The Novel Roles of MAP Kinase-Interacting Serine/Threonine-Protein Kinase 1 (MNK1) in Melanoma Progression

Elie Khoury

Experimental Medicine

McGill University, Montreal

December 2019

A thesis submitted to McGill University in fulfillment of the requirements of the degree of Master of Science.

Table of contents

Abstract

In cutaneous melanoma, two of the signaling pathways that are most frequently dysregulated are the MAPK and PI3K-AKT pathways. The most common mutation in cutaneous melanoma is *BRAF*^{V600E}, which results in 500-fold activation of the MAPK pathway. Studying how the MAPK and PI3K pathways converge and cross-talk can lead us to further understand melanoma progression and develop novel therapeutics. Downstream of the MAPK pathway we find the mitogen-activated protein kinase (MAPK) interacting protein kinases (MNK1/2). MNK1/2 has many roles, but arguably its best studied role is its ability to phosphorylate the eukaryotic translation initiation factor 4E (eIF4E) at serine 209. The phosphorylation of eIF4E on serine 209 leads to the selective translation of pro-tumourigenic and pro-invasive mRNAs, and is thus essential for tumourigenesis and cancer progression. MNK1/2 is known to shuttle into and out of the nucleus, however little is described about the nuclear functions of MNK1. The impact of MNK1 on the regulation of gene transcription, for example, remains unknown. In our research we showed that cells expressing a constitutively active form of MNK1 resulted in an increase in the expression of mRNAs that code for proteins responsible for invasion, tumourigenesis, and proliferation. Conversely, cells devoid of MNK1, generated using CRISPR/Cas9, showed less expression of these same MNK1-regulated target genes. One of the most upregulated genes in cells expressing the constitutively active MNK1, was angiopoetin-like 4 (*ANGPTL4*). Cells that were devoid of MNK1 expressed less ANGPTL4 than their wild-type counterparts. Overexpression of ANGPTL4 in melanoma cells leads to a more invasive phenotype via the upregulation of matrix metalloproteinases (MMPs). Melanoma cell invasion and MMP9 levels, and activity, were decreased with the knockdown of *ANGPTL4*. *In vivo* studies using syngeneic mouse models of melanoma, showed that cells devoid of MNK1 resulted in smaller tumors and a decrease in lung

metastases. The MNK1/2 inhibitor SEL201 was shown to be able to recapitulate the *in vitro* and *in vivo* results that were obtained using cells devoid of MNK1. Finally, when we interrogated publicly available patient data, we observed a correlation between the expression of *MKNK1* (MNK1 gene name) and *ANGPTL4* in $BRAF^{V600E}$ primary melanomas.

Résumé

Dans le mélanome cutané, il y a deux voies de signalisation qui sont le plus souvent dérégulées; la voie de signalisation MAPK et la voie de signalisation PI3K-AKT. La mutation la plus commune dans le mélanome cutané est $BRAF^{V600E}$ qui résulte en une augmentation de l'activation catalytique de 500 fois de la voie de signalisation MAPK. En étudiant comment ces deux voies de signalisations convergent et communiquent peut nous mener à mieux comprendre la progression du mélanome et le développement de nouveaux thérapeutiques. En aval de la voie MAPK, nous trouvons la protéine mitogen-activated protein kinase (MAPK) interacting protein kinase (MNK1/2). MNK1/2 joue plusieurs rôles, cependant son rôle le plus étudié est son habilité à phosphoryler le eukaryotic translation initiation factor 4E (eIF4E) sur la sérine 209. La phosphorylation de eIF4E sur la sérine 209 mène à une traduction sélective d'ARNm pro-tumorigènes et pro-invasifs, ce qui justifie pourquoi MNK1/2 est essentiel pour la progression du cancer en général. MNK1/2 est aussi connu pour le sa localisation nucléaire, cependant, très peu est décrit sur ses autres fonctions nucléaires. L'impact que MNK1 joue sur la régulation de la transcription de gènes par exemple, est encore inconnu. Dans notre recherche, nous montrons que les cellules qui expriment une forme constitutivement active de MNK1 résulte démontre une augmentation dans l'expression d'ARNm qui codent pour des protéines responsables pour l'invasion, la prolifération et la progression du cancer. Au contraire, les cellules sans MNK1 générées par CRISPR/Cas9 montrent une diminution de l'expression de ces gènes réguler par MNK1. Un des gènes des plus positivement régulés dans les cellules avec MNK1 constitutivement actif qui était très intéressant est angiopoetin-like 4 (ANGPTL4). Les cellules sans la présence de MNK1 expriment moins de ANGPTL4 comparativement aux cellules contrôles. La surexpression de ANGPTL4 dans les cellules du mélanome montre un phénotype plus invasif via la régulation

positive des matrices métalloprotéinases (MMPs). L'invasion des cellules et les niveaux et l'activité de MMP9 sont diminués avec le knockdown de ANGPTL4. Dans les études *in vivo,* nous utilisons un modèle murin syngénique du mélanome qui montre que les cellules sans MNK1 mènent à des tumeurs plus petites et une diminution du nombre de métastases dans les poumons. Nous étions en mesure de répliquer les résultats obtenus dans les cellules sans MNK1 en utilisant un inhibiteur pharmacologique de MNK1/2, SEL201. Finalement, nous avons analysé des données disponibles publiquement de patients atteints de mélanome. Nous observons une forte corrélation dans l'expression de *MKNK1* (nom du gène de MNK1) et *ANGPTL4* dans les tumeurs primaires ayant une mutation somatique $BRAF^{V600E}$.

List of abbreviations

4E-BPs- Eukaryotic translation initiation factor 4E binding proteins

α-MSH Alpha-melanocyte–

stimulating hormone

AKT- Protein kinase B

ALM- Acral lentiginous melanoma

AM- Amelanotic melanoma

AML- Acute myeloid leukemia

ANGPTL4- Angiopoetin-like 4

BAD- Bcl-2-associated death promoter

BCL-2- B-cell lymphoma 2

BIRC2- Baculoviral IAP Repeat Containing 2

BRAF- Raf murine sarcoma viral oncogene homolog B

CCL2-7- C-C Motif Chemokine Ligand 2

CDK4- Cyclin-dependent kinase 4

CDKN2A- Cyclin-dependent kinase Inhibitor 2A

cKit -kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog

c-MYC -myc avian myelocytomatosis viral oncogene homolog

CTLA-4- Cytotoxic T lymphocyte antigen-4

DLBCL- Diffuse large B-cell lymphoma

DNA- Deoxyribonucleic acid

DM- Desmoplastic melanoma

EGFR- Epidermal growth factor receptor

eIF4E- Eukaryotic translation initiation factor 4E

eIF4F- Eukaryotic translation initiation factor 4F

ERK- Extracellular signal-regulated kinase

GAB1- GRB2-associated-binding protein 1

GAPDH- Glyceraldehyde-3-phosphate dehydrogenase

GDP- Guanosine diphosphate

GTP- Guanosine triphosphate

HNSCC- Head and neck squamous cell carcinoma

HnRNP A1- Heterogeneous Nuclear Ribonucleoprotein A1

KIT- tyrosine-protein kinase KIT

LMM- Lentigo maligna melanoma

MAPK- Mitogen- activated protein kinases

MC1R- Melanocortin 1 receptor

MCL-1- Induced myeloid leukemia cell differentiation protein Mcl-1

MEK Mitogen- activated protein/extracellular signal-regulated kinase kinase

MITF Microphthalmia-associated transcription factor

MMPs- Matrix metalloproteinases

MNKs- Mitogen-activated protein kinase (MAPK)-interacting kinases

mRNA- Messenger Ribonucleic Acid

mTOR- Mammalian target of rapamycin

NF-1 Neurofibromin 1

NM- Nodular melanoma

NRAS Neuroblastoma RAS viral (v-ras) oncogene homolog

p16INK4A- cyclin-dependent kinase inhibitor 2A, multiple tumor suppressor 1

p38 MAPK Mitogen-activated protein kinase 14, MAPK14

PD-1 Programmed death 1

PD-L1 Programmed death-ligand 1

PSF- PTB-associated splicing factor

PI3K- Phosphoinositide 3-kinase

PIP2- Phosphatidylinositol diphosphate

PIP3- Phosphatidylinositol triphosphate

PTEN- Phosphatase and tensin homologue

Rb- Retinoblastoma

RAF- Rapidly Accelerated Fibrosarcoma kinase

ROS- Reactive oxygen species

RTKs- Receptor tyrosine kinases

SSM- Superficial spreading melanoma

TGFβ- Transforming growth factor beta

TNFα- Tumor necrosis factor alpha

TP53- Tumor protein p53

TSC2- Tuberous sclerosis complex 2

UVR- Ultraviolet radiation

VEGF- Vascular endothelial growth factor

WHO- World health organization.

Acknowledgments

Having the experience to obtain a Master's degree from McGill at the Lady Davis Institute is something out of this world. Obtaining this degree in the Miller/Del Rincon lab has taught me many things. I have learned to be more patient, resilient and a lot more passionate for my work. Working in such an amazing and supportive environment helped me develop many scientific techniques and critical thinking.

I would first like to start by thanking both my supervisors Dr. Sonia del Rincon and Dr. Wilson H. Miller for giving me the opportunity to embark on this wonderful journey. I would also thank them for their guidance and time they invested in me.

I would like to give a special and warm thank you to Dr. Sonia del Rincon. The time and energy she spent into my degree is immense and greatly appreciated. She has always been there whenever I had any questions about my project, science in general or even just life advice. She always pushed me to question my research and nudged me in the right direction with questions that kept my mind awake and brought me back on track when I got lost. I want to thank her for her patience with me throughout my Master's degree. Without her, all this work would not have been possible.

I would also like to thank all the lab members from the del Rincon/Miller/Mann lab that supported me through my Masters. I want to single out Michael Dahabieh that was always a brotherly and parental type of figure around me in the lab, challenged my ideas, kept me on track and helped me whenever I needed guidance. I also want to thank Vivienne, Sam, Fan, Sath and Saktee for their help, their support and their friendship during my time in the lab. I would like to thank especially William, an old Master's student that mentored me while I was doing my internship in the lab, helped me do some of my experiments and guided my scientific thinking. I

also want to thank Christophe who was always willing to help guide me through new experiments and techniques as well as helping me optimize some protocols. I can't forget Audrey and Dany that helped with bioinformatic analysis and animal work. I would also thank Dr. Koren Mann and all the members in her lab for their feedback whenever we discussed my project and all the laughs we shared while working near each other.

I could not have done all this work if it wasn't for my family and their constant support in all aspects as well as my friends that helped me whenever I was feeling down.

I would finally like to thank all the members on my committee. All the feedback they gave me on my work got me where I am now. I would like to thank Dr. Stephanie Lehoux, Dr. Nathalie Lamarche-Vanne and Dr. Kostas Pantopoulos for their guidance.

Contribution of authors

The overwhelming majority of the experiments were conducted and designed by the Master's student. However, some parts were done in collaboration with other colleagues:

- Figure 2.4.1: B, F-G was done with contributions and help from William Yang.
- All the rest is the work of Elie Khoury.

1. Introduction

1.1 Clinical relevance of melanoma

1.1.1 Introduction to melanoma etiology and risk factors

Melanoma's incidence worldwide has been rapidly increasing through the years; in fact, it is one of the fastest growing cancers (1). The melanoma prevalence growth rate is mostly seen in developed countries, such as the United States, Canada and most notably Australia (1). Although melanoma is one of the rarest types of skin cancer, accounting for only around 1% of skin cancers, it is paradoxically the leading cause of death in all skin cancers (2).

Melanoma is a cancer that arises in the pigment-producing melanocytes (3). These melanocytes are usually found in the epidermis of the skin; they produce melanin which serves to protect the DNA from being damaged by UVR (3). Melanocytes are also present in multiple tissues of the body such as the eyes, gastrointestinal mucosa, genital mucosa, inner ear, meninges, heart and hair bulbs. Melanoma that arises in those types of tissues can have different genetic signatures, which incidentally increases the complexity of this already complex disease.

The most important and dangerous risk factor for the development of cutaneous melanoma is UVR. Many epidemiological studies show the significant effect of UVR on the skin in the development of this deadly cancer. Clinical and epidemiologic evidence show a higher incidence of melanoma in people with repeated exposure to sunlight (4). Phenotypic traits such as family history, number of nevi (moles), age, colour of the skin and even being male are some of the many risk factors associated with melanoma development (5).

1.1.2 Epidemiology and in-depth look at factors

Melanoma is the fifth leading cancer in the United States in both men and women (6). Supporting the claim that fairness of the skin is a major factor in the development of melanoma, incidence in Caucasian Europe jumped from 3.0 cases/100,000 inhabitants per year in the 1970s to around 15.0 cases/100,000 inhabitants per year in the early 2000s. When comparing these statistics to the rest of the world, we see a major difference in rate of change, wherein the incidence went from 2.2 cases/100,000 inhabitants per year to just 3.0 cases/100,000 inhabitants per year in the same time-lapse (6). The incidence of melanoma in Queensland, Australia at 72.0 cases/100,000 inhabitants per year, is the highest in the world. **Figure 1** supports the claim that mainly Caucasian countries have a higher incidence of melanoma. Fairness of the skin causes higher UV sensitivity which leads to an increase in melanoma (7). Conversely, countries with inhabitants having darker skin seem to be more resistant to melanoma (7).

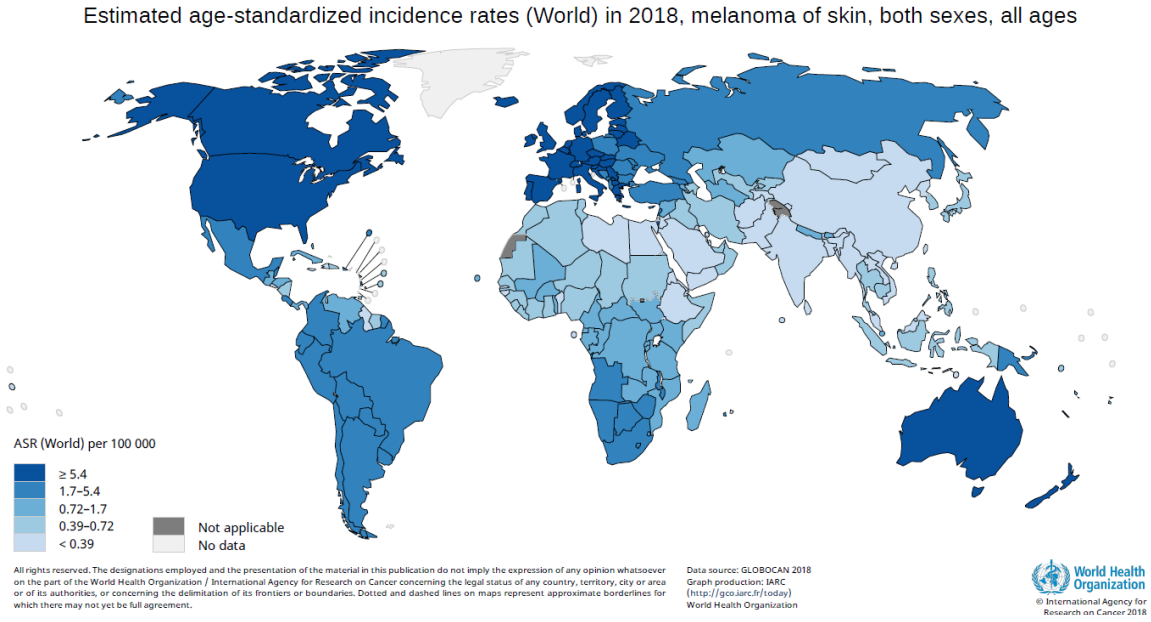


Figure 1. Prevalence of melanoma in the world. Highest melanoma prevalence is seen in developed countries.

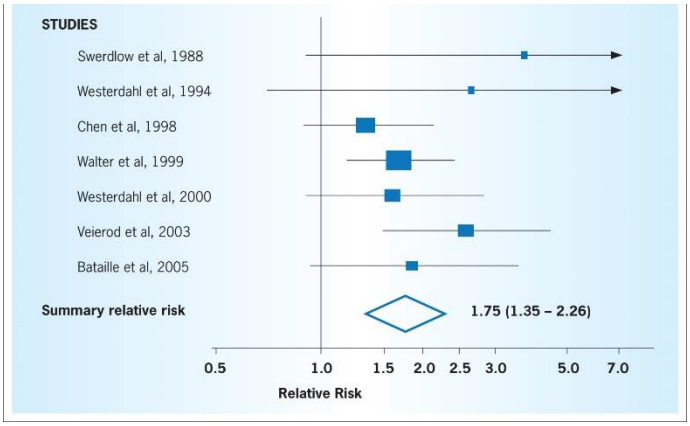
Generated from the data available from World Health Organisation (2019)

Melanin or eumelanin is produced by the melanocytes under UVR, more specifically UVA and UVB. Harmful UVR penetrates the epidermis to the dermis and causes DNA-damaging ROS (8). Eumelanin acts as a barrier to protect the skin by absorbing the harmful radiation. Eumelanin also plays a role in the scavenging of ROS. This, in turn, prevents ROS-mediated DNA damage (8). One of the key regulators of pigmentation is MC1R. When melanocytes are exposed to UVR, they produce melanocortin peptides which bind to MC1R and lead to the production of melanin through the upregulation of MITF (9). In people with fair skin, a mutation occurs in the *MC1R* gene which leads to the production of a type of melanin called pheomelanin. Pheomelanin is a subtype of melanin that leads to fairer skin and red hair. This subtype is weaker than eumelanin in the protection for UVR and in the scavenging of ROS (10). Eumelanin, on the other hand, leads to darker skin and affords stronger protection to DNA from UVR. Higher *MC1R* leads to a higher eumelanin/pheomelanin ratio (10). Independent of UVR, mutation in *MC1R*, leads to increased melanoma incidence. This is due to less scavenging of ROS by eumelanin (11). The genetic component of melanoma is not to be underestimated. Around 10% of melanomas occur in patients with a family history (10). Studies have also shown that within the same living environments, people with fairer skin generally sustain a higher rate of melanoma. Even though melanoma incidence seems to be growing uncontrollably, this disease is preventable. The genetic aspect of it cannot be obviated, but the environmental aspect is controllable.

Tanning and sunbathing is an ever-growing activity in western culture (12). These activities – if not protected by sunscreen – lead to an increase risk of developing melanoma. Lifestyle and social status dictate our day-to-day activities. People with a higher socio-economic status tend to be more prone to melanoma due to more solar exposure (13). Those factors are preventable and, when curtailed conscientiously, greatly decrease the risk of melanoma. An epidemiological study conducted by the World Health Organization shows that removing UVR as a risk factor yields a 93% decrease in the incidence of melanoma (14). To that effect, UVR is a very controllable aspect of everyday life; sunscreen and clothing are some of the

Figure 2. Relative risk of melanoma. Studies detailing the relative risk of melanoma incidence

Adapted from Heckman *et.* al (2012) (12)



Estimated age-standardized incidence rates (World) in 2018, melanoma of skin, all ages

	Males	Females
	Incidence	Incidence
Oceania	33.8	23.5
North America	14.7	11.1
Europe	11.5	11.3
World	3.5	2.9
Latin America and the Caribbean	2.5	2.3
Africa	0.86	0.93
Asia	0.43	0.39

Figure 3. Incidence of melanoma in populations. Oceania and North America show more incidence of melanoma compared to countries with darker skin.

Generated from the data available from the World Health Organization (2019)

examples which can lower exposure to UVR. In fact, 50-90% of all melanomas can be prevented with the reduction of exposure to UVR (14).

That said, there is also a genetic component to melanoma. Namely, men are usually more prone to developing melanoma (15). Melanoma is also more prevalent in people with a family history. In fact, they are two to three times as likely to develop melanoma than people with no family history of melanoma (15). Another factor that comes into play is the state of the immune system (15). With a suppressed immune system, due to medication or disease, melanoma is more prone

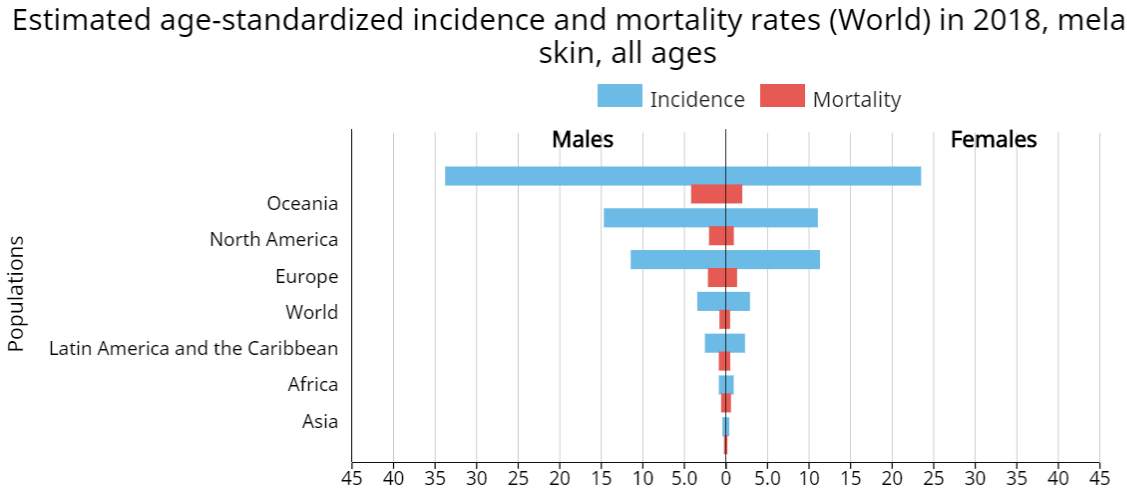


Figure 4. Incidence and mortality of melanoma in populations. Men in Oceania and North America show more incidence and mortality of melanoma compared to females. Generated from the data available from the World Health Organization (2019).

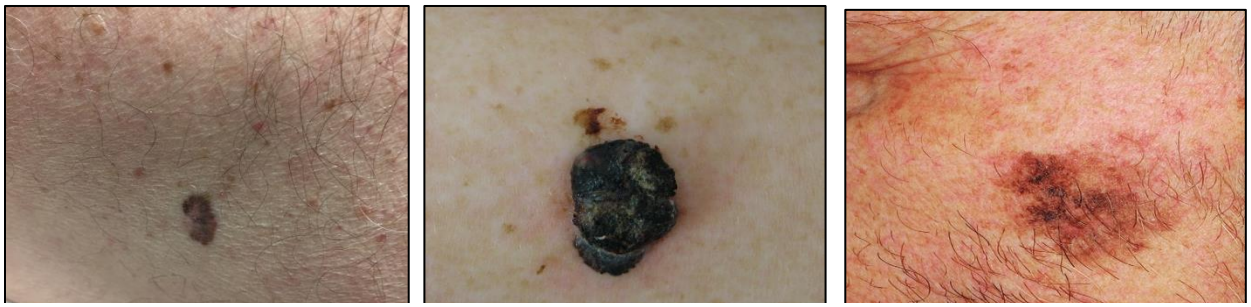
to occur (15). Mutations also occur more frequently with age, such that age is another important factor for melanoma (15).

What we can conclude from the studies conducted on the risk factors of melanoma is that the most dangerous risk factor, is also the most preventable. Less UVR exposure is directly linked to a better chance of not developing melanoma.

1.1.3 Subtypes of melanoma

Cutaneous melanoma is divided in four main subtypes and two other rarer subtypes: superficial spreading melanoma (SSM), which accounts for around 70% of melanomas, nodular melanoma (NM), which represents 5%, lentigo maligna melanoma (LMM), which hovers between 4-15% of melanomas, acral lentiginous melanoma (ALM), around 5%, amelanotic melanoma (AM) and much rarer, desmoplastic melanoma (DM) at less than 4% (16, 17).

Figure 5. Subtypes of melanoma



Superficial spreading melanoma Nodular melanoma Lentigo maligna melanoma

1.1.4 Detection of melanoma

Melanoma is the most aggressive type of skin cancer, able to quickly spread to distant sites in the body, which is why early detection is a very important factor in overall survival. Many visual aspects of a nevus can help in assessing the melanoma risk (18). In North America, what is usually employed are the ABCDEs of melanoma, an acronym that represents Asymmetry, Border, Color, Diameter and Evolution (18). More specifically:

- **A**symmetry: Regular benign moles are usually symmetrical. If one is irregular/asymmetric, it is cause for closer attention to that specific mole.
- **B**order: Contour of the mole (definition of the border, smoothness etc.) If the border is rugged, it is cause for closer attention to that specific mole.

- **C**olor: A uniform mole is a good sign. Uneven colours or different shades are a sign of melanoma.
- **D**iameter: A diameter over 6mm.
- **E**volution: Any change in ABCD over time.

Having one or more of the ABCDE criteria does not guarantee detection of melanoma, but it gives a clearer idea and can help with early detection (18). On the other hand, moles can also be malignant even if they do not fit the ABCDE criterion (19). Dermatologists can screen melanoma much more efficiently using dermascopes, which act as microscopes without removing the lesion (19). A closer look at the suspicious nevus shows more definitely whether the mole is benign or malignant (19). In cases where the mole is deemed malignant or suspicious, a biopsy is performed to confirm the initial diagnosis (20).

While early detection is a crucial factor in patient survival, early screening has not been shown to help to reduce mortality from that deadly cancer (21). In fact, early screening leads to more unnecessary biopsies, more anxiety in patients, overtreatment and often misdiagnosis (22).

1.1.5 Progression of melanoma

Melanoma is classified from stage 0 (benign nevus) to stage IV (distant metastasis) (23).

- Stage I: Very low risk. No metastasis.
- Stage II: High risk of recurrence. No metastasis.
- Stage III: Regional metastasis. Lymph node metastasis.
- Stage IV: Distant metastases.

Melanoma stages can also be subclassified under the TNM staging system which give more detail on the progression of the disease: the primary tumour (T), the regional lymph nodes (N) and the distant metastases (M) (23). Table 1, 2, and 3 illustrate in further detail the subdivisions mentioned earlier.

N	No. of Metastatic Nodes	Nodal Metastatic Burden
N0	0	NA
N1	1	a: Micrometastasis*
		b: Macrometastasis†
N2	2-3	a: Micrometastasis*
		b: Macrometastasis†
		c: In transit metastases/satellites without metastatic nodes
N3	4+ metastatic nodes, or matted nodes, or in transit metastases/satellites with metastatic nodes	

Table 1. TNM staging for superficial melanoma. Number of metastatic nodes and size of the metastatic burden.

Adaptated from Bech *et.* al (2012) (23)

Classification		Thickness (mm)	Ulceration Status/Mitoses
T			
	Tis	NA	NA
	T1	≤ 1.00	a: Without ulceration and mitosis < 1/mm^2
			b: With ulceration or mitoses ≥ 1/mm^2
	T2	1.01-2.00	a: Without ulceration
			b: With ulceration
	T3	2.01-4.00	a: Without ulceration
			b: With ulceration
	T4	> 4.00	a: Without ulceration
			b: With ulceration

Table 2. TNM staging for superficial melanoma. Size of the primary tumour (T) and location of the metastases (M)

Adaptated from Bech *et.* al (2012) (23)

M	Site	Serum LDH
M0	No distant metastases	NA
M1a	Distant skin, subcutaneous, or nodal metastases	Normal
M1b	Lung metastases	Normal
M1c	All other visceral metastases	Normal
	Any distant metastasis	Elevated

	Clinical Staging*				Pathologic Staging†				Clinical Staging*				Pathologic Staging†		
	T	N	M		T	N	M		T	N	M		T	N	M
0	Tis	N0	M0	0	Tis	N0	M0								
IA	T1a	N0	M0	IA	T1a	N0	M0								
IB	T1b	N0	M0	IB	T1b	N0	M0					IIIB	T1-4b	N1a	M0
	T2a	N0	M0		T2a	N0	M0						T1-4b	N2a	M0
IIA	T2b	N0	M0	IIA	T2b	N0	M0						T1-4a	N1b	M0
	T3a	N0	M0		T3a	N0	M0						T1-4a	N2b	M0
IIB	T3b	N0	M0	IIB	T3b	N0	M0						T1-4a	N2c	M0
	T4a	N0	M0		T4a	N0	M0					IIIC	T1-4b	N1b	M0
IIC	T4b	N0	M0	IIC	T4b	N0	M0						T1-4b	N2b	M0
III	Any T	N > N0	M0	IIIA	T1-4a	N1a	M0						T1-4b	N2c	M0
					T1-4a	N2a	M0						Any T	N3	M0
								IV	Any T	Any N	M1	IV	Any T	Any N	M1

Table 3. TNM staging for superficial melanoma. Seven stages of melanoma progression (T; primary tumour, N; number of metastases, M; location of distant metastases)

Adaptated from Bech *et.* al (2012) (23)

1.1.6 Genetic subtypes of melanoma

Melanoma is one of the best models to study how multiple mutations can transition a benign nevus from non-malignant to malignant and highly invasive melanoma (24). In fact, melanoma is the cancer with the most mutations due to the UVR damage (24). Although there are many mutations in melanoma, the majority are non-tumourigenic, thereby generating the puzzle for experts to determine which genes are drivers of melanoma and which ones are not (24).

The development of the benign nevus is the first step towards invasive melanoma (25). For the first step to happen, a mutation in *NRAS* or *BRAF* usually occurs, leading to an overactivation of the mitogen-activated protein kinase (MAPK) pathway (25). Mutations in *NRAS* occur in 20% of melanoma patients, while *BRAF* mutations arise in more than 50% of patients (25). The most common *BRAF* mutation, accounting for around 90%, is the $BRAF^{V600E}$ mutation.

This mutation leads to the overactivation of MAPK by around 500-fold (25). Overactivation of this pathway alone leads to oncogene-induced senescence. Therefore, single mutations in either of those genes can only produce a benign nevus (25).

For a benign nevus to transition to malignant melanoma, a dysplastic nevus is the next step. Beyond the BRAF mutation, a second genetic defect needs to appear, and this defect is usually seen in the loss-of-function mutations of *CDKN2A, PTEN* or *TP53* (26). *CDKN2A* encodes for two important tumour suppressors, $p16^{INK4A}$ and $p19^{ARF}$ (26). $p16^{INK4A}$ is a known inhibitor of the CDK4 kinase which plays an important role in cell cycle signaling. An increase in CDK4 leads to an increase in phosphorylation of Rb, a tumour suppressor, which leads to its inactivation. This, in turn, facilitates the transition of cell cycling and an increase in proliferation. $p19^{ARF}$ also plays a major role in the cell cycle; it is a regulator of the G2/M checkpoint and it manages the p53 pathway. $p19^{ARF}$, in turn, assures that cells with DNA damage undergo apoptosis instead of continuing mitosis (27). *PTEN* is a tumour suppressor gene that acts on the PI3K-AKT pathway. PTEN loss leads to an overactivation of this pathway by not dephosphorylating phosphatidylinositol (3,4,5)-trisphosphate (PIP3), which leads to an increase in the AKT activity (28).

The *NRAS* mutation is another important subtype of melanoma. *NRAS* mutations are predominantly Q61K or Q61R (70%) (29). Those mutations lead to an overactivation of both the RAF-MEK-ERK pathway and the PI3K-AKT pathway (29). Mutations that usually are concurrent are the $p16^{INK4A}$ mutation, TP53 and an AKT overactivation overall (29). Mutations in *BRAF* and *NRAS* are generally mutually exclusive in cutaneous melanoma.

A third subtype of melanoma is the *NF1*-mutant melanoma. *NF1* mutations represent around 10-15% of all melanomas (30). *NF1* is a tumour suppressor gene which encodes a RAS

GTPase activation protein, which negatively regulates RAS overactivation by preventing RAS GDP from becoming the active RAS GTP (30). Mutation of NF1 leads to an overactivation of both pathways downstream of RAS, RAF-MEK-ERK and PI3K-AKT (30).

The fourth subtype of melanoma is the triple wild-type melanoma, which does not harbour a *BRAF*, *NRAS* or *NF1* mutation (31). Triple wild-type melanomas generally harbour mutations in *KIT* (22%), which encodes for a receptor tyrosine kinase (RTK) (32). *KIT* mutations most often occur in acral and mucosal melanoma (32). Constitutive activation of C-KIT via a *KIT* mutation leads to a downstream upregulation of the RAF-MEK-ERK MAPK pathway and the PI3K-AKT-mTOR pathway (32). *KIT* mutations are usually mutually exclusive with the other melanoma subtypes, such as *BRAF* and *NRAS* (32). *KIT* melanoma distinguishes itself by a poorer clinical outcome than its other melanoma mutation counterparts (33).

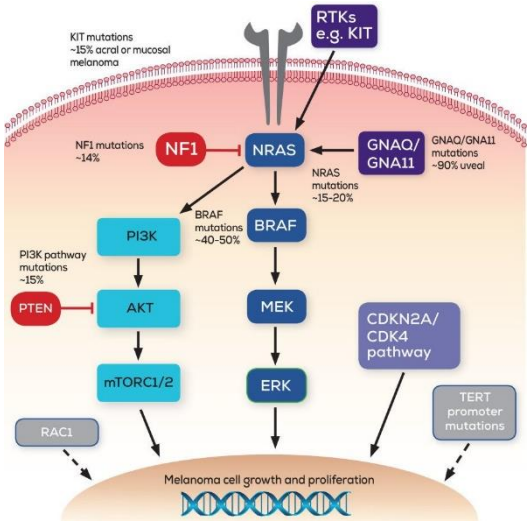


Figure 6. Mutations in melanoma. The MAPK pathway and its link to the PI3K-AKT pathway show the major mutations in melanoma such as NRAS, BRAF and KIT.

Adapted from Davis *et* al. (2018) (24)

Mutation Subtypes	*BRAF*	*RAS*	*NF1*	Triple Wild-Type
[1]MAPK pathway	[1]*BRAF* V600, K601	[1]*(N/H/K) RAS* G12, G13, Q61	[1]*NF1* LoF mut; (*BRAF* non-hot-spot mut)	[1]*KIT* COSMIC mut/amp, *PDGFRa* amp, *KDR* (*VEGFR2*) amp; rare COSMIC *GNA11* mut, *GNAQ* mut
[2]Cell-cycle pathway	*CDKN2A* mut/del/h-meth (~60%); [2](CDK4 COSMIC mut)	*CDKN2A* mut/del/h-meth (~70%); *CCND1* amp (~10%), [2](*CDK4* COSMIC mut)	*CDKN2A* mut/del/h-meth (~70%); *RB1* mut (~10%)	*CDKN2A* mut/del/h-meth (~40%); *CCND1* amp (~10%), [2]*CDK4* amp (15%)
[3]DNA damage response and cell death pathways	*TP53* mut (~10%); [3] (note: *TP53* wild-type in ~90% of *BRAF* subtype)	*TP53* mut (20%)	*TP53* mut (~30%)	[3]*MDM2* amp (~15%); [3]BCL2 upregulation
[4]PI3K/Akt pathway	[4]*PTEN* mut/del (~20%); [4] (rare *AKT1/3* and *PIK3CA* COSMIC mut)	[4]AKT3 overexpression (~40%); [4](rare *AKT1/3* and *PIK3CA* COSMIC mut)	[4]AKT3 overexpression (~30%)	[4]AKT3 overexpression (~20%)
[5]Epigenetics	[5]*IDH1* mut, [5](rare *EZH2* COSMIC mut); [5]*ARID2* mut (~15%)	[5]*IDH1* mut, [5](rare *EZH2* COSMIC mut); [5]*ARID2* mut (~15%)	[5]*IDH1* mut, [5] (*EZH2* mut); [5] *ARID2* mut (~30%)	[5]*IDH1* mut, [5](rare *EZH2* COSMIC mut)
Telomerase pathway	Promoter mut (~75%)	Promoter mut (~70%)	Promoter mut (~85%)	Promoter mut (< 10%); *TERT* amp (~15%)
Other pathways	*PD-L1* amp, *MITF* amp, *PPP6C* mut (~10%)	*PPP6C* mut (~15%)		
[6]High immune infiltration (pathology)	~30%	~25%	~25%	~40%

Table 4. Mutations in melanoma. All the subtypes of melanoma mutations and their incidence in patients.

1.1.7 Melanoma treatments

Melanoma treatments have greatly improved throughout the years. Early excision of the tumour remains the best way to ensure overall survival (34). However, as melanoma progresses, excision of the primary tumour does not remove the entire melanoma and it becomes an unpractical therapeutic tool.

Targeted therapies – mostly focusing upon the MAPK pathway – have been developed in order to combat this. Patients that have *BRAF* mutations have mainly benefited from MAPK-targeted therapies (35).

BRAF inhibitors such as vemurafenib or dabrafenib were developed to treat patients with *BRAF* mutations such as the most prominent $BRAF^{V600E}$. $BRAF^{V600E}$-harbouring patients showed dramatic improved overall survival when treated with vemurafenib or dabrafenib (36). However, resistance is acquired quickly by patients in the first year (36). In combination with MEK inhibitors such as trametinib and cobimetinib, patient survival was greatly improved in comparison to single agent BRAF inhibitors (36). Despite the improvement, resistance is acquired in the majority of patients on BRAF plus MEK inhibitor combination therapy (36). This acquired resistance can be due to a reactivation of the MAPK pathway through BRAF amplification, mutations in *NRAS* or *MAP2K1/2*, or loss of NF1, amongst several other recently described mechanisms (36). In general, targeted therapy greatly improves survival in the short term, but in the long-term, relapse and progression are inevitable which is why combinations with other types of treatments could be essential (35).

One of those other types of treatments that is on the rise is immunotherapy. Malignant melanomas are highly immunogenic, especially melanomas with many mutations. CTLA4, PDL1, and PD1 are receptors known as immune checkpoints that downregulate immune responses (37). PD1 is a cell surface protein that binds to the PDL1, which can be present on tumor cells. The anti-cancer activity of T-cells can be activated by CTLA4 inhibition and PD1/PDL1 inhibition (37). Ipilimumab, which targets CTLA4, shows a 20% survival rate after three years. Tumour cells also very often over-express PDL1 which helps in evading the immune system (38). Nivolumab and pembrolizumab both target PDL1 and help reactivate T-cells (38). PDL1 inhibitors show an overall

survival of 35% after five years (38). Contrary to targeted therapies, immunotherapy seems to have long-term clinical benefits; in fact, around 75% of melanoma patients on combination therapies such as the combination of nivolumab and ipilimumab are still alive after 2 years (39).

Finally, radiotherapy is also sometimes used in the treatment of some melanomas. While melanoma has mostly been shown to be radioresistant, some studies have shown that the combination of targeted therapy and radiotherapy can lead to better outcome (40). Treatment with vemurafenib sensitizes radioresistant cells which leads to this combination being effective in some scenarios (40).

Mutation Subtypes	*BRAF*	*RAS*	*NF1*	Triple Wild-Type
Class 1: Clinically actionable	[3]MDM2/p53 interaction inhibitors			[3]MDM2/p53 interaction inhibitors
	[4]PI3K/Akt/mTOR inhibitors	[4]PI3K/Akt/mTOR inhibitors	[4]PI3K/Akt/mTOR inhibitors	[4]PI3K/Akt/mTOR inhibitors
	[6]immunotherapies (mAb against immune checkpoint proteins, high dose bolus IL-2, interferon-α2b)			
Class 2: Translationally actionable	[1]ERK inhibitors	[1]ERK inhibitors	[1]MEK inhibitors; [1] ERK inhibitors	
	[5]IDH1 inhibitors	[5]IDH1 inhibitors	[5]IDH1 inhibitors	[5]IDH1 inhibitors
	[5]EZH2 inhibitors	[5]EZH2 inhibitors	[5]EZH2 inhibitors	[5]EZH2 inhibitors
	(PPP6C) Aurora kinase inhibitors	(PPP6C) Aurora kinase inhibitors		
Class 3: Pre-clinical	[5]ARID2 chromatin remodelers (synthetic lethality)	[5]ARID2 chromatin remodelers (synthetic lethality)	[5]ARID2 chromatin remodelers (synthetic lethality)	[3](BCL2) BH3 mimemitcs

Table 5. Melanoma treatments. Range of melanoma treatments between class 1 and class 3 depending on the mutation involved.

1.1.8 The RAS-RAF-MEK-ERK MAPK Signaling Pathway

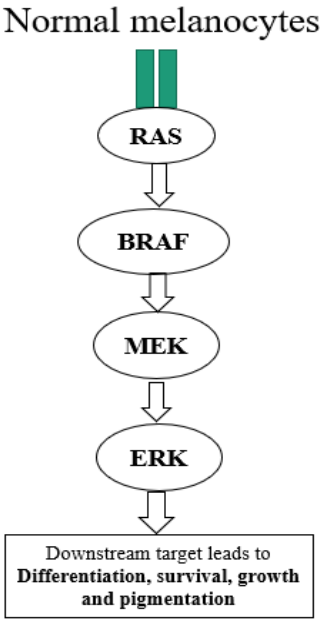


Figure 7. Progression of normal melanocytes

The MAPK pathway is a key pathway in melanoma progression and initiation (25). Normal melanocytes utilize the MAPK pathway for downstream differentiation, survival, growth and pigmentation. In normal MAPK signaling, growth factors bind to the RTK which activates the tyrosine kinase domain in the RTK, which in turn, results in the autophosphorylation and activation of signaling downstream of the RTK (41). SOS (son of sevenless) is a Ras-specific guanine nucleotide exchange factor. When this factor comes in contact with RAS, RAS-GDP, the inactive substrate, is converted to RAS-GTP RAS-GTP will then activate BRAF through phosphorylation. Active BRAF in turn phosphorylates MEK, which further along the signaling cascade will phosphorylate ERK (42). ERK plays multiple roles in the cytoplasm, as well as in the nucleus. In the nucleus, phospho-ERK can phosphorylate transcription factors responsible for normal cell development, proliferation and survival (43). However, an overactivation of this pathway leads to an increase in those transcription factors and potential malignant development. Some of the transcription factors regulated by phospho-ERK are ETS and Myc. The role ERK plays in the cytoplasm is also two-faceted; it can post-translationally modify BAD, MCL-1 and BCL-2 that are proteins responsible for cell apoptosis (44). ERK can also continue the MAPK cascade by phosphorylating the MAP Kinase-Interacting Serine/Threonine-Protein Kinases 1 and 2, MNK1/2 (45). MNK1/2 are the only known kinases for one of their many substrates, eukaryotic translation initiation factor 4E (eIF4E) (45). eIF4E is an important mRNA translation initiation factor responsible for normal protein synthesis, but also responsible for the

translation of many mRNAs that code for proteins having roles in cancer, when its levels and/or phosphorylation become deregulated. In $BRAF^{V600E}$-mutated melanoma, the mutation of a valine to a glutamate (V → E) leads to the constitutive activation of RAF-MEK-ERK signaling (45). This constitutive overactivation leads to uncontrolled cell proliferation and cancer survival (45). Therefore, this pathway is regularly targeted by many inhibitors.

While *BRAF* mutations are very common on their own, NRAS mutations also affect the MAPK signaling. As mentioned earlier, *NRAS* mutations also cause constitutive activation of the MAPK pathway. Furthermore, *NRAS* and *BRAF* mutations are mutually exclusive, which means that patients with a BRAF mutation never harbour an NRAS mutation, and vice-versa (29).

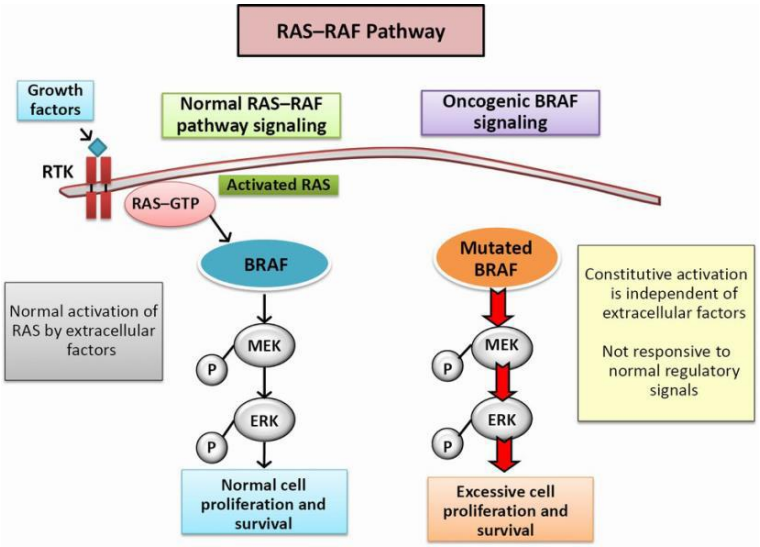


Figure 8. MAPK pathway and its role in melanoma progression. In normal melanocytes, it leads to differentiation, survival, growth and proliferation. With a mutated BRAF that causes constitutive activation, we get an excess of cell proliferation and survival which leads to tumourigenesis.

Adapted from Munoz-Counselo *et* al. (2015) (43)

1.1.9 The PI3K-AKT-mTOR Signaling Pathway

Whilst an overactivation on the MAPK pathway is an important factor for melanoma progression, aberrant MAPK signaling can often cooperate with other signaling pathways. The overactivation of the PI3K-AKT pathway is the second ¨hit¨ required for melanoma development, progression, and resistance to therapy in some cases (46). PI3K-AKT signaling can be activated via one of two avenues: either by regular growth factors that bind to RTKs or by cross-talk with the MAPK pathway by the intermediary RAS (46). PI3K is divided in two smaller subunits, p110 and p85 (46). p110 acts as the catalytic subunit while p85 is the regulatory subunit. p110 catalyzes the signaling cascade by converting phosphatidylinositol (4,5)-diphosphate (PIP_2), an inactive substrate, to its active counterpart, PIP_3, which serves to recruit and activate AKT (28). Notably, AKT is an important regulator of survival and proliferation pathways downstream of the PI3K pathway through activation of *survivin* and *BCL-2.* AKT plays an important role in cancer by activation of the mTOR complex which results in subsequent activation of eIF4E (28). AKT can also be regulated by its inhibitor and an important tumour suppressor, PTEN (28). PTEN dephosphorylates PIP_3 to PIP_2 which inactivates the whole signaling pathway (28). In melanoma, *PTEN* is often mutated, which leads to this pathway being overactive (28). mTOR is an important complex downstream of the activated PI3K-AKT pathway. This complex is subdivided in two units, mTORC1 and mTORC2 (28). The mTORC1 complex is composed of mTOR itself and the scaffolding protein Raptor. The activation of the mTORC1 complex leads to the phosphorylation of 4EBP-1 (28). 4EBP-1, when dephosphorylated, is bound to eIF4E, resulting in an inhibition of mRNA translation (28). 4EBP-1 binding to eIF4E prevents the scaffolding protein eIF4G from binding to eIF4E. Without eIF4E-eIF4G binding, MNK1/2 cannot phosphorylate eIF4E. Phosphorylated eIF4E is responsible for the selective translation of tumourigenic mRNAs such as

cyclin D1, *c-myc* and *VEGF* (28). The mTORC2 complex on the other hand plays a synchronized role of promoting a positive feedback loop by phosphorylating AKT (28). AKT phosphorylation creates a positive feedback loop which causes further cancer progression by an increase in survival, proliferation and invasion. Interestingly, the *NRAS* mutation leads to an activation in both the PI3K-AKT pathway as well as the MAPK pathway.

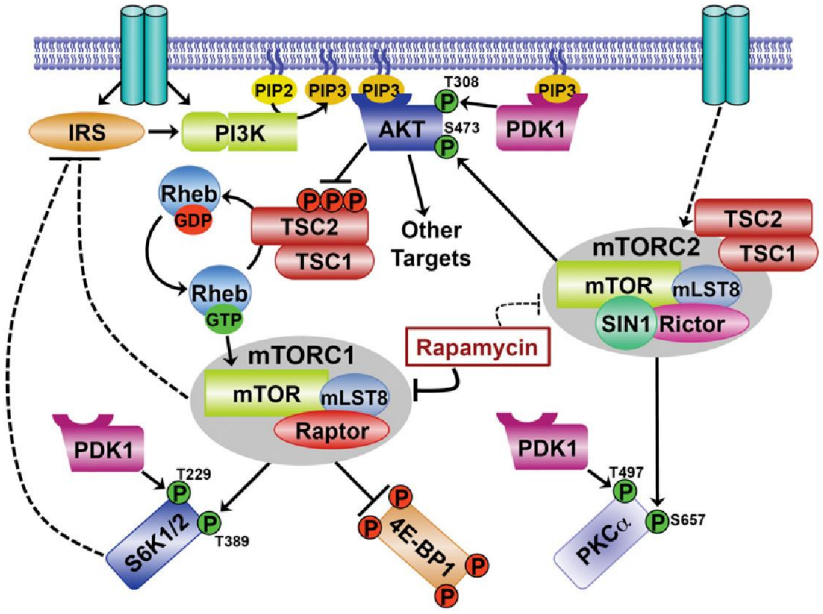


Figure 9. PI3K-AKT pathway. PI3K-AKT pathway overactivation is commonly seen in melanoma patients. Mutations in PTEN, NRAS and AKT amplification cause aberrant activation of PI3K-AKT pathway.

Adapted from Huang *et* al. (2009) (60)

1.1.10 Molecular cross-talk in melanoma

While studying each pathway responsible in melanoma progression is already complex, when we account for cross-talk between the pathways, understanding the molecular biology becomes ever more arduous. The two major pathways involved in melanoma progression are undoubtedly the PI3K-AKT pathway and the MAPK pathway. Both pathways often co-operate in

the progression of melanoma by cross-talking (46). Increased ERK activity leads to the phosphorylation of the GAB1 protein (46). GAB1 acts as an inhibitor in this situation by blocking PI3K in its interactions (46). Usually, PI3K communicates with the epidermal growth factor receptor (EGFR) to increase activation of the PI3K-AKT pathway, but with phospho-GAB1, this interaction is inhibited, thereby stifling downstream signaling (46). Furthermore, overactive AKT leads to the phosphorylation of RAF on its inhibitory sites, which dampens the activation of the MAPK pathway (46). Surprisingly, while we would have expected this inhibition to attenuate melanoma progression, the dampening of the MAPK pathway actually leads to less senescence (46). Cells as retaliation to the overactive MAPK pathway will normally undergo oncogene-induced senescence which blocks the cells from becoming malignant and proliferative, yet with the dampening of this effect, progression continues (46). Cross-inhibition leads to melanoma progression, but the contrary is also true: cross-activation leads to neoplastic progression, as well (46). As mentioned earlier, activation of RAS, with its active form, RAS-GTP, binds directly to the P110 subunit of PI3K to over-activate both pathways. Phospho-ERK is responsible in the phosphorylation of the TSC2 protein in the PI3K-AKT pathway. The un-phosphorylated form of TSC2 is an inhibitor of the PI3K-AKT pathway; with its activation, there is an increase in the downstream signaling which leads to the PI3K-AKT pathway cascade being reactivated.

Not only is there cross-talk between those two pathways, but as mentioned earlier, there is also convergence occurring. With an overactivation of the MAPK pathway, we see the downstream effect on MNK1/2. MNK1/2 is phosphorylated by upstream phospho-ERK (35). Phospho-MNK1/2, in turn, phosphorylates eIF4E, an important translation factor and constituent of the larger eIF4F complex (35). Phosphorylation of eIF4E leads to translation of a specific subset of mRNAs responsible for oncogenesis including cyclin D3 and cyclin E1(35).

In parallel, overactivation of the PI3K-AKT pathway leads to mTOR hyperphosphorylation of 4EBP-1 (45). 4EBP-1, when dephosphorylated, is bound to eIF4E and blocks it from joining the eIF4F complex (45). Phosphorylation of 4EBP-1 releases eIF4E and the latter is then free to join the eIF4F complex and get phosphorylated by MNK1/2 (45).

Activation of both pathways might be crucial for eIF4E to become phosphorylated by MNK1/2; and therefore MNK1/2 is an interesting target to further study, an enterprise that we will undertake in the next section.

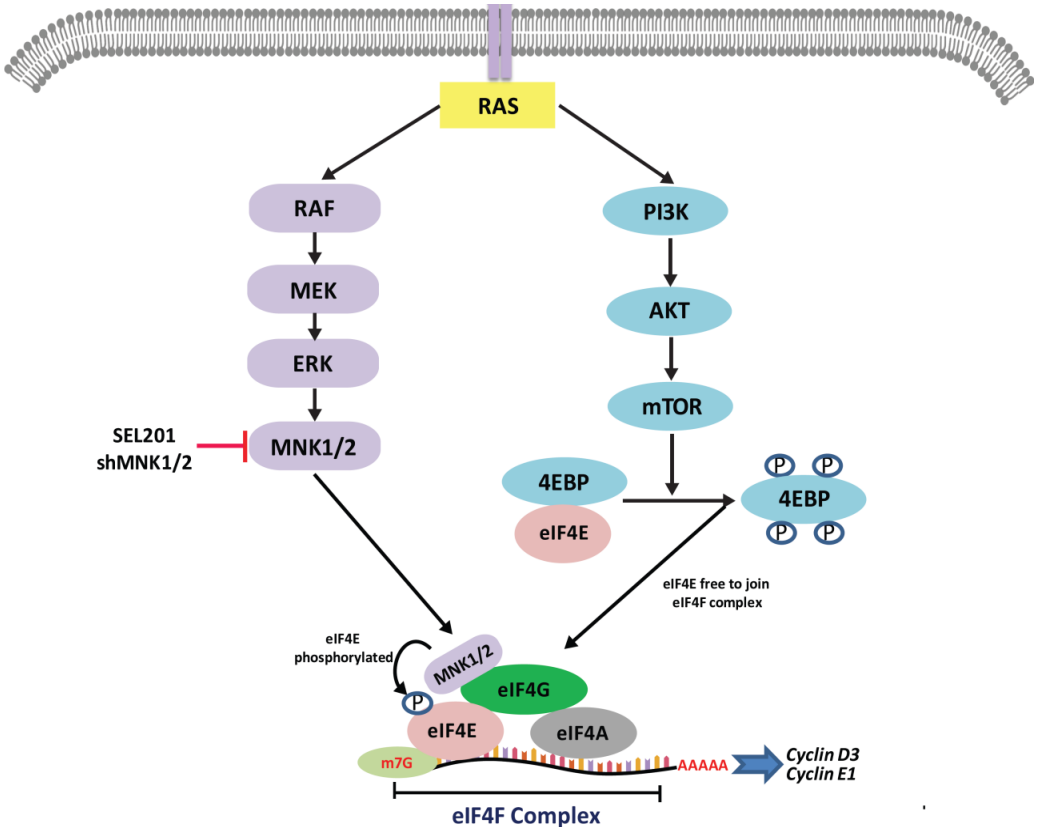


Figure 10. PI3K-AKT and MAPK pathways both converge on eIF4E. Both pathways converge on eIF4E to induce its phosphorylation and increase tumourigenesis.

Adapted from Zhan *et* al (2017) (44)

1.1.11 Molecular biology of MNK1/2

MNK1 and MNK2 kinases (i.e. MNK1/2) lie downstream of the MAPK pathway and are phosphorylated by ERK and p38 (41). MNK1/2 are well known for their ability to phosphorylate eIF4E and other proteins such as Sprouty2, hnRNPA1, PSF and cPLA2 (41). MNK1/2 in humans are subdivided into four isoforms, MNK1a, MNK1b, MNK2a and MNK2b, while the mouse counterpart only has two identified isoforms, MNK1 and MNK2 (41). Whereas all the isoforms have a nuclear localization signal (NLS) at the N-terminus that allows them to enter the nucleus, MNK1a is the only isoform to possess a nuclear export signal (NES) on the C-terminus which permits MNK1a to exit the nucleus (47). This means that MNK1a is primarily located in the cytoplasm as compared to MNK1b, MNK2a and MNK2b. The N-terminus also contains an eIF4G-binding domain that allows all MNK isoforms to bind to eIF4G, a component of the eIF4F complex (47). Another domain that is common between all isoforms is the catalytic domain, the kinase domain (47). MNK1a and MNK1b have an identical kinase domain and MNK2a and MNK2b also feature an identical kinase domain (47). The homology between the kinase domains on all four isoforms is very similar (47). MNK1a and MNK2a are the only isoforms to have a MAPK binding site (47).

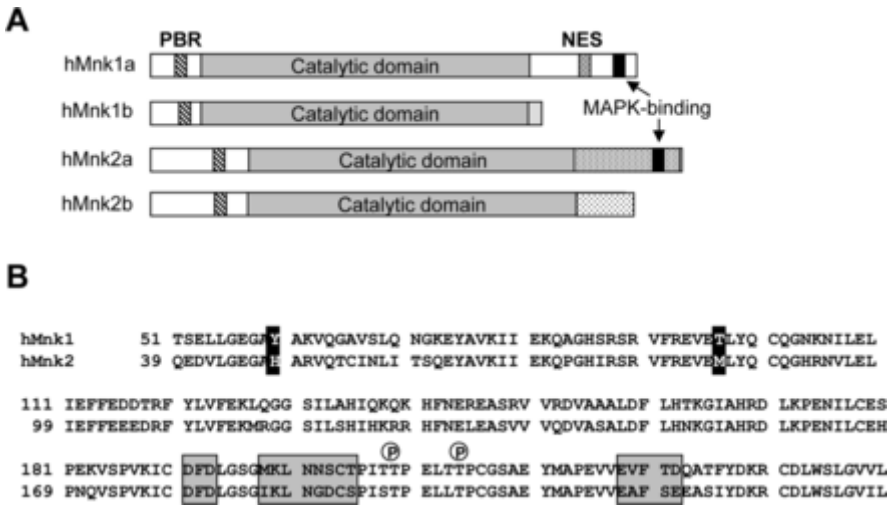


Figure 11. Isoforms of MNK1/2. Human MNK has four isoforms; MNK1a, MNK1b, MNK2a and MNK2b, each one acts in a different way. Panel A shows the different catalytic domains of human MNK. Panel B highlights the amino acids involved in the catalytic domains. Adapted from Goto *et* al (2009) (47)

MNK2a has a high basal activity and is not stimulated under stress nor regular activation of the MAPK pathway (47). On the other hand, MNK1a has a low basal activity, but can be activated by ERK or p38 (47). MNK1b, by contrast, has slightly higher basal activity than MNK1a, but is not regulated by an increase in factors (47).

Isoform	Basal activity	Regulation of activity	Subcellular localization	Comments (see also Figure 1A)
Mnk1a	Low	Markedly increased by ERK/p38 MAPKs	Mainly cytoplasmic	Long C-terminus contains NES and MAPK-binding site
Mnk1b	High	No marked regulation	Partly nuclear	Very short C-terminus lacks NES and MAPK-binding site
Mnk2a	Very high	Slightly increased by ERK/p38 MAPKs	Mainly cytoplasmic	Long C-terminus contains no NES; C-terminus impairs access to NLS; can bind activated ERK
Mnk2b	Very low	Not known	Partly nuclear	Shorter C-terminus lacks NES or MAPK-binding site

Table 6. Isoforms of MNK1/2. The four MNK isoforms are in different compartments of the cell. MNK1a is the only isoform to have a nuclear export signal (NES).

Adapted from Goto *et* al. (2009) (47)

1.1.12 Known downstream targets of MNK1/2

MNK1/2 are the only known kinases for eIF4E. MNKs phosphorylate eIF4E at serine 209 which ultimately leads to oncogenesis (48). [This will be discussed in more detail in the next section.] MNKs have also been shown to phosphorylate Sprouty2 (48). Sprouty2 plays two distinct roles: 1-inhibition of ERK and 2-EGFR activation (48). The inhibition of ERK is dependant on the phosphorylation of Sprouty2 by MNK1/2 (49). On the other hand, Sprouty2 phosphorylation leads to increased stability of EGFR (49). Both effects are antagonistic, however. While the inhibition of ERK leads to less tumour invasiveness, activation of EGFR leads to cancer growth and proliferation (48). MNKs also play a role in the phosphorylation of HnRNP A1 (RNA-binding protein) and PSF (PTB-associated splicing factor) (50).

1.1.13 Detailed look at the MNK-eIF4E axis

As mentioned earlier, both the MAPK and PI3K-AKT pathways converge upon eIF4E and act on its phosphorylation with the help of MNK1/2 (45). Along the MAPK pathway, MNK1/2 are phosphorylated by ERK at threonines 197 and 202 (51). In addition to activation of the ERK-MAPK pathway, under stress conditions, the p38-MAPK pathway is activated and also leads to MNK1/2 phosphorylation (51). Activated MNK1/2 then phosphorylate eIF4E at serine 209, which in turn causes specific translation of mRNAs responsible for oncogenesis, notably *MMP3*, *Snai1*, *MMP9*, *Cyclin D1*, and *VEGF* (52).

Three components constitute the eIF4F complex: the helicase eIF4A, the scaffolding protein eIF4G and the cap binding protein eIF4E (53). Most mRNAs rely on eIF4E to be recruited to the ribosomes for translation (53). The initiation of mRNA translation debuts when eIF4E binds

to the 7-methylguanosine (m7G) cap of the mRNA (53). eIF4A resolves the secondary structures of mRNAs during translation which increases its efficiency (53). Increased eIF4F activity in cancer cells allows some of the more tumourigenic mRNAs to be translated, while the translation of housekeeping genes remain unchanged, which leads to tumour progression and invasion (53).

1.1.14 Role of eIF4E in melanoma

eIF4E is a key regulator of many tumourigenic mRNAs such as *c-myc* and *MMP*s (53). Studies have also shown that regulation of eIF4E does not affect housekeeping genes such as *GAPDH* or *Actin* (53). Experiments have shown that the PRTE (pyrimidine-rich translation element) is needed to control the translation of mRNAs responsible for cancer progression (53). However, the details of how PRTE regulatory elements cooperate with eIF4E to control mRNA translation remain to be identified (53).

eIF4E activity is overactivated via oncogenes such as Myc by increasing the amount of eIF4F components (53). Furthermore, eIF4E is responsible for the translation of c-myc, which leads to a positive feedback loop between c-myc and increased activity of eIF4E (53). Increased levels of eIF4E are seen in many cancers such as lung cancer, hepatomas, lymphomas and melanomas (53). Cells overexpressing eIF4E have been shown to be more proliferative than their wild-type counterparts (53). Indeed, studies in fibrosarcoma have shown that knockdown of eIF4E by siRNA results in less proliferation (53). *In vivo*, the same result was seen when cells were depleted of eIF4E, such that less cancer progression occurred. In fact, the dose of eIF4E present to be recruited to the eIF4F complex is crucial in cancer progression. Multiple studies have shown

that with a 50% decrease in eIF4E levels, tumour progression was halted (54). On the other hand, a mere two-fold increase in the same levels was enough to induce oncogenesis (54).

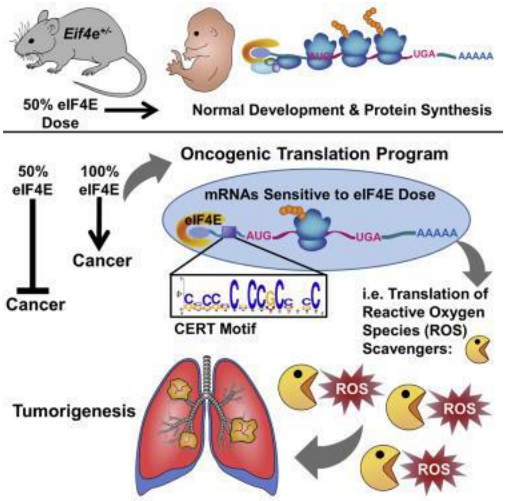


Figure 12. eIF4E regulation affects tumourigenesis. eIF4E decrease (50%), can inhibit the progression to a tumour.

Adapted from Truitt *et* al (2015) (54)

High levels of eIF4E have been shown to be implicated in vemurafenib resistance in melanoma cells (56). Vemurafenib inhibits the phosphorylation of 4EBP-1, leading to a stronger eIF4E-4EBP1 association, thereby preventing eIF4E from translating mRNAs responsible for oncogenesis. However, in vemurafenib-resistant melanoma cells, there is an increase in 4EBP-1 phosphorylation, leading to decreased eIF4E-4EBP1 association, thereby allowing mRNA translation of oncogenes responsible for tumourgenesis and drug resistance (56).

Not only is proliferation affected with an increase of eIF4E activity, but importantly metastatic potential is also altered. When mice were injected subcutaneously with breast cancer cells, primary tumours formed, and cells metastasized. The cells that metastasized had a higher expression of eIF4E compared to the primary tumours (53,55). Likewise, eIF4E decrease concomitantly yields a decrease in pro-invasive proteins, such as MMPs (55).

Furthermore, when eIF4E is inhibited, there is a reduction in the epithelial-to-mesenchymal transition – a transition which is proposed to facilitate metastasis (55).

1.1.15 Overview of metastasis in melanoma

Progression of a solid tumour to a metastatic disease is a multi-step process (57). The basics of melanoma progression can be summarized in five points (58):

1. Infiltration of the melanoma cells in the stroma.
2. Intravasation of the melanoma cells in the blood vessel/lymphatic vessels.
3. Dissemination of the cells through the vessels and survival of the cells.
4. Extravasation of the cells.
5. Recolonization of distant organs.

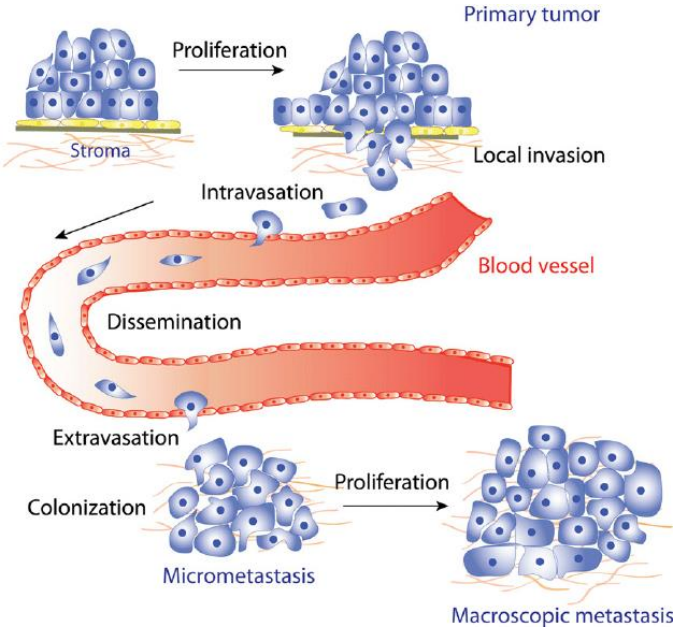


Figure 13. Steps of metastasis. Melanoma cells detach from the primary tumour spot to ultimately create a metastatic niche.

Adapted from Saxeena *et* al (2013) (57)

In step 1, although melanoma cells are not epithelial in origin, they can undergo a process akin to the epithelial-to-mesenchymal transition (EMT), which confers plasticity to the cells and gives it mesenchymal-like features (58). EMT cells exhibit a loss of E-cadherin which leads to the detachment of cells from the primary tumor site and facilitates the movement of cells. EMT alone can lead to the start of the invasion, however, in melanoma degradation of the extracellular matrix (ECM) is required for the process of metastasis to move to the next step (58). Factors such as MMP2, MMP9 and others are secreted, and cell invasion is then greatly facilitated (58).

Many factors are responsible for the progression of a solid tumour to a metastatic niche. MMPs are upregulated in almost every type of cancer, which makes them a hallmark in metastasis (58). MMPs can be secreted by the tumour micro-environment and aggressive cancer cells, they act by cleaving cell adhesion molecules, degrading ECM proteins and the degradation of cytokines and growth factors (58). ANGPTL4 is a secreted protein that is directly involved in lipid metabolism (59). ANGPTL4 has also been shown to be involved in angiogenesis (59). MMPs have been previously shown to be regulated by angiopoietin-like 4 (ANGPTL4) (59). High ANGPTL4 expression has been shown in many cancers and is associated with metastasis (59). Because of their ubiquitous expression in melanoma, MMPs are important potential therapeutic targets to block metastasis, and this could be achieved through strategies that downregulate ANGPTL4.

1.1.16 Role of eIF4E in other malignancies

As mentioned earlier, an increase in the levels of MNK activity (ultimately eIF4E phosphorylation) does not increase all mRNAs, but rather only those responsible in oncogenesis. Interestingly, mice lacking expression of MNK1/2 have no developmental issues and are healthy (60). This leads us to believe that normal cells lacking MNK1/2 can easily survive and proliferate normally while limiting the production of pro-oncogenic mRNAs in cancer cells. Similarly, $eIF4E^{S209A/S209A}$ knock-in transgenic mice, in which eIF4E cannot be phosphorylated, are also healthy (60,61). Thus, inhibiting the MNK1/2-eIF4E axis is potentially a worthy therapeutic target in cancer.

Additionally, $eIF4E^{S209A/S209A}$ knock-in mice that are deficient in phospho-eIF4E are resistant to prostate cancer initiation, and eIF4E phosphorylation mechanistically causes the upregulation of mRNAs that are important for prostate cancer progression (53). These mRNAs include *MMP3* and *MMP9* (responsible for prostate cancer metastasis), *Ccl2* and *Ccl7* (catalyzing prostate cancer proliferation), VEGF (growth factor in cancer), and BIRC2 (inhibitor of apoptosis). In fact, it is interesting to note that levels of phospho-eIF4E directly correlate with prostate cancer progression (53)

The same trend replicates in breast cancer, as the aforementioned $eIF4E^{S209A/S209A}$ knock-in mice did not lead to significant cancer progression (53). At the mechanistic level, TGF-beta was shown to induce an increase in eIF4E phosphorylation, leading to activation of the non-canonical TGF-beta pathway (54). The non-canonical TGF-beta pathway causes phosphorylation of ERK and p38, and these subsequently induce activation of MNK1 to ultimately result in the phosphorylation of eIF4E. In breast cancer, the phosphorylation of eIF4E causes mRNA

translation of *SNAIL* and *MMP-3*, yielding proteins responsible for breast cancer metastasis and EMT.

1.1.17 MNK inhibitors

Along the years, many MNK1/2 inhibitors have been created, notably CGP57380 and cercosporamide (53). Both drugs inhibit MNK1/2 activity and cause a decrease in proliferation with the induction of apoptosis (53). Specifically, in melanoma, cercosporamide administration reveals an inhibition of phosphorylation of eIF4E, which leads to blocking proliferation and lung metastasis (53). MNK1/2 inhibitors show promising effects in AML, breast cancer and melanoma (53). On the other hand, cercosporamide has many off-target effects and lacks specificity (62). This non-specific MNK1/2 inhibitor can inhibit other kinases such as Janus kinase 3, glycogen synthase kinase-3β, activin-like kinase-4 and Pim1 (62). However, the efficiency of MNK1/2 inhibitors can be greatly improved upon when used in combination with other drugs upstream of the MAPK pathway, such as MEK inhibitors or BRAF inhibitors (63).

Although MNK1/2 inhibitors have many positive effects on cancer cells, the main problem with the earlier models of this drug was the extremely high concentration needed to produce the effect required on the inhibition of phosphorylation of eIF4E (64). Newer MNK1/2 inhibitors such as EFT508 or SEL201 are much more promising in terms of dosing and concentration (65). In fact, EFT508 has already shown great potential in the treatment of Diffuse large B-cell lymphoma (DLBCL) (65). SEL201 has also been shown to inhibit cell invasion, cell migration and metastasis (44, 66, 67).

1.2 Rationale and objectives

Previous studies have shown the importance of the phosphorylation of eIF4E in melanoma, where the increased expression of phospho-eIF4E is associated with poor overall patient survival (68). My Masters project focused on examining the role of MNK1 in melanoma progression. Previous data in our lab showed that expression of a constitutively active MNK1 in murine melanoma cells could promote invasion *in vitro*. Moreover, previous *in vitro* work from our team showed that murine melanoma cells that were devoid of MNK1 using CRISPR/Cas9 technology were less invasive and proliferated less well than their control counterparts. I hypothesized that there would be a differential gene expression signature associated with melanoma cells expressing a constitutively active MNK1. I thus sought to characterize the expression of genes, using RNA-seq, that were altered in response to the constitutive activation of MNK1 in melanoma. My thesis shows a group of genes whose expression are changed by modulating MNK1 expression or activity, and that are responsible for promoting invasion and metastasis. I also sought to create, and characterize, human melanoma cell lines that (1) were devoid of MNK1 using CRISPR/Cas9 technology or (2) expressed a constitutively active form of MNK1. Finally, I tested whether pharmacologic inhibition of MNK1/2 would be a potential new therapeutic avenue in *BRAF*-mutated melanoma.

2. The role of MNK1 in melanoma cells

2.1 Abstract

The $BRAF^{V600E}$ mutation occurs in more than 50% of cutaneous melanomas, and results in the constitutive activation of the mitogen-activated protein kinases (MAPK) pathway. MAP kinase-interacting serine/threonine-protein kinase 1 and 2 (MNK1/2) are downstream effectors of the activated MAPK pathway, and important molecular targets in invasive and metastatic cancer. Despite the well-known role of MNK1 in regulating mRNA translation, little is known concerning the impact of its aberrant activation on gene transcription. Here, we show that changes in the activity, or abundance, of MNK1 result in changes in the expression of pro-oncogenic and pro-invasive genes. Among the MNK1-upregulated genes, we identify Angiopoietin-like 4 (ANGPTL4), which in turn promotes an invasive phenotype via its ability to induce the expression of matrix metalloproteinases (MMPs). Using a pharmacologic inhibitor of MNK1/2, SEL201, we demonstrate that BRAFV600E-mutated cutaneous melanoma cells are reliant on MNK1/2 for invasion and lung metastasis.

2.2 Introduction

Recent advances in melanoma research have led to several FDA-approved MAPK-targeted and immuno-therapies (1, 2, 3, 4). Although the current treatments are effective at tumor-killing in melanoma patients, there is an unmet medical need for novel therapeutics that target tumor metastasis (5). Compared to other forms of skin cancer, melanoma is considered the deadliest due to its highly metastatic and aggressive nature (6). The Cancer Genome Atlas (TCGA) Network has recently classified cutaneous melanoma into four genomic subtypes: B-Raf proto-oncogene (BRAF), NRAS proto-oncogene (NRAS), neurofibromin 1 (NF1), and triple-wild-type (7). BRAFV600E is the most common mutation in cutaneous melanoma, leading to overactivation of the mitogen-activated protein kinase (MAPK) pathway. In approximately 30% of all BRAFV600E-mutant melanomas, there is paralleled activation of the phosphoinositide 3-kinase-Protein kinase B (PI3K-AKT) pathway, caused by loss-of-function mutations in the tumour suppressor protein phosphatase and tensin homolog (PTEN).

MNK1 and MNK2 are two kinases that lie immediately downstream of constitutive MAPK and PI3K pathway activation, driven by BRAFV600E and mutated PTEN. Although we have previously identified MNK1/2 as novel therapeutic targets in KIT-driven acral melanomas, whether other melanoma subtypes can be therapeutically targeted with MNK1/2 inhibitors remains unknown. MNK1/2 have been shown to facilitate invasive and metastatic disease via promoting the phosphorylation of eIF4E and inducing the translation of mRNAs such as CCNE1, SNAI1, and MMP3 (8, 9, 10, 11). Moreover, MNK1/2 are implicated in the invasive progression of breast ductal carcinoma *in situ* via the induction of NODAL, a morphogen essential for embryonic patterning, which is often re-expressed in breast cancer (12).

Although MNK1/2 inhibitors have reached Phase I/II clinical trials, the mechanisms that underpin the role of MNK1/2 in cancer progression, independent of mRNA translation, are still not well understood. The activity of MNK1 is inducible by the upstream kinases (ERK and p38), while MNK2 exhibits a basal kinase activity that is independent of the intercellular or intracellular signaling. Herein, we describe the impact of increased MNK1 activity on the progression of BRAF-mutant melanoma, via a novel mechanism that involves robust changes in the transcription of genes that are required for invasion and metastasis, such as ANGPTL4, early growth response protein 1 (EGR1), and several matrix metalloproteinases (MMPs) (13). Furthermore, we show that the invasiveness of cutaneous melanoma can be blocked by inhibiting MNK1 or ANGPTL4; a gene previously unknown to be regulated by MNK1.

2.3 Material and methods

2.3.1 Cells and Reagents

D4m.3a (BRAF mutant, PTEN mutant) murine melanoma cells were kindly provided by Dr. Constance Brinckerhoff (Geisel School of Medicine, Dartmouth College). A375 (BRAF mutant, CDKN2A mutant) human melanoma cells were purchased from Plexxikon Inc. LWT1 (BRAF mutant) murine melanoma cells were kind gifts from Dr. Mark Smyth (QIMR Berghofer Medical Research Institute). D4m.3a was cultured in advanced DMEM media containing 5% fetal bovine serum (FBS), 5ml Glutamax (100x) and antibiotics. A375 cells were maintained in DMEM media containing 10% FBS and antibiotics. LWT1 cells were cultured in RPMI-1640 containing 10% FBS and antibiotics. All cell lines were maintained at 37°C in a humidified incubator with 5% CO_2. MNK1/2 inhibitor SEL201 was obtained from Selvita S.A. (Poland).

2.3.2 Generation of MNK1 Knockout Cell Lines using CRISPR-CAS9 Technology

CRISPR/Cas9-mediated knockout of MNK1 in D4m.3a or A375 cells was accomplished using a CRISPR/Cas9 KO plasmid purchased from GenScript, Piscataway, NJ, USA. D4m.3a cells were transfected with either Cas9-GFP control or MNK1 sgRNA/Cas9-GFP plasmids. Individual GFP positive clones were sorted into single cells in 96-well plates 48 h after transfection. Single cell clones were expanded and Western blot analysis confirmed the MNK1 KO status of the clones.

2.3.3 Clonogenic Assay

300 cells/well were plated in 6-well plates and were treated as indicated. New media and drugs were replenished every other day. After 7 days, colonies were stained with 0.5% (W/V) crystal violet in 70% ethanol and quantified using a Gel Count colony counter (Oxford Optronix, Milton, England).

2.3.4 Western Blot Analysis

Cell pellets were lysed in RIPA buffer (50 mM Tris-HCl, pH 8.0, with 150 mM sodium chloride, 1.0% Igepal CA-630 (NP-40), 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulphate) as described previously (8). Equal amounts of proteins were loaded and separated on a 10% SDS-PAGE acrylamide gel, and subsequently transferred to nitrocellulose membrane (BioRad, Hercules, CA, USA). MNK1, eIF4E, phospho-eIF4E, ANGPTL4 and MMP3 were probed with corresponding antibodies, and β-Actin was probed to confirm equal loading. Detailed antibody information is listed in Table 1.

Target	**Supplier**	**Catalog number**
MNK1	Cell signaling	#2195S
eIF4E	BD transduction laboratory	#610269
Phospho-eIF4E	Cell signaling	#9714S
ANGPTL4	Thermo Fisher Scientific	#40–9800
MMP3	Abcam	#ab52915
β-Actin	Cell signaling	#3700

Table 1: Antibodies used

2.3.5 Plasmids, Virus Production, Stable Cell Selection

pBABE and caMNK1 plasmids were kind gifts from Dr. Jonathan Cooper (Fred Hutchinson Cancer Research Center, University of Washington Seattle). Plasmids were transfected into 293FT cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) to generate viral particles according to manufacturer's instruction. Viral supernatants were harvested 48 h post-transfection and were used to infect D4m.3a or A375 cells. Cells were subsequently selected with puromycin (1μg/ml) to generate pBABE and caMNK1 clones as previously described (8).

2.3.6 RNA Interference

200,000 cells were seeded in 10 cm dishes. Scramble siRNA (Qiagen AllStars Negative Control siRNA, Hilden, Germany) or ANGPTL4 siRNA (IDT, Coralville, IA, USA) were introduced into the cells using lipofectamine RNAi Max reagents (Invitrogen) following manufacturer's instructions.

ANGPTL4 siRNA sequence 1: rArUrArArArArGrCrArArCrCrUrCrArGrArArCrArCrUrUTG,

ANGPTL4 siRNA sequence 2:

rCrArArArGrUrGrUrUrCrUrGrArGrGrUrUrGrCrUrUrUrUrArUrUrC

2.3.7 RNA Isolation, cDNA Synthesis and qPCR Analysis

Total mRNA was isolated with Omega Bio-Tek E.Z.N.A total RNA kit (Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer's instructions. RNA concentration was quantified (Thermo Fisher scientific Nanodrop 1000, Waltham, MA, USA) and cDNA was synthesized using iScript™ cDNA Synthesis Kit (BioRad) following the manufacturer's instructions. qPCR was performed with SYBR Green reagents (BioRad) as previously described (8). Primer sequences are listed in Table 2.

Table 2: Primer sequences

Mouse *ANGPTL4*	CTG AAT ATC ACT TCT CGC CTA CC (FWD Mouse) CCT GTC TCC AGT CAG TCA ATA TG (REV Mouse)
Mouse *MMP1a*	GGA CTT ATA TGG ACC TTC CCC A (FWD Mouse) AAT TGA GCT CAG GTT CTG GC (REV Mouse)
Mouse *MMP3*	GGT TGT GTG CTC ATC CTA CCC (FWD Mouse) ACC CGA GGA ACT TCT GCA TTT (REV Mouse)
Mouse *MMP9*	AAA GAC CTG AAA ACC TCC AAC CT (FWD Mouse) TGT AAC CAT AGC GGT ACA AGT ATG C (REV Mouse)
Mouse *MMP10*	CCA GCT AAC TTC CAC CTT TCT (FWD Mouse) GAC AGA CAA CAC AGG AAC CA (REV Mouse)
Mouse *RGS5*	CCA GAA CAG CTA TGG ATT TGC C (FWD Mouse) ATT TGC TTT GCC TTC TCC GC (REV Mouse)
Mouse *CLU*	GAG AAG GCG CTA CAG GAA TAC (FWD Mouse) CCT CCC AGA CAC TCC TAC ATA (REV Mouse)
Mouse *EGR1*	AGG AGT GAT GAA CGC AAG AG (FWD Mouse) GGA TGG GTA AGA AGA GAG TGA AG (REV Mouse)
Human *ANGPTL4*	ACG AAA GAC GGT GAC TCT TG (FWD Human) TCT TCT CTG TCC ACA AGT TTC C (REV Human)
Human *MMP1a*	TGT CAG GGG AGA TCA TCG GG (FWD Human) TGG GCC TGG TTG AAA AGC AT (REV Human)
Human *MMP3*	ACC CAC CTT ACA TAC AGG ATT G (FWD Human) GTC ACC TCT TCC CAG ACT TTC (REV Human)

Human *MMP9*	TTC AGG GAG ACG CCC ATT TC (FWD Human) AAC CGA GTT GGA ACC ACG AC (REV Human)
Human *MMP10*	GGC CCT CTC TTC CAT CAT ATT T (FWD Human) CCT GCT TGT ACC TCA TTT CCT (REV Human)
Human *RGS5*	AGG AAA GGC ATC CCA GAC AG (FWD Human) GCA AGT CCA TCT TTC CAG GCA T (REV Human)
Human *CLU*	CAG CCC TTC CTT GAG ATG ATA C (FWD Human) TCG CCT TCT CGT ATG AAT TCT G (REV Human)
Human *EGR1*	TGA CCG CAG AGT CTT TTC CT (FWD Human) GTG GTT TGG CTG GGG TAA CT (REV Human)

2.3.8 Migration and Invasion Assay

200,000 melanoma cells were seeded per 10 cm dish on day 1 in complete media, then switched to serum-free media on day 2 and starved overnight. On day 3, the transwells were coated with Matrigel (Corning) (100 μg/ml) as previously described (8). 30,000 cells were then seeded into the transwells on the top and were allowed to invade for 24 h. Invaded cells were fixed with 5% glutaradehyde and stained with 0.5% crystal violet. Representative images were taken and invaded cells stained with crystal violet were counted for analysis (8)

2.3.9 Gelatin zymography assays to detect MMP9 activity

MMP9 activity in cell culture supernatant were detected by gelatin zymography as previously described (26). Briefly, a 10ml cell culture supernatant was concentrated to 0.2 ml by using Amicon Ultra-15 centrifugal spinning units (Millipore, Burlington, MA, USA). Concentrated

supernatants were subsequently separated with 7.5 % acrylamide gels containing 0.1 % gelatin A (Thermo Fisher Scientific) and MMP9 bands were revealed with 0.25% Coomassie blue staining.

2.3.10 Animal Studies

All animal care and experiments were carried out according to rules and regulations established by the Canadian Council of Animal Care, and protocols were approved by the McGill University Animal Care Committee. 8-week old male C57BL/6 mice or 12-week old NOD/SCID mice were purchased from Charles River Laboratories. 100,000 D4m.3a cells (in any condition) or 1 million A375 cells were suspended in PBS and injected subcutaneously into the flank of the mice. Tumour volume was determined by caliper measurements using the following formula: (4/3×(3.14159)×(L/2) ×(W/2)^2), in which L refers to the diameter of the longest axis and W refers to the diameter of the shortest axis. At endpoint, tissues were fixed in 10% formalin for immunohistochemistry (IHC) assessment. For intravenous injections, 300 000 A375 melanoma cells or 500 000 D4m.3a cells were injected into NOD-SCID mice or C57BL/6 mice respectively, through the tail vein, and lung metastasis were quantified as previously reported (8).

2.3.11 RNA-seq Analysis

Total RNA from D4M.3A pBABE and caMNK1 (n=3, each) was prepared using RNeasy Plus Mini Kit (Qiagen). RNA QC, library preparation and sequencing were performed at the McGill University and Genome Quebec Innovation Center (Montreal, QC). RNA-seq libraries were prepared using Illumina's TruSeq® Stranded Total RNA LT kit following the manufacturer's protocol. RNA quality was assessed by Agilent 2100 Bioanalyzer (Agilent Technologies). Libraries were multiplexed, six samples per lane, and sequenced. Illumina HiSeq 4000 was used to obtain 100 bp paired-end reads at an average of 57 million reads per sample. Trimmomatic v0.32 was used to trim reads, including removal of low-quality bases at the end of reads (phred33 < 30), clipping of the first four bases and clipping of Illumina adaptor sequences using the palindrome mode. We executed quality trimming with a sliding window, cutting once the average quality of a window of four bases fell below 30. We discarded reads shorter than 30 base pairs after trimming. Quality control was orchestrated using metrics obtained with FASTQC v0.11.2. The resulting high-quality RNAseq reads were aligned to the mouse reference genome mm10 using STAR v2.5.3a. We obtained a uniquely mapping rate average higher than 90%.

2.3.12 Differential gene expression (DE) analysis

Aligned reads were summarized through *featureCounts* v1.5.3 with the gene model from Ensembl (Mus_musculus.GRCm38.Ensembl83.gtf) at gene level: specifically, the uniquely mapped reads that overlapped with an exon (feature) by at least 1bp were counted and then the counts of all exons annotated to a gene name (meta-features) were summed into a single number. All analyses described below were performed in the R statistical computing environment (http://www.R-project.org). Exploratory analysis and visualization was done prior to differential gene expression

analysis using DESeq2 v1.18.1 package. The rlog transformation was applied to obtain homoskedastic data, i.e. where the variance of the count is stabilized across the mean, allowing principal components analysis (PCA) and distance clustering calculation in order to detect outliers. After withdrawal of the outlier sample, DESeq2 was applied to the raw counts to estimate the log2 fold change (LFC) between caMNK and pBABE by adapting the independent filtering threshold to our FDR (False Discovery Rate, alpha = 0.05). We then applied the adaptive shrinkage from the ashr (Adaptive Shrinkage) v2.0.5 package as regularization method to remove the noise and preserve large differences. This method employs an Empirical Bayes approach to multiple testing in order to determine FDR by using effect sizes and their standard errors to summarize each measurement. Genes with statistically significant FDR (Padj < 0.05), large LFC (fold change > 2.0) and above the determined expression threshold (average normalized expression across samples > 100) were selected as gene of interest for further analysis. Heatmaps and volcano plots were produced using the pheatmap v1.0.8 with RColorBrewer v1.1-2 and ggplot2 v2.2.1 packages, respectively.

2.3.13 Functional analysis

For functional enrichment analyses, goseq v1.30.0 and GAGE were used. Background was matched to DE gene list using the genefinder command of the genefilter v1.60.0 package. A weight of 20 and the Manhattan method was used to recruit at least 10 background genes for each DE gene. Then the weighting function of goseq was applied using gene length as the bias against which to normalize. The analysis was conducted through Gene Ontology (GO) and KEGG pathways. The dotplot was designed using ggplot2 v2.2.1 package and barcode plots were drawn using the barcodeplot function of the limma v3.34.5 package.

For protein–protein interaction (PPI) network analysis, network topology-based analysis of proteins that encoded by DEGs to BIOGRID database were retrieved using the WEB-based Gene Set Analysis Toolkit (WebGestalt, http://www.webgestalt.org/). The PPI network was visualized using Cytoscape (http://www.cytoscape.org/). Functional annotations of clusters were retrieved using Search Tool for the Retrieval of Interacting Genes/Proteins (STRING, http://string-db.org/).

2.3.14 Statistical analysis

Prism software (GraphPad) was used to perform statistical analysis. Three biological independent experiments were performed for all *in vitro* work unless otherwise stated. The significance of differences between groups was determined by applying either unpaired Student's t test, one-way ANOVA, two-way ANOVA, and spearman's correlation, as appropriate. The sample size and specific statistical analysis for each figure is listed in Table 3. P values < 0.05 were considered significant, individual P values and details of statistical analysis are also presented in figure captions. All animal studies were randomized and blinded. Analysis of invasion assays and tissue slide staining were also blinded.

Table 3: Detailed statistical analysis

1A	$N = 2$ (pBABE) $N = 3$ (caMNK)	See RNA-Seq Analysis, differential gene expression (DE) analysis and functional analysis section of "Methods"
1B	$N = 2$ (pBABE) $N = 3$ (caMNK)	See RNA-Seq Analysis, differential gene expression (DE) analysis and functional analysis section of "Methods"
1C	$N = 2$ (pBABE) $N = 3$ (caMNK)	See RNA-Seq Analysis, differential gene expression (DE) analysis and functional analysis section of "Methods"
1D		See functional analysis section of "Methods"
1E	Three independent experiments for each qPCR	Wilcoxon–Mann Whitney test
2A	Three independent experiments for each migration and invasion experiment	T test (Do not assume same SD)
2B	Three independent experiments for each migration and invasion experiment	One-way ANOVA
2C	D4M.3a CTL $n = 6$ D4M.3a MNK1 KO $n = 4$	T test (Do not assume same SD)
2D	A375 CTL $n = 6$ A375 MNK1 KO $n = 8$	T test (Do not assume same SD)
2E	Number of cells D4M.3a CTL $n = 3$ D4M.3a MNK1 KO $n = 3$ Tumor outgrowth D4M.3a CTL $n = 5$ D4M.3a MNK1 KO $n = 8$	Two-way ANOVA

2F	Number of cells A375 CTL $n = 3$ A375 MNK1 KO $n = 3$ Tumor outgrowth A375 CTL $n = 8$ A375 MNK1 KO $n = 7$	Two-way ANOVA
3A	Three independent experiments for each qPCR	Wilcoxon–Mann Whitney test
3C	Three independent experiments for each qPCR	Wilcoxon–Mann Whitney test
3D	Three independent experiments	One-way ANOVA
3G	Three independent experiments	One-way ANOVA
4B	Three independent experiments for each migration and invasion experiment	*T* test (Do not assume same SD)
4C	Three independent experiments for each qPCR	Wilcoxon–Mann Whitney test
4D	D4M.3a Vehicle $n = 7$ D4M.3a SEL201 $n = 7$	*T* test (Do not assume same SD)
5A	TCGA Skin Cutaneous Melanoma Data, $BRAF^{V600E}$ patient cohort (37 patients)	Linear regression
5B	TCGA Skin Cutaneous Melanoma Data, $BRAF^{V600E}$ patient cohort (164 patients)	Student's *t* test

2.4 Results

2.4.1 Increased MNK1 signaling is associated with the elevated expression of genes involved in tumour progression

MNK1 activation can mediate some of its pro-invasive effects via modulation of mRNA translation. However, less is known about the impact of aberrant MNK1 activity on transcriptional

reprogramming (14). We thus examined whether the expression of a constitutively active MNK1 in melanoma cells, induces changes in transcriptional landscapes. The changes in gene expression that we identify might explain the observed differences in invasion and metastasis associated with modulating MNK1 level or activity. We performed RNA sequencing (RNA-seq) analysis on pBABE- (control cell line) versus caMNK1-expressing ($MNK1^{T332D}$, a MNK1 variant with constitutive catalytic activity which we term caMNK1) D4M.3a melanoma cells. D4M.3a cells were originally derived from a melanoma that outgrew from an inducible-$BRAF^{V600E}$/$PTEN^{null/null}$ melanoma mouse model (15). We characterized changes in the genetic landscape upon constitutive activation of MNK1 (Figure 1A, Supplementary Figure 1). To obtain a robust caMNK1 gene signature, we only considered genes that had an adjusted p-value (p_{adj}-value) of <0.05 and fold change ≥2.0. This led us to a high confidence signature of 85 genes that were differentially expressed between pBABE versus caMNK1 cells, wherein 62 genes were upregulated, while 23 genes were downregulated in melanoma cells expressing caMNK1 (Figure 1A). The volcano plot shows the significantly upregulated and downregulated differentially expressed genes (DEGs) in the caMNK1 group (Figure 1B). Our analysis revealed a subset of genes with established roles in cancer progression that were significantly upregulated in caMNK1 cells, including *MMP9, EGR1, ANGPTL4, MMP10, MMP1a, MMP3,* Clusterin (*CLU*), and Regulator of G-protein signaling 5 (*RGS5*) (Figure 1B). MMPs are known to degrade components of the ECM to facilitate cancer migration and invasion (13). Strikingly, ANGPTL4 (16), a critical upstream regulator of MMPs, was amongst the most upregulated genes in our analysis ($p=7.09x10^{-25}$).

To identify relevant pathways associated with the differential gene expression, we performed gene ontology and KEGG analyses, finding that caMNK1 cells were enriched for cellular functions and molecular pathways involved in the regulation of cellular adhesion (GO:

0007155), remodeling of the extracellular matrix (ECM) (GO:0031012) and protein degradation and digestion (GO:0005578). These results suggest that MNK1 modulates melanoma invasion through degradation of extracellular proteins and loss of cellular adhesion (Figure 1C). To further demonstrate the genetic interactions between the top-ranked genes, we used gene clustering analysis (Figure 1D). Cluster 1 showed the most extensive genetic network and most notably illustrated that MNK1 directly, or indirectly, regulates genes involved in melanoma progression such as *MMP9, EGR1, ANGPTL4, MMP10, MMP3* and *EGR1* (Figure 1D, genes of interest highlighted in purple).

To validate the DEGs that were regulated when MNK1 activity is increased, we performed qPCR assays. We focused our validation on the genes which we identified as being most significantly upregulated in D4M.3a-caMNK1 cells, compared to pBABE controls, and having roles in cancer, including: degradation of the extracellular matrix (*MMP10, MMP1a, MMP3, MMP9*) (17), angiogenesis (*ANGPTL4, RGS5)* (18, 19), drug resistance (*CLU*) (20), and tumour growth (*EGR1*) (21). qPCR analyses confirmed that all the above DEGs were upregulated in caMNK1-expressing cells (Figure 1E). Additionally, ANGPTL4 and MMP3 protein levels were elevated in D4M.3a caMNK1-expressing cells as examined by western blot (Figure 1F). We also performed a gelatin-based zymography assay routinely used to evaluate MMP9 activity, and consistent with the mRNA expression data, MMP9 expression is elevated in D4M.3a caMNK1-expressing cells (Figure 1F). These data demonstrate that increased levels of MNK1 activity lead to changes in the transcriptional landscape that facilitate melanoma progression towards invasive disease.

2.4.2 MNK1 facilitates melanoma metastasis and outgrowth

Based on the DEGs identified by the RNA-seq (Figure 1B), we hypothesized that the pro-invasive transcriptional signatures induced by MNK1 (Figure 1), would promote a more aggressive phenotype in $BRAF^{V600E}$ tumor cells. To test the impact of constitutively activating MNK1 on melanoma invasiveness, we used murine D4M.3a and human A375 $BRAF^{V600E}$ mutant melanoma cells engineered to express caMNK1. Expression of caMNK1 in both D4M.3a and A375 cells was validated to be functional by detecting increased phosphorylation of one of its best-studied substrates, eIF4E (Figure 2B). Both caMNK1-expressing (1) D4M.3a and (2) A375 cells invaded significantly more compared to their D4M.3a pBABE control counterparts (Figure 2B). We next utilized CRISPR/Cas9 technology to knock out MNK1 (MNK1 KO) in D4M.3a and A375 cells. Two independent MNK1 KO clones for each cell line were impaired in their ability to invade (Figure 2A). Given the robust *in vitro* effects that we observed upon modulating MNK1 expression or activity, we next inoculated (1) D4M.3a MNK1 KO and (2) A375 MNK1 KO cells intravenously into C57BL/6 and NOD-SCID mice, respectively. The animals inoculated with MNK1 KO melanoma cells had significantly less lung metastases ($p<0.0001$), compared to their counterpart CTL cells in both D4m.3a and A375 models (Figure 2C,D). Collectively, our *in vitro* and *in vivo* data are consistent with activated MNK1-expressing melanoma cells acquiring an invasive transcriptional signature (Figure 1).

We also assessed the effect of MNK1 loss on clonogenic outgrowth, cell proliferation and primary tumour outgrowth. Both D4M.3a and A375 cell lines devoid of MNK1 showed reduced clonogenic outgrowth and cell proliferation (Figure 2E, F). Next, to assess the impact of loss of MNK1 on melanoma outgrowth *in vivo*, we subcutaneously injected MNK1 null-D4M.3a or -A375 cells into syngeneic and immune deficient mice, respectively. The melanomas derived in both

MNK1 KO groups were significantly smaller compared to their respective control groups ($p<0.001$) (Figure 2E,F), demonstrating that MNK1 loss has a negative impact on tumor outgrowth. Conversely, caMNK1 cells showed an increase in clonogenicity and proliferation compared to their pBABE counterpart (Figure 2G). In summary, modulating MNK1 activity, or abundance, in *BRAF*^{V600E} mutant melanoma cells resulted in altered invasion and metastasis both *in vitro* and *in vivo*.

2.4.3 ANGPTL4 upregulates the expression of MMPs and increases invasion, downstream of MNK1

caMNK1-expressing melanoma cells show an upregulation of genes involved in invasion and metastasis. Conversely, in MNK1-deficient cells, we hypothesized that the same set of pro-invasive genes would be downregulated. We thus performed qPCR assays to measure the expression of *MMP10, MMP1a, MMP3, MMP9, ANGPTL4, RGS5, CLU,* and *EGR1* in D4M.3a and A375 MNK1 KO cell lines. The majority of the aforementioned genes that were upregulated in the caMNK1 expressing cells, were downregulated in D4M.3a and A375 cells depleted of MNK1, with the exception of *CLU* in A375 cells and *EGR1* in both cell lines (Figure 3A). Moreover, ANGPTL4 protein levels were repressed in MNK1 KO cells, as demonstrated by western blot (Figure 3B). Finally, MMP9 activity is significantly reduced in D4M.3a MNK1 KO cells as shown by zymography (Figure 3B).

Previous reports have shown that ANGPTL4 functions as a master regulator of MMP expression (16, 22), thus we examined the relationship between MNK1, ANGPTL4, and MMPs. Transient downregulation of *ANGPTL4,* using siRNA, significantly reduced the mRNA expression for *MMP1a*, *MMP3*, *MMP9*, and *MMP10* (Figure 3C). Moreover, caMNK1-expressing cells transfected with siRNA against *ANGPTL4* were less invasive (Figure 3D) and expressed less MMP9 (Figure 3E), compared to siRNA control. We next assessed whether the defect in invasion observed in MNK1 KO cells could be rescued using recombinant ANGPTL4 (Figure 2). Adding recombinant ANGPTL4 to MNK1 KO cells partially overcame the reduced invasion upon MNK1 depletion (Figure 3F-G). Together, these data demonstrate that MNK1 acts upstream of ANGPTL4; inducing its expression, and thus enabling the subsequent upregulation in MMP expression to ultimately promote melanoma cell invasion.

2.4.4 MNK1/2 can be used as therapeutic targets to treat malignant melanoma

Given the robust biological outcomes that accompany the loss of MNK1 in melanoma cells (Figure 2), we next investigated whether pharmacological inhibition of MNK1/2 would block melanoma progression. Treatment of D4M.3a cells with the novel and orally bioavailable MNK1/2 inhibitor SEL201 (8, 12) resulted in a robust decrease in MNK1/2 activity, as determined by reduced expression of phospho-eIF4E (Figure 4A). In keeping with the role of MNK1/2 as potent regulators of cell invasion, SEL201 significantly decreased the invasive capacity of three *BRAF*^{V600E} melanoma cell lines D4M.3a, LWT1 and A375 (Figure 4B). The decreased invasive abilities of melanoma cell lines treated with SEL201 are associated with a transcriptional downregulation of *ANGPTL4* and *MMP9* mRNAs (Figure 4C). To further explore the ability of SEL201 to block metastasis, we injected D4M.3a melanoma cells intravenously into syngeneic C57BL/6 mice and randomized the animals to either control or SEL201 treatment groups. SEL201 decreased the metastatic tumour burden in the lungs compared to the vehicle control (Figure 4D), demonstrating that SEL201 is a potent inhibitor of melanoma metastasis.

2.4.5 MNK1 mRNA levels correlate with pro-invasive gene signatures in TCGA patient samples

We next used TCGA melanoma patient data to interrogate whether any positive correlation exists between *MKNK1* mRNA expression and the expression of downstream MNK1-regulated genes that were identified via RNA Seq (Figure 1). The results indicated that MNK1 levels positively correlated with the expression of *ANGPTL4, MMP9, MMP3* and *MMP1* in primary $BRAF^{V600E}$-mutant human melanoma samples ($p<0.01$) (Figure 5A). Finally, our survey of TCGA data shows that *MKNK1* is more abundant in primary melanoma tumours compared to metastatic tumours ($p<0.01$) (Figure 5B), suggesting that MNK1 overexpression is an early event in tumour dissemination and metastasis. Together, our results suggest that MNK1 regulates a network of previously unknown genes that contribute to melanoma invasion and progression (Figure 5C).

2.5 Discussion

Our present study has defined a novel gene signature that correlates with high MNK1 activity, with several genes having roles in invasion and extracellular processes such as the expression of genes responsible for ECM degradation. We have also shown that MNK1 regulates the expression of genes with known roles in proliferation, such as *EGR1, RGS5* and drug-resistance genes such as *Clu,* which encodes for the protein clusterin.

Clu has previously been implicated in therapy resistance in a variety of cancers including renal, bladder, pancreatic and lung adenocarcinomas (23). *Clu* expression prevents chemotherapy-mediated cell death, and therefore many patients expressing high levels of *Clu* in advanced disease experience therapy resistance (20). Future investigations regarding the role of MNK1/2 inhibitors in the context of therapy-resistant melanoma are ongoing in our lab.

Considering that several ongoing phase II clinical trials are investigating MNK1/2 inhibitors as potential anti-cancer therapies, it is imperative to understand the breadth of functions associated with MNK1/2 in tumour biology. This study reveals a novel MNK1-driven transcriptional programme that helps promote the progression of melanoma to invasive and metastatic disease. Previous studies have largely focused on the role of MNK1 in oncogenic mRNA translation (8). However, our study proposes a new paradigm for MNK1-mediated invasion, via positively regulating the expression of *ANGPTL4* and *MMP*s that encode for proteins with known roles in invasion and metastasis. Our experiments have indicated that elevated MNK1 activity leads to increased invasion, suggesting that upstream signals in melanoma, such as pathologic MAPK signaling, can lead to disease progression via MNK1. Decreased MNK1 activity, using genetic ablation or pharmacological interventions, has previously been reported to be effective in blocking *KIT* mutant melanoma metastasis and hindering the transition from ductal

carcinoma *in situ* to invasive disease (8, 24). In this study, we expand the therapeutic repertoire of therapeutically blocking MNK1/2 to include $BRAF^{V600E}$-mutant melanoma, which is the most common form of cutaneous melanoma (7, 25).

Our proposed mechanism suggests that MNK1 might act early in the metastatic cascade. We predict that increased expression and/or activity of MNK1 would lead to an upregulation of ANGPTL4. This in turn orchestrates the subsequent upregulation of MMPs needed to degrade the ECM and enable tumour cells to intravasate (Figure 5C). Once a tumor cell seeds a metastatic site, while MNK1 expression may no longer be needed for MNK1-mediated ECM remodeling, perhaps MNK1 expression is needed for tumor cell outgrowth. Finally, future work in our lab will focus on using MNK1 inhibitors such as SEL201 in combination with immunotherapies to induce a synergistic effect to prevent both tumor metastasis and stimulate anti-tumor immunity in melanoma patients.

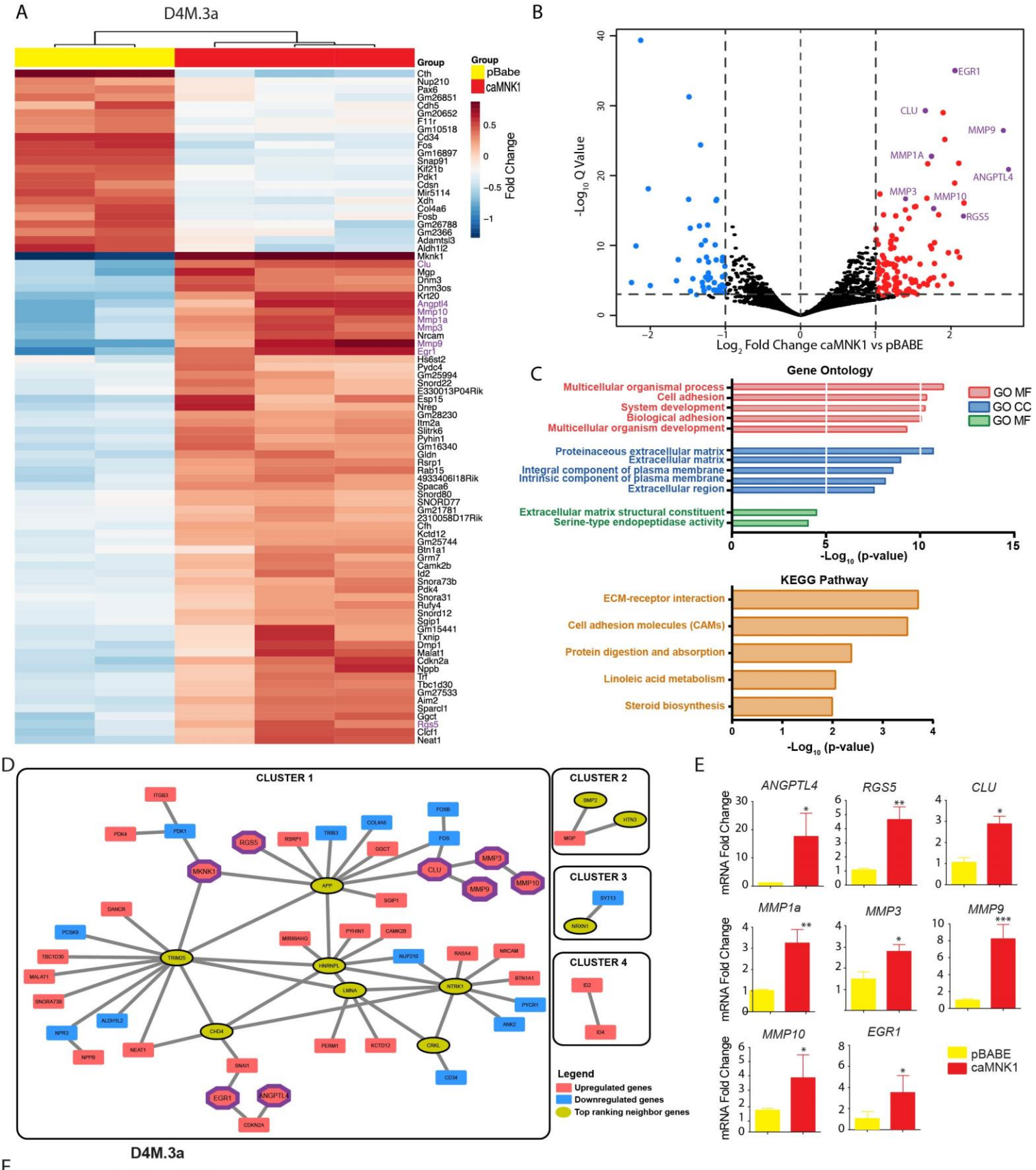

A
D4M.3a
Group
pBabe
caMNK1
Fold Change
Cth
Nup210
Pax6
Gm26851
Cdh5
Gm20652
F11r
Gm10518
Cd34
Fos
Gm16897
Snap91
Kif21b
Pdk1
Cdsn
Mir5114
Xdh
Col4a6
Fosb
Gm26788
Gm2366
Adamtsl3
Aldh1l2
Mknk1
Clu
Mgp
Dnm3
Dnm3os
Krt20
Angptl4
Mmp10
Mmp1a
Mmp3
Nrcam
Mmp9
Egr1
H6st2
Pydc4
Gm25994
Snord22
E330013P04Rik
Esp15
Nrep
Gm28230
Itm2a
Slitrk6
Pyhin1
Gm16340
Gldn
Rsrp1
Rab15
4933406I18Rik
Spaca6
Snord80
SNORD77
Gm21781
2310058D17Rik
Cfh
Kctd12
Gm25744
Btn1a1
Grm7
Camk2b
Id2
Snora73b
Pdk4
Snora31
Rufy4
Snord12
Sgip1
Gm15441
Txnip
Dmp1
Malat1
Cdkn2a
Nppb
Ttf
Tbc1d30
Gm27533
Aim2
Sparcl1
Ggct
Rgs5
Clcf1
Neat1
B
-Log10 Q Value
Log2 Fold Change caMNK1 vs pBABE
EGR1
CLU
MMP9
MMP1A
ANGPTL4
MMP3
MMP10
RGS5
C
Gene Ontology
Multicellular organismal process
Cell adhesion
System development
Biological adhesion
Multicellular organism development
Proteinaceous extracellular matrix
Extracellular matrix
Integral component of plasma membrane
Intrinsic component of plasma membrane
Extracellular region
Extracellular matrix structural constituent
Serine-type endopeptidase activity
GO MF
GO CC
GO MF
-Log10 (p-value)
KEGG Pathway
ECM-receptor interaction
Cell adhesion molecules (CAMs)
Protein digestion and absorption
Linoleic acid metabolism
Steroid biosynthesis
-Log10 (p-value)
D
CLUSTER 1
CLUSTER 2
CLUSTER 3
CLUSTER 4
ITGB3
PDK4
PDK1
RGS5
RSRP1
TRIB3
COL4A6
FOSB
FOS
GGCT
MMP3
CLU
MMP10
MMP9
MKNK1
APP
SGIP1
DANCR
PCSK9
TBC1D30
MALAT1
SNORA73B
TRIM25
MIR99AHG
PYHIN1
CAMK2B
HNRNPL
NUP210
RASA4
NRCAM
BTN1A1
LMNA
NTRK1
PYCR1
ANK2
ALDH1L2
NPR3
NPPB
CHD4
NEAT1
SNAI1
EGR1
ANGPTL4
CDKN2A
PERM1
KCTD12
CRKL
CD34
BMP2
HTN3
MGP
SYT13
NRXN1
ID2
ID4
Legend
Upregulated genes
Downregulated genes
Top ranking neighbor genes
D4M.3a
E
ANGPTL4
RGS5
CLU
MMP1a
MMP3
MMP9
MMP10
EGR1
mRNA Fold Change
pBABE
caMNK1
F
pBABE
caMNK1
ANGPTL4
MMP3

Fig. 1. MNK1/2 mediates transcription of downstream targets involved in tumour progression. A. Heatmap showing differential gene expression between D4M.3a pBABE and caMNK cells. **B.** Volcano plot showing genes significantly upregulated and downregulated in D4M.3a caMNK cells relative to pBABE cells. **C.** GO and KEGG pathway analysis regulated in D4M.3a caMNK cells compared to pBABE cells. **D.** The sub-network module obtained from the PPI network. Red node stands for upregulated gene and blue node stands for downregulated gene, while yellow node stands for top-ranked neighbour gene. Purple circle genes were selected for validation. **E.** Overexpression of caMNK1 in D4M.3a cells increases the mRNA levels of pro-tumourigenic genes, including ANGPTL4, MMP1a, MMP3, MMP9, MMP10, RGS5, CLU, and EGR1. **F.** MMP3 and ANGPTL4 levels are increased in caMNK cells compared to pBABE cells, as gauged by Western blot. MMP9 activity is similarly increased in caMNK cells as gauged by zymography.

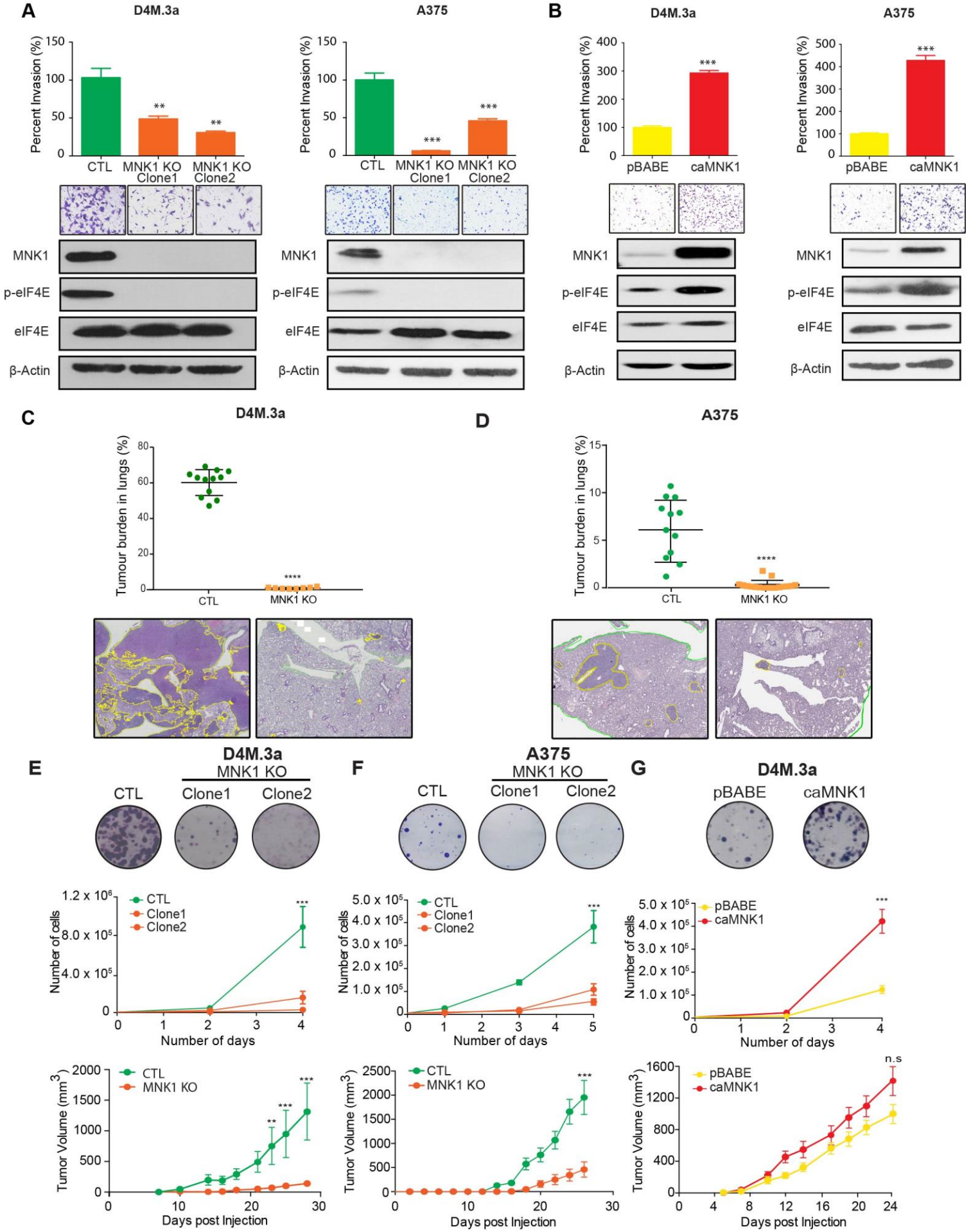

A
D4M.3a
A375
Percent Invasion (%)
CTL
MNK1 KO Clone1
MNK1 KO Clone2
MNK1
p-eIF4E
eIF4E
β-Actin
B
pBABE
caMNK1
C
D4M.3a
Tumour burden in lungs (%)
CTL
MNK1 KO
D
A375
E
D4M.3a
MNK1 KO
Clone1
Clone2
F
A375
MNK1 KO
G
D4M.3a
pBABE
caMNK1
Number of cells
Number of days
Tumor Volume (mm3)
Days post Injection
n.s

Fig. 2. **MNK1/2 promote melanoma invasion, metastasis and proliferation. A**. D4M.3a and A375 MNK1/2 double KO clones showed significant decrease in invasion compared to their parental controls. One-way ANOVA test was used. **B.** D4M.3a and A375 cells with caMNK displayed an increased level of invasion compared to pBABE control cells. Student's unpaired t-test was used. **C.** D4M.3a KO cells have less lung metastasis compared to control cells in an experimental model of melanoma metastasis (n=6 for WT, n=4 for MNK1 KO; 2 lung step-sections were analyzed per animal). Student's unpaired t-test was used. **D.** A375 KO cells have less lung metastasis compared to control cells in an experimental model of melanoma metastasis (n=6 for WT, n=8 for MNK1 KO, 2 lung step-sections were analyzed per animal). Student's unpaired t-test was used. **E, F.** MNK1/2 deficiency inhibits colony formation and cell proliferation of D4M.3a and A375 cells. (n=1 for clonogenics, n=3 for cell proliferation assay). D4M.3a (WT n=5 animals; MNK KO n=8 animals) and A375 MNK1 KO (WT n=8 animals; MNK KO n=7 animals) tumours present with growth disadvantage compared to control tumours. Two-way ANOVA analysis was used. **G**. caMNK1 overexpression enhances D4M.3a colony formation and cell proliferation (n=1 for clonogenics, n=3 for cell proliferation assay). caMNK1-expressing D4M.3a tumours show slight growth advantage over pBABE control tumours (n=10 each group, not significant). Two-way ANOVA analysis was used. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. All experiments were conducted in biological triplicate unless otherwise stated. Error bars represent SEM.

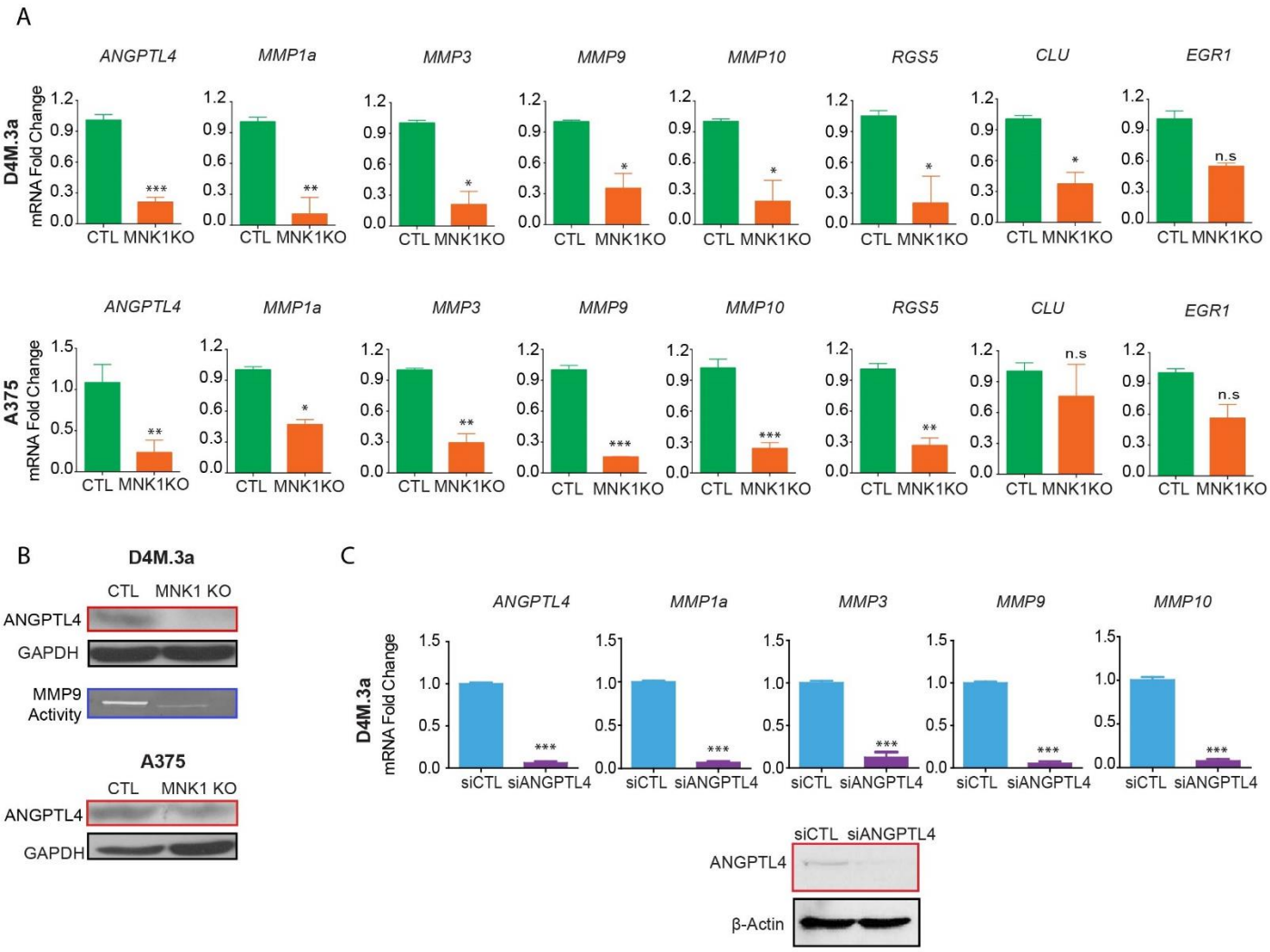

A
ANGPTL4
MMP1a
MMP3
MMP9
MMP10
RGS5
CLU
EGR1
D4M.3a
A375
mRNA Fold Change
CTL MNK1KO
n.s
B
D4M.3a
CTL MNK1 KO
ANGPTL4
GAPDH
MMP9 Activity
A375
C
siCTL siANGPTL4
β-Actin
D
Percent Invasion (%)
pBABE
caMNK1
E
pBABE
caMNK
F
rANGPTL4
G

Fig. 3. MNK regulates expression of pro-invasive genes in melanoma. A. MNK1 KO in D4M.3a and A375 cells decreases the mRNA levels of pro-tumourigenic genes. Student's unpaired t-test was used. **B.** MMP9 and ANGPTL4 protein levels were examined by zymography, and their levels were downregulated upon MNK KO. **C.** Knockdown of ANGPTL4 using siRNA significantly decreased mRNA levels of pro-tumourigenic genes. Student's unpaired t-test was used. **D.** Knockdown of ANGPTL4 using siRNA in pBABE and caMNK melanoma cells significantly decreased cancer cell invasion. One-way ANOVA analysis was used. **E.** D4M.3a pBABE and caMNK with ANGPTL4 knockdown displayed a significant decrease in MMP9 activity as examined by zymography. **F,G.** Invasion defects in D4M.3a MNK KO cells were rescued by addition of recombinant ANGPTL4 protein. One-way ANOVA analysis was used. *p <0.05, **p < 0.01, ***p < 0.001. ****p <0.0001. All experiments conducted in biological triplicate. All error bars represent SEM.

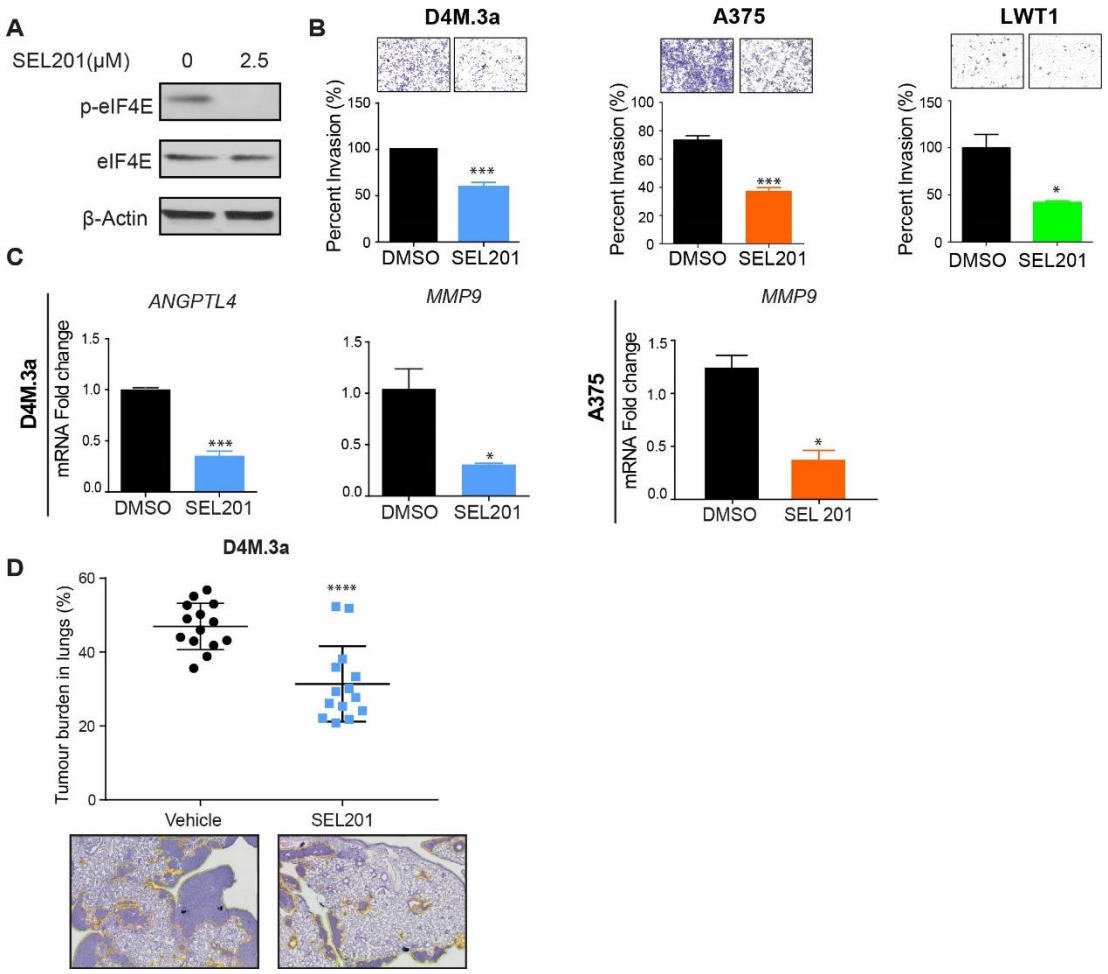
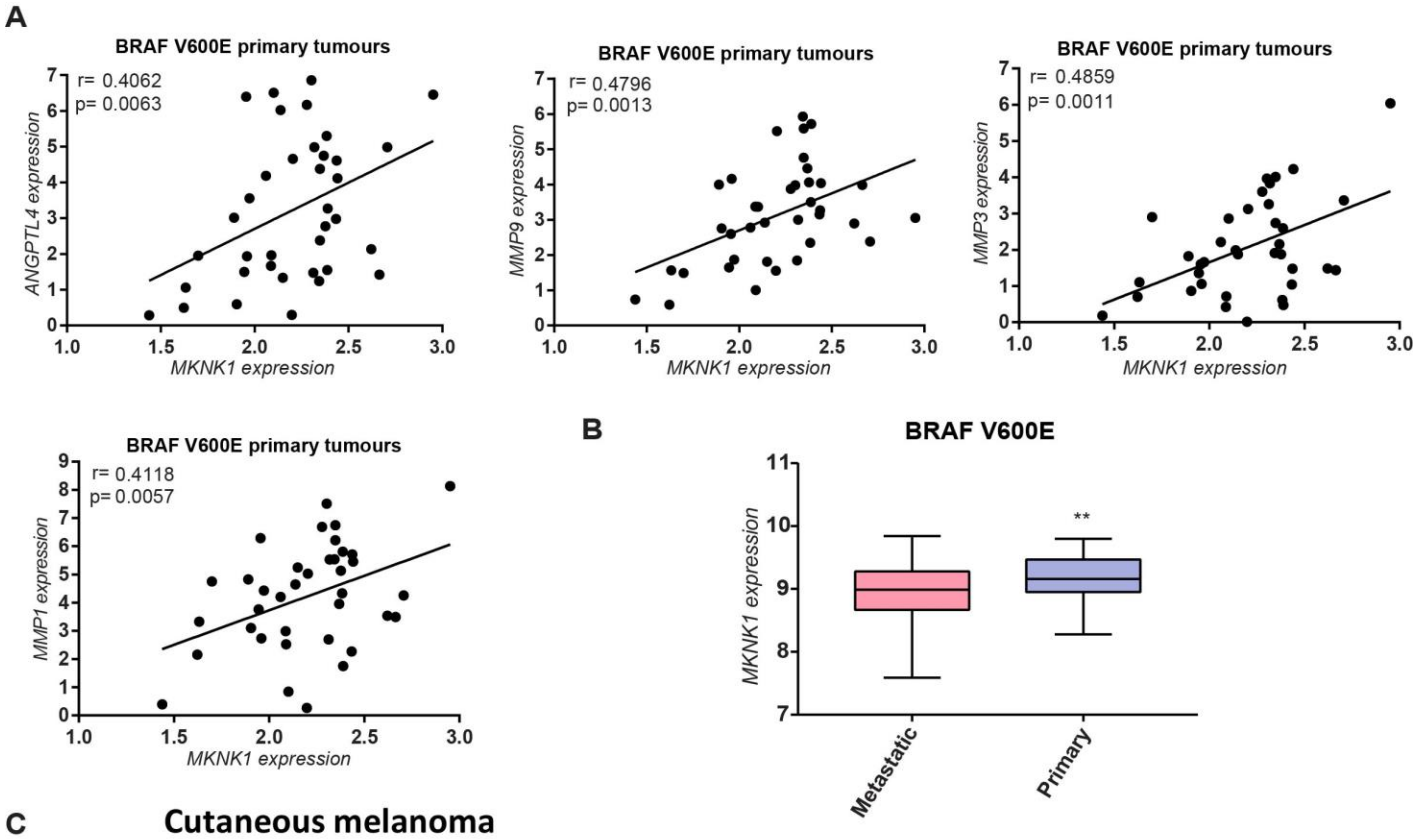


Fig. 4. SEL201 treatment decreases invasion in mouse and human melanoma models. A. SEL201 significantly inhibits MNK function, as shown by decreased levels of phospho-eIF4E in D4M.3a cells. **B.** SEL201 decreases invasion *in vitro* in D4M.3a ,A375 and LWT1 melanoma cells. 2.5μM of SEL201 was used for treatment. Student's unpaired t-test was used. **C**. SEL201 decreases ANGPTL4 and MMP9 mRNA levels in melanoma cells. 2.5μM of SEL201 was used for treatment. Student's unpaired t-test was used. **D.** Animals treated with SEL201 had significantly lower lung metastatic burden compared to control groups in D4M.3a experimental model of melanoma metastasis (n=7 animals for each group, 2 lung step-sections analyzed per animal). Student's unpaired t-test was used. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. All experiments conducted in biological triplicate unless otherwise stated. Error bars represent SEM.


A
BRAF V600E primary tumours
r= 0.4062
p= 0.0063
ANGPTL4 expression
MKNK1 expression
BRAF V600E primary tumours
r= 0.4796
p= 0.0013
MMP9 expression
MKNK1 expression
BRAF V600E primary tumours
r= 0.4859
p= 0.0011
MMP3 expression
MKNK1 expression
BRAF V600E primary tumours
r= 0.4118
p= 0.0057
MMP1 expression
MKNK1 expression
B
BRAF V600E
MKNK1 expression
**
Metastatic
Primary

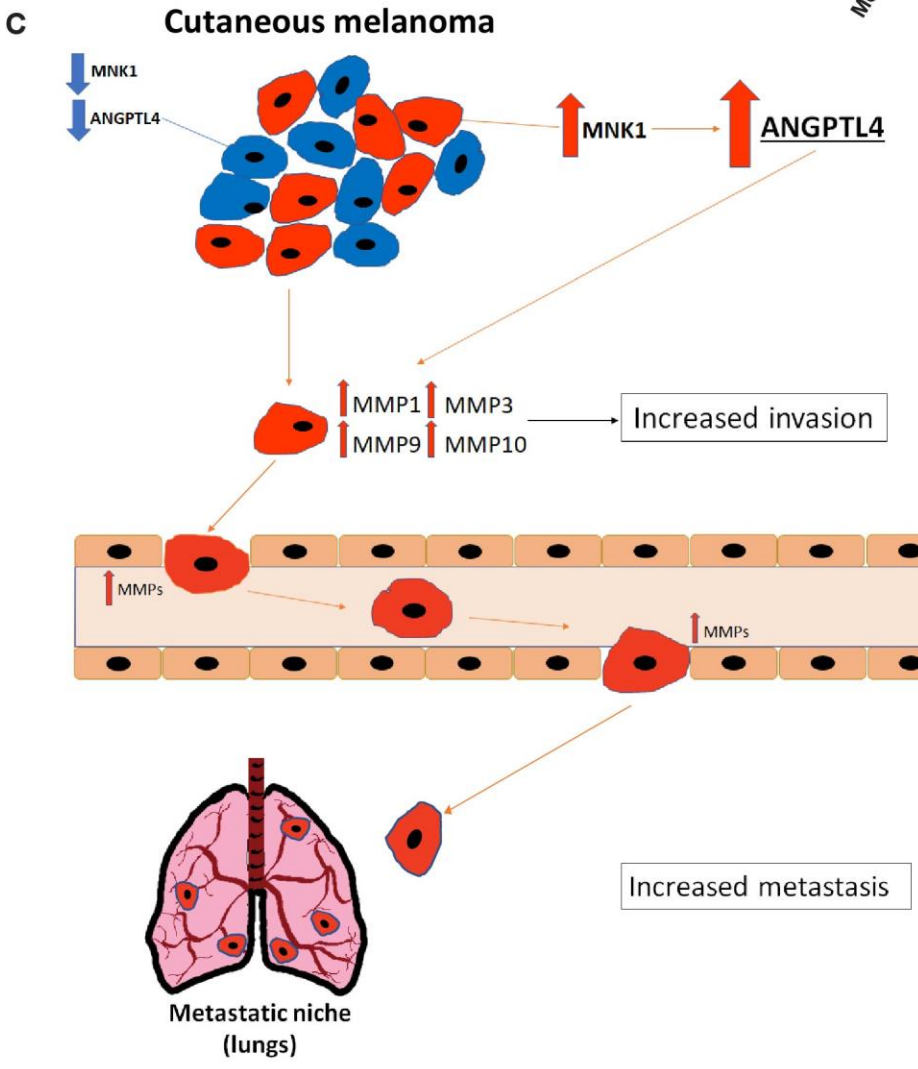
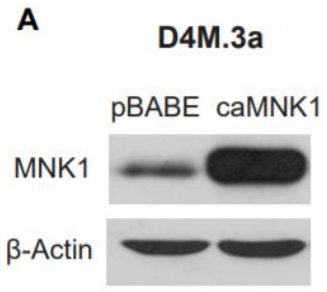

C
Cutaneous melanoma
MNK1
ANGPTL4
MNK1
ANGPTL4
MMP1
MMP3
MMP9
MMP10
Increased invasion
MMPs
MMPs
Increased metastasis
Metastatic niche
(lungs)

Fig. 5. Clinical relevance of MNK1/2 in melanoma progression. A. TCGA analysis revealed that ANGPTL4, MMP9, MMP3, and MMP1 expression in melanoma patients directly correlates with MNK1 expression. **B.** MNK1 is more highly expressed in primary BRAF mutant melanoma tumours compared to tumours from a metastatic site (**$p<0.01$). **C.** A model depicting the role of MNK1 in regulating melanoma metastasis through ANGPTL4 and MMP activation.



Supplementary Figure 1. Western blot analysis of pBABE and caMNK1 expressing D4M.3a cells. A. caMNK1 cells with MNK1^{T332D} variant have significantly higher levels of MNK1 compare to pBABE control cells.

2.6 References

1 Larkin J, Ascierto PA, Dreno B, Atkinson V, Liszkay G, Maio M *et al*. Combined vemurafenib and cobimetinib in BRAF-mutated melanoma. *N Engl J Med* 2014; 371: 1867-1876.

2 Robert C, Karaszewska B, Schachter J, Rutkowski P, Mackiewicz A, Stroiakovski D *et al*. Improved Overall Survival in Melanoma with Combined Dabrafenib and Trametinib. *New Engl J Med* 2015; 372: 30-39.

3 Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB *et al*. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med* 2010; 363: 711-723.

4 Larkin J, Hodi FS, Wolchok JD. Combined Nivolumab and Ipilimumab or Monotherapy in Untreated Melanoma. *N Engl J Med* 2015; 373: 1270-1271.

5 Domingues B, Lopes JM, Soares P, Populo H. Melanoma treatment in review. *Immunotargets Ther* 2018; 7: 35-49.

6 Geller AC, Annas GD. Epidemiology of melanoma and nonmelanoma skin cancer. *Semin Oncol Nurs* 2003; 19: 2-11.

7 Cancer Genome Atlas N. Genomic Classification of Cutaneous Melanoma. *Cell* 2015; 161: 1681-1696.

8 Zhan Y, Guo J, Yang W, Goncalves C, Rzymski T, Dreas A *et al*. MNK1/2 inhibition limits oncogenicity and metastasis of KIT-mutant melanoma. *The Journal of clinical investigation* 2017; 127: 4179-4192.

9 Robichaud N, Del Rincon SV, Huor B, Alain T, Petruccelli LA, Hearnden J *et al*. Phosphorylation of eIF4E promotes EMT and metastasis via translational control of SNAIL and MMP-3. *Oncogene* 2015; 34: 2032-2042.

10 Ramalingam S, Gediya L, Kwegyir-Afful AK, Ramamurthy VP, Purushottamachar P, Mbatia H *et al*. First MNKs degrading agents block phosphorylation of eIF4E, induce apoptosis,

inhibit cell growth, migration and invasion in triple negative and Her2-overexpressing breast cancer cell lines. *Oncotarget* 2014; 5: 530-543.

11 Guo Z, Peng G, Li E, Xi S, Zhang Y, Li Y *et al*. MAP kinase-interacting serine/threonine kinase 2 promotes proliferation, metastasis, and predicts poor prognosis in non-small cell lung cancer. *Sci Rep* 2017; 7: 10612.

12 Guo Q, Li VZ, Nichol JN, Huang F, Yang W, Preston SEJ *et al*. MNK1/NODAL Signaling Promotes Invasive Progression of Breast Ductal Carcinoma In Situ. *Cancer Res* 2019; 79: 1646-1657.

13 Deryugina EI, Quigley JP. Matrix metalloproteinases and tumor metastasis. *Cancer Metastasis Rev* 2006; 25: 9-34.

14 Joshi S, Platanias LC. Mnk kinase pathway: Cellular functions and biological outcomes. *World J Biol Chem* 2014; 5: 321-333.

15 Dankort D, Curley DP, Cartlidge RA, Nelson B, Karnezis AN, Damsky WE, Jr. *et al*. Braf(V600E) cooperates with Pten loss to induce metastatic melanoma. *Nat Genet* 2009; 41: 544-552.

16 Liu L, Zhuang X, Jiang M, Guan F, Fu Q, Lin J. ANGPTL4 mediates the protective role of PPARgamma activators in the pathogenesis of preeclampsia. *Cell Death Dis* 2017; 8: e3054.

17 Gialeli C, Theocharis AD, Karamanos NK. Roles of matrix metalloproteinases in cancer progression and their pharmacological targeting. *FEBS J* 2011; 278: 16-27.

18 Hu K, Babapoor-Farrokhran S, Rodrigues M, Deshpande M, Puchner B, Kashiwabuchi F *et al*. Hypoxia-inducible factor 1 upregulation of both VEGF and ANGPTL4 is required to promote the angiogenic phenotype in uveal melanoma. *Oncotarget* 2016; 7: 7816-7828.

19 Wang JH, Huang WS, Hu CR, Guan XX, Zhou HB, Chen LB. Relationship between RGS5 expression and differentiation and angiogenesis of gastric carcinoma. *World J Gastroenterol* 2010; 16: 5642-5646.

20 Redondo M, Tellez T, Roldan MJ. The role of clusterin (CLU) in malignant transformation and drug resistance in breast carcinomas. *Adv Cancer Res* 2009; 105: 21-43.

21 Baron V, Adamson ED, Calogero A, Ragona G, Mercola D. The transcription factor Egr1 is a direct regulator of multiple tumor suppressors including TGFbeta1, PTEN, p53, and fibronectin. *Cancer Gene Ther* 2006; 13: 115-124.

22 Liao YH, Chiang KH, Shieh JM, Huang CR, Shen CJ, Huang WC *et al*. Epidermal growth factor-induced ANGPTL4 enhances anoikis resistance and tumour metastasis in head and neck squamous cell carcinoma. *Oncogene* 2017; 36: 2228-2242.

23 Djeu JY, Wei S. Clusterin and chemoresistance. *Adv Cancer Res* 2009; 105: 77-92.

24 Guo Q, Li VZ, Nichol JN, Huang F, Yang W, Preston SEJ *et al*. MNK1/NODAL Signaling Promotes Invasive Progression of Breast Ductal Carcinoma In Situ. *Cancer Res* 2019.

25 Ascierto PA, Kirkwood JM, Grob JJ, Simeone E, Grimaldi AM, Maio M *et al*. The role of BRAF V600 mutation in melanoma. *J Transl Med* 2012; 10: 85.

26 Pettersson F, Del Rincon SV, Emond A, Huor B, Ngan E, Ng J *et al*. Genetic and pharmacologic inhibition of eIF4E reduces breast cancer cell migration, invasion, and metastasis. *Cancer Res* 2015; 75: 1102-1112.

3. Future Directions, Discussion and Conclusion

If we are to have a take-home message from my research, it is that MNK1 expression and activity play an important role in melanoma progression via the upregulation of pro-invasive and pro-tumourigenic genes. We show through various *in vitro* methods, that cells with increased MNK1 activity have a more invasive phenotype. We confirm the importance of MNK1 for metastatic progression and primary tumour outgrowth using an *in vivo* model. We show that decreased levels of MNK1, either genetically of pharmacologically, negatively impacted primary $BRAF^{V600E}$ melanoma outgrowth, and metastasis to the lungs in syngeneic and xenograft models of melanoma. These important pieces of data gave us the reasoning needed to further analyze how those phenotypes were being produced.

Previously, MNK1 has been well-studied to phosphorylate eIF4E and promote tumourigenesis through that avenue. However, additional roles for MNK1 are just beginning to be investigated, and we wanted to see whether an overactivation of MNK1 in melanoma cells would impact gene expression. We proceeded to perform RNA sequencing analysis on melanoma cells expressing a constitutively active MNK1, or their pBABE control counterparts in the D4M.3a cell line, since this is a syngeneic murine model of melanoma that we routinely use in the lab for in vitro and in vivo studies. Our analysis of the RNA sequencing data showed that cells with overactive MNK1 have an overexpression of genes responsible in invasion and many extracellular processes. When we looked more in details at those genes, we found that constitutively active MNK1 promoted the expression of genes responsible in ECM degradation such as *MMP1a, MMP3, MMP9, MMP10* and *ANGPTL4*. *ANGPTL4* was previously unknown to us in the context of melanoma, nonetheless it has been shown to be a key regulator of the MMPs, notably *MMP1, MMP3, MMP9* and *MMP10* (59, 67). On the other hand, ANGPTL4 expression has been shown

to inversely correlate with MMP9 and MMP2 inhibitors, tissue inhibitor of metalloproteinase-1 (TIMP-1) and tissue inhibitor of metalloproteinase-2 (TIMP-2) (87). With those data in hand, we pursued the novel hypothesis that MNK1 is upstream of ANGPTL4, and that ANGPTL4 in turn regulates MMP expression. Not only did we identify genes responsible in invasion, but we also identified genes in ca-MNK1 expressing cells which were responsible in proliferation, such as *EGR1* and *RGS5*. This also supported our previous data that melanoma cells lacking MNK1 showed less proliferation both *in vivo* and *in vitro*. Our data, however, showed no significant difference in the tumour volume between the D4M.3a pBABE-experssing and D4M.3a caMNK1-expressing cells. It is possible that MNK1 activity is at its peak in D4M.3a cells, and thus increasing the activity of the kinase is unable to result in a further increase on tumor outgrowth. It is also plausible that some feedback mechanism, yet to be identified, is responsible for limiting the activity of a constitutively active MNK1 *in vivo*.

Another point of our study was to show that MNK1 is an important potential therapeutic target to treat *BRAF*-mutant melanoma, the most common form of cutaneous melanoma. By using SEL201 we were able to show, using an experimental model of metastasis, that the inhibition of MNK1/2 blocks tumor cell colonization in the lungs of mice. Moreover, we used publicly available data from the TCGA (Figure 5) to show that *MKNK1* expression correlates with our genes of interest; *ANGPTL4, MMP9, MMP1A* and *MMP3*. Together our data support the proposition to further investigate MNK1/2 as a novel therapeutic target in melanoma, perhaps in combination with other drugs. Recent studies have in fact shown great promise in the combination of kinase inhibitors and PD1/PDL1 inhibitors for example (84). For instance, continuous inhibition of receptor tyrosine kinase EGFR in non-small cell lung cancer leads to an increase in the expression of PDL1 which leads to resistance to the tyrosine kinase inhibitors and cancer progression (85).

This resistance can in theory be overcome with anti-PDL1/PD1 treatment. Activation of RAS/RAF/MEK/ERK kinases have also been shown to play inhibitory roles on T-cells activity which further supports combination with immunotherapy (84). Combination of immunotherapy and kinase inhibitors have also shown great pre-clinical synergy in renal cell carcinoma and head and neck squamous cell carcinoma (HNSCC) (84). Interestingly, the expression of PDL1 is under translational control (86). Ongoing work in our group is looking at combining MNK1/2 inhibitors with anti-PDL1/PD1 treatment in our pre-clinical mouse models of cancer.

Work is currently ongoing in the lab to determine the mechanistic link between ANGPTL4 and MNK1. One venue we will be pursuing is to assess the role of PPARγ downstream of MNK1. Previous findings have linked the regulation of ANGPTL4 with the activity of PPARγ (67). Many experiments could be interesting to address whether the regulation of *ANGPTL4* by MNK1 is PPARγ dependent. We could start by looking at PPARγ levels in the MNK1 KO cell lines and determine whether its expression is downregulated, compared to cells expressing MNK1. Furthermore, we could investigate whether knockdown of PPARγ in cells with MNK1 replicates what is seen in the MNK1KO cells in terms of decreased *ANGPTL4* expression. Moreover, we can assess the functional role that PPARγ has in our MNK1-modified cell lines. For example, we can assess the impact of treating the MNK1-modified cells with PPAR antagonists and PPAR agonists, and looking at the regulation of *ANGTPL4*. We would predict that treatment of caMNK1-expressing cells with a PPAR antagonist might cause a downregulation of *ANGPTL4* expression, if PPARγ activity is important for the link between MNK1 and ANGPTL4. With all those experiments lined up, we hypothesize that MNK1 may play a role in the regulation of PPARγ, which in turn acts upon *ANGPTL4* expression.

Another path our research leads into, is the role of ANGPTL4 in tumor biology. We showed that MNK1 knockout and expression of caMNK1 in melanoma cells caused drastic changes in tumor phenotypes both *in vivo* and *in vitro*. However, how much of the MNK1-induced effects are attributable to ANGPTL4, is a question we will try to answer by regulating the activity of ANGPTL4. We could generate cell lines devoid of ANGPTL4 and confirm how much this knockout abrogates metastasis compared to their control counterparts. Conversely, we could assess the impact of overexpressing ANGPTL4 in the MNK1KO cells, to determine whether ANGPTL4 alone can rescue the observed defects in invasion and metastasis. Studying ANGPTL4 alone can further lead us to understand how MNK1 affects cancer progression.

Furthermore, we have yet but scratched the surface on the many roles of MNK1. Another aspect we will investigate in our lab which is a direct link to my research, is the specific role MNK1 has in the nucleus. In fact, we have already started creating the cellular tools necessary in order to study the role of MNK1 in the nucleus. MNK1 KO cells will be modulated to express MNK1 constructs that have restricted expression in the cytoplasm or the nucleus. MNK1 will be mutated in the following way: a single point mutation in either the nuclear localization signal (NLS, R,26,27,28A) or in the nuclear export signal (NES, L390S). Those single point mutations will give us cytoplasmic MNK1, as MNK1 cannot enter the nucleus without this signal, and nuclear MNK1 as MNK1 cannot exit the nucleus without this signal, respectively. Those tools will help us isolate the effects that MNK1 can have in the nucleus, and the cytoplasm, and uncover novel roles for this kinase.

To conclude, our data shows that MNK1/2 is critical in the formation of metastases and tumour growth in melanoma. We also show that MNK1/2 is a regulator of many pro-oncogenic genes.

Finally, blocking MNK1/2 with novel inhibitors and genetically, drastically reduced tumourigenicity and cancer progression.

4. Master references

1. Halpern A, Marghoob A, Reiter O. Melanoma Overview Skin Cancer Foundation2019 [July 2019]. Available from: https://www.skincancer.org/skin-cancer-information/melanoma/#panel1-5.

2. Leonardi GC, Falzone L, Salemi R, Zanghi A, Spandidos DA, McCubrey JA, et al. Cutaneous melanoma: From pathogenesis to therapy (Review). Int J Oncol. 2018;52(4):1071-80.

3. Dubey S, Roulin A. Evolutionary and biomedical consequences of internal melanins. Pigment Cell Melanoma Res. 2014;27(3):327-38.

4. Rastrelli M, Tropea S, Rossi CR, Alaibac M. Melanoma: epidemiology, risk factors, pathogenesis, diagnosis and classification. In Vivo. 2014;28(6):1005-11.

5. Society AC. Risk Factors for Melanoma Skin Cancer 2016 [updated 20 may 2016July 2019]. Available from: https://www.cancer.org/cancer/melanoma-skin-cancer/causes-risks-prevention/risk-factors.html.

6. Curiel-Lewandrowski C. Risk factors for the development of melanoma. In: Corona R, editor. Melanoma. UpToDate2019.

7. Whiteman DC, Green AC, Olsen CM. The Growing Burden of Invasive Melanoma: Projections of Incidence Rates and Numbers of New Cases in Six Susceptible Populations through 2031. J Invest Dermatol. 2016;136(6):1161-71.

8. Vincensi MR, d'Ischia M, Napolitano A, Procaccini EM, Riccio G, Monfrecola G, et al. Phaeomelanin versus eumelanin as a chemical indicator of ultraviolet sensitivity in fair-skinned subjects at high risk for melanoma: a pilot study. Melanoma Res. 1998;8(1):53-8.

9. Mitra D, Luo X, Morgan A, Wang J, Hoang MP, Lo J, et al. An ultraviolet-radiation-independent pathway to melanoma carcinogenesis in the red hair/fair skin background. Nature. 2012;491(7424):449-53.

10. Lucas RM, McMichael AJ, Armstrong BK, Smith WT. Estimating the global disease burden due to ultraviolet radiation exposure. Int J Epidemiol. 2008;37(3):654-67.

11. Beaumont KA, Liu YY, Sturm RA. The melanocortin-1 receptor gene polymorphism and association with human skin cancer. Prog Mol Biol Transl Sci. 2009;88:85-153.

12. Heckman CJ, Coups EJ, Manne SL. Prevalence and correlates of indoor tanning among US adults. J Am Acad Dermatol. 2008;58(5):769-80.

13. Schadendorf D, Hauschild A. Melanoma in 2013: Melanoma--the run of success continues. Nat Rev Clin Oncol. 2014;11(2):75-6.

14. Armstrong BK, Kricker A. The epidemiology of UV induced skin cancer. J Photochem Photobiol B. 2001;63(1-3):8-18.

15. Ferlay J, Colombet M, Soerjomataram I, Mathers C, Parkin DM, Pineros M, et al. Estimating the global cancer incidence and mortality in 2018: GLOBOCAN sources and methods. Int J Cancer. 2019;144(8):1941-53.

16. Whiteman DC, Pavan WJ, Bastian BC. The melanomas: a synthesis of epidemiological, clinical, histopathological, genetic, and biological aspects, supporting distinct subtypes, causal pathways, and cells of origin. Pigment Cell Melanoma Res. 2011;24(5):879-97.

17. Schadendorf D, Fisher DE, Garbe C, Gershenwald JE, Grob JJ, Halpern A, et al. Melanoma. Nat Rev Dis Primers. 2015;1:15003.

18. Halpern AM, A., Reiter O. Melanoma Warning Signs 2019 [updated April 2019. Available from: https://www.skincancer.org/skin-cancer-information/melanoma/melanoma-warning-signs-and-images/.

19. Grob JJ, Bonerandi JJ. The 'ugly duckling' sign: identification of the common characteristics of nevi in an individual as a basis for melanoma screening. Arch Dermatol. 1998;134(1):103-4.

20. Vestergaard ME, Macaskill P, Holt PE, Menzies SW. Dermoscopy compared with naked eye examination for the diagnosis of primary melanoma: a meta-analysis of studies performed in a clinical setting. Br J Dermatol. 2008;159(3):669-76.

21. Elwood JM. Screening for melanoma and options for its evaluation [see comment]. J Med Screen. 1994;1(1):22-38.

22. Hansson J, Bergenmar M, Hofer PA, Lundell G, Mansson-Brahme E, Ringborg U, et al. Monitoring of kindreds with hereditary predisposition for cutaneous melanoma and dysplastic nevus syndrome: results of a Swedish preventive program. J Clin Oncol. 2007;25(19):2819-24.

23. Balch CM, Gershenwald JE, Soong SJ, Thompson JF, Atkins MB, Byrd DR, et al. Final version of 2009 AJCC melanoma staging and classification. J Clin Oncol. 2009;27(36):6199-206.

24. Davis E, Johnson D, Sosman J, Chandra S. Melanoma: What do all the mutations mean? Cancer. 2018;14(17):3490-9.

25. Shain AH, Bastian BC. From melanocytes to melanomas. Nat Rev Cancer. 2016;16(6):345-58.

26. Read J, Wadt KA, Hayward NK. Melanoma genetics. J Med Genet. 2016;53(1):1-14.

27. Box NF, Vukmer TO, Terzian T. Targeting p53 in melanoma. Pigment Cell Melanoma Res. 2014;27(1):8-10.

28. Cheaib B, Auguste A, Leary A. The PI3K/Akt/mTOR pathway in ovarian cancer: therapeutic opportunities and challenges. Chin J Cancer. 2015;34(1):4-16.

29. Munoz-Couselo E, Adelantado EZ, Ortiz C, Garcia JS, Perez-Garcia J. NRAS-mutant melanoma: current challenges and future prospect. Onco Targets Ther. 2017;10:3941-7.

30. Johannessen CM, Reczek EE, James MF, Brems H, Legius E, Cichowski K. The NF1 tumor suppressor critically regulates TSC2 and mTOR. Proc Natl Acad Sci U S A. 2005;102(24):8573-8.

31. Iida Y, Salomon MP, Hata K, Tran K, Ohe S, Griffiths CF, et al. Predominance of triple wild-type and IGF2R mutations in mucosal melanomas. BMC Cancer. 2018;18(1):1054.

32. Cancer Genome Atlas N. Genomic Classification of Cutaneous Melanoma. Cell. 2015;161(7):1681-96.

33. Beadling C, Jacobson-Dunlop E, Hodi FS, Le C, Warrick A, Patterson J, et al. KIT gene mutations and copy number in melanoma subtypes. Clin Cancer Res. 2008;14(21):6821-8.

34. Haigh PI, DiFronzo LA, McCready DR. Optimal excision margins for primary cutaneous melanoma: a systematic review and meta-analysis. Can J Surg. 2003;46(6):419-26.

35. Ascierto PA, Kirkwood JM, Grob JJ, Simeone E, Grimaldi AM, Maio M, et al. The role of BRAF V600 mutation in melanoma. J Transl Med. 2012;10:85.

36. Tang T, Eldabaje R, Yang L. Current Status of Biological Therapies for the Treatment of Metastatic Melanoma. Anticancer Res. 2016;36(7):3229-41.

37. Larkin J, Hodi FS, Wolchok JD. Combined Nivolumab and Ipilimumab or Monotherapy in Untreated Melanoma. N Engl J Med. 2015;373(13):1270-1.

38. Sullivan RJ, Flaherty KT. Resistance to BRAF-targeted therapy in melanoma. Eur J Cancer. 2013;49(6):1297-304.

39. Hodi FS, Chesney J, Pavlick AC, Robert C, Grossmann KF, McDermott DF, et al. Combined nivolumab and ipilimumab versus ipilimumab alone in patients with advanced melanoma: 2-year overall survival outcomes in a multicentre, randomised, controlled, phase 2 trial. Lancet Oncol. 2016;17(11):1558-68.

40. Espenel S, Vallard A, Rancoule C, Garcia MA, Guy JB, Chargari C, et al. Melanoma: Last call for radiotherapy. Crit Rev Oncol Hematol. 2017;110:13-9.

41. Parra-Palau JL, Scheper GC, Wilson ML, Proud CG. Features in the N and C termini of the MAPK-interacting kinase Mnk1 mediate its nucleocytoplasmic shuttling. J Biol Chem. 2003;278(45):44197-204.

42. Chin L. The genetics of malignant melanoma: lessons from mouse and man. Nat Rev Cancer. 2003;3(8):559-70.

43. Munoz-Couselo E, Garcia JS, Perez-Garcia JM, Cebrian VO, Castan JC. Recent advances in the treatment of melanoma with BRAF and MEK inhibitors. Ann Transl Med. 2015;3(15):207.

44. Zhan Y, Guo J, Yang W, Goncalves C, Rzymski T, Dreas A, et al. MNK1/2 inhibition limits oncogenicity and metastasis of KIT-mutant melanoma. J Clin Invest. 2017;127(11):4179-92.

45. Hay N. Mnk earmarks eIF4E for cancer therapy. Proc Natl Acad Sci U S A. 2010;107(32):13975-6.

46. Mendoza MC, Er EE, Blenis J. The Ras-ERK and PI3K-mTOR pathways: cross-talk and compensation. Trends Biochem Sci. 2011;36(6):320-8.

47. Goto S, Yao Z, Proud CG. The C-terminal domain of Mnk1a plays a dual role in tightly regulating its activity. Biochem J. 2009;423(2):279-90.

48. Mason JM, Morrison DJ, Bassit B, Dimri M, Band H, Licht JD, et al. Tyrosine phosphorylation of Sprouty proteins regulates their ability to inhibit growth factor signaling: a dual feedback loop. Mol Biol Cell. 2004;15(5):2176-88.

49. Yim DGR, Ghosh S, Guy GR, Virshup DM. Casein kinase 1 regulates Sprouty2 in FGF–ERK signaling. Oncogene. 2014;34:474.

50. Lemieux B, Blanchette M, Monette A, Mouland AJ, Wellinger RJ, Chabot B. A Function for the hnRNP A1/A2 Proteins in Transcription Elongation. PLoS One. 2015;10(5):e0126654.

51. Joshi S, Platanias LC. Mnk kinase pathway: Cellular functions and biological outcomes. World J Biol Chem. 2014;5(3):321-33.

52. Hou J, Lam F, Proud C, Wang S. Targeting Mnks for cancer therapy. Oncotarget. 2012;3(2):118-31.

53. Pelletier J, Graff J, Ruggero D, Sonenberg N. Targeting the eIF4F translation initiation complex: a critical nexus for cancer development. Cancer Res. 2015;75(2):250-63.

54. Truitt ML, Conn CS, Shi Z, Pang X, Tokuyasu T, Coady AM, et al. Differential Requirements for eIF4E Dose in Normal Development and Cancer. Cell. 2015;162(1):59-71.

55. Pettersson F, Del Rincon SV, Emond A, Huor B, Ngan E, Ng J, et al. Genetic and pharmacologic inhibition of eIF4E reduces breast cancer cell migration, invasion, and metastasis. Cancer Res. 2015;75(6):1102-12.

56. Zhan Y, Dahabieh MS, Rajakumar A, Dobocan MC, M'Boutchou MN, Goncalves C, et al. The role of eIF4E in response and acquired resistance to vemurafenib in melanoma. J Invest Dermatol. 2015;135(5):1368-76.

57. Saxena, Meera & Christofori, Gerhard. Rebuilding cancer metastasis in the mouse. Molecular oncology. 7. (2013).10.1016

58. Van Zijl F, Krupitza G, Mikulits W. Initial steps of metastasis: cell invasion and endothelial transmigration. Mutat Res. 2011;728(1-2):23–34.

59. Liao, Y., Chiang, K., Shieh, J. et al. Epidermal growth factor-induced ANGPTL4 enhances anoikis resistance and tumour metastasis in head and neck squamous cell carcinoma. Oncogene (2017) 36, 2228–2242

60. Ueda T, Watanabe-Fukunaga R, Fukuyama H, Nagata S, Fukunaga R. Mnk2 and Mnk1 are essential for constitutive and inducible phosphorylation of eukaryotic initiation factor 4E but not for cell growth or development. Mol Cell Biol. 2004; 24:6539–49. 10.1128/MCB.24.15.6539-6549.2004.

61. Furic L, Rong L, Larsson O, Koumakpayi IH, Yoshida K, Brueschke A, et al. eIF4E phosphorylation promotes tumorigenesis and is associated with prostate cancer progression. Proceedings of the National Academy of Sciences. 2010;107(32):14134-9.

62. Diab S., Kumarasiri M., Yu M., Teo T., Proud C., Milne R., Wang S. MAP kinase-interacting kinases - Emerging targets against cancer (2014) Chemistry and Biology, 21 (4) , pp. 441-452.

63. Lock R, Ingraham R, Maertens O, Miller AL, Weledji N, Legius E, et al. Cotargeting MNK and MEK kinases induces the regression of NF1-mutant cancers. J Clin Invest. 2016;126(6):2181-90.

64. Kardos GR, Robertson GP. Therapeutic interventions to disrupt the protein synthetic machinery in melanoma. Pigment Cell Melanoma Res. 2015;28(5):501-19.

65. Reich SH, Sprengeler PA, Chiang GG, Appleman JR, Chen J, Clarine J, et al. Structure-based Design of Pyridone-Aminal eFT508 Targeting Dysregulated Translation by Selective

Mitogen-activated Protein Kinase Interacting Kinases 1 and 2 (MNK1/2) Inhibition. J Med Chem. 2018;61(8):3516-40.

66. Guo Q, Li VZ, Nichol JN, Huang F, Yang W, Preston SEJ *et al*. MNK1/NODAL Signaling Promotes Invasive Progression of Breast Ductal Carcinoma In Situ. *Cancer Res* 2019; 79: 1646-1657.

67. Dankort D, Curley DP, Cartlidge RA, Nelson B, Karnezis AN, Damsky WE, Jr. et al. Braf(V600E) cooperates with Pten loss to induce metastatic melanoma. Nat Genet 2009; 41: 544-552.

68. Carter, J.H., *et al.* Phosphorylation of eIF4E serine 209 is associated with tumour progression and reduced survival in malignant melanoma. *British Journal Of Cancer* (2016) **114**, 444.

69. Larkin J, Ascierto PA, Dreno B, Atkinson V, Liszkay G, Maio M et al. Combined vemurafenib and cobimetinib in BRAF-mutated melanoma. N Engl J Med 2014; 371: 1867-1876.

70. Robert C, Karaszewska B, Schachter J, Rutkowski P, Mackiewicz A, Stroiakovski D et al. Improved Overall Survival in Melanoma with Combined Dabrafenib and Trametinib. New Engl J Med 2015; 372: 30-39.

71. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB et al. Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med 2010; 363: 711-723.

72. Domingues B, Lopes JM, Soares P, Populo H. Melanoma treatment in review. *Immunotargets Ther* 2018; 7: 35-49.

73. Geller AC, Annas GD. Epidemiology of melanoma and nonmelanoma skin cancer. *Semin Oncol Nurs* 2003; 19: 2-11.

74. Robichaud N, Del Rincon SV, Huor B, Alain T, Petruccelli LA, Hearnden J *et al*. Phosphorylation of eIF4E promotes EMT and metastasis via translational control of SNAIL and MMP-3. *Oncogene* 2015; 34: 2032-2042.

75. Ramalingam S, Gediya L, Kwegyir-Afful AK, Ramamurthy VP, Purushottamachar P, Mbatia H *et al*. First MNKs degrading agents block phosphorylation of eIF4E, induce apoptosis, inhibit cell growth, migration and invasion in triple negative and Her2-overexpressing breast cancer cell lines. *Oncotarget* 2014; 5: 530-543.

76. Guo Z, Peng G, Li E, Xi S, Zhang Y, Li Y *et al*. MAP kinase-interacting serine/threonine kinase 2 promotes proliferation, metastasis, and predicts poor prognosis in non-small cell lung cancer. *Sci Rep* 2017; 7: 10612.

77. Guo Q, Li VZ, Nichol JN, Huang F, Yang W, Preston SEJ *et al*. MNK1/NODAL Signaling Promotes Invasive Progression of Breast Ductal Carcinoma In Situ. *Cancer Res* 2019; 79: 1646-1657.

78. Deryugina EI, Quigley JP. Matrix metalloproteinases and tumor metastasis. *Cancer Metastasis Rev* 2006; 25: 9-34.

79. Gialeli C, Theocharis AD, Karamanos NK. Roles of matrix metalloproteinases in cancer progression and their pharmacological targeting. FEBS J 2011; 278: 16-27.

80. Hu K, Babapoor-Farrokhran S, Rodrigues M, Deshpande M, Puchner B, Kashiwabuchi F et al. Hypoxia-inducible factor 1 upregulation of both VEGF and ANGPTL4 is required to promote the angiogenic phenotype in uveal melanoma. Oncotarget 2016; 7: 7816-7828

81. Baron V, Adamson ED, Calogero A, Ragona G, Mercola D. The transcription factor Egr1 is a direct regulator of multiple tumor suppressors including TGFbeta1, PTEN, p53, and fibronectin. Cancer Gene Ther 2006; 13: 115-124.

82. Djeu JY, Wei S. Clusterin and chemoresistance. Adv Cancer Res 2009; 105: 77-92.

83. Guo Q, Li VZ, Nichol JN, Huang F, Yang W, Preston SEJ et al. MNK1/NODAL Signaling Promotes Invasive Progression of Breast Ductal Carcinoma In Situ. Cancer Res 2019.

84. García-Aranda M, Redondo M. Targeting Protein Kinases to Enhance the Response to anti-PD-1/PD-L1 Immunotherapy. Int J Mol Sci. 2019;20(9):2296. Published 2019 May 9. doi:10.3390/ijms20092296

85. Jiang, Li et al. "Continuous targeted kinase inhibitors treatment induces upregulation of PD-L1 in resistant NSCLC." Scientific reports vol. 9,1 3705. 6 Mar. 2019, doi:10.1038/s41598-018-38068-3

86. Xu Y, Poggio M, Jin HY, et al. Translation control of the immune checkpoint in cancer and its therapeutic targeting. Nat Med. 2019;25(2):301–311. doi:10.1038/s41591-018-0321-2

87. Liu L, Zhuang X, Jiang M, Guan F, Fu Q, Lin J. ANGPTL4 mediates the protective role of PPARγ activators in the pathogenesis of preeclampsia. Cell Death Dis. 2017;8(9):e3054. Published 2017 Sep 21. doi:10.1038/cddis.2017.419