected set of established and short-term patient derived CMM cells with MTH1 inhibitor TH1579 and observed an induction of DNA damage (here seen by elevated levels of p-H2AX),downregulation of PMS2 protein, loss of cell viability and significantly ($p<0.05$) reduced tumor spheroid size after treatment with TH1579. FACS analysis of tumor cells treated with TH1579 also showed induction of apoptotic cell death (*Paper IV, Figure 4a-f, Paper III, Figure 3G*). Finally we showed that by silencing MTH1 and PMS2 concurrently, we could invoke a significant induction in apoptotic cell death as compared to silencing either MTH1 or PMS2 alone (*Paper IV, Figure 4g*).

4.4.2 Discussion: In this study we have highlighted the role of DNA repair with regards to treatment response to clinical therapy in CMM. As per our knowledge, this is the first study demonstrating that co-expression of MTH1 and PMS2 is observed in clinically advanced CMM tumors and that it is associated with shorter PFS in patients treated with immunotherapy.

Abberant genetic alterations, especially gene amplifications of chromosome 7 has been associated to be a marker of metastatic melanoma (Bastian, LeBoit et al. 1998, Udart, Utikal et al. 2001). Both *MTH1* and *PMS2* are located on chromosome 7. Although no previous studies have shown amplifications in *MTH1* and *PMS2* in association with cancer, in this study we have shown correlation between PMS2 and MTH1 CNV (*Paper IV, Figure 1a*). We also found a similar correlation at the mRNA level (*Paper IV, Figure 1c-e*). Several studies have demonstrated an upregulation of MTH1 expression in several cancers including CMM (Wang, Liu et al. 2017). However, the role of PMS2 is relatively unknown. Previous data indicates that PMS2 deficiency increases the incidence of several cancers like breast, stomach, prostate (Kasela, Nystrom et al. 2019) on one hand, whereas another study has shown that an increase in PMS2 expression is associated with prostate tumor aggressiveness (Wilczak, Rashed et al. 2017). Yet another study in ovarian cancer evaluating the prognostic value of DNA repair proteins for patients treated with platinum based chemotherapy showed

that those patients with higher PMS2 had a favorable OS (Zhao, Li et al. 2018). In this study we have shown that patients treated with immunotherapy who have high PMS2 and MTH1 mRNA expression have a significantly worse PFS ($p<0.001$) (*Figure 19,Paper II, Figure 2a*). Furthermore, patients treated with targeted or immunotherapy show a increased expression of PMS2 and MTH1 as well as co-expression of the two proteins at relapse (*Paper III, Figure 3*). These data indicate that for CMM, co-expression of MTH1 and PMS2 has a poor prognostic value. Therefore we propose that co-silencing of MTH1 and PMS2 could be a better therapeutic strategy since concurrent silencing of both these proteins led to increased cytotoxicity in CMM short-term patient derived cells (*Paper IV, Figure 4*).

In summary we show that MTH1 and PMS2 is co-expressed in advanced CMM tumors (Stage III/IV) and in refractory tumors. We therefore elucidate that though patients may initially respond to therapy, those who harbor clones of MTH1 and PMS2 positive tumor cells have risks of relapse (*Figure 20*). However, to which extent these two proteins play a role in attributing towards relapse cannot be deduced from this study. Moreover we show that patient derived CMM cells (including cells from a relapsed patient) are sensitive to TH1579 (Karonudib). TH1579 was able to ablate growth of tumor spheroids established from patients undergoing immunotherapy. TH1579 is currently in phase 1 clnical trials for solid and haematological malignancies. Based on our findings we suggest that combining immunotherapy with TH1579 might be an attractive option for improving therapy efficacy in CMM treatment.

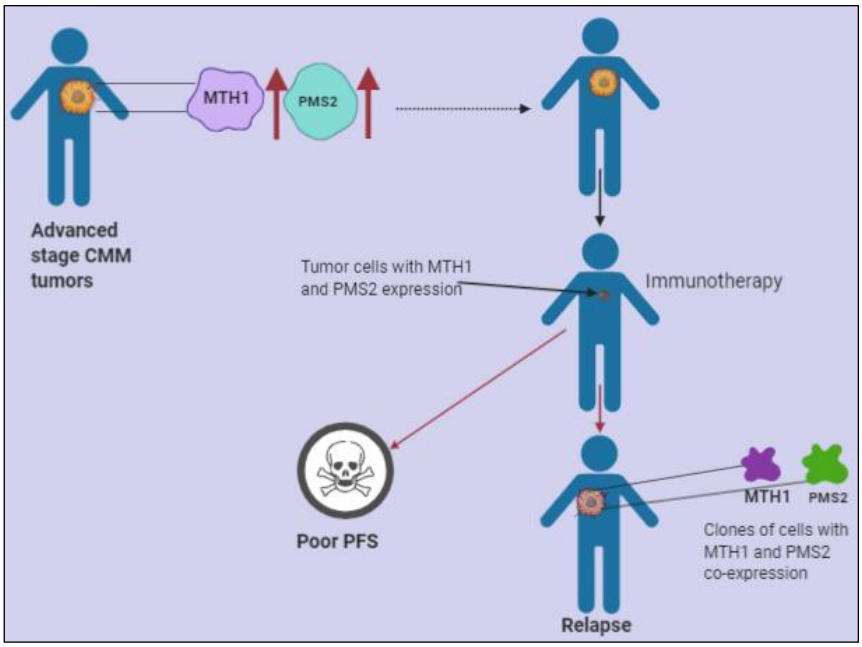

Figure 20: Schematic illustration showing association of MTH1 and PMS2 expression to CMM disease stage and therapy response

5. CONCLUSIONS AND FUTURE PRESPECTIVES

Understanding the molecular mechanisms behind melanoma onset and pathogenesis has been fundamental when developing new therapeutic strategies for CMM treatment. Although treatment of advanced CMM with immunotherapy and targeted therapy has significantly improved the overall survival, there are still patients who do not respond to either treatments, making it an unmet need to develop further therapy options for such patients. Moreover, a deeper understanding of the biology of the disease and response to therapies is warranted to identify subgroups who would have long-term benefit of the current therapies and also to be able to further develop alternative treatment options. The objective of this thesis was to uncover novel therapeutic alternatives for CMM and to unravel new druggable molecular targets for an improved clinical efficacy.

In paper I we elucidated the therapeutic potential of afatinib and crizotinib as a combination treatment strategy for CMM. We showed that the novel combination treatment caused loss of cell viability, cell proliferation, migration, invasion and induced cell death in melanoma cells. In line with our *in vitro* studies, we were also able to show that the combination treatment significantly abrogated tumor growth rate in a A375 xenograft mouse model without any severe toxic effects. Lastly, we showed that downregulation of WEE1 and IGF1R expression was associated with the broad phenotypic effects of the combination treatment. Future studies are warranted to combine WEE1 and or IGF1R inhibitors either in combination with each other, with MAPKi or with afatinib and crizotinib to see if these combinations can elicit a further cytotoxic response in CMM cells. WEE1 and IGF1R expression in association to therapy response also needs to be further studied in CMM clinical samples to understand more lucidly their roles in disease relapse.

In paper II we expanded our study from paper I. To do so, we performed an in depth analysis to unravel key molecular mediators underlying the broad phenotypic effects of afatinib and crizotinib in combination. We saw that PI3K/AKT, mTOR, Insulin signaling and DNA damage pathways were deregulated by the combination therapy. Taking into consideration our *in vitro* and *in vivo* data, we decided to focus on the mTOR and insulin pathways. We found that IRS-1, RPS6KB1 and RPS6 were among the most significantly downregulated proteins by combination treatment both *in vitro* and *in vivo.* Staining of these proteins in

CMM clinical samples showed that RPS6KB1 and RPS6 expression was higher in more advanced disease. We also found a discernable nuclear IRS-1 signal in ~50% of stage III/IV tumors compared to Stage I/II where almost no nuclear localization was observed. Furthermore we observed that IRS-1 mRNA expression was higher in *NRAS* mutant tumors and that higher IRS-1 expression was associated with significantly shorter OS in CMM patients on immunotherapy. This study provides a platform for future exploratory studies since we also found other proteins associated with cell cycle, DNA damage and metabolism which we infer as interesting candidates that have in part already been shown to play a role in melanoma etiology. A better understanding behind the association of these candidates and the their role in different stages of melanoma would help develop novel targeted therapies which could provide clinical benefit.

In paper III we investigated the therapeutic potential of MTH1 inhibitor TH1579 in CMM. We found that CMM cells were sensitive to TH1579 independent of *BRAF/NRAS* mutation status. TH1579 treatment elicited DNA damage, ROS accumulation, mitotic arrest and cell death in CMM cells. AXL high CMM cells were particularly sensitive to TH1579 and by overexpressing AXL in AXL low cell lines, we could sensitize them to TH1579 treatment. We also observed that in vemurafenib resistant cell line A375VR4, CAV-1 played a role in mediating sensitivity to the inhibitor. Lastly we also saw that by combining BRAF inhibitors with TH1579, we could enhance the sensitivity of *BRAF* mutant CMM cells. This observation was made both *in vitro* and in an orthotopic zebrafish model system. This study advocates combining BRAFi with TH1579, thereby providing an alternative therapy regime for refractory tumors where resistance to BRAFi has developed. Further studies are required to understand how AXL and CAV-1 may play a role in TH1579 mediated cytotoxic effects and whether combining AXL inhibitors with TH1579 might provide opportunities for enhanced therapeutic efficacy.

In paper IV we studied the significance of MTH1 and PMS2 in relation to therapy response and disease stage. We found that patients who received immunotherapy with concomitantly high PMS2 and MTH1 mRNA expression in their pre-treatment tumors had a significantly shorter PFS. We observed that refractory tumors comprised of clones of cells that co-expressed MTH1 and PMS2. Moreover we also saw that CMM cells derived from patients administered with immunotherapy were sensitive to MTH1 inhibitor TH1579 when cultured as spheroid models. Lastly we found that co-silencing MTH1 and PMS2 evoked cell death more significantly than when either MTH1 or PMS2 was silenced alone. In order to validate

this hypothesis and hopefully bring it into clinical practice, validation studies with larger patient cohorts are required in the future.

6. THESIS SUMMARY: A POPULAR SCIENCE VERSION

Employing novel treatment approaches to combat the deadliest skin cancer

Melanoma is the deadliest form of skin cancer with an increasing incidence rate worldwide. It is easier to cure melanoma if detected early, but once it has advanced, it could be a serious problem to treat. Currently targeted therapy and immunotherapy, activating the immune system, are employed which have significantly improved the survival for many patients with advanced melanoma. However, only a subgroup of these patients has sustained benefit of the treatment. The majority of the patients do not respond or have a relapsed disease during treatment or upon treatment cessation because they are or become unresponsive to the treatment. A number of studies are ongoing to find biomarkers to be able to identify patients who are likely to have long-term benefit from the current treatments as well as finding novel treatments for the subgroups who do not respond or have short-term benefit (personalized medicine). However, we still have a long way to go before personalized treatment becomes a reality. My thesis therefore focuses on finding alternative therapy options for melanoma patients.

We have explored broadly two novel treatment strategies with the aim to find effective novel treatments for melanoma. For the first approach, we have tested two drugs in combination (afatinib and crizotinib), which have been clinically approved for lung cancer but not for melanoma treatment. In the other approach we have investigated a drug TH1579 (karonudib) alone or in combination with drugs clinically approved for *BRAF* mutant melanoma. TH1579 is under investigation in clinical studies to assess the tolerance in patients with different types of tumors. We show that afatinib and crizotinib in combination or TH1579 alone or in combination with clinically approved drugs used to treat melanoma patients is able to provide an anti-tumor effect that not only manages to reduce the tumor growth, but is also able to restrict the spread of the tumor, a major challenge in melanoma. These therapeutic strategies are also ultimately able to kill the cancer cells.

The effects of TH1579 and the afatinib and crizotinib drug combination were also studied in animal models. We found that the melanoma cells were successfully killed by the drugs, supporting our proof of concept. We here suggest potential novel targeted therapeutic strategies for patients with melanoma to study further with a hope that they would be beneficial for treatment of melanoma in the future.

7. ACKNOWLEDGEMENTS

My journey as a graduate student has been a great one and through mostly ups, but also some downs, here I am almost towards the finish line. I have come to know several people during this beautiful phase and I thank them all for being there with me. I would also like to thank all the patients who have kindly given consent to use material for the research included in this thesis.

Suzanne, my main supervisor. Words are not enough to express how grateful I feel that I had such a wonderful, kind hearted, understanding supervisor. Before the start of my PhD, I had always heard from peers that although it is important to get a lab with good projects, how much more important it is to share good dynamics with your supervisor. You have completely redefined for me of how a typical supervisor is. From having supported me during the initial phase where we had some issues about my PhD registration, to hearing several non- academic related complaints of mine (some complaints about projects too), to encouraging me to go on conferences, our daily conversations, fika (though the frequency reduced once Rainer left 😊), calming me down when the first paper took time to get published, your non-stop encouragement and support is much more than I could ever ask for. I would also like to apologize for having been very impatient and pushy at times, whether it had to do with experiments, discussing about manuscripts or submitting a manuscript. But I guess you would understand that the real reason behind it all was just to complete everything on schedule. I would like to thank you for constantly guiding me through all projects, for motivating me and for always being available when I needed to discuss results with you. You have inspired me with your scientific intellect, have always trusted me and given me the freedom to design my own experiments, troubleshoot and learn from my mistakes. Thank you for making my PhD journey so smooth and easy 😊.

Johan, my co-supervisor, thank you for all your encouragement and support during these years, especially when it was really difficult for me with the PhD registration process. Thank you for coming with me to meetings to discuss about the progress of my PhD registration and for always keeping my spirit high. I have always looked up to you as an excellent group leader and an intelligent man brimming with deep and vast scientific knowledge. Thank you for your invaluable comments during project presentations and for my manuscripts.

Rainer, my co-supervisor and my go to knowledge guru. I think it's pretty safe to say that those who know you and are reading this thesis, have to agree that you are one person you

can ask just about anything. I've always considered you as my friend whom I could discuss and share almost anything with. Ankit and I had a great time when we visited your beautiful home. Thank you so much for inviting us, although after that we realized that you are a great cook and hence all my insane demands for your homemade cakes commenced after that. I must add a sentence about your dance moves too—soo groovy :D! Thank you for always encouraging me, although sometimes I think you were too kind and praised me way more than I deserved, but truly, that helped me to strive to be better. Thank you for your help during my PhD registration. Our scientific discussions throughout my PhD have been extremely fruitful for me and has definitely enabled me to think out of the box (though I have a long way to go before I catch up with you 😊). Things are really quiet at the office now that you have left and we all miss having discussion about science, life and worldly affairs with you. I heard that have plans to come back to the lab during summer—so I'm definitely looking forward to that.

Ulrika, I got to know you almost through serendipity and I'm so grateful that you agreed to be my co-supervisor. It has been a lot of fun working under your supervision and your lab has a terrific energy level which I enjoyed 😊. Your drive and passion for science is something every young researcher can learn from. I don't know if I've ever mentioned to you, but I really appreciated your honestly towards me where you told me outright if there wasn't something that you liked or if you wanted me to improve figures in my manuscript. This experience has definitely been enriching. It was almost amazing how we both landed up in the same Indian state for our vacation at the same time. I do hope you enjoyed that trip a lot, and that you visit India once again.

Veronica, thank you for all your help with the ampliSEQ data analysis which has found its way through mostly all my projects 😊. I really admire your strong personality, and I've always seen you smile even though your surrounding situations might not have been the best. I applaud your drive towards all these challenging races that you participate in. Thank you for bringing a smile to all our faces.

Karin, my office and lunch partner, especially during these days when it's insanely quiet at Bioclinicum. Thank you for all your energy that you bring to the group. Thank you for planning all the wonderful events that you do during the entire year. I would've probably missed out on tasting traditional Swedish Julboard had it not been for our annual Christmas dinners. They have always been at fantastic locations. Thanks for bringing our office space

to life. Amidst the white blank walls, your corner stands out with all the pictures and plants. I got inspired and tried to decorate my desk with some toys as well 😊.

Muyi aka Bruce, thanks for your company in the lab and for our discussions. It was a lot of fun to have you around. Thanks for sometimes eating sweet treats on my behalf :D. I wish you all the very best for your PhD. I'm sure you'll do great. Don't stress much.

Alireza, thanks for being a great senior. I learned a lot from you during the initial start of my PhD. Thanks for generating the vemurafenib resistant cell lines. I think both Bruce and I have really made the maximum use of them, especially A375VR4 😊.

Current members and past members of the Hansson lab: **Fernanda, Hildur, Diana, Johan F., Kalle, Hanna, Stefano, Marianne**, thank you all for your constant help and support. Thanks to **Rodolfo** (exchange researcher) for help with IHC and scoring.

Thomas, thank you for all your help and support including suggesting challenging experiments that proved to answer important clinical questions. I would also like to thank you for suggestions for labs during my post-doc application phase.

Svetlana, my PhD mentor. It is safe to say that your role in my PhD journey started much before. Without your help I wouldn't have reached this point at all. I still sometimes read the email that you drafted for me when I was seeking a position here at Karolinska. I would like to thank you from the bottom of my heart for trusting someone you barely knew and helping me out so much.

Sara, my dear friend. Thank you for endless support during the tough times with my PhD registration. I can't express to you how grateful I am for all your help. You were so easy to talk to and you lend an ear to whatever I had to say. Thank you for all your efforts to help me with my registration process 😊.

Katja, where do I start. You are such a fun person to be around, so caring and always very enthusiastic. Thank you for keeping the fika tradition alive and thanks for all the delicious Russian desserts that you brought. You are always brimming with energy and I always feel rejuvenated in your presence😊.

Anna, thank you for making tons of blocks, sections and also helping me with staining optimizations. I really appreciate all your help and the fact that you never turned me down

whenever I seeked your aid. You always said, ‘yes, ofcourse’ with a smile on your face. Thank you for learning how to make zebrafish cryosections for me.

Juan, thank you for helping me with some of my FACS optimizations and for help with cell cycle analysis. Thank you for trusting me with the responsibility to take care of the FACS core the days when you were away.

Friends at the Onk-pat administration: **Hanna and Erika**: Thank you for all your help which I received soo promptly. Hanna, you have a beautiful smile and you are such a lovely person to be around. I hope you always stay like this forever.

Andor and Enikö, thanks for the great evening we had at City Hall during Ankit’s convocation ceremony. It was an amazing experience and I will always remember it. **Enikö**, I will also cherish memories of sitting down and having lunch with you during the IID conference in Florida, wondering if we would really find alligators around the hotel premises or not :D.

My friends at CCK/ Bioclinicum:

Ewa, somehow we never spoke so much when you were at CCK, but things took a different turn when we met again in Bioclinicum. I think I’ve gotten to know you quite well over these last few months that we’ve interacted and I honestly have had a great time to go out with you for lunches and dinners. I’m soo happy that we continued to have our rendezvous even when you moved away and joined a new job. I still feel you are very much a part of Bioclinicum and it was really a lot of fun to have you around. May we continue our little food adventures whether it be Sweden or USA 😊 and ofcourse I need to visit your home to have the wine flavored tea 😆. Good luck with your job 😊.

Nicole, I miss you. We used to hang out much more often when we were in CCK. All our talks in the cell culture room. Remember I helped you with this workshop in Stockholm University? That was a lot of fun actually, although I had a terrible time finding the place amidst all the construction. I wish we could’ve hung out together more often when you stayed at Kungshamra. I still need to visit your new home, but perhaps we can plan that when you return. **Noah**, is really such a cute bundle of joy. Keep sending me pictures and keep me updated. Good luck with your PhD. You’ll do just fab 😊.

Aishwarya, it was so nice to have you around, although it was for a very short time. Thanks for keeping me company during lunch. It was great to see the enthusiasm and energy that a

young researcher like yourself possesses. I admire your tenacity and never give up attitude. I do hope you succeed in whatever career path you choose to uptake.

Aravindh, thank you for your company, especially during our bus rides to and from home. I hope you do well in your future postdoc appointments. **Yuan,** thanks for being my friend. All the times we teased each other - it was great fun. Keep smiling always and good luck with both your manuscripts. **Pedro, Aarren, Mathilde, Ioannis, Nathan, Monika** thanks so much for being my friend and keeping me company during conference breaks. **Vicky**, thanks for preparing the abstract book for OnkPat kick off, 2019 ☺. **Ziqing**, thanks for all your kind help with the Incucyte. **Alessandro,** thanks for all the good times, especially during organizing the pubs. Alas it didn't really continue, but we had a lot of fun, especially the time we made sandwiches together. **Hao**, thanks for your company during cell-culture. It was amazing to see how many cell culture flasks you can balance in one go :D. **Tom**, thanks for being a very dependable fellow doctoral student representative. I wish you all the best for your half-time and your PhD. **Amineh**, thanks for being my friend, for helping me set up the Incucyte the first time and also for our conversations that we had sometimes during lunch. Also, thanks for being agreeing to being a part of committees (like kick off and also pub). It was a lot of fun organizing events with you, including cleaning the horribly dirty storage room in CCK ---well maybe not that much fun :D. Good luck for your PhD. **Huaitao**, you really need to stop living in the lab :O. Thanks for being my friend and for sharing with me stories about your friends in UK, especially your Bengali friends. Thanks for being so enthusiastic about Indian food :D. **Weng-Onn**- thank you for the fruitful discussions about Stanford. **Anaya**, although we did not spend much time together, yet I feel I've known you for a long time. It was lovely to go out for coffee with you and engage in our long discussions. I do hope that things work out for you for the best. **Patricia and Dalel**, oh I do miss you ladies since the 'pink panther' group has said goodbye to Onk-Pat and CCK. Patricia, it has been so lovely to interact with you and more so to hear such good things from your friend whom I met completely accidentally in France. Out lunch/ dinner is still pending. **Evelyn**, you are like a light beam, always glowing. How do you really manage to have a smile on your face at all times? Thanks for being my friend and I wish you good luck for your future career. **Linnea**, thanks for being such an easy going person. It was superb to organize events with you, especially the international dinner that we had on the 5th floor in CCK. I look forward to meeting you again in SF.

Friends at Helleday lab:

Helge, thank you so much for teaching my all the new techniques and for helping out so much for one of the MTH1 papers. You have always been such a dependable person and it has been a pleasure to get to know you and to work with you.

Linda, well what can I say. I really thank my stars that we met during the course and we got to talking and actually thanks for proposing the zebrafish model for my project. It was a brilliant idea. Thanks for all your efforts that you put in 😊 and I hope you have a fabulous start to the new phase in your life.

Lars, thank you for helping me with the remaining zebrafish experiments once Linda left the lab. Thank you for being the positive person that you are. It has been lovely to work with you.

Ingrid, thanks for all your help and input for the drug synergy and *in vitro* kinase assays. Also a big thank you to you for preparing cells for the zebrafish transplantation experiment when I couldn't do it since our lab space was shut down due to the move.

Kumar, thanks for all your conversations in Bengali. It made me feel at home 😊.

Friends in CMM:

Harry, Bo & Liya- thank you for your amazing friendship. Bo, I admire your strength (both mental and physical). Our trips to Annalena's summer house, our trip to Copenhagen, they were all fabulous. **Liya**, you are a sweetheart and it has been awesome to come over and play with you, whether it be in Stockholm or Gothenburg. **Bo**, I do hope that you can plan a trip to USA soon 😊.

Irene and Lucas, it was so lovely to revisit Sunderban and Darjeeling with you guys. Irene, thanks for all the amazing dinners we've had together after swimming. Honestly, that was my motivation for some exercise 😉.

Annalena, Lorenzo, Kunal, Mansi, Manika, Dong, Elisa, Hano, Sissi- thank you for all the good times we've shared together.

Other friends and collaborators

I would like to thank all friends from the KAW consortium, especially **Fan** and **Prof.Lars Gunnar Larsson** for fruitful discussions about senescence and melanoma. Thank you to **Maggan** for all your help with the mouse xenograft experiments and also a big thanks to

Gianluca for your help with the mass-spec studies. Thanks **Nick** for helping with some of the bioinformatics.

Indian friends in Stockholm:

Shawon di & Sajit bhaiya, thank you for always being there for me and for all the lovely times we had together during our summer adventures, camping trips and travels. I will never forget Shawon di's cooking- it's simply delicious. Shawon di can perhaps take the hint 😉. **Madhur, Ankur & Aayank**- it has been awesome to know you and I will always cherish our friendship. I will never forget the birthday celebration where we cut the macroon infront of Eiffel tower on a rainy day 😊. **Sumonto da and Ruchi**- I feel privileged to know you both. Thank you for all the good times we had in Stockholm and in Kiruna facing the challenges, battling the storm to catch a glimpse of the northern lights. **Suhas bhai,Ashwini bhabhi** & sweet little **Parth**- thank you for being our friends and for inviting us soo many times to your home. I've watched Parth grow into a very sweet and handsome young man. Please plan a trip to California soon😊. **Swapnali, Sachin S., Sachin T., Monali, Gargi, Daya, Neha, Daksh, Satya, Soniya, Harkamal, Prajakta, Deepak Ji, Suvarna 'bhabhi ji', Aarush, Rushab, Vivek, Gayatri, Ganesh, Anuj, Shahul, Romanico**- thank you all for being there.

Friends from outside Sweden:

Sudipta, Subrata, Moumita, Ankan, Debosree, Rukmini, Kaushik, Nipa didi, Arpita, Sayoni, Neilli, Puneet, Ankit Kapoor, Ammu, Supraja, Nisha, Christy, Jaya, Ambi, Anna, Gaurav, Ani - my buddies from childhood (before school), school, undergraduation and postgraduation- thanks for being there for me. **Sudipta and Subrata**- thanks for the lovely time we had in Australia. It was truly an unforgettable experience for Ankit and me. Even though we might meet less than once a year, I always cherish the good times we've had before which keeps me going when I'm not in such a good mood. I would really like to specifically mention my postgraduate days which happened to be my first experience of living outside my hometown and it was the best experience of my life. Those two years are irreplaceable. Thanks guys for making it soo special for me. **Yuhan** and **Linging** (aka '**stickyorange**') - my friends in USA, thanks for all the fun and adventure. I miss you guys a lot.

I would also like to thank all my previous mentors who have guided me and encouraged me to do my PhD.

Family:

My heartfelt gratitude to all my relatives for their support and belief in me during the entire course of my studies.

Ma and baba- I know you must be very proud and ma must be sighing with relief now that my PhD is almost over 😉. Your love, support, help, encouragement, kindness, belief in me has been tremendous ever since I was a child. You have always taught me to be patient- a virtue I hope I was able to uphold atleast during most parts of my thesis work. It really does mean a lot to me that you have been my constant pillar of strength and support and I can't thank you enough for the love that you've bestowed upon me. A big part of whatever I have achieved today is because of you. You both mean the world to me and I love you both a lot.

Mummy, Shanu, Shipra di, Rajeev jija- thank you for being a part of my family. Shanu and mummy- thank you for all your love, blessings, support and understanding.

Ankit- time for the truth 😉. You have been one of my dearest friends (since I don't believe in the term 'best friend') ever since our post-graduation days. Whether we are in two different cities, continents or we are in the same place, your support and love for me has always been unconditional and undivided. No matter how bad the situation has been for me, be it my struggles in USA or here in Sweden with my PhD registration, you have always been there for me and never once have you lost faith in me. I really love your optimism towards life. You have managed to instill in me some of the optimistic attitude which has definitely helped me in many ways. You have been the person I have always gone to for help and you have always tried your best to help me out. As you've said in your thesis, the same also holds true for me that I'm writing this thesis because of you and I hope that whatever challenges life throws at us, we will be able to combat it together- as a '**team**'. I love you.

8. REFERENCES

Abel, E. V., K. J. Basile, C. H. Kugel, 3rd, A. K. Witkiewicz, K. Le, R. K. Amaravadi, G. C. Karakousis, X. Xu, W. Xu, L. M. Schuchter, J. B. Lee, A. Ertel, P. Fortina and A. E. Aplin (2013). "Melanoma adapts to RAF/MEK inhibitors through FOXD3-mediated upregulation of ERBB3." J Clin Invest **123**(5): 2155-2168.

Adachi, E., K. Sakai, T. Nishiuchi, R. Imamura, H. Sato and K. Matsumoto (2016). "Different growth and metastatic phenotypes associated with a cell-intrinsic change of Met in metastatic melanoma." Oncotarget **7**(43): 70779-70793.

Agosto-Arroyo, E., M. Rosa, A. Chau and L. Khazai (2017). "Concurrent BRAF and PTEN mutations in melanoma of unknown origin presenting as a breast mass." SAGE Open Med Case Rep **5**: 2050313X17711064.

Alver, T. N., T. J. Lavelle, A. S. Longva, G. F. Oy, E. Hovig and S. L. Boe (2016). "MITF depletion elevates expression levels of ERBB3 receptor and its cognate ligand NRG1-beta in melanoma." Oncotarget **7**(34): 55128-55140.

Alvino, E., F. Passarelli, E. Cannavo, C. Fortes, S. Mastroeni, S. Caporali, J. Jiricny, G. C. Cappellini, A. Scoppola, P. Marchetti, A. Modesti and S. D'Atri (2014). "High expression of the mismatch repair protein MSH6 is associated with poor patient survival in melanoma." Am J Clin Pathol **142**(1): 121-132.

Ardito, F., M. Giuliani, D. Perrone, G. Troiano and L. Lo Muzio (2017). "The crucial role of protein phosphorylation in cell signaling and its use as targeted therapy (Review)." Int J Mol Med **40**(2): 271-280.

Aristizabal Prada, E. T., M. Orth, S. Nolting, G. Spottl, J. Maurer and C. Auernhammer (2017). "The MTH1 inhibitor TH588 demonstrates anti-tumoral effects alone and in combination with everolimus, 5-FU and gamma-irradiation in neuroendocrine tumor cells." PLoS One **12**(5): e0178375.

Arlauckas, S. P., C. S. Garris, R. H. Kohler, M. Kitaoka, M. F. Cuccarese, K. S. Yang, M. A. Miller, J. C. Carlson, G. J. Freeman, R. M. Anthony, R. Weissleder and M. J. Pittet (2017). "In vivo imaging reveals a tumor-associated macrophage-mediated resistance pathway in anti-PD-1 therapy." Sci Transl Med **9**(389).

Atefi, M., E. von Euw, N. Attar, C. Ng, C. Chu, D. Guo, R. Nazarian, B. Chmielowski, J. A. Glaspy, B. Comin-Anduix, P. S. Mischel, R. S. Lo and A. Ribas (2011). "Reversing melanoma cross-resistance to BRAF and MEK inhibitors by co-targeting the AKT/mTOR pathway." PLoS One **6**(12): e28973.

Azimi, A., S. Caramuta, B. Seashore-Ludlow, J. Bostrom, J. L. Robinson, F. Edfors, R. Tuominen, K. Kemper, O. Krijgsman, D. S. Peeper, J. Nielsen, J. Hansson, S. Egyhazi Brage,

M. Altun, M. Uhlen and G. Maddalo (2018). "Targeting CDK2 overcomes melanoma resistance against BRAF and Hsp90 inhibitors." Mol Syst Biol **14**(3): e7858.

Azimi, A., R. Tuominen, F. Costa Svedman, S. Caramuta, M. Pernemalm, M. Frostvik Stolt, L. Kanter, P. Kharaziha, J. Lehtio, C. Hertzman Johansson, V. Hoiom, J. Hansson and S. Egyhazi Brage (2017). "Silencing FLI or targeting CD13/ANPEP lead to dephosphorylation of EPHA2, a mediator of BRAF inhibitor resistance, and induce growth arrest or apoptosis in melanoma cells." Cell Death Dis **8**(8): e3029.

Aziz, S. A., M. Davies, E. Pick, C. Zito, L. Jilaveanu, R. L. Camp, D. L. Rimm, Y. Kluger and H. M. Kluger (2009). "Phosphatidylinositol-3-kinase as a therapeutic target in melanoma." Clin Cancer Res **15**(9): 3029-3036.

Balaji, K., S. Vijayaraghavan, L. Diao, P. Tong, Y. Fan, J. P. Carey, T. N. Bui, S. Warner, J. V. Heymach, K. K. Hunt, J. Wang, L. A. Byers and K. Keyomarsi (2017). "AXL Inhibition Suppresses the DNA Damage Response and Sensitizes Cells to PARP Inhibition in Multiple Cancers." Mol Cancer Res **15**(1): 45-58.

Barriuso, J., R. Nagaraju and A. Hurlstone (2015). "Zebrafish: a new companion for translational research in oncology." Clin Cancer Res **21**(5): 969-975.

Bastian, B. C., P. E. LeBoit, H. Hamm, E. B. Brocker and D. Pinkel (1998). "Chromosomal gains and losses in primary cutaneous melanomas detected by comparative genomic hybridization." Cancer Res **58**(10): 2170-2175.

Bean, J., C. Brennan, J. Y. Shih, G. Riely, A. Viale, L. Wang, D. Chitale, N. Motoi, J. Szoke, S. Broderick, M. Balak, W. C. Chang, C. J. Yu, A. Gazdar, H. Pass, V. Rusch, W. Gerald, S. F. Huang, P. C. Yang, V. Miller, M. Ladanyi, C. H. Yang and W. Pao (2007). "MET amplification occurs with or without T790M mutations in EGFR mutant lung tumors with acquired resistance to gefitinib or erlotinib." Proc Natl Acad Sci U S A **104**(52): 20932-20937.

Beaumont, K. A., N. Mohana-Kumaran and N. K. Haass (2013). "Modeling Melanoma In Vitro and In Vivo." Healthcare (Basel) **2**(1): 27-46.

Behrend, L., G. Henderson and R. M. Zwacka (2003). "Reactive oxygen species in oncogenic transformation." Biochem Soc Trans **31**(Pt 6): 1441-1444.

Beuret, L., E. Flori, C. Denoyelle, K. Bille, R. Busca, M. Picardo, C. Bertolotto and R. Ballotti (2007). "Up-regulation of MET expression by alpha-melanocyte-stimulating hormone and MITF allows hepatocyte growth factor to protect melanocytes and melanoma cells from apoptosis." J Biol Chem **282**(19): 14140-14147.

Boyiadzis, M. M., J. M. Kirkwood, J. L. Marshall, C. C. Pritchard, N. S. Azad and J. L. Gulley (2018). "Significance and implications of FDA approval of pembrolizumab for biomarker-defined disease." J Immunother Cancer **6**(1): 35.

Brautigam, L., L. Pudelko, A. S. Jemth, H. Gad, M. Narwal, R. Gustafsson, S. Karsten, J. Carreras Puigvert, E. Homan, C. Berndt, U. W. Berglund, P. Stenmark and T. Helleday

(2016). "Hypoxic Signaling and the Cellular Redox Tumor Environment Determine Sensitivity to MTH1 Inhibition." Cancer Res **76**(8): 2366-2375.

Brookes, S., J. Rowe, M. Ruas, S. Llanos, P. A. Clark, M. Lomax, M. C. James, R. Vatcheva, S. Bates, K. H. Vousden, D. Parry, N. Gruis, N. Smit, W. Bergman and G. Peters (2002). "INK4a-deficient human diploid fibroblasts are resistant to RAS-induced senescence." EMBO J **21**(12): 2936-2945.

Buchbinder, E. I. and K. T. Flaherty (2016). "Biomarkers in Melanoma: Lessons from Translational Medicine." Trends Cancer **2**(6): 305-312.

Bucheit, A. D., G. Chen, A. Siroy, M. Tetzlaff, R. Broaddus, D. Milton, P. Fox, R. Bassett, P. Hwu, J. E. Gershenwald, A. J. Lazar and M. A. Davies (2014). "Complete loss of PTEN protein expression correlates with shorter time to brain metastasis and survival in stage IIIB/C melanoma patients with BRAFV600 mutations." Clin Cancer Res **20**(21): 5527-5536.

Cerami, E., J. Gao, U. Dogrusoz, B. E. Gross, S. O. Sumer, B. A. Aksoy, A. Jacobsen, C. J. Byrne, M. L. Heuer, E. Larsson, Y. Antipin, B. Reva, A. P. Goldberg, C. Sander and N. Schultz (2012). "The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data." Cancer Discov **2**(5): 401-404.

Chalmers, Z. R., F. W. Huang, L. M. Gay, S. M. Ali, J. Chmielecki, J. S. Ross, V. A. Miller, S. Ganesan, P. Stephens, L. A. Garraway and G. M. Frampton (2016). "Analysis of tumor mutation burden (TMB) in >51,000 clinical cancer patients to identify novel non-coding PMS2 promoter mutations associated with increased TMB." Journal of Clinical Oncology **34**(15_suppl): 9572-9572.

Chamcheu, J. C., T. Roy, M. B. Uddin, S. Banang-Mbeumi, R. N. Chamcheu, A. L. Walker, Y. Y. Liu and S. Huang (2019). "Role and Therapeutic Targeting of the PI3K/Akt/mTOR Signaling Pathway in Skin Cancer: A Review of Current Status and Future Trends on Natural and Synthetic Agents Therapy." Cells **8**(8).

Chan, X. Y., A. Singh, N. Osman and T. J. Piva (2017). "Role Played by Signalling Pathways in Overcoming BRAF Inhibitor Resistance in Melanoma." Int J Mol Sci **18**(7).

Chen, B., L. Yang, R. Zhang, Y. Gan, W. Zhang, D. Liu, H. Chen and H. Tang (2017). "Hyperphosphorylation of RPS6KB1, rather than overexpression, predicts worse prognosis in non-small cell lung cancer patients." PLoS One **12**(8): e0182891.

Chen, K. G., R. D. Leapman, G. Zhang, B. Lai, J. C. Valencia, C. O. Cardarelli, W. D. Vieira, V. J. Hearing and M. M. Gottesman (2009). "Influence of melanosome dynamics on melanoma drug sensitivity." J Natl Cancer Inst **101**(18): 1259-1271.

Chen, S., G. A. Crabill, T. S. Pritchard, T. L. McMiller, P. Wei, D. M. Pardoll, F. Pan and S. L. Topalian (2019). "Mechanisms regulating PD-L1 expression on tumor and immune cells." J Immunother Cancer **7**(1): 305.

Cheng, H., V. Chua, C. Liao, T. J. Purwin, M. Terai, K. Kageyama, M. A. Davies, T. Sato and A. E. Aplin (2017). "Co-targeting HGF/cMET Signaling with MEK Inhibitors in Metastatic Uveal Melanoma." Mol Cancer Ther **16**(3): 516-528.

Ciolczyk-Wierzbicka, D., D. Gil, M. Zarzycka and P. Laidler (2020). "mTOR inhibitor everolimus reduces invasiveness of melanoma cells." Hum Cell **33**(1): 88-97.

Clark, J. I., J. Singh, M. S. Ernstoff, C. D. Lao, L. E. Flaherty, T. F. Logan, B. Curti, S. S. Agarwala, B. Taback, L. Cranmer, J. Lutzky, T. L. Luna, S. Aung and D. H. Lawson (2018). "A multi-center phase II study of high dose interleukin-2 sequenced with vemurafenib in patients with BRAF-V600 mutation positive metastatic melanoma." J Immunother Cancer **6**(1): 76.

Cooper, Z. A., V. R. Juneja, P. T. Sage, D. T. Frederick, A. Piris, D. Mitra, J. A. Lo, F. S. Hodi, G. J. Freeman, M. W. Bosenberg, M. McMahon, K. T. Flaherty, D. E. Fisher, A. H. Sharpe and J. A. Wargo (2014). "Response to BRAF inhibition in melanoma is enhanced when combined with immune checkpoint blockade." Cancer Immunol Res **2**(7): 643-654.

Corcoran, R. B., H. Ebi, A. B. Turke, E. M. Coffee, M. Nishino, A. P. Cogdill, R. D. Brown, P. Della Pelle, D. Dias-Santagata, K. E. Hung, K. T. Flaherty, A. Piris, J. A. Wargo, J. Settleman, M. Mino-Kenudson and J. A. Engelman (2012). "EGFR-mediated re-activation of MAPK signaling contributes to insensitivity of BRAF mutant colorectal cancers to RAF inhibition with vemurafenib." Cancer Discov **2**(3): 227-235.

Cox, J. and M. Mann (2008). "MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification." Nat Biotechnol **26**(12): 1367-1372.

Cox, J., N. Neuhauser, A. Michalski, R. A. Scheltema, J. V. Olsen and M. Mann (2011). "Andromeda: a peptide search engine integrated into the MaxQuant environment." J Proteome Res **10**(4): 1794-1805.

Curtin, J. A., J. Fridlyand, T. Kageshita, H. N. Patel, K. J. Busam, H. Kutzner, K. H. Cho, S. Aiba, E. B. Brocker, P. E. LeBoit, D. Pinkel and B. C. Bastian (2005). "Distinct sets of genetic alterations in melanoma." N Engl J Med **353**(20): 2135-2147.

Czyz, M. (2018). "HGF/c-MET Signaling in Melanocytes and Melanoma." Int J Mol Sci **19**(12).

Das, I., H. Gad, L. Brautigam, L. Pudelko, R. Tuominen, V. Hoiom, I. Almlof, V. Rajagopal, J. Hansson, T. Helleday, S. Egyhazi Brage and U. Warpman Berglund (2020). "AXL and CAV-1 play a role for MTH1 inhibitor TH1579 sensitivity in cutaneous malignant melanoma." Cell Death Differ.

Das, I., H. Gad, L. Bräutigam, L. Pudelko, R. Tuominen, V. Höiom, I. Almlöf, V. Rajagopal, J. Hansson, T. Helleday, S. Egyházi Brage and U. Warpman Berglund (2020). "AXL and CAV-1 play a role for MTH1 inhibitor TH1579 sensitivity in cutaneous malignant melanoma." Cell Death & Differentiation.

Das, I., M. Wilhelm, V. Hoiom, R. Franco Marquez, F. Costa Svedman, J. Hansson, R. Tuominen and S. Egyhazi Brage (2019). "Combining ERBB family and MET inhibitors is an effective therapeutic strategy in cutaneous malignant melanoma independent of BRAF/NRAS mutation status." Cell Death Dis **10**(9): 663.

Das, I., M. Wilhelm, V. Höiom, R. Franco Marquez, F. Costa Svedman, J. Hansson, R. Tuominen and S. Egyhàzi Brage (2019). "Combining ERBB family and MET inhibitors is an effective therapeutic strategy in cutaneous malignant melanoma independent of BRAF/NRAS mutation status." Cell Death & Disease **10**(9): 663.

Dasari, A. and W. A. Messersmith (2010). "New strategies in colorectal cancer: biomarkers of response to epidermal growth factor receptor monoclonal antibodies and potential therapeutic targets in phosphoinositide 3-kinase and mitogen-activated protein kinase pathways." Clin Cancer Res **16**(15): 3811-3818.

Davies, M. A., K. Stemke-Hale, E. Lin, C. Tellez, W. Deng, Y. N. Gopal, S. E. Woodman, T. C. Calderone, Z. Ju, A. J. Lazar, V. G. Prieto, K. Aldape, G. B. Mills and J. E. Gershenwald (2009). "Integrated Molecular and Clinical Analysis of AKT Activation in Metastatic Melanoma." Clin Cancer Res **15**(24): 7538-7546.

Deken, M. A., J. Gadiot, E. S. Jordanova, R. Lacroix, M. van Gool, P. Kroon, C. Pineda, M. H. Geukes Foppen, R. Scolyer, J. Y. Song, I. Verbrugge, C. Hoeller, R. Dummer, J. B. Haanen, G. V. Long and C. U. Blank (2016). "Targeting the MAPK and PI3K pathways in combination with PD1 blockade in melanoma." Oncoimmunology **5**(12): e1238557.

Dexheimer, T. S. (2013). DNA Repair Pathways and Mechanisms. DNA Repair of Cancer Stem Cells. L. A. Mathews, S. M. Cabarcas and E. M. Hurt. Dordrecht, Springer Netherlands**:** 19-32.

Di Meglio, P., G. K. Perera and F. O. Nestle (2011). "The multitasking organ: recent insights into skin immune function." Immunity **35**(6): 857-869.

Diem, S., B. Kasenda, L. Spain, J. Martin-Liberal, R. Marconcini, M. Gore and J. Larkin (2016). "Serum lactate dehydrogenase as an early marker for outcome in patients treated with anti-PD-1 therapy in metastatic melanoma." Br J Cancer **114**(3): 256-261.

Domingues, B., J. M. Lopes, P. Soares and H. Populo (2018). "Melanoma treatment in review." Immunotargets Ther **7**: 35-49.

Easty, D. J., S. G. Gray, K. J. O'Byrne, D. O'Donnell and D. C. Bennett (2011). "Receptor tyrosine kinases and their activation in melanoma." Pigment Cell Melanoma Res **24**(3): 446-461.

Einarsdottir, B. O., J. Karlsson, E. M. V. Soderberg, M. F. Lindberg, E. Funck-Brentano, H. Jespersen, S. F. Brynjolfsson, R. O. Bagge, L. Carstam, M. Scobie, T. Koolmeister, O. Wallner, U. Stierner, U. W. Berglund, L. Ny, L. M. Nilsson, E. Larsson, T. Helleday and J. A. Nilsson (2018). "A patient-derived xenograft pre-clinical trial reveals treatment responses and a resistance mechanism to karonudib in metastatic melanoma." Cell Death Dis **9**(8): 810.

Eroglu, Z. and A. Ribas (2016). "Combination therapy with BRAF and MEK inhibitors for melanoma: latest evidence and place in therapy." Ther Adv Med Oncol **8**(1): 48-56.

Eshtad, S., Z. Mavajian, S. G. Rudd, T. Visnes, J. Bostrom, M. Altun and T. Helleday (2016). "hMYH and hMTH1 cooperate for survival in mismatch repair defective T-cell acute lymphoblastic leukemia." Oncogenesis **5**(12): e275.

Flaherty, K., M. A. Davies, J. J. Grob, G. V. Long, P. D. Nathan, A. Ribas, C. Robert, D. Schadendorf, D. T. Frederick, M. R. Hammond, J. Jane-Valbuena, X. J. Mu, M. Squires, S. A. Jaeger, S. R. Lane, B. Mookerjee and L. A. Garraway (2016). "Genomic analysis and 3-y efficacy and safety update of COMBI-d: A phase 3 study of dabrafenib (D) + trametinib (T) vs D monotherapy in patients (pts) with unresectable or metastatic BRAF V600E/K-mutant cutaneous melanoma." Journal of Clinical Oncology **34**(15_suppl): 9502-9502.

Frederick, D. T., R. A. Salas Fragomeni, A. Schalck, I. Ferreiro-Neira, T. Hoff, Z. A. Cooper, R. Haq, D. J. Panka, L. N. Kwong, M. A. Davies, J. C. Cusack, K. T. Flaherty, D. E. Fisher, J. W. Mier, J. A. Wargo and R. J. Sullivan (2014). "Clinical profiling of BCL-2 family members in the setting of BRAF inhibition offers a rationale for targeting de novo resistance using BH3 mimetics." PLoS One **9**(7): e101286.

Freeman, A. K., D. A. Ritt and D. K. Morrison (2013). "The importance of Raf dimerization in cell signaling." Small GTPases **4**(3): 180-185.

Gad, H., T. Koolmeister, A. S. Jemth, S. Eshtad, S. A. Jacques, C. E. Strom, L. M. Svensson, N. Schultz, T. Lundback, B. O. Einarsdottir, A. Saleh, C. Gokturk, P. Baranczewski, R. Svensson, R. P. Berntsson, R. Gustafsson, K. Stromberg, K. Sanjiv, M. C. Jacques-Cordonnier, M. Desroses, A. L. Gustavsson, R. Olofsson, F. Johansson, E. J. Homan, O. Loseva, L. Brautigam, L. Johansson, A. Hoglund, A. Hagenkort, T. Pham, M. Altun, F. Z. Gaugaz, S. Vikingsson, B. Evers, M. Henriksson, K. S. Vallin, O. A. Wallner, L. G. Hammarstrom, E. Wiita, I. Almlof, C. Kalderen, H. Axelsson, T. Djureinovic, J. C. Puigvert, M. Haggblad, F. Jeppsson, U. Martens, C. Lundin, B. Lundgren, I. Granelli, A. J. Jensen, P. Artursson, J. A. Nilsson, P. Stenmark, M. Scobie, U. W. Berglund and T. Helleday (2014). "MTH1 inhibition eradicates cancer by preventing sanitation of the dNTP pool." Nature **508**(7495): 215-221.

Gad, H., O. Mortusewicz, S. G. Rudd, A. Stolz, N. Amaral, L. Brautigham, L. Pudelko, K. Sanjiv, C. Kaldéren, A.-S. Jemth, I. Almlöf, T. Visnes, N. Schultz, J. Boström, J. M. Calderon Montano, A. Hagenkort, P. Groth, O. Loseva, C. Gokturk, T. Koolmeister, P. Wakchaure, E. Homan, C. E. Ström, M. Scobie, H. Bastians, U. W. Berglund and T. Helleday (2019). "MTH1 promotes mitotic progression to avoid oxidative DNA damage in cancer cells." bioRxiv: 575290.

Gao, J., B. A. Aksoy, U. Dogrusoz, G. Dresdner, B. Gross, S. O. Sumer, Y. Sun, A. Jacobsen, R. Sinha, E. Larsson, E. Cerami, C. Sander and N. Schultz (2013). "Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal." Sci Signal **6**(269): pl1.

Gao, J., J. F. Ward, C. A. Pettaway, L. Z. Shi, S. K. Subudhi, L. M. Vence, H. Zhao, J. Chen, H. Chen, E. Efstathiou, P. Troncoso, J. P. Allison, C. J. Logothetis, Wistuba, II, M. A.

Sepulveda, J. Sun, J. Wargo, J. Blando and P. Sharma (2017). "VISTA is an inhibitory immune checkpoint that is increased after ipilimumab therapy in patients with prostate cancer." Nat Med **23**(5): 551-555.

Gao, M. Z., H. B. Wang, X. L. Chen, W. T. Cao, L. Fu, Y. Li, H. T. Quan, C. Y. Xie and L. G. Lou (2019). "Aberrant modulation of ribosomal protein S6 phosphorylation confers acquired resistance to MAPK pathway inhibitors in BRAF-mutant melanoma." Acta Pharmacol Sin **40**(2): 268-278.

Giribaldi, M. G., A. Munoz, K. Halvorsen, A. Patel and P. Rai (2015). "MTH1 expression is required for effective transformation by oncogenic HRAS." Oncotarget **6**(13): 11519-11529.

Girotti, M. R., M. Pedersen, B. Sanchez-Laorden, A. Viros, S. Turajlic, D. Niculescu-Duvaz, A. Zambon, J. Sinclair, A. Hayes, M. Gore, P. Lorigan, C. Springer, J. Larkin, C. Jorgensen and R. Marais (2013). "Inhibiting EGF receptor or SRC family kinase signaling overcomes BRAF inhibitor resistance in melanoma." Cancer Discov **3**(2): 158-167.

Gopal, Y. N., W. Deng, S. E. Woodman, K. Komurov, P. Ram, P. D. Smith and M. A. Davies (2010). "Basal and treatment-induced activation of AKT mediates resistance to cell death by AZD6244 (ARRY-142886) in Braf-mutant human cutaneous melanoma cells." Cancer Res **70**(21): 8736-8747.

Gusenbauer, S., P. Vlaicu and A. Ullrich (2013). "HGF induces novel EGFR functions involved in resistance formation to tyrosine kinase inhibitors." Oncogene **32**(33): 3846-3856.

Hanahan, D. and R. A. Weinberg (2011). "Hallmarks of cancer: the next generation." Cell **144**(5): 646-674.

Haq, R., S. Yokoyama, E. B. Hawryluk, G. B. Jonsson, D. T. Frederick, K. McHenry, D. Porter, T. N. Tran, K. T. Love, R. Langer, D. G. Anderson, L. A. Garraway, L. M. Duncan, D. L. Morton, D. S. Hoon, J. A. Wargo, J. S. Song and D. E. Fisher (2013). "BCL2A1 is a lineage-specific antiapoptotic melanoma oncogene that confers resistance to BRAF inhibition." Proc Natl Acad Sci U S A **110**(11): 4321-4326.

Havel, J. J., D. Chowell and T. A. Chan (2019). "The evolving landscape of biomarkers for checkpoint inhibitor immunotherapy." Nat Rev Cancer **19**(3): 133-150.

Hegde, P. S., V. Karanikas and S. Evers (2016). "The Where, the When, and the How of Immune Monitoring for Cancer Immunotherapies in the Era of Checkpoint Inhibition." Clin Cancer Res **22**(8): 1865-1874.

Herbst, R. S., P. Baas, D. W. Kim, E. Felip, J. L. Perez-Gracia, J. Y. Han, J. Molina, J. H. Kim, C. D. Arvis, M. J. Ahn, M. Majem, M. J. Fidler, G. de Castro, Jr., M. Garrido, G. M. Lubiniecki, Y. Shentu, E. Im, M. Dolled-Filhart and E. B. Garon (2016). "Pembrolizumab versus docetaxel for previously treated, PD-L1-positive, advanced non-small-cell lung cancer (KEYNOTE-010): a randomised controlled trial." Lancet **387**(10027): 1540-1550.

Hertzman Johansson, C., A. Azimi, M. Frostvik Stolt, S. Shojaee, H. Wiberg, E. Grafstrom, J. Hansson and S. Egyhazi Brage (2013). "Association of MITF and other melanosome-

related proteins with chemoresistance in melanoma tumors and cell lines." Melanoma Res **23**(5): 360-365.

Holderfield, M., M. M. Deuker, F. McCormick and M. McMahon (2014). "Targeting RAF kinases for cancer therapy: BRAF-mutated melanoma and beyond." Nat Rev Cancer **14**(7): 455-467.

Hu-Lieskovan, S., S. Mok, B. Homet Moreno, J. Tsoi, L. Robert, L. Goedert, E. M. Pinheiro, R. C. Koya, T. G. Graeber, B. Comin-Anduix and A. Ribas (2015). "Improved antitumor activity of immunotherapy with BRAF and MEK inhibitors in BRAF(V600E) melanoma." Sci Transl Med **7**(279): 279ra241.

Hugo, W., J. M. Zaretsky, L. Sun, C. Song, B. H. Moreno, S. Hu-Lieskovan, B. Berent-Maoz, J. Pang, B. Chmielowski, G. Cherry, E. Seja, S. Lomeli, X. Kong, M. C. Kelley, J. A. Sosman, D. B. Johnson, A. Ribas and R. S. Lo (2016). "Genomic and Transcriptomic Features of Response to Anti-PD-1 Therapy in Metastatic Melanoma." Cell **165**(1): 35-44.

Ikejiri, F., Y. Honma, T. Kasukabe, T. Urano and J. Suzumiya (2018). "TH588, an MTH1 inhibitor, enhances phenethyl isothiocyanate-induced growth inhibition in pancreatic cancer cells." Oncol Lett **15**(3): 3240-3244.

Inoue, D. and J. Wittbrodt (2011). "One for all--a highly efficient and versatile method for fluorescent immunostaining in fish embryos." PLoS One **6**(5): e19713.

Ishikawa, K., K. Takenaga, M. Akimoto, N. Koshikawa, A. Yamaguchi, H. Imanishi, K. Nakada, Y. Honma and J. Hayashi (2008). "ROS-generating mitochondrial DNA mutations can regulate tumor cell metastasis." Science **320**(5876): 661-664.

Ji, Z., Y. Erin Chen, R. Kumar, M. Taylor, C. N. Jenny Njauw, B. Miao, D. T. Frederick, J. A. Wargo, K. T. Flaherty, G. Jonsson and H. Tsao (2015). "MITF Modulates Therapeutic Resistance through EGFR Signaling." J Invest Dermatol **135**(7): 1863-1872.

Johnson, D. B. and J. A. Sosman (2013). "Update on the targeted therapy of melanoma." Curr Treat Options Oncol **14**(2): 280-292.

Jonsson, A., R. Tuominen, E. Grafstrom, J. Hansson and S. Egyhazi (2010). "High frequency of p16(INK4A) promoter methylation in NRAS-mutated cutaneous melanoma." J Invest Dermatol **130**(12): 2809-2817.

Kalal, B. S., D. Upadhya and V. R. Pai (2017). "Chemotherapy Resistance Mechanisms in Advanced Skin Cancer." Oncol Rev **11**(1): 326.

Karbowniczek, M., C. S. Spittle, T. Morrison, H. Wu and E. P. Henske (2008). "mTOR is activated in the majority of malignant melanomas." J Invest Dermatol **128**(4): 980-987.

Kasela, M., M. Nystrom and M. Kansikas (2019). "PMS2 expression decrease causes severe problems in mismatch repair." Hum Mutat **40**(7): 904-907.

Kawamura, T., M. Kawatani, M. Muroi, Y. Kondoh, Y. Futamura, H. Aono, M. Tanaka, K. Honda and H. Osada (2016). "Proteomic profiling of small-molecule inhibitors reveals dispensability of MTH1 for cancer cell survival." Sci Rep **6**: 26521.

Kennedy, C. H., R. Cueto, S. A. Belinsky, J. F. Lechner and W. A. Pryor (1998). "Overexpression of hMTH1 mRNA: a molecular marker of oxidative stress in lung cancer cells." FEBS Lett **429**(1): 17-20.

Ketteler, J. and D. Klein (2018). "Caveolin-1, cancer and therapy resistance." Int J Cancer **143**(9): 2092-2104.

Koketsu, S., T. Watanabe and H. Nagawa (2004). "Expression of DNA repair protein: MYH, NTH1, and MTH1 in colorectal cancer." Hepatogastroenterology **51**(57): 638-642.

Korabiowska, M., U. Brinck, H. Dengler, J. Stachura, A. Schauer and M. Droese (2000). "Analysis of the DNA mismatch repair proteins expression in malignant melanomas." Anticancer Res **20**(6B): 4499-4505.

Kozar, I., C. Margue, S. Rothengatter, C. Haan and S. Kreis (2019). "Many ways to resistance: How melanoma cells evade targeted therapies." Biochim Biophys Acta Rev Cancer **1871**(2): 313-322.

Krepler, C., M. Xiao, K. Sproesser, P. A. Brafford, B. Shannan, M. Beqiri, Q. Liu, W. Xu, B. Garman, K. L. Nathanson, X. Xu, G. C. Karakousis, G. B. Mills, Y. Lu, T. A. Ahmed, P. I. Poulikakos, G. Caponigro, M. Boehm, M. Peters, L. M. Schuchter, A. T. Weeraratna and M. Herlyn (2016). "Personalized Preclinical Trials in BRAF Inhibitor-Resistant Patient-Derived Xenograft Models Identify Second-Line Combination Therapies." Clin Cancer Res **22**(7): 1592-1602.

Kuzu, O. F., R. Gowda, A. Sharma, M. A. Noory, G. Kardos, S. V. Madhunapantula, J. J. Drabick and G. P. Robertson (2018). "Identification of WEE1 as a target to make AKT inhibition more effective in melanoma." Cancer Biol Ther **19**(1): 53-62.

Kwong, L. N. and M. A. Davies (2013). "Navigating the therapeutic complexity of PI3K pathway inhibition in melanoma." Clin Cancer Res **19**(19): 5310-5319.

Lans, H., J. H. J. Hoeijmakers, W. Vermeulen and J. A. Marteijn (2019). "The DNA damage response to transcription stress." Nat Rev Mol Cell Biol **20**(12): 766-784.

Larkin, J., V. Chiarion-Sileni, R. Gonzalez, J. J. Grob, P. Rutkowski, C. D. Lao, C. L. Cowey, D. Schadendorf, J. Wagstaff, R. Dummer, P. F. Ferrucci, M. Smylie, D. Hogg, A. Hill, I. Marquez-Rodas, J. Haanen, M. Guidoboni, M. Maio, P. Schoffski, M. S. Carlino, C. Lebbe, G. McArthur, P. A. Ascierto, G. A. Daniels, G. V. Long, L. Bastholt, J. I. Rizzo, A. Balogh, A. Moshyk, F. S. Hodi and J. D. Wolchok (2019). "Five-Year Survival with Combined Nivolumab and Ipilimumab in Advanced Melanoma." N Engl J Med **381**(16): 1535-1546.

Lasithiotakis, K. G., T. W. Sinnberg, B. Schittek, K. T. Flaherty, D. Kulms, E. Maczey, C. Garbe and F. E. Meier (2008). "Combined inhibition of MAPK and mTOR signaling inhibits

growth, induces cell death, and abrogates invasive growth of melanoma cells." J Invest Dermatol **128**(8): 2013-2023.

Lawrence, M. S., P. Stojanov, P. Polak, G. V. Kryukov, K. Cibulskis, A. Sivachenko, S. L. Carter, C. Stewart, C. H. Mermel, S. A. Roberts, A. Kiezun, P. S. Hammerman, A. McKenna, Y. Drier, L. Zou, A. H. Ramos, T. J. Pugh, N. Stransky, E. Helman, J. Kim, C. Sougnez, L. Ambrogio, E. Nickerson, E. Shefler, M. L. Cortes, D. Auclair, G. Saksena, D. Voet, M. Noble, D. DiCara, P. Lin, L. Lichtenstein, D. I. Heiman, T. Fennell, M. Imielinski, B. Hernandez, E. Hodis, S. Baca, A. M. Dulak, J. Lohr, D. A. Landau, C. J. Wu, J. Melendez-Zajgla, A. Hidalgo-Miranda, A. Koren, S. A. McCarroll, J. Mora, B. Crompton, R. Onofrio, M. Parkin, W. Winckler, K. Ardlie, S. B. Gabriel, C. W. M. Roberts, J. A. Biegel, K. Stegmaier, A. J. Bass, L. A. Garraway, M. Meyerson, T. R. Golub, D. A. Gordenin, S. Sunyaev, E. S. Lander and G. Getz (2013). "Mutational heterogeneity in cancer and the search for new cancer-associated genes." Nature **499**(7457): 214-218.

Lee, J. H., G. V. Long, S. Boyd, S. Lo, A. M. Menzies, V. Tembe, A. Guminski, V. Jakrot, R. A. Scolyer, G. J. Mann, R. F. Kefford, M. S. Carlino and H. Rizos (2017). "Circulating tumour DNA predicts response to anti-PD1 antibodies in metastatic melanoma." Ann Oncol **28**(5): 1130-1136.

Letrado, P., I. de Miguel, I. Lamberto, R. Diez-Martinez and J. Oyarzabal (2018). "Zebrafish: Speeding Up the Cancer Drug Discovery Process." Cancer Res **78**(21): 6048-6058.

Liu-Smith, F., C. Poe, P. J. Farmer and F. L. Meyskens, Jr. (2015). "Amyloids, melanins and oxidative stress in melanomagenesis." Exp Dermatol **24**(3): 171-174.

Lord, C. J. and A. Ashworth (2012). "The DNA damage response and cancer therapy." Nature **481**(7381): 287-294.

LoRusso, P. M., K. Schalper and J. Sosman (2019). "Targeted Therapy and Immunotherapy: Emerging Biomarkers in Metastatic Melanoma." Pigment Cell Melanoma Res.

Luchini, C., F. Bibeau, M. J. L. Ligtenberg, N. Singh, A. Nottegar, T. Bosse, R. Miller, N. Riaz, J. Y. Douillard, F. Andre and A. Scarpa (2019). "ESMO recommendations on microsatellite instability testing for immunotherapy in cancer, and its relationship with PD-1/PD-L1 expression and tumour mutational burden: a systematic review-based approach." Ann Oncol.

Lugowska, I., P. Teterycz and P. Rutkowski (2018). "Immunotherapy of melanoma." Contemp Oncol (Pozn) **22**(1A): 61-67.

Luke, J. J., K. T. Flaherty, A. Ribas and G. V. Long (2017). "Targeted agents and immunotherapies: optimizing outcomes in melanoma." Nat Rev Clin Oncol **14**(8): 463-482.

Lunardi, A. and P. P. Pandolfi (2015). "A co-clinical platform to accelerate cancer treatment optimization." Trends Mol Med **21**(1): 1-5.

Magnussen, G. I., R. Holm, E. Emilsen, A. K. Rosnes, A. Slipicevic and V. A. Florenes (2012). "High expression of Wee1 is associated with poor disease-free survival in malignant melanoma: potential for targeted therapy." PLoS One **7**(6): e38254.

Maleki Vareki, S. (2018). "High and low mutational burden tumors versus immunologically hot and cold tumors and response to immune checkpoint inhibitors." J Immunother Cancer **6**(1): 157.

Malins, D. C., N. L. Polissar and S. J. Gunselman (1996). "Progression of human breast cancers to the metastatic state is linked to hydroxyl radical-induced DNA damage." Proc Natl Acad Sci U S A **93**(6): 2557-2563.

McDonell, L. M., K. D. Kernohan, K. M. Boycott and S. L. Sawyer (2015). "Receptor tyrosine kinase mutations in developmental syndromes and cancer: two sides of the same coin." Hum Mol Genet **24**(R1): R60-66.

McWilliams, R. R., E. D. Wieben, K. G. Rabe, K. S. Pedersen, Y. Wu, H. Sicotte and G. M. Petersen (2011). "Prevalence of CDKN2A mutations in pancreatic cancer patients: implications for genetic counseling." Eur J Hum Genet **19**(4): 472-478.

Meyskens, F. L., Jr., P. J. Farmer, S. Yang and H. Anton-Culver (2007). "New perspectives on melanoma pathogenesis and chemoprevention." Recent Results Cancer Res **174**: 191-195.

Miller, A. J. and M. C. Mihm, Jr. (2006). "Melanoma." N Engl J Med **355**(1): 51-65.

Mooradian, M. J., A. Reuben, P. A. Prieto, M. Hazar-Rethinam, D. T. Frederick, B. Nadres, A. Piris, V. Juneja, Z. A. Cooper, A. H. Sharpe, R. B. Corcoran, K. T. Flaherty, D. P. Lawrence, J. A. Wargo and R. J. Sullivan (2018). "A phase II study of combined therapy with a BRAF inhibitor (vemurafenib) and interleukin-2 (aldesleukin) in patients with metastatic melanoma." Oncoimmunology **7**(5): e1423172.

Morgillo, F., J. K. Woo, E. S. Kim, W. K. Hong and H. Y. Lee (2006). "Heterodimerization of insulin-like growth factor receptor/epidermal growth factor receptor and induction of survivin expression counteract the antitumor action of erlotinib." Cancer Res **66**(20): 10100-10111.

Muller, J., O. Krijgsman, J. Tsoi, L. Robert, W. Hugo, C. Song, X. Kong, P. A. Possik, P. D. Cornelissen-Steijger, M. H. Geukes Foppen, K. Kemper, C. R. Goding, U. McDermott, C. Blank, J. Haanen, T. G. Graeber, A. Ribas, R. S. Lo and D. S. Peeper (2014). "Low MITF/AXL ratio predicts early resistance to multiple targeted drugs in melanoma." Nat Commun **5**: 5712.

Nakabeppu, Y. (2014). "Cellular levels of 8-oxoguanine in either DNA or the nucleotide pool play pivotal roles in carcinogenesis and survival of cancer cells." Int J Mol Sci **15**(7): 12543-12557.

Nakabeppu, Y., E. Ohta and N. Abolhassani (2017). "MTH1 as a nucleotide pool sanitizing enzyme: Friend or foe?" Free Radic Biol Med **107**: 151-158.

Nestle, F. O., P. Di Meglio, J. Z. Qin and B. J. Nickoloff (2009). "Skin immune sentinels in health and disease." Nat Rev Immunol **9**(10): 679-691.

Nonomura, Y., A. Otsuka, C. Nakashima, J. A. Seidel, A. Kitoh, T. Dainichi, S. Nakajima, Y. Sawada, S. Matsushita, M. Aoki, T. Takenouchi, T. Fujimura, N. Hatta, S. Koreeda, S. Fukushima, T. Honda and K. Kabashima (2016). "Peripheral blood Th9 cells are a possible pharmacodynamic biomarker of nivolumab treatment efficacy in metastatic melanoma patients." Oncoimmunology **5**(12): e1248327.

Park, J., A. H. Talukder, S. A. Lim, K. Kim, K. Pan, B. Melendez, S. D. Bradley, K. R. Jackson, J. S. Khalili, J. Wang, C. Creasy, B. F. Pan, S. E. Woodman, C. Bernatchez, D. Hawke, P. Hwu, K. M. Lee, J. Roszik, G. Lizee and C. Yee (2017). "SLC45A2: A Melanoma Antigen with High Tumor Selectivity and Reduced Potential for Autoimmune Toxicity." Cancer Immunol Res **5**(8): 618-629.

Pasquali, S., A. V. Hadjinicolaou, V. Chiarion Sileni, C. R. Rossi and S. Mocellin (2018). "Systemic treatments for metastatic cutaneous melanoma." Cochrane Database Syst Rev **2**: CD011123.

Patel, A., D. G. Burton, K. Halvorsen, W. Balkan, T. Reiner, C. Perez-Stable, A. Cohen, A. Munoz, M. G. Giribaldi, S. Singh, D. J. Robbins, D. M. Nguyen and P. Rai (2015). "MutT Homolog 1 (MTH1) maintains multiple KRAS-driven pro-malignant pathways." Oncogene **34**(20): 2586-2596.

Peeper, D. S. (2011). "Oncogene-induced senescence and melanoma: where do we stand?" Pigment Cell Melanoma Res **24**(6): 1107-1111.

Pelster, M. S. and R. N. Amaria (2019). "Combined targeted therapy and immunotherapy in melanoma: a review of the impact on the tumor microenvironment and outcomes of early clinical trials." Ther Adv Med Oncol **11**: 1758835919830826.

Pieper, N., A. Zaremba, S. Leonardelli, F. N. Harbers, M. Schwamborn, S. Lubcke, B. Schrors, J. Baingo, A. Schramm, S. Haferkamp, U. Seifert, A. Sucker, V. Lennerz, T. Wolfel, D. Schadendorf, B. Schilling, A. Paschen and F. Zhao (2018). "Evolution of melanoma cross-resistance to CD8(+) T cells and MAPK inhibition in the course of BRAFi treatment." Oncoimmunology **7**(8): e1450127.

Pudelko, L., S. Edwards, M. Balan, D. Nyqvist, J. Al-Saadi, J. Dittmer, I. Almlof, T. Helleday and L. Brautigam (2018). "An orthotopic glioblastoma animal model suitable for high-throughput screenings." Neuro Oncol **20**(11): 1475-1484.

Pudelko, L., P. Rouhi, K. Sanjiv, H. Gad, C. Kalderen, A. Hoglund, M. Squatrito, A. J. Schuhmacher, S. Edwards, D. Hagerstrand, U. W. Berglund, T. Helleday and L. Brautigam (2017). "Glioblastoma and glioblastoma stem cells are dependent on functional MTH1." Oncotarget **8**(49): 84671-84684.

Ramsey, M. R. and N. E. Sharpless (2006). "ROS as a tumour suppressor?" Nat Cell Biol **8**(11): 1213-1215.

Rankin, E. B. and A. J. Giaccia (2016). "The Receptor Tyrosine Kinase AXL in Cancer Progression." Cancers (Basel) **8**(11).

Reuveni, H., E. Flashner-Abramson, L. Steiner, K. Makedonski, R. Song, A. Shir, M. Herlyn, M. Bar-Eli and A. Levitzki (2013). "Therapeutic destruction of insulin receptor substrates for cancer treatment." Cancer Res **73**(14): 4383-4394.

Revach, O. Y., O. Sandler, Y. Samuels and B. Geiger (2019). "Cross-Talk between Receptor Tyrosine Kinases AXL and ERBB3 Regulates Invadopodia Formation in Melanoma Cells." Cancer Res **79**(10): 2634-2648.

Riaz, N., J. J. Havel, V. Makarov, A. Desrichard, W. J. Urba, J. S. Sims, F. S. Hodi, S. Martin-Algarra, R. Mandal, W. H. Sharfman, S. Bhatia, W. J. Hwu, T. F. Gajewski, C. L. Slingluff, Jr., D. Chowell, S. M. Kendall, H. Chang, R. Shah, F. Kuo, L. G. T. Morris, J. W. Sidhom, J. P. Schneck, C. E. Horak, N. Weinhold and T. A. Chan (2017). "Tumor and Microenvironment Evolution during Immunotherapy with Nivolumab." Cell **171**(4): 934-949 e916.

Ribas, A., I. Puzanov, R. Dummer, D. Schadendorf, O. Hamid, C. Robert, F. S. Hodi, J. Schachter, A. C. Pavlick, K. D. Lewis, L. D. Cranmer, C. U. Blank, S. J. O'Day, P. A. Ascierto, A. K. Salama, K. A. Margolin, C. Loquai, T. K. Eigentler, T. C. Gangadhar, M. S. Carlino, S. S. Agarwala, S. J. Moschos, J. A. Sosman, S. M. Goldinger, R. Shapira-Frommer, R. Gonzalez, J. M. Kirkwood, J. D. Wolchok, A. Eggermont, X. N. Li, W. Zhou, A. M. Zernhelt, J. Lis, S. Ebbinghaus, S. P. Kang and A. Daud (2015). "Pembrolizumab versus investigator-choice chemotherapy for ipilimumab-refractory melanoma (KEYNOTE-002): a randomised, controlled, phase 2 trial." Lancet Oncol **16**(8): 908-918.

Rivera Vargas, T. and L. Apetoh (2019). "Can Immunogenic Chemotherapies Relieve Cancer Cell Resistance to Immune Checkpoint Inhibitors?" Frontiers in Immunology **10**(1181).

Rizos, H., A. P. Darmanian, E. A. Holland, G. J. Mann and R. F. Kefford (2001). "Mutations in the INK4a/ARF melanoma susceptibility locus functionally impair p14ARF." J Biol Chem **276**(44): 41424-41434.

Rizos, H., A. M. Menzies, G. M. Pupo, M. S. Carlino, C. Fung, J. Hyman, L. E. Haydu, B. Mijatov, T. M. Becker, S. C. Boyd, J. Howle, R. Saw, J. F. Thompson, R. F. Kefford, R. A. Scolyer and G. V. Long (2014). "BRAF inhibitor resistance mechanisms in metastatic melanoma: spectrum and clinical impact." Clin Cancer Res **20**(7): 1965-1977.

Rizvi, N. A., M. D. Hellmann, A. Snyder, P. Kvistborg, V. Makarov, J. J. Havel, W. Lee, J. Yuan, P. Wong, T. S. Ho, M. L. Miller, N. Rekhtman, A. L. Moreira, F. Ibrahim, C. Bruggeman, B. Gasmi, R. Zappasodi, Y. Maeda, C. Sander, E. B. Garon, T. Merghoub, J. D. Wolchok, T. N. Schumacher and T. A. Chan (2015). "Cancer immunology. Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer." Science **348**(6230): 124-128.

Robert, C., J. J. Grob, D. Stroyakovskiy, B. Karaszewska, A. Hauschild, E. Levchenko, V. Chiarion Sileni, J. Schachter, C. Garbe, I. Bondarenko, H. Gogas, M. Mandala, J. Haanen, C. Lebbe, A. Mackiewicz, P. Rutkowski, P. D. Nathan, A. Ribas, M. A. Davies, K. T.

Flaherty, P. Burgess, M. Tan, E. Gasal, M. Voi, D. Schadendorf and G. V. Long (2019). "Five-Year Outcomes with Dabrafenib plus Trametinib in Metastatic Melanoma." N Engl J Med **381**(7): 626-636.

Roskoski, R., Jr. (2012). "ERK1/2 MAP kinases: structure, function, and regulation." Pharmacol Res **66**(2): 105-143.

Saleh, J. (2018). "Murine models of melanoma." Pathol Res Pract **214**(9): 1235-1238.

Schadendorf, D., D. E. Fisher, C. Garbe, J. E. Gershenwald, J. J. Grob, A. Halpern, M. Herlyn, M. A. Marchetti, G. McArthur, A. Ribas, A. Roesch and A. Hauschild (2015). "Melanoma." Nat Rev Dis Primers **1**: 15003.

Schwartz, P. A. and B. W. Murray (2011). "Protein kinase biochemistry and drug discovery." Bioorg Chem **39**(5-6): 192-210.

Scurr, L. L., G. M. Pupo, T. M. Becker, K. Lai, D. Schrama, S. Haferkamp, M. Irvine, R. A. Scolyer, G. J. Mann, J. C. Becker, R. F. Kefford and H. Rizos (2010). "IGFBP7 is not required for B-RAF-induced melanocyte senescence." Cell **141**(4): 717-727.

Seidel, J. A., A. Otsuka and K. Kabashima (2018). "Anti-PD-1 and Anti-CTLA-4 Therapies in Cancer: Mechanisms of Action, Efficacy, and Limitations." Front Oncol **8**: 86.

Serrano, M., A. W. Lin, M. E. McCurrach, D. Beach and S. W. Lowe (1997). "Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a." Cell **88**(5): 593-602.

Shain, A. H. and B. C. Bastian (2016). "From melanocytes to melanomas." Nat Rev Cancer **16**(6): 345-358.

Simeone, E., A. M. Grimaldi, L. Festino, D. Giannarelli, V. Vanella, M. Palla, M. Curvietto, A. Esposito, G. Palmieri, N. Mozzillo and P. A. Ascierto (2017). "Correlation between previous treatment with BRAF inhibitors and clinical response to pembrolizumab in patients with advanced melanoma." Oncoimmunology **6**(3): e1283462.

Snyder, A., V. Makarov, T. Merghoub, J. Yuan, J. M. Zaretsky, A. Desrichard, L. A. Walsh, M. A. Postow, P. Wong, T. S. Ho, T. J. Hollmann, C. Bruggeman, K. Kannan, Y. Li, C. Elipenahli, C. Liu, C. T. Harbison, L. Wang, A. Ribas, J. D. Wolchok and T. A. Chan (2014). "Genetic basis for clinical response to CTLA-4 blockade in melanoma." N Engl J Med **371**(23): 2189-2199.

Somasundaram, R. and M. Herlyn (2012). "Melanoma exosomes: messengers of metastasis." Nat Med **18**(6): 853-854.

Sun, C., L. Wang, S. Huang, G. J. Heynen, A. Prahallad, C. Robert, J. Haanen, C. Blank, J. Wesseling, S. M. Willems, D. Zecchin, S. Hobor, P. K. Bajpe, C. Lieftink, C. Mateus, S. Vagner, W. Grernrum, I. Hofland, A. Schlicker, L. F. Wessels, R. L. Beijersbergen, A. Bardelli, F. Di Nicolantonio, A. M. Eggermont and R. Bernards (2014). "Reversible and adaptive resistance to BRAF(V600E) inhibition in melanoma." Nature **508**(7494): 118-122.

Takagi, Y., D. Setoyama, R. Ito, H. Kamiya, Y. Yamagata and M. Sekiguchi (2012). "Human MTH3 (NUDT18) protein hydrolyzes oxidized forms of guanosine and deoxyguanosine diphosphates: comparison with MTH1 and MTH2." J Biol Chem **287**(25): 21541-21549.

Tarhini, A. and R. R. Kudchadkar (2018). "Predictive and on-treatment monitoring biomarkers in advanced melanoma: Moving toward personalized medicine." Cancer Treat Rev **71**: 8-18.

Ten Broeke, S. W., H. M. van der Klift, C. M. J. Tops, S. Aretz, I. Bernstein, D. D. Buchanan, A. de la Chapelle, G. Capella, M. Clendenning, C. Engel, S. Gallinger, E. Gomez Garcia, J. C. Figueiredo, R. Haile, H. L. Hampel, J. L. Hopper, N. Hoogerbrugge, M. von Knebel Doeberitz, L. Le Marchand, T. G. W. Letteboer, M. A. Jenkins, A. Lindblom, N. M. Lindor, A. R. Mensenkamp, P. Moller, P. A. Newcomb, T. A. M. van Os, R. Pearlman, M. Pineda, N. Rahner, E. J. W. Redeker, M. J. W. Olderode-Berends, C. Rosty, H. K. Schackert, R. Scott, L. Senter, L. Spruijt, V. Steinke-Lange, M. Suerink, S. Thibodeau, Y. J. Vos, A. Wagner, I. Winship, F. J. Hes, H. F. A. Vasen, J. T. Wijnen, M. Nielsen and A. K. Win (2018). "Cancer Risks for PMS2-Associated Lynch Syndrome." J Clin Oncol **36**(29): 2961-2968.

Tiwary, S., M. Preziosi, P. G. Rothberg, N. Zeitouni, N. Corson and L. Xu (2014). "ERBB3 is required for metastasis formation of melanoma cells." Oncogenesis **3**: e110.

Trujillo, J. A., J. J. Luke, Y. Zha, J. P. Segal, L. L. Ritterhouse, S. Spranger, K. Matijevich and T. F. Gajewski (2019). "Secondary resistance to immunotherapy associated with beta-catenin pathway activation or PTEN loss in metastatic melanoma." J Immunother Cancer **7**(1): 295.

Udart, M., J. Utikal, G. M. Krahn and R. U. Peter (2001). "Chromosome 7 aneusomy. A marker for metastatic melanoma? Expression of the epidermal growth factor receptor gene and chromosome 7 aneusomy in nevi, primary malignant melanomas and metastases." Neoplasia **3**(3): 245-254.

Vido, M. J., K. Le, E. J. Hartsough and A. E. Aplin (2018). "BRAF Splice Variant Resistance to RAF Inhibitor Requires Enhanced MEK Association." Cell Rep **25**(6): 1501-1510 e1503.

Villanueva, J., A. Vultur and M. Herlyn (2011). "Resistance to BRAF inhibitors: unraveling mechanisms and future treatment options." Cancer Res **71**(23): 7137-7140.

Vogelstein, B., N. Papadopoulos, V. E. Velculescu, S. Zhou, L. A. Diaz, Jr. and K. W. Kinzler (2013). "Cancer genome landscapes." Science **339**(6127): 1546-1558.

Volonte, D., A. R. Vyas, C. Chen, S. Dacic, L. P. Stabile, B. F. Kurland, S. R. Abberbock, T. F. Burns, J. G. Herman, Y. P. Di and F. Galbiati (2018). "Caveolin-1 promotes the tumor suppressor properties of oncogene-induced cellular senescence." J Biol Chem **293**(5): 1794-1809.

Wagner, V. and J. Gil (2018). "An Epigenetic Switch: From Senescent Melanocytes to Malignant Melanoma (and Back)." Cancer Cell **33**(2): 162-163.

Wang, J. Y., L. Jin, X. G. Yan, S. Sherwin, M. Farrelly, Y. Y. Zhang, F. Liu, C. Y. Wang, S. T. Guo, H. Yari, T. La, J. McFarlane, F. X. Lei, H. Tabatabaee, J. Z. Chen, A. Croft, C. C. Jiang and X. D. Zhang (2016). "Reactive Oxygen Species Dictate the Apoptotic Response of Melanoma Cells to TH588." J Invest Dermatol **136**(11): 2277-2286.

Wang, J. Y., G. Z. Liu, J. S. Wilmott, T. La, Y. C. Feng, H. Yari, X. G. Yan, R. F. Thorne, R. A. Scolyer, X. D. Zhang and L. Jin (2017). "Skp2-mediated stabilization of MTH1 promotes survival of melanoma cells upon oxidative stress." Cancer Res.

Wang, J. Y., G. Z. Liu, J. S. Wilmott, T. La, Y. C. Feng, H. Yari, X. G. Yan, R. F. Thorne, R. A. Scolyer, X. D. Zhang and L. Jin (2017). "Skp2-Mediated Stabilization of MTH1 Promotes Survival of Melanoma Cells upon Oxidative Stress." Cancer Res **77**(22): 6226-6239.

Wang, Z., N. Wang, P. Liu, F. Peng, H. Tang, Q. Chen, R. Xu, Y. Dai, Y. Lin, X. Xie, C. Peng and H. Situ (2015). "Caveolin-1, a stress-related oncotarget, in drug resistance." Oncotarget **6**(35): 37135-37150.

Waris, G. and H. Ahsan (2006). "Reactive oxygen species: role in the development of cancer and various chronic conditions." J Carcinog **5**: 14.

Warpman Berglund, U., K. Sanjiv, H. Gad, C. Kalderen, T. Koolmeister, T. Pham, C. Gokturk, R. Jafari, G. Maddalo, B. Seashore-Ludlow, A. Chernobrovkin, A. Manoilov, I. S. Pateras, A. Rasti, A. S. Jemth, I. Almlof, O. Loseva, T. Visnes, B. O. Einarsdottir, F. Z. Gaugaz, A. Saleh, B. Platzack, O. A. Wallner, K. S. Vallin, M. Henriksson, P. Wakchaure, S. Borhade, P. Herr, Y. Kallberg, P. Baranczewski, E. J. Homan, E. Wiita, V. Nagpal, T. Meijer, N. Schipper, S. G. Rudd, L. Brautigam, A. Lindqvist, A. Filppula, T. C. Lee, P. Artursson, J. A. Nilsson, V. G. Gorgoulis, J. Lehtio, R. A. Zubarev, M. Scobie and T. Helleday (2016). "Validation and development of MTH1 inhibitors for treatment of cancer." Ann Oncol **27**(12): 2275-2283.

Weiss, S. A., J. D. Wolchok and M. Sznol (2019). "Immunotherapy of Melanoma: Facts and Hopes." Clin Cancer Res **25**(17): 5191-5201.

Werner, H., R. Sarfstein and I. Bruchim (2019). "Investigational IGF1R inhibitors in early stage clinical trials for cancer therapy." Expert Opin Investig Drugs **28**(12): 1101-1112.

Wilczak, W., S. Rashed, C. Hube-Magg, M. Kluth, R. Simon, F. Buscheck, T. S. Clauditz, K. Grupp, S. Minner, M. C. Tsourlakis, C. Moller-Koop, M. Graefen, M. Adam, A. Haese, C. Wittmer, G. Sauter, J. R. Izbicki, H. Huland, T. Schlomm, S. Steurer, T. Krech and P. Lebok (2017). "Up-regulation of mismatch repair genes MSH6, PMS2 and MLH1 parallels development of genetic instability and is linked to tumor aggressiveness and early PSA recurrence in prostate cancer." Carcinogenesis **38**(1): 19-27.

Wittgen, H. G. and L. C. van Kempen (2007). "Reactive oxygen species in melanoma and its therapeutic implications." Melanoma Res **17**(6): 400-409.

Wu, Y., J. Xu, C. Du, Y. Wu, D. Xia, W. Lv and J. Hu (2019). "The Predictive Value of Tumor Mutation Burden on Efficacy of Immune Checkpoint Inhibitors in Cancers: A Systematic Review and Meta-Analysis." Front Oncol **9**: 1161.

Xin, H., M. Lei, Z. Zhang, J. Li, H. Zhang, X. Luo, A. Wang and F. Deng (2018). "Suppression of IGF1R in Melanoma Cells by an Adenovirus-Mediated One-Step Knockdown System." Mol Ther Nucleic Acids **12**: 861-871.

Yamaguchi, H., S. S. Chang, J. L. Hsu and M. C. Hung (2014). "Signaling cross-talk in the resistance to HER family receptor targeted therapy." Oncogene **33**(9): 1073-1081.

Yan, C., Q. Yang, D. Do, D. C. Brunson and D. M. Langenau (2019). "Adult immune compromised zebrafish for xenograft cell transplantation studies." EBioMedicine **47**: 24-26.

Yan, Y., C. Robert, J. Larkin, P. A. Ascierto, B. Dreno, M. Maio, C. Garbe, P. B. Chapman, J. A. Sosman, M. J. Wongchenko, J. J. Hsu, I. Chang, I. Caro, I. Rooney, G. McArthur and A. Ribas (2016). "Genomic features of complete responders (CR) versus fast progressors (PD) in patients with BRAFV600-mutated metastatic melanoma treated with cobimetinib + vemurafenib or vemurafenib alone." Annals of Oncology **27**: vi379.

Zaretsky, J. M., A. Garcia-Diaz, D. S. Shin, H. Escuin-Ordinas, W. Hugo, S. Hu-Lieskovan, D. Y. Torrejon, G. Abril-Rodriguez, S. Sandoval, L. Barthly, J. Saco, B. Homet Moreno, R. Mezzadra, B. Chmielowski, K. Ruchalski, I. P. Shintaku, P. J. Sanchez, C. Puig-Saus, G. Cherry, E. Seja, X. Kong, J. Pang, B. Berent-Maoz, B. Comin-Anduix, T. G. Graeber, P. C. Tumeh, T. N. Schumacher, R. S. Lo and A. Ribas (2016). "Mutations Associated with Acquired Resistance to PD-1 Blockade in Melanoma." N Engl J Med **375**(9): 819-829.

Zhang, K., P. Wong, J. Duan, B. Jacobs, E. C. Borden and B. Bedogni (2013). "An ERBB3/ERBB2 oncogenic unit plays a key role in NRG1 signaling and melanoma cell growth and survival." Pigment Cell Melanoma Res **26**(3): 408-414.

Zhang, Z., J. C. Lee, L. Lin, V. Olivas, V. Au, T. LaFramboise, M. Abdel-Rahman, X. Wang, A. D. Levine, J. K. Rho, Y. J. Choi, C. M. Choi, S. W. Kim, S. J. Jang, Y. S. Park, W. S. Kim, D. H. Lee, J. S. Lee, V. A. Miller, M. Arcila, M. Ladanyi, P. Moonsamy, C. Sawyers, T. J. Boggon, P. C. Ma, C. Costa, M. Taron, R. Rosell, B. Halmos and T. G. Bivona (2012). "Activation of the AXL kinase causes resistance to EGFR-targeted therapy in lung cancer." Nat Genet **44**(8): 852-860.

Zhao, C., S. Li, M. Zhao, H. Zhu and X. Zhu (2018). "Prognostic values of DNA mismatch repair genes in ovarian cancer patients treated with platinum-based chemotherapy." Arch Gynecol Obstet **297**(1): 153-159.

Zhao, S., J. Huang and J. Ye (2015). "A fresh look at zebrafish from the perspective of cancer research." J Exp Clin Cancer Res **34**: 80.

Zhou, W., L. Ma, J. Yang, H. Qiao, L. Li, Q. Guo, J. Ma, L. Zhao, J. Wang, G. Jiang, X. Wan, M. Adam Goscinski, L. Ding, Y. Zheng, W. Li, H. Liu, Z. Suo and W. Zhao (2019). "Potent and specific MTH1 inhibitors targeting gastric cancer." Cell Death Dis **10**(6): 434.