PhD-FSTC-2017-45
The Faculty of Sciences, Technology and Communication

DISSERTATION

Defense held on 05/09/2017 in Luxembourg

to obtain the degree of

DOCTEUR DE L'UNIVERSITÉ DU LUXEMBOURG

EN BIOLOGIE

by

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MOLECULAR MECHANISMS OF KINASE INHIBITOR RESISTANCE IN MELANOMA

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Affidavit

I hereby confirm that the PhD thesis entitled “Molecular mechanisms of kinase inhibitor resistance in melanoma” has been written independently and without any other sources than cited.

Luxembourg, 3rd August 2017 Giulia Cesi

Only who wanders finds new paths.

Norwegian proverb

Acknowledgements

When I moved to Luxembourg four years ago, I did not know many things about myself. During this time, I have realized how ambitious and determined I can be. As many PhD students, I had difficult times but exactly those moments made me grow as a person and as a scientist.

I found out how exciting and satisfying research can be if you are willing to work hard and if you never give up.

At the end of this path, I would like to express my sincere gratitude to all those who have helped and inspired me during my doctoral studies. This thesis would not have been possible without the continuous support from many people.

First, I would like to thank my supervisor Dr. Stephanie Kreis for giving me the opportunity to work on this challenging but really fascinating topic. Most importantly, I would like to thank her for believing in me, encouraging my research and for mentoring and allowing me to grow as a research scientist.

I would like to thank Prof. Dr. Serge Haan, Dr. Bassam Janji, Prof. Dr. Dagmar Kulms and Prof. Dr. Frits Alan Thorsen for accepting to be part of my Dissertation Defense Committee. Furthermore, I am very grateful to the members of my Dissertation Supervisory Committee, Dr. Stephanie Kreis, Dr. Jochen Schneider and Dr. Laurent Vallar for having accompanied my PhD project and for having provided constructive criticism.

Special thanks go to Dr. Claude Haan, who contributed to many discussions that helped to shape this project. Most importantly, I would like to thank him for inspiring me with his enthusiasm and for sharing with me knowledge and expertise.

I would also like to thank Prof. Dr. Iris Behrman for giving me the opportunity to work in the Signal Transduction group.

I would also like to express my sincere gratitude to Florence Servais, Ines Kozar, Demetra Philippidou and Catherine Rolvering for being always there answering my questions and helping as they could. It was a great pleasure working with them. Besides the lab, they have become good friends.

These acknowledgements would not be complete without mentioning current and former members of the Signal Transduction group, Dr. Christiane Margue, Dr. Andreas Zimmer, Dr.

Geoffroy Walbrecq, Dr. Anke Baldacchino and Dr. Mélanie Kirchmeyer for their encouragement, support and friendship.

I would also like to thank our collaborators Dr. Guillaume Van Niel from the Marie Curie Institute for giving me the opportunity to visit the Marie Curie Institute and for useful advice, Dr. Yeoun Jin Kim from NantOmics and Francois Bernardin from the Luxembourg Institute of Health for performing mass spectrometry, Dr. Aurélien Ginolhac and Dr. Elisabeth Letellier from the University of Luxembourg for bioinformatics support and for productive discussions respectively and the Genomics Research Group at the Luxembourg Institute of Health, especially Laurent Valler, Dr. Petr Nazarov and Nathalie Nicot for performing microarray analysis.

Thanks also to my current and former fellow PhD students, especially Raffaella, Pit, Nicolas, Mafalda, Deborah for sharing this time with me, for their help, advice and for being always very kind to me.

I would like to express my gratitude to all the different funding sources that have supported my PhD project, the University of Luxembourg, the Doctoral School in Systems and Molecular Biomedecine, as well as the Fondation du Pélican de Mie et Pierre Hippert-Faber.

A special thanks to my family. Words cannot express how grateful I am to my mom, my dad and my brother for being as they are and for supporting all my choices even from far away.

Finally, I would like to thank my amazing husband Tommaso who spent endless weekends at home waiting for me, and was always my support in the moments when I felt lost. To him I dedicate this thesis.

Thank you all

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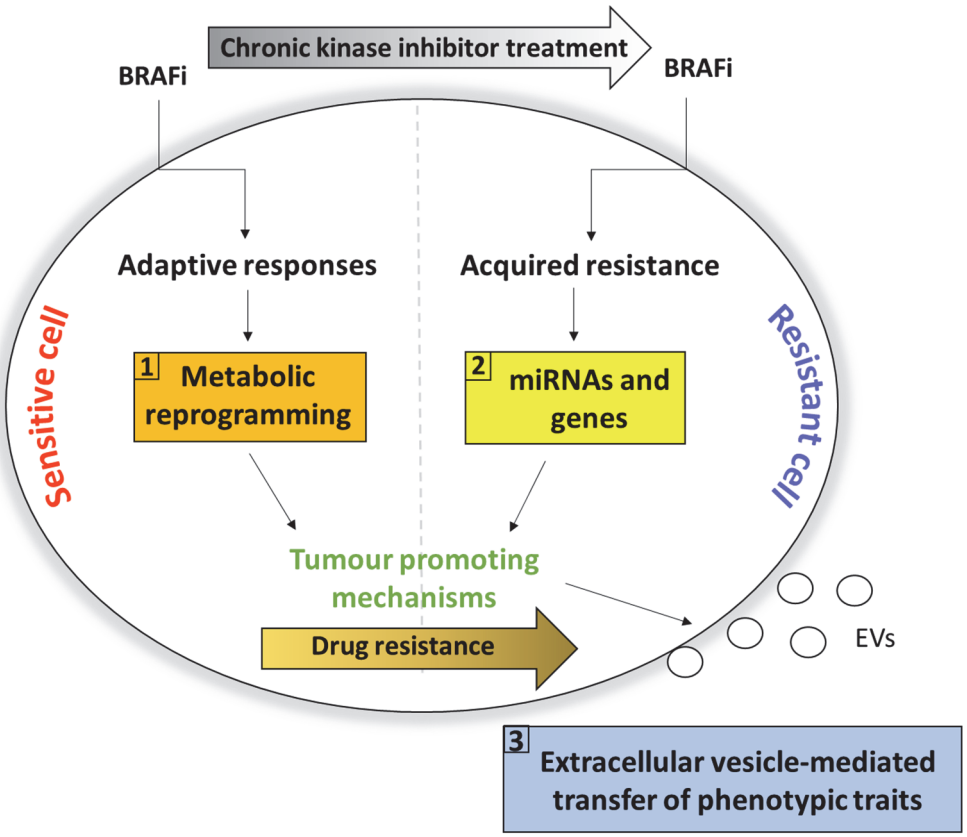
List of abbreviations

3'UTR	3' Untranslated Region
5'UTR	5' Untranslated Region
Acetyl-CoA	Acetyl Coenzyme A
Ago	Argonaute
ALK	Anaplastic Lymphoma Kinase
AMPK	AMP-Activated Protein Kinase
α-MSH	α-Melanocyte Stimulating Hormone
ATP	Adenosine Triphosphate
BRAF	B-Rapidly Accelerated Fibrosarcoma
BRAFi	BRAF Inhibitor
BRAFWT	BRAF Wild Type
CAF	Cancer-Associated Fibroblast
CDKN2A	Cyclin-Dependent Kinase Inhibitor 2A
CO2	Carbon Dioxide
COT	Cancer Osaka Thyroid
CTLA4	Cytotoxic T Lymphocyte Antigen 4
EGFR	Epidermal Growth Factor Receptor
EMT	Epidermal Mesenchymal Transition
ERK	Extracellular-Signal Regulated Kinase
ESCRT	Endosomal Sorting Complex Required for Transport
EV	Extracellular Vesicle
FAD	Flavin Adenine Dinucleotide
FDA	Food Drug Administration
FDR	False Discovery Rate
GDP	Guanosine Diphosphate
GLUT	Glucose Transporter
Grb2	Growth Factor Receptor Docking 2
GSH	Glutathione
GTP	Guanosine Triphosphate
H2O2	Hydrogen Peroxide
HIF	Hypoxia Inducible Factor
JAK	Janus Kinase
KRAS	Kirsten Rat Sarcoma Virus
LDH	Lactate Dehydrogenase
LDHA	Lactate DeHydrogenase A
MAPK	Mitogen-Activated Protein Kinase

MCT4	Monocarboxylate Transporter 4
mdr1	Multi-Drug Resistance 1 Gene
MEK	MAP/ERK Kinase
miRNA	microRNA
MITF	Microphthalmia-Associated Transcription Factor
MTOR	Mechanistic Target of Rapamycin
MVB	Multivesicular Body
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NF1	Neurofibromin 1
NOX	NAPDH Oxidase
NRAS	Neuroblastoma Rat Sarcoma
NRF2	Nuclear Factor-Erythroid 2-Related Factor-2
nSMase2	Neutral Sphingomyelinase
P-body	Processing Body
PD1	Programmed Death Receptor
PDH	Pyruvate Dehydrogenase
PDK	Pyruvate Dehydrogenase Kinase
PET	Positron Emission Tomography
PI3K	Phosphatidylinositol 3 Kinase
POMC	Pro-Opio-Melanocortin
PP2A	Protein Phosphatase 2A
PPP	Pentose Phosphate Pathway
pre-miRNA	Precursor microRNA
pri-miRNA	Primary microRNA
PTEN	Phosphatase and Tensin homolog
RAF	Rapidly Accelerated Fibrosarcoma
RAS	Rat Sarcoma
RISC	RNA-Induced Silencing Complex
ROS	Reactive Oxygen Species
RTK	Receptor Tyrosine Kinases
SH2	Src Homology 2 Domain
SNP	Single Nucleotide Polymorphism
SOCS	Suppressor of Cytokine Signaling
SOD	Superoxide Dismutase
STAT	Signal Transducer and Activator of Transcription
TCA	Tricarboxylic Acid
VEGF	Vascular Endothelial Growth Factor

Graphical abstract

During my PhD studies, I focused on three different aspects of drug resistance in melanoma, as graphically outlined below, which resulted in two published papers, a review article and a manuscript in preparation.



Articles:

1) "ROS production induced by BRAF inhibitor treatment rewires metabolic processes affecting cell growth of melanoma cells" (Cesi et al., Mol Cancer, 2017)
2) "Impact of BRAF kinase inhibitors on the miRNomes and transcriptomes of melanoma cells" (Kozar et al., BBA, 2017)
3) a) "Overexpressed ALK transported by extracellular vesicles confers drug resistance to sensitive melanoma cells: a novel mechanism of acquired resistance" (Cesi et al., in preparation);

   b) "Transferring intercellular signals and traits between cancer cells: extracellular vesicles as "homing pigeons"" (Review Cesi et al., Cell Communication and Signaling, 2016).

Chapter 1 Introduction

In my thesis, I elucidated several aspects of melanoma biology, all related to the influence of targeted therapies in both responding and resistant cells. To better understand the MAPK signalling pathway, the impact of BRAF inhibitors on metabolic alterations as well as the connection between BRAF inhibitors and the onset of drug resistance was investigated. This introduction is focused on four topics: i) melanoma, ii) cancer metabolism, iii) miRNAs and iv) extracellular vesicles. First, melanoma biology including incidence rates, etiology, canonical and altered signalling pathways, therapies and resistance mechanisms will be introduced. The second part of the introduction will concentrate on metabolic alterations in the context of cancer and their implication on proliferation and survival. Thirdly, miRNAs and extracellular vesicles will be illustrated providing insights into their role in cancer development and especially drug resistance.

1.1 Melanoma biology

1.1.1 Molecular basis of carcinogenesis

Cancer is one of the major causes of mortality throughout the world and despite the remarkable efforts in understanding the biology of cancer, and the amount of money devoted over the past several decades, successful eradication and control of advanced disease is still to come (Siegel et al., 2016). Indeed, the cellular complexity of the disease, its dynamic and evolutionary character challenge successful cancer therapies. Carcinogenesis is a multi-step process characterized by the dysfunction of the mechanisms involved in the control of cell proliferation. The disease develops in three stages:

1) *Initiation*: cells acquire irreversible genetic damage due to external carcinogens or inherited genetic lesions. These mutations mostly affect oncogenes and tumour suppressor genes that normally are key regulators of physiological processes such as proliferation, cell death, differentiation and senescence. The activation of an oncogene generally involves “a gain of function”, which it is dominant; on the contrary mutations in tumour suppressor genes which determine “a loss of function” are generally recessive (Lodish et al., 2000). Some types of cancers occur early in life and are associated with mutations in a single gene. Retinoblastoma, for example, a childhood form of retinal cancer, is caused by mutations in the tumour suppressor RB1. However, more often, cancer is a multistep process and single

mutations are generally not sufficient to transform a cell. Several mutations in different genes are needed for a cell to lose control on its own cell cycle (Paige, 2003).

2) *Promotion*: this stage is characterized by the selective clonal expansion of "initiated cells". Within this period, the process can be altered by chemotherapeutic agents that can affect growth rates. Promotion is the phase between a pre-malignant lesion and the development of invasive cancer. Despite their monoclonal origin, tumours tend to become heterogeneous masses where cells accumulate different mutations that best fit the specific microenvironment (Weston and Harris, 2003).
3) *Progression*: cells acquire further genetic and epigenetic alterations which confer more aggressive features and a more invasive behavior leading to metastasis (Weston and Harris, 2003).

Further, genome instability during this multistep development induces cancers to acquire six biological characteristics. They include sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis and activating invasion and metastasis (Hanahan and Weinberg, 2011). In the last decade, two additional cancer hallmarks have been identified: metabolic reprogramming and evading immune responses. Moreover, in addition to cancer cells, the tumour microenvironment has been gradually recognized as a key contributor for cancer progression enhancing the complexity of neoplastic diseases (Hanahan and Weinberg, 2011).

1.1.2 Cancer metastasis and tumour microenvironment

In spite of significant advances in cancer treatment, metastatic disease still remains incurable and responsible for the great majority of cancer-associated deaths (Chaffer and Weinberg, 2011). Metastasis is the result of a multistep process, where cells from the primary tumour spread to distant organs. The acquisition of genetic and epigenetic alterations can drive cancer cells to: i) break down the surrounding extracellular matrix and locally invade, ii) intravasate into the blood vessels, iii) survive the transport through the vasculature, iv) arrest at distant organ sites, v) extravasate into the parenchyma of distant tissues, vi) initially survive and proliferate in a foreign microenvironment in order to form micrometastases (Valastyan and Weinberg, 2011). This process, collectively named the "metastatic cascade", is extremely inefficient. Fortunately, most cancer cells fail to undergo metastasis and less than 0.01% of tumour cells entering the bloodstream acquire all the properties necessary to ultimately develop into metastasis (Valastyan and Weinberg, 2011). According to Stephen Paget's hypothesis, postulated in 1889, metastasis is not a random colonization of a secondary tumour site but is mostly dependent on the cross-talk between the cells

(the seeds) and the host microenvironment (the soil). In particular, many studies demonstrated that tumour-secreted factors and tumour shed extracellular vesicles are responsible for the generation of a suitable microenvironment in distant organs often called the “pre-metastatic niche” (Peinado et al., 2017).

The tumor microenvironment (TME) has been increasingly recognized as a key factor at multiple stages of cancer progression, particularly in drug resistance, immune escape and distant metastasis (Chen et al., 2015). The TME consists of extracellular matrix as well as myofibroblasts and other cellular players, such as fibroblasts, adipose cells, immune-inflammatory cells, and the blood and lymphatic vascular networks (Chen et al., 2015). These components can influence malignant cell growth by releasing extracellular matrix proteins, growth factors and cytokines. At the same time, cancer cells themselves secrete growth factors, vesicles and proteases that are able to modify the local microenvironment making it more permissive for cell motility and adhesion (Finger and Giaccia, 2010). In addition, a key microenvironmental factor associated with tumor progression is hypoxia. When cells are exposed to a lower oxygen tension, Hypoxia Inducible Factors (HIFs) induce transcriptional programs involved in the adaptation of cells to the lack of oxygen but also in many different processes, including angiogenesis, glucose metabolism, and autophagy (Semenza, 2012). Furthermore, hypoxia has been reported to play a key role in protecting tumour cells from immune cell attack either by promoting immune suppression, or by inducing many other oncogenic events and/or resistance mechanisms in cancer cells, allowing the tumour to escape from immune cell attack (reviewed in Noman et al., 2015).

1.1.3 Melanocytes in human skin

Human skin is the largest organ of the body with a range of functions that support survival. It is organized in three main layers: the outer epidermis, the dermis and the subcutaneous fat. The epidermis itself is divided in layers such as the stratum corneum, the granular cell layer, the spinous cell layer, and the basal cell layer. **Keratinocytes**, the most abundant cell type of the epidermis, are characterized by the presence of keratin and by the formation of desmosomes and tight junctions with each other to form a physico-chemical barrier against environmental damage (Hirobe, 2014). The epidermis also contains **dendritic (Langerhans) cells**, which are antigen presenting cells important for first line defense against exogenous pathogens. Only a small percentage of the epidermis is represented by **melanocytes** (Hirobe, 2014). Melanocytes are the cells responsible for the production of a complex mixture of pigments, which exert protective functions against UV rays and which determine the skin color. Melanocytes together with neurons, glial cells, cardiac cells, cartilage, connective tissues and bone morrow originate from embryonic neural crest cells and are

present not only in the basal layer of the epidermis and hair follicles but also in the inner ears, eyes, nervous system and the heart (Mort et al., 2015). In the skin, melanocytes provide neighboring keratinocytes with melanin pigment, which is synthesized within specific organelles called melanosomes. The production of melanin is triggered by UV radiation-induced DNA damage to keratinocytes, which consequently produce pro-opio-melanocortin (POMC) in a p53-dependent manner (Figure 1). POMC is further processed and secreted as α-melanocyte stimulating hormone (α-MSH), which binds to the melanocortin 1 receptor (MC1R), expressed on neighboring melanocytes resulting in a signal cascade that enhances the levels of MITF transcription factor (microphthalmia-associated transcription factor). MITF, then, regulates the expression of genes required for melanin synthesis (tyrosinase and tyrosinase-related protein). In addition to increased pigmentation, α-MSH also stimulates melanocyte proliferation, a process which is supposed to enhance photoprotection (Shain and Bastian, 2016). Although sun exposure has positive effects on human health triggering vitamin D synthesis, frequent and intense exposure to UV radiation represents a major risk factor for the development of skin cancer, notably melanoma.

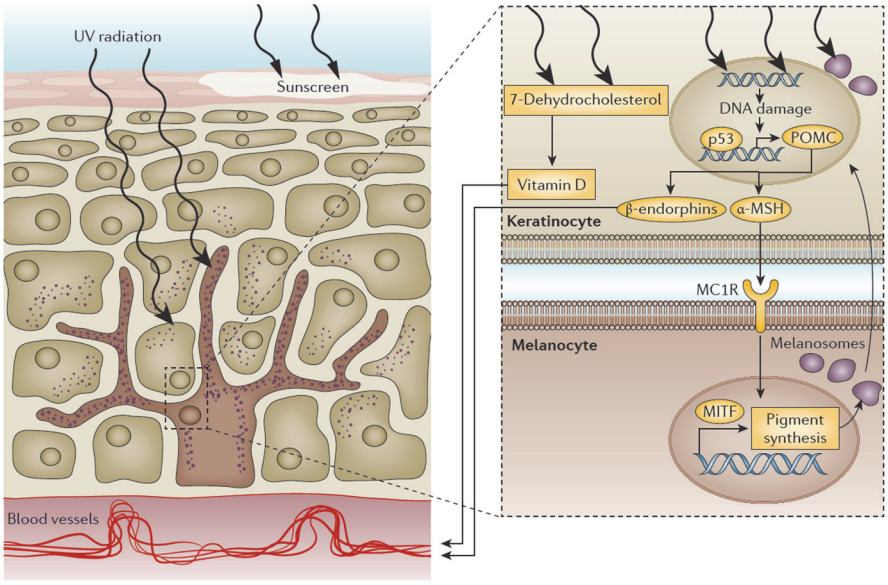


Figure 1. UV radiation triggers the production of melanin pigments.
In keratinocytes, UV rays induce DNA damage and p53 activation. P53 induces the transcription of pro-opio-melanocortin (POMC), which is post-translationally processed and secreted as two bioactive peptides, α-melanocyte-stimulating hormone (α-MSH) and β-endorphins. α-MSH binds to the melanocortin 1 receptor (MC1R) located in the plasma membrane of melanocytes inducing a cyclic AMP-mediated signalling that stimulates MITF to induce pigment synthesis within melanosomes. Melanosomes are then transferred from melanocytes to keratinocytes where they become positioned in the perinuclear area of each keratinocyte, providing protection against further DNA damage. Image taken from Schadendorf et al., 2015.

1.1.4 Cutaneous melanoma

The malignant transformation of melanocytes into cancer cells requires a complex synergy of both environmental (exogenous) and genetic (endogenous) factors (Bandarchi et al., 2010). Due to the presence of melanin pigment, cutaneous melanoma can often be accurately diagnosed earlier than other malignancies and surgically resected. However, advanced stages of the disease are generally associated with poor patient survival. Melanoma is classified according to the thickness, the depth of penetration, and the degree to which the melanoma has spread (Balch et al., 2009; Vultur and Herlyn, 2013) (Figure 2). The stage is most often determined using the TNM system. It is based on the combination of three factors: 1) tumour depth (T), described by Breslow's thickness (expressed in millimeters), 2) lymph node status (N) and 3) distant metastasis (M).

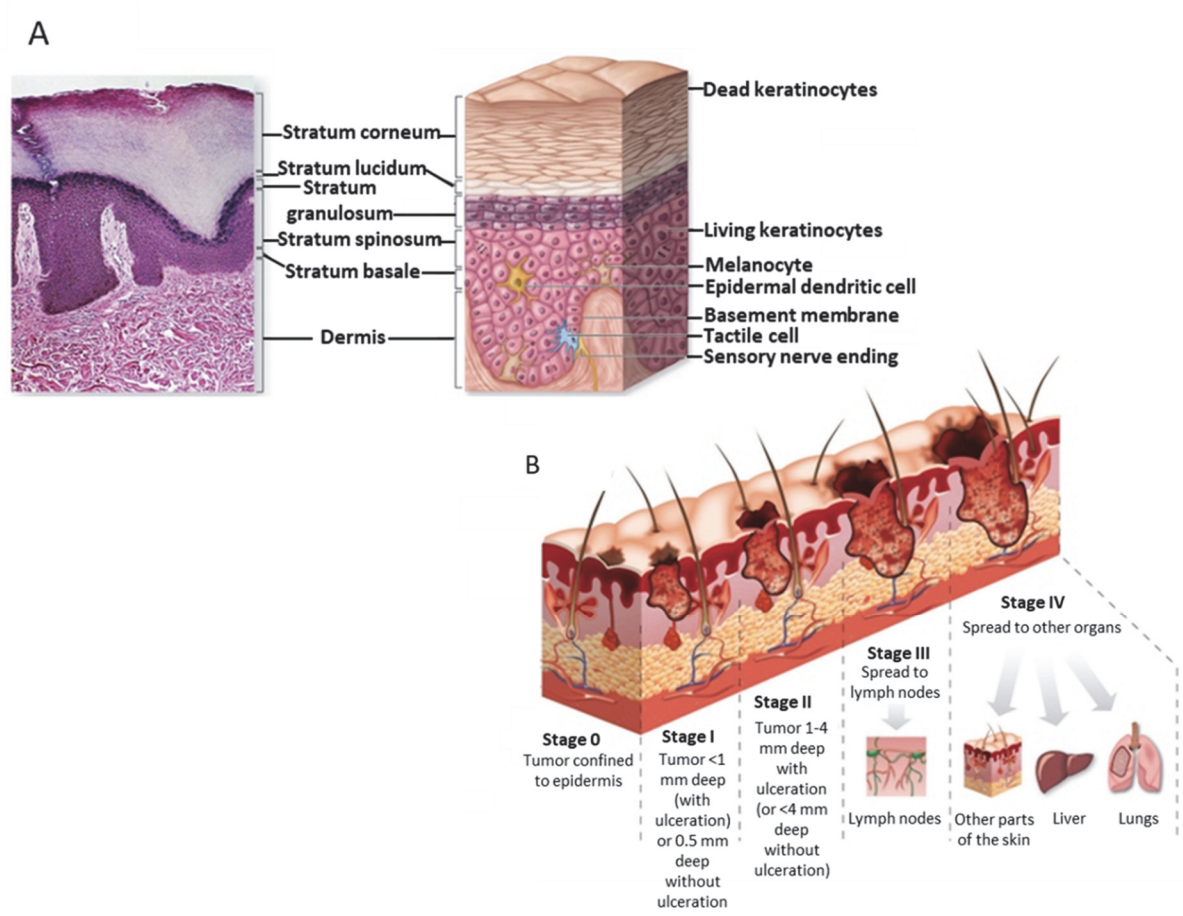


Figure 2. Stages of melanoma.

The stage is based on the results of physical and histological examination and it depends on the size or thickness of the tumour, whether it has spread to the lymph nodes or other organs, and other characteristics, such as growth rate. Picture A taken from Mescher, 2009, picture B taken from www.skindermatologists.com/melanoma.html.

Their combination defines four stages. Early melanomas (stages 0 and I) are localized; stage 0 tumours are *in situ*, meaning that they are non-invasive and have not penetrated below the surface of the skin, while stage I tumours have invaded the skin but are small and are growing at a slow mitotic rate. Stage II tumours, though localized, are larger (generally over 1 mm thick) and/or may be ulcerated or have a faster mitotic rate; they are considered intermediate melanomas. More advanced melanomas (stages III and IV) have spread (metastasized) to other parts of the body such as lymph nodes, liver, brain and lungs. The staging is used to determine treatment. For stage 0, I and II patients, surgical excision represents the first line treatment. Stage III and IV patients are often hard to cure; however, in recent years, the treatment of melanomas has changed as newer forms of immunotherapy and targeted drugs have been shown to be more effective than chemotherapy (Luke et al., 2017) (See 1.1.7 and 1.1.11).

1.1.5 Epidemiology and risk factors

Melanoma but also non-melanoma skin cancers are the most common types of cancer in Caucasians (Schadendorf et al., 2015) with an **increasing incidence** rate worldwide but a stable or decreasing mortality rate (Schadendorf et al., 2015). Although melanoma accounts for only 1% of all skin cancers, it is responsible for the majority of skin cancer-related deaths (American Cancer Society) (https://www.cancer.org/research/cancer-facts-statistics). According to the US National Cancer Institute, the estimated number of new cases of melanoma in 2016 was 76.380, which represented 4.5% of all new cancer cases. Cutaneous melanoma mainly affects the light skinned population whereas more pigmented populations, which are less prone to develop the disease, mostly suffer from acral and mucosal melanoma (Schadendorf et al., 2015). Australia and New Zealand have reported the highest incidence rate worldwide with 60 cases per 100.000 inhabitants per year (Schadendorf et al., 2015). Compared to other solid cancers, melanoma exhibits an extremely high prevalence of somatic mutations (Alexandrov et al., 2013), which is almost entirely attributable to UV light (UV mutational signature). Sun-derived UV rays, a mixture of UV-A and UV-B, may exert deleterious effects on cells, damaging the DNA and causing genetic mutations (Brenner and Hearing, 2008). UV-B rays, for example, strongly induce the formation of thymine dimers, with two adjacent pyrimidines forming a double covalent bond (Zaidi et al., 2012). The typical “UV-B signature mutation” is characterized by cytosine to thymine (C to T) and cytosine-cytosine to thymine-thymine (CC to TT) transitions. On the other hand, UV-A rays generate reactive oxygen species (ROS) responsible for the formation of photodimers in the genome. Oxidation of nucleotides promotes mispairing and therefore mutagenesis (Zaidi et al., 2012). In addition to UV light, mutations in specific genes contribute to the onset of melanoma. A familiar background

occurs in 10% of all melanoma patients (Tsao et al., 2012) and of those, 40% harbor high-penetrance germline mutations within the cyclin-dependent kinase inhibitor 2A (CDKN2A) (Schadendorf et al., 2015), a gene which encodes two distinct tumour-suppressor genes: p16 and p14ARF. The most common type of melanoma in Caucasians arises in areas chronically exposed to the sun, such as the head, ears, neck and lower extremities (Shain and Bastian, 2016). Signs of cumulative exposure to UV light, typically solar elastosis, are often associated with this type of melanoma which develops in > 60 year old people. Melanomas from chronic sun damage are usually characterized by high mutation rates affecting genes such as neurofibromin NF1, KIT, $BRAF^{nonV600E}$ (Bastian, 2014). In contrast, melanomas associated with intermittent sun exposure do not show solar elastosis and are more common in younger people. In this case, the disease is very aggressive and it is characterized by a moderate mutation rate with $BRAF^{V600E}$ being the most frequent alteration (Bastian, 2014).

1.1.6 The Mitogen-Activated Protein Kinase signalling pathway

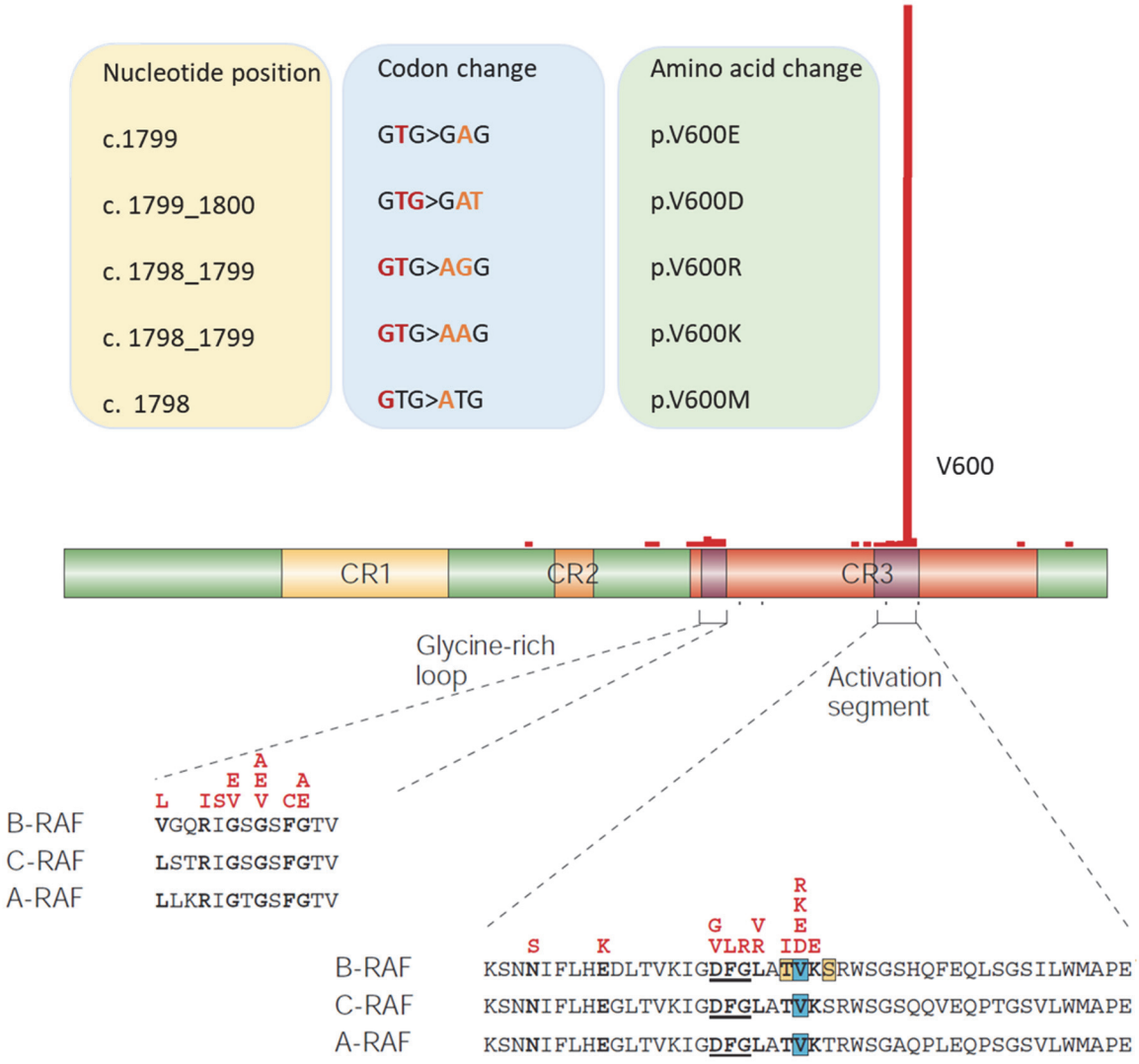
The Mitogen-Activated Protein Kinase (MAPK) signalling pathway is currently the most oncogenic cascade in melanoma. Its high therapeutic relevance arises from the fact that it plays a fundamental role in many key cellular processes such as proliferation, survival, differentiation and metabolism (Plotnikov et al., 2011). The MAPK pathway can be activated through receptor tyrosine kinases (RTKs). Ligands induce RTK dimerization and *in trans* receptor autophosphorylation on tyrosine residues, resulting in receptor activation. These phosphorylated tyrosine residues hook adaptor proteins, such as Shc (Src Homology 2 Domain-Containing proteins), which contain the Src (sarcoma) homology 2 (SH2) domain. Shc proteins, in turn, recruit the Growth Factor Receptor-Bound 2 (Grb2) together with the guanine nucleotide exchange factor (Son of Sevenless) leading to the conversion of inactive RAS-GDP to active RAS-GTP. Subsequently, RAS-GTP interacts with and activates the serine/threonine RAF kinase family (Roskoski, 2010). The RAF kinase family includes three isoforms: ARAF, BRAF and CRAF. The three RAF isoforms share three conserved regions (CR) (Figure 3). The CR1 contains the RAS-binding domain and the cysteine-rich domain which are important for RAF membrane recruitment, CR2 connects CR1 and CR3 and contains binding sites for the dimer 14-3-3 protein and CR3, which contains the catalytic domain. In absence of RAS-GTP, the closed and inactive conformation of RAF is stabilized by a 14-3-3 dimer binding to phosphorylated serine residues (in CR2 and CR3). Dephosphorylation of these residues at the cell membrane by specific protein phosphatases (PP2A, PP1) releases the protein 14-3-3 and allows a conformational change, which enables RAS binding and membrane recruitment. RAS promotes RAF dimerization and activation via phosphorylation of serine residues present in the negatively charged N region (located N-terminal to the kinase domain) and in the activation segment. SRC family

kinases seem to be responsible for serine phosphorylation in the N region while it is unclear which kinase is responsible for the phosphorylation in the activation domain. However, recently, cis-autophosphorylation has been proposed (Lavoie and Therrien, 2015). Fully activated RAF proteins can then phosphorylate and activate MEK1 and MEK2, which in turn phosphorylate their targets ERK1 and ERK2 (Figure 4). Activated ERK phosphorylates and activates a variety of nuclear and cytoplasmatic substrates that mediate the pleiotropic effects of the pathway. ERK signalling also activates a very important negative feedback loop phosphorylating several inhibitory sites in RAF and causing a release from RAS and the disruption of RAF dimers, which terminate the signalling.

1.1.7 The $BRAF^{V600E}$ mutation

The MAPK signalling pathway is hyperactivated in 30% of human cancers (Burotto et al., 2014). Mutations in the Ser/Thr-kinase BRAF have been found in 10% of all human cancers with the highest prevalence observed in melanoma patients (> 50%), making BRAF one of the most mutated cancer-associated genes (Davies et al., 2002; Holderfield et al., 2014). In the inactive BRAF conformation, the phenylalanine side chain of the conserved DFG motif (aspartate, phenylalanine, and glycine) present in the activation segment of the kinase domain occupies the nucleotide binding pocket preventing ATP binding in the catalytic cleft (refer to Figure 5). In addition, the LAT motif residues (leucine, alanine and threonine) directly adjacent to the DFG, are engaged in hydrophobic interactions with the P-loop (glycine-rich ATP-phosphate-binding loop). Interestingly, the P-loop and the activation segment form two clusters of mutations (Figure 3). Thus, the interference with these interactions plays an important role in oncogenic BRAF activation.

Figure 3. Schematic representation of RAF protein and frequent mutations.
The glycine-rich loop and the activation segment can be frequently mutated. The most common amino acid substitutions are reported in red above the sequences. The frequency of the different mutations is indicated by the red lines above the protein structure. V600 is the most frequent mutated site in BRAF. The phosphorylation sites present in the activation segment of BRAF (T599 and S602) are highlighted in yellow while V600 is in blue. The DFG motif is underlined. The top panels contain details about nucleotide position, codon and amino acid change of the most recurrent mutations at V600 position. Modified from Wellbrock et al., 2004.



Physiologically, to switch into its active form, BRAF must form dimers and be phosphorylated at two key residues (T599 and S602) located in the BRAF activation segment. Phosphorylation of the activation segment results in the destabilization of these hydrophobic interactions by introducing a negative charge, resulting in a conformational change of the DFG segment into its active state and allowing access to the catalytic cleft. Although more than 40 different mutations have been identified in the BRAF gene, in 90% of the cases, BRAF mutations involve a thymine to adenine single-base change at position 1,799 which results in an amino acid change from valine (V) to glutamic acid (E) at residue 600 (p.V600E) (Figure 3) (Davies et al., 2002). Glutamic acid is a negatively charged amino acid, which mimics phosphorylation of the activation segment by RAS, destabilizing the conformation that normally maintains the inactive orientation of the DFG motif. This mutation renders $BRAF^{V600E}$ constitutively active with increased and constitutive kinase activity (Figure 4). Opposed to BRAF, mutations within ARAF and CRAF are rather rare. This is probably due to the fact that these two proteins require additional phosphorylation of residues present in the N

region of the kinase domain for full activation while BRAF already contains negative charges in its N region (Roskoski, 2010). Hence, a single mutation is sufficient to activate BRAF while at least two distinct events would be necessary to constitutively activate ARAS and CRAS (Wellbrock and Arozarena, 2016).

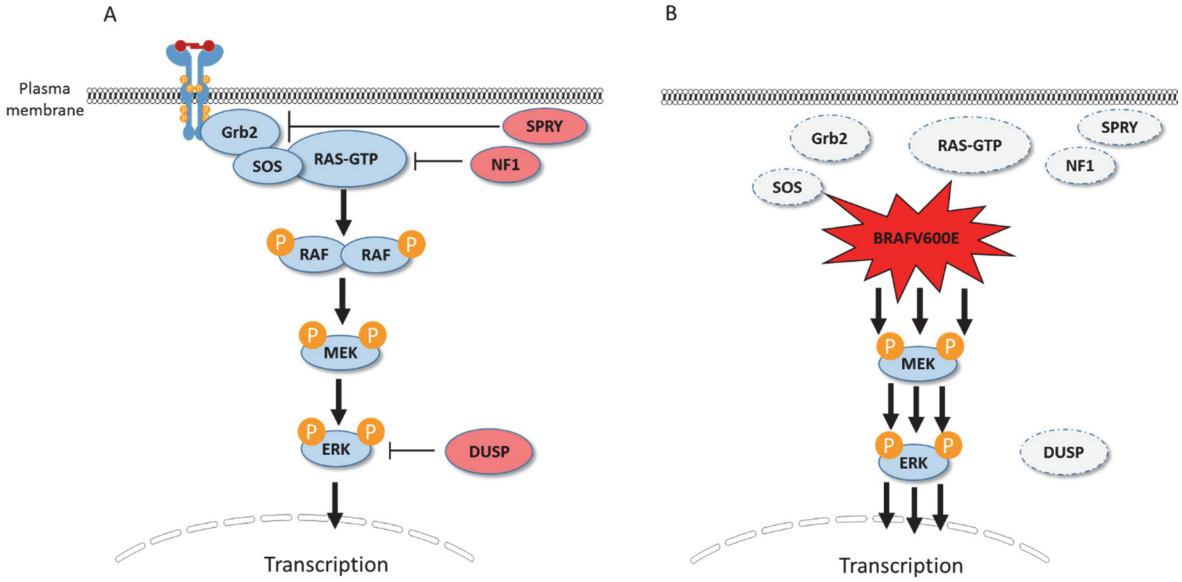


Figure 4. The BRAF^{V600E} mutation constitutively activates the MAPK signalling pathway.
(A) Under physiological conditions, the binding of a ligand to the receptor tyrosine kinase induces its activation and consequently the activation of the RAF-MEK-ERK pathway. The pathway is switched off by negative regulators such as NF1, SPRY and DUSP. (B) Mutant BRAF is constantly active and leads to the overexpression of ERK target genes promoting proliferation and survival.

1.1.8 Targeting the BRAF^{V600E} mutation in metastatic melanoma

The identification of mutations in the BRAF gene and the development of a targeted therapy for it revolutionized the treatment of patients with advanced melanoma. The principle behind targeted therapy is the interference with specific molecules necessary for tumour growth and progression. Traditional cytotoxic chemotherapies usually kill all rapidly dividing cells in the body by blocking cell division with severe side effects (McKnight, 2003). A primary goal of targeted therapies, on the other hand, is to selectively target proteins, which are mutated in cancer cells but not in normal surrounding cells, with potentially fewer side effects. Initial therapeutic strategies to inhibit oncogenic BRAF used sorafenib, which targets multiple tyrosine kinases with poor efficacy in BRAF-mutated melanomas (Eisen et al., 2006; Hauschild et al., 2009). For this reason, a second generation of inhibitors was developed to specifically target only BRAF^{V600E}. Vemurafenib (also known as

PLX4032) and Dabrafenib (also known as GSK2118436) are two small molecules designed through a crystallographic approach. Both inhibitors bind to the ATP-binding pocket and are classified as type I kinase inhibitors because they favor the active conformation of the protein, induced by the mutation (Holderfield et al., 2014) (Figure 5).

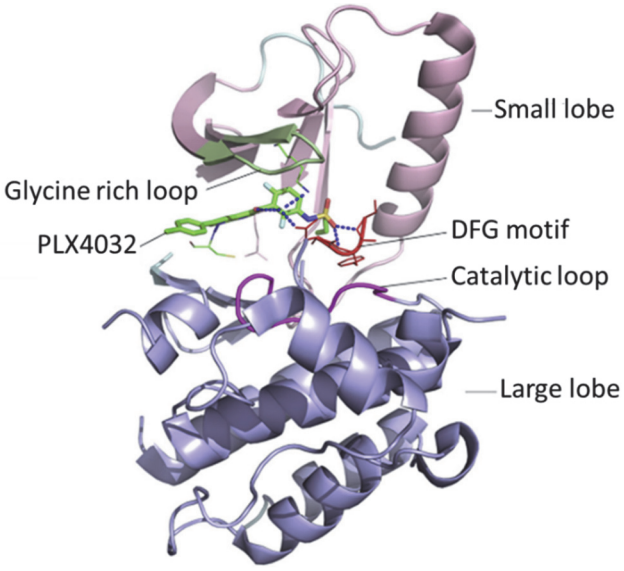


Figure 5. Structure of BRAF kinase domain bound to BRAF inhibitor PLX4032 (Vemurafenib).
The inhibitor binds the kinase in its active conformation interacting with the ATP binding site by forming hydrophobic bonds in and around the adenine regions where the adenine ring of ATP usually binds. Image taken from Rahman et al., 2014.

These inhibitors bind with high affinity to the mutated form of BRAF (V600E and the less common BRAF mutations V600K, V600D, V600R and V600M where valine at position 600 is replaced by lysine, aspartic acid, arginine or methionine respectively) (Menzies et al., 2012; Rubinstein et al., 2010). The drugs have been approved by the Food and Drug Administration (FDA) in 2011 for clinical use and have successfully been administered as monotherapy in a subset of late stage melanoma patients showing impressive results at first (Flaherty et al., 2010) (Figure 6). However, despite the initial promising results, most patients relapse and develop **drug resistance** within six months (Hartsough et al., 2014). Drug resistance is often achieved by bypassing BRAF inhibition through downstream activation of MEK (Trunzer et al., 2013). Therefore, a combination therapy targeting BRAF V600 mutations (Dabrafenib or Vemurafenib) together with MEK (Trametinib or Cobimetinib) has been approved in 2015 for use in stage III and stage IV melanoma patients (Garbe et al., 2016;

Spain et al., 2016). Overall, median survival has increased with the combination therapy from 18.7 months to 25.1 months (Garbe et al., 2016; Spain et al., 2016).

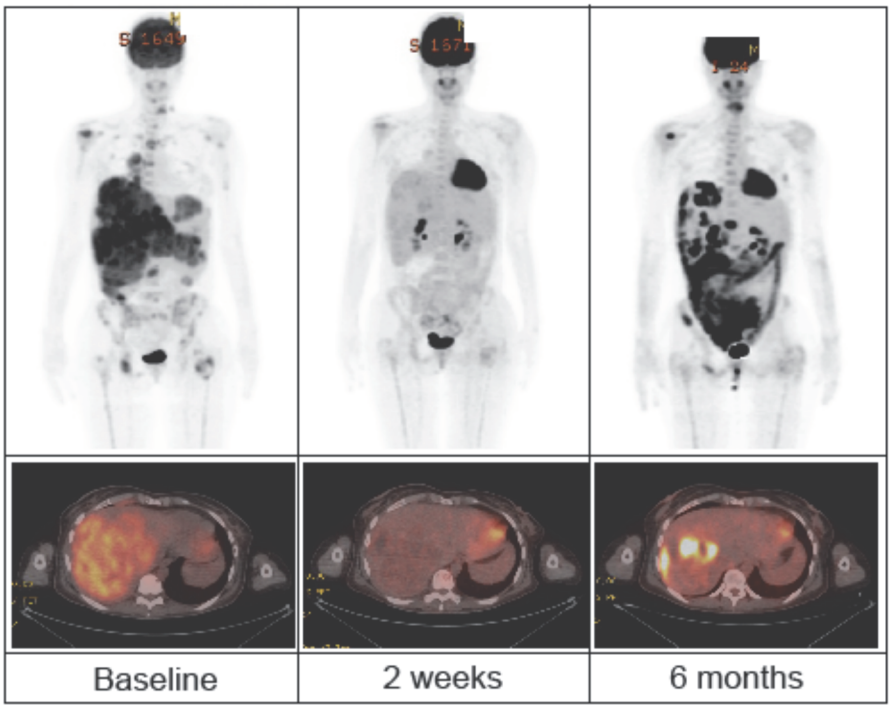


Figure 6. BRAF inhibitors are effective in late stage melanoma patients harboring BRAF^{V600E}.
Comparison of patient PET scans after Vemurafenib treatment. Considerable tumour shrinkage is visible after 2 weeks of treatment suggesting effective response. Unfortunately, drug resistance and further tumour progression is often observed after 6 months. Image from Finn et al., 2012.

1.1.9 The BRAF inhibitor paradox

As mentioned above, BRAF inhibitors profoundly contributed to extended survival of patients with BRAF^{V600E} metastatic melanoma (Chapman et al., 2011; Menzies et al., 2012; Song et al., 2015). These drugs do not inhibit wild-type BRAF but rather confer a paradoxic growth advantages to those cells (Halaban et al., 2010; Joseph et al., 2010). The so called "BRAF inhibitor paradox" was extensively investigated in the past few years. Biochemical evidence suggests that the effects of BRAF inhibitors strongly depend on both the mutational status of BRAF and on the levels of RAS-GTP. In wild-type BRAF cells, elevated RAS-GTP promotes the dimerization and the membrane localization of RAF proteins. The presence of non-saturating concentrations of BRAF inhibitor bound to one molecule of the dimer induces an allosteric change that transactivates the other drug-free molecule of the dimer, which can then activate the ERK pathway (Hatzivassiliou et al., 2010; Poulikakos et al., 2010). In cells expressing mutant BRAF, RAS-GTP levels are insufficient to induce

dimer formation and RAF proteins mostly exist as monomers. In addition, unlike wild-type BRAF, monomeric $BRAF^{V600E}$ is hyperactive and capable of activating the ERK pathway by itself. BRAF inhibitors efficiently bind and inhibit monomeric $BRAF^{V600E}$, suppressing downstream signalling (Freeman et al., 2013; Poulikakos et al., 2011) (Figure 7). This model suggests that any mutation able to increase RAS-GTP (RAS mutation or activation of RTKs), can enhance RAF dimerization and promote drug resistance against BRAF kinase inhibitors (Poulikakos et al., 2010).

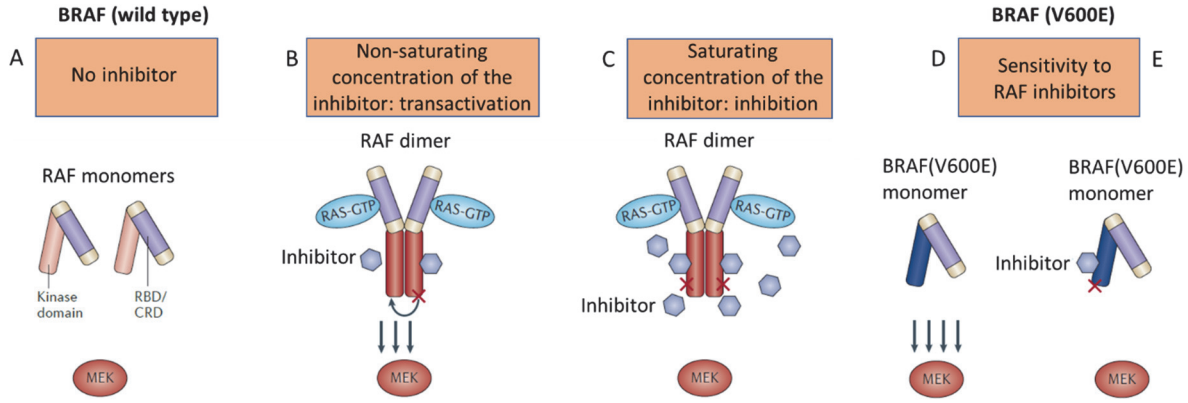


Figure 7. The effects of BRAF inhibitors depend on the mutational status of BRAF and on the levels of RAS-GTP.

(A) In BRAF wild-type cells, when the levels of RAS-GTP are low, RAF proteins are inactive monomers. (B) Elevated levels of RAS-GTP promote RAF dimerization. BRAF inhibitor, at non-saturating concentration, binds to one monomer which in turn transactivates the other member of the dimer. (C) At saturating concentrations of the inhibitor, both monomers are inhibited and RAF activity is inhibited. (D) In BRAF V600E cells, when the levels of RAS-GTP are low, all RAF isoforms mostly exist as hyperactive monomers. (E) BRAF inhibitor efficiently binds and suppresses monomeric RAF suppressing its activity and downstream pathway. Modified from Samatar and Poulikakos, 2014.

1.1.10 Drug resistance in cancer: a clinical challenge

The recent development of targeted therapies represents one of the most important steps forward in cancer treatment. Many targeted cancer therapies have been approved by the FDA to treat different types of cancer (such as skin, lung, colorectal and breast cancer, lymphoma, leukemia etc.). Others are in clinical trials, and many more are in preclinical testing. Unfortunately, the positive effects of targeted therapy are often challenged by the emergence of rapid drug resistance. There are two categories of drug resistance: intrinsic and acquired. Intrinsic resistance refers to the lack of drug responsiveness probably due to the presence of pre-existing factors in the tumour, which make a certain therapy an unavailing attempt (Holohan et al., 2013). Acquired resistance, on the other hand, develops during the treatment, which was previously effective and it could be

interpreted as a protective adaptation mechanism of the cancer cell to get rid of the cytotoxic agent (Friedman, 2016). Additional mutations acquired during the treatment affecting alteration of drug transport, drug metabolism, cell death and DNA repair as well as the activation of alternative compensatory signalling pathways are behind the onset of this type of resistance (Holohan et al., 2013) (Figure 8).

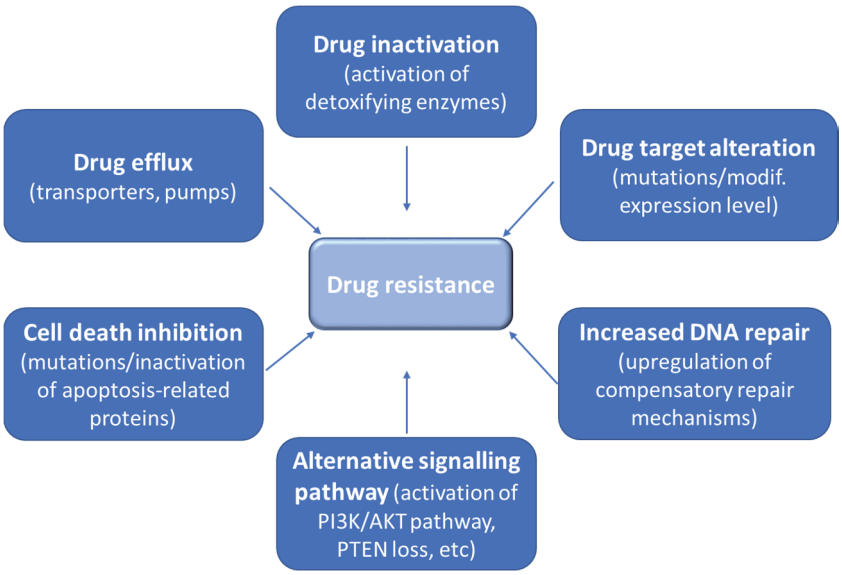


Figure 8. Mechanisms that can promote drug resistance in human cancer cells.

These mechanisms might act independently or in combination resulting in drug tolerance and drug resistance.

In addition, tumours are highly heterogeneous, thus drugs could select for intrinsically resistant subpopulations present in the original tumours (Swanton, 2012). Strategies targeting multiple pathways and/or combination of different therapeutic approaches (targeted therapy, immunotherapy) will probably be necessary to successfully eliminate these subpopulations and to obtain sustained drug responses (Rebecca and Smalley, 2011).

1.1.11 Resistance to BRAF inhibitors

Several mechanisms of acquired resistance to BRAF inhibitors have already been characterized and in most cases the re-activation of the MAPK pathway enables the cells to bypass BRAF inhibition and to resume proliferation (Villanueva et al., 2011; Winder and Virós, 2017). For instance, a subset of BRAF inhibitor-resistant melanoma cells can restore the MAPK activity by expressing truncated forms of BRAF or by overexpressing BRAF, CRAF, or COT1 (Corcoran et al., 2010; Johannessen et al., 2010; Montagut et al., 2008; Poulikakos et al., 2011). Similarly, activating mutations in NRAS (Q61K/R) and MEK1 (C121S) trigger and stimulate the MAPK signaling in BRAF inhibitor–resistant cell lines and clinical samples (Nazarian et al., 2010; Wagle et al., 2011). Moreover, the upregulation

of other receptor tyrosine kinases (RTKs) is another common mechanism of resistance to targeted inhibitors. The RTK platelet-derived growth factor receptor-β (PDGFRβ) and the Insulin Like Growth Factor 1 Receptor (IGF-1R) confer BRAF inhibitor resistance in a MAPK-independent manner that activates the phosphatidylinositol 3-kinase (PI3K)/AKT pathway (Nazarian et al., 2010; Shi et al., 2014; Villanueva et al., 2011) (Figure 9). In addition, intrinsic resistance to BRAF inhibitors in cells harboring a BRAF mutation is often due to the loss or inactivation of genes implicated in the regulation of proliferation and survival pathways such as the negative regulator of the PI3K-AKT pathway, PTEN (phosphatase and tensin homolog), the recessive tumour suppressor CDKN2A and the negative regulator of RAS, NF1 (Palmieri et al., 2015).

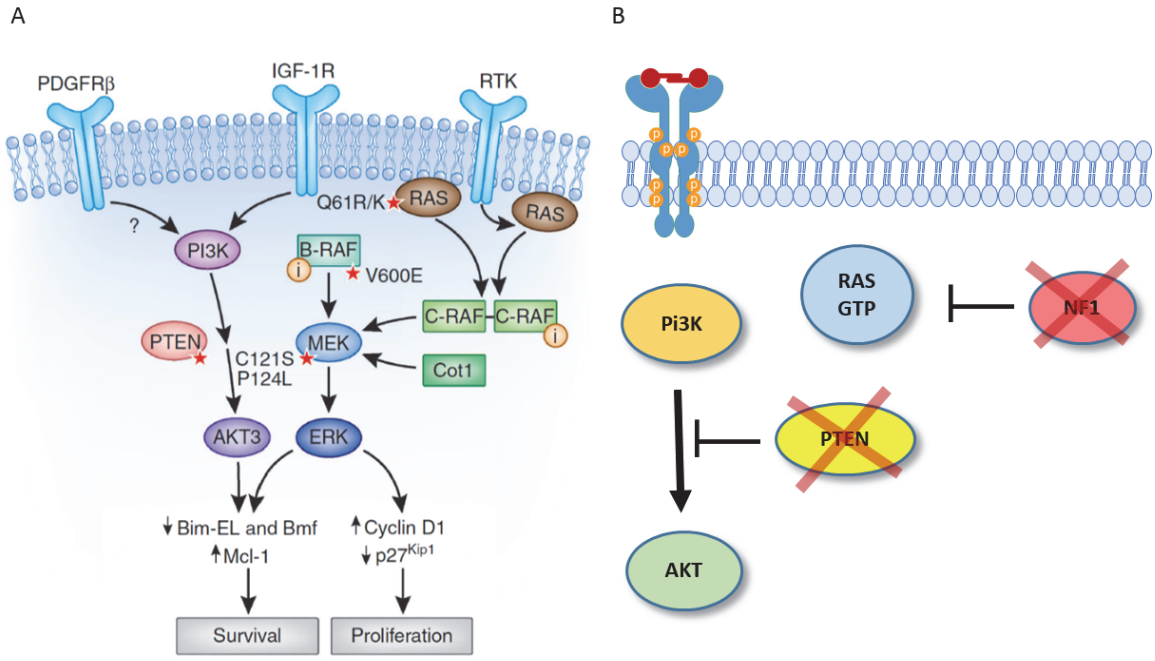


Figure 9. Selection of possible mechanisms of resistance to BRAF inhibitors.

(A) Acquired resistance to BRAF inhibitors can be due to mutations in NRAS and/or upregulation of receptor tyrosine kinases, which enhance RAS activity and activation of CRAF. The re-activation of the MAPK pathway can also occur via upregulation of the kinase COT. In parallel, PTEN loss can induce the upregulation of the PI3K/AKT pathway. Together these pathways promote proliferation and survival by promoting expression of the anti-apoptotic protein, Mcl-1, as well as by down-modulating levels of the pro-apoptotic BH3-only proteins, Bim-EL, and Bmf. (B) Intrinsic resistance is mostly due to the loss of tumour suppressors PTEN and NF1. Picture A from Aplin et al., 2011.

1.1.12 Immunotherapy in melanoma

Immune-based therapies, which are used regardless of the tumour genotype, represent another breakthrough in cancer and also in melanoma therapeutics. Immunotherapy aims to help the immune system to identify and attack cancer cells and in melanoma it could be used as a first-line therapy, particularly for patients with wild-type BRAF melanoma or NRAS mutant and as a second-line treatment if kinase inhibitors are not effective or once patients become resistant (Johnson et al., 2015). Immune checkpoints inhibitors are monoclonal antibodies targeting negative regulators of T cell immune responses resulting in increased activation of the immune system response towards the cancer cells. In particular, ipilimumab (anti-cytotoxic T lymphocyte antigen 4 (CTLA4)) and nivolumab (anti-programmed death receptor 1 (PD1)) used as monotherapy or combined provide survival benefits in a subset of melanoma patients (Larkin et al., 2015). Despite encouraging results obtained with immunotherapies, responses to such treatments are quite heterogeneous: the immune response is dynamic and changing in each patient depending on environmental and genetic factors as well as on previous clinical treatment (Sharma et al., 2017). The majority of patients do not benefit (intrinsic resistance) or develop drug resistance after initial response. Resistance mechanisms are mostly due to the lack of T cell recognition of tumour cells, which might not express tumour antigens or might display alterations in the antigen-presenting machinery or develop escape mutation variants (Luke et al., 2017; Sharma et al., 2017). Combinations of BRAF inhibitors and immunotherapies, especially for patients with very high disease burdens, are currently under investigation (Luke et al., 2017).

1.2 Cancer metabolism

In the past decade, cancer metabolism has become a topic of renewed interest. Understanding mechanisms and functional consequences of tumour-associated metabolic alterations in different cancers, have expanded current knowledge on the physiology of the disease and it will progressively support the development of new strategies to treat human cancer. To study metabolic alterations under selective pressure, the impact of BRAF inhibitors on metabolic pathways in melanoma has been explored.

1.2.1 Metabolic reprogramming in cancer

The connection between cancer cells and altered metabolism has become a cancer hallmark ever since many studies reported the observation of recurring metabolic changes in several types of cancers. The first observation that cancer cells alter their metabolism was made in 1924 by Otto

Warburg. He noticed that cancer cells, unlike most other cells, predominantly depend on aerobic glycolysis and produce large amounts of lactate. The reason why cancer cells would prefer a less efficient strategy to produce energy, was long debated. Initially, it was thought that this metabolic switch was a consequence of mitochondrial dysfunction. Nowadays, it is recognized that cancer cells primarily need to enhance glycolysis to produce metabolic intermediates for nucleotide synthesis and to produce NADPH for antioxidant defense. Most of the ATP in cancer cells is still produced by oxidative phosphorylation driven by e.g. glutamine anaplerosis into the TCA cycle.

The term "metabolic reprogramming" is used to describe a change occurring in a classical metabolic pathway which results in its increased or inhibited activity (DeBerardinis and Chandel, 2016). Generally, these metabolic alterations fulfil the needs of fast proliferating cells, such as cancer cells, supporting rapid ATP generation to fuel biochemical reactions, increased biosynthesis of macromolecules and maintenance of a balanced redox status (Cairns et al., 2011). Although it was initially thought that these metabolic alterations were the result of an adaptive process that was trying to keep the pace with a high proliferation rate, it became clear that many metabolic changes are instead driven by oncogenes or due to the loss of tumour suppressors. The PI3K/AKT pathway, for instance, is very often altered in cancers. In addition to survival advantages, the activated pathway has also effects on cellular metabolism. AKT stimulates glycolysis by increasing the expression of glucose transporters and by phosphorylating important glycolytic enzymes such as hexokinase and phosphofructokinase 2 (Elstrom et al., 2004; Robey and Hay, 2009). AKT also activates mTOR which, in turn, stimulates protein and lipid biosynthesis in response to sufficient nutrients and energy (Guertin and Sabatini, 2007). Another example of metabolic reprogramming is mediated by HIF1. In addition of being activated under hypoxia, HIF1 can also be activated by oncogenes under normoxic conditions (Courtnay et al., 2015; Hudson et al., 2002). Activated HIF1 induces the transcription of glucose transporters and activates the pyruvate dehydrogenase kinases (PDKs) which, in turn, inactivate the pyruvate dehydrogenase complex slowing down the tricarboxylic acid cycle (TCA) and thereby reducing oxidative phosphorylation (Kim et al., 2006). Furthermore, many cancer cells show a dysregulation of the AMP-activated protein kinase (AMPK) signalling. Normally, in response to decreased ATP, AMPK inhibits cell proliferation counteracting the effects triggered by AKT and inhibiting mTOR (Kuhajda, 2008; Shackelford and Shaw, 2009). LKB1, the upstream kinase important for AMPK activation is often mutated in some cancers (Ji et al., 2007; Wingo et al., 2009). Besides, the loss of p53 might also induce the acquisition of a glycolytic phenotype. Indeed, p53 inhibits the glycolytic pathway by upregulating the expression of TP53-induced glycolysis and apoptosis regulator (TIGAR), which acts as a negative regulator of glycolysis by lowering intracellular levels of Fructose-2,6-bisphosphate, resulting in the pentose

phosphate pathway (PPP) activation and NADPH production (Bensaad et al., 2006). P53 also promotes oxidative phosphorylation inducing the activation of SCO2, involved in the biogenesis of cytochrome c oxidase subunit II (Matoba et al., 2006). Mutant KRAS and BRAF, often present in melanoma as well as in colorectal, thyroid and pancreatic cancer cells, have also been associated with an enhanced expression of glucose transporters and glycolytic enzymes such as hexokinase 1 and 2 and phosphofructokinase 1 (Parmenter et al., 2014; Yun et al., 2009). Together, these alterations favor an increased glucose uptake and glycolytic activity (Figure 10).

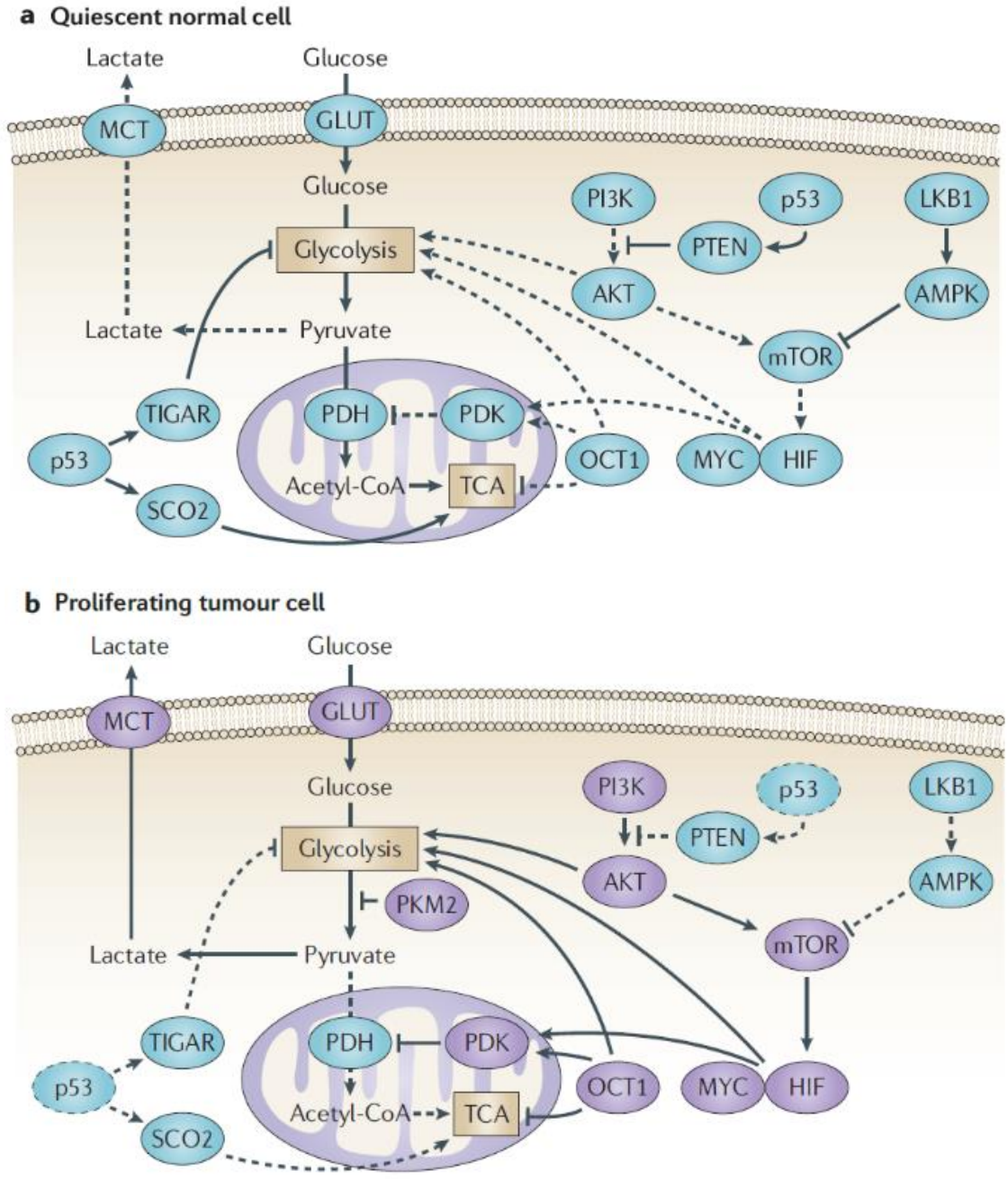
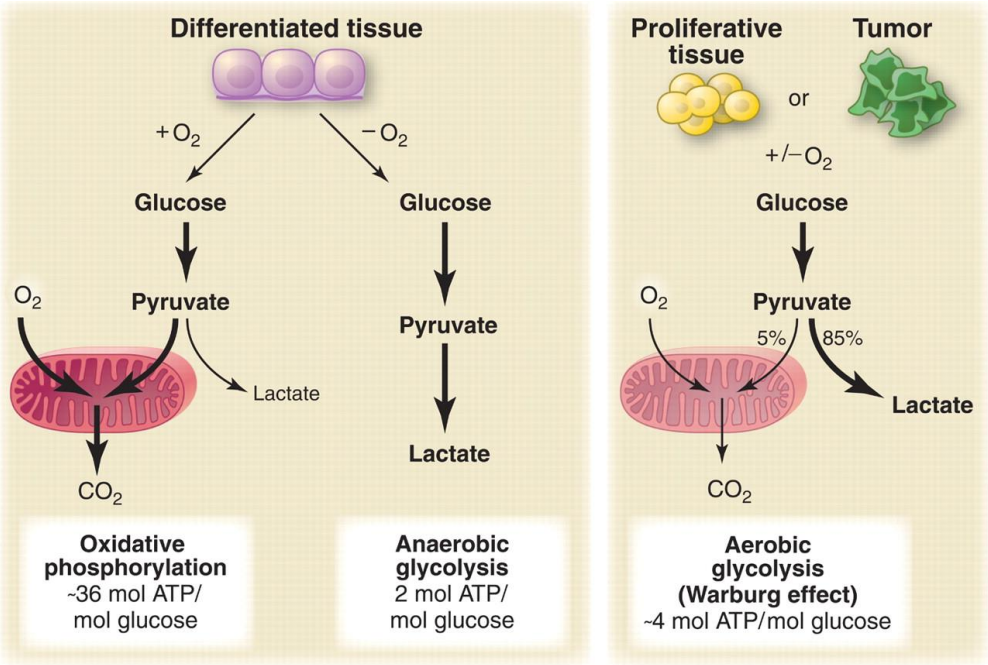


Figure 10. Signalling pathways reprogram cancer cell metabolism.
Compared to normal cells (a), the activation of oncogenic pathways in cancer cells leads to a shift towards aerobic glycolysis (b). Activated AKT stimulates glycolysis by both regulating glycolytic enzymes and by activating mTOR, which enhances the activity of HIF1. HIF1 increases the expression of glucose transporters, glycolytic enzymes and pyruvate dehydrogenase kinase 1 (PDK1) that blocks the entry of pyruvate into the TCA cycle. MYC cooperates with HIF1 and enhances glycolytic enzymes and mitochondrial metabolism. The loss of p53 leads to a reduction of TIGER and SCO2 promoting aerobic glycolysis. Figure from Cairns et al., 2011.

1.2.2 Glucose and glutamine in cancer metabolism

In normal cells, glucose is oxidized to pyruvate, which can be further converted to acetyl-CoA to fuel the TCA cycle. The TCA cycle generates ATP as well as both NADH and $FADH_2$ which provide the mitochondrial respiratory chain with electrons for energy production. This is an efficient system to produce energy since each glucose molecule can provide up to 36 ATP molecules, mostly coming from mitochondrial respiration. In normal cells, anerobic glycolysis takes place only when oxygen supply is limited. In cancer cells, despite the presence of oxygen, glycolysis is increased and pyruvate is rather converted into lactate (Cairns et al., 2011). This process referred to as aerobic glycolysis (or Warburg effect) severely limits the amount of ATP formed. Although it was initially hypothesized that the aerobic glycolysis was a consequence of mitochondrial impairments, this hypothesis has been disproven: many tumours still retain a fully operational TCA and a functional oxidative phosphorylation (Coller, 2014; Lu et al., 2015) (Figure 11).

Figure 11. The Warburg effect.
In the presence of oxygen, non-proliferating (differentiated) cells first convert glucose into pyruvate via glycolysis. Most of the pyruvate is completely oxidized in the mitochondria to CO_2 during the process of oxidative phosphorylation. The final electron acceptor to completely oxidize the glucose is oxygen, which is essential for this process. When oxygen is low, pyruvate is converted into lactate (anaerobic glycolysis), resulting in lower ATP production compared to the oxidative phosphorylation. On the contrary, cancer cells as well as fast proliferating cells convert the majority of pyruvate into lactate, despite the presence of oxygen and functional mitochondria (aerobic glycolysis). Picture from Vander Heiden et al., 2009.



Aerobic glycolysis very efficiently fuels rapid proliferative cells. Importantly, glycolysis provides cancer cells with not only energy but also intermediate metabolites for biosynthesis and reduction equivalents for antioxidant defense. Therefore, several glycolytic metabolites are diverted into other metabolic pathways. For instance, glucose-6-phosphate and fructose-6-phosphate are often consumed by the pentose phosphate pathway to synthesize nucleotides and NADPH (a major reducing agent important for redox homeostasis and drug detoxifying reactions) (X. Chen et al., 2015). 3-phosphoglycerate can feed the serine biosynthesis pathway. In addition, serine and glycine (which is produced from serine) are precursors for glutathione synthesis and donate one-carbon units to the folate cycle, which is essential for *de novo* synthesis of adenosine, guanosine and thymidylate and can contribute to NADPH production (Yang and Vousden, 2016). Glutathione and NADPH are important defense strategies against the deleterious effects of reactive oxygen species. Taken together, glycolysis can be considered as an important source of "building blocks" required for fast proliferating cells and especially for cancer cells (Vander Heiden et al., 2009). Furthermore, the high amounts of lactate produced during aerobic glycolysis are not simply a waste product. Experimental evidence suggests that lactate is a real "oncometabolite" with pro-tumourigenic functions (Hirschhaeuser et al., 2011). Lactate is secreted into the tumour microenvironment via MCT4 transporter and it might fuel other cancer cells in the tumour microenvironment (Whitaker-Menezes et al., 2011). Besides, secreted lactate reduces the extracellular pH. Acidic pH can both kill healthy cells and activate pH-sensitive metalloproteinases, which breakdown the extracellular matrix promoting tumour invasion (Li et al., 2016). Lactate might as well have immune-suppressive

functions by interfering with the metabolism of cytotoxic T cells, which strongly rely on glycolysis (Fischer et al., 2007).

Apart from glucose, cancer cells display an increased dependence on glutamine (Wise and Thompson, 2010). Glutaminolysis is a series of biochemical reactions catabolizing glutamine into downstream metabolites such as glutamate and α-ketoglutarate. Glutamine is an anaplerotic substrate; indeed, the products of glutaminolysis provide carbon to maintain pools of the TCA cycle intermediates. These intermediates are used for the synthesis of lipids, cholesterol, amino acids and other essential metabolites. In addition, glutamine can also provide cancer cells with NADPH through the activity of malic enzyme which catalyses the oxidative decarboxylation of malate to pyruvate, with the concomitant release of CO_2 and conversion of $NADP^+$ to NADPH (DeBerardinis et al., 2007), which is essential in maintaining redox homeostasis.

1.2.3 The pyruvate dehydrogenase complex

The pyruvate dehydrogenase complex (PDH) is a key regulatory enzyme in cellular metabolism as it links glycolysis with the TCA cycle and subsequent oxidative phosphorylation. It is a multi-enzyme complex localized in the mitochondrial matrix, catalyzing the conversion of pyruvate, the end-product of the glycolysis, to acetyl coenzyme A (acetyl-CoA). Acetyl-CoA then feeds the TCA, resulting in the formation of citrate. The PDH complex is constituted of three catalytic subunits: the pyruvate dehydrogenase (E1), the dihydrolipoamide aceryltransferase (E2) and the dihydrolipoamide dehydrogenase (E3). An additional component is present in eukaryotes, the E3 binding protein (E3BP) (Patel et al., 2014). Since the PDH is an important interface with a central role in cellular energy regulation and the supply of intermediates for many biosynthesis processes, its activity is tightly regulated. A reduction of PDH activity occurs through reversible phosphorylation of the PDH-E1α subunit on any of the three serine residues S293, S300 or S232 by kinases of the pyruvate dehydrogenase kinase (PDKs) family (PDK1, PDK2, PDK3, PDK4) (Korotchkina and Patel, 1995; Patel and Korotchkina, 2001), which show a tissue-specific expression pattern and differential regulation of their activity (Patel and Korotchkina, 2006; Sugden and Holness, 2006). The re-activation of PDH is achieved through de-phosphorylation of the PHD-E1α subunit, catalyzed by the pyruvate dehydrogenase phosphatases (PDP1 and PDP2), which also display differences regarding their tissue distribution, regulation and activity (Huang et al., 1998; Patel and Korotchkina, 2006). In the last few years, PDH has received much attention especially because its misregulation could contribute to several diseases (Patel et al., 2012). In cancer cells, for instance, PDK inhibition and PDH activation trigger mitochondrial oxidative phosphorylation and consequently ROS production which, if excessive, causes cell death. The aerobic glycolysis gives the

cancer cells the possibility to avoid cellular oxidative stress that would be produced by mitochondrial oxidative phosphorylation for glucose metabolism.

1.2.4 Reactive oxygen species in cancer

For a long time, reactive oxygen species (ROS) have been considered to be lethal by-products of the cellular metabolism. However, in recent years, several studies have revealed an unexpected role for ROS as signaling molecules (Sabharwal and Schumacker, 2014). ROS are intracellular chemical species which contain oxygen: the superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\cdot}$) (Murphy, 2009). The main sources of their production are both the mitochondria and the membrane-bound NAPDH oxidases (NOXs) (Reczek and Chandel, 2017). In mitochondria, electrons flow through the electron transport chain to finally react with oxygen and protons to form water. The leakage of electrons during this process might cause the reaction of these electrons with O_2 to produce $O_2^{\cdot-}$. $O_2^{\cdot-}$ is released into the mitochondrial matrix and converted into H_2O_2 by superoxide dismutase 2 (SOD2). In addition, H_2O_2 can also be produced in the cytoplasm from both NOX- and mitochondria-derived superoxide by the cytosolic SOD. H_2O_2 can subsequently be detoxified to water by cytosolic and mitochondrial antioxidant scavengers. In particular, peroxiredoxins undergo H_2O_2-mediated oxidation of their active site cysteines (Reczek and Chandel, 2017). Oxidized peroxiredoxins are then reduced by thioredoxins, thioredoxin reductases and NADPH. Glutathione peroxidases also convert H_2O_2 to water by oxidizing reduced glutathione to glutathione disulfide. The latter is reduced back to glutathione by glutathione reductases and NADPH. High levels of ROS, if not properly neutralized, can cause oxidative damage and cell death. Compared to normal cells, cancer cells have higher rates of ROS production most probably due to oncogene activation, tumour suppressor loss, increased metabolic activity or limited nutrients or oxygen (Schieber and Chandel, 2014). To maintain ROS homeostasis, cancer cells must increase their antioxidant capacity (Gorrini et al., 2013). For instance, cells activate the transcription factor NRF2 (nuclear factor erythroid 2-related factor-2) which, in turn, induces the transcription of antioxidant proteins such as SODs, peroxiredoxins, glutathione peroxidases and enzymes that increase cytosolic NADPH (DeNicola et al., 2011; Gorrini et al., 2013). NADPH is also produced in multiple pathways such as the pentose phosphate, folate metabolism and by malic enzyme. By neutralizing excess ROS, cancer cells manage to maintain ROS at sustainable levels that allow for the activation of protumourigenic downstream effects (Figure 12).

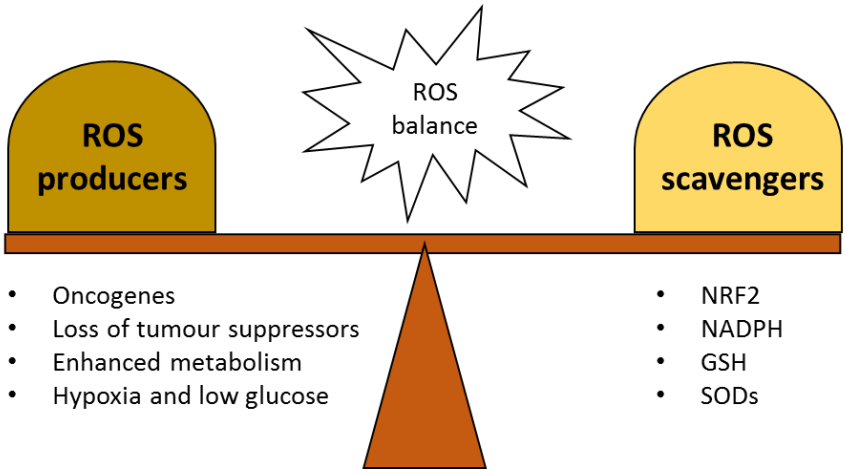


Figure 12. Levels of ROS are tightly regulated in cancer cells.
Activation of oncogenes, loss of tumour suppressor genes, metabolic reprogramming and hypoxia induce higher levels of ROS, which are counteracted by an upregulation of antioxidant genes and cofactors. By increasing their antioxidant capacity, cancer cells can survive potential oxidative damage.

It has been shown that low levels of ROS, especially H_2O_2, oxidize cysteine residues of proteins involved in cellular proliferation. For instance, H_2O_2 activates the PI3K/AKT/mTOR pathway by oxidizing and inactivating the negative regulator PTEN (Lee et al., 2002). ROS also oxidize and inactivate MAPK phosphatases causing an extended duration of MAPK activities (Son et al., 2011). An hypoxic microenvironment, often found in highly proliferating tumours, promotes ROS that can stabilize the HIF1α subunit, allowing its dimerization with the HIF1β subunit. The complex can then translocate into the nucleus and activate the transcription of genes involved in survival and angiogenesis (Reczek and Chandel, 2017). Tumour invasion can also be stimulated by ROS, promoting the formation of invadopodia (Diaz et al., 2009). Overall, ROS levels are tightly regulated in cancer cells since they are a double-edged sword: low and controlled levels of ROS provide beneficial effects for tumour growth, however once a certain threshold is reached, ROS can become fatal and induce cancer cell death. This delicate balance exclusively depends on their amount.

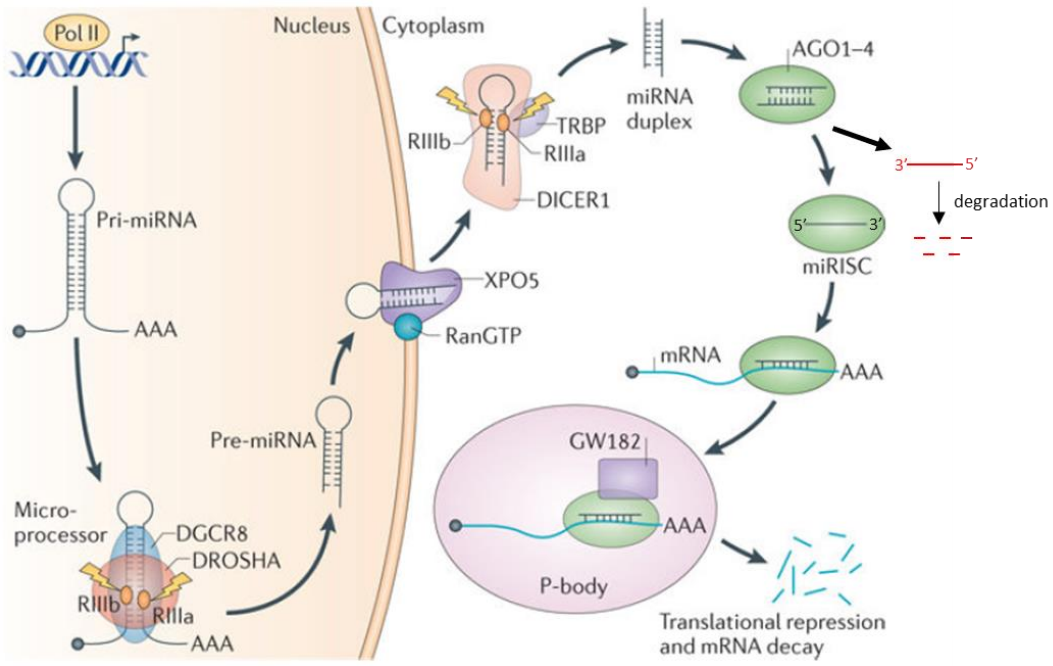
1.3 MiRNAs

MiRNAs have been shown to play an important role in key cellular processes and are therefore also implicated in almost all diseases including cancer. Furthermore, their potential as biomarkers has become an intense area of research. To better understand their potential role in the context of drug resistance, miRNA expression profiles in both sensitive and resistant melanoma cell lines have been investigated.

1.3.1 MiRNA biogenesis and function

MiRNAs are small non-coding RNA molecules, ~22 nucleotides long that play key roles in the regulation of gene expression. Acting at the post-transcriptional level, these molecules fine-tune the expression of ~60% of protein-coding genes in the human genome (Friedman et al., 2009). MiRNA biogenesis is a complex and tightly regulated process which takes place both in the nucleus and in the cytoplasm (Figure 13). In humans, most of miRNA sequences are encoded by introns of different coding and non-coding transcripts; some are also located in exonic regions (Y. Lee et al., 2002). MiRNA transcription is generally carried out by RNA polymerase II, although in some cases miRNAs can also be generated by RNA polymerase III (Borchert et al., 2006; Lee et al., 2004). Following transcription, the primary miRNA (pri-miRNA) which is 5'capped and 3' polyadenylated, undergoes cleavage by a microprocessor complex (formed by RNA-binding protein DiGeorge Critical Region 8 (DGCR8) and the type III RNAse Drosha) forming a stem-loop structure called precursor miRNA (pre-miRNA). Thereafter, the pre-miRNA is exported from the nucleus to the cytoplasm by Ran/GTP/Exportin 5 complex where it is cleaved by Dicer into a small RNA duplex which is subsequently loaded onto an Argonaute (AGO) protein to form the pre-RNA-induced silencing complex (pre-RISC). The passenger strand of the miRNA duplex is quickly degraded whereas the guide strand is selected to be part of the functional RISC complex. Strand selection depends on the stability of the 5' terminal ends and on the nucleotide sequence: strands with an uracil at position 1 and with an unstable 5' terminus seem to be preferentially selected as guide strands (Hu et al., 2009; Schwarz et al., 2003). However, in some cases, both strands can be active (MacFarlane and Murphy, 2010).

Figure 13. MiRNA biogenesis pathway.
The pri-miRNA is transcribed by RNA Polymerase II in the nucleus. The microprocessor (Drosha and DGCR8) processes the pri-miRNA into a pre-miRNA by cleaving the 5' and 3' extremities. The pre-miRNA translocates from the nucleus to the cytoplasm via exportin-5 where it is cleaved by Dicer in a short miRNA duplex. The functional strand of the mature miRNA (in black) is loaded together with Argonaute (Ago2) proteins into the RNA-induced silencing complex (RISC). This complex then binds to target mRNAs and induces cleavage, translational repression or deadenylation, while the passenger strand (in red) is degraded. Adapted from Lin and Gregory, 2015.



In addition to the canonical biogenesis pathway, alternative pathways exist as well. For instance, the so called “mirtrons” can bypass the Drosha-mediated processing step by direct splicing from intronic sequences (Berezikov et al., 2007). In addition, a Dicer-independent mechanism has been observed for miR-451. The precursor miRNA escapes Dicer and is directly loaded into the RISC complex (Cifuentes et al., 2010). It has been estimated that human cells express nearly 2000 miRNAs, each one potentially capable of binding to hundreds of messenger RNAs (mRNAs) (Friedländer et al., 2014). Nevertheless, only a small fraction of these interactions has experimentally been validated. In general, a specific “seed region” located between nucleotide 2 and 7 at the 5’end of the mature miRNA binds to the 3’ untranslated region (3’UTR) of a target mRNA and induces its degradation (in case of perfect complementarity) or inhibits its translation (partial complementarity) (Winter et al., 2009). However, Helwak et al. recently demonstrated that less than 40% of miRNA-mRNA interactions follow these canonical binding rules. They identified additional non-canonical interactions, which are independent of the miRNA seed region by using a method called CLASH (crosslinking and sequencing of hybrids) (Helwak et al., 2013). By applying this technique, interacting miRNAs and mRNAs are cross-linked and co-precipitated with AGO and finally sequenced. Regardless of the interaction complexity, miRNAs seem to play a role in many biological processes, including development, cell proliferation and metabolic regulation (Ameres and Zamore, 2013). Dysregulation of miRNA expression levels has been associated with several pathological conditions including cancer (Mendell and Olson, 2012).

1.3.2 MiRNAs in cancer

Considerable evidence indicates that miRNAs and their biogenesis machinery are involved in the development of cancer. Several miRNAs can act either as **oncomiRs** or as **tumour suppressor miRNAs** and depending on which gene or pathway they regulate, miRNAs can enhance or limit cancer development (Svoronos et al., 2016). OncomiRs mostly inhibit tumour suppressor genes and thus, they are generally overexpressed in cancer. On the contrary, tumour suppressor miRNAs target oncogenes resulting in their downregulation. In some cases, the classification of a certain miRNA as oncomiR or tumour suppressor has led to conflicting reports. However, given the large number of genes influenced by an individual miRNA, it is not surprising that some miRNAs can function as oncomiRs or as tumour suppressors in different scenarios or cell types (Svoronos et al., 2016). Aberrant expression of miRNAs can originate from several mechanisms including genomic amplification, deletion and/or translocation, epigenetic factors as well as alterations in some components of the miRNA biogenesis pathway (Lee and Dutta, 2009). Interestingly, modifications of the biogenesis pathway can affect many miRNAs resulting in a dramatic change of the whole miRNome of cancer cells (Hesse and Arenz, 2014).

An example of a widely studied oncomiR, which is upregulated in different cancers is miR-21 (Chan et al., 2005; Schetter et al., 2008) whose tumourigenic potential has been demonstrated *in vitro* and *in vivo* (Medina et al., 2010; Yan et al., 2008). Well characterized targets of miR-21 are the programmed cell death protein 4 (PDCD4), a protein involved in apoptosis and metastasis and often downregulated in several cancers (Asangani et al., 2008) and the tumour suppressor PTEN (Peralta-Zaragoza et al., 2016). Among the miRNAs downregulated in cancer, the miR-34 family has received considerable attention. This miRNA family is transcriptionally regulated by the tumour suppressor p53 during the DNA damage response, and p53 and the DNA damage response are often altered in cancer cells (Chang et al., 2007; Okada et al., 2014). Loss of miR-34 family members seems to block apoptosis and to drive cancer cell proliferation (Chang et al., 2007).

Tissue-specificity of miRNA effects is illustrated by miR-105. In endothelial cells, which normally express low levels of miR-105, breast cancer-derived miR-105 effectively reduced tight junction ZO-1 protein expression and disrupted the barrier function of these cells, thereby promoting metastasis both *in vitro* and in animal models (Zhou et al., 2014). Interestingly, augmented levels of miR-105 in the serum of breast cancer patients correlated with increased metastasis. In contrast, Honeywell et al. (Honeywell et al., 2013) described miR-105 to act as a tumour suppressor in prostate cancer indicating that tissue-specific mechanisms controlling the function of miRNAs might also be involved (Zhou et al., 2014).

The high stability and the tissue/disease-specific expression patterns of miRNAs suggested that their expression levels could harbor diagnostic, prognostic, and therapeutic potential as **biomarkers** (Iorio and Croce, 2009). It has been demonstrated that miRNAs can be passively released from broken cells and apoptotic bodies or be actively secreted as RNA-protein complexes or via cell-derived microvesicles (Turchinovich et al., 2011). The presence of regulatory miRNAs within extracellular vesicles has raised a strong interest ever since Valadi et al. showed for the first time that miRNAs in mast cell-derived extracellular vesicles can be transferred to other mast cells and be functional (Valadi et al., 2007). In contrast to protein-associated miRNAs (the uptake of which might be feasible but was never really demonstrated), those contained within microvesicles can be transferred to recipient cells, modulate gene expression and trigger functional effects (Stoorvogel, 2012). Interestingly, Melo and colleagues recently demonstrated that extracellular vesicles display a cell-independent capacity to process precursor miRNAs to their mature form: extracellular vesicles derived from breast cancer cells contained pre-miRNAs, along with Dicer and AGO2, and in a time-course experiment the six pre-miRNAs examined were inversely proportional to their corresponding mature form, suggesting an ongoing maturation process (Melo et al., 2014). This finding suggests that AGO-associated miRNAs might directly be functional in recipient cells.

In addition, recent reports provide evidence that miRNAs delivered by extracellular vesicles can regulate target gene expression in recipient cells. In an elegant study, Zhuang et al. found that the tumour-derived miR-9 transported by extracellular vesicles was functionally active in recipient cells: exogenous miR-9 effectively enhanced the JAK/STAT pathway by reducing SOCS5 levels, a negative regulator of the pathway (Zhuang et al., 2012). The so up-regulated signalling cascade then promoted endothelial cell migration and tumour angiogenesis. Albeit existing evidence of the importance of secreted miRNAs, it remains uncertain whether such miRNAs are really functional in a physiological environment and whether the concentration of secreted individual miRNAs would be sufficient to mediate measurable endocrine effects. Furthermore, it is still unclear how widely this process occurs *in vivo* and whether it is restricted to certain cell types, physiological conditions or diseases or whether it is a ubiquitous way of cell-to-cell communication.

1.3.3 MiRNAs in drug resistance

Despite significant progress has been made in cancer therapies, drug resistance often prevents full recovery of patients. Several studies have reported that aberrant miRNA expression is strongly implicated in the onset of drug resistance in several cancers (Fattore et al, 2017). Many miRNAs have been identified as important regulators of drug transporter genes in cancer cells. For instance, miR-451 was found to be downregulated in breast cancer cells resistant to doxorubicin. The authors

showed that this miRNA regulates the expression of the multi-drug resistance 1 gene (mdr1), a crucial mediator of drug resistance (Kovalchuk et al., 2008). In another context, Liu et al. have shown that, in small-cell lung cancer (SCLC), low levels of miR-7 were closely correlated with chemotherapy resistance, in addition to shorter overall survival. They demonstrated that miR-7 mediates chemoresistance by repressing drug efflux MRP1/ABCC1 genes (Liu et al., 2015). Apart from modulating the expression of transporters, miRNAs can also modulate specific signaling pathways. Stark et al. showed that miR-514a modulates the sensitivity to BRAF inhibitors in melanoma by regulating the tumour suppressor neurofibromin 1 (NF1). NF1 negatively regulates RAS activity and therefore the MAPK pathway. Consequently, increased levels of miR-514a inhibit the expression of NF1, which correlates with the increased survival of $BRAF^{V600E}$ cells treated with Vemurafenib (Stark et al., 2015). Another example is miR-214, which was recently shown to be associated with ovarian cancer cell survival and cisplatin resistance by regulating PTEN levels and consequently activating the AKT pathway (Yang et al., 2008). Furthermore, researchers have started to explore the possibility that miRNAs packaged in extracellular vesicles can also contribute to the maintenance and onset of drug resistance. MiRNAs loaded in these vesicles might be transferred to recipient cells to exert genome-wide regulation of gene expression (reviewed in (Cesi et al., 2016)). Although more and more studies have highlighted their functional role in *in vitro* studies, it still remains to be shown how these secreted miRNAs can contribute to the development of drug resistance in the context of the tumour microenvironment.

1.4 Extracellular vesicles

Extracellular vesicles have been shown to be vehicles for transfer of miRNAs, proteins, DNA between cells and to partake in intercellular communication in physiological and pathological conditions. To examine whether drug resistance could be transferred from resistant to sensitive cells, the content and the functions of extracellular vesicles released by resistant cells have been studied here.

1.4.1 Extracellular vesicle biogenesis and function

Extracellular vesicles (EVs) are a heterogeneous group of membrane-surrounded vesicles secreted from all types of cells of many different organisms, from prokaryotes to eukaryotes (Colombo et al., 2014). Considered at the beginning simply as “garbage bins” to remove unwanted molecules from secreting cells (Harding and Stahl, 1983; Pan and Johnstone, 1983), it turned out that these tiny vesicles play a role in cell-to-cell communication carrying a real “message in a bottle”. Generally, EVs are classified according to their biogenesis pathway and their size (Abels and Breakefield, 2016).

There are three main classes of EVs: i) **Apoptotic bodies**: generally large vesicles ranging between 50–5000 nm produced exclusively from cells undergoing cell death by apoptosis. These large vesicles are characterized by the presence of organelles (Elmore, 2007). In addition, smaller vesicles (50–500 nm) probably coming from membrane blebbing, are also released during this process (Coleman et al., 2001). Most apoptotic bodies are cleared up by macrophages, which recognize specific changes in the composition of the apoptotic cells' membrane such as phosphatidylserine which is translocated to the outer leaflet of the lipid layer (Martínez and Freyssinet, 2001). ii) **Microvesicles**: 50–1000 nm vesicles, which originate from direct outward budding of the plasma membrane (Akers et al., 2013). Membrane budding occurs following the interaction between specific phospholipids and cytoskeletal proteins. In a first step, phosphatidylserine is translocated to the outer-membrane leaflet (Hugel et al., 2005). Then, the budding process is completed through the contraction of cytoskeletal structures by actin-myosin interactions (Muralidharan-Chari et al., 2009). iii) **Exosomes**: generally defined as small vesicles, ~30 -120 nm in size, with an endosomal origin. Their biogenesis starts when endocytic vesicles fuse with early endosomes which incorporate their content. Early endosomes mature into late endosomes also known as multivesicular bodies (MVBs), which are characterized by small intraluminar vesicles following the inward budding of the limiting membrane. Many MVBs fuse with lysosomes for content degradation. Some can fuse with the plasma membrane releasing the vesicular content in the extracellular space (Colombo et al., 2014). The best characterized mechanism for MVB formation is mediated by the endosomal sorting complex required for transport (ESCRT). Moreover, other ESCRT-independent mechanisms have been described. Edgar et al. observed that CD63 molecules were sufficient to generate intraluminal vesicles within the MVB (Edgar et al., 2014). In addition, two lipid metabolic enzymes (neutral sphingomyelinase and phospholipase D2) have been shown to induce inward budding of the MVB and thus formation of intraluminal vesicles (Ghossoub et al., 2014; Trajkovic et al., 2008) (Figure 14).

Despite this theoretical classification, current isolation methods do not allow such a precise separation of vesicle groups. Some vesicles share a similar size and morphology but once they have been released their origin cannot be tracked anymore because unique markers for the different vesicle types have not been defined yet (Raposo and Stoorvogel, 2013). Because of the difficulties to experimentally classify isolated vesicles, the International Society of Extracellular Vesicles suggested to use the generic term "extracellular vesicles (EVs)" to describe vesicles isolated from the extracellular milieu (Lötvall et al., 2014).

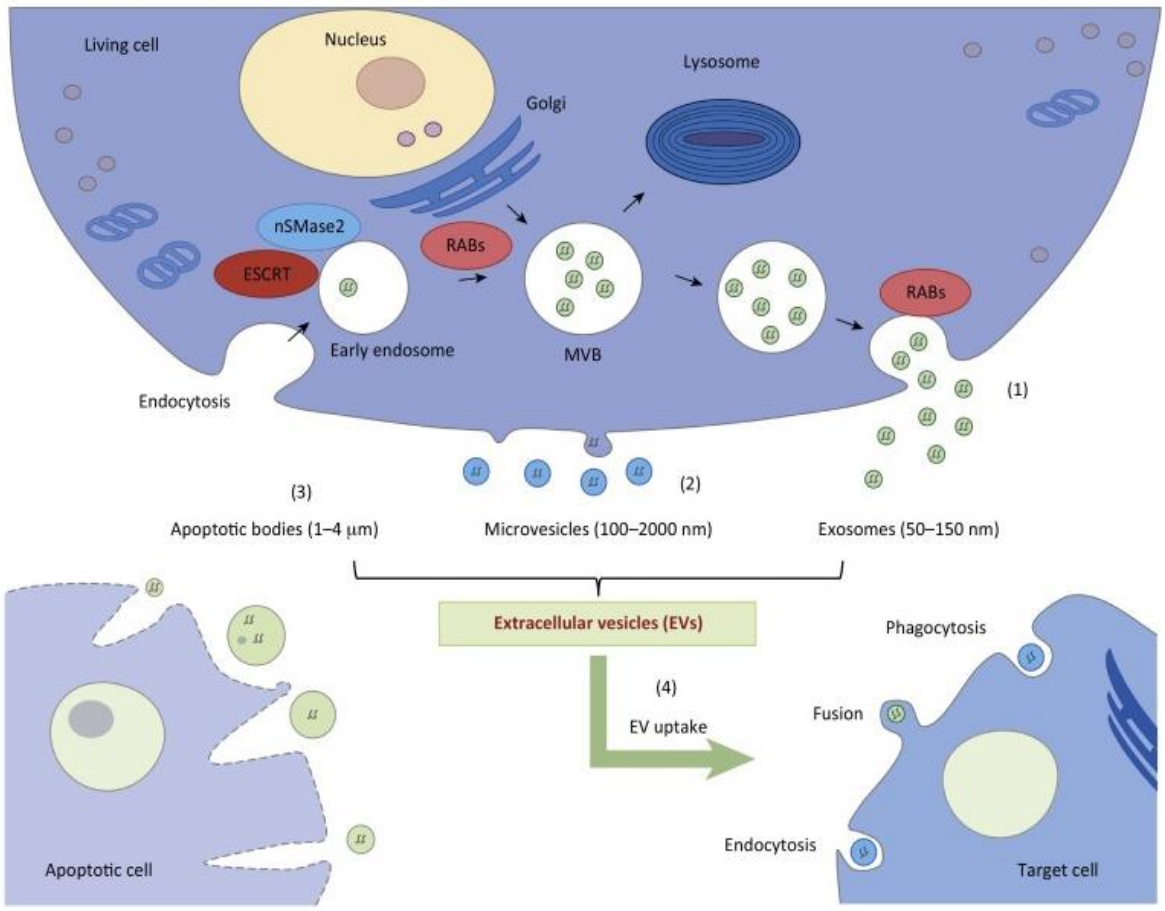


Figure 14. Different biogenesis pathways give rise to different populations of extracellular vesicles.
(1) Exosomes derive from the endocytic pathway. Endocytic vesicles mature into early endosomes and then into late endosomes or MVBs. MVBs can either be sorted for lysosomal degradation or they can fuse with the plasma membrane and be released as exosomes. Neutral sphingomyelinase (nSMase2) is an important factor in the formation of the intraluminal vesicles within early endosomes. Rab GTPases regulate MVB fusion with the plasma membrane and release of exosomes. (2) Microvesicles derive from the outward budding of plasma membrane, which is controlled by regulatory proteins and cytoskeleton elements that promote membrane curvature at ceramide-enriched domains. (3) Cells undergoing apoptosis produce large membrane blebs, known as apoptotic bodies. (4) EVs can transfer their contents to target cells through different mechanisms. Figure taken from Fujita et al., 2015.

1.4.2 Uptake of EVs

The possibility of transferring molecules to recipient cells strongly depends on the capability of EVs to interact with target cells. Depending on the recipient cell, EV uptake might occur through different mechanisms. The mode of EV entry into cells will determine their functional effects. **Direct fusion** of EVs would cause the direct release of the content into the cytoplasm of the recipient cell. However, some forms of **endocytosis** seem to be more frequent (Mulcahy et al., 2014). In

particular, actin polymerization-dependent phagocytosis (Feng et al., 2010); receptor-mediated endocytosis (Morelli et al., 2004); clathrin-dependent endocytosis (Frühbeis et al., 2013); cholesterol- and lipid raft-dependent endocytosis (Svensson et al., 2013); or caveolae-dependent endocytosis (Nanbo et al., 2013) have been described. If EVs enter the endocytic pathway, their cargo must find a way out as MVBs either fuse with lysosomes or with the plasma membrane (Abels and Breakefield, 2016). Although many studies clarified the mechanisms behind the release and the uptake of EVs *in vitro*, it is still quite difficult to translate these findings to *in vivo* settings and to understand the fate of EVs once released by specific cells. EVs are found in many biological fluids such as saliva, blood and urine suggesting their frequent release in the body. Moreover, some studies tried to elucidate potential destinations for EVs once injected in animals. Melanoma-derived EVs were found in lung, liver and bone marrow (Peinado et al., 2012; Takahashi et al., 2013) and EVs from a pancreatic adenocarcinoma cell line were recovered in pancreas, lung, and kidney (Rana et al., 2013) suggesting that EVs from different origins specifically target different organs *in vivo*.

1.4.3 Content of EVs

Many studies have attempted to elucidate the content of EVs. However, due to the lack of standard protocols for their isolation, variations in culture conditions and different cell types used for profiling, it remains technically difficult to exactly determine EV content. Overall, the content of EVs mostly depends on their "parental cells" of origin. Generally, EVs from different cell types contain proteins associated with their biogenesis mechanism such as endosome-associated proteins (e.g., Rab GTPase, SNAREs, Annexins, flotillin or Alix and Tsg101) (Van Niel et al, 2006). Membrane proteins including tetraspanins (e.g., CD63, CD81, CD82, CD53, CD37 and CD9), heat shock proteins, MHC complexes, growth factors and many others are also present (Tickner et al., 2014). How exactly proteins are sorted into EVs is still under investigation. The lipid composition of EVs has also been studied. EVs carry lipids of a similar composition than found in the plasma membrane of the parental cells (such as cholesterol, ceramide and sphingomyelin) (Kharaziha et al., 2012). The RNA content of EVs is enriched in small RNAs especially miRNAs (Kharaziha et al., 2012). Although the sorting mechanisms are not fully understood, recent evidence suggests that the composition of miRNAs present in EVs differs from the one of the cell of origin suggesting that mRNA are not randomly secreted into EVs but that a selective sorting mechanism exists. In this context, Villarroya-Beltri et al. (Villarroya-Beltri et al., 2013) identified the presence of a four nucleotide motif (GGAG) enriched in miRNAs present in EVs. They hypothesized that the unique sequence could prone miRNAs for sorting into EVs. They also demonstrated that the sumoylated heterogeneous nuclear ribonucleo-protein A2B1 binds miRNAs through their "EXO-motifs" and controls their loading into

EVs, thus providing an explanation for the specific sorting of certain miRNAs into EVs. On the contrary, interesting findings from Squadrito et al. (Squadrito et al., 2014) suggest a passive mechanism for miRNA sorting modulated by cell activation-dependent changes of miRNA target levels. EV miRNA secretion might be a mechanism by which cells remove unwanted miRNAs, which are overexpressed compared to their corresponding targets to re-establish miRNA/mRNA homeostasis. Additionally, posttranscriptional modifications of the 3'end of miRNAs appear to contribute to direct miRNA sorting into EVs. In particular, uridylated miRNAs seem to be enriched in EVs whereas the adenylated miRNAs are more abundant in cells (Koppers-Lalic et al., 2014). Last but not least, EVs can contain single-stranded DNA and transposable elements (Balaj et al., 2011).

1.4.4 EVs in cancer

One of the most studied aspects of EVs is their pathological role in tumour biology. Emerging evidence suggests that tumour-derived EVs are implicated in many aspects of tumourigenesis by promoting tumour growth, angiogenesis, metastasis and immune cell escape (Tkach and Théry, 2016). Cancer EVs differ from those released by healthy cells in terms of their quantity and content. An increased secretion of EVs has been reported in different cancer cell lines and cancer patients (King et al., 2012; Logozzi et al., 2009). Acidic pH (Federici et al., 2014; Parolini et al., 2009) and hypoxia (King et al., 2012; Wang et al., 2014) often present in tumours, might be responsible for the intensification of EV production. In this context, leukemia cells seem to secrete more EVs under hypoxic versus normoxic conditions and this affected angiogenesis as exemplified by enhanced tube formation of endothelial cells (Tadokoro et al., 2013). Furthermore, an increased expression of miRNA-210 was detected, which down-regulated the receptor tyrosine kinase ligand, Ephrin-A3 (EFNA3) and thereby also contributed to tube formation. It has been recently shown that hypoxic stress increases the formation of EVs in a HIF- dependent manner in breast cancer cell lines. The increased EV secretion was strictly correlated with HIF-dependent expression of RAB22A. Indeed, RAB proteins, which are membrane-bound GTPases, play a key role in vesicle formation, trafficking and membrane fusion and seem to be direct HIF-1α and HIF-2α target genes (Wang et al., 2014).
Cancer-derived EVs deliver their pathogenic components to their microenvironment (other cancer cells, fibroblasts, immune cells, endothelial cells) to support tumour growth but also to distant cells to prepare niches that support metastasis. For instance, glioblastoma-derived EVs can transfer the epidermal growth factor receptor variant III (EGFRvIII, a truncated and oncogenic form of EGFR) to cancer cells lacking EGFRvIII. The horizontal propagation of this oncogene promotes the progression of the malignancy (Al-Nedawi et al., 2008). Peinado et al. reported that EVs from highly metastatic melanoma cells increased the metastatic behavior of primary tumours by permanently "educating"

bone marrow progenitor cells toward a pro-vasculogenic and pro-metastatic phenotype via the MET receptor (Peinado et al., 2012). Interestingly, EVs can also modulate cancer cell metabolism. In particular, Zhao et al. showed that EVs isolated from prostate cancer patient-derived cancer-associated fibroblasts (CAFs) were able to reprogram prostate cancer cells by inhibiting oxidative phosphorylation and by upregulating glucose metabolism and increasing reductive glutamine. In addition, they identified within the EVs a pool of metabolite cargo such as TCA cycle metabolites, amino acids and lipids (Zhao et al., 2016).

Taken together, a better understanding of the resistance mechanisms against different therapeutic agents will be helpful to identify second line treatments. In this context, cancer-derived EVs might provide useful information. Currently, there are three frequently described EV-mediated drug resistance mechanisms: EV-mediated drug export, EV-mediated miRNA export, EV-mediated efflux pump transport (reviewed in Cesi et al., 2016, see chapter 4.4).

Chapter 2 Aims

Although considerable progress in the field of melanoma biology has been made in the last decade, advanced disease is still associated with poor outcome for patients. Therapies blocking the MAPK signalling pathway by targeting both BRAF and MEK, have improved overall survival and about 90% of patients experience tumour regression at first (Winder and Virós, 2017). However, despite encouraging clinical results, most patients acquire drug resistance, which leads to rapid tumour progression. As a result, many studies have tried to elucidate the molecular mechanisms driving acquired forms of resistance. Surprisingly, not much attention has been given to what happens during the early phase of treatment, when patients still respond to the treatment with inhibition of the MAPK pathway. In addition, new escape mechanisms as well as post-transcriptional changes by non-coding RNAs that can lead to drug resistance are in the focus of attention. Considering this background, the main objectives of this thesis can be divided into three main parts (Figure 15):

1) ***Impact of BRAF inhibitors on metabolic pathways in melanoma***.

   Mutant BRAF has been associated with altered cellular metabolism. In this part, the effects of BRAF and MEK inhibitors on metabolic proteins at early time points after treatment have been analyzed in mutant and wild-type BRAF cell lines providing evidence of adaptive metabolic responses taking place under selective pressure.

2) ***Analysis of the miRNome and transcriptome in both sensitive and resistant melanoma cells.***

   The implication of deregulated miRNAs in BRAF inhibitor resistance has not been studied thoroughly. In this study, the expression patterns of miRNAs that might play a role in drug resistance or could be used as biomarkers have been investigated in different cell lines. Despite heterogeneous responses to long term treatment with BRAF inhibitors in the different cell lines, several miRNAs and genes were differentially expressed in drug-resistant versus drug-sensitive cell lines.

3) ***Role of extracellular vesicles in melanoma drug resistance.***

   Extracellular vesicles have been identified as new messengers in transferring drug resistance to still sensitive cells contributing to tumour progression. To better explore this

phenomenon in the context of melanoma, this project part was focusing on the functional properties of extracellular vesicles and on their capability of transferring drug resistance.

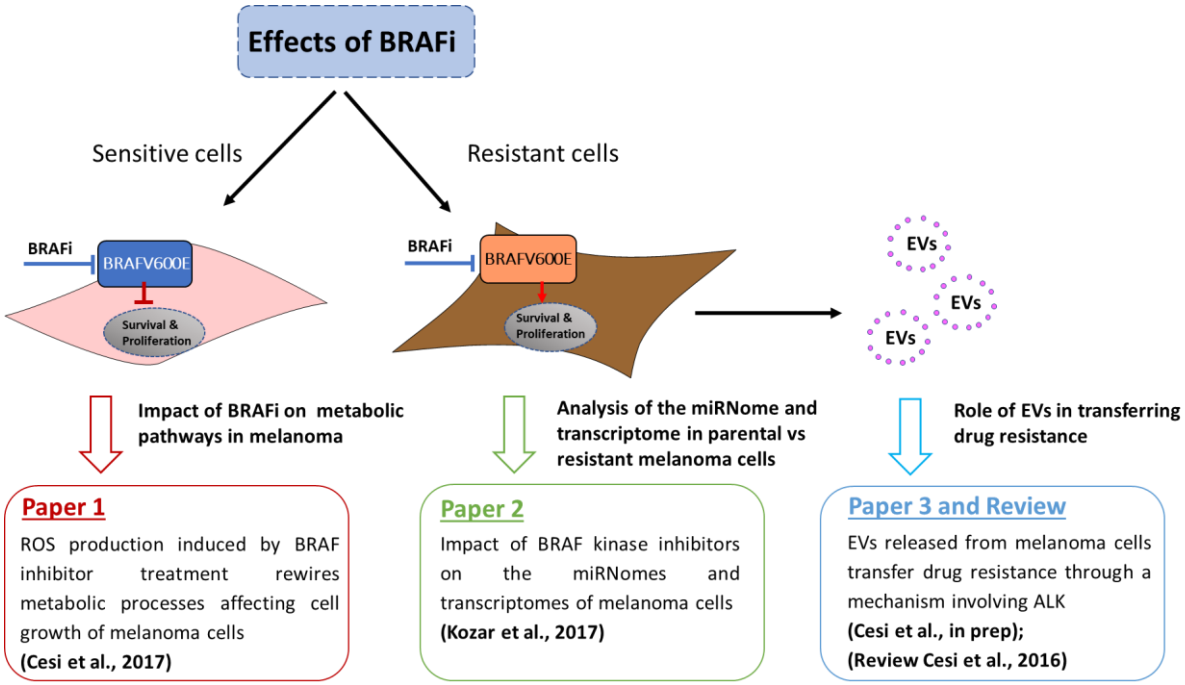


Figure 15. Project overview.

Summary of the different subparts of the thesis and corresponding publications.

Chapter 3 Materials and Methods

All methods used in the scope of the three project parts are extensively explained in the corresponding manuscripts (Cesi et al., 2017 in chapter 4.1; Kozar et al., 2017 in chapter 4.2; Cesi et al, in prep in chapter 4.3). The following section only includes materials and methods, which were used to obtain additional results.

3.1 Total RNA extraction from extracellular vesicles and quality control

Total RNA from 200 μl of EV suspension was extracted using the miRNeasy serum/plasma kit (Quiagen) according to the manufacturer's instructions. As an internal calibrator, a mix of C. elegans miRNAs cel-39, cel-54 and cel-238 exogenous controls were spiked into the samples. RNA was eluted with 14 μl of RNAse-free water. Quality control of extracted RNA was performed by RT-qPCR using primers for:

- Cel-39, Cel-45 and Cel-238 spikes to control for variations in recovery and amplification efficiency between samples
- miRTC which is an internal miRNA reverse transcription control provided in the kit

Briefly, 4 μl of eluted total RNA were reverse transcribed in a 10 μl reaction volume with the miScript RT II kit (Qiagen) following the supplied protocol using Hispec buffer, which specifically amplifies mature miRNAs. Real time PCR detection of the above-mentioned controls was carried out on a CFX96 Detection System (Bio Rad) using 1 μl of 1:10 diluted cDNA, 2x iQ SYBR Green Supermix (Bio-Rad) and 10x miRNA-specific primer assay (Qiagen) as described previously (Margue et al., 2015).

3.2 miRNA profiling of extracellular vesicles by qPCR arrays

MiRNAs extracted from extracellular vesicles were profiled with human whole miRNome miScript miRNA qPCR arrays (Qiagen, v16, 1066 miRNAs). The 1:5 diluted cDNA was pre-amplified with the miScript PreAmp PCR kit (Qiagen) using the corresponding primer mixes (whole miRNome primer mix for whole miRNome qPCR arrays). Pre-amplification control experiments were performed by RT-qPCR using primer assay for cel-39 and miRTC. Quality-controlled pre-amplified cDNA was diluted 1:5 and further used for miScript whole miRNome according to the supplied protocol. Real-time PCR detection on the qPCR arrays was carried out on a CFX384 Detection System (Bio-Rad). Specificity of the qPCR primers was assessed by qPCR melting curve analysis.

3.3 Quality control of qPCR melting curves

To assess the quality of melting curves from the whole miRNome qPCR, our lab has previously developed a Support Vector Machine-based program within the R package (Margue et al., 2015). The tool is able to predict "good" and "bad" curves, based on their shapes and curve feature such as Cq values, the melting temperature, the peak height and the starting and ending temperatures. The script also calculates the probability that the prediction is reliable (the closer to 1, the more reliable). If this probability is closer to 0, the label of the curve becomes questionable meaning that the curve had to be inspected manually. Good melt curves mean specific product amplification whereas bad melt curves mean non-specific or non-product amplification. The tool was applied to all RT-qPCR array amplifications and only high-quality amplifications were kept.

3.4 Data analysis

For qPCR array data analysis, baselines and thresholds were adjusted as recommended by the supplier and Cq values were exported for analysis. Cq values obtained with the cel-39 primers were used to calibrate the data sets: the Cq mean for cel-39 for each sample was calculated, the highest Cq mean of all samples was determined and the difference (correction factor) with the other samples was established. This correction factor was added to all Cq values of a sample. Calibrated Cq values greater than 30, as well as primers with bad melting curves were considered as not detected (N/A). These lower cut-off Cq values, recommended by the supplier, are due to the additional 12 PCR cycles during pre-amplification. Because of the lack of established house-keeping genes for extracellular vesicles for data normalization, we used means of commonly expressed miRNAs (for whole miRNome qPCR arrays).

3.5 Microarray analysis

For microarray analysis, cells were seeded for 48 hours in the presence or absence of 30 μg of extracellular vesicles. Total RNA was extracted with miRNeasy kit (Qiagen) in triplicates following the manufacturer's instructions. RNA quality was further assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies). Microarray analyses were performed at the Luxembourg Institute of Health (LIH) in Luxembourg. The Affymetrix miRNA 4.0 and Affymetrix HuGene 2.0 ST platforms were used for miRNA and mRNA microarrays, respectively. The commercial software Partek Genomic Suite was used for data pre-processing using Robust Multiarray Analysis (RMA) with GC-content correction. The Log2-transformed intensities were imported into the R environment. Differential expression of genes and miRNAs in the drug-resistant cell lines, as compared to the drug-sensitive, was determined using the R/Bioconductor package limma, which

adopts a linear modeling approach with empirical Bayesian statistics. The multiple correction was performed using the Benjamini-Hochberg's FDR (false discovery rate or adjusted P-value). Genes and miRNAs with FDR<0.01 and at least 1.5-log fold change were considered as differentially expressed.

Chapter 4 Results

4.1 Impact of kinase inhibitor treatment on cellular metabolism of melanoma cells

4.1.1 Preamble

Reactive oxygen species (ROS), initially considered to be harmful side products of cellular respiration, are now recognized as novel signal mediators involved in growth, differentiation, progression, and death of the cell (DeBerardinis and Chandel, 2016). Cancer cells exhibit higher levels of ROS compared to normal cells, mostly due to genetic and metabolic alterations. Since the redox equilibrium plays a crucial role in cell survival, increased ROS levels are compensated by an elevated antioxidant defense (Gorrini et al., 2013), which works as salvage mechanisms avoiding ROS to reach toxic levels.

In this first project part, we focused on early adaptive responses to BRAF kinase inhibitor treatment in melanoma cells. In particular, we have demonstrated that BRAF inhibition in melanoma cells induces ROS upregulation both in mitochondria and in the cytosol within hours of treatment. The link between MAPK pathway and ROS are multiple: activating BRAF mutations have been associated with enhanced glycolytic activity (Parmenter et al., 2014). Therefore, BRAF inhibition suppresses glycolysis and NADPH production influencing the flux of pyruvate through the TCA cycle and ultimately the flux through the electron transport chain. Interestingly, every disturbance to the steady state of the electron transport chain generates ROS (Schieber and Chandel, 2014). Thus, inhibition of BRAF might enhance ROS by altering the flux through the TCA and the electron transport chain. In addition, the MAPK and the PI3K/AKT pathway have been described to activate the transcription of genes involved in antioxidant defense (Espinosa-Diez et al., 2015). Accordingly, BRAF inhibition should increase ROS by suppressing the transcription of these antioxidant genes.

In this part of the thesis, we could show that: i) MAPK pathway suppression by BRAF inhibition, MEK1 inhibition or ERK1/2 knock-down leads to phosphorylation of PDH in $BRAF^{V600E}$ mutant cells; ii) BRAF inhibition in melanoma cells induces ROS upregulation both in mitochondria and in the cytosol; iii) inhibition of PDK1 (with a recently described inhibitor used at micromolar concentration) caused growth retardation in BRAF mutant and BRAF inhibitor- resistant melanoma cells. These data have implications for a combined inhibition of the BRAF/MEK/ERK signalling pathway together with inhibiting specific metabolic features.

Authors' contributions are explained at the end of the article.

Cesi *et al. Molecular Cancer* (2017) 16:102
DOI 10.1186/s12943-017-0667-y

Molecular Cancer

RESEARCH Open Access

ROS production induced by BRAF inhibitor treatment rewires metabolic processes affecting cell growth of melanoma cells

Giulia Cesi, Geoffroy Walbrecq, Andreas Zimmer, Stephanie Kreis*† and Claude Haan†

Abstract

Background: Most melanoma patients with $BRAF^{V600E}$ positive tumors respond well to a combination of BRAF kinase and MEK inhibitors. However, some patients are intrinsically resistant while the majority of patients eventually develop drug resistance to the treatment. For patients insufficiently responding to BRAF and MEK inhibitors, there is an ongoing need for new treatment targets. Cellular metabolism is such a promising new target line: mutant $BRAF^{V600E}$ has been shown to affect the metabolism.

Methods: Time course experiments and a series of western blots were performed in a panel of $BRAF^{V600E}$ and $BRAF^{WT}$/$NRAS^{mut}$ human melanoma cells, which were incubated with BRAF and MEK1 kinase inhibitors. siRNA approaches were used to investigate the metabolic players involved. Reactive oxygen species (ROS) were measured by confocal microscopy and AZD7545, an inhibitor targeting PDKs (pyruvate dehydrogenase kinase) was tested.

Results: We show that inhibition of the RAS/RAF/MEK/ERK pathway induces phosphorylation of the pyruvate dehydrogenase PDH-E1α subunit in $BRAF^{V600E}$ and in $BRAF^{WT}$/$NRAS^{mut}$ harboring cells. Inhibition of BRAF, MEK1 and siRNA knock-down of ERK1/2 mediated phosphorylation of PDH. siRNA-mediated knock-down of all PDKs or the use of DCA (a pan-PDK inhibitor) abolished PDH-E1α phosphorylation. BRAF inhibitor treatment also induced the upregulation of ROS, concomitantly with the induction of PDH phosphorylation. Suppression of ROS by MitoQ suppressed PDH-E1α phosphorylation, strongly suggesting that ROS mediate the activation of PDKs. Interestingly, the inhibition of PDK1 with AZD7545 specifically suppressed growth of BRAF-mutant and BRAF inhibitor resistant melanoma cells.

Conclusions: In $BRAF^{V600E}$ and $BRAF^{WT}$/$NRAS^{mut}$ melanoma cells, the increased production of ROS upon inhibition of the RAS/RAF/MEK/ERK pathway, is responsible for activating PDKs, which in turn phosphorylate and inactivate PDH. As part of a possible salvage pathway, the tricarboxylic acid cycle is inhibited leading to reduced oxidative metabolism and reduced ROS levels. We show that inhibition of PDKs by AZD7545 leads to growth suppression of BRAF-mutated and -inhibitor resistant melanoma cells. Thus small molecule PDK inhibitors such as AZD7545, might be promising drugs for combination treatment in melanoma patients with activating RAS/RAF/MEK/ERK pathway mutations (50% BRAF, 25% $NRAS^{mut}$, 11.9% $NF1^{mut}$).

Keywords: Melanoma, Metabolism, RAS/RAF/MEK/ERK pathway, NRAS, BRAF inhibitors, MEK inhibitors, Pyruvate dehydrogenase, Pyruvate dehydrogenase kinases, Reactive oxygen species, PDK inhibitors

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Background

Mutations in the Ser/Thr-kinase BRAF have been found in 10% of all human cancers with the highest prevalence observed in melanoma patients (>50%), making BRAF one of the most mutated cancer-associated genes [1, 2]. The increased kinase activity of BRAF is due to somatic point mutations, such as V600E, which result in the constitutive activation of the MAP kinase signaling pathway [3]. The mutation dysregulates cellular proliferation and confers survival advantages to cancer cells. In melanoma, activating mutations in the RAS/RAF/MEK/ERK pathway are found in many patients (50% BRAF, 25% $NRAS^{mut}$, 11.9% $NF1^{mut}$) while only a small part are "triple WT" and do not present one of the aforementioned hotspot mutations. Small molecule inhibitors (such as Vemurafenib also known as PLX4032 or Dabrafenib also known as GSK2118436), which bind with high affinity to the mutated form of BRAF have been successfully used as monotherapy in melanoma patients [4, 5]. However, despite the initial promising results, most patients relapse and develop drug resistance within 6 months [6]. The onset of drug resistance is often achieved by bypassing BRAF inhibition through downstream activation of MEK [7]. Therefore, a combination therapy targeting BRAF V600 mutations (Dabrafenib or Vemurafenib) as well as MEK (Trametinib or Cobimetinib) has been approved in 2015 for use in stage III and stage IV melanoma patients [8, 9]. Overall, median survival has increased with the combination therapy from 18.7 to 25.1 months [8, 9]. Thus, there is a need for novel therapies that can target melanoma efficiently as a monotherapy or delay resistance mechanisms to occur as part of a combination therapy.

Metabolic reprogramming can contribute to cancer cell survival and it is often driven by activated oncogenes or inactivated tumor suppressors such as c-Myc, HIF1α, TP53, AMPK, PI3K/AKT as well as Ras-related genes [10]. It has been shown that mutations of proteins in the RAS/RAF/MAPK pathway promote glycolysis [11] and the expression of cell surface glucose transporter 1 (GLUT1) in both colorectal cancer cell lines [12] and in melanoma [13] indicating that glucose metabolism might be important for BRAF-driven tumourigenesis. The pyruvate dehydrogenase complex (PDH) is the gatekeeper enzyme connecting glycolysis and the tricarboxylic acid (TCA) cycle. It is a multi-enzyme complex localised in the mitochondrial matrix, catalyzing the conversion of pyruvate, the end-product of the glycolysis, to acetyl coenzyme A (acetyl-CoA). Acetyl-CoA then feeds the TCA, resulting in the formation of citrate. Since the PDH is an important interface with a central role in cellular energy regulation and the supply of intermediates for many biosynthesis processes, its activity is tightly regulated. A reduction of PDH activity occurs through reversible phosphorylation of the PDH-E1α subunit on any of the three serine residues S293, S300 or S232 by kinases of the pyruvate dehydrogenase kinase (PDKs) family (PDK1, PDK2, PDK3, PDK4) [14, 15], which show a tissue-specific expression pattern and differential regulation of their activity [16, 17]. The reactivation of PDH is achieved through de-phosphorylation of the PHD-E1α subunit, catalyzed by the pyruvate dehydrogenase phosphatases (PDP1 and PDP2), which also display differences regarding their tissue distribution, regulation, and activity [16, 18]. In cancer cells, PDK inhibition and PDH activation trigger mitochondrial oxidative phosphorylation (Oxphos) and consequently ROS production which, if excessive, causes cell death. The aerobic glycolysis (also known as Warburg effect) gives the cancer cells the possibility to avoid cellular oxidative stress that would be produced by mitochondrial Oxphos for glucose metabolism.

In this study, we investigate the effects of BRAF and MEK inhibitors on metabolic proteins at early time points after treatment and analyse how BRAF-mutant cells attempt to survive under selective pressure. In particular, we demonstrate that the treatment of BRAF mutant melanoma cells with BRAF kinase inhibitors (PLX4032 or GSK2118436) increases the phosphorylation of the PDH-E1α subunit, while this is not the case in cells harboring $BRAF^{WT}$/$NRAS^{mut}$, a process which is facilitated by PDKs. PDH phosphorylation was also induced following inhibition of MEK1 and knock-down of Erk1/2 suggesting that downstream targets of mutant BRAF are responsible rather than off target effects of the BRAF inhibitor. In addition an activation of AMP-activated protein kinase (AMPK) was observed. Interestingly, PDH phosphorylation correlated with the appearance of an altered redox state in both mitochondria and the cytosol and was also inducible by H_2O_2 treatment. Finally, we show that a new PDK1 inhibitor, AZD7545, leads to efficient suppression of cell growth in cells harboring $BRAF^{V600E}$ mutation suggesting itself as potential combination treatment with BRAF-and MEK targeting kinase inhibitors.

Methods

Reagents and antibodies

Selective BRAF inhibitors PLX4032 (Vemurafenib) and GSK2118436 (Dabrafenib) were purchased from Selleckchem. BRAF inhibitors were dissolved in DMSO according to the manufacturer's instructions and stored at −80 °C. Working aliquots were diluted in 100% ethanol at a concentration of 1 mM for PLX4032 and 100 μM for GSK2118436 and stored at −20 °C. The MEK inhibitor GSK1120212 (Trametinib) was purchased from Selleckchem and was dissolved in DMSO at a concentration of 10 mM and stored at −20 °C. Dichloroacetic acid (DCA) was purchased from Sigma-Aldrich and dissolved

in distilled water before use. AZD7545 was purchased from Selleckchem and was dissolved in 100% ethanol according to the manufacturer's instructions. 10 mM working aliquots were stored at −20 °C. Mitoquinone mesylate (MitoQ) was purchased from Medkoo Biosciences, dissolved in DMSO according to the manufacturer's instructions and stored at −20 °C. The following antibodies were used for western blot detection: anti phospho-PDH E1α (Merck Millipore); anti PDK1 (Enzo Life Sciences); anti phospho-ERK1/2 (Cell Signaling); anti ERK1/2 (Santa Cruz); anti cleaved-PARP (Cell Signaling); anti PARP (Cell Signaling); anti α-tubulin (Santa Cruz); anti HIF1α (Abcam); anti phospho- AMPK (Cell Signaling).

Cell lines and cell culture

All melanoma cells were purchased from ATCC while 501Mel was obtained from Dr. Ruth Halaban (Dermatology department, Yale School of Medicine, USA). All cell lines were cultured in RPMI 1640 medium containing ultraglutamine (Lonza BioWhittaker), supplemented with 10% FCS (Foetal Calf Serum, GIBCO) and 1% PS (10′000 U/ml Penicillin and 10′000 U/ml Streptomycin, Lonza BioWhittaker) and grown at 37 °C in a humidified atmosphere at 5% CO2. Cells were regularly tested to be mycoplasma free. Drug-resistant cells were generated by culturing parental A375 cells in presence of 1 μM PLX4032 for 4–6 weeks. Twenty surviving clones were picked and then combined in equal proportions to obtain an heterogeneous pool of resistant cells.

Plasmids

The plasmid encoding the cytosolic (cyto)-human glutaredoxin 1 (Grx1)-roGFP2 was obtained by amplifying the cDNA Grx1-roGFP2 sequence without the Peroxisomal Targeting Sequence 1 (PTS1) from the plasmid encoding the peroxisomal (po)-Grx1-roGFP2 vector [19] by PCR (primers; pCyto-EcoRI-5′ 5′-gga gga gga tca gga gga gaa ttc gtg agc aag ggc gag gag-3′ (forward) and pCyto-XbaI-3′ 5′-ctc gac tta tct aga tta ctt gta cag ctc gtc-3′ (reverse)) and subcloned into PCR2.1-TOPO (Invitrogen). The resulting plasmid was digested with EcoRI and XbaI, prior to subcloning into the EcoRI/XbaI digested pcDNA3.1-Grx1-roGFP2-PTS1 vector to obtain the p-cyto-Grx1-roGFP2. The plasmid was verified by DNA sequencing.

Small interfering RNAs and transfection

The ERK1/2 siRNAs were obtained from GE Dharmacon (siGenome Human). siRNA transfections were performed using 3 μL HiPerfect transfection reagent (Qiagen) per reaction according to the manufacturer's instructions. The final concentration of siRNA was 50 nM for each ERK1/2 and 100 nM for scrambled control. ERK1 and ERK2 siRNA transfections were performed 48 h prior to the 24 h incubation with PLX4032 (1 μM) and/or Trametinib (3 nM).

The PDK 1–4 siRNAs were obtained from GE Dharmacon (ON-TARGETplus Human). siRNA transfections were performed using 1.5 μL Lipofectamine RNAiMAX (Invitrogen) per reaction according to the manufacturer's instructions. The final concentration of siRNA was 25 nM for each PDK (PDK1–4) and 100 nM for scrambled control. PDK1–4 siRNA transfections were performed 24 h prior to 24 h incubation with PLX4032 (1 μM).

Western blot analysis and antibodies

Cell lysis was performed at 4 °C using ice cold buffers. Cells were lysed on the dish with lysis buffer containing 30 mM Tris/HCl pH 6.7, 5% glycerol, 2.5% mercaptoethanol, 1% SDS. Protein extracts were further analysed by SDS-PAGE and Western blotting. ECL signals were detected as described before [20]. Before reprobing, blots were stripped as described before [21]. All experiments were performed in three biological replicates and one representative replicate is shown. Western blot quantification of ECL signals was performed using both the Image Lab 4.0.1 software from Bio-RAD and Bio1D analysis package (Vilber).

Quantitative PCR procedure

Total RNA was extracted using the Quick-RNA™ miniprep kit (Zymo Research) according to the manufacturer's instructions and the concentration and quality was determined using a NanoDrop Spectrophotometer. 250 or 500 ng of total RNA was reverse-transcribed with the miScript II RT kit (Qiagen) in a volume of 10 μL, according to the manufacturer's instructions. Quantitative real time PCR (qPCR) was carried out on a CFX96 Detection System (BioRad) in a total volume of 10 μL (10 pmol of each primer and containing cDNA corresponding to 50 ng RNA template). The housekeeping genes PPIA, HPRT and the target genes were assayed in parallel for each sample. Melting curve analysis was performed to guarantee the specificity of the qPCR primers as previously described [22]. All samples were run in biological triplicates each consisting of three technical replicates.

Gene-specific qPCR primers for PDK1, PDK3, PDK4 and HIF1a were purchased from Eurogentec (Belgium). For the detection of PDK2 a RT2 qPCR primer assays from Qiagen were used. The geometric mean of two housekeeping genes was calculated and a normalization factor for each sample was generated using geNorm (VBA add-in for Microsoft Excel). The normalization factor was used to calculate the relative amount of each target mRNA in each sample. Each sample was normalized to the untreated control.

Detection of ROS by confocal microscopy

Cells were transfected with a plasmid coding for Grx1-roGFP2 or mitochondrial (mito)-roGFP2 (pMF1762) as previously described [23], using Lipofectamine LTX2000 (Invitrogen). Two days post transfection the cells were either subjected to a 1 h pretreatment with mitoQ (150 nM), a treatment with PLX4032 (3 h for A375; 10 h for 501 Mel or IGR37) or a combination thereof. roGFP2 is a genetically engineered GFP, which contains two cysteine residues that can be oxidized. Depending on the oxidation state, roGFP changes its excitation spectrum (reduced: 488/530 nm; oxidized: 400/530 nm). By measuring the ratio (400/488 nm) of the fluorescence emitted by the two excitation states of the roGFP2, the redox status of a subcellular compartment can be monitored [24, 25]. The higher this ratio, the higher is the redox state. The analysis was performed using the ImageJ software on pictures taken using an Andor Revolution W1 spinning disc confocal microscope, mounted on a Nikon Ti microscope (60× oil objective). Between 10 and 20 ROIs (regions of interest) were analysed per cell with a minimum of 12–20 cells analysed for each condition. The excitation record time was set to 250 msec or 300 msec for the 400 nm channel and 50 msec or 60 msec for the 488 nm channel for the Grx1-roGFP2 or mito-roGFP2 respectively. Two independent experiments were performed.

Real-time proliferation assays

50×10^3 cells/well of 5 melanoma cell lines were seeded in 12-well plates and 24 h later stimulated with 10 μM of AZD7545. Cellular growth was monitored in the IncuCyte ZOOM live cell microscope (Essen BioScience) and images were taken in phase contrast every 3 h for a total of 90 h. Proliferation assay were carried out for three biological replicates and for each figure one representative replicate is shown.

Preparation of the A375-iRFP cell line

A375 cells were plated in 12 well plates in complete medium and allowed to adhere overnight. Cells were transduced with lentiviral particles (LV-iRFP-P2A-Puro) containing near-infrared fluorescent protein (iRFP) linked to the puromycin resistance gene (Puro) via a P2A cleavage peptide (Imanis Life Sciences). Virus was prepared in serum-free medium and added to the cells with a multiplicity of infection (MOI) of 10. To increase transduction efficiency, the plate was centrifuged at 300 g for 30 min and placed in the incubator. Four hours after transduction, complete medium was added to the cells. Forty-eight hours after transduction, cells were checked on the Odyssey Infrared imaging system (LI-COR Bioscience) for iRFP expression and selection with puromycin (Invivogen) at 1 μg/ml was initiated. After several rounds of puromycin selection, cells were analysed on the Odyssey Infrared imaging system (LI-COR Bioscience) and on a FACSCanto II flow cytometer using FACSDiva (BD Bioscences) software to confirm that 100% of the cell population is expressing iRFP.

Long term proliferation assay

The A375-iRFP cells were used to test the combination of 1 μM PLX4032 and 10 μM AZD7545 versus PLX4032 alone. 10,000 cells were seeded per well (6 well plates) and were treated with the inhibitors for 3 weeks. Medium was changed twice a week. After 3 weeks of treatment, cells were scanned and the intensity of the iRFP signal was measured using the LI-COR Odyssey instrument (LI-COR Biosciences). The iRFP signal was quantified using the Image Studio lite version 4.0 software (LI-COR Biosciences).

3D spheroid growth assay

A375 cells were plated in round-bottom ultra-low attachment 96-well plates in 90 μl of serum-free DMEM-F12 supplemented with B-27 (1X; Invitrogen), Insulin (4 U/L; Sigma), Heparin (4 μg/ml; Sigma), EGF (20 ng/ml; Biomol); bFGF (20 ng/ml: Miltenyi Biotec), and penicillin/streptomycin (1X, Lonza). After 2 days of sphere formation, drugs were added. Sphere growth was monitored in the IncuCyte ZOOM live cell microscope (Essen BioScience) by measuring four different spheroid diameters after 3 days. Spheroid assays were carried out in three biological replicates.

Results

BRAF inhibitors lead to an increase of PDH phosphorylation in BRAF mutant (V600E) but not in BRAF wild-type melanoma cells

In the wake of recent publications investigating the role of the BRAF oncogene on metabolic processes, we wanted to explore the effects of BRAF inhibitors on melanoma cells. To this end, time course experiments were performed in a panel of $BRAF^{V600E}$ and $BRAF^{WT}/NRAS^{mut}$ human melanoma cells, which were incubated with two BRAF kinase inhibitors, PLX4032 and GSK2118436, for the indicated time points (Fig. 1). As expected, BRAF inhibition caused the down-regulation of phosphorylated ERK in the $BRAF^{V600E}$ positive cell lines A375, IGR37 and 501Mel already after 30 min (Fig. 1a). On the contrary and as previously described [26, 27], PLX4032 and GSK2118436 lead to an upregulation of ERK phosphorylation in cells expressing $BRAF^{WT}/NRAS^{mut}$ (Fig. 1b). Total PARP and cleaved PARP detections were performed to assess the induction of apoptosis which seems to occur only 48 and 72 h after treatment of the $BRAF^{V600E}$ positive cells. Interestingly, we found an increased phosphorylation of PDH-E1α on S_{300} from around 7 h post inhibitor treatment in

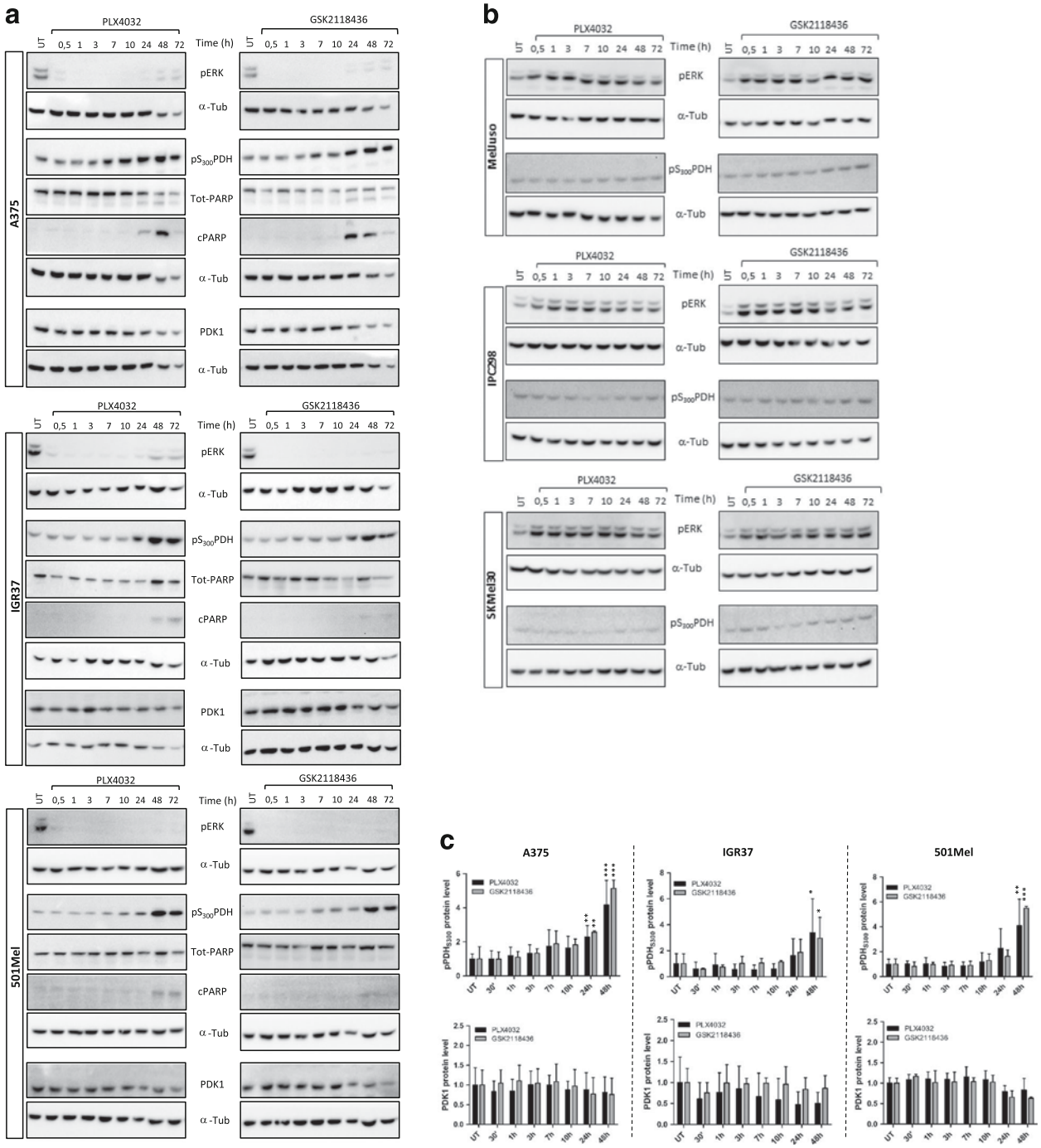

Fig. 1 BRAF inhibitors induce upregulation of PDH-E1α phosphorylation in $BRAF^{V600E}$ melanoma cells but not in wild-type BRAF melanoma cells. Western blot analysis of A375, IGR37 and 501 Mel cells ($BRAF^{V600E}$) (**a**) and MelJuso, IPC298 and SKMel30 cells ($BRAF^{WT}$/$NRAS^{mut}$) (**b**) treated with 1 μM of PLX4032 and 100 nM of GSK2118432 for the indicated time points. α-Tubulin was used as loading control; representative blots of three biological replicates are shown. **c** Quantification of pPDH and PDK1 levels for A375, IGR37 and 501Mel, normalized to the untreated control. Error *bars* represent the standard deviation of three biological replicates. Statistical significance was determined using one-way ANOVA coupled with Dunnett's multiple comparisons tests. *$p > 0.05$, **$p > 0.01$, ***$p > 0.001$

the $BRAF^{V600E}$ positive cell lines A375, IGR37 and 501 Mel (Fig. 1a and c), increasing at 24 h and generally peaking at 48 h. In the $BRAF^{WT}$/$NRAS^{mut}$ cells, the situation was again different and no change in the phosphorylation of PDH was observed over time. Phosphorylation of PDH-E1α at S_{300} is associated with an inhibition of the activity

of the pyruvate dehydrogenase complex (PDH), which converts pyruvate into acetyl-CoA and thus regulates the entry of metabolites from glycolysis into the TCA cycle. Our data suggest that treatment with BRAF inhibitors induces changes in the metabolism of cells harboring the $BRAF^{V600E}$ mutation in a different way than in cell expressing $BRAF^{WT}$.

PDH phosphorylation is mediated by Erk

To rule out that the PDH phosphorylation is an off target effect of the BRAF inhibitor and since $BRAF^{V600E}$ are known to activate ERK1/2 via MEK1, we investigated the involvement of ERK1/2 and MEK1 in mediating PDH-E1α phosphorylation in A375 cells. Using an siRNA approach targeting both ERK1 and ERK2 in combination with PLX4032 we could show that a knock-down of ERK1/2 induces an upregulation of PDH-E1α phosphorylation, comparable to what was achieved with PLX4032 (Fig. 2a). In addition, the same upregulation was obtained when the cells were treated with a MEK1 inhibitor (Trametinib), which also leads to the down-regulation of pERK. Of note, neither of the

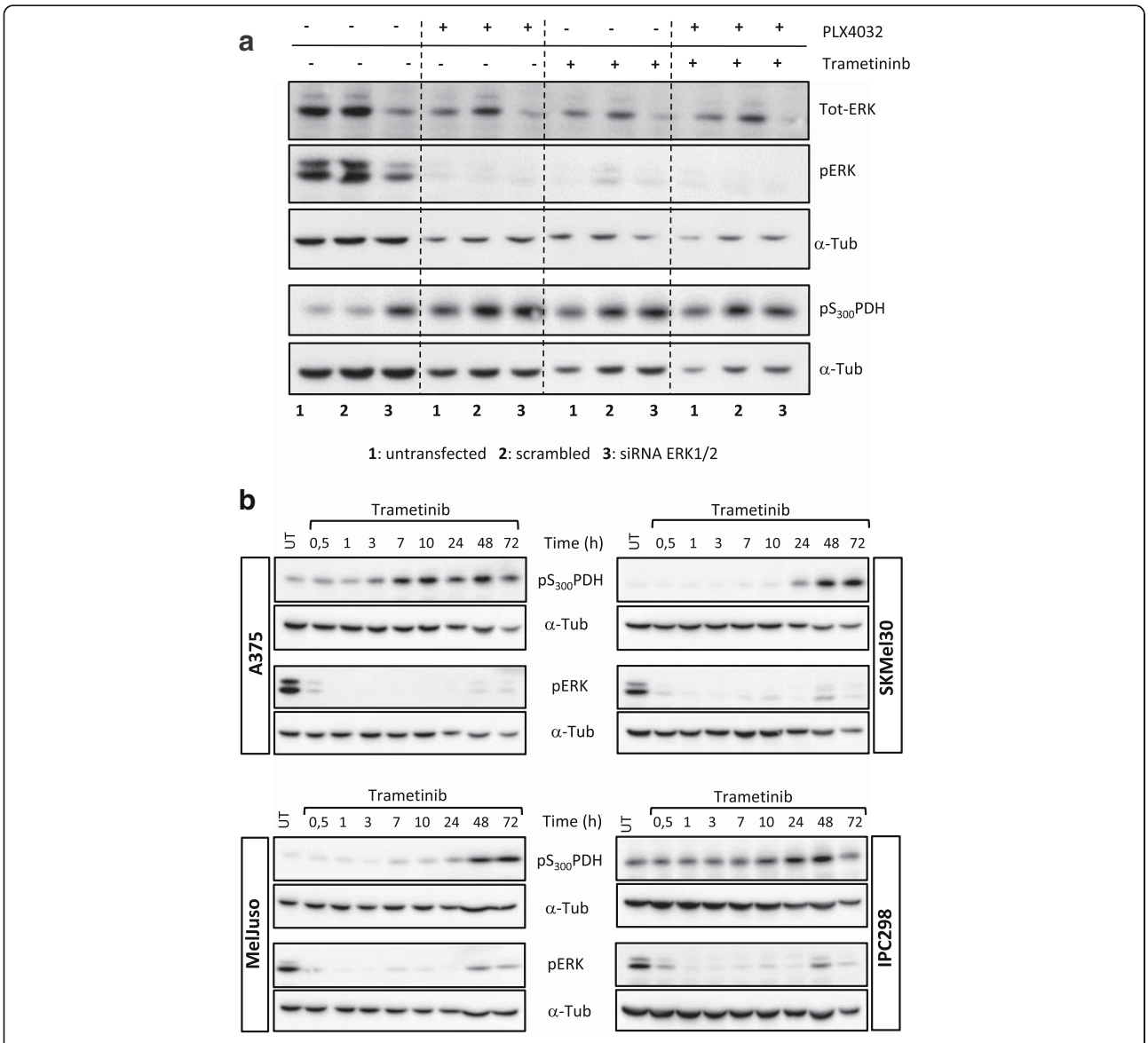

Fig. 2 Knock-down of ERK1/2 by siRNAs and treatment with a MEK1 inhibitor induces $pS_{300}PDH$. **a** A375 cells were transfected with siRNA against ERK1/2 (50 nM each siRNA) or a scrambled control (100 nM) for 72 h. 48 h prior to collection, the cells were incubated with either PLX4032 (1 μM) or Trametinib (3 nM) or both drugs. One representative of three biological replicates is shown. **b** Western blot analysis of A375 ($BRAF^{V600E}$), MelJuso, IPC298 and SKMel30 cells ($NRAS^{mut}$) treated with 5 nM of Trametinib for the indicated time points. α-Tubulin was used as loading control; representative blots of three biological replicates are shown. 1: *Untransfected*; 2: *scrambled*; 3: *ERK1/2 siRNA*

effects could be increased by combination treatment with both BRAF and MEK inhibitor. To further explore the link between RAS/RAF/MEK/ERK pathway and PDH phosphorylation we used $NRAS^{mut}$ cells (SKMel30, MelJuso, IPC298) (which did not upregulate pPDH upon BRAF inhibitor treatment) and treated them with Trametinib for the indicated time points. As expected, MEK inhibition caused a down-regulation of ERK phosphorylation already after 30 min (Fig. 2b) also in $NRAS^{mut}$ cells. Phosphorylation of PDH-E1α on S_{300} was observed in those cells from around 24 h onwards. These results suggest that the phosphorylation of PDH is a specific effect mediated by the BRAF/MEK/ERK pathway and not an off target effect of BRAF inhibitors and that it occurs in all melanoma cells presenting activating mutations in this pathway.

Pyruvate dehydrogenase kinase (PDK) mRNA levels are upregulated upon BRAF inhibitor treatment

Since PDKs are known to be responsible for PDH phosphorylation [14, 15], we next analysed mRNA levels of the four PDK isoforms in three $BRAF^{V600E}$ cell lines. HIF1α was included in the study since this transcription factor induces PDK1 expression and has been shown to be upregulated by conditions other than hypoxia [28], notably also by activation of the RAS/MAPK pathway [29]. Furthermore, constitutive HIF1α activity has been described in malignant melanoma [30]. Surprisingly, an upregulation of the mRNA levels of PDK1 and HIF1α was detected in all cell lines at 12 and 24 h after PLX4032 treatment (Fig. 3). However, validation of these results showed that although HIF1α mRNA was upregulated upon treatment, this effect was not detectable at the protein level (Additional file 1: Figure S1). This finding was corroborated by the fact that PDK1 protein levels, which are regulated by HIF1α protein, were also not upregulated upon BRAF inhibitor treatment. Interestingly, the mRNAs of the other PDK family members PDK3 and PDK4 were upregulated upon BRAF inhibitor treatment, while PDK2 was not detected in any cell line. The protein levels of PDK3 and PDK4 could not be investigated since the available antibodies were not specific for these proteins (data not shown). Thus, mRNA levels of the PDKs are upregulated following BRAF inhibitor treatment, although for PDK1 no increase in protein levels was detectable.

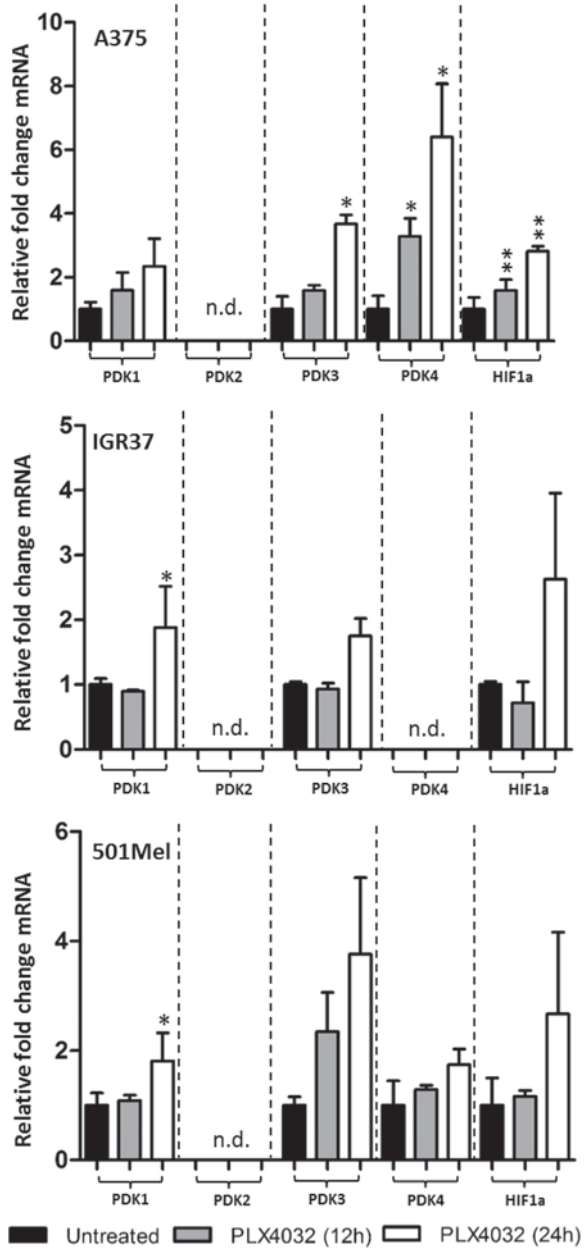

Fig. 3 PLX4032 treatment induces upregulation of PDK mRNA in $BRAF^{V600E}$ melanoma cells. Quantitative RT-PCR of PDKs and HIF-1α mRNA. The fold change was calculated relative to untreated controls. Error *bars* represent the standard deviation of three biological replicates. PDK2 was not detectable in *tested cell lines* (Cq ≥ 30) while PDK4 (Cq ≥ 30) was not detectable in IGR37 cells only. Error *bars* represent the standard deviation of three biological replicates. Statistical significance was determined in comparison to the untreated control using paired Student's *t*-tests. *$p > 0.05$, **$p > 0.01$, ***$p > 0.001$

PDKs are crucial for the observed PDH-E1α phosphorylation

The activity of PDKs can also be regulated by alternative post-translational mechanisms [31], and for enzymes the regulation of their activity is often more important than their mere expression level. Therefore, we investigated the importance of PDKs in our inhibitor-mediated PDH-E1α phosphorylation by knocking down all four PDK isoforms using an siRNA approach. The down-regulation of mRNAs for all isoforms ranged between ~10–40% compared to both

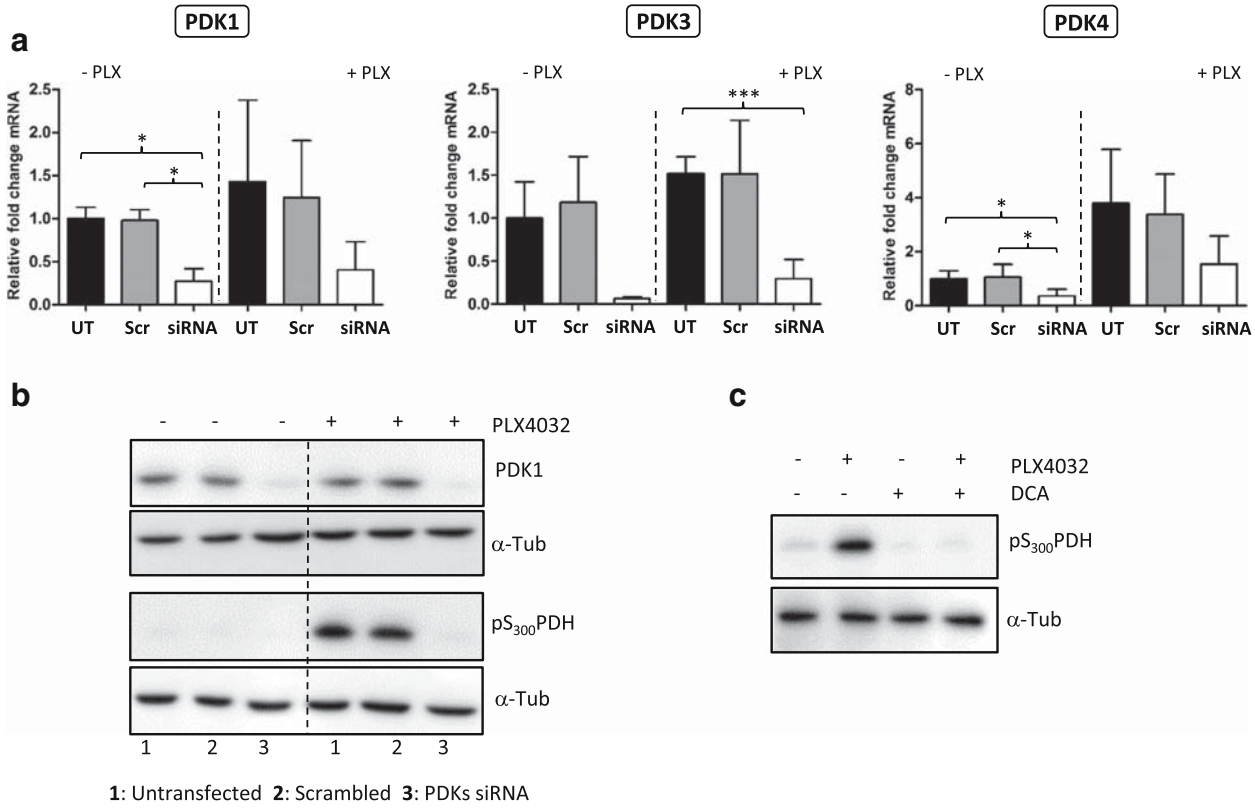

Fig. 4 PDKs are crucial for PDH phosphorylation. A375 cells were transfected with siRNA against all four PDK isoforms (PDK1–4; 25 nM siRNA for each isoform) or 100 nM of scrambled control; after 24 h cells were incubated with PLX4032 (1 μM) for another 24 h. **a** qPCR analysis of PDK1–4 mRNA levels to show the efficiency of the siRNA knock-down of each siRNA and (**b**) corresponding western blot analysis. **c** Western blot analysis of A375 treated with PLX4032 (1 μM for 24 h), DCA (20 mM for 48 h) or both drugs. Error *bars* represent the standard deviation of three biological replicates. For each western blot experiment, one representative of three biological replicates is shown. Statistical significance was determined using paired Student's *t*-tests. *$p > 0.05$, **$p > 0.01$, ***$p > 0.001$. 1: *Untransfected*; 2: *scrambled*; 3: *PDKs siRNA*

untreated and scrambled controls (Fig. 4a). As for PDK1 protein levels, a strong reduction following the siRNA treatment could be observed too (Fig. 4b). Following the knock-down of the PDKs, a drastic decrease in the phosphorylation levels of PDH-E1α at serine 300 residue in A375 cells was detected, despite the presence of PLX4032 (Fig. 4b). To further support these results, we used dichloroacetic acid (DCA), a pan-PDK inhibitor, which also resulted in a decreased phosphorylation level of PDH-E1α in A375 (Fig. 4c). In summary, these data indicate that PDK isoforms mediate the PLX4032/GSK118436-induced phosphorylation of PDH-E1α.

BRAF inhibitors also trigger the phosphorylation of AMP-activated protein kinase (AMPK)

AMPK phosphorylation and its subsequent activation upon BRAF inhibitor treatment has previously been described in melanoma and colorectal cancer cells [32, 33]. Since AMPK has indirectly been implicated in mediating PDH phosphorylation [34], we explored the phosphorylation status of AMPK in our setting. In accordance with previous publications [32, 33], we also observed an up-regulation of AMPK phosphorylation on T_{172} following inhibition of $BRAF^{V600E}$ with both PLX4032 and GSK2118436 (Fig. 5). However, the phosphorylation occurred generally at later time points (24 or 48 h after treatment) compared to the onset of PDH phosphorylation. For this reason, we exclude the possibility that the observed PDH phosphorylation following BRAF inhibitor treatment was indirectly induced by AMPK.

Reactive oxygen species (ROS) are upregulated upon PLX4032 treatment and correlate with PDH phosphorylation

Mitochondrial ROS production after PLX4032 exposure has recently been described [35]. Since we and others have previously demonstrated that the induction of ROS can stimulate the phosphorylation of PDH [34, 36], we investigated a possible connection between ROS and pPDH after PLX4032 treatment in the context of melanoma. In order to measure the redox state in both mitochondria and the cytosol after PLX4032 treatment,

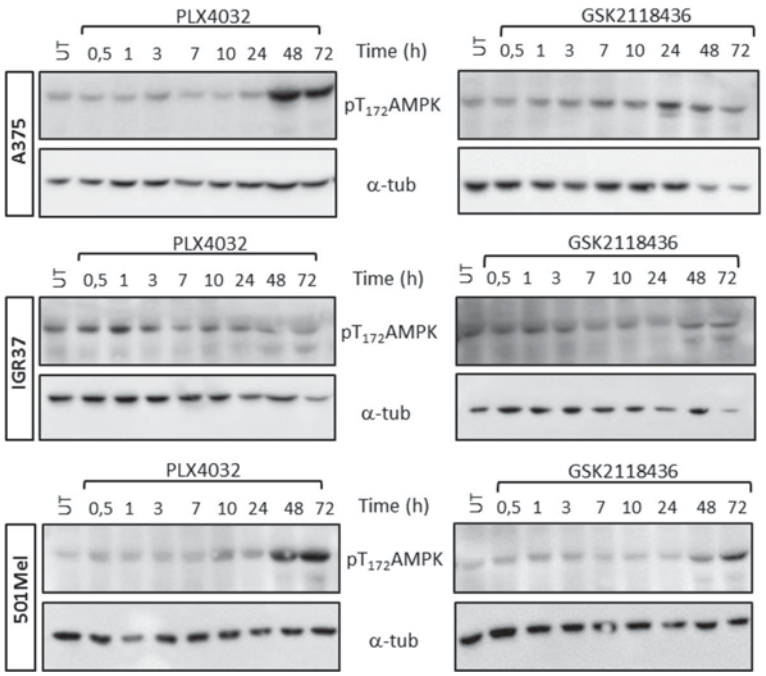

Fig. 5 PLX4032 and GSK2118436 induce upregulation of AMPK phosphorylation in $BRAF^{V600E}$ melanoma cells. Western blot analysis of A375, IGR37 and 501 Mel cells ($BRAF^{V600E}$) treated with 1 μM of PLX4032 and 100 nM of GSK2118432 for the indicated time points. α-Tubulin was used as loading control, and one representative blot is shown

A375, IGR37 and 501Mel cells were transfected with a plasmid expressing either the mito-roGFP2 or the Grx1-roGFP2, which can be used to monitor the mitochondrial and the cytosolic redox state, respectively. Both redox states after PLX4032 treatment were compared to untreated controls. In particular, we treated A375 cells with the BRAF inhibitor for 3 h whereas IGR37 and 501Mel were tested for 10 h. The time points were chosen according to the onset of pS_{300}PDH after PLX4032 treatment, in particular just before the phosphorylation was becoming prominent. Furthermore, we also used a mitochondria-targeted antioxidant (mitoQ) in order to prevent or decrease oxidative stess induced by PLX4032. Melanoma cells were pre-treated with 150 nM of the antioxidant, mitoquinone mesylate (MitoQ), followed by PLX4032 treatment. For all cell lines, both the mitochondrial and the cytosolic redox status was significantly increased after exposure to PLX4032 and decreased in case of mitoQ pre-treatment (Fig. 6a). We could also observe a decreased phosphorylation of PDH-E1α on S_{300} when the cells were pre-incubated with MitoQ suggesting that ROS are activating PDKs, which in turn leads to inhibition of PDH by phosphorylation (Fig. 6b). As an additional confirmation, cells were treated with 100 μM H_2O_2, which also induced an upregulation of PDH-E1α phosphorylation (Fig. 6c).

AZD7545 (PDK inhibitor) leads to efficient growth suppression in cells harboring BRAF and NRAS mutations as well as in inhibitor-resistant melanoma

Generally a metabolic shift from glycolysis to glucose oxidation has been associated with an increase in the electron transport chain activity and ROS production [37] and oxidative stress in form of increased ROS levels drives cells into apoptosis [38]. Based on this, we hypothesize that the inhibition of PDKs during acute BRAF inhibitor treatment and during early development of resistance might be beneficial for patients as such that sufficient levels of ROS could be generated, which might drive cancer cells into apoptosis and thus delay or prevent resistance. In this context, it has previsouly been shown that the PDK inhibitor DCA negatively affects the growth of melanoma cells, which are sensitive or resistant to BRAF inhibition by suppressing glycolysis [39]. First we tested a PDK inhibitor with low micromolar cellular potency, AZD7545, as single treatment on both $BRAF^{V600E}$ and $BRAF^{WT}$/$NRAS^{mut}$ human melanoma cells. Interestingly, we detected a 30–40% decrease in cell growth in $BRAF^{V600E}$ A375 and IGR37 cell lines (Fig. 7a). Interestingly the AZD7545-mediated suppression of growth could also be observed in BRAF/$NRAS^{mut}$ cells, although, at later time points. This might be attributed to the slower growth of those cell lines (Fig. 7b), which also signal via the MAPK pathway. These data suggest that AZD7545,

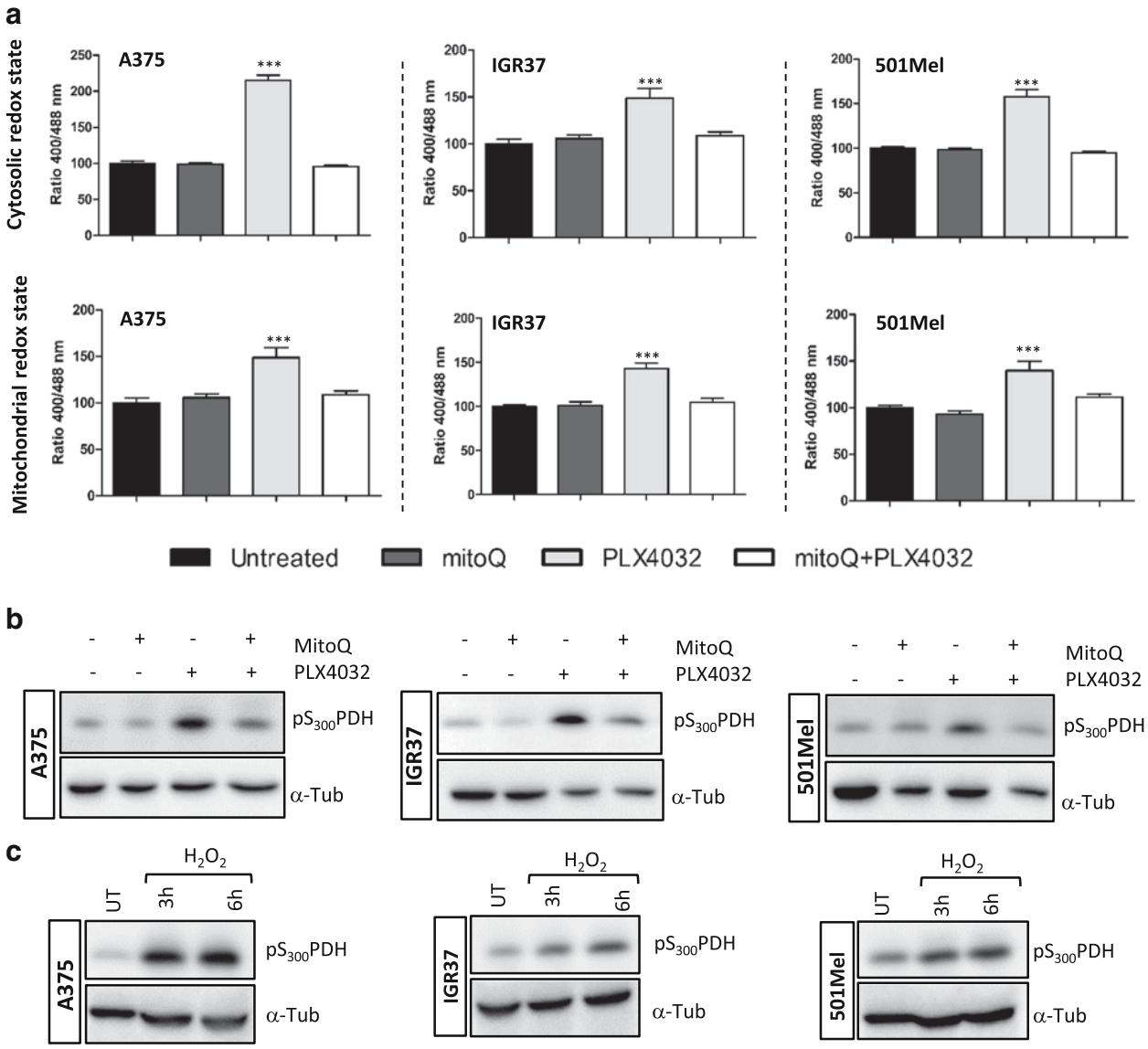

Fig. 6 ROS are induced after PLX4032 treatment of melanoma cells. **a** A375, 501 Mel or IGR37 cells were transiently transfected with plasmids encoding Grx1-roGFP2 or mito-roGFP2. Two days post transfection the cells were either subjected to a 1 h pretreatment with mitoQ (150 nM), a treatment with PLX4032 (3 h for A375; 10 h for 501Mel or (IGR37) or a combination thereof. Following this, the redox status was measured in the cytosol or mitochondria. Data were normalized to untreated cells, followed by one-way ANOVA coupled with Dunnett's multiple comparisons tests. Representative graphs of two biological replicates are shown. **b** Western blot analysis of A375, IGR37 and 501Mel pre-treated for 1 h with mitoQ (150 nM) followed by 24 h of 1 μM PLX4032 treatment. Results are shown for one representative of three biological replicates. **c** Western blot analysis of A375, IGR37 and 501 Mel treated with hydrogen peroxide (H_2O_2; 100 μM) for the indicated time points. Results are shown for one representative of three biological replicates. *$p > 0.05$, **$p > 0.01$, ***$p > 0.001$

which we used at 10 μM, might be a valid alternative to Dichloroacetate (DCA), another PDK inhibitor used at millimolar concentrations. To investigate if AZD7545 could delay or influence the occurrence of BRAF inhibitor resistance we tested the combination of the two inhibitors on melanoma cell growth. A375 melanoma cells were transduced with lentiviral particles containing iRFP and were treated for 3 weeks either with 1 μM of PLX4032 or with 1 μM of PLX4032 in combination with 10 μM AZD7545. By measuring the intensity of the iRFP signal, we observed that cells treated with both inhibitors showed a more pronounced reduction in cellular growth (Fig. 8a). This drug combination was also tested on sphere models with similar results (Fig. 8b). It is tempting to speculate that the targeting of PDKs in combination with BRAF inhibitors could be a promising strategy to more efficiently

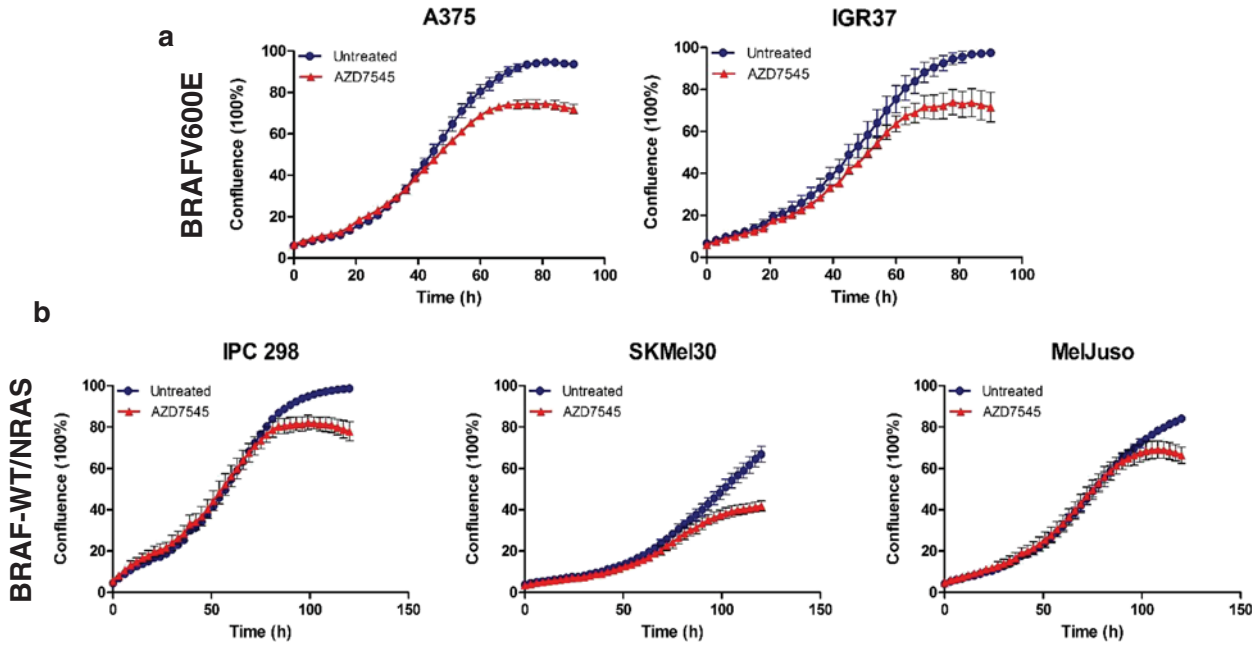

Fig. 7 AZD7545 mediated growth suppression of $BRAF^{V600E}$ mutant and $NRAS^{mut}$ melanoma cells. Twenty-four hours after plating, both A375 and IGR37 *cell lines* ($BRAF^{V600E}$) (**a**) and SKMel30, IPC298 and MelJuso *cell lines* ($NRAS^{mut}$) (**b**) were treated with 10 μM of AZD7545. The plates were imaged using an IncuCyte ZOOM live cell microscope (Essen BioScience) and images were taken every 3 h for a total of 90 h ($BRAF^{V600E}$) and 120 h ($NRAS^{mut}$). Results are shown for one representative of three biological replicates

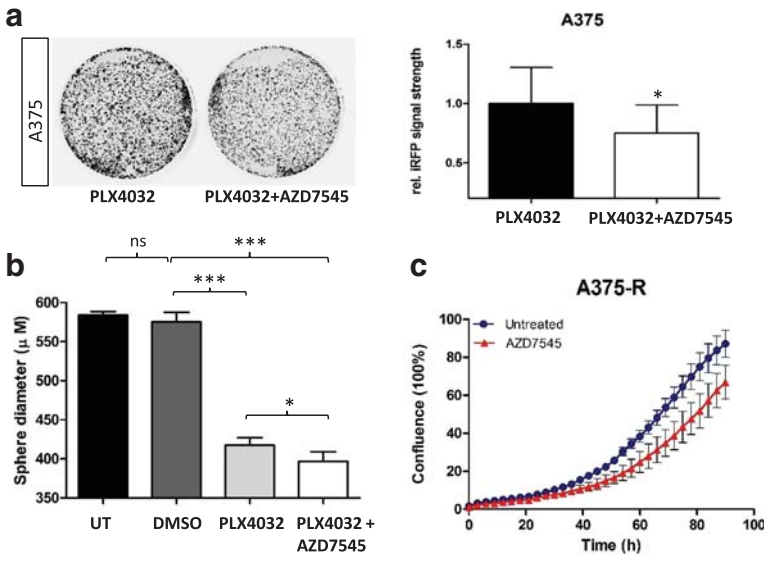

Fig. 8 Combination of AZD7545 and PLX4032 more efficiently suppresses melanoma growth compared to each compound alone. **a** Represenative experiment of A375 melanoma cells expressing iRFP treated either with 1 μM of PLX4032 or with 1 μM of PLX4032 in combination with 10 μM AZD7545 for 3 weeks. The intensity of red fluorescence was quantified and the bar diagram represents three biological replicates with their standard deviation. **b** Spheroid cultures of A375 melanoma cells were treated with DMSO control, with 1 μM of PLX4032 or with 1 μM of PLX4032 in combination with 10 μM AZD7545. After 3 days sphere diameters were measured and represented as bar diagrams. Error *bars* represent the standard deviation of a minimum of four technical replicates of one representative experiment of three biological replicates. **c** Twenty-four hours after plating, BRAFi-resistant A375 melanoma cell (A375-R) were stimulated with 10 μM of AZD7545. The plates were imaged using an IncuCyte ZOOM live cell microscope (Essen BioScience) and images were taken every 3 h for a total of 90 h. Results are shown for one representative of three biological replicates. Statistical significance was determined using paired Student's *t*-tests. *$p > 0.05$, **$p > 0.01$, ***$p > 0.001$

hit melanoma cells or to delay the onset of drug resistance. In addition BRAFi-resistant A375 melanoma cells also showed reduction of growth upon AZD7445 treatment (Fig. 8c).

Discussion

Metabolic reprogramming, often driven by activated oncogenes, is a well known feature of cancer cells. Recent studies have shown a link between oncogenic BRAF signaling and metabolic reprogramming in melanoma (for a comprehensive review see [40]), making the targeting of metabolic pathways a potentially interesting therapeutic strategy. Melanoma has been described to be highly glycolytic, due to upreguation of glucose transporters and lactate dehydrogenase-A (LDH-A) [41–43]. Inhibition of $BRAF^{V600E}$ suppresses GLUT1/3 and Hexokinase 2 protein levels leading to reduced levels of lactate and ATP, thus showing that BRAF inhibition counteract the Warburg effect [13]. In the present study, we demonstrate that administration of BRAF inhibitors induces phosphorylation of proteins involved in the cellular metabolism, notably via PDH. Furthermore, inhibition of MEK1 alone or in combination with BRAF inhibitors as well as siRNA knock-down of ERK1/2 also mediated phosphorylation of PDH, indicating that it is not an off target effect of the BRAF inhibitor but an effect mediated by the RAS/RAF/MEK/ERK pathway (Figs. 1, 2a and b). PDH is the key enzyme linking glycolysis to the TCA. PDH-E1α phosphorylation at the serine residues 293, 300, and 232 is known to be responsible for the down-regulation of its activity. Thereby, conversion of pyruvate to acetyl-CoA is prevented, which contributes to a down-regulation of the TCA and Oxphos.

We demonstrate that PDKs are required for the observed effect since siRNA-mediated knock-down of the PDKs clearly impairs the inhibitor-mediated upregulation of phosphorylation. Administration of the PDK inhibitor DCA had the same effect. Interestingly, the expressed PDK1, PDK3 and PDK4 proteins are regulated either by HIF-mediated transcription (PDK1), increasing ATP levels (PDK3) or amino acid starvation (PDK4). Here, we did not observe changes in HIF1α or PDK1 protein levels. ATP has been described to be decreased upon BRAF inhibition [13] and it is thus unlikely to account for PDK2 activation. In our short term 2D cell culture (the effect is seen from 7 h post inhibitor treatment) experiment, nutrient deprivation can hardly be responsible for PDK4 activation [44]. Thus alternative activation mechanisms have to be taken into account. Protein kinases can be redox-sensitive [45] and can either be activated or inactivated by ROS. We have recently shown that PDKs were activated by ROS in the first hours of hypoxic conditions [36] and that PDH phosphorylation can be mediated by ROS-dependent activation of PDKs [34, 36]. In the current study, we detected an increased mitochondrial and cytosolic redox state upon administration of PLX4032 in BRAF-mutant positive melanoma cells and we also observed that exogenously applied H_2O_2 was sufficient to increase PDH phosphorylation in melanoma cells, indicating that ROS might indeed be responsible for the observed effect. In the presence of a mitochondria-targeted ROS scavenger, MitoQ, the BRAF inhibitor failed to induce PDH phosphorylation, which strongly points to an activation of PDKs by ROS.

Cancer cells produce higher levels of ROS compared to nontransformed cells, due to metabolic reprogramming or to changing fluxes of metabolites or to nutrient and oxygen fluctuations in the tumour microenvironment. Interestingly, any disturbance of the steady state of the electron transport chain (ETC.) seems to generate ROS [46, 47]. As one example, increasing and decreasing oxygen levels both induce the production of more ROS by the ETC. In the same way, activation of BRAF by mutation in melanoma should lead to the induction of genes involved in antioxidant defence, since MAPK activation was described to induce such a response [48]. As a consequence, inhibition of BRAF might increase ROS levels by suppressing the transcription of these antioxidant genes. In addition NRF2, a transcription factor regulating antioxidant defence, is known to be activated by ERK, other MAPKs and Akt. Thus, BRAF mutations also activate antioxidant defence downstream of the NRF2 pathway. Again, BRAF inhibitor treatment might also lead to increases in ROS by this mechanism.

Melanoma cells have been discussed to have a highly oxidative metabolism and thus treatment with BRAF or MEK inhibitors, increase oxidative stress within the cancer cell by upregulating ROS (as we show here). The increased phosphorylation of PDH by PDKs that we describe can be interpreted as a protective response of the cancer cells to prevent further ROS production. The oxidative activation of PDKs, due to increasing ROS levels, prevent pyruvate from entering the TCA and the ETC. (which is the major source of ROS) and stop ROS from reaching toxic levels. PDK inhibitors thus release the block on pyruvate entry into the TCA and consequently lead to an increase in ROS levels that can be toxic to cells and contribute to cancer cell death (Fig. 9). BRAF inhibitor treatment inhibits the TCA cycle and contributes to the decrease in ATP production, which was recently reported by several studies [13, 49] and by our preliminary measurements (data not shown). Interestingly, we observed that in PLX4032-resistant melanoma cells the PDH phosphorylation upregulation was overcome (Additional file 2: Fig. S2) suggesting a metabolic adaptive process occurring during drug treatment (Fig. 9).

This ROS-mediated regulation of kinases is an interesting emerging field in cancer cell biology. It seems that

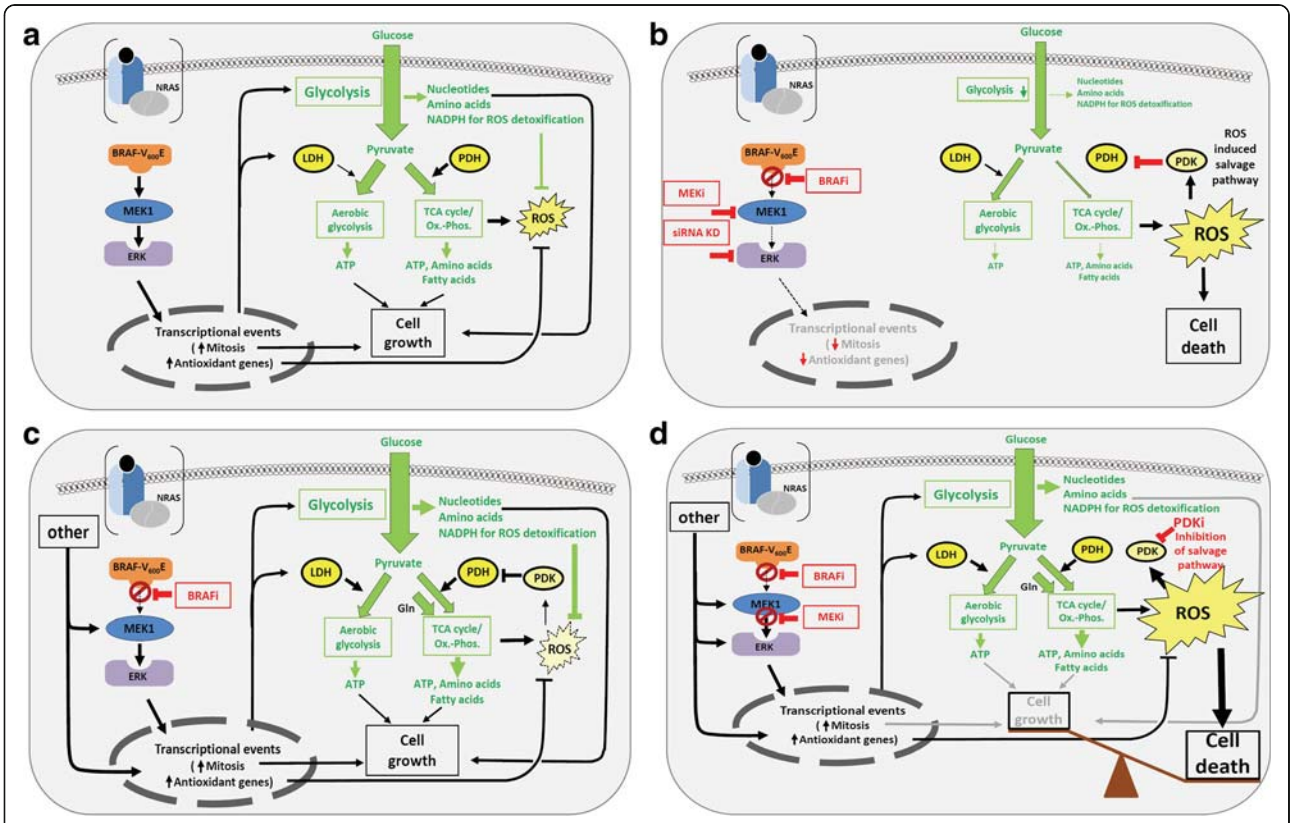

Fig. 9 Model summarising metabolic changes in sentitive versus resistant melanoma cells upon BRAF inhibition. **a** $BRAF^{V600E}$, in addition to its well known effects on the cell cycle and anti-apoptosis, leads to upregulation of glucose uptake and LDH-A, promoting aerobic glycolysis and cell growth. Moreover, activation of BRAF, leads to the induction of genes involved in antioxidant defense. **b** Upon treatment with BRAF inibitors, or other MEK/ERK pathway inhibitors, glycolysis is inhibited. Changes in metabolic fluxes through the TCA cycle together with the inhibition of the transcription of antioxidant genes, induce ROS. The increasing levels of ROS activate PDKs, which in turn inactivate PDH, thus reducing pyruvate use in the TCA cycle, which in a negative regulatory loop, inhibit further ROS production. **c** In BRAF inhibitor resistance, the MAPK pathway is reactivated by compensatory mechanisms and as a consequence glycolysis and antioxidant-defence genes are reactivated. Glycolysis produces ATP, nucleotides (via the pentose phosphate pathway (PPP)), NADPH (for antioxidant defense via PPP) and amino acids. The cells become addicted to an oxidative metabolism with glutamine (Gln) feeding anaplerotically into the TCA cycle to produce NADH, amino acids, ATP (via Oxphos), fatty acids (via AcetylCoA) and ROS. These ROS can be kept in check by NADPH, which is produced by the PPP and by antioxidant gene transcription downstream of the RAS/RAF/MEK/ERK pathway. At need, PDKs are activated by ROS and PDH is inhibited. This prevents pyruvate from entering the TCA and ROS to reach toxic levels. **d** In BRAF inhibitor resistance of melanoma, pharmacological inhibition of PDKs releases the brake on pyruvate entry into the TCA and causes unchecked pyruvate use, associated with an increase in ROS production. This increase in ROS tilts the balance towards cell death. Thus potent nonomolar PDK inhibitors could efficiently reduce the viability of BRAFi-resistant cells or might prevent or delay BRAFi resistance

ROS can either activate or inactivate kinases by oxidation. Interestingly, ROS regulate some kinases directly involved in metabolic processes, which are also involved in regulation of ROS itself. One such mechanism we now describe in this paper: The ROS-mediated activation of PDKs lead to phosphorylation of PDH, which in turn limits influx of pyruvate into the TCA and the Oxphos (or the ETC.). This represents a negative regulatory loop limiting the generation of more ROS. In the same way, ROS inhibit PKM2 (a cancer-expressed isoform of pyruvate kinase), leading to accumulation of glycolytic intermediates, which feed into the pentose phosphate pathway to generate NADPH. NADPH in turn plays an essential role in ROS detoxification [50]. Along the same lines, ROS have been described to activate AMPK, a kinase regulating mitophagy/autophagy, which can be interpreted as a pathway restricting further ROS production [51, 52].

Interestingly, the $BRAF^{V600E}$ oncogene signaling regulates PDH phosphorylation in very different ways in Oncogene Induced Senescence (OIS) [53] and in BRAF inhibitor resistance (this paper and [13]), which occur in different steps of oncogenesis or treatment (see also Fig. 9). In a melanoma mouse model, OIS in $BRAF^{V600E}$ positive cells was mediated by down-regulation of the TCA and Oxphos. In this system, PDH down-regulation by phosphorylation was mediated by PDK1 down-regulation and PDP2 upregulation at the protein

level. Loss of OIS, characteristic of progression to cancer leads to a reversal of these effects [53].

DCA has been tested in multiple cell culture and rodent models of cancer, and PDK1 knock-down has been described to enhance the sensitivity of $BRAF^{V600E}$ positive melanoma to BRAF inhibitors. We tested a pan-PDK inhibitor, AZD7545, which interferes with the lypoyl binding pocket of PDKs for its capacity to inhibit the growth of $BRAF^{V600E}$ and $NRAS^{mut}$ positive cells. We observed that AZD7545 suppressed growth of $BRAF^{V600E}$ positive cells and kinase inhibitor-resistant cells when applied in μM concentrations. Interestingly, AZD7545 had no effect on keratinocytes (HaCaT) and normal fibroblasts, cell types which constitute the cutaneous microenvironment of melanoma tumors (data not shown), indicating selective effects on BRAF/NRAS-mutated or resistant cancer cells. Finally, we show that the combination of BRAF inhibitors with PDK inhibitors is more efficient in tumor growth suppression than the single treatment suggesting that the simultaneus targeting of metabolic pathways might indeed be beneficial for melanoma patients.

Conclusions

Current guidelines for advanced stage melanoma foresee the use of targeted therapy followed by immunotherapy if kinase inhibitor treatments are not effective or once patients become resistant. Appropriate first line treatments are selected based on specific features of the patient, including BRAF mutation status. For BRAF-mutated melanoma patients, targeted combination therapy with BRAF and MEK inhibitors is recommended, a treatment line, which has been approved in 2015. Until then, BRAF inhibitors were given alone but combined BRAF and MEK inhibition was shown to improve the overall survival [54]. Despite these encouraging results, double drug resistance is likely to occur in some patients [55]. Triple target therapy approaches are currently under investigation, especially those targeting pathways other than the RAS/RAF/MEK/ERK. Furthermore, about 50% of patients do not carry the BRAF mutation and not all of those might be suitable candidates for immunotherapy. Finally, intrinsic resistance exists in a group of melanoma patients carrying BRAF mutations but not responding to BRAF and MEK inhibitors. For this large group of melanoma patients, alternative therapeutic targets need to be identified. A deeper understanding of metabolic changes under selective pressure may contribute to the identification of such novel target lines.

In this study, we report that RAS/RAF/MEK/ERK pathway inhibition induces increased ROS levels, which activate PDKs. PDKs then phosphorylate and thereby inhibit PDH, which reduces further ROS production by the TCA cycle. Consequently, PDK inhibitors should increase ROS production significantly by preventing blockade of pyruvate entry into the TCA cycle. The use of a specific PDK inhibitor combined with BRAF inhibition or with BRAF and MEK inhibitors might thus increase ROS production to levels, which initiate cell death and in this way delay or prevent BRAF inhibitor resistance. Given the early onset of resistance to BRAF inhibitors in virtually all treated patients together with the still limited success rates of immune therapy [56], there is a pressing need for efficient therapies in late stage melanoma patients, which either prevent or considerably delay drug resistance by targeting other aberrant signaling pathways. More potent PDK inhibitors with nanomolar cellular potency targeting the lipoyl- or the ATP-binding pockets of the kinase domains, might be a promising inhibitor to add in combination with BRAF and/orMEK kinase inhibitors.

Additional files

Additional file 1: Figure S1. PLX4032 and GSK2118436 do not induce up-regulation of HIF-1α protein in BRAFV600E melanoma cells. Western blot analysis of A375, IGR37 and 501Mel cells (BRAFV600E) treated with 1 μM of PLX4032 and 100 nM of GSK2118432 for the indicated time points. HIF-1α protein was not detectable in all three cell lines. Positive control: A375 short term hypoxia. (PDF 155 kb)

Additional file 2: Figure S2. BRAF inhibitors do not induce phosphorylation of PDH in resistant melanoma cells. Western blot analysis of untreated A375, A375 cells resistant to Vemurafenib (A375-R) and under constant presence of 1 μM of PLX4032 and A375 cells stimulated with 1 μM of PLX4032 for 24 h. α-Tubulin was used as loading control; representative blots of three biological replicates are shown. (PDF 102 kb)

Abbreviations

AcetylCoA: Acetyl coenzyme A; AMPK: AMP-ctivated Protein Kinase; BRAF: B-Rapidly accelerated fibrosarcoma; DCA: Dichloroacetic acid; ERK: Extracellular signal regulated kinase; LDH-A: Lactate dehydrogenase-A, Gln, glutamine; mTOR: Mechanistic target of rapamycin; Oxphos: Oxydative phosphorylation; PDC: Pyruvate dehydrogenase complex; PDH: Pyruvate dehydrogenase; PDKs: Pyruvate dehydrogenase kinases; PDP1 and PDP2: Pyruvate dehydrogenase phosphatases; ROS: Reactive oxygen species; TCA: Tricarboxylic acid cycle

Acknowledgments

We thank Prof. Bernard Knoops, Sarah Becker and André Clippe (Université catholique de Louvain, Belgium) for help in generating the p-cyto-Grx1-roGFP2 and Marc Fransen (KU Leuven, Belgium) for providing the plasmid encoding mito-roGFP2.

Funding

GC is supported by funding from an IRP grant (IRP13MB) by the University of Luxembourg.

Availability of data and materials

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

Authors' contributions

GC performed the experiments, interpreted the data, prepared the figures and wrote the manuscript; GW carried out the ROS measurements and had input on the manuscript; AZ helped to conceive experiments and

interpreted data; SK and CH supervised, interpreted the data and wrote the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Consent for publication
Not applicable.

Ethics approval and consent to participate
Not applicable.

Publisher's Note

Received: 21 September 2016 Accepted: 24 May 2017
Published online: 08 June 2017

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Supplementary material

Figure S1

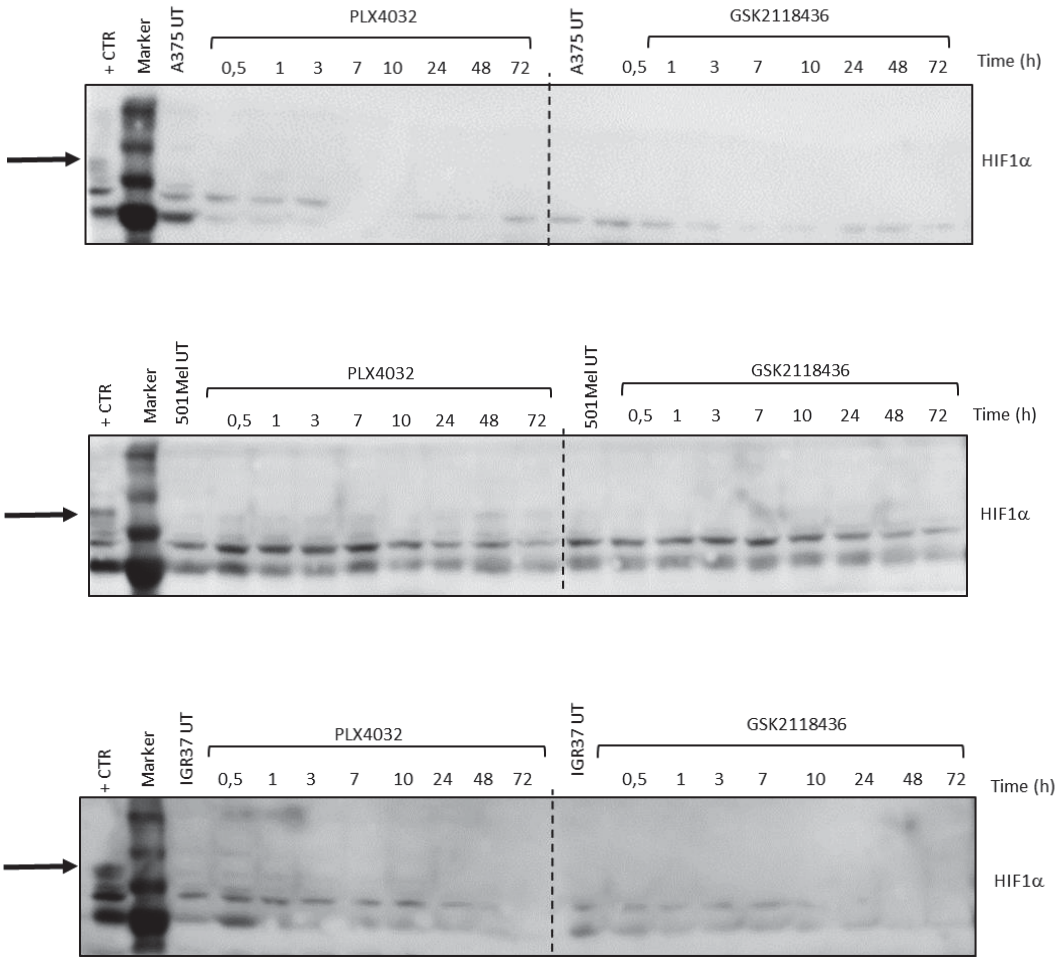
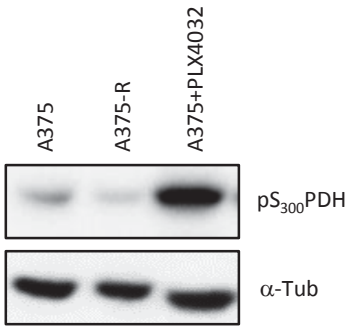


Figure S2



4.2 Impact of BRAF kinase inhibitor treatment on the miRNomes and transcriptomes of resistant melanoma cells

4.2.1 Preamble

Acquired resistance to BRAF inhibitors represents a significant obstacle to the successful control of melanoma. Usually, acquired resistance is characterized by the reactivation of the MAPK pathway (Trunzer et al., 2013). Furthermore, the scenario might be complicated by the paradoxical development of secondary skin tumours, arising from the BRAF inhibitors-induced activation of MAPK pathway in wild-type BRAF cells present in same tumour (Gibney et al., 2013). For these reasons, the gold standard therapy for BRAF mutated melanoma patients has recently become a combination of BRAF and MEK inhibitors (Garbe et al., 2016; Spain et al., 2016). This combination, although it significantly increases the overall survival of patients, does not prevent the onset of resistance and thus is not an appropriate long term treatment for most patients (Eroglu and Ribas, 2016).

Many studies have elucidated several key genetic changes responsible for acquired resistance; nevertheless, in some cases, new mutations have not been found (Shi et al., 2014), suggesting a potential involvement of epigenetic and post transcriptional changes. MiRNAs are important post transcriptional regulators of gene expression, often dysregulated in many tumours (Svoronos et al., 2016). So far, not much is known about the implication of miRNAs in BRAF inhibitor-resistant melanomas.

In the scope of this article, we aimed at a better understanding of the mechanisms underlying the resistance to BRAF inhibitors, with a special focus on the potential role of miRNAs. We used *in vitro* melanoma cell models consisting of drug-sensitive and drug-resistant cells, which have been generated in our laboratory. We performed microarray analyses with the aim to describe changes in the transcriptome and the miRNome that might play a role in resistance. On the whole, this study provides insights into a complex and very heterogeneous response to BRAF inhibitors. Nevertheless, several genes and miRNAs were differentially regulated in the drug-resistant versus -sensitive cell lines, which might be considered as prognostic and/or diagnostic resistance biomarkers in melanoma drug resistance.

Kozar et al. is a joint first author paper and authors' contributions are explained at the end of the article.

Supplementary tables can be found at the following address:
http://www.sciencedirect.com/science/article/pii/S0304416517301307?via%3Dihub

ARTICLE IN PRESS

Contents lists available at ScienceDirect

BBA - General Subjects

BBA - General Subjects xxx (xxxx) xxx–xxx

journal homepage: www.elsevier.com/locate/bbagen

Impact of BRAF kinase inhibitors on the miRNomes and transcriptomes of melanoma cells☆

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ARTICLE INFO

Keywords:
Melanoma
Drug resistance
miRNA
BRAF inhibitors
Targeted therapy

ABSTRACT

Background: Melanoma is an aggressive skin cancer with increasing incidence worldwide. The development of BRAF kinase inhibitors as targeted treatments for patients with BRAF-mutant tumours contributed profoundly to an improved overall survival of patients with metastatic melanoma. Despite these promising results, the emergence of rapid resistance to targeted therapy remains a serious clinical issue.

Methods: To investigate the impact of BRAF inhibitors on miRNomes and transcriptomes, we used in vitro melanoma models consisting of BRAF inhibitor-sensitive and -resistant cell lines generated in our laboratory. Subsequently, microarray analyses were performed followed by RT-qPCR validations.

Results: Regarding miRNome and transcriptome changes, the long-term effects of BRAF inhibition differed in a cell line-specific manner with the two different BRAF inhibitors inducing comparable responses in three melanoma cell lines. Despite this heterogeneity, several miRNAs (e.g. miR-92a-1-5p, miR-708-5p) and genes (e.g. DOK5, PCSK2) were distinctly differentially expressed in drug-resistant versus -sensitive cell lines. Analyses of coexpressed miRNAs, as well as inversely correlated miRNA-mRNA pairs, revealed a low MITF/AXL ratio in two drug-resistant cell lines that might be regulated by miRNAs.

Conclusion: Several genes and miRNAs were differentially regulated in the drug-resistant and -sensitive cell lines and might be considered as prognostic and/or diagnostic resistance biomarkers in melanoma drug resistance.

General significance: Thus far, only little information is available on the significance and role of miRNAs with respect to kinase inhibitor treatments and emergence of drug resistance. In this study, promising miRNAs and genes were identified and associated to BRAF inhibitor-mediated resistance in melanoma. This article is part of a Special Issue entitled "Biochemistry of Synthetic Biology - Recent Developments" Guest Editor: Dr. Ilka Heinemann and Dr. Patrick O'Donoghue.

1. Introduction

Melanoma is an aggressive malignancy, which has a poor prognosis once the tumour has metastasized [1,2]. Together with lung cancer, melanoma is one of the most unstable and highly mutated tumours with approximately 200 non-synonymous mutations per lesion and an average mutation rate of 16.8 mutations/Mb [3–5]. In spite of this high genetic heterogeneity, > 50% of melanoma patients harbour activating mutations in the B-Raf proto-oncogene serine/threonine kinase (BRAF), with V600E being the predominant amino acid change [4,6]. Mutated BRAF leads to a constitutive activation of the Mitogen Activated Protein Kinase (MAPK) pathway, impairs normal cell proliferation and triggers sustained cell survival [7,8]. Consequently, BRAF kinase inhibitors (e.g. vemurafenib/PLX4032 and dabrafenib/GSK2118436) were developed as targeted treatment options for patients with BRAF-mutant tumours. These drugs are in widespread clinical use since 2011 (vemurafenib) and 2013

Abbreviations: ASNS, asparagine synthetase; AXL, proto-oncogene tyrosine kinase receptor AXL; BRAF, B-Raf proto-oncogene serine/threonine kinase/v-raf murine sarcoma viral oncogene homolog B; CCL2, chemokine C-C motif ligand 2; CE, coexpression coefficient; CHAC1, ChaC cation transport regulator homolog 1; CTLA4, cytotoxic T-lymphocyte associated protein 4; CYR61, cysteine-rich angiogenic inducer 61; DCT, dopachrome tautomerase; DOK5, docking protein 5; EDIL3, EGF-like repeats and discoidin I-like domains 3; EMT, epithelial-mesenchymal transition; GP, dabrafenib/GSK2118436-resistant pool; HIF-1α, hypoxia-inducible factor 1α; HRE, hypoxia-response element; MAPK, mitogen activated protein kinase; MITF, microphthalmia-associated transcription factor; NF1, neurofibromin 1; PC2, protein convertase 2; PCA, principle component analysis; NRAS, neuroblastoma RAS viral oncogene homolog; PCSK2, proprotein convertase subtilisin/kexin type 2; SESN2, sestrin 2; WFDC1, WAP four disulfide core 1; XP, vemurafenib/PLX4032-resistant pool

☆ This article is part of a Special Issue entitled "Biochemistry of Synthetic Biology - Recent Developments" Guest Editor: Dr. Ilka Heinemann and Dr. Patrick O'Donoghue.

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http://dx.doi.org/10.1016/j.bbagen.2017.04.005
Received 14 December 2016; Received in revised form 1 March 2017; Accepted 6 April 2017

(dabrafenib) and contributed profoundly to the increase of the overall survival of patients with metastatic melanoma [9–12]. Despite these promising results, the rapid emergence of resistance to BRAF inhibitor-mediated targeted therapy remains a serious clinical issue. Consequently, research has extensively focused on the identification and description of components and pathways involved in drug resistance. Various mechanisms contributing to resistance to BRAF inhibition have been described, ranging from reactivation of the MAPK pathway through activating MEK mutations, activation of alternative signalling pathways to the overexpression of tyrosine kinase receptors on the cell surface [13–16]. In order to suspend the MAPK-mediated acquired resistance to BRAF inhibition, BRAF inhibitors are now combined with MEK inhibitors (e.g. vemurafenib/cobimetinib, dabrafenib/trametinib), which is reported to delay the onset of resistance and to increase the progression-free survival by ~3 months as well as the overall survival by 20% when compared to BRAF inhibitor monotherapy [17–21].

Large-scale analyses of resistance mechanisms have revealed a tremendously heterogeneous response to kinase inhibitors and triggered an interest in alternative approaches to study resistance including epigenetic and post-transcriptional drug-induced changes [22,23]. As a result, miRNAs have been associated with the development of resistance to targeted therapy [23]. miRNAs are small non-coding RNA molecules that play an important role in post-transcriptional gene regulation [24]. As miRNAs exhibit tissue-specific as well dynamic expression patterns over time [25] and many tumours show aberrant miRNA levels [26,27], these small RNA molecules are considered promising biomarkers to predict disease progression, response and emerging resistance to treatment [28–31]. Moreover, miRNAs can modulate the sensitivity to BRAF inhibitors in melanoma. As an example, the overexpression of miR-514a inhibited the expression of the negative regulator of the MAPK pathway, neurofibromin 1 (NF1), which correlated with increased survival of BRAF-mutant cells treated with vemurafenib [32]. Also, the inhibition of CCL2-induced miR-34a, miR-100, and miR-125b was shown to bypass resistance to vemurafenib in melanoma cell lines and might be used as prognostic factors [33]. Apart from this, not much is known about the implication of miRNAs in BRAF inhibitor-resistant melanomas. To further elucidate the expression patterns and the role of miRNAs in drug-resistance, we used melanoma cell lines as in vitro models consisting of parental BRAF inhibitor-sensitive cell lines A375, IGR37, and 501Mel, and the corresponding vemurafenib- and dabrafenib-resistant cells. MiRNA and mRNA microarrays were generated and changes in the miRNome and transcriptome of drug-sensitive versus -resistant cell lines were analysed, followed by RT-qPCR validations of interesting candidates. This study shows that responses to long-term BRAF inhibition occur mainly in a cell-line specific manner, and that the BRAF inhibitors vemurafenib and dabrafenib induced a similar response in melanoma cell lines. Regardless of the discernible heterogeneity, we identified miRNAs and genes that were predominantly up- or down-regulated in most of the resistant cell lines as compared to their parental counterpart (e.g. miR-708-5p, PCSK2), hence they might be considered as potential prognostic/diagnostic biomarkers in melanoma drug resistance.

2. Methods

2.1. BRAF inhibitors

The BRAF-mutant specific inhibitors vemurafenib (PLX4032) and dabrafenib (GSK2118436) were purchased from Selleck Chemicals. They were dissolved in DMSO according to the manufacturer's instructions and stored at −80 °C. Working aliquots were diluted in 100% ethanol at a concentration of 1 mM for PLX4032 and 100 µM for GSK2118436 and stored at −20 °C.

2.2. Cell lines

The BRAF-mutant melanoma cell lines (Fig. S1) A375 and IGR37 were purchased from ATCC and DSMZ respectively, and 501Mel was obtained from Dr. Ruth Halaban (Dermatology department, Yale School of Medicine, USA). All cell lines were cultured in RPMI 1640 medium containing ultraglutamine (Lonza BioWhittaker), supplemented with 10% FCS (Foetal Calf Serum, GIBCO) and 1% PS (10,000 U/mL Penicillin and 10,000 U/mL Streptomycin, Lonza BioWhittaker) and grown at 37 °C in a humidified atmosphere at 5% CO_2. Cells were regularly tested to be mycoplasma free. Drug-resistant melanoma cell pools were generated from parental A375, IGR37, and 501Mel cells by long-term culturing under continuous presence of 5 µM vemurafenib (PLX4032) or 100 nM dabrafenib (GSK2118436). Inhibitor-containing media were exchanged three times a week. Pools resistant to vemurafenib or dabrafenib were denoted _XP or _GP, respectively.

2.3. RNA extraction and quality control

Total RNA was extracted with the AllPrep DNA/RNA kit (Qiagen) from 5×10^6 cells in triplicates and per cell line, following the manufacturer's instructions. For qPCR validations of the microarray data, total RNA was extracted in three biological replicates each consisting of three technical replicates using the Quick-RNA™ Mini-Prep Kit (ZYMO Research Corp). Briefly, 10^6 cells were collected in triplicates and lysed in 600 µL RNA-Lysis Buffer. RNA purity and quality were assessed using the NanoDrop2000 Spectrophotometer (Thermo Scientific).

2.4. Microarray data analysis and visualization

For the microarray analyses, RNA quality was further assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies). Microarray analyses were performed at the Luxembourg Institute of Health (LIH) in Luxembourg. The Affymetrix miRNA 4.0 and Affymetrix HuGene 2.0 ST platforms were used for miRNA and gene microarrays, respectively. The raw microarray data are accessible in the ArrayExpress database (https://www.ebi.ac.uk/arrayexpress/) under the accession numbers E-MTAB-5510 (miRNA dataset) and E-MTAB-5511 (gene expression dataset). The commercial software Partek Genomic Suite was used for data pre-processing using Robust Multiarray Analysis (RMA) with GC-content correction at the Luxembourg Institute of Health. The log2-transformed intensities were imported into the R environment. Differential expression of genes and miRNAs in the drug-resistant cell lines, as compared to the drug-sensitive, was determined using the R/Bioconductor package *limma*, which adopts a linear modeling approach with empirical Bayesian statistics. The multiple correction was performed using the Benjamini-Hochberg's FDR (false discovery rate or adjusted P-value). Genes and miRNAs with FDR < 0.01 and at least 1.5-log fold change were considered as differentially expressed. The data were filtered and further analysed using the R packages *dplyr* and *tidyr*. Most figures were generated using functions from the R package *ggplot2*, Fig. 1 was generated using the R package *UpSetR*, and the correlation matrix (function: http://sablab.net/scripts/xCorrMatrix.r) was visualized using the function heatmap.2 from the *gplots* package.

2.5. Coexpression analysis

The coexpression patterns of miRNAs and mRNAs across the samples were examined using CoExpress, a software tool for concurrent expression analysis of miRNA and mRNA microarray data (available at www.bioinformatics.lu/CoExpress). The expression is determined by computing the Pearson coefficient, thus a positive or negative coexpression coefficient (CE) indicate a positive correlation or inverse correlation, respectively. The input for CoExpress is a tab-separated text file with log2 expression values. First, a coexpression matrix is calculated for both the miRNA and gene expression datasets. Next, a network analysis was perfomed based on coexpression. Only coexpression events with a correlation coefficient higher than 0.9 (positive correlation) or lower than −0.9 (inverse correlation) were taken into

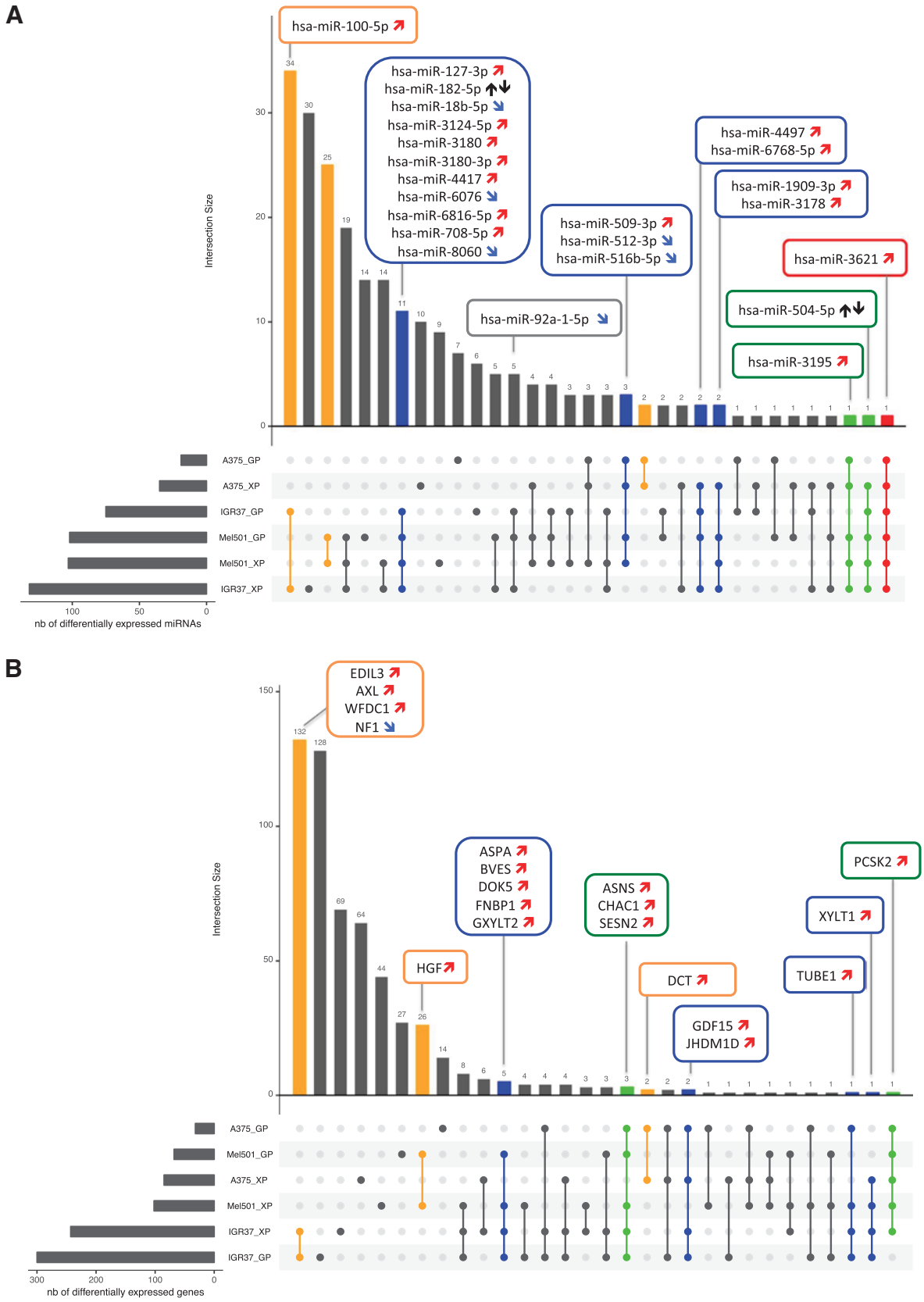

Fig. 1. Differentially expressed miRNAs and genes across drug-resistant melanoma cell lines. The number of significantly differentially expressed (FDR < 0.01, at least 1.5-log fold change) miRNAs (A) and genes (B) is illustrated by the horizontal bars. Vertical bars illustrate the number of significantly differentially expressed miRNAs or genes that appear in one or more drug-resistant cell lines. Bars highlighted in orange show the number of candidates that are exclusively differentially expressed in the resistant cell lines originating from the same parental drug-sensitive cell line. Blue bars indicate the number of genes or miRNAs differentially expressed in four out of six cell lines, green bars indicate those present in five out six resistant cell lines, and the red bar marks candidates present in all six resistant cell lines. Red and blue arrows indicate up- or downregulated candidates, respectively, and the black up and down arrows signify that the expression varies from cell line to cell line.

consideration. Finally, a comparison with predicted miRNA-mRNA targets was performed. The databases used for target prediction were TargetScanHuman (predicted miRNA-target interactions) [34] and miRTarBase (experimentally validated miRNA-target interactions) [35]. Gene set enrichement analysis was performed using Enrichr [36].

2.6. Quantitative PCR

Briefly, 500 ng of total RNA were reverse-transcribed using the miScript kit (Qiagen) in a total reaction volume of 10 µL, according to the manufacturer's instructions. Quantitative real time PCR (qPCR) was carried out on a CFX96 Detection System (Bio-Rad) in a total volume of 10 µL (10 pmol of each primer and cDNA corresponding to 5 ng and 50 ng RNA template for miRNA and mRNA, respectively). The reference miRNAs (RNU1A, RNU5A, SCARNA17) and genes (HPRT, TBP, PPIA), as well as the target miRNAs and genes were assayed in parallel for each sample. All samples were run in triplicates.

miRNA-specific primers were purchased from Qiagen and qPCR primers for gene amplification were purchased from Eurogentec (Belgium). Gene amplification qPCR primers used herein: AXL (F: 5′ GAGACCCGTTATGGAGAAGTGT 3′; R: 5′ CTGATGCCCAGGCTGTTCAAG 3′), CTLA4 (F: 5′ AGGCTGATGAGTGGTGCAAA 3′; R: 5′ CCAGTGGCCAGTAAGTCAGG 3′), DOK5 (F: 5′ GCAGTTAGTTCGGGGTTGT 3′; R:5′ CACATTCTGGCTCTGTTGGG 3′), EDIL3 (F: 5′ CCAGTTCGGCAAAGGTGATA 3′; R: 5′ AGGGACCAACCTCCACAA3′), NF1 (F: 5′ ATAAGCCCTCACAACAACCA 3′, R: 5′ ACTCGGTGCCATTCGTATT 3′), PCSK2 (F: 5′ CCTCCAACTATAATGCCGA 3′; R: 5′ AACCAGTCATCTGTGTACCG 3′). The geometric mean of three reference genes was calculated and a normalization factor for each sample was generated using geNorm (VBA add-in for Microsoft Excel [37]) for each sensitive-resistant cell line pair separately. The normalization factor was used to calculate the relative amount of each target miRNA and mRNA in each sample. Statistical significance between the resistant cell lines and their corresponding parental drug-sensitive cell line was determined using a two-tailed, unpaired Student's *t*-test (GraphPad Prism).

2.7. Western blot analysis

Cell lysis was performed at 4 °C using ice cold lysis buffer containing 30 mM Tris/HCl pH 6.7, 5% glycerol, 2.5% mercaptoethanol, and 1% SDS. Protein extracts were analysed by SDS-PAGE and Western blotting. ECL signals were detected as described before [38]. All experiments were performed in three biological replicates, of which one representative replicate is shown. The following antibodies were used for western blot: anti-AXL C89E7 (Cell Signaling), anti-MITF C5 (Sigma), anti-PC2 D1E1S (Cell Signaling) and anti-α-tubulin (Santa Cruz).

2.8. Growth assay

5000 A375, A375_XP, A375_GP, 501Mel, 501Mel_XP, 501Mel_GP cells, and 8000 IGR37, IGR37_XP, and IGR37_GP cells were seeded in a 96-well plate in complete medium. The BRAF inhibitors vemurafenib and dabrafenib were serially diluted at a ratio of 1:3, starting at 10 µM and 300 nM, respectively, and supplemented to the cells in a total reaction volume of 100 µL. A blank control (medium only), as well as an untreated control for each cell line, were included. Upon 72 h, cell viability was assessed using PrestoBlue® cell viability reagent (ThermoFisher Scientific) and fluorescence was measured using the microplate reader CLARIOstar (BMG-LABTECH). Growth was expressed as percentage of living cells upon BRAF inhibitor treatment as compared to the untreated control (relative cell viability (%)). The experiment was performed in technical and biological triplicates.

2.9. Sanger sequencing

$BRAF^{V600}$, $NRAS^{G13}$, and $NRAS^{Q61}$ were Sanger sequenced at GATC Biotech (Konstanz, Germany) using the following primers: $BRAF^{V600}$ (F: 5′ CATCCTAACACATTTCAAGCC 3′; R: 5′ GTAACTCAGCAGCATCTCAGG 3′), $NRAS^{G13}$ (F: 5′ ACTATGGCCTGTGTTTCTCATGTA 3′; R: 5′ AATCCGGTGTTTTTGCGTTC 3′), and $NRAS^{Q61}$ (F: 5′ TTCCAAGTCATTCCCAGTAGCA 3′; R: 5′ CAGCACAAATAAAACAGTCCAGT 3′).

3. Results

3.1. Responses to BRAF inhibition are mainly cell line-specific

miRNA and gene expression analysis has been performed on the BRAF-mutant drug-sensitive (A375, IGR37, 501Mel), vemurafenib–resistant (A375_XP, IGR37_XP, 501Mel_XP) and dabrafenib–resistant (A375_GP, IGR37_GP, 501Mel_GP) cell lines (Fig. S1). A correlation matrix was calculated and visualized for the miRNA and gene expression datasets, showing a perfect correlation for duplicate samples (Fig. S2A, S2B). There was no clear segregation between the drug-sensitive (green) and drug-resistant (red) cell lines with only few obvious differences. The highest similarity was observed between the resistant cells and their corresponding parental drug-sensitive cell line. Additionally, the principle component analysis (PCA) showed that vemurafenib and dabrafenib have a comparable impact on melanoma cell lines. Therefore, most observed differences responsible for the emergence of resistance appear to be cell line-specific rather than being influenced by different BRAF inhibitor treatments (Fig. S1 J–O, Fig. S2C, S2D). Interestingly, the cell line-specific response was more pronounced in the transcriptome as compared to the miRNA data.

In order to investigate the global impact of BRAF inhibition on miRNomes and transcriptomes, the number of differentially expressed candidates (FDR < 0.01, at least 1.5-log fold change) as well as the most common miRNAs and genes emerging in the resistant versus sensitive cells, were examined (Tables S1, S2). Overall, resistant IGR37 and 501Mel cell lines had much higher numbers of differentially expressed miRNAs than resistant A375 (Fig. 1A). Changes on the miRNome level were heterogeneous with only miR-3621 being consistently upregulated in all resistant cell lines (red box). Additionally, miR-3195 and miR-504-5p were differentially expressed in five out of six resistant cell lines (green box), however, the overall expression of miR-504-5p varied between up- and down-regulation events in the different resistant cell lines. Also, miR-100-5p, which has previously been described to be involved in melanoma drug resistance [33], was upregulated in resistant IGR37 cells.

On gene level, the resistant IGR37 cell lines had also much higher numbers of differentially expressed genes compared to resistant 501Mel and A375 cell lines. Four genes were consistently upregulated in five out of six resistant lines (green boxes, Fig. 1B): the proprotein convertase subtilisin/kexin type 2 (PCSK2), asparagine synthetase (ASNS), the ChaC cation transport regulator homolog 1 (CHAC1), and sestrin 2 (SESN2).

Interestingly, the vast majority of events scored under drug resistance were clear upregulation of genes and miRNAs, with much fewer downregulations (Tables S1, S2). Also, based on the intersection size (Fig. 1A, B), the number of differentially expressed analytes that are unique to each cell line, or to resistant cell lines originating from the same parental drug-sensitive cell line was much higher than the amount of analytes that commonly appear in the majority of resistant cell lines. This observation once again supports the notion that the miRNA and gene expression responses to BRAF inhibition are mainly cell line-specific.

Since BRAF inhibitor-mediated changes were mostly cell-line specific, we decided to focus on the top significantly differentially expressed (highest up- or downregulation) miRNAs (Fig. 2) and genes (Fig. 3). MiR-509-3p was significantly increased (~3–5 log fold change) in four of six resistant cell lines (Fig. 1) and was also among the top upregulated miRNAs in A375_XP, A375_GP, 501Mel_XP and 501Mel_GP (Fig. 2A, B). MiR-708-5p was distinctly upregulated in both resistant

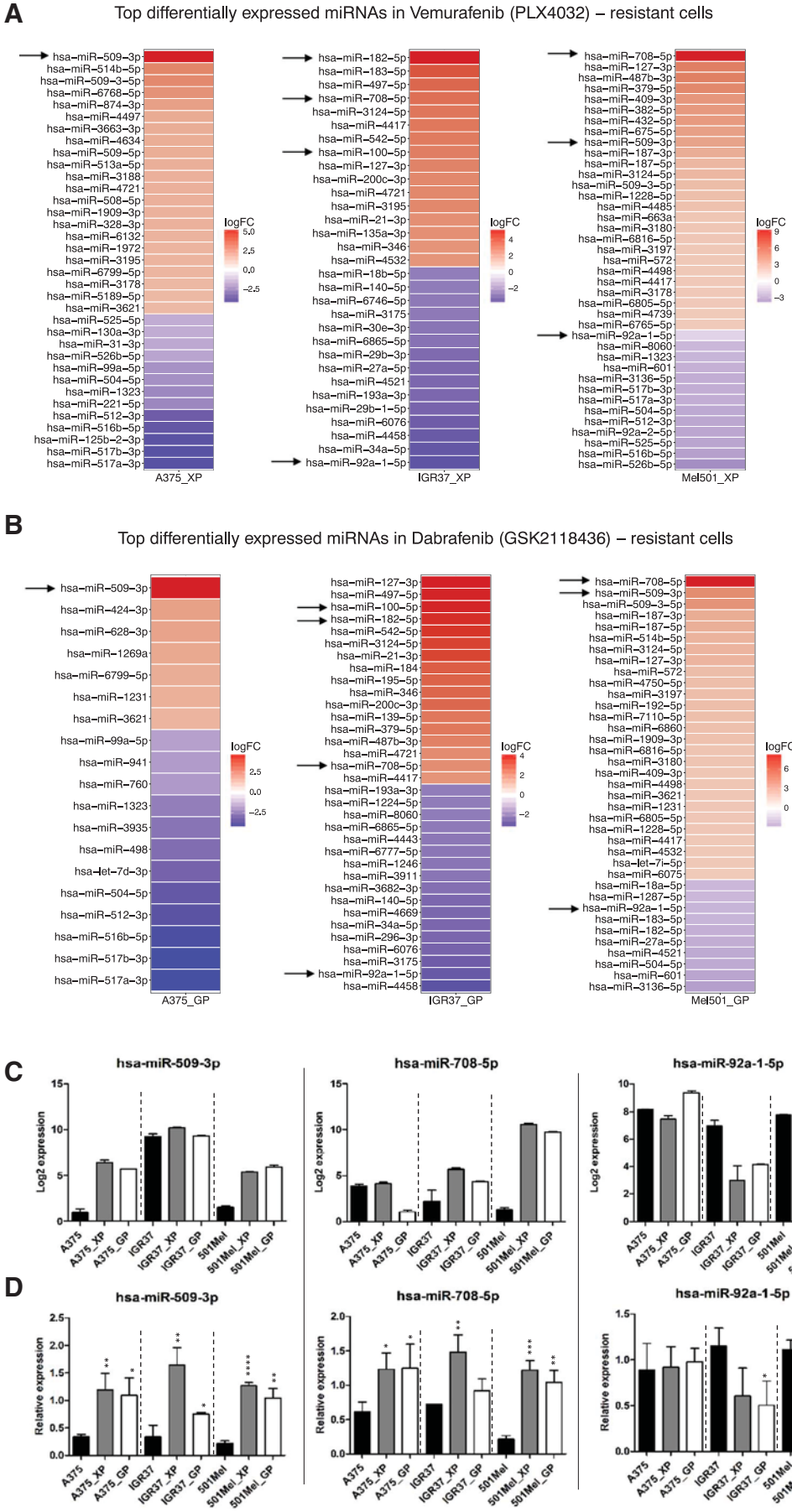

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Fig. 2. Top differentially expressed miRNAs. Differentially expressed miRNAs (FDR < 0.01 and at least 1.5-log fold change) in vemurafenib (XP)- (A) and dabrafenib (GP)- (B) resistant cell lines. Upregulated and downregulated miRNAs are shown in red and blue, respectively. (C) Log2 expression values of selected miRNAs from microarray data. (D) Validation of selected miRNA expression levels by RT-qPCR. Error bars represent the standard deviation of three biological replicates. The expression threshold for the microarray data was set to 5, thus all candidates with a log2 expression below 5 were considered as not expressed. Statistical significance was determined in comparison to the sensitive cells using two-tailed unpaired *t*-test and p-values < 0.05 were regarded as significant (p-value < 0.05*, p-value < 0.01**, p-value < 0.001***).

501Mel and IGR37 cells (~2–9 log fold change). Moreover, miR-92a-1-5p was downregulated in 501Mel_GP, IGR37_XP and IGR37_GP (~3 log fold change). The expression levels of miR-509-3p, miR-708-5p and miR-92a-1-5p measured by microarrays (Fig. 2C) were confirmed by RT-qPCR (Fig. 2D). Also, miR-512-3p, miR-516b-5p, miR-517a-3p, and miR-517b-3p were significantly downregulated (~3 log fold change) in A375_XP and A375_GP, and their expression levels were also low in 501Mel_XP and 501Mel_GP. Finally, among the differentially expressed miRNAs in the resistant IGR37 cell lines, miR-182-5p, which has previously been described to be involved in melanoma metastasis, and miR-100-5p, which was linked to melanoma drug resistance, showed increased expression levels (~4 log fold change) [33,39,40]. The expression of miR-100 has previously been described to be induced by the chemokine C-C motif ligand 2 (CCL2), which conferred resistance to vemurafenib in melanoma patients [33]. The upregulation of CCL2 (~3 log fold change) and miR-100-5p was confirmed in the resistant cell line IGR37_GP (Figs. 2, 3). Table 1 lists the miRNAs that have so far been described in connection with melanoma drug resistance: miR-100, -514, -192, and 509-3p were upregulated in resistant cells in previous as well as in our study whereas for miR-34a, miR-200c and miR-125b we found opposing results.

Considering the gene expression analysis, PCSK2 was remarkably upregulated between 14 and 22 fold (~4 log fold change) in 501Mel_GP and 501Mel_XP and between 5 and 20 fold (~2–4 log fold change) in A375_GP and A375_XP as compared to the corresponding sensitive cell lines (Fig. 3A, B). Strongly increased expression of PCSK2 from the microarray analysis (Fig. 3C) was confirmed by RT-qPCR (Fig. 3D) and western blot (PC2 (protein convertase 2) encoded by the PCSK2 gene, Fig. 3E). Also, docking protein 5 (DOK5), which was shown to enhance the metastatic potential of melanoma cells [41], displayed augmented expression (~3 log fold change) in resistant IGR37 and 501Mel cells (Fig. 3A, B). Furthermore, resistant A375 cells had higher levels (~2.5 log fold change) of Dopachrome Tautomerase (DCT), an enzyme that is involved in melanin synthesis and that has been associated with drug and radiation therapy resistance [42]. Additional interesting candidates were differentially expressed in the resistant IGR37 cell lines, namely WAP four disulfide core 1 (WFDC1) (~5 log fold change), EGF-like repeats and discoidin I-like domains 3 (EDIL3) (~4.5 log fold change), and cysteine-rich angiogenic inducer 61 (CYR61) (~5 log fold change). WFDC1 was shown to play a tumour suppressive role in melanoma cell lines [43–45], but has not yet been linked to drug-resistance in melanoma. EDIL3 is involved in epithelial-mesenchymal transition (EMT) and was shown to play a role in metformin-resistance of prostate cancer cells [46]. Furthermore, increased levels of CYR61, which were also detected in resistant IGR37 cell lines, appear to be associated to PLX4032-resistance in melanoma cell lines [47].

3.2. Coexpression analysis

Next, a coexpression analysis was performed with the aim to identify co-regulated groups of miRNAs as well as inversely correlated miRNA-mRNA pairs, indicative of a potential interaction between miRNAs and their target genes (Tables S3 and S4). MiRNAs with similar expression trends across the different cell lines (Fig. 4A) and their common target genes (pie charts, Fig. 4B) as well as biological processes where they might be implicated in (Fig. 4C) were investigated using CoExpress, TargetScanHuman [34], and Enrichr [36], respectively. Members of the miRNA-506-514 cluster [48–50], located on chromosome X, were coexpressed. Among those was miR-509-3p, one of the top differentially expressed miRNAs present in most resistant cell lines. Members of this cluster have 217 target genes in common, which are implicated in hematopoietic cell differentiation, nitric oxide biosynthesis, or negative regulation of receptor activity (Gene Ontology Biological Processes, Fig. 4C). Moreover, miR-1228-5p, miR-3621, miR-4467, miR-6805-5p and miR-6816-5p were not only highly upregulated (~2 log fold change) in the resistant cell lines but they also shared remarkably similar expression patterns. The subsequent analysis revealed 49 common target genes, indicating that indeed, these miRNAs, although not expressed in one genomic cluster or transcription unit, might be co-regulated to work in concert on a shared set of target genes. Additionally, a chromosome 19-linked set of miRNAs (miR-512-3p, miR-516b-5p, miR-517-3p, miR-525-5p, miR-526b-5p, miR-1323) with an overall lower expression but downregulated in most resistant cell lines also had a strikingly similar expression pattern across the different samples. This group of miRNAs had 39 common target genes that were predominantly implicated in post-transcriptional gene regulation. Finally, another group of similarly down-regulated miRNAs across the resistant cell lines (miR-3911, miR-4298, miR-4459, miR-4669, miR-6831-5p, miR-7107-5p, miR-8060) had only four common target genes, indicating a rather coincidental co-regulation, which might not result in a shared functional output.

Plotting inverse correlations of miRNA and gene expression levels (CoExpress) can provide first insights into functional interactions between miRNAs and potential target genes. Fig. 5 shows examples of inverse expression patterns (Fig. 5A–D) and co-expressed miRNA-gene pairs (Fig. 5D–E). An interesting candidate pair was miR-92a-1-5p (Fig. 2) and DOK5 (Fig. 3), which were down- and upregulated, respectively (Fig. 5A) and confirmed by RT-qPCR (Fig. 2D, Fig. S3). According to standard target gene prediction algorithms, DOK5 had no binding site for miR-92a. However, miR-92a has recently been shown to not follow canonical binding rules as it can also bind the target mRNAs in a non-complementary manner (absence of seed sequence), hence most target genes of this miRNA are not predicted by current methods [51]. DOK5 and miR-92a-1-5p could be an example of such a non-canonical interaction.

Consequently, we also focused on miRNA-gene pairs that have not necessarily been predicted to interact directly, but that might still be functionally relevant. Levels of cytotoxic T-lymphocyte associated protein 4 (CTLA4), which is mainly expressed on the surface of CD4 + and CD8 + T cells [52] where it is a target of immunotherapy against melanoma and also lung cancer, were inversely correlated with miR-3180-3p (Fig. 5B). Interestingly, IGR37 and 501Mel moderately expressed CTLA4 at mRNA level (Fig. S3), although it was thought to be exclusively present on T cells. Furthermore, CTLA4 was downregulated in the corresponding cell lines resistant to vemurafenib and dabrafenib. Several histones showed an obvious inverse correlation with miR-509-3p (Fig. 5C), although only HIST1H3A has predicted binding sites for this miRNA. Moreover, the expression of the negative regulator of the MAPK pathway, NF1, was inversely correlated with three miRNAs, and positively correlated with miR-296-3p (Fig. 5D); downregulation of NF1 was confirmed by RT-qPCR (Fig. S3). NF1 is frequently mutated in melanoma patients and loss of function mutations or absence of NF1 expression due to post-transcriptional downregulation by miRNAs is an alternative way to activate canonical MAPK signalling [4,50,53]. NF1 showed significant inverse correlation with WFDC1, which was one of

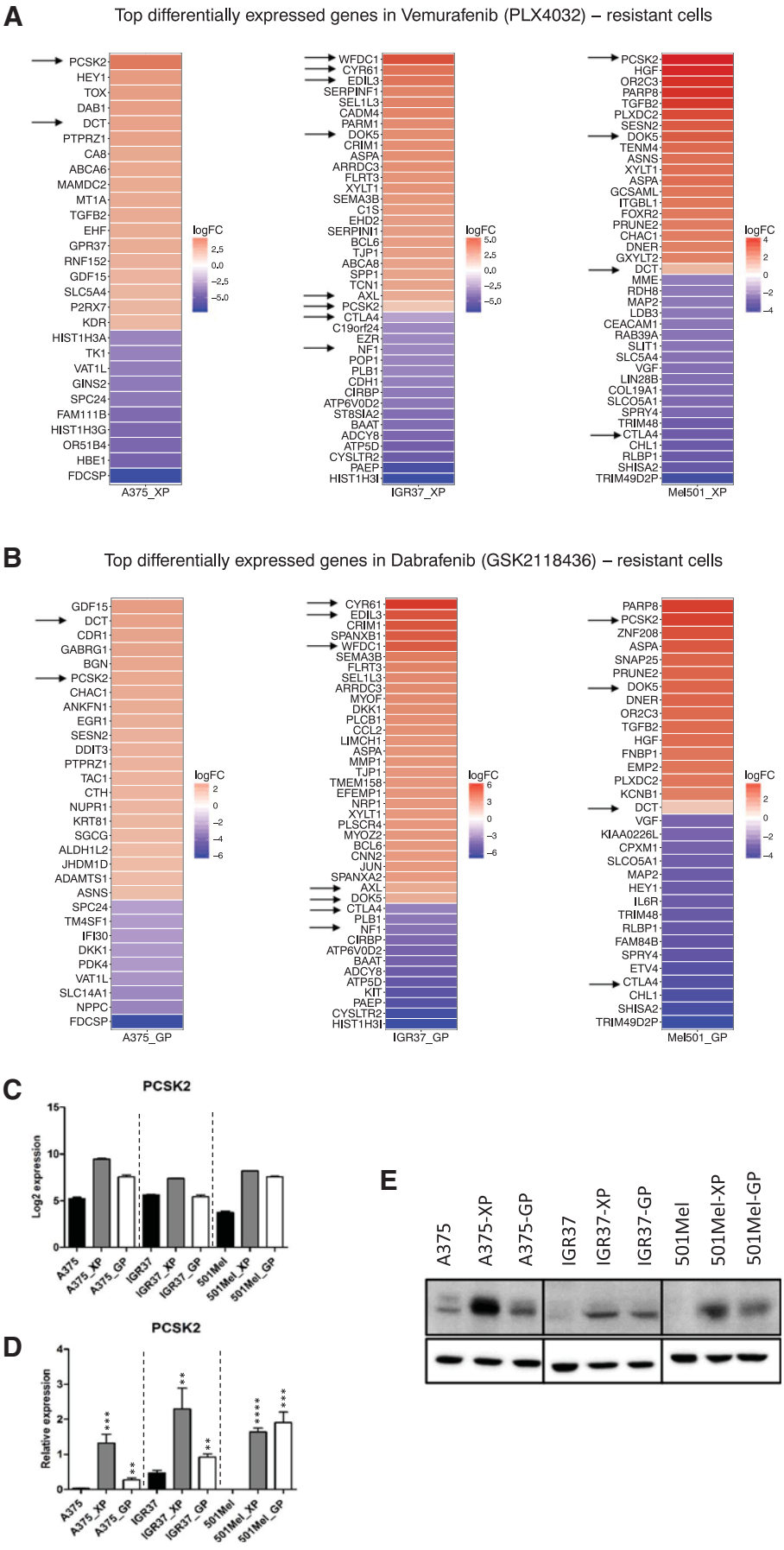

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Fig. 3. Top differentially expressed mRNAs. Differentially expressed mRNAs (FDR < 0.01 and at least 1.5-log fold change) in vemurafenib (XP)- (A) and dabrafenib (GP)- (B) resistant cell lines. Upregulated and downregulated mRNAs are shown in red and blue, respectively. (C) Log2 expression values of PCSK2 from the microarray data. (D) Relative expression determined by RT-qPCR as described in Fig. 2D. Statistical significance was determined as compared in Fig. 2. (E) Protein levels of PC2 (encoded by the gene PCSK2) were determined by western blot and α-tubulin was used as loading control. One representative western blot experiment of three biological replicates is shown. The expression threshold for the microarray data was set to 5, thus all candidates with a log2 expression below 5 were considered as not expressed. Statistical significance was determined in comparison to the sensitive cells using two-tailed unpaired *t*-test and p-values < 0.05 were regarded as significant (p-value < 0.05*, p-value < 0.01**, p-value < 0.001***).

the top upregulated genes in the resistant IGR37 cell lines, and described to have a tumour suppressive role in melanoma [43–45]. WFDC1 displayed similar expression patterns as miR-542-5p and miR-346 but was inversely correlated with miR-296-3p (Table S4). Although these candidates have distinct chromosomal localisations and are involved in different biological processes, their expression patterns suggest a potential co-regulation in IGR37_XP and IGR37_GP. Finally, the expression patterns of the receptor tyrosine kinase oncogene AXL correlated strongly with that of miR-100-5p, and both were upregulated in IGR37_XP and IGR37_GP (Figs. 2, 3, 5F).

3.3. MITF/AXL ratio potentially fine-tuned by miRNAs

A low microphthalmia-associated transcription factor/AXL (MITF/AXL) ratio has recently been reported to predict the development of resistance to targeted therapy in melanoma [54], and cells within melanoma tumours seem to display either high MITF or high AXL expression levels [55]. As AXL was upregulated in the resistant IGR37_XP and IGR37_GP (Fig. 3), the expression of MITF on mRNA (Fig. 6A, B) and protein level (Fig. 6C) was examined. Based on the microarray (Fig. 6A) and RT-qCPR results (Fig. 6B), MITF was not differentially expressed in the resistant cell lines. Interestingly, this was not reflected on protein level (Fig. 6C), which largely varied between cell lines and between sensitive and resistant lines in case of IGR37, indicating that MITF levels might be regulated post-translationally. However, MITF and AXL protein levels showed indeed perfect inverse expression patterns in almost all samples (Fig. 6C). Additionally, a positive correlation was detected between miR-100-5p and the tyrosine kinase AXL in IGR37_XP and IGR37_GP (Fig. 5E) in microarrays and by RT-qPCR (Fig. 6A, B). As expected, a potential interaction between AXL and miR-100-5p was not predicted by common tools (e.g. TargetScan, miRTarBase), hence, we hypothesise that either miR-100-5p targets and down-regulates an AXL inhibitor, or that AXL leads directly or indirectly to the overexpression of miR-100-5p. Furthermore, miR-182-5p, which is known to target MITF [39], was upregulated in IGR37_XP and IGR37_GP (Fig. 6A, B). Interestingly and as mentioned before, the MITF protein levels in the same samples were strikingly low. Consequently, the high levels of miR-182-5p might indeed cause the downregulation of the MITF protein in IGR37_XP and IGR37_GP. On the whole, our data support the hypothesis that the MITF/AXL ratio plays a role in the response to targeted therapy in melanoma, and we propose that this ratio might be fine-tuned by miR-182-5p and miR-100-5p in IGR37_XP and IGR37_GP (summarised in Fig. 6D). Additional experiments will have to be implemented in order to investigate their exact role in resistance and to elucidate the interactions in a potential network consisting of miR-182-5p, miR-100-5p, MITF, and AXL.

4. Discussion

Advances in immunotherapy and targeted therapy triggered a substantial progress in clinical treatment of melanoma patients [56]. The introduction of immune checkpoint inhibitors (PD-1 [57] and CTLA4 [58]), as well as BRAF kinase inhibitor monotherapy (vemurafenib [9] and dabrafenib [10]) or in combination with MEK inhibitors

Table 1
List of miRNAs described to be involved in melanoma drug resistance. Red and blue arrows represent up- and downregulated miRNAs as reported in literature as well as in our study. miRNAs that were differentially expressed in drug-resistant melanoma cell lines based on literature, but were not differentially expressed in our resistant melanoma cell lines, are marked with an x. The lower part of the table shows miRNAs potentially associated to melanoma drug resistance that were derived from our study, and which, to our knowledge, have not previously been described regarding resistance in melanoma [68–73].

	miRNAs involved in melanoma drug resistance						
	miRNA	Expression in resistant cells	Drug	Model	Target/effect	Ref.	Our study
Previously described miRNAs involved in melanoma drug resistance	miR-200c	↓	Vemurafenib	Cell lines	BMI-1, acquisition of EMT features	[68,69]	↑
	miR-7	↓	Vemurafenib	Cell lines	EGFR, IGF-1R, CRAF	[60]	x
	miR-193b	↓	Vemurafenib	Cell lines, Patients	Mcl-1	[70]	x
	miR-132	↓	Vemurafenib	Patients	/	[23]	x
	miR-579-3p	↓	Vemurafenib, Trametinib	Cell lines, Patients	BRAF, MDM2, E3 ubiquitin ligase	[71]	x
	miR-222	↑	Ipilimumab	Patients	ICAM1	[72]	x
	miR-3151	↑	Vemurafenib	Cell lines	TP53	[73]	x
	miR-100	↑	Vemurafenib	Cell lines, Patients	CCL2-induced	[33]	↑
	miR-514	↑	Vemurafenib, Cisplatin	Cell lines	NF1	[32,60]	↑
	miR-192	↑	/	Cell lines	prognostic factors/no before after	[23]	↑
	miR-509-3p	↑	Vemurafenib	Cell lines	/	[60]	↑
	miR-34a	↑	Vemurafenib	Cell lines, Patients	CCL2-induced	[33]	↓
	miR-125b	↑	Vemurafenib	Cell lines, Patients	CCL2-induced	[33]	↓
New miRNAs	miR-92a-1-5p, miR-517a-3p, miR-517a-3b, miR-512-3p, miR-4458, miR-526b-5p, miR1323, miR-3911, miR-4669, miR-8060						↓
	miR-708-5p, miR-182-5p, miR-497-5p, miR-4467, miR-3621, miR-1228, miR-183-5p, miR-187-5p, miR-127-3p						↑

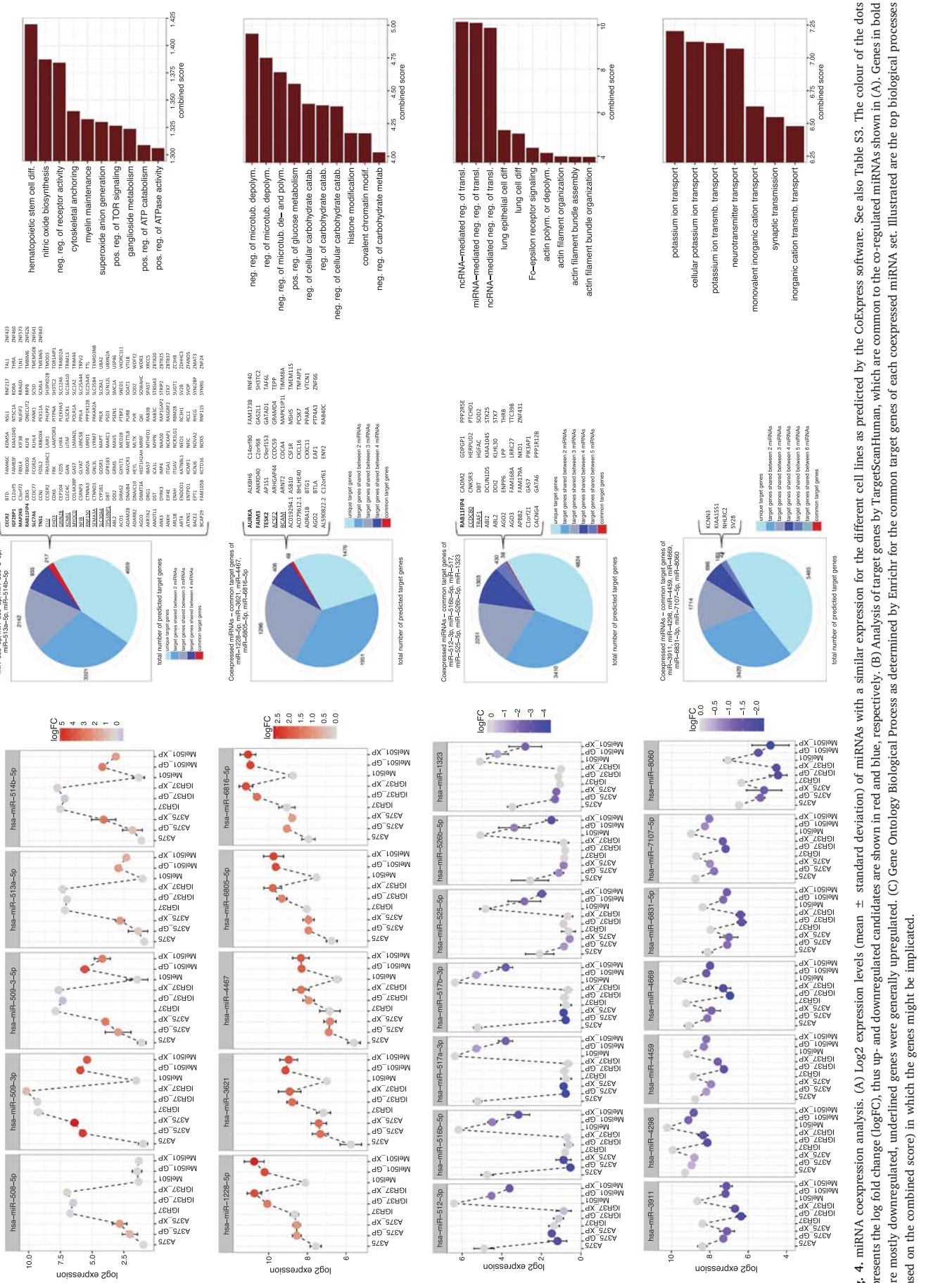

Fig. 4. miRNA coexpression analysis. (A) Log2 expression levels (mean ± standard deviation) of miRNAs with a similar expression for the different cell lines as predicted by the CoExpress software. See also Table S3. The colour of the dots represents the log fold change (logFC), thus up- and downregulated candidates are shown in red and blue, respectively. (B) Analysis of target genes by TargetScanHuman, which are common to the co-regulated miRNAs shown in (A). Genes in bold were mostly downregulated, underlined genes were generally upregulated. (C) Gene Ontology Biological Process as determined by Enrichr for the common target genes of each coexpressed miRNA set. Illustrated are the top biological processes (based on the combined score) in which the genes might be implicated.

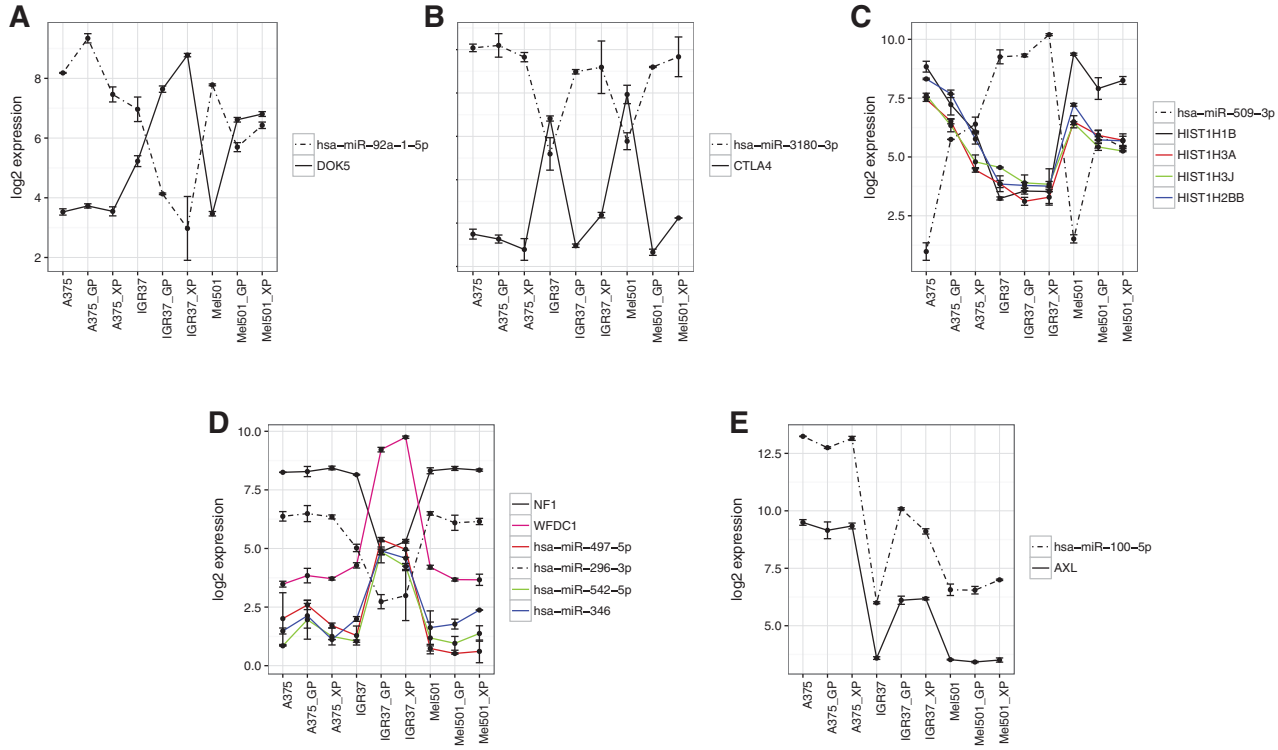

Fig. 5. Coexpressed miRNA-gene pairs. (A–E) Genes and miRNAs with inverse (CE < -0.9) or positive (CE > 0.9) correlations. Shown are the mean log2 expression values ± standard deviation. Correlation analysis with the CoExpress software predicts potential functional interactions between miRNAs and target genes based on expression patterns. See also Table S4.Coexpressed miRNA-gene pairs. (A–E) Genes and miRNAs with inverse (CE < -0.9) or positive (CE > 0.9) correlations. Shown are the mean log2 expression values ± standard deviation. Correlation analysis with the CoExpress software predicts potential functional interactions between miRNAs and target genes based on expression patterns. See also Table S4.

(vemurafenib/cobimetinib [17] and dabrafenib/trametinib [19]), contributed profoundly to an improved overall survival of patients with metastatic melanoma. However, despite the initial response to treatment, the efficacy of these drugs is limited by the rapidly emerging resistance to therapy, which remains a serious clinical issue [16].

Here, we investigated different mechanisms that may contribute or confer resistance to targeted treatment with BRAF inhibitors, with a focus on miRNAs, as their role in the emergence of drug resistance has not been studied thoroughly. We generated drug-resistant melanoma cell lines and performed miRNA and mRNA microarray analyses to obtain insights into the changes occurring upon BRAF inhibitor resistance.

Our data show that the responses to BRAF inhibition mainly occur in a cell line-specific manner, as the resistant cell lines were more similar to their parental sensitive lines than to other resistant cell lines (Fig. 1, Fig. S2). In addition, vemurafenib and dabrafenib, both of which are routinely given to metastatic melanoma patients in clinical settings had comparable effects by inducing differential expression of the same miRNAs and genes (Figs. 2, 3) and only few changes appeared to be drug-specific.

The highest number of differentially expressed miRNAs and genes was found in resistant IGR37 cell lines, followed by resistant 501Mel. The relatively few changes on miRNome and transcriptome level in resistant A375 cells as compared to resistant IGR37 and 501Mel cell lines might be explained by the fact that A375_XP and A375_GP harboured acquired activating heterozygous NRAS mutations ($NRAS^{G13R}$ and $NRAS^{Q61K}$ respectively), which we confirmed by Sanger sequencing (Fig. S1). Consequently, the emergence of resistance in A375 could possibly be explained by NRAS-mediated reactivation of the MAPK or alternative pathways. To obtain a more dynamic view on the genetic and gene regulatory events, we have started to collect data on short-term BRAF inhibitor treatment.

The top differentially expressed miRNAs in most resistant cell lines were miR-509-3p, miR-708-5p, and miR-92a-1-5p (Fig. 2). miR-509-3p is known to be highly expressed in late stage melanoma [49,50,59], and was also shown to be upregulated in vemurafenib-resistant melanoma cell lines (Table 1, [60]). Furthermore, several members of the chromosome X-linked miR-506-514 cluster, including miR-509-3p, were co-expressed. Streicher and colleagues [49] have shown that members of this cluster can trigger oncogenesis. Therefore, these miRNAs are particularly interesting for follow-up validation experiments to elucidate their role in drug resistance. Another potentially promising candidate is miR-708-5p, which has not been connected to drug resistance yet. Interestingly, in a previous study we have detected high levels of both miR-708-5p and miR-509-3p in whole blood of stage III and IV melanoma patients [27], making these miRNAs potentially promising biomarkers for melanoma drug-resistance.

Finally, miR-92a-1-5p, which was downregulated in half of the BRAF inhibitor-resistant cell lines, had reduced expression levels in late stage melanoma patient-derived cell populations under hypoxia [61]. Qin et al. have recently demonstrated the involvement of hypoxia in BRAF inhibitor-mediated resistance in melanoma [62], while Cao et al. have shown that drug treatment can trigger the expression of hypoxia-inducible factor 1α (HIF-1α) even under normoxia [63]. Furthermore, PCSK2, one of the herein top upregulated candidates in drug-resistant cells, contains two hypoxia-response elements (HRE) in its regulatory region and was inducible by HIF-1α upregulation [64]. It is tempting to speculate that HIF-1α-mediated upregulation of PCSK2 might contribute to acquired resistance to vemurafenib and dabrafenib of melanoma cell lines.

A low MITF/AXL ratio has been connected to the emergence of drug-resistance in melanoma [54]. Inverse expression levels of AXL and MITF were observed in all sensitive and resistant cell lines, which confirms the two previously described distinct expression states: (1) high AXL—low MITF expression, or (2) high MITF—low AXL expression [55]. Additionally, several of our resistant cell lines displayed a

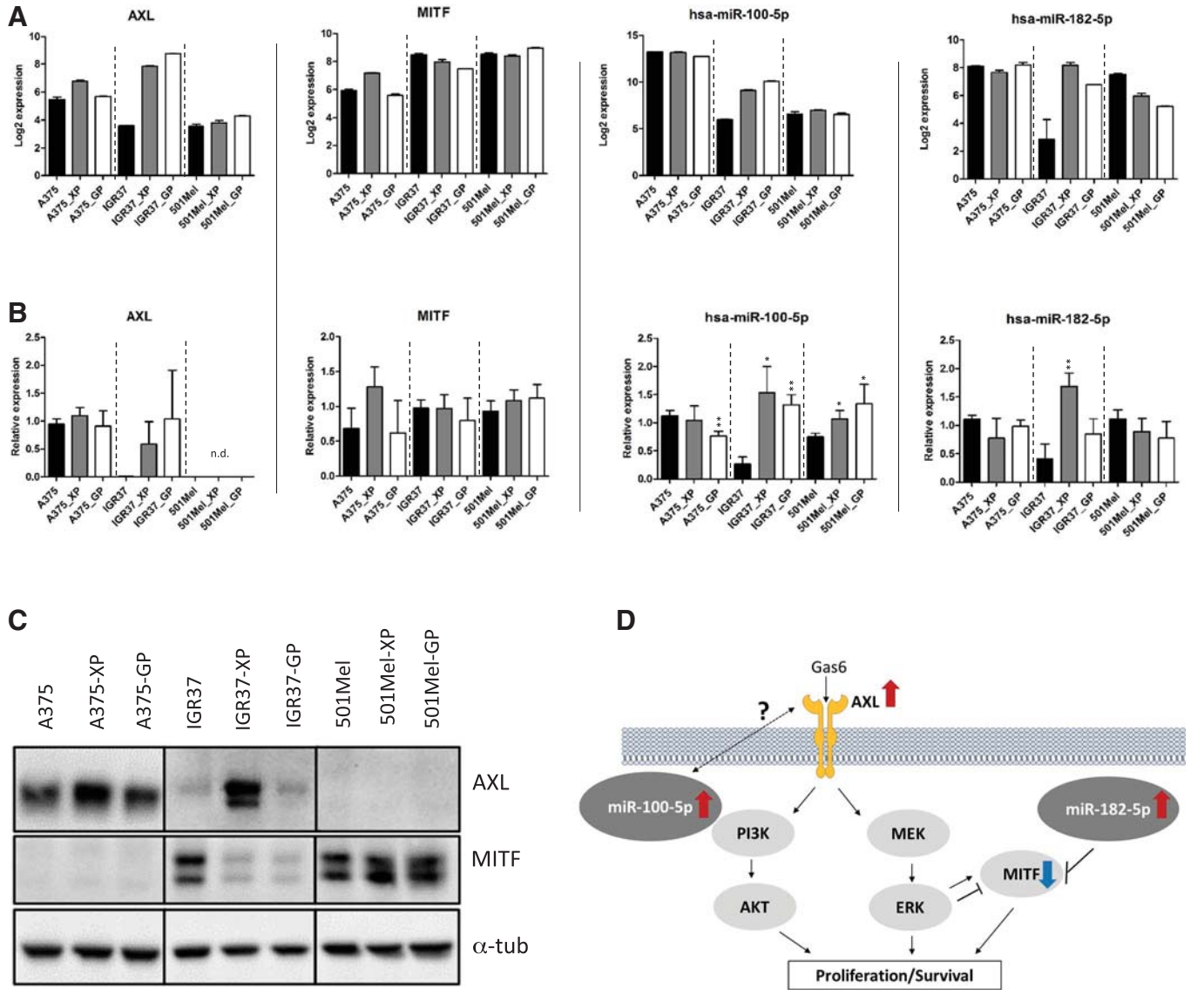

Fig. 6. Potential involvement of the MITF/AXL ratio in melanoma drug resistance. (A) Log2 expression values of AXL, MITF, miR-100-5p and miR-182-5p genes from the microarray data. (B) Confirmation of relative expression levels by RT-qPCR where error bars represent the standard deviation of three biological replicates (n.d.: not detected). Statistical significance was determined as compared in Fig. 2. (C) Protein levels of AXL and MITF in sensitive and resistant melanoma cell lines. For each western blot experiment, one representative of three biological replicates is shown. (D) Schematic representation of potential fine-tuning of the MITF/AXL ratio by miR-100-5p and miR-182-5p in resistant IGR37 cell lines. Prolonged BRAF inhibitor treatment leads to the upregulation of AXL, miR-100-5p and miR-182-5p as well as to the downregulation of MITF, which altogether could trigger the activation of the MAPK and/or PI3K/AKT signalling cascade leading to persistent cell proliferation and survival.

low MITF/AXL ratio, which was strongly connected to the high expression of miR-100-5p and miR-182-5p (Fig. 6). These observations suggest that the MITF/AXL ratio might be fine-tuned by miRNAs and support its implication in the development or persistence of BRAF inhibitor-induced resistance.

Classic target gene prediction failed to identify promising miRNA-mRNA pairs supported by expression patterns in our data sets. In this context, a recent study demonstrated that < 40% of miRNA-mRNA interactions followed canonical binding rules, with 7 matching or almost matching seed nucleotides in the 3′ UTR sequence of targets [51]. Helwak and colleagues developed the CLASH technology (cross-linking and sequencing of hybrids), relying on the simultaneous identification of miRNAs crosslinked to their target sequence, which could be mRNAs, rRNAs, tRNAs, lncRNAs, other miRNAs and pseudogenes [51,65]. Control experiments revealed that targets lacking a canonical seed match were only regulated half as efficiently as the seed-containing targets, nevertheless such interactions were frequent and not predicted by currently available target gene prediction software tools [66]. There is accumulating evidence that such “seed-less” miRNA-mRNA interactions are functionally relevant and the vast majority of them has so far been missed as they are not predicted by widely applied algorithms, which search for seed pairing, conservation or energy interaction scores [67]. Here, we first applied standard prediction tools (Targetscan, miRTarBase) to identify potential targets of miRNAs that were differentially regulated in drug-resistant versus -sensitive melanoma cells. Only few interactions were predicted, therefore we also analysed miRNA-mRNA pairs with statistically significant inverse correlation, i.e. miRNAs or groups of miRNAs with coregulated expression levels and mRNAs with a correspondingly low or inverse expression pattern. We found several interesting pairs that all lack a canonical binding sequence in the target 3′ UTRs but might nevertheless be functionally connected (Figs. 4 and 5, Tables S3 and S4).

In summary, our data provide an overview of the changes in the miRNome and transcriptome of different BRAF inhibitor-resistant melanoma cells. We found that (i) changes are mostly cell line-specific rather than treatment-dependent, (ii) both BRAF inhibitors had a

comparable impact on miRNome and transcriptome levels, (iii) few miRNA-mRNA pairs were characterised by canonical seed binding but rather by inverse correlations, which might be indicative of functional interactions; their exact way of binding and interaction will have to be elucidated in future, (iv) several of our BRAF inhibitor-resistant cell lines displayed a low MITF/AXL ratio, which seems to be regulated by miR-100-5p and miR-182-5p, suggesting their implication in the development of drug-resistance, and (v) several interesting miRNAs and mRNAs, which could either serve as biomarkers of drug resistance (miR-708-5p, miR-509-3p, miR-3621) or be functionally involved in development of resistance have been identified (PCSK2, DOK5, AXL, miR-92a-1-5p, miR-100-5p) and will be further followed up experimentally in melanoma patient samples.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2017.04.005.

Authors' contributions

IK and GC performed array and validation experiments and data analysis. CM, DP, SK generated resistant cell lines. SK conceived the study. IK, GC, and SK wrote the manuscript with editorial input from CM and DP. All authors read and approved the final manuscript.

Disclosure of potential conflicts of interest

The authors indicate no potential conflicts of interest.

Transparency document

The http://dx.doi.org/10.1016/j.bbagen.2017.04.005 associated with this article can be found, in online version.

Acknowledgments

We thank Dr. Petr Nazarov from the Luxembourg Institute of Health for providing invaluable advice on the microarray and coexpression data analysis. This study was supported by a grant from the Fondation Cancer (Luxembourg). IK is funded by the PRIDE Doctoral Research Program by Fond National de la Recherche (Luxembourg) in the scope of the Doctoral Teaching Unit—"CANBIO".

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Figure S1

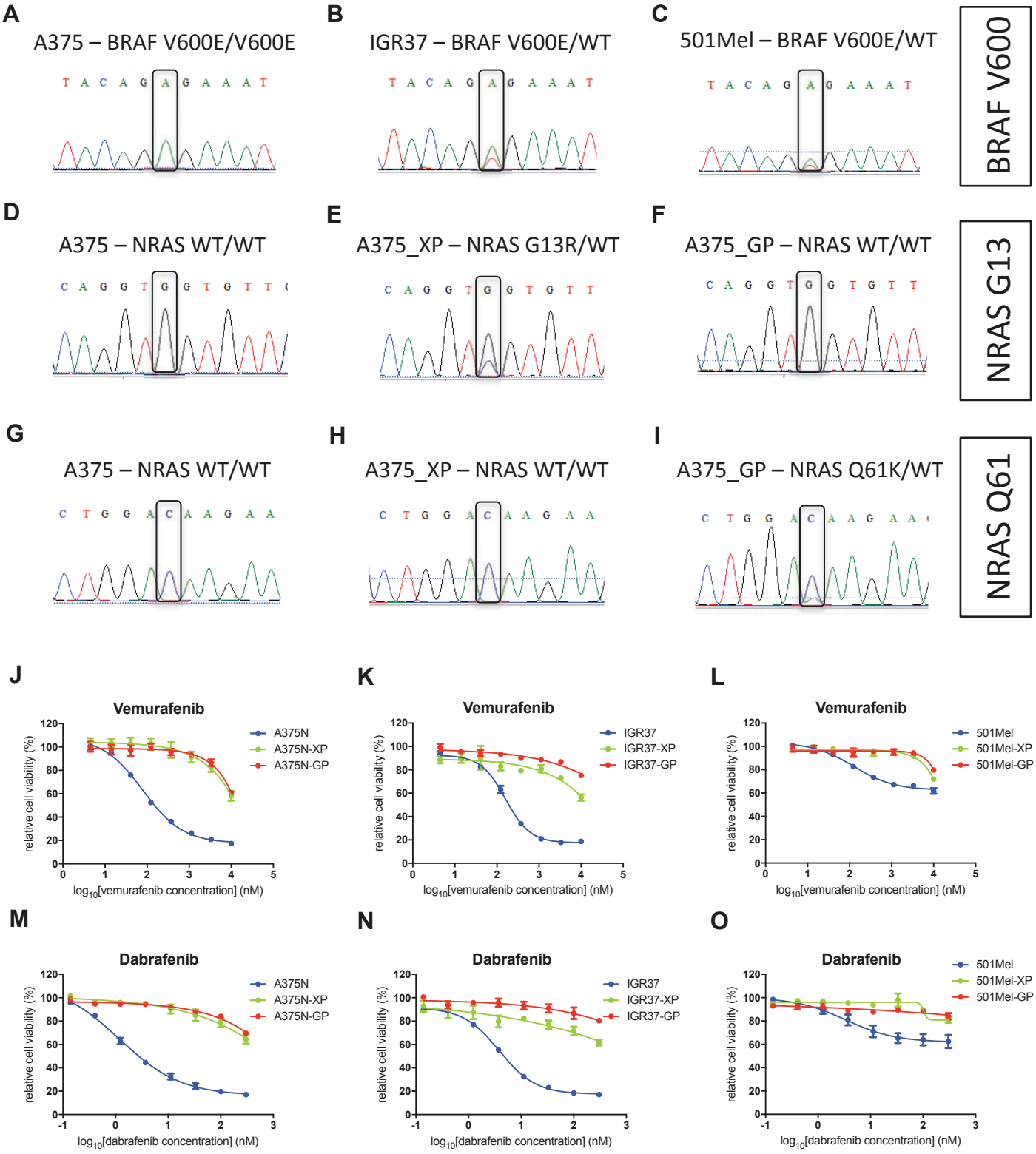
A
A375 – BRAF V600E/V600E
B
IGR37 – BRAF V600E/WT
C
501Mel – BRAF V600E/WT
BRAF V600
D
A375 – NRAS WT/WT
E
A375_XP – NRAS G13R/WT
F
A375_GP – NRAS WT/WT
NRAS G13
G
A375 – NRAS WT/WT
H
A375_XP – NRAS WT/WT
I
A375_GP – NRAS Q61K/WT
NRAS Q61
J
Vemurafenib
A375N
A375N-XP
A375N-GP
relative cell viability (%)
log10[vemurafenib concentration] (nM)
K
Vemurafenib
IGR37
IGR37-XP
IGR37-GP
relative cell viability (%)
log10[vemurafenib concentration] (nM)
L
Vemurafenib
501Mel
501Mel-XP
501Mel-GP
relative cell viability (%)
log10[vemurafenib concentration] (nM)
M
Dabrafenib
A375N
A375N-XP
A375N-GP
relative cell viability (%)
log10[dabrafenib concentration] (nM)
N
Dabrafenib
IGR37
IGR37-XP
IGR37-GP
relative cell viability (%)
log10[dabrafenib concentration] (nM)
O
Dabrafenib
501Mel
501Mel-XP
501Mel-GP
relative cell viability (%)
log10[dabrafenib concentration] (nM)

Figure S1. Sanger sequencing chromatograms and drug dose response curves. Screening of the $BRAF^{V600E}$ mutation in the BRAF-mutant cell lines showing a homozygous BRAF mutation in A375 **(A)** and a heterozygous BRAF mutation in IGR37 **(B)** and 501Mel **(C)**. Screening of the $NRAS^{G13R}$ **(D-F)** and $NRAS^{Q61K}$ **(G-I)** in A375, A375_XP, and A375_GP showing the acquisition, upon development of drug resistance, of a heterozygous $NRAS^{G13R}$ and $NRAS^{Q61K}$ mutation in A375_XP and A375_GP, respectively. WT = wild-type. Vemurafenib **(J-L)** and dabrafenib **(M-O)** dose response curves in the parental and BRAF inhibitor-resistant cell lines showing the cell viability relative to the untreated control (%) upon increasing drug concentrations. Illustrated are the mean Âś standard deviation of one representative biological replicate out of three.

Figure S2

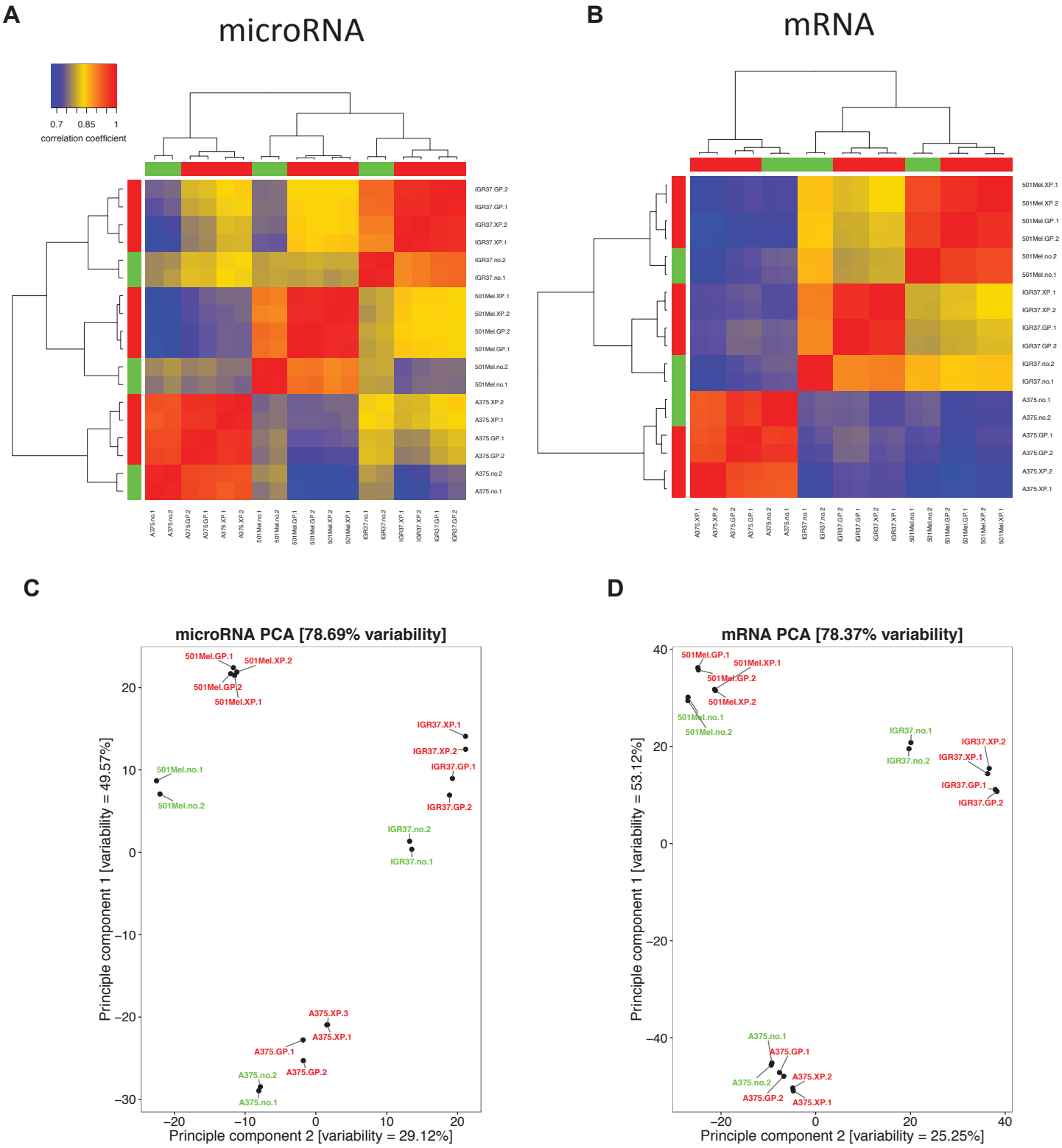

Figure S2. Correlation matrix and principle component analysis (PCA). Correlation matrix and PCA for miRNA **(A, C)** and mRNA **(B, D)** datasets. Duplicate samples were analysed. The BRAF inhibitor-sensitive and -resistant cell lines are marked in green and red, respectively.

Figure S3

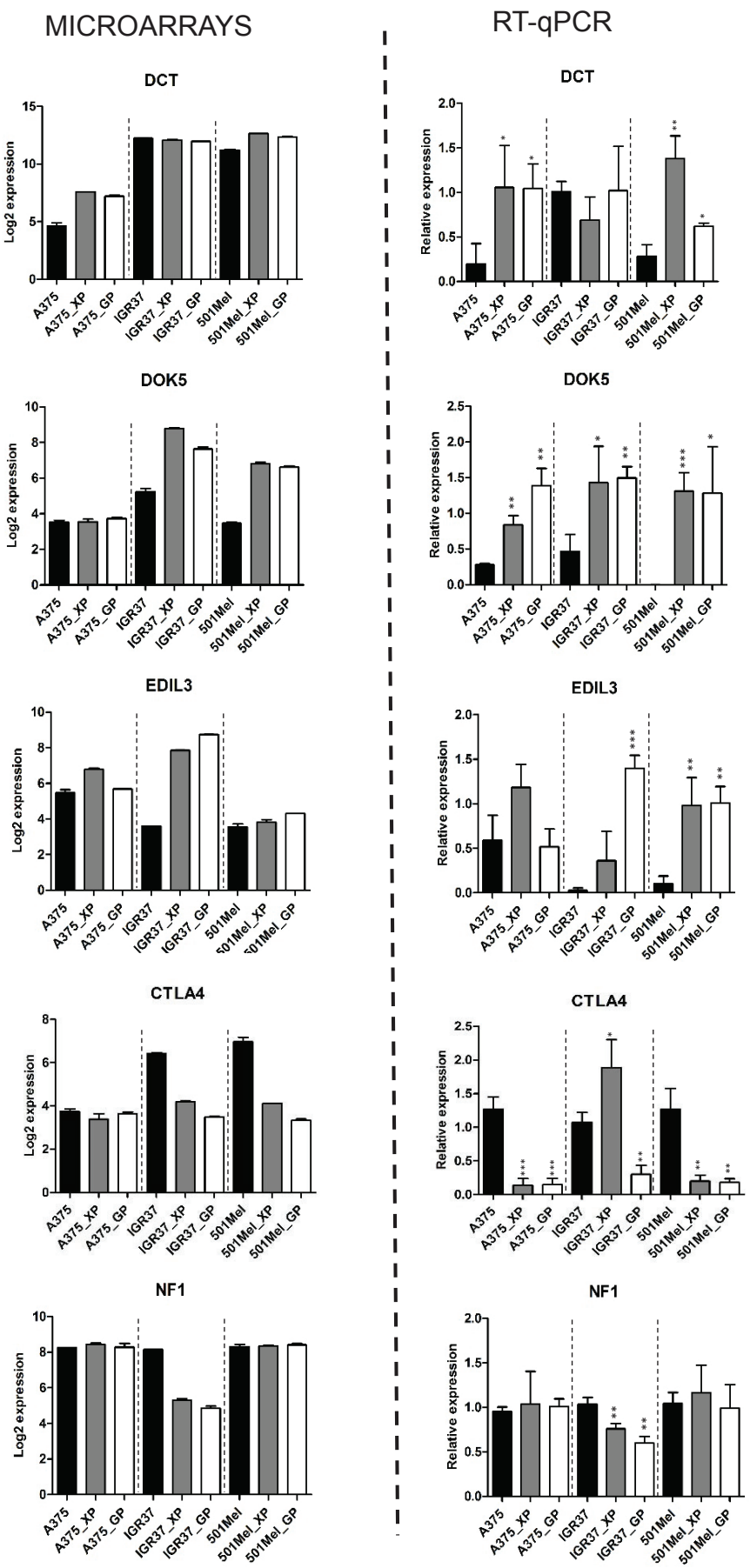

Figure S3. RT-qPCR validations of selected candidates from the mRNA microarray dataset. **(A)** Log2 expression values of DCT, DOK5, EDIL3, CTLA4, and NF1 from the microarray data in duplicates, and **(B)** relative expression in three biological replicates determined by RT-qPCR.

4.2.3 Additional results

Transcriptional changes induced by short-term BRAF inhibition

Having investigated the long-term adaptation to vemurafenib treatment in fully resistant cell lines, we asked whether this long-term adaptive response could be compared to the short-term response. To gain insights into the changes induced by a short-term BRAF inhibition, we treated A375 melanoma cells with Vemurafenib for 48 hours. Already after this short period of time, an enormous number of differentially expressed mRNAs and miRNAs was observed (Figure 16 and 17). Although some of the differentially expressed mRNAs or miRNAs were also identified in the resistant cell lines (such as PCSK2, DCT, TGFβ2, miR4497, miR-1909-3p and miR92a-1-5p), we could identify a distinct expression profile after short-term treatment possibly due to a BRAF inhibition-induced early adaption phase. For instance, the cell adhesion molecule ALCAM (activated leukocyte cell adhesion molecule) has been found to be upregulated upon BRAF inhibitor treatment. Donizy and colleagues demonstrated that high ALCAM expression in primary tumour cancer cells is strongly correlated with unfavourable prognosis as compared with patients with lower ALCAM and it may indicate a more invasive phenotype of cancer cells (Donizy et al., 2015). As another example, TP53INP1 protein (Tumour Protein P53 Inducible Nuclear Protein 1) was also upregulated. This protein is a key stress protein with antioxidant-associated tumour suppressive function (Saadi et al., 2015). As for miRNAs, miR-3185 was upregulated. Misra et al. described an increased expression of hsa-miR-3185 upon induction of the oxidation state (Misra et al., 2014). In this context, validation of some candidates in time course experiments in more cell lines will follow. Along these lines, Lunavat et al. have very recently show that short-term vemurafenib treatment induces upregulation of several miRNAs in melanoma cells, notably miR211, which in turn reduces the sensitivity of cells to BRAF inhibition (Lunavat et al., 2017).

These data suggest the presence of adapting changes in melanoma cells under selective pressure. In addition, the early and late responses to BRAF inhibition share some alterations that might play a role in the development of drug resistance.

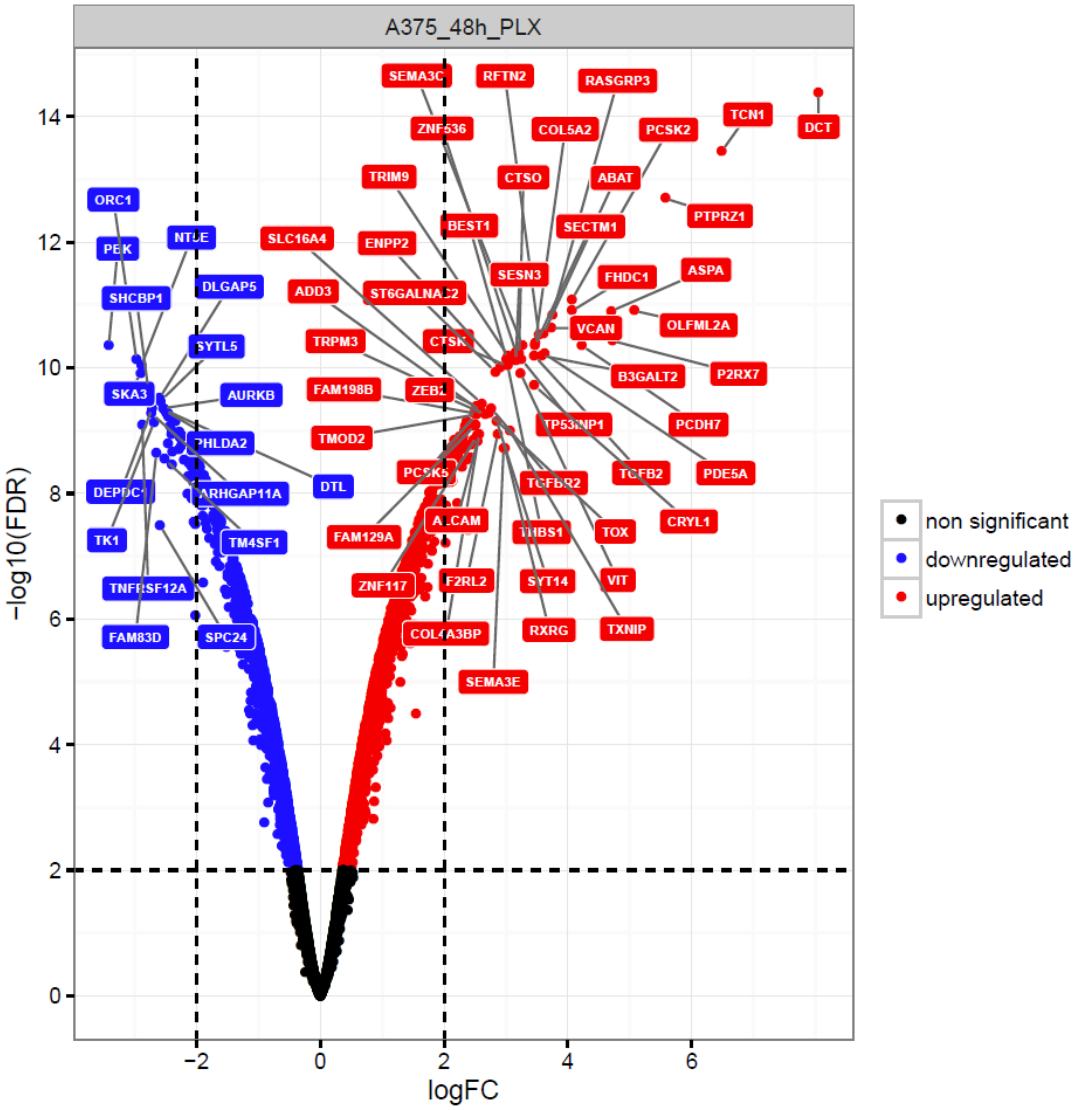

Figure 16. Top differentially expressed genes.

Vulcano plot illustrating the top differentially mRNAs with an FDR<0.01 and ads (logFC)>1.5 in A375 upon 48 hours treatment with PLX4032. Upregulated and downregulated mRNAs and miRNAs are shown in red and blue respectively. ALCAM and TP53INP1, among others are upregulated

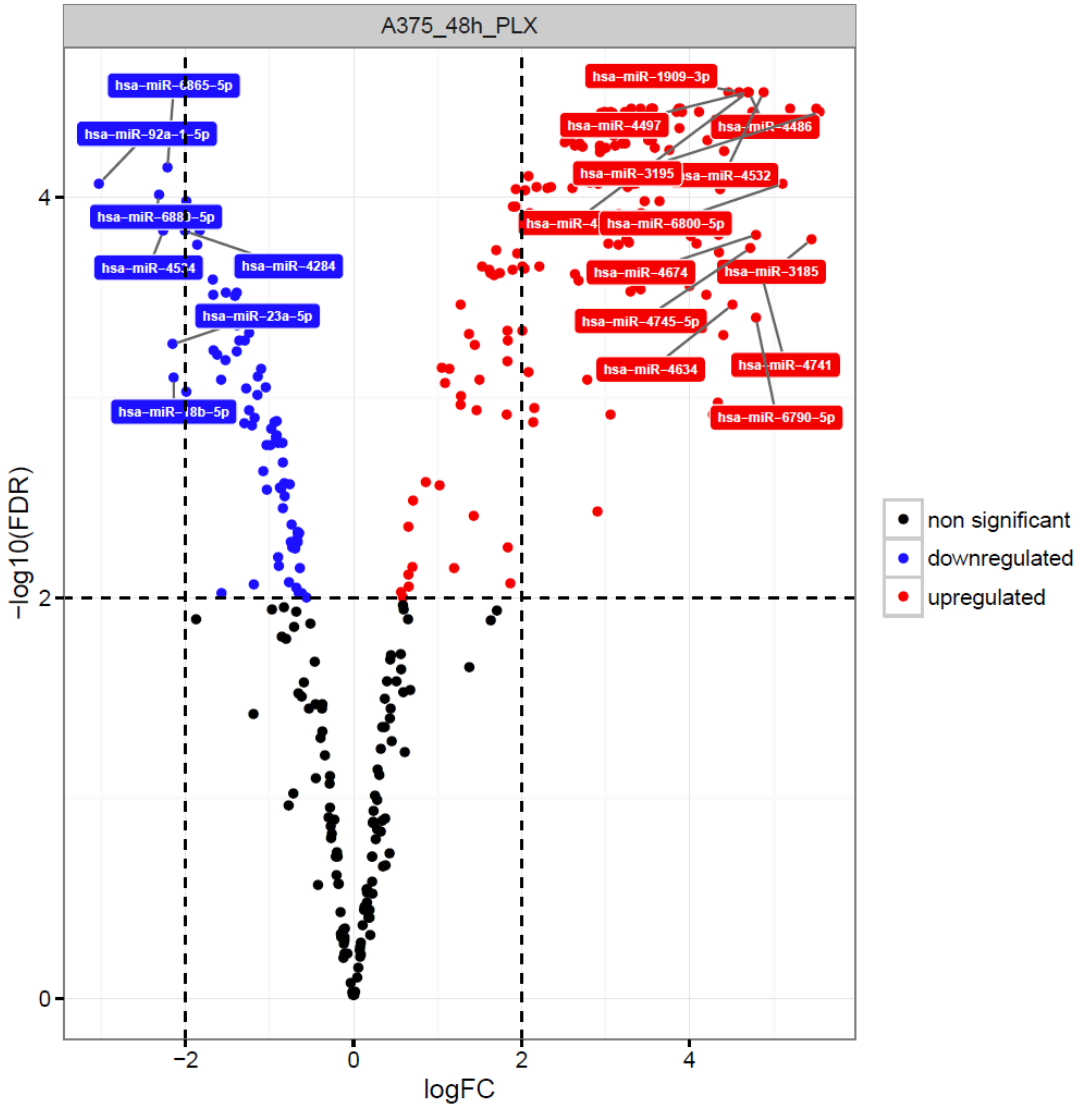

Figure 17. Top differentially expressed miRNA.

Vulcano plot illustrating the top differentially microRNAs with an FDR<0.01 and ads (logFC)>1.5 in A375 upon 48 hours treatment with PLX4032. Upregulated and downregulated mRNAs and miRNAs are shown in red and blue respectively. Among others, miR-3185 is upregulated.

Additional validation for CTLA4

To further explore the role of CTLA4 on melanoma cells, we validated its presence by western blot analysis. The presence of CTLA4 on melanoma cells was unexpected since this protein is normally expressed on T cells where it plays a negative regulatory role on T cell activation. Interestingly, Laurent et al. observed that patient-derived melanoma cells and tissues constitutively express the CTLA-4 molecule, which can interact and respond to ipilimumab (Laurent et al., 2013). In particular, ipilimumab is able to trigger an antibody dependent cellular cytotoxicity engaging the receptor FcγRIIIA on primary NK cells. In our datasets, we could find CTLA4 mRNA present in the sensitive cells, which was generally reduced in resistant cells with some discrepancies between microarray and qPCR validation probably due to higher sensitivity of the qPCR technique and to primer design (Figure S3, page 73). In the paper, we also showed that the levels of CTLA4 were inversely correlated with miR-3180-3p (Figure 5B), suggesting a potential interaction which requires further investigation. We additionally validated CTLA4 at the protein level (Figure 18) where CTLA4 was slightly reduced only in the resistant IGR37-GP cells. Long half-life and/or increased protein stability might explain the lack of correlation between mRNA and protein levels. To further investigate this discrepancy, CTLA4 expression will be followed up in a detailed time course experiment. Moreover, the specificity of the antibody will be tested by using an siRNA approach. Given the ability of ipilimumab to interact with CTLA4 present on melanoma cells and to trigger cellular cytotoxicity (Laurent et al., 2013), the potential downregulation of CTLA4 in resistant melanoma cells might be considered as an additional mechanism favouring the survival of resistant melanoma cells.

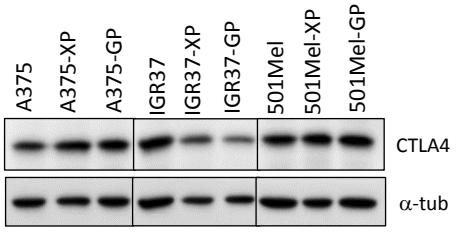

Figure 18: CTLA4 expression in melanoma cells.

Western blot analysis of CTLA4 in parental and corresponding resistance cells lines. α-Tubulin was used as loading control.

4.3 A novel mechanism of BRAF kinase inhibitor drug resistance in melanoma cells involving ALK transported by extracellular vesicles (manuscript in preparation)

4.3.1 Preamble

Recent studies have shown an increased complexity of melanoma tumours formed by different subclones, which respond differently to BRAF inhibition therapies (Tirosh et al., 2016) with drug-resistant subclones present before the treatment or emerging as a result of targeted therapies.

Another pressing issue in clinical care of melanoma and other cancer patients is the lack of biomarkers that can reliably predict the onset of drug resistance or biomarkers that could help to tailor targeted therapies in a more efficient way (Samatov et al., 2016). There is evidence to suggest that extracellular vesicles (EVs) might be a useful source of biomarkers because they contain unique proteins and non-coding RNAs reflecting the phenotype of the secreting cells (Barile and Vassalli, 2017).

In the previous paper, we used pools of resistant cells to mimic the cellular heterogeneity also present in human tumours. Here, and in order to get a clearer picture of important mechanisms driving resistance to BRAF inhibitors, we focused on a particular clone that exhibited the fastest growth rate under kinase inhibitor treatment.

In the scope of this project, microarray analyses were performed with the aim to describe the changes in the transcriptome that might play a role in resistance and we identified, among others, a novel isoform of the Anaplastic Lymphoma Kinase (ALK) to be upregulated. This finding was corroborated by the fact that ALK has been already identified in melanoma patients (Wiesner et al., 2015). Next, the molecular impact of this upregulated ALK was investigated. Finally, we asked whether the resistant phenotype could be transferred between cells through soluble factors. Co-culture experiments have been performed to understand the potential involvement of resistant EVs in the “spreading” of drug resistance.

Interestingly, we could show that sensitive melanoma cells acquire the drug resistant phenotype if co-cultured with EVs released by resistant cells. Proteomic analysis performed on EV populations revealed a panel of proteins enriched in the “resistant EVs” compared to “sensitive EVs”, and again ALK was identified to be upregulated. Our results suggest that “resistant EVs” have functional properties capable of making sensitive melanoma cells more resistant to BRAF inhibitors through a mechanism involving the transport of ALK.

Currently, we are further investigating the functional consequences of the upregulated ALK in melanoma and we are in the process of stably transducing several ALK-negative melanoma cells lines with the herein identified ALK isoform to confirm that ALK is indeed conferring drug resistance. Additionally, we plan to perform *in vivo* experiments injecting those cells into immunocompromised mice: the ability of ALK-positive cells to form tumours compared to ALK-negative cells and *in vivo* therapeutic responses to pharmacological inhibition of ALK in combination with BRAF inhibitors will be tested. We plan to finish these additionally experiments by the end of the year and submit the paper beginning of 2018.

The following manuscript is in preparation and for better readability, figures have been inserted in the text where they thematically belong.

Personal contribution to this manuscript

- Contributed to the study design
- Performed experiments and generated Fig 1, Fig 2C and H, Fig 3, Fig 4, Fig 5A and C, Fig 6, Fig 7A and C, Fig 9 and 10
- Analyzed data and wrote the manuscript

4.3.2 Third publication (Cesi et al., in preparation)

Overexpressed ALK transported by extracellular vesicles confers drug resistance to sensitive melanoma cells: a novel mechanism of acquired resistance

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Abstract

For melanoma patients, drug resistance is an unsolved clinical issue. Despite the promising initial results obtained with BRAF kinase inhibitors (vemurafenib and dabrafenib), it soon became evident that these specific inhibitors were not able to provide durable responses, as resistance to treatment develops within months in virtually all patients. Often resistance results from re-activation of the MEK/ERK signalling pathway due to acquired mutations in genes such as NRAS, COT, CRAF. Here, we report a novel mechanism of acquired drug resistance, which involves the activation of a truncated form of the anaplastic lymphoma kinase (ALK). ALK knock down and inhibitors targeting ALK re-sensitized resistant cells to BRAF inhibition and induced apoptosis. Interestingly, overexpressed ALK was secreted into extracellular vesicles (EVs) and we show that EVs were the vehicle for transferring drug resistance: sensitive melanoma cells acquired the drug resistant phenotype if co-cultured with extracellular vesicles released by resistant cells, which carried ALK to sensitive cells. To our knowledge, this is the first report to demonstrate the functional involvement of EVs in drug resistance by transporting a truncated but functional form of ALK, which activated the MAPK signalling pathway in target cells. This novel mechanism in acquired drug resistance adds potential as clinical target (ALK) and as potential biomarkers for emerging drug resistance (EVs).

Introduction

Melanoma is an aggressive and highly metastatic cancer, associated with poor outcome once advanced stages have been reached. Compared to other solid cancers, melanoma exhibits an extremely high prevalence of somatic mutations [1, 2], which is almost entirely attributable to UV light exposure. Despite this high genetic heterogeneity, more than 50% of melanoma patients carry mutations in the Ser/Thr-kinase BRAF (most often V600E), which renders the BRAF kinase and the downstream MAPK signalling pathway constitutively active [3]. The introduction of specific kinase inhibitors for melanoma patients carrying this BRAF mutation has revolutionized melanoma care. In 2011, specific BRAF inhibitors were FDA-approved showing convincing results at first [4, 5] and since 2015 a combined inhibition of BRAF and MEK kinases is recommended [6, 7], which has increased median survival from 18.7 to 25.1 months [8, 9]. However, despite these unprecedented clinical responses, drug resistance arises rapidly within 3-12 months [10, 11] leaving very little treatment options. Most of the time acquired resistance is driven by secondary mutations which re-activate the MAPK signalling pathway resuming proliferation.

Anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase, that is normally involved in the development of the nervous system [12]. In differentiated tissues, ALK can be activated by translocations or mutations becoming an oncogene in a variety of malignancies, such as non-small cell lung cancer, anaplastic large cell lymphoma, neuroblastoma and many more [13]. Additionally, in 2015, Wiesner and colleagues identified in 11% of melanoma tissues a truncated ALK transcript starting from intron 19 and resulting in a smaller protein, which was shown to be oncogenic [14].

Here, we identified the overexpression of another truncated form of ALK as a new mechanism driving acquired drug resistance in melanoma cells. In particular, we demonstrate that the treatment of the ALK-expressing resistant melanoma cells with siRNA or ALK inhibitors in combination with either BRAF or MEK inhibitors, leads to efficient cell growth suppression and apoptosis suggesting this combination to be an interesting clinical option for patients harboring both $BRAF^{V600E}$ and expressing ALK. Moreover, we show for the first time that the overexpressed ALK is secreted into extracellular vesicles (EVs) and transferred to sensitive, ALK-negative melanoma cells. There, ALK is functional in activating the MAPK signalling pathway and thus mediating the transfer of drug resistance. In addition, the presence of ALK

within EVs could be further explored as biomarker for monitoring resistance mechanisms against BRAF kinase inhibitors.

Experimental procedures

Reagents and antibodies

All inhibitors used in this study were purchased from Selleckchem and were dissolved in DMSO at a concentration of 10 mM and stored at -20°C. The following antibodies were used for western blot: anti-ALK (Cell signaling), anti phospho-ERK1/2 (Cell Signaling), pAKT (Cell Signaling), anti-ERK1/2 (Santa Cruz), tot-AKT (Santa Cruz), anti □-tubulin (Santa Cruz), CD9 (System Biosciences), CD81 (System Biosciences), TSG101 (Abcam).

Cell lines and cell culture

A375 melanoma cells were purchased from ATCC and cultured in RPMI 1640 medium containing ultraglutamine (Lonza BioWhittaker), supplemented with 10% FCS (Foetal Calf Serum, GIBCO) and 1% PS (10'000 U/ml Penicillin and 10'000 U/ml Streptomycin, Lonza BioWhittaker) and grown at 37°C in a humidified atmosphere at 5% CO2. Cells were regularly tested to be mycoplasma free. Drug-resistant clones were generated by culturing parental A375 cells in presence of 1μM PLX4032 for 4-6 weeks. 20 different clones were picked and grown independently under constant PLX4032 treatment. A375X1 was selected for further experiments.

Microarray analysis

Total RNA was extracted with the miRNeasy kit (Qiagen) in triplicates following the manufacturer's instructions. RNA quality was further assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies). Microarray analyses were performed at the Luxembourg Institute of Health (LIH) by using the Affymetrix HuGene 2.0 ST platform. The commercial software Partek Genomic Suite was used for data pre-processing using Robust Multiarray Analysis (RMA) with GC-content correction. The log2-transformed intensities were imported into the R environment. Differential expression of genes in the drug-resistant cell line, as compared to the drug-sensitive, was determined using the R/Bioconductor package Limma, which adopts a linear modeling approach with empirical Bayesian statistics. A multiple correction was performed using the Benjamini-Hochberg's FDR (false discovery rate or

adjusted P-value). Genes with FDR<0.01 and at least 1.5-log fold change were considered differentially expressed.

5'RACE and sequencing of amplified products

5'RACE was performed according to the manufacturer's instructions using the GeneRacer™ kit (Invitrogen) and ALK specific primers binding to exon 21 and to the junction between exon 24 and 25 were designed. Reverse21: 5' AGGGGGCTTGGGTCGTTGGGCATT 3' and Reverse24-25: 5' TGTCTCGGTGGATGAAGTGGTTTTCC 3'. The final product was sequenced at GATC Biotech (Konstanz, Germany).

In addition, ALK was fully sequenced using the following primers. ALK-Forward1: 5' TGATGGAAGGCCACGGG 3'and ALK-Reverse1: 5' TCAGGCAGCGTCTTCACA 3', ALK-Forward2: 5' CCTCATTCGGGGTCTGG 3' and ALK-Reverse2: 5' CCCTTTCTATAGTAGCTCGCC 3', ALK-Forward3: 5' AACTGCCTCTTGACCTGTCC 3' and ALK-Reverse3: 5' TTTTGCCTGTTGAGAGACCA 3', ALK-Forward4: 5' GGAAGAGAAAGTGCCTGTGAG 3' and ALK-Reverse4: 5' AAGAGAAGTGAGTGTGCGACC 3'.

PCR

ALK was amplified using the following primers. ALK-CTRL-Forward21: 5' GGGGAGGTGTATGAAGGC 3' and ALK-CTRL-Reverse24: 5' CGGTGGATGAAGTGGTTTT 3'. The fusion between MMLV and ALK was amplified using MMLV-Forward: 5' CAGGCAGTGATGGAAGGC 3' and ALK-CTRL-Reverse24: 5' CGGTGGATGAAGTGGTTTT 3'.

Quantitative PCR procedure

Total RNA was extracted using the Quick-RNA™ miniprep kit (Zymo Research) according to the manufacturer's instructions and the concentration and quality was determined using a NanoDrop Spectrophotometer. 250 or 500 ng of total RNA was reverse-transcribed with the miScript II RT kit (Qiagen) in a volume of 10 µL, according to the manufacturer's instructions. Quantitative real time PCR (qPCR) was carried out on a CFX96 Detection System (BioRad) in a total volume of 10 µL (10 pmol of each primer and containing cDNA corresponding to 50 ng RNA template). The housekeeping genes PPIA, HPRT and the target genes were assayed in parallel for each sample. Melting curve analysis was performed to guarantee the specificity of

the qPCR primers as previously described [15]. The following primers were used to amplify ALK: Forward 5' GCATTGTGTCACCCACCC 3' and Reverse 5' CATGGCTTGCAGCTCCTG 3'.

ALK immunoprecipitation

ALK was precipitated from lysates of A375X1 cells. Cells were lysed in RIPA buffer and incubated with an anti-ALK antibody (Cell Signalling) overnight at 4° on an overhead shaker. The next day, lysates were incubated with protein G sepharose™ (GE Healthcare), which was previously washed with the lysis buffer for 1 hour at 4° on an overhead shaker. After three washing steps, the protein was released by heat treatment in 2x Laemmli buffer and separated by SDS-PAGE.

Small interfering RNAs and transfection

Three different ALK siRNAs were obtained from GE Dharmacon (ON-TARGETplus Human). siRNA transfections were performed using 1.5 µL Lipofectamine RNAiMAX (Invitrogen) per reaction according to the manufacturer's instructions. The final concentration of both ALK siRNA and scrambled control was 100 nM. siRNA transfections were performed 24 hours prior to 48 or 72 hours incubation with PLX4032 (1µM), Trametinib (5nM) or MK2206 (1µM).

Real-time proliferation assays

25 X 10^3 cells/well of A375X1 melanoma cells were seeded in 24-well plates and 24 hours later treated with both scrambled and ALK siRNA. Next, cells were incubated with PLX4032 (1µM), Trametinib (5nM) and MK2206 (1µM). Cellular growth was monitored in the IncuCyte ZOOM live cell microscope (Essen BioScience) and images were taken in phase contrast every 3 hours for a total of 90 hours.

Western blot analysis and antibodies

Cell lysis was performed at 4°C using ice cold lysis buffer containing 30 mM Tris/HCl pH6.7, 5% glycerol, 2.5% mercaptoethanol, and 1% SDS. Protein extracts were analysed by SDS-PAGE and Western blotting. ECL signals were detected as described before [16]. All experiments were performed in three biological replicates, of which one representative replicate is shown.

Dose-response analysis

Black 96-well plates with transparent bottom were used. In case of ALK inhibitors, 5000 cells/well of resistant A375X1 cells have been seeded in RPMI medium. In order to determine the dose response, the kinase inhibitors were serially diluted at a ratio of 1:3, starting at 10µM, in a total reaction volume of 100µl. We included a blank control (RPMI 1640 medium only), as well as an untreated control for each cell line. After 72 hours, cell viability was measured. For dose-response to vemurafenib, 3500 cells/well of resistant A375X1 cells were seeded and treated with 1µM of ALK inhibitors. Twenty-four hours after the pre-treatment, Vemurafenib was serially diluted at a ratio of 1:3, starting at 10 µM and added to the cells in a total reaction volume of 100 µl for 72 hours.

For drug resistance transfer, 1000 cells/well of sensitive A375 were seeded in RPMI medium. The day after, EVs at a concentration of 10 µg/ml were added to the cells. 24 hours later, Vemurafenib was serially diluted at a ratio of 1:3, starting at 10 µM, in a total reaction volume of 100 µl. A blank control and untreated control were included as well. Cell viability was measured 72 hours later.

For all experiments, cell viability was measured using the CyQuant proliferation assay. Fluorescence intensity was measured using the microplate reader CLARIOstar^{R} (BMG-LABTECH). The blank corrected values were exported as Microsoft Excel files and analysed. Experiment were performed in technical and biological triplicates. Dose-response curves were generated using GraphPad Prism 5.

Caspase-3 activity assay

To measure apoptosis in A375 cwlls, 10000 cells/well were seeded in black 96-well plates with a transparent bottom and treated with 1µM of single inhibitors (PLX4032 or ALK inhibitors) or combined. Cells treated with etoposide (50nM) were included as internal positive control for apoptosis. 24 hours later, cells were lysed with a lysis buffer containing dithiothreitol (6mM) and DEVD-AFC substrate (AFC: 7-amino-4-trifluoromethyl coumarin) (Alfa Aesar) for 30 minutes at 37°. Upon cleavage of the substrate by caspases, free AFC emits fluorescence, which can be quantified using a microplate reader (400nm excitation and 505nm emission). Additionally, we included a blank control (RPMI 1640 medium only), an untreated control as well as a negative control represented by cells treated with DEVD-CHO (Alfa Aesar), a synthetic tetrapeptide inhibitor for Caspase-3.

Extracellular vesicles isolation

Donor cells (both A375 and A375X1) were slowly adapted to serum-free medium (UltraCulture, Lonza BioWhittaker). Culture supernatants (100ml) were harvested, centrifuged 2x10 minutes at 400g, followed by 20 minutes at 2000g to remove cells and cell debris. Extracellular vesicles were isolated by ultracentrifugation (70 minutes at 110000g, 4°C) by using a MLA-55 fixed rotor followed by flotation on an Optiprep cushion (Axis-Shield, 17%) for 75 minutes at 100000g at 4°C using a swinging MLS-50 rotor. After a PBS wash (110000g, 70 minutes), extracellular vesicles were re-suspended in PBS and frozen at -80°C.

Extracellular vesicle labelling

To label extracellular vesicles, culture supernatants were processed as mentioned above. After ultracentrifugation at 110000g, the pellet was resuspended in 250µl of PBS and stained with 5µl of PKH67 (Sigma) for 30 minutes at 37°. To remove excess dye, this suspension was loaded on the Optiprep cushion which was followed by a PBS washing step.

Visualization of EVs

For electron microscopy, a drop of extracellular vesicles suspended in PBS was deposited on Formvar-carbon-coated electron microscopy grids. The samples were fixed with 2% PFA, labelled with anti-CD63 (Abcam) and immunogold-labelled using protein A coupled to 10nM gold (PAG10).

Preparation of the Exosome Proteome

An exosome sample containing 100 µg of protein was reduced with 10 mM DTT in the presence of 1% sodium deoxycholate (SDC) (100 mM Tris buffer, pH8) for 1 hour at 37°C. After reduction, proteins were alkylated with 25 mM iodoacetamide for 1hour at 37°C, in the dark, until quenched by 10 mM N-acetyl cysteine for 30 min, at room temperature, in the dark. Then, protein extraction was performed using a methanol/chloroform precipitation method. Briefly, 150 µL of exosome sample were mixed with 1 mL of a methanol:chloroform:water (2:1:2) solution, and vortexed before centrifugation for 5 min, at 5000 g. The upper layer of the solution was removed and 600 µL methanol were added. After vortexing followed by centrifugation for 30 min at 20000 g, the supernatant was removed to recover the protein pellet. The air-dried pellet was reconstituted for trypsin digestion in 1% SDC (100 mM Tris

buffer, pH 8.8) for 16 hrs, at 37 °C. After trypsin digestion, SDC was precipitated by acidification using 1% formic acid (FA). The white pellet formed after acidification was removed by centrifugation, for 20 min at 20000 g. The supernatant was cleaned up using a C18 cartridge (Sep-Pak®, 1cc, Waters Corporation) and dried in a speedvac concentrator (Savant SPD 111V, Thermo Fischer Scientific). The dried peptide mixture was reconstituted with 100 µL of 0.1% FA / 5% acetonitrile (ACN) for LC-MS analysis.

LC-MS/MS Analysis

The reconstituted sample was further diluted (5 times) and mixed with 7.5 fmol of a peptide-retention-time calibration mixture (Thermo Fisher Scientific) for retention time control of the data. Peptides were separated with a reverse-phase liquid chromatography (LC) system using an Ultimate 3000 RSLCnano (Thermo Fisher Scientific) equipped with an Acclaim PepMap RSLC column (15 cm × 75 µm, C18, 2 µm, 100 Å) (Thermo Fisher Scientific). 1 µL of each sample was used for proteomic analysis. Peptide elution was performed by applying a mixture of solvents A and B to the LC system. Solvent A was an aqueous solution with 0.1% FA, and solvent B was ACN with 0.1% FA. A linear gradient of 2–35% solvent B at 300 nL/min was applied over 48 min followed by a washing step (5 min at 90% solvent B) and an equilibration step (10 min at 2% solvent B). Mass spectrometry analysis was performed using a Q-Exactive Plus mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with a nano-electrospray source. For ionization, uncoated SilicaTips (12 cm, 360 µm o.d., 20 µm i.d., 10 µm tip i.d.) were used with application of 1500 V of liquid junction voltage and 250 °C of capillary temperature. For MS/MS analysis, data dependent acquisition (DDA) was employed with a top-12 mode at a resolving power of 17500 (at 200 m/z). A target automatic gain control (AGC) value of 1e6, and a maximum fill time of 60 ms were used.

Protein Identification

For protein identification of LC-MS/MS files, a protein database search was performed using the MASCOT search engine with 1% FDR. Carbamidomethylcysteine was set as fixed modification. Methionine oxidation, deamidation, and N-terminal pyroglutamate were set as variable modifications. Proteome Discoverer (version 1.4, Thermo Scientific) was used for database search and data analysis.

EV transfer experiments

For the transfer assays, 25000 cells in 24 well plates were seeded in RPMI medium. The day after, following 1 hour pre-treatment with 1µM of PLX4032, increasing concentrations of resistant EVs were added to the cells. After 7 hours, cells were collected for western blot analysis.

Immunofluorescence

For immunofluorescence, A375 or A375X1 cells grown on glass coverslips were treated with 10µg of EVs for 24 hours. Cells were washed with PBS and fixed with 4% paraformaldehyde in PBS for 10 minutes at room temperature. The coverslips were washed three times in PBS-Tween (0.05 % Tween 20). Then, cells were permeabilized with PBS 0.5% Triton X-100 for 10 minutes at room temperature, and blocked in PBS 2% bovine serum albumin (BSA) for 15 minutes. Cells were incubated with anti-ALK antibody (Cell Signalling), diluted in PBS 2% BSA, for 1 hour at room temperature. Coverslips were washed 3 times with PBS and treated with Alexa Fluor 4681 donkey anti-rabbit IgG (Invitrogen) for 1 hour at room temperature. Coverslips were washed and mounted with Gold antifade reagent with DAPI (Invitrogen). The cells were visualized by Andor Revolution Spinning Disk confocal microscopy.

Protein extraction and quantification

EV pellets were lysed in RIPA buffer (50mM Tris-HCl, 150mM NaCl, 1% sodium deoxycholate, 1% NP40) in presence of protease inhibitor cocktail (Sigma). Bovine serum albumin (BSA) diluted in RIPA buffer were prepared as protein standards (4, 2, 1, 0.5, 0.25, 0 µg/ml). Protein quantification was performed using Pierce™ BCA Protein Assay Kit (Termo Fisher) in 96 well plates according to the manufacturer's instructions. The absorbance was read at 562 nm with the microplate reader CLARIOstar^{R} (BMG-LABTECH).

Results

Characterization of vemurafenib-sensitive and -resistant A375 melanoma cells

$BRAF^{V600E}$ A375 cells were made resistant to 1μM PLX4032 over a period of six weeks with constant exposure to the drug. Twenty different resistant clones were isolated in order to investigate new mechanisms of resistance. The fastest growing clone under PLX4032 treatment, named A375X1, was selected for further experiments. The resistance of the established cell clone was examined by dose-responses analysis (Fig 1A) and by growth assays (Fig 1B) showing that resistant cells have similar growth rates under PLX4032 compared to untreated parental cells. The resistant cells also displayed an increased pAKT compared to parental cells suggesting an activation of the Pi3K/AKT pathway (Fig 1C).

To elucidate underlying mechanisms of resistance, we first performed gene expression analysis on drug sensitive and resistant A375 cells. Differentially expressed candidates emerging in the resistant versus sensitive cells (FDR<0.01, at least 1.5-log fold change) were plotted (Fig 2A). In accordance with our previous data [17], several genes were upregulated in the resistant A375X1 such as the Proprotein Convertase Subtilisin/Kexin type 2 (PCSK2), the Dopachrome Tautomerase (DCT), the Matrix Metallopeptidase 8 (MMP8) (Fig 2B). Surprisingly, we also identified the Anaplastic Lymphoma Kinase (ALK). As ALK has recently been described to be present in an oncogenic form in melanoma patients [14], we focused our attention of this gene.

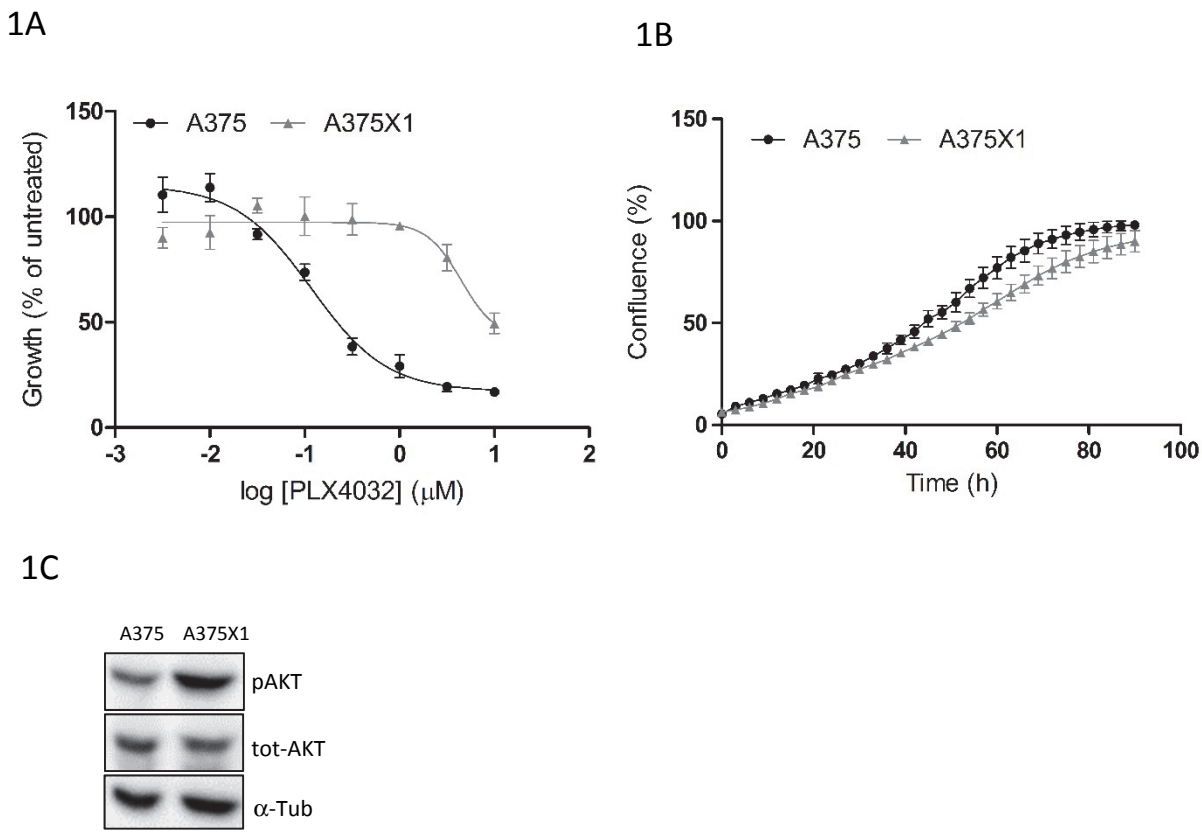


Fig 1. Characterization of resistant melanoma cells. **(A)** Vemurafenib dose-response analysis in sensitive A375 (black) and resistant A375X1 cells (grey). **(B)** Growth comparison between untreated sensitive cells versus resistant cells under constant PLX4032 treatment (1μM). **(C)** Western blot analysis of A375 and A375X1. α-Tubulin was used as loading control; representative blots of three biological replicates are shown.

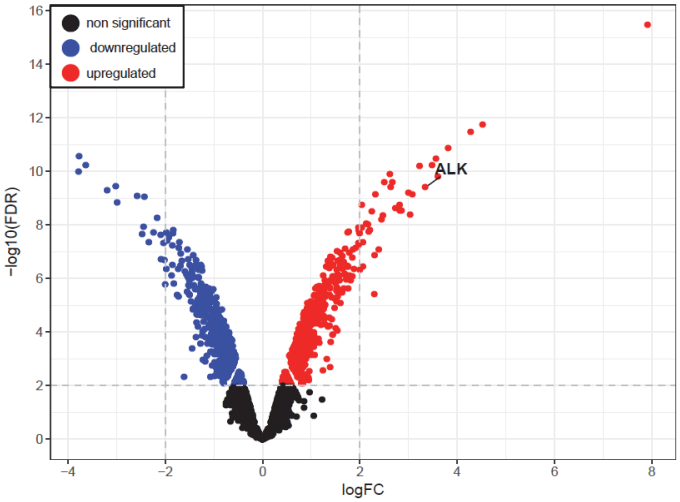
Characterization of ALK

ALK is known to be rearranged or mutated in several malignancies [13]. ALK was validated by western blot (Fig 2C), which showed the presence of a smaller protein (multiple bands around 70 KDa) compared to ALK full size (200 KDa). On the wake of the novel ALK isoform (ALK^{ATI}) identified in melanoma patients, to better characterize this protein, we performed 5'-rapid amplification of cDNA ends (5'-RACE) followed by Sanger sequencing. Results identified a truncated ALK starting from exon 18 (Fig 2D) fused to a sequence aligning to murine leukemia virus (MMLV). ALK was additionally fully sequenced confirming the presence of a transcript starting from exon 18 to exon 29 (Fig S1). PCR amplification confirmed the presence of ALK in our resistant cells. EML4-ALK positive lung cancer cells were used as positive control (Fig 2E). The amplification of this unusual fusion gave a positive signal exclusively in A375X1 (Fig 2F).

Next, several melanoma cells lines were screened for ALK, which was identified exclusively in the resistant A375X1 (Fig 2G). The ORF finder software predicted one possible ORF starting with the first methionine in the exon 18 of ALK. Furthermore, we also performed immunoprecipitation (Fig 2H) followed my mass spectrometry and we could confirm the absence of any viral protein fused to ALK. Taken together, these data suggest the existence of an unusual fusion between the C-terminus of ALK and a MMLV sequence at the mRNA level but not at the protein level. In addition, the ALK antibody detected two bands on western blots. Mass spectrometry confirmed that both correspond to ALK suggesting the possible presence of two different isoforms. Treatment of the immunoprecipitated ALK with glycosidases, especially PNGaseF, showed the presence of glycosylation residues present in the transmembrane domain (Fig 2H).

Fig 2 (on next page). Differentially expressed genes in drug-resistant A375X1 melanoma cells. **(A)** Vulcano plot showing differentially expressed genes in resistant compared to sensitive melanoma cells (FDR<0.01, at least 1.5-log fold change). **(B)** Top differentially expressed mRNAs in resistant cells. **(C)** Western blot analysis detecting ALK only in resistant A375X1 cells. α-Tubulin was used as loading control; representative blots of three biological replicates are shown. **(D)** Alignment after Sanger sequencing of the 5'-RACE-cDNA fragments confirming the starting of ALK from exon 18 (red arrow). The asterisks indicate a perfect match with the reference sequence present in the NCBI database. **(E)** PCR amplification of ALK in sensitive and resistant cells. EMLA4-ALK positive cell line was used as positive control. **(F)** PCR amplification of the fusion MMLV-ALK present exclusively in the resistant cells. **(G)** Quantitative RT-PCR of ALK mRNA in different melanoma cell lines. Error bars represent the standard deviation of three technical replicates. **(H)** ALK immunoprecipitation pulls down two different proteins, both corresponding to ALK, probably two different isoforms. The shift of the upper band after PNGaseF treatment reveals the presence of a transmembrane domain in one of the isoform.

2A



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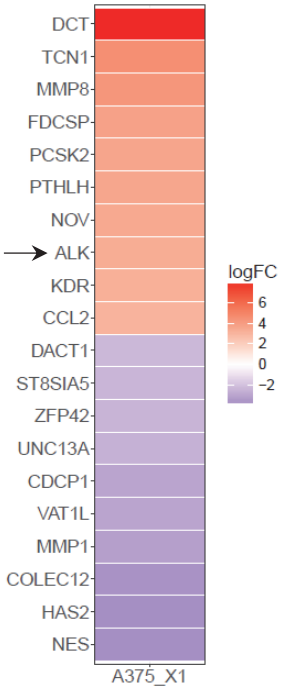

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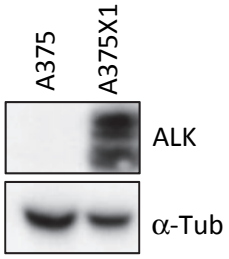

2D

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A375X1            ------------------------------------------------------------

ALK_NP_004295.2   KGIQIWKVPATDTYSISGYGAAGGKGGKNTMMRSHGVSVLGIFNLEKDDMLYILVGQQGE
A375X1            ------------------------------------------------------------

ALK_NP_004295.2   DACPSTNQLIQKVCIGENNVIEEEIRVNRSVHEWAGGGGGGGGATYVFKMKDGVPVPLII
A375X1            ----------------------------------------XGVFHLG--------X
                                                           **::

ALK_NP_004295.2   AAGGGGRAYGAKTDT--FHPERLENNSSVLGLNGNSGAAGGGGGWNDNTSLLWAGKSLQE
A375X1            RPGSGDPCPG-TTDPPPGDPGKLTAL-------IESVLITHQPTWDDCQQLLGTL--L-T
                    *.*. . * .**    .* :*          :*       *:*  .** :   *

ALK_NP_004295.2   GATGGHSCPQAMKKWGWETRGGFGGGGGGCSSGGGGGGYIGGNAASNNDPEMDGEDGVSF
A375X1            G----EE----KQRVLLEARKAVRGDDG------------------RPTQLPNEVDAAF
                  *    ..     ::   *:* .. *..*                  .  :: .* ..:*

ALK_NP_004295.2   ISPLGILYTPALKVMEGHGEVNIKHYLNCSHCEVDECHMDPESHKVICFCDHGTVLAEDG
A375X1            PLERPDWDYTTQAVMEGHGEVNIKHYLNCSHCEVDECHMDPESHKVICFCDHGTVLAEDG
                         : **************************************************

ALK_NP_004295.2   VSCIVSPTPEPHLPLSLILSVVTSALVAALVLAFSGIMIVYRRKHQELQAMQMELQS
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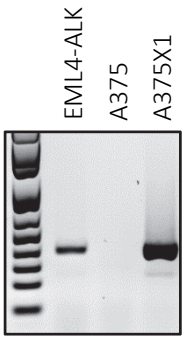
2E



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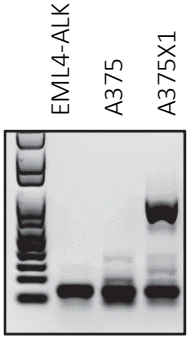

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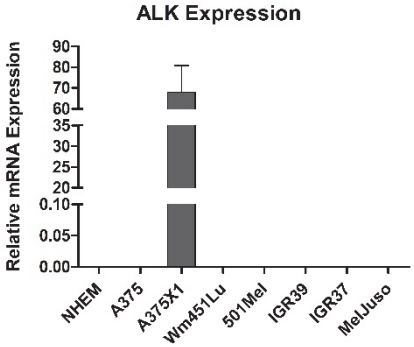
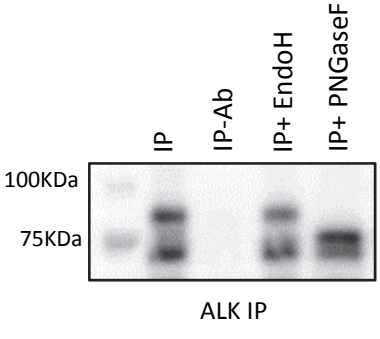


2H



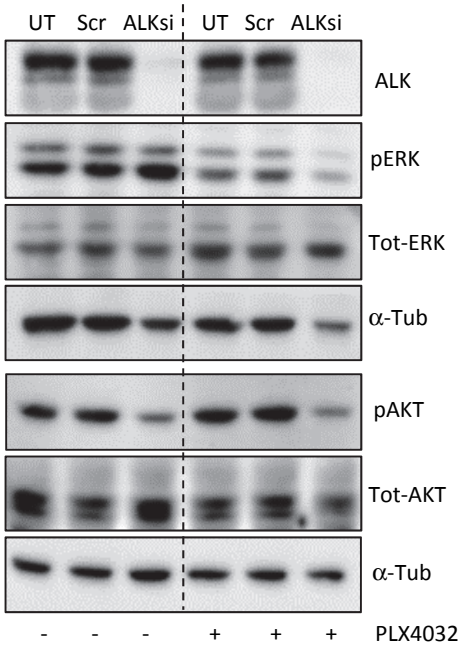
ALK can confer acquired resistance to melanoma cells

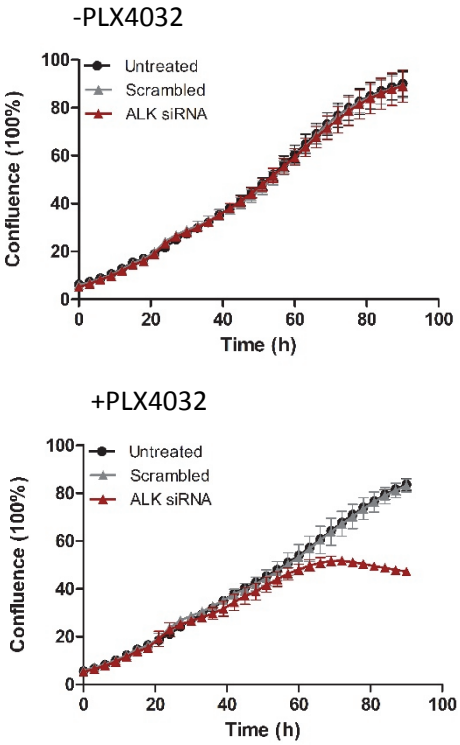
Many driving mutations have already been described in melanoma drug resistance [18]; but so far, ALK has not been implicated. Therefore, we investigated the involvement of ALK in mediating drug resistance by knocking down ALK using an siRNA approach. Western blot analysis and growth assays were performed in cells treated with both scrambled and ALK siRNA in the absence or presence of PLX4032.

Strong reduction of ALK expression levels following the siRNA treatment could be observed (Fig 3A). Following the down regulation of ALK, a decrease in pERK was detected in presence of PLX4032 while no change was observed in absence of the drug. In addition, lower levels of pAKT were detected under both conditions (Fig 3A). No change in growth behavior was observed in the absence of PLX4032 whereas growth reduction was detected when cells were treated with ALK siRNA in combination with PLX4032 (Fig 3A). Similar results were obtained when cells were treated with a MEK inhibitor (Fig 3B). Interestingly, the concomitant down regulation of ALK and pAKT did not influence growth of A375X1 cells (Fig 3A and B) suggesting that the observed growth inhibition is mostly due to the down regulation of pERK after BRAF or MEK inhibitor treatment. To confirm these observations, cells were additionally treated with ALK siRNA in combination with an AKT inhibitor (MK2206). As expected, although pAKT was reduced when cells were treated with both siRNA alone and MK2206 (Fig 3C), no effects were observed on cellular growth (Fig 3C). Altogether, these results indicate that ALK is mediating acquired resistance by activating the MAPK pathway. In the absence of ALK, cells respond again to both BRAF and MEK inhibitors.

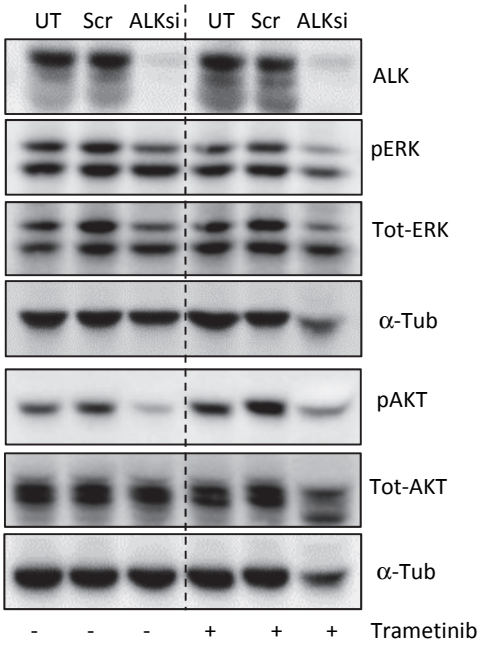
Fig 3 (on next page). Knock down of ALK re-sensitizes resistant cells to BRAF inhibition. A375X1 cells were transfected with three different siRNAs against ALK or scrambled control (100nM) for 72 hours. 48 hours prior to collection, the cells were incubated with either PLX4032 (1μM) **(A)** or Trametinib (3nM) **(B)** or MK2206 (1μM) **(C)**. α-Tubulin was used as a loading control and one representative of three biological replicates is shown. **(A-C)** Corresponding growth assays on the right. The plates were imaged every 3 hours using an IncuCyte ZOOM live cell microscope (Essen BioScience) and images were taken for a total of 90 hours. Results are shown for one representative of three biological replicates.

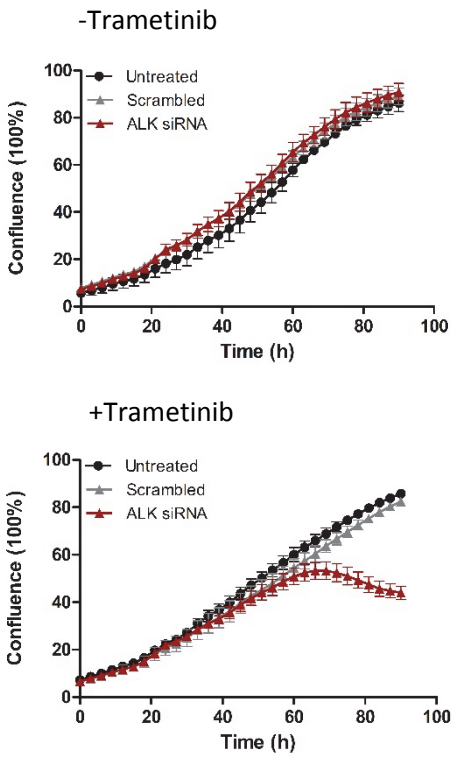
3A


UT Scr ALKsi UT Scr ALKsi
ALK
pERK
Tot-ERK
α-Tub
pAKT
Tot-AKT
α-Tub
- - - + + + PLX4032

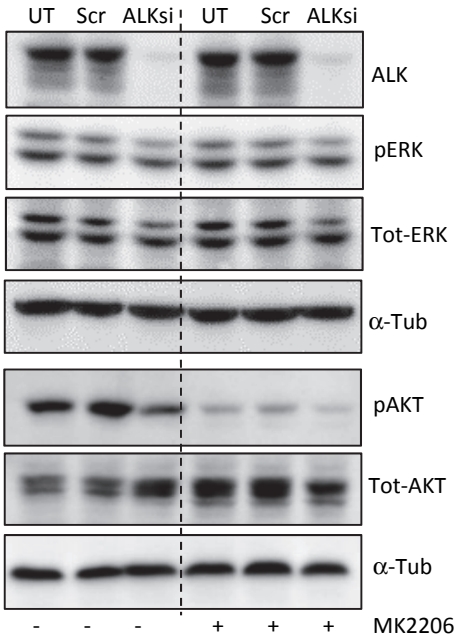

-PLX4032
Untreated
Scrambled
ALK siRNA
Confluence (100%)
Time (h)
+PLX4032
Untreated
Scrambled
ALK siRNA
Confluence (100%)
Time (h)

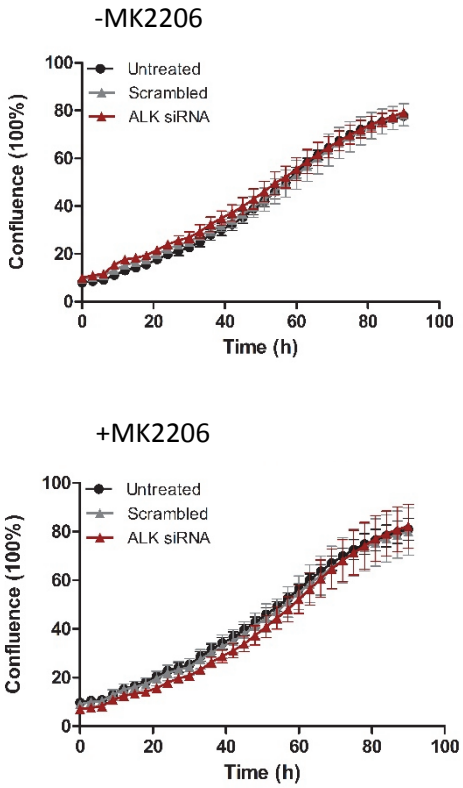
3B


UT Scr ALKsi UT Scr ALKsi
ALK
pERK
Tot-ERK
α-Tub
pAKT
Tot-AKT
α-Tub
- - - + + + Trametinib


-Trametinib
Untreated
Scrambled
ALK siRNA
Confluence (100%)
Time (h)
+Trametinib
Untreated
Scrambled
ALK siRNA
Confluence (100%)
Time (h)

3C





Combination of ALK inhibitors with Vemurafenib induces apoptosis in resistant melanoma cells

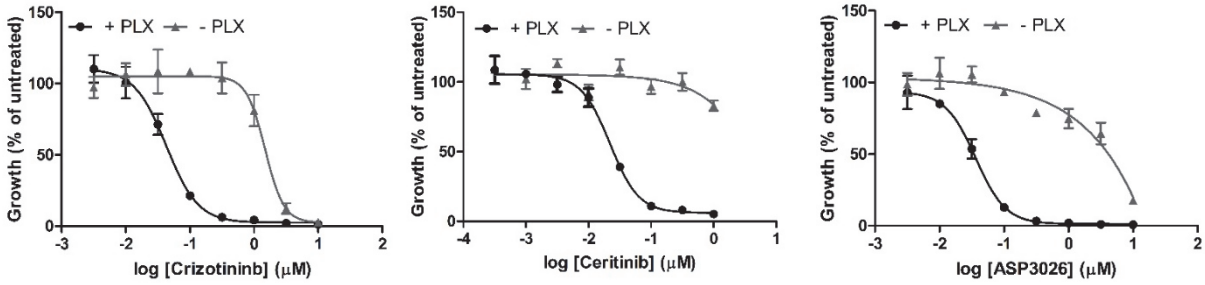
To determine whether the dependence of A375X1 melanoma cells on ALK could be exploited to overcome BRAF inhibitor resistance, we treated the cells with three different ALK inhibitors (Crizotinib, Ceritinib and ASP3026) alone or in combination with PLX4032. Dose-response analysis showed that ALK inhibitors combined with the BRAF inhibitor were much more efficient in suppressing cellular proliferation (Fig 4A) compared to single treatments. In addition and importantly, by pre-treating the cells with 1µM of ALK inhibitors, resistant cells regained sensitivity to PLX4032 (Fig 4B).

To further explore whether the combination of inhibitors was exclusively inhibiting growth or whether it could also induce cell death of resistant melanoma cells, apoptosis assays were carried out. As expected, no apoptosis was scored when the resistant cells were treated either with PLX4032 alone or with the three ALK inhibitors. However, combination treatment with both types of inhibitors induced a significant increase in apoptosis (Fig 4C).

These data clearly indicate that the combination of BRAF and ALK inhibitors could be a promising strategy to overcome drug resistance in patients carrying both $BRAF^{V600}$ and expressing ALK.

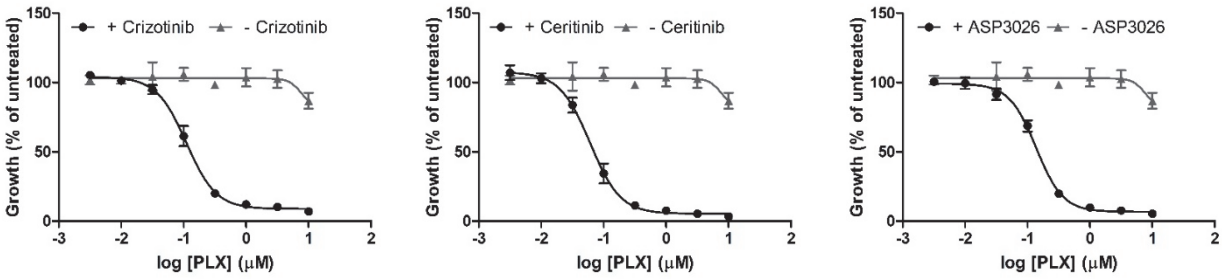
4A

ALKi IC_{50}



4B

PLX4032 IC_{50}



4C

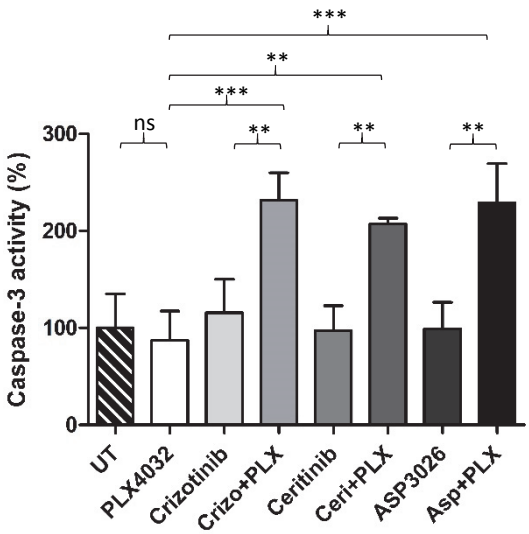


Fig 4. The combination of ALK and PLX4032 inhibitors is efficient in resistant melanoma cells. **(A)** ALK inhibitors (Crizotinib, Ceritinib and ASP3026) dose-response in resistant A375X1 cells cultured in the absence or presence of 1μM of PLX4032. **(B)** PLX4032 dose-response in

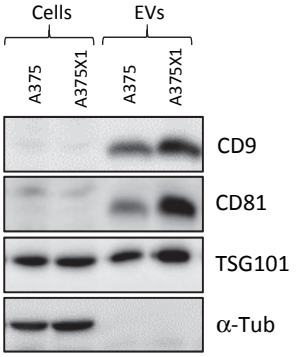
resistant cells cultured with or without 1µM of ALK inhibitors. **(C)** Apoptosis assays showing the activity of caspase-3 in resistant cells treated either with single inhibitors or with a combination of ALK and BRAF inhibitors, normalized to the untreated control. Error bars represent the standard deviation of three technical replicates of three biological replicates. Statistical significance was determined with a one-way ANOVA coupled with Tukey's multiple comparison tests. *p > 0.05, **p > 0.01, ***p > 0.001

Characterization of EVs secreted from vemurafenib-sensitive and -resistant A375 melanoma cells

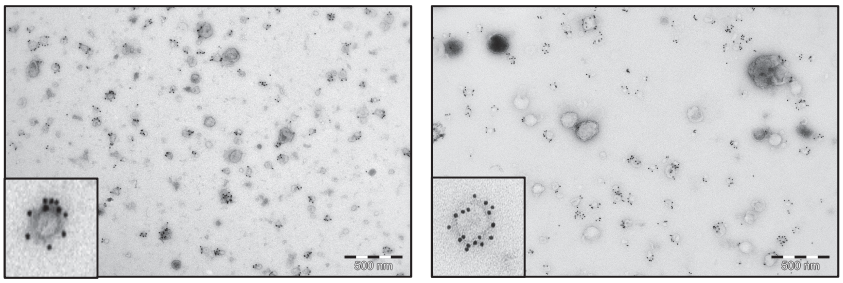
EV-mediated intercellular communication has recently been described as an important mechanism to propagate drug resistance [19]. To investigate such a potential transfer of drug resistance in our model, EVs were isolated from A375 parental and A375X1 resistant cell supernatants. The purity of isolated EVs was assessed by western blot analysis to detect the presence of generic and well known EV markers. As expected, CD9, CD81 and TSG101 were enriched in EV preparations (Fig 5A). Electron microscopic visualization of EVs revealed their characteristic and artificial cup-shaped morphology. Furthermore, immunogold labelling was positive for CD63 (Fig 5B). To study vesicle uptake by melanoma cells, purified EVs from resistant cells were labeled with a green fluorescent dye (PKH67) and incubated with sensitive A375 melanoma cells for 24 hours illustrating that sensitive A375 take up resistant EVs (Fig 5C).

Fig 5 (on next page). Characterization of extracellular vesicles isolated from both sensitive and resistant melanoma cells. **(A)** Western blot analysis of 20µg lysates from sensitive A375 and resistant A375X1 cells and corresponding EVs. Results are shown for one representative of two biological replicates. **(B)** Transmission electron microscopy pictures of CD63 immunogold labelled EVs isolated from both cell lines. Results are shown for one representative of two biological replicates. **(C)** Resistant EVs were labeled with PKH67 before OptiPrep cushion separation. A375 cells were co-cultured with 10µg of labeled EVs and fixed after 24 hours. Upper panel, untreated sensitive A375 cells; Lower panel, sensitive A375 co-cultured with "resistant EVs". Images are shown for one representative of two biological replicates. Blue: nucleus; red: actin; green: EVs

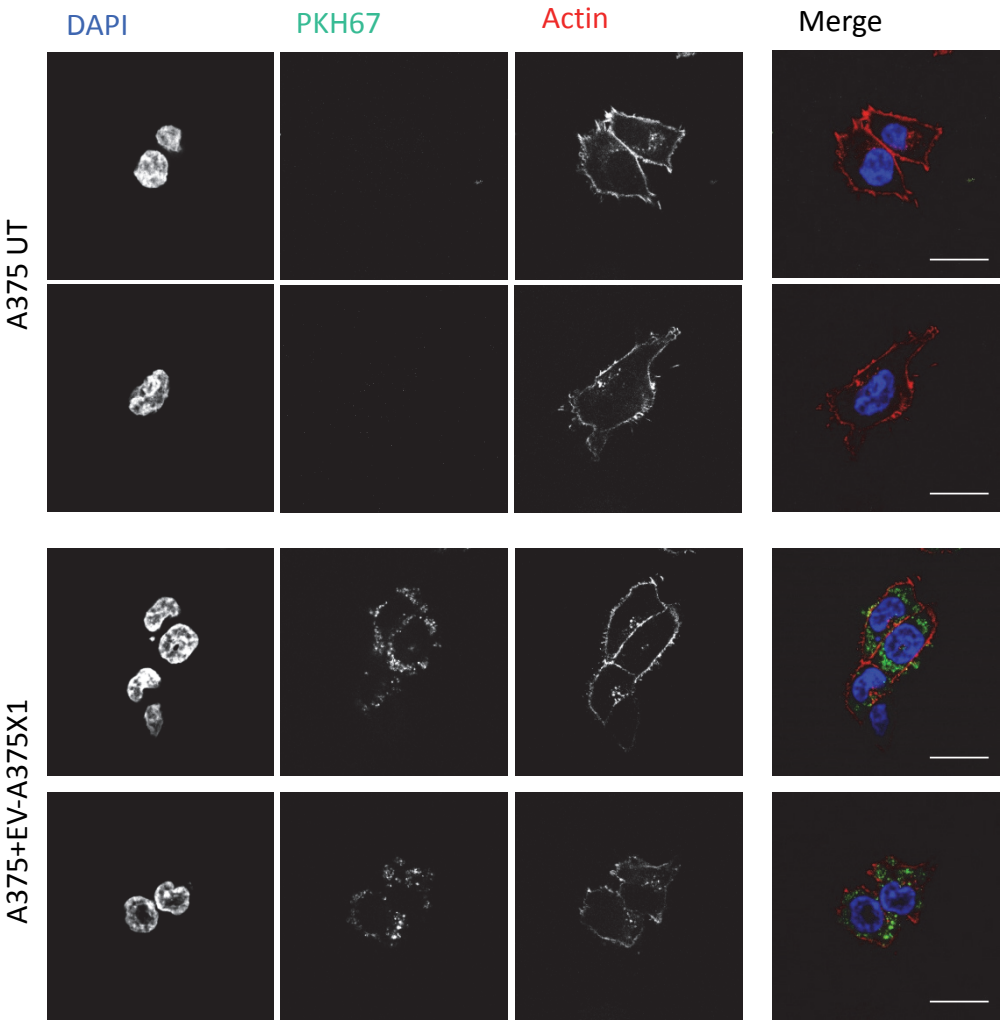
5A


Cells
EVs
A375
A375X1
A375
A375X1
CD9
CD81
TSG101
α-Tub

5B


Sensitive A375 cells

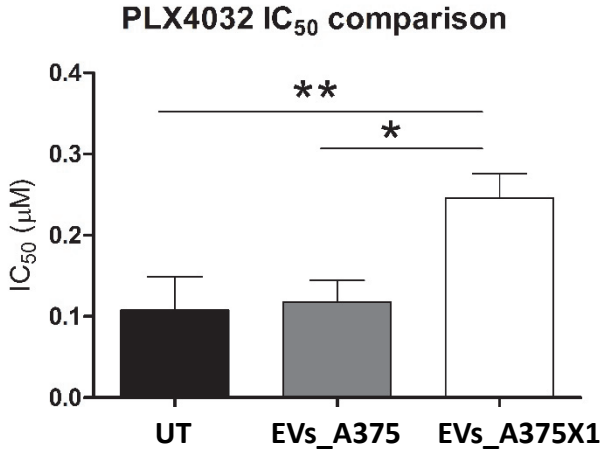
5C


DAPI
PKH67
Actin
Merge
A375 UT
A375+EV-A375X1

Drug resistance can be transmitted by EVs

To study the capability of EVs isolated from resistant cells to transfer the acquired drug resistance to sensitive ones, we first determined the dose response to PLX4032 following EV uptake (Fig 6A). 50% cell growth inhibition (IC_{50}) was calculated to assess differences in drug response between A375 cells, A375 cells pre-incubated with EVs isolated from the same A375 cells or pre-incubated with EVs isolated from resistant A375X1 cells. We did not observe any significant difference when sensitive cells were incubated with their own EVs while significantly higher IC_{50} were observed when cells were incubated with resistant-EVs (Fig 6A and 6B). Taken together, these results show that the uptake of resistant EVs makes the sensitive cells more resistant to PLX4032 suggesting a transfer of the drug resistance phenotype.

6A



6B

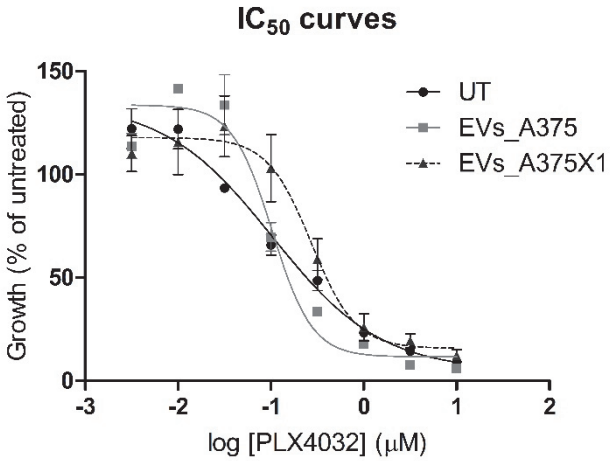


Fig 6. EVs can transfer functional properties. **(A)** Sensitive A375 melanoma cells were co-cultured with both EV-A375 and EV-A375X1 (10µg/ml). After 24 hours, Vemurafenib dose-response analysis was performed to calculate the IC_{50}. Error bars represent the standard deviation of three biological replicates. **(B)** Representative dose-response curves of sensitive A375 (black), sensitive A375 plus EV-A375 (grey) and sensitive A375 plus EV-A375X1 (dotted line). Statistical significance was determined using paired Student's t-tests. *p>0.05, **p>0.01, ***p>0.001.

Overexpressed ALK in resistant cells is present in corresponding EVs

Next, we characterized the protein content of EVs to identify potential shuttled players, including ALK, involved in transferring drug resistance. Proteomic analysis of sensitive and resistant EVs, isolated from the supernatants of the corresponding cell lines identified about 1400 proteins. Of these, 962 were common in both, 254 were unique for sensitive-EVs and 196 were unique for resistant-EV (Fig 7A). Interestingly, overexpressed ALK was exclusively detected in the resistant-EVs (Fig 7B). We confirmed its presence by western blotting in both resistant cells and the corresponding EVs (Fig 7B) while it was not detectable in sensitive cells and theirs EVs.

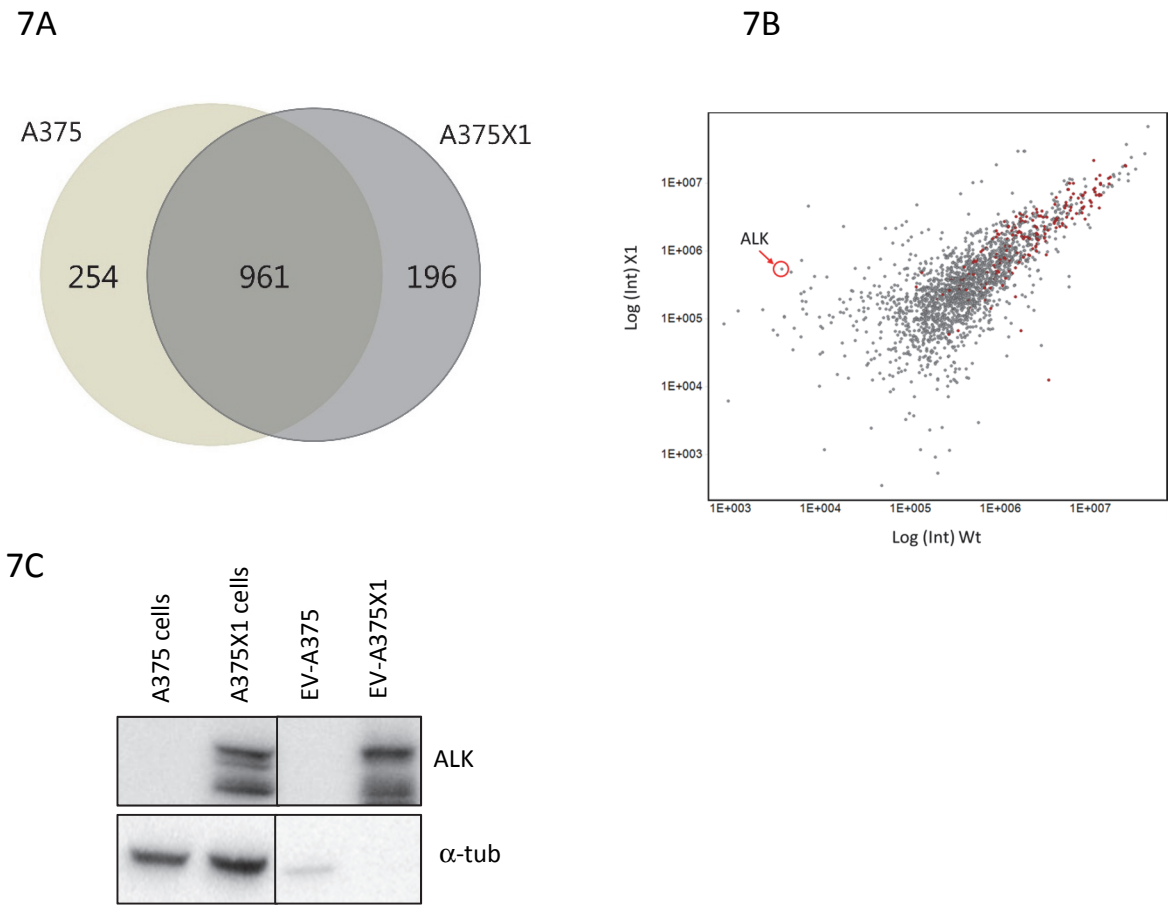


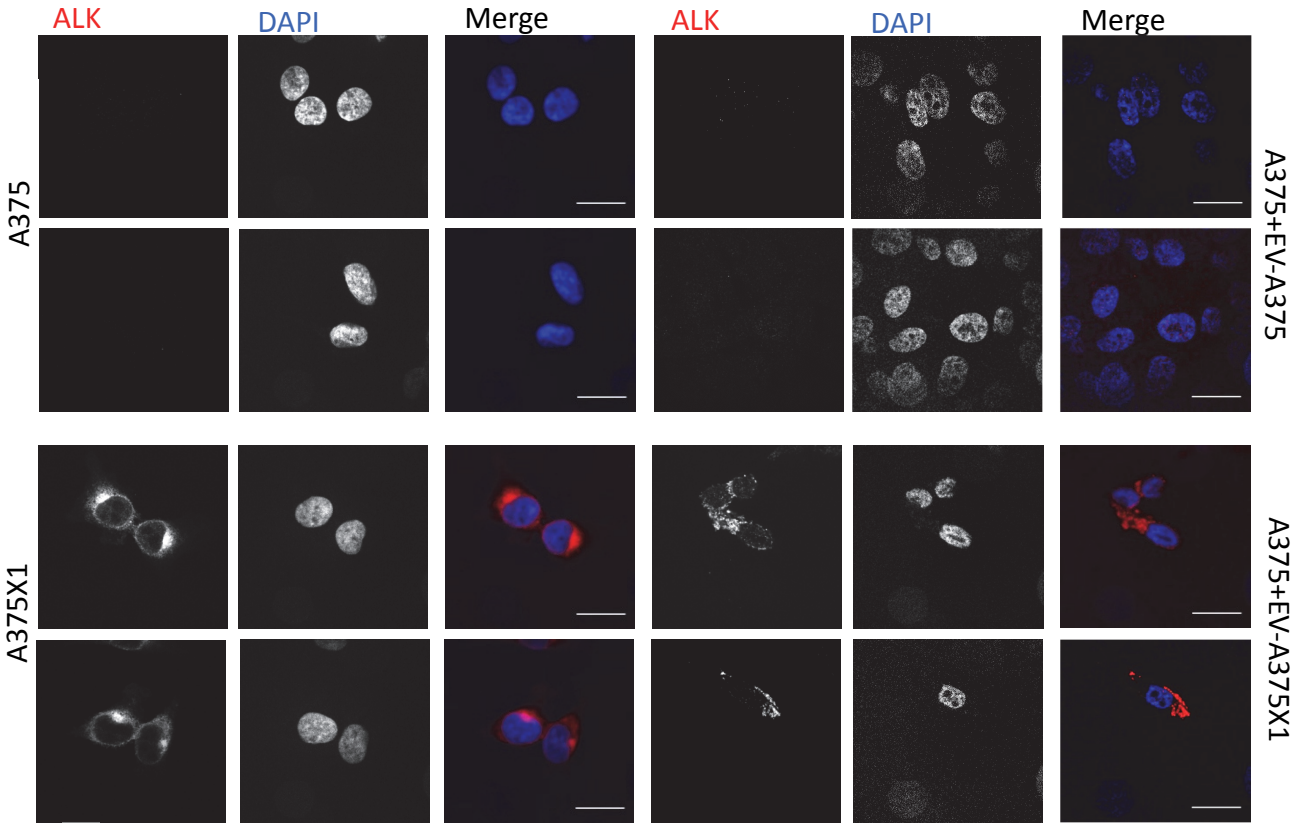
Fig 7. Proteomic profiling of EVs. **(A)** Venn diagram showing unique and shared proteins identified by mass spectrometry inEVs isolated from both sensitive A375 and resistant A375X1 cells. **(B)** Scatter plot on protein intensities identified in both populations of EVs. Anaplastic lymphoma kinase (ALK) was exclusively present in EVs isolated from resistant cells. Red dots indicate proteins listed in the top 100 proteins found in EVs according to the ExoCarta database. **(C)** ALK western blot analysis of 20µg lysates from sensitive and resistant cells and corresponding EVs.

ALK can be transferred through EVs

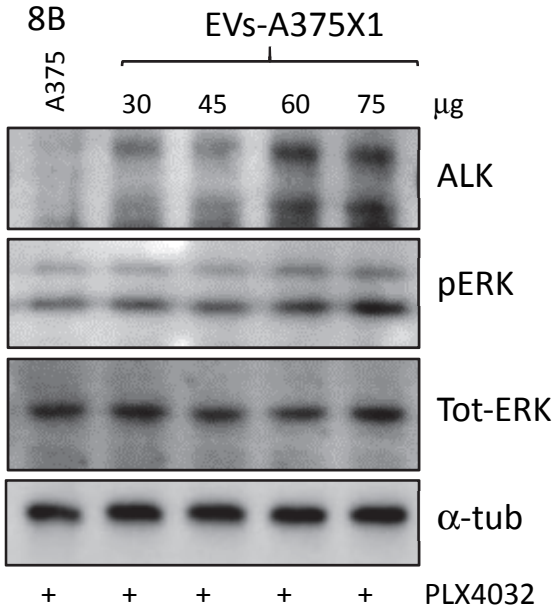
EVs are known to promote horizontal transfer of different molecules to recipient cells. However, the transfer of phenotypic traits and functional properties by EVs and their content is often difficult to establish. Given the importance of ALK in mediating drug resistance in our cells, we asked whether ALK could be transferred through EVs and remain functional in recipient cells. In this regard, confocal microscopy pictures of immunofluorescence staining for ALK showed its presence in sensitive cells after 24 hours treatment with resistant EVs (Fig 8A) suggesting the successful transfer of ALK between cells. To investigate whether transferred ALK is functional, we analysed whether the addition of ALK-containing resistant-EVs could activate the MAPK pathway. Sensitive A375 melanoma cells were initially treated with 1μM of PLX4032 to reduce their basal level of pERK. Next, increasing concentration of resistant-EVs were added to the cells over 6 hours. Increasing levels of pERK in accordance with increased concentration of EVs could be detected (Fig 8B and C) suggesting an activation of ERK by EV-transferred ALK.

Fig 8 (on next page). ALK is transferred to sensitive cells via EVs where it is functional. **(A)** Sensitive A375 melanoma cells were co-cultured with 10μg of both EV-A375 and EV-A375X1. After 24 hours, untreated A375 cells, resistant A375X1 cells and A375 co-cultured with both types of EVs were fixed and stained for ALK. Images were captured by fluorescence confocal microscopy. Representative images of two biological replicates. Scale bar, 20mm. Blue: nucleus; red: ALK. **(B)** Sensitive A375 cells were treated with 1μM of PLX4032. After 1 hour, increasing concentrations of resistant EVs were added to the cells for additional 6 hours. α-Tubulin was used as a loading control; representative blots of three biological replicates are shown. **(C)** Quantification of pERK levels, normalized to the untreated control. Error bars represent the standard deviation of three biological replicates. Statistical significance was determined using one-way ANOVA coupled with Dunnett's multiple comparisons tests. *p > 0.05, **p > 0.01, ***p > 0.001

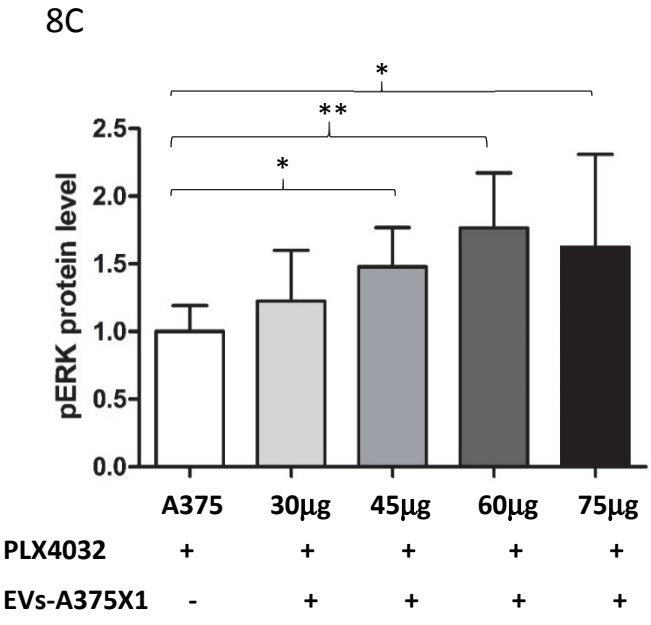
8A


ALK
DAPI
Merge
ALK
DAPI
Merge
A375
A375+EV-A375
A375X1
A375+EV-A375X1

8B


EVs-A375X1
A375
30
45
60
75
µg
ALK
pERK
Tot-ERK
α-tub
+ + + + +
PLX4032

8C


*
**
*
pERK protein level
2.5
2.0
1.5
1.0
0.5
0.0
A375
30µg
45µg
60µg
75µg
PLX4032 + + + + +
EVs-A375X1 - + + + +

In vivo experiments are planned.

Discussion

The use of serine/threonine-protein kinase BRAF inhibitors (as monotherapy) or in combination with MEK inhibitors in the treatment of BRAF-mutant melanoma is limited by both acquired and intrinsic drug resistance [11]. The re-activation of the MAPK signalling pathway following a secondary mutation is one of the key mechanisms driving acquired resistance. Nevertheless, melanoma treatments are rapidly changing and improving. Current efforts aim for an improved efficacy of the existing treatments and/or for delaying the onset of drug resistance. However, a lack of second line treatments for patients who have developed drug resistance urgently requires the introduction of new strategies. Promising new drugs that are effective in both intrinsically and acquired resistant cells and xenografts have recently been identified [20-22]. In our previous publication, we also identified a new PDK1 inhibitor AZD7545 to be effective in resistant melanoma cells [23]. A deeper understanding of the re-activation mechanisms of the MAPK pathway will aid the selection of appropriate therapies to improve survival. Importantly, different mechanisms of drug resistance can coexist simultaneously making tumours extremely heterogeneous [24]. Single tumour cell studies in melanoma patients revealed the presence of subclones, which express molecular patterns that make them less likely to respond to therapy and prone to selection during disease progression [25].

In this study, we report ALK as a new mechanism driving resistance in a subclone of BRAF-resistant cells. Translocations, mutations or amplifications make ALK oncogenic in different cancer types [13]. So far, 22 different genes have been described to be fused with the C-terminal part of ALK making the ALK locus particularly prone to activating translocations [13]. The various translocations normally produce constitutively activated ALK fusion proteins, which can signal through the MAPK signalling pathway, the PI3K/AKT pathway or the JAK/STAT pathway contributing to cell proliferation and survival [12]. ALK fusion proteins have been, for instance, identified in non-small cell lung cancer (EMLA4-ALK), diffuse large cell lymphoma (NPM-ALK) and inflammatory myofibroblastic tumour (TPM3-ALK). In addition, a new ALK transcript consisting of a fragment of intron 19 followed by exons 20–29 that resulted from an alternative transcription initiation was identified in 11% of melanoma patients [14]. In our model, we observed an activating translocation with a murine leukemia viral sequence, which

leads to a truncated protein lacking the N-terminal part (exons 1-17). We assume that this MMLV was stably inserted in our A375 cells and currently whole genome sequencing of the cells is being performed to establish the presence of the sequence in the genome. The identification of MMLV has been reported for many cancer cell lines, including melanoma, across several laboratories [26, 27] suggesting MMLV as a regular resident in cancer cells. However, the activation of ALK by a murine retrovirus suggests that other sequences from human retroviruses or their closely related human retrotransposons could activate this oncogene in human settings.

Most of the ALK variants described so far (overexpressed wild-type ALK, EML4-ALK, NPM-ALK, ALK^{ATI}, ALK^{R1275Q}, ALK^{F1174L}) were shown to trigger proliferation and tumorigenesis and to be sensitive to ALK inhibitors [14, 28-30]. To determine therapeutic responses, we tested three different ALK inhibitors: crizotinib (first-generation inhibitor) and ceritinib and ASP2036 (second-generation) in combination with BRAF inhibitor. Interestingly, both ALK knock down and ALK inhibition did not have any effect *per se* on the growth of resistant cells, only the combination with BRAF inhibition were able to suppress growth and induce apoptosis. Taken together, our data show that ALK modulates sensitivity to BRAF inhibition: the re-activation of the MAPK pathway due to the overexpression of ALK makes melanoma cells resistant to BRAF inhibitors (Fig 9A); however, the inhibition of ALK restores sensitivity to BRAF inhibition (Fig 9B). This drug combination could be of clinical relevance for patients who acquired secondary mutations within ALK or for those who carry $BRAF^{V600E}$ and overexpress ALK simultaneously and show intrinsic resistance to BRAF inhibitor monotherapy.

Interestingly, the presence of ALK in resistant cells was reproduced in the corresponding EVs, providing further evidence that circulating vesicles can be used as a diagnostic tool to monitor resistance mechanisms. Furthermore, we also showed a critical role for ALK in the transfer of drug resistance to sensitive recipient cells (Fig 9C). The transfer of drug resistance through EVs is an emerging and interesting field of research. Peinado and colleagues showed that melanoma-derived EVs could transfer the MET receptor tyrosine kinase to bone marrow progenitors [31]; Al-Nedawi et al. provided evidence that EGFRvIII can be transferred by EVS between glioma cells [32]. Here, we describe a functional transfer of a kinase being involved in the propagation of the drug resistance phenotype by EVs.

To achieve more effective and personalized treatments of melanoma, understanding the individual mechanisms of drug resistance is crucial. Our findings describe a novel mechanism

driving resistance in melanoma, which has significant consequences in the spread of the drug resistant phenotype. To the best of our knowledge, this is the first study demonstrating: i) the association of ALK with drug resistance; ii) the presence of a functional kinase within EVs, which mediates the transfer of drug resistance; iii) the transfer of drug resistance in the context of melanoma.

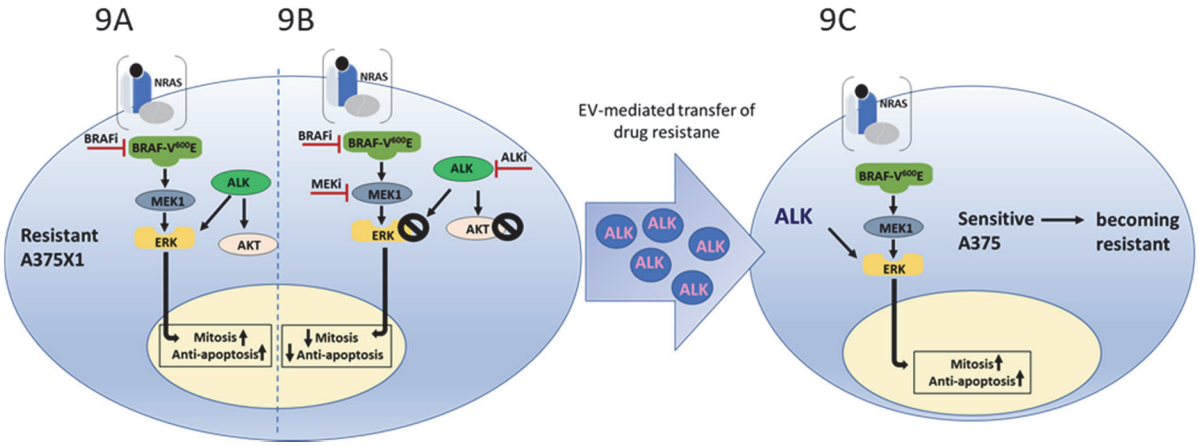


Fig 9. Model summarizing the ALK mediated drug resistance. **(A)** Despite the presence of BRAF inhibitors, A375X1 cells are resistant to it. ALK is mediating resistance, activating the MAPK signalling pathway. **(B)** By knocking down ALK or by targeting ALK with specific inhibitors, BRAF inhibitors are effective again and can shut down the MAPK pathway. **(C)** Overexpressed ALK is additionally secreted into extracellular vesicles which, in turn, can be taken-up by sensitive cells. Once in sensitive cells, ALK is functional and can activate the MAPK pathway mediating drug resistance transfer.

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Figure S1

Fig S1. ALK sequencing results. The protein sequence of ALK expressed in A375X1 cells (blue) was aligned to the NCBI Reference Sequence (NM_004304.4). The amino acidic substitution at position 489 is reported as a SNP (https://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?geneId=238).

4.3.3 Additional results

Mutations in ALK and BRAF can co-exist

In the manuscript in preparation, we identified ALK as a novel mechanism of resistance and we showed that the double treatment with ALK and BRAF inhibitors is effective *in vitro*. As previously mentioned, a truncated form of ALK has been identified in 11% of melanoma patients (Wiesner et al., 2015). Moreover, ALK can also be activated by somatic mutations (Hallberg and Palmer, 2013). To broaden the scope of our findings and to better understand if the proposed drug combination could be of clinical relevance for patients, we looked for ALK somatic mutations in melanoma patients. Especially, we wanted to determine the simultaneous presence of mutations in ALK and BRAF in melanoma patients. 467 melanoma patients present in the TCGA database were screened and 17 patients were found to have somatic missense mutations in both ALK and BRAF (Figure 19). Although none of the identified mutations in ALK seem to occur in the kinase domain, they might still activate the protein. This observation requires further investigation, nevertheless it suggests that pharmacological inhibition of ALK combined with BRAF inhibitors might represent an interesting therapeutic opportunity for a subset of melanoma patients.

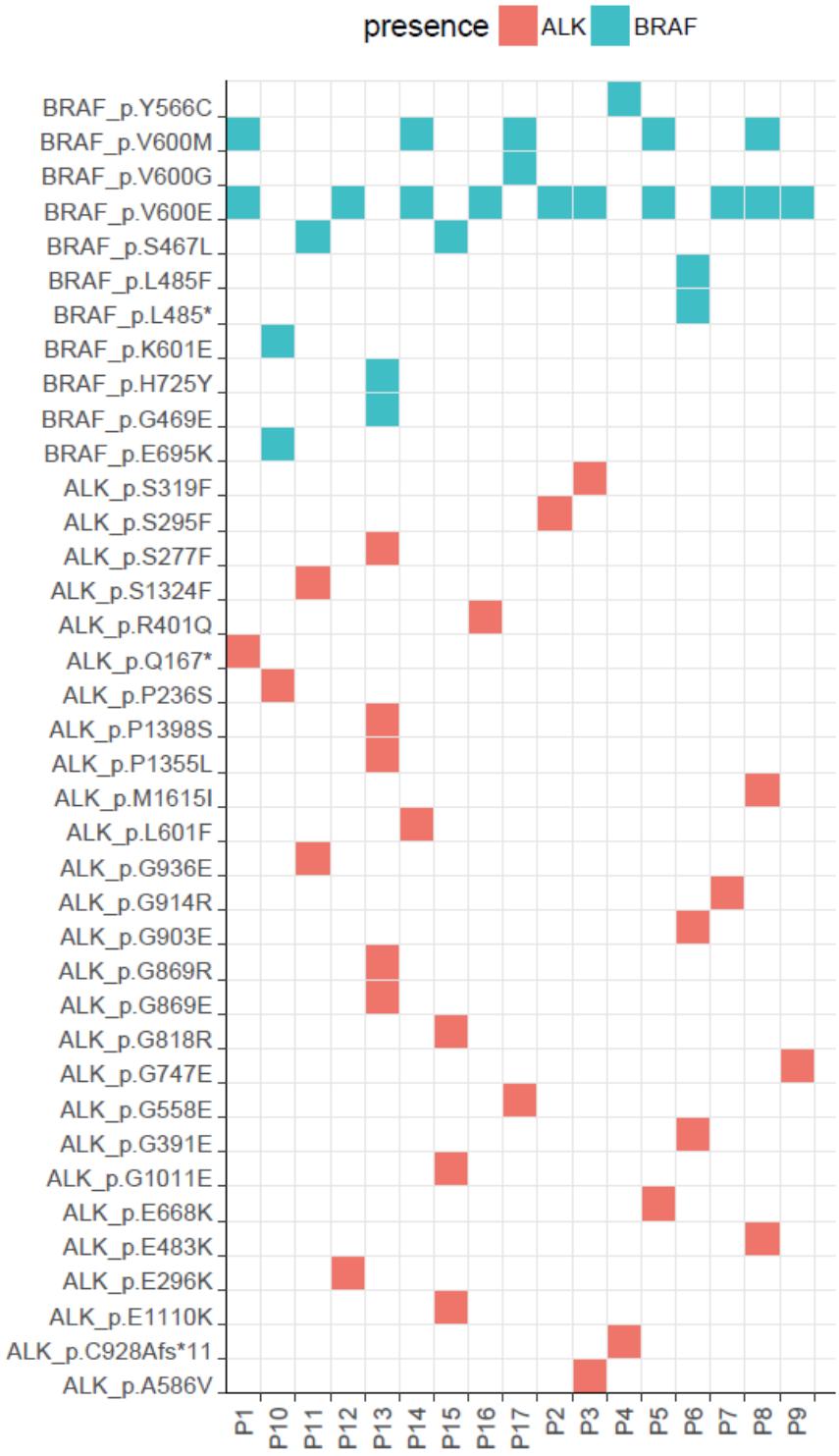


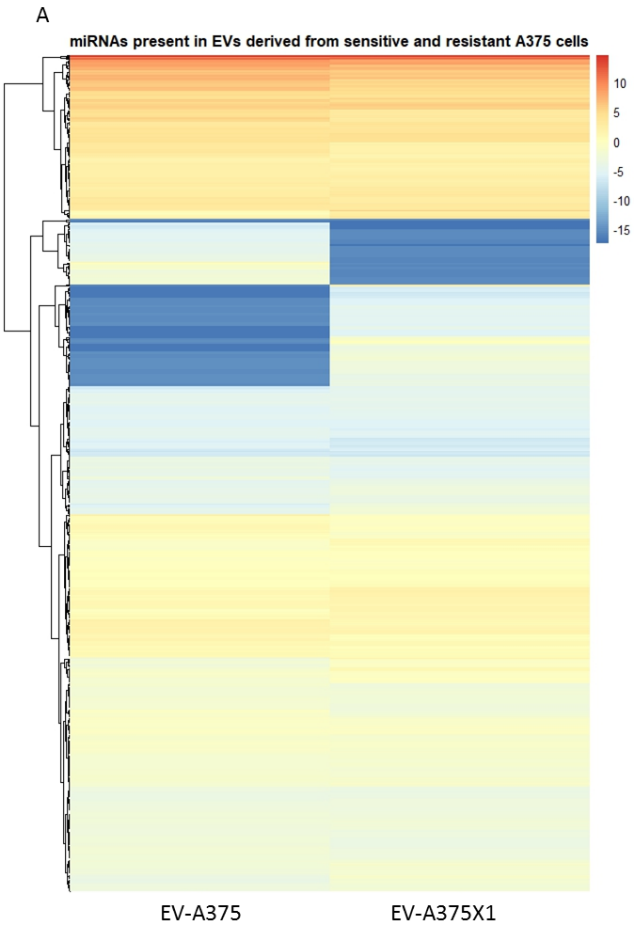
Figure 19. Somatic missense mutations present in both ALK and BRAF genes in melanoma patients.

Table made using R, R studio and ggplot2 package.

Comparison of miRNAs secreted in sensitive versus resistant EVs

As previously mentioned, EVs contain miRNAs. Apart from monitoring the protein content, we also analyzed miRNAs present in both sensitive and resistant EV populations in order to find potential players in drug resistance. The miRNA content of EVs was analyzed by qPCR arrays (method described on page 34). The heatmap in Figure 20A shows an overview of whole miRNome data sets for sensitive and resistant melanoma EVs. The two EV populations have remarkably similar expression patterns with only few exceptions. The Figure 20B shows the top-upregulated miRNA in resistant EVs compared to sensitive. Interestingly, the most upregulated miRNA was let-7g. This miRNA belongs to the let7 family which has been described to act as crucial tumour suppressor able to inhibit diverse oncogenes such as RAS (reviewed in Lee et al., 2016). It is tempting to speculate that resistant cells selectively export this miRNA out. Moreover, miRNA-509-3p was also identified in the resistant EVs. This miRNA was previously identified in several resistant cell lines (Kozar et al, 2017) suggesting its potential use as diagnostic resistance biomarker.

In this context, further experiments are planned to investigate the functional involvement of such miRNAs in the onset of drug resistance and in the transfer of it.



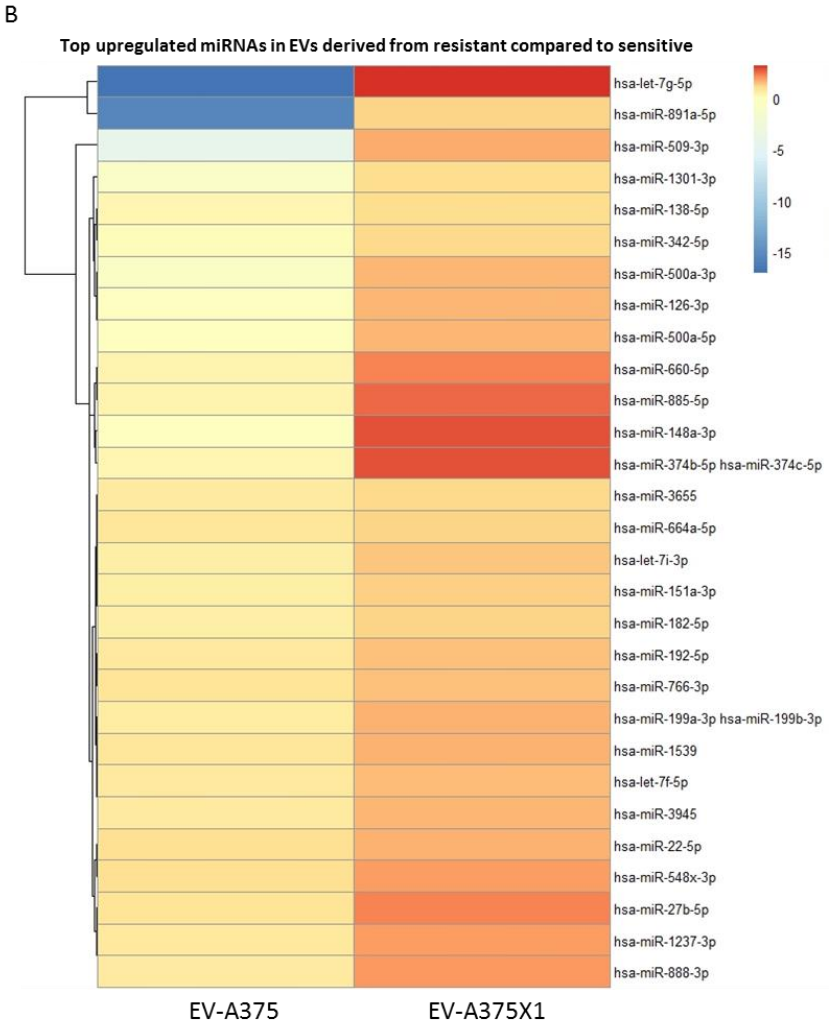


Figure 20. miRNA profiling of EVs.

A) Heatmap of whole miRNome qPCR array data of the two EV populations normalized by global plate mean (miRNAs absent across all samples are not depicted). B) Top upregulated miRNAs in resistant EVs.

MiRNA microarray analysis after EV treatment

In order to study the EV-mediated transfer of miRNAs, microarray analysis was performed on drug-sensitive A375, drug-resistant A375X1, A375 co-cultured for 48 hours with 30μg of EVs isolated from sensitive A375 and with EVs isolated from resistant A375X1. The number of differentially expressed candidates (FDR < 0.01, 1-log fold change) in the resistant versus sensitive cells and in sensitive cells co-cultured with resistant EVs versus cells co-cultured with sensitive EVs were examined. Interestingly, we could confirm the presence of miR-509-3p in resistant cells which was

significantly increased (~3 log fold change) compared to sensitive cells (Kozar et al., 2017) (Figure 21). However, when we compared the sensitive cells treated with the different populations of EVs, we could not identify any differentially expressed miRNAs, therefore no heatmap could be generated. Although some miRNAs were enriched in resistant-EVs by qPCR arrays (Figure 20), microarrays analysis revealed that there was no difference between the treatment. While this finding requires further confirmation and validation, it nevertheless provides a first indication that the quantity of EV-transferrable miRNAs might not be sufficient to possibly detect differences.

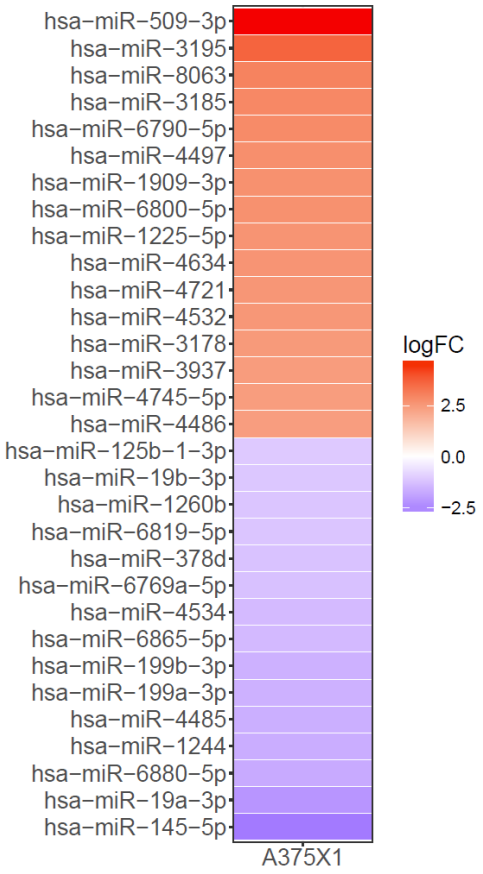


Figure 21. Top differentially expressed miRNAs.

Differentially expressed miRNAs (FDR < 0.01 and 1 log fold change) in vemurafenib X1-resistant cells compared to sensitive A375.

4.4 Transferring intercellular signals and traits between cancer cells: extracellular vesicles as “homing pigeons” (Review)

4.4.1 Preamble

The topic of extracellular vesicles was new in our laboratory. Given the emerging evidence on the involvement and importance of EVs in several aspects of cancer biology, especially intercellular communication, we began with a thorough literature review on the topic. In addition, we wanted to gain some expertise in the field of EVs in order to study their content and investigate their potential ability to “ship” ALK to ALK-negative cells and mediate drug resistance.

Therefore, in this review, we summarized findings on the involvement of EVs in transferring traits of cancer cells to their surroundings. As mentioned already, a pressing issue in cancer treatment is the onset of resistance to many initially efficient drug therapies. Here, we review studies investigating the role of EVs in this phenomenon together with a summary of the technical challenges that this new field is still facing. Finally, emerging areas of research such as oncosomes (a larger type of vesicle), the analysis of the lipid composition on EVs, and cutting-edge techniques to visualize the trafficking of these small vesicles are discussed.

Authors' contributions are at the end of the article.

Cesi *et al. Cell Communication and Signaling* (2016) 14:13
DOI 10.1186/s12964-016-0136-z

Cell Communication and Signaling

REVIEW Open Access

Transferring intercellular signals and traits between cancer cells: extracellular vesicles as "homing pigeons"

Giulia Cesi, Geoffroy Walbrecq, Christiane Margue and Stephanie Kreis*

Abstract

Extracellular vesicles are cell-derived vesicles, which can transport various cargos out of cells. From their cell of origin, the content molecules (proteins, non-coding RNAs including miRNAs, DNA and others) can be delivered to neighboring or distant cells and as such extracellular vesicles can be regarded as vehicles of intercellular communication or "homing pigeons". Extracellular vesicle shuttling is able to actively modulate the tumor microenvironment and can partake in tumor dissemination. In various diseases, including cancer, levels of extracellular vesicle secretion are altered resulting in different amounts and/or profiles of detectable vesicular cargo molecules and these distinct content profiles are currently being evaluated as biomarkers. Apart from their potential as blood-derived containers of specific biomarkers, the transfer of extracellular vesicles to surrounding cells also appears to be involved in the propagation of phenotypic traits. These interesting properties have put extracellular vesicles into the focus of many recent studies.

Here we review findings on the involvement of extracellular vesicles in transferring traits of cancer cells to their surroundings and briefly discuss new data on oncosomes, a larger type of vesicle. A pressing issue in cancer treatment is rapidly evolving resistance to many initially efficient drug therapies. Studies investigating the role of extracellular vesicles in this phenomenon together with a summary of the technical challenges that this field is still facing, are also presented. Finally, emerging areas of research such as the analysis of the lipid composition on extracellular vesicles and cutting-edge techniques to visualise the trafficking of extracellular vesicles are discussed.

Keywords: Extracellular vesicles, Intercellular communication, Imaging, Cancer, Drug resistance

Background

According to an advanced PubMed search, "exosome" has become the most cited term in publications describing any kind of vesicle [1]. However, the term exosome only refers to vesicles generated by the inward budding of the endosomal compartments, of which several are forming so-called multivesicular bodies (MVBs). Many MVBs fuse with lysosomes whereas others may fuse with the plasma membrane, resulting in secretion of their intraluminal vesicles [2]. Exosomes are generally distinguished from microvesicles by size and by origin: exosomes are ~30 -120 nm in size, with an endosomal origin whereas microvesicles are >100-1000 nm and originate from the plasma membrane (Fig. 1a). Current purification methods unfortunately do not allow to precisely discriminate between the two populations as it is very likely that microvesicles with a size of 30–120 nm exist as well. Protein aggregates and lipoproteins might also contaminate and confound the sample preparation. Furthermore, once the vesicles have been released, their origin cannot be identified as unique markers for the different vesicle types have not been defined yet [3–5]. Because of the difficulty to specifically discriminate exosomes from other circulating vesicles, the International Society of Extracellular Vesicles suggested to use the generic term "extracellular vesicles (EVs)" to describe vesicles isolated from the extracellular milieu [1]. For clarity, in this review, the term exosome mentioned in the majority of the cited publications, will be replaced by the term "extracellular vesicles" to be in accordance with the new guidelines.

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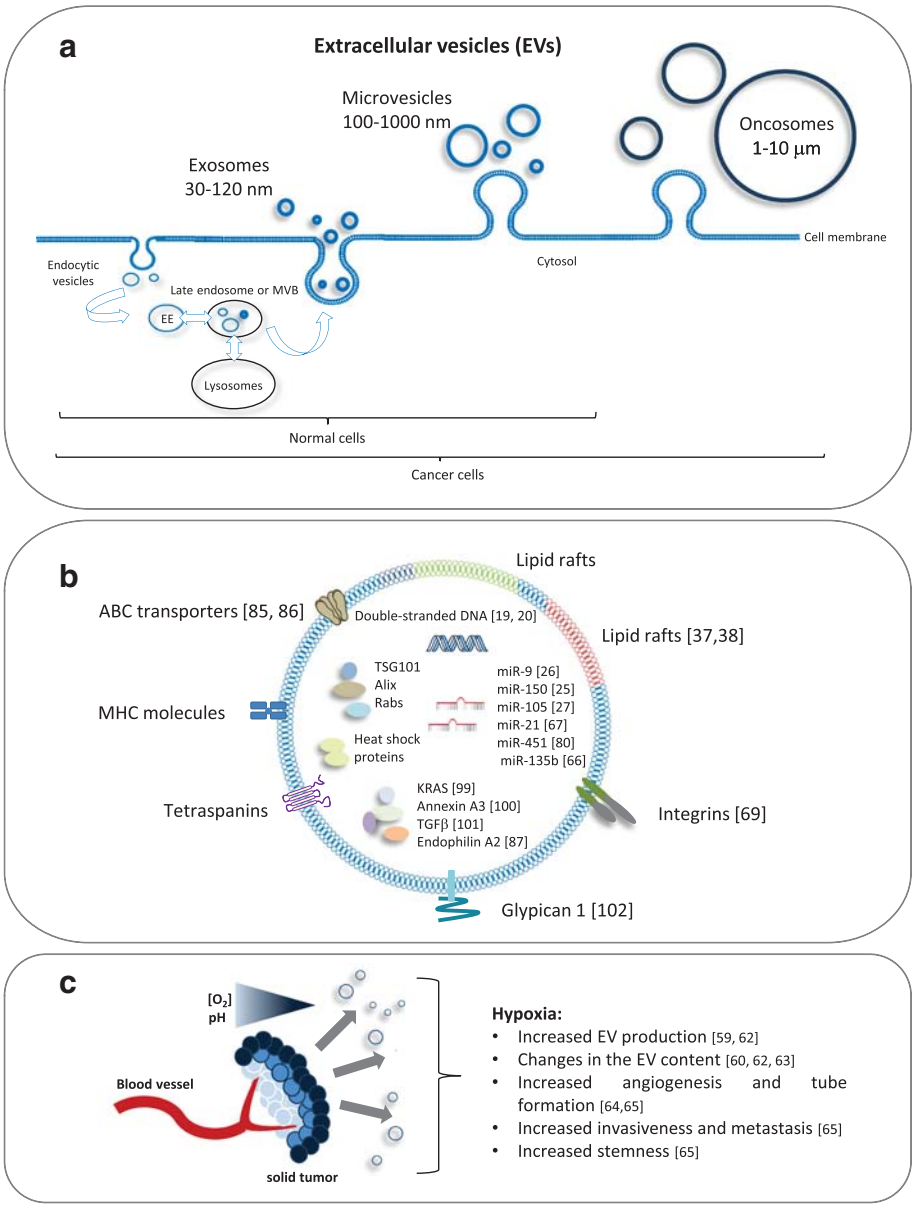

Fig. 1 a EV repertoire. Cells secrete distinct sub-populations of EVs and although of different origins, they overlap in size or density and often co-purify. Exosomes are vesicles generated by the inward budding of the endosomal compartments (endocytic vesicles), which become early endosomes (EE), several of which are forming so-called multivesicular bodies (MVBs). MVBs either fuse with lysosomes or with the plasma membrane, which results in their secretion. In addition, cancer cells can produce larger vesicles named "large oncosomes". Together with exosomes and microvesicles, oncosomes contain abundant bioactive molecules, which can transfer cancer traits or be used as biomarkers. **b** Relative to the general EV content of normal cells (tetraspanins, MHC molecules, proteins involved in the MVB biogenesis, heat shock proteins), cancer EVs are often enriched in specific miRNAs or proteins. Furthermore, the membrane of cancer EVs is characterised by specific lipid species localised in lipid rafts. **c** Cancer cells secrete more EVs than the corresponding healthy cells. Acidic pH and hypoxia, which often characterise the tumor microenvironment, stimulate an increased secretion of EVs and influence the EV content, which in turn supports angiogenesis and metastasis. Additional references not previsouly cited in the text: KRAS [99], Annexin A3 [100], TGFβ [101], Glypican 1 [102]

Although various isolation methods exist, differential ultracentrifugation followed by density gradient separation, electron microscopy together with protein composition analysis is generally considered the best so far available workflow to isolate and characterise EVs [6]. Nevertheless, some studies where EVs were isolated by using the less accepted "precipitation method" are also cited here, in order to provide a complete overview of the different topics covered in this review.

EVs were initially thought to be an expedient for cells to remove unwanted components [7, 8]. However, recent findings have shown that these nano-vesicles are surely

more than just garbage bins. As EVs can be released by "donor" cells and taken up by "recipient" cells, it has been hypothesised that these vesicles broaden the cells' repertoire to communicate and exchange signals. Several studies have already confirmed that EVs are important players in influencing both physiological and pathological conditions by delivering molecules such as cytokines, growth factors, proteins, mRNAs, miRNAs and other non-coding RNAs to other tissues and cells [3]. The discovery that EVs have a wide range of regulatory functions and carry various endogenous cellular components made them the most studied vesicles in recent years [9] and several reviews covering different aspects of extracellular vesicle biology and function have been published [9–13].

The current review focuses on new developments in EV characterisation (lipid profiling of cancer-derived EVs) and visualisation (imaging of EV traffic) as well as on the potential involvement of EVs in propagating tumorigenic properties, in particular drug resistance. Finally, technical limitations that impede a full understanding of EV biology and functions will be summarised.

Characterisation of EVs

Cargo and composition of EVs

The content profiles of EVs depend mainly on their parental cells. The ExoCarta database (www.exocarta.org) provides information about the EV content in different organisms and cell types. Generally, EVs from different cell types contain endosome-associated **proteins** (e.g. Rab GTPase, SNAREs, Annexins, and flotillins), some of which are involved in the biogenesis of MVBs (e.g. Alix and Tsg101) [14]. Membrane proteins including tetraspanins (e.g. CD63, CD81, CD82, CD53, CD37 and CD9), heat shock proteins, MHC complexes, growth factors and many others are also present [15]. How exactly proteins are sorted into EVs is still under investigation. The role of ubiquitination seems controversial: in most cases, ubiquitination targets the proteins destined for degradation (upon fusion of the MVB with lysosomes) while proteins to be exported show no ubiquitination [16]. In some other cases, EV proteins appear to be highly ubiquitinated [17]. EVs have also been shown to contain single-stranded **DNA** and transposable elements [18] as well as double-stranded genomic DNA which might reflect the mutational status of the parental tumor cell [19, 20].

The **RNA** content of EVs is enriched in small non-coding RNAs including **miRNAs** [2]. Although the sorting mechanisms are not fully understood, recent evidence suggests that the composition of EV miRNAs differs from the one of the cell of origin suggesting a selective sorting of miRNAs into EVs. In this context, Villarroya-Beltri et al. [21] identified the presence of unique sequence motifs that could prone miRNAs for sorting into EVs or for intracellular localisation. They also demonstrated that the sumoylated heterogeneous nuclear ribonucleoprotein A2B1 binds miRNAs through their "EXO-motifs" and controls their loading into EVs, thus providing an explanation for the specific packing of certain miRNAs into EVs. In contrast, interesting findings from Squadrito et al. [22] suggest a passive mechanism for miRNA export modulated by cell activation-dependent changes of miRNA target levels: EV miRNA secretion might be a mechanism by which cells remove miRNAs in excess of their corresponding targets to re-establish miRNA/mRNA homeostasis. More recently McKenzie and collegues identified Ago2 protein as a possible major player in miRNA sorting. Indeed, they demonstrated that phosphorylation of Ago2 promoted by KRAS suppressed its secretion into EVs and thereby the sorting of specific miRNAs [23].

The presence of regulatory miRNAs within EVs has raised a strong interest ever since Valadi et al. [24] showed for the first time that miRNAs in mast cell-derived EVs can be transferred to other mast cells and be functional. Since then, fascinating examples of intercellular communication via miRNAs between cells in culture have been provided [25–28]. Albeit accumulating evidence for the importance of miRNAs in EVs, it remains uncertain whether such miRNAs are really functional in a physiological environment and whether the concentration of secreted individual miRNAs would be sufficient to mediate measurable endocrine effects. Furthermore, it is still unclear how widely this process occurs *in vivo* and whether it is restricted to certain cell types, physiological conditions or diseases or whether it is a ubiquitous way of cell-to-cell communication. For Williams et al. [29] the concentration of miRNAs in biological fluids is significantly lower than in the surrounding cells and might be below the threshold for triggering any significant function *in vivo*. The work of Chevillet et al. [30] argues along these lines. By using a stochiometric approach, they performed quantitative assessments of miRNAs within EVs isolated from five different sources. Less than one copy of a given miRNA per EV was observed by absolute quantification through real time PCR. These data would suggest that standard EV preparations might not carry biologically significant numbers of miRNAs. In accordance with this, we made a similar observation. After successful transfer of detectable levels of miR-211-5p via EVs isolated from melanoma patient serum samples to miR-211-5p-negative melanoma recipient cells, we could not detect any down-regulation of previously confirmed target genes (unpublished data). Interestingly, in the same cellular model 5 nM of miR-211 mimic was able to effectively down-regulate those target genes (RAB22A, AP1S2, M6PR) [31] suggesting that amounts of transferred miRNAs isolated from patient sera were not sufficient to evoke downstream effects. Moreover and apart from quantities, other factors play a role: it is still difficult to clearly discriminate between secreted miRNAs indicative

of malignant processes from "contaminating" miRNAs derived from platelets, erythrocytes, lymphocytes or normal cell death [32]. Among all the components of EVs, miRNAs are one of the most controversial but also interesting players in intercellular signaling and tumor progression and their potential involvement in acquisition and transfer of cancer cell resistance to drug treatments is discussed in more detail below.

EVs carry **lipids** of a similar composition as found in the plasma membrane of the parental cells (such as cholesterol, ceramide and sphingomyelin) [2]. An emerging field in vesicle research and more specifically in the context of cancer, is "**lipidomics**" which, apart from general lipid profiling, also studies alterations in lipid compositions. Changes in lipid metabolism and in particular activation of *de novo* lipogenesis have already been described for several cancers [33–35]. Recently, Marien and colleagues identified a distinct lipid signature in non-small cell lung cancer. By using a mass spectrometry-based phospho-lipidomics approach, the authors identified 91 phospholipid species differentially expressed in cancer versus normal tissues [36]. The distinct lipid composition of EVs coupled with the capability of EVs to travel in biological fluids, puts lipid profiling on the list for novel biomarker discovery. Interestingly, an enrichment in certain lipid species in the membrane of EVs has been reported in several publications. In this context, Llorente et al. [37] observed a specific sorting of lipids into EVs compared to the secreting cells. Lipid composition analysis of metastatic prostate cancer cells and corresponding EVs revealed an enrichment in glycosphingolipids, cholesterol, sphingomyelin and phosphatidylserine in EVs compared to parental cells. However, the authors did not compare the lipid composition of these EVs to those released from normal prostate cells. The enrichment of specific lipids within the membrane of EVs has also been described in colorectal cancer cells [38]. Furthermore, Schlaepfer and colleagues observed that hypoxia triggered triglyceride accumulation in prostate cancer cells and corresponding EVs due to the activation of lipogenesis-related enzymes [39]. Overall, lipidomics of EVs has gained attention in recent years but to this day, it remains controversial which lipids are involved in EV-mediated cell-to-cell communication [40], also because it is a challenge to produce pure EV preparations and to avoid cellular lipoparticle contaminations, potentially leading to misinterpretations. Nevertheless, standardised and well-controlled lipid profiling of EV membranes might be useful for the identification of new biomarkers and for a better understanding of the biology of EV secretion.

Visualisation of EVs and EV traffic

The most common methods used to detect and characterise EVs are electron microscopy (EM), dynamic light scattering (DLS), nanoparticle tracking analysis (NTA), fluorescence microscopy and flow cytometry (FCM). Two standard methods are used to assess the quality of the EV preparation: EM and either DLS or NTA. EM has the advantage that it provides the highest resolution compared to the other methods. In addition, EM combined with immuno-gold labeling allows for recognition of protein markers on the surface of EVs. DLS and NTA both measure the size of particles using Brownian molecular movement but NTA has, additionally, a camera documenting the movement and light scattering of the samples [41]. Unlike previous methods, which only enable physical characterisation of EVs in fixed samples, fluorescence microscopy visualises labelled EVs in live cell conditions/assays. Several fluorescent membrane dyes are used to label purified EVs such as the PKH-67 (green) or PKH-26 (red) linker dyes. One disadvantage of the labelling dyes is their long half-life *in vivo* (from 5 to >100 days), which hinders the dynamic tracking of EVs *in vivo* [42].

An alternative to labelling the membranes of EVs is to link their protein content to TAMRA-NHS (carboxytetramethylrhodamine succinimidyl ester, Biotum) [43]. In order to label EVs released by the cells *in vitro* and *in vivo*, EV protein marker or membrane localisation tags (e.g. palmitoylation signal) have been fused to fluorescent proteins [44, 45]. Moreover, EVs from melanoma cells were visualised *in vivo* using multiphoton microscopy in orthotopic tumors using *Gaussia* luciferase (Gluc) [46]. Gluc was also fused to biotin (GlucB) on the surface of the EVs, facilitating the conjugation of labeled streptavidin in order to see the labeled EVs *in vivo* using fluorescence mediated tomography (FMT) [42]. In addition to FMT, GlucB can also be visualised using magnetic resonance imaging (MRI) or positron emission photography (PET) [47]. Finally, advances in flow cytometry (FCM) enhanced the sensitivity of this technique to detect EVs. A recent, improved method allows for detection of PKH-67-labelled EVs with a comparable detection threshold as compared to NTA [48]. FCM can also be coupled with a camera in order to discriminate the EVs from false positive results [49, 50] and has been applied to characterise EVs released by mesenchymal stromal cells (MSC) using antibodies against MSC marker proteins [51].

Lai et al. have recently succeeded to show the dynamics of EV-mediated communication by taking advantage of the different combination possibilities offered by fusing enhanced green fluorescent protein (eGFP) and tandem dimer Tomato (td Tomato) to a palmitoylation signal (PalmGFP, PalmtdTomato) and GlucB [52]. First, EV exchange between 2 populations of cancer cells was visualised by labelling one with PalmGFP and the other with PalmtdTomato [52]. Then, by combining Gluc-labelled EV [42] and PalmtdTomato, EV uptake and EV-mRNA translation was tracked in the recipient cells [52].

Next, EV-packaged mRNA was monitored by tagging the transcripts encoding PalmtdTomato to a MS2 RNA binding sequence fused with eGFP, allowing to simultaneously visualise EV-packaged mRNA and EVs themselves. Finally, by combining PalmtdTomato and EV-GlucB, the dynamics of EV uptake and EV-mRNA translation were monitored [52]. Applying completely different systems to track EV traffic, several elegant studies by two different groups have visualised EV uptake in living cells using both β-galactosidase and the Cre/LoxP system. Ridder et al. used LacZ gene as reporter gene and β-galactosidase expression was induced in recipient cells by EV transfer. This transfer was demonstrated in mouse tumor models and between hematopoietic system and brain *in vivo* [53, 54]. This method was the first approach to analyse the physiological transfer of EVs *in vivo* and represents a step forward in avoiding potential artifacts introduced by submitting cells to an excess amount of isolated and labelled EVs [55]. By using a similar approach, the expression of green fluorescent protein (GFP) was triggered in cells, which took up EVs produced by tumor cells expressing the Cre recombinase [55, 56]: Cre-expressing melanoma cells injected into mice were releasing EVs containing Cre mRNA, which were then transferred to non-tumour cells *in vivo* [56]. In the target cells, Cre mRNA was translated into Cre protein and induced the expression of GFP [56]. However, this method cannot be used to assess the precise quantification of the uptake of EVs in recipient cells [56] as it does not allow for characterisation of transferred EVs or the uptake mechanism of EVs [57].

Taken together, the visualisation and tracking of EV movements has seen rapid and promising developments in recent years. Nevertheless, all these advances will need appropriate controls and to some degree standardisation of protocols in order to substantiate new findings on EV dynamics, characteristics and transfer of oncogenic traits in physiologic contexts and their potential clinical applications. As such, these novel imaging techniques could be combined with gene deletion or mutation strategies in order to better understand the role of specific molecules, which are transferred into EVs or are involved in the loading of cargo into EVs or in the uptake of EVs by target cells.

EVs in cancer

Cancer EVs differ from those released by healthy cells in terms of content and quantity. An increased secretion of EVs has been reported for different cancer cell lines and patients [58, 59]. Some typical proteins, miRNAs and other molecules described to be augmented in EVs released from cancer cells are presented in Fig. 1b. Acidic pH [60, 61] and **hypoxia** [59, 62], hallmark properties of many solid tumors, might be responsible for the intensification of EV production and for their altered content. Several recent studies have investigated this phenomenon under hypoxic conditions. An enhanced secretion of microvesicles from mesenchymal stem cells in response to hypoxia was reported by Zhang et al. [63]. Along these lines, Kucharzewska et al. [64] showed that EVs derived from glioblastoma cells grown under hypoxic conditions were potent inducers of angiogenesis *in vitro* through phenotypic modulation of endothelial cells: glioblastoma-derived hypoxic EVs induced endothelial cells to secrete several potent growth factors and cytokines and to stimulate the PI3K/AKT signaling pathway. In addition, EVs from hypoxic prostate cancer cells enhanced invasiveness and stemness of prostate cancer cells under normoxia and promoted the cancer-associated fibroblast phenotype in prostate stromal cells by targeting adherent junction molecules [65]. Umezu and colleagues [66] provided evidence that in endothelial cells, hypoxia-driven accelerated tube formation was attributable to miRNA-135b in EVs shed from hypoxia-resistant multiple myeloma cells. Interestingly, the EV transfer of miRNA-135b resulted in the suppression of FIH-1, a negative regulator of HIF-1α suggesting that the upregulation of HIF-1α could enhance angiogenesis. More recently, Li et al. observed increased levels of miR-21 in EVs isolated from hypoxic oral squamous cell carcinoma. The transfer of this miRNA in normoxic cells induced migration and invasion both *in vitro* and *in vivo* [67]. Although the mentioned studies of Umezu and Li provide solid evidence for the reported biological effects, they did not follow the generally accepted EV isolation procedures, increasing the possibility of precipitating contaminants.

It is also worth mentioning that hypoxic, but not normoxic tumor-derived EVs impaired NK cell function by delivering both TGFβ and miRNA-23a [68]. In conclusion, EVs secreted from hypoxic cancer cells seem to carry a cargo, which supports angiogenesis and thus metastasis as well as immunosuppression.

Cancer EVs are taken up by "recipient cells" but whether this process occurs in a specific manner or at random is poorly understood. Interesting findings by Hoshino et al. showed that integrins inserted in the membrane of EVs dictate their adhesion to specific cells in specific organs [69]. Tumor-derived EVs taken up by specific cells based on their integrin expression profile, were able to promote pro-migratory and pro-inflammatory S100 gene upregulation and by doing so, initiated the pre-metastatic niche *in vivo*. More studies will be necessary to confirm a potential specificity in the cellular uptake of EVs.

EVs are not the only vehicles to transfer oncogenic information. Recently, a new class of microvesicles named **oncosomes** has been described. Oncosomes differ from nano-sized EVs in terms of size (oncosomes are much larger with a diameter of 1–10 μm) and in the biogenesis pathway (oncosomes derive from the plasma membrane

of cells that have acquired an amoebotic phenotype, Fig. 1a). Di Vizio et al. [70] first introduced the term "large oncosomes" to describe large vesicles originating from amoeboid prostate cancer cells. The shedding of these vesicles could be induced by EGF while bleb formation in both normal prostate epithelial and stromal cells was modest and unresponsive to EGF. Indeed, only tumor cells seem to release quantifiable amounts of large oncosomes that were directly correlated with their rate of aggressiveness [71, 72]. Oncosomes, like EVs, contain mRNAs, miRNAs and proteins. Caveolin 1, a serum biomarker of metastatic prostate cancer, was detected in oncosomes and thus correlated with prostate tumor progression in mice and discriminated patients with metastatic disease from those with organ-confined disease [71]. Furthermore, the oncosome-associated miR-1227 produced by prostate cancer cells was able to induce migration of cancer-associated fibroblasts [72]. Whether nano-sized EVs and oncosomes share some of their molecular cargo is still under investigation. Nevertheless, recent findings from Minciacchi et al. revealed a different protein content in the two vesicle populations suggesting a specific selection of proteins destined for both vesicle types [73]. So far only a limited number of studies are available on oncosomes and more will be required in order to better characterise the two vesicle classes in terms of unique markers, content and function.

Contribution of EVs to drug resistance

Drug resistance of cancer cells represents a challenge in most anti-neoplastic treatments. The development of a resistant phenotype is considered to be multi-factorial and mainly due to decreased drug accumulation, increased efflux, increased biotransformation, drug compartmentalisation, acquired genetic modification of drug targets and/or defects in cellular pathways [74]. Recently, EVs have been identified as new players in passing resistance onto still sensitive cells [75, 76], which in turn might "gain" drug resistance traits as illustrated in Fig. 2.

EV-mediated drug export

Apart from the up-regulation of efflux pumps, which will be described below, in the establishment of drug resistance, the direct sequestration of drugs into lysosomal vesicles and EVs has also been reported. Safaei et al. [77] have shown the lysosomal compartment to be notably reduced in size in cisplatin-resistant human ovarian carcinoma cells with more EVs exporting cisplatin via this route compared to sensitive cells.

Pulse-chase experiments with doxorubicin, a fluorescent anticancer drug, confirmed the hypothesis that drug expulsion can occur via EVs. In MCF-7 cells, doxorubicin localised in the nucleus immediately after the drug was administered. Twenty-four hours later, nuclear fluorescence was significantly decreased and most of the visible doxorubicin was present in EVs associated with the cell periphery indicating the active sorting of drugs into vesicles [78]. Furthermore, Federici et al. [61] demonstrated that EVs purified from supernatants of melanoma cells treated with cisplatin contained detectable levels of the drug. HPLC analysis indicated that cisplatin within EVs was in its unmetabolised form suggesting that EVs might incorporate the drug immediately after the uptake by the cell. More recently Koch et al. were able to detect anthracyclines in EVs from diffuse large B cell lymphoma cells lines. Interestingly, knocking down the ATP-transporter A3 (ABCA3) augmented intracellular retention of the drugs, thus increasing their cytostatic effects [79].

EV-mediated miRNA export

In addition to the above-mentioned mechanisms, miRNAs packaged within EVs can also contribute to the onset and maintenance of drug resistance. Pigati et al. [80] have observed that mammary epithelial cells released a different subset of miRNAs compared to the ones which were retained. They found that nearly 30 % of the released miRNAs *in vitro* did not reflect the cellular profile, indicating that miRNAs are retained or released selectively. In particular, the malignant mammary epithelial cells released most of their miRNA-451 into the environment. This concurs with findings from Kovalchuk et al. [81] who reported that miRNA-451 targets the multidrug resistant gene (mdr1), thereby down-regulating P-gp expression. Indeed, transfection of the doxorubicin-resistant MCF-7 cells with miRNA-451 resulted in an increased sensitivity of breast cancer cells to the drug. It is tempting to speculate that cancer cells have selective mechanisms to export certain miRNAs in order to retain higher levels of P-gp necessary to shuttle chemotherapeutic drugs out.

In this context, Chen et al. [82] showed that MCF-7 cells acquired an increased survival potential through EVs released by corresponding docetaxel-resistant lines. Microarray analysis revealed once again a specific subset of miRNAs (including miRNA-222 and miRNA-452) in the "resistant EVs". The incubation of sensitive MCF-7 cells with resistant EVs resulted in a reduction of intracellular PTEN and APC4 mRNAs known to be targeted by miRNA-222 and miRNA-452, respectively. Although protein levels had unfortunately not been analysed, the authors speculated that these oncomiRs act by down-regulating tumor suppressors. Furthermore, miRNA-21 and miRNA-155 from EVs were identified as important players in the cross-talk between neuroblastoma cells and monocytes. Co-culture experiments showed that miRNA-21 released from neuroblastoma cells led to a TLR8- and NF-κB-dependent secretion of EV-containing miRNA-155 from monocytes. Once taken up by neuroblastoma cells, miRNA-155 targeted the telomeric repeat-binding factor 1

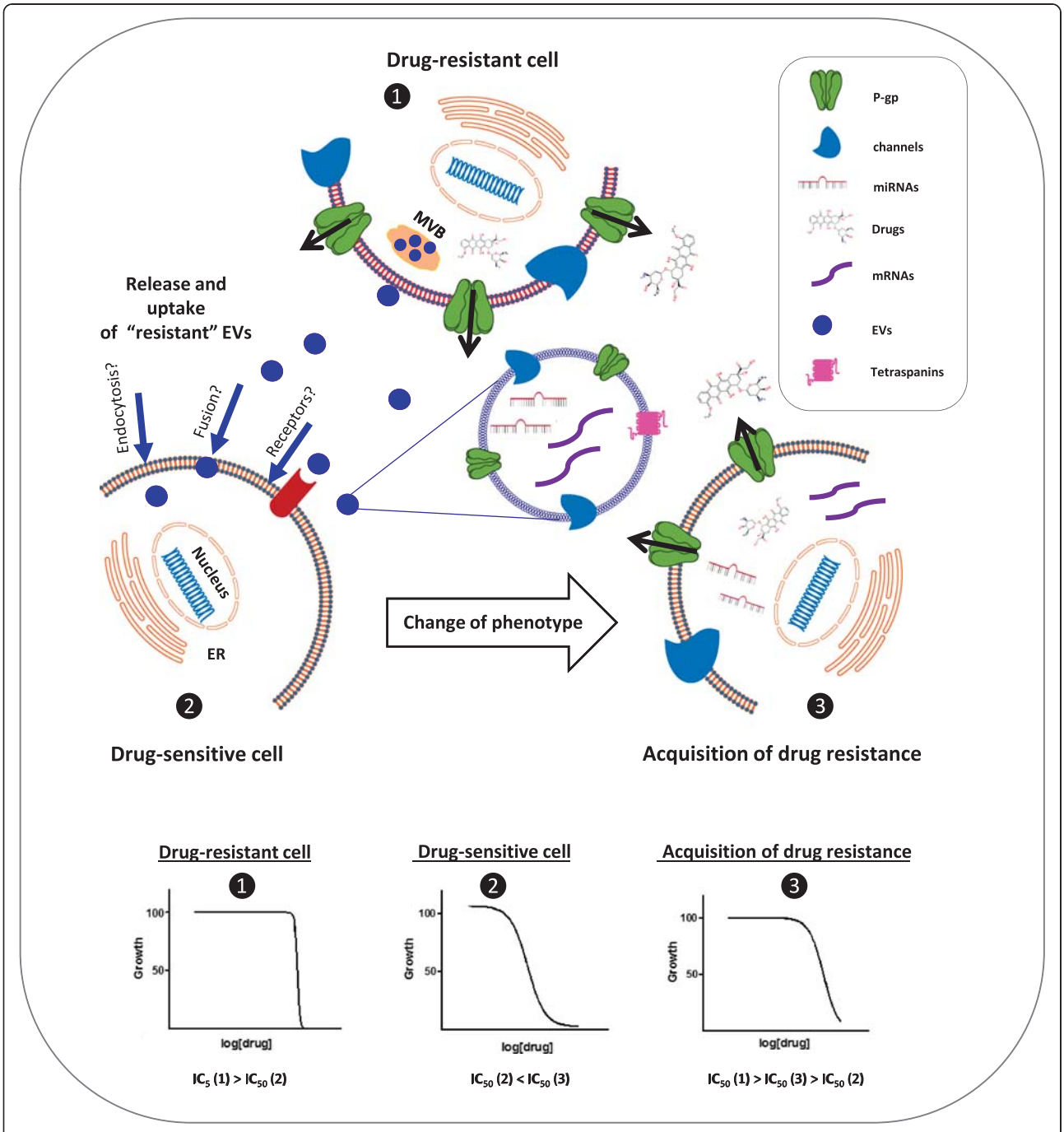

Fig. 2 Drug-resistant cells can transfer the resistant phenotype through EVs. EVs released by drug-resistant cells contain proteins and miRNAs, which partake in propagating resistance. Drug-sensitive cells become gradually resistant when they incorporate "resistant EVs": Resistant cells (1) then over-express efflux pumps (P-gp) to eliminate anti-cancer drugs and produce more EVs, which again reflect the resistant phenotype of the secreting cell. Once in the extracellular environment, these EVs can be taken up by sensitive recipient cells (2) through fusion, endocytosis or binding to surface receptors. The released content acts on these cells, which in turn might also become drug-resistant (3). The lower part shows example trends of dose–response curves to a cytotoxic drug, representative of drug-resistant cells (1), drug-sensitive cells (2) and the same sensitive cells, which are becoming resistant after incubation with "resistant EVs" (3). The effectiveness of the drug to inhibit a specific biological function in the cells (exemplified by the inhibition of cell growth) is expressed by the IC_{50} value. The higher this value, the more resistant the cells. The IC_{50} value of cells which are acquiring the resistant phenotype would be in between the IC_{50} values of the other two conditions: IC_{50} (1) > IC_{50} (3) > IC_{50} (2)

(TERF1) inducing an increased growth when the cells were treated with cisplatin [83].

Drug efflux pumps

Among the different reasons for drug resistance in malignancies, the up-regulation of efflux pumps such as ABC transporters is often responsible for transporting drugs out of cells [84]. Bebawy and colleagues [85] demonstrated for the first time by flow cytometry that EVs transfer functional P-glycoproteins (P-gp), well characterised ABC transporters, from drug-resistant to drug-sensitive human acute lymphoblastic leukemia cells. Corcoran et al. [86] described the potential role of EVs in transferring phenotypic changes associated with docetaxel-resistance in prostate cancer cells: an induced resistance in sensitive prostate cancer cells cultured in the presence of EVs derived from resistant cells was scored. The authors suggested P-gp to be potentially involved in the newly-acquired resistance as P-gp was expressed by both resistant prostate cells and in corresponding EVs whereas it was undetectable in the sensitive parental cells. Likewise, proteomic analysis of EVs secreted from sensitive prostate cancer cells compared to those from docetaxel-resistant cells revealed a different profile, with "resistant EVs" being once again enriched in P-gp and endophilin A [87]. The presence of these proteins was also detected in the serum of a small cohort of docetaxel-resistant patients. Hence, these EV-transported proteins were proposed as predictive biomarkers for therapeutic response or development of drug resistance [87].

The relevance of P-gp delivery through EVs in the process of transferring drug resistance was also confirmed in breast cancer cells [88]. Here, P-gp was not directly transported by EVs, but its transcription was activated by the calcium permeable channel Transient Receptor Protein Channel 5 (TrpC5) in EVs released from adriamycin-resistant breast cancer cells. Uptake of these vesicles allowed the sensitive recipient cells to acquire the TrpC5 channel, leading to increased Ca^{2+}-entry and the activation of the Ca^{2+}-dependent transcription factor NFATc3 (nuclear factor of activated T cells isoform c3), which in turn was responsible for increased P-gp transcription [89]. Taken together, ABC transporters carried or induced by EVs seem to play a prominent role in the development of drug resistance by providing the cell with means to rid themselves from drugs.

Several studies demonstrated the capability of EVs to confer drug resistance; however little is known about the role of EVs in inhibition of cancer cell proliferation during chemotherapy. Bovy and colleagues [90] showed that endothelial EVs taken up by breast cancer cells were able to impair growth. In response to chemotherapeutic agents, endothelial cells released EVs containing miRNA-503. The presence of this miRNA within breast cancer cells induced a reduction of their growth and invasion potential by targeting cyclins D2 and D3. Twenty-two additional up-regulated miRNAs were detected both in resistant cells and corresponding EVs in the context of breast cancer chemoresistance [91], 12 of which were significantly up-regulated in biopsies taken after neoadjuvant chemotherapy. Similar to miRNAs, long non coding RNAs (lncRNAs) have also been described to transfer drug resistance traits. The lncRNA linc-VLDLR enriched in EVs released from HCC cells was able to modulate chemotherapeutic response to sorafenib in recipient cancer cells by upregulating the ABC transporters [92]. To date, it remains to be proven whether inhibiting EV secretion might be a therapeutic option to avoid responsive cancer cells to become unresponsive.

Limitations of the functional analysis of EVs

Although there is little doubt that EVs and their cargo can be transferred to be functionally active in recipient cells, there are several, mostly technical issues which need to be addressed to take this field one step further Standard and generally accepted procedures and protocols should be developed for:

- **Sample preparation**. EVs can be isolated by different methods (ultracentrifugation, density gradient ultracentrifugation and precipitation reagents) and there is no generally accepted procedure yet. In this context, Van Deun et al. have clearly shown that the purification method of choice will influence the purity of the vesicle population and downstream results [6]. Although it is well accepted by the community to isolate EVs through density gradient ultracentrifugation, there is an ongoing effort to find alternatives especially for complex body fluids such as plasma or urine where also the volume is a limiting factor. Moreover, density gradient ultracentrifugation is time-consuming and difficult to implement on a daily basis in clinical routine. Size exclusion chromatography, ultrafiltration and immunoprecipitation with specific antibodies have recently been tested by several groups [93–95] and might become convincing EV isolation methods in future.
- **Purity of the isolated EVs**. The essential requirements to define an EV population are: i) providing a general overview of the protein composition including proteins that should not be present in EVs, ii) performing transmission electron microscopy and nanoparticle-tracking analysis and/or flow cytometry to understand the purity of the isolates and using proper controls in functional studies.

- **Quantification**. The number of EV particles, micrograms of proteins, nanograms of EV-RNA need to be accurately determined.
- **Sensitivity of visualisation** of EV trafficking should be further improved to allow for analysis of EV functions under physiological conditions.
- **Characterisation of EV cargos** in different cellular settings with a focus on protein, miRNA and lipid profiles. The next task in this field will be to develop sensitive and specific tools to overcome the above issues. Only then will we be able to completely understand the potential of EVs as new targets in anti-neoplastic treatments and/or as new biomarkers for early detection of pathological conditions.

Conclusions

The discovery of EVs as multi-component signaling complexes mediating intercellular communication through the delivery of molecules such as miRNAs and proteins has raised a particular interest for the use of these microvesicles as potential cancer biomarkers. Indeed, EVs present in body fluids might represent a snapshot of the status of the cancer cell at a specific time point providing highly sensitive and specific cancer markers. The simultaneous production of different subpopulations of EVs has been confirmed in many publications [96–98]. In this context, the biggest challenge the field is currently facing is the isolation and precise characterisation of the different vesicle populations and their corresponding functions. Questions such as i) different EV production and concentration in diseased versus healthy cells, ii) a clear discrimination between cancer cell-released EVs from surrounding stromal or healthy cells as well as iii) specificity of EV uptake will all have to be addressed in future studies. Only when most of these points are elucidated, can we begin to target certain subpopulation of vesicles for therapeutic purposes.

Taken together in order to exploit EVs as potential biomarkers or therapeutical targets, several technical obstacles will have to be tackled in the near future: the procedures for EV isolation and quantification need to be standardised to avoid the observed discrepancies and to dissect which other molecule classes are present in EVs and whether they get sorted into vesicles by chance or by targeted yet unknown processes. In addition, further studies on lipid composition and alteration of EVs will provide a more comprehensive understanding of their role in the biological function of EVs and also their potential impact on recipient cells. Nevertheless and in support of the above reviewed evidence, EVs can be regarded as interesting and important "homing pigeons" carrying specific messages from one place to the other. It remains to be shown how the cargo is selected and sorted and whether these processes are generally targeted or coincidental.

Abbreviations

AP1S2, adaptor-related protein complex 1, sigma 2 subunit; APC4, anaphase promoting complex subunit 4; DLS, dynamic light scattering; EFNA3, Ephrin-A3; EGF, epidermal growth factor; eGFP, enhanced green fluorescent protein; EM, electron microscopy; EVs, extracellular vesicles; FCM, flow cytometry; FMT, fluorescence mediated tomography; HIF1a, Hypoxia-inducible factor 1-alpha; HMGA2, high-mobility group AT-hook 2; HPLC, high-performance liquid chromatography; JAK-STAT, Janus kinase, signal transducer and activator of transcription; lncRNA, long non coding RNA; M6PR, Mannose-6-Phosphate Receptor; mdr1, multidrug resistant gene 1; MHC, major histocompatibility complex; MRI, magnetic resonance imaging; MVBs, multivesicular bodies; NFATc3, nuclear factor of activated T cells isoform c3; NTA, nanoparticle tracking analysis; PET, positron emission photography; P-gp, P-glycoprotein; PI3K, Phosphoinositide 3-kinase; PTEN, phosphatase and tensin homolog; RISC, RNA-induced silencing complex; SNAREs, soluble NSF attachment protein receptor; SOCS5, Suppressor of cytokine signaling 5; TGFβ, transforming growth factor β; TrpC5, Transient Receptor Protein Channel 5; ABC transporters, ATP-binding cassette transporters; TSG101, tumor susceptibility gene; EE, early endosomes

Acknowledgments

We thank the Fondation Cancer (Luxembourg) for generous funding and the Pélican Foundation for a personal grant to GC and Iris Behrmann for critically reading of the manuscript.

Authors' contributions

GC wrote most of the article and generated the 2 figures. CM suggested paragraphs and performed editorial work, GW wrote the paragraphs on imaging. SK decided on the content, had editorial input on all sections and designed the layout of figures. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 12 April 2016 Accepted: 2 June 2016
Published online: 10 June 2016

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Chapter 5 Discussion and perspectives

The work in this thesis aimed to extend current knowledge on the influence of BRAF kinase inhibitors on several aspects of melanoma biology including early adaptive responses to the treatment and drug resistance mechanisms. In the following section, the main findings of the project parts will be briefly summarized and discussed including a short outlook on future experiments. However, to avoid a mere repetition of the individual papers, this discussion will mostly focus on three key topics, which cover the four manuscripts completed during this thesis. In particular, section 5.1 and 5.2 will focus on metabolic rewiring in melanoma and the tumour microenvironment (based on chapter 4.1); section 5.3 will focus on mechanisms of drug resistance (based on chapter 4.3) and section 5.4 on potential biomarkers of drug resistance (based on chapters 4.2, 4.3 and 4.4). Finally, I will end my dissertation, putting our main findings in a more clinical context (section 5.5).

5.1 Metabolic rewiring in melanoma

Cutaneous melanoma is the deadliest form of skin cancer that emerges from the uncontrolled proliferation of melanocytes (Rajkumar and Watson, 2016). Although melanoma displays an extreme molecular heterogeneity, the most common mutation identified in > 50% of patients is a single amino acid substitution at position 600 in BRAF (most often V600E), which results in a constitutively active kinase that drives MEK/ERK signalling leading to cellular hyper-proliferation. The advent of targeted therapies and immunotherapies extended progression-free and overall survival of melanoma patients considerably (Garbe et al., 2016; Luke et al., 2017). However, poor responses and/or the development of resistance to treatments represents major critical challenges. Nevertheless, these clinical issues leave room for developing new and more effective strategies by targeting, among others, metabolic pathways that are often altered in cancer cells (DeBerardinis and Chandel, 2016).

The role of ROS

Mutant proteins such as $BRAF^{V600E}$ have been shown to affect the metabolism by enhancing glycolysis and the tricarboxylic acid (TCA) cycle activity (Parmenter et al., 2014). The pyruvate dehydrogenase complex (PDH) is an important gatekeeper enzyme linking glycolysis to the TCA and oxidative phosphorylation, thereby strongly influencing the metabolic phenotype of a cell.

In the **first publication (Cesi et al., 2017)**, we showed that BRAF inhibition leads to ROS production. We postulate that this increased ROS production is an early adaptive response, which activates a negative regulatory loop that limits the generation of further ROS. Indeed, we could show that ROS activates PDKs, which in turn phosphorylate and inactivate PDH limiting the flux of pyruvate into the TCA and into the oxidative phosphorylation and consequently the generation of more ROS, which might be lethal to the cell. In a similar way, for instance, ROS inhibits pyruvate kinase M2 (PKM2, a cancer-expressed isoform of pyruvate kinase), leading to accumulation of glycolytic intermediates, which feed into the pentose phosphate pathway to generate NADPH. NADPH in turn plays an essential role in ROS detoxification (Anastasiou et al., 2011). As another example, ROS accumulation activates AMPK (Zhao et al., 2017). AMPK generally promotes a more oxidative metabolism and inhibits biosynthetic pathways (Zhao et al., 2017). In the context of ROS detoxification, AMPK also stimulates fatty acid oxidation and limits the fatty acid synthesis, thus also saving NADPH (used in fatty acid synthesis). AMPK inhibits AKT/mTOR activity leading to protein synthesis inhibition and increasing autophagy and it also promotes FOXO activity to maintain the redox balance through enhanced antioxidant production and glucose metabolism (Zhao et al., 2017) (Figure 22).

Taken together, ROS-mediated regulation of kinases is an exciting emerging field in biochemistry as it appears that ROS can either activate or inactivate kinases by oxidation. Interestingly, ROS regulate some kinases, which are directly involved in metabolic processes and vice versa regulate ROS itself.

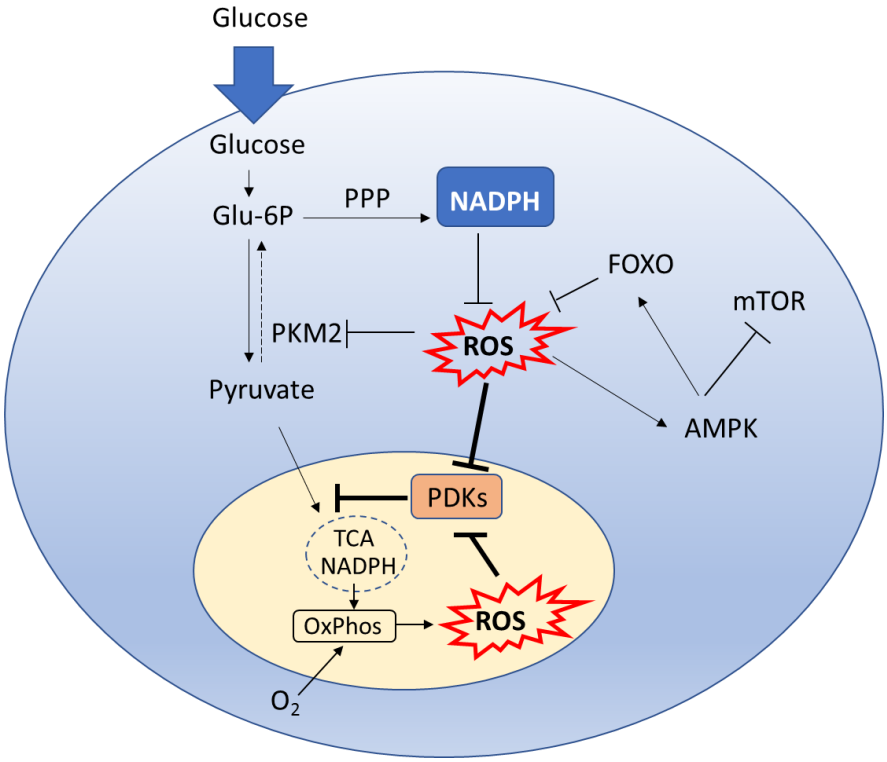

Figure 22. Salvage mechanisms activated by ROS signalling.
ROS themselves protect the cell against ROS damage by inducing different antioxidant responses and re-establishing or maintaining redox homeostasis. Thicker lines represent a new regulatory mechanism in which increased levels of ROS (induced by BRAF inhibition) activate PDKs, phosphorylating and thus inhibiting PDH. Consequently, the TCA, oxidative phosphorylation and further ROS production is reduced.

Given the lethal potential of ROS, inhibitors enhancing the oxidative stress might be beneficial in melanoma therapy. Indeed, in our study we observed that inhibition of pyruvate dehydrogenase kinases (PDKs) leads to reduced growth in both sensitive and resistant melanoma cells by a mechanism involving the generation of ROS exceeding a certain threshold. This spawns the hypothesis that metabolic enzymes or other proteins involved in antioxidant defense might be useful targets for melanoma or other cancer treatments. As NRF2 (a master regulator of antioxidant responses) promotes cell survival under stress, it is tempting to speculate that increased NRF2 activity could be tumour promoting by being protective for cancer cells, especially in the context of melanoma drug resistance. Some studies have described an NRF2-mediated drug resistance mechanism in several cancer cell lines (Homma et al., 2009; Hong et al., 2010).
In this context, we are in the process of investigating the role of the antioxidant transcription factor NRF2 in chemoresistance development in melanoma. We have stably knocked down NRF2 in both resistant and sensitive melanoma cells and we plan to test the vulnerability of the cells to several inhibitors, especially BRAF, MEK but also PDK inhibitors.

Metabolic rewiring driven by MITF

Metabolic rewiring has also been described to be driven by MITF. The BRAF/MEK1/ERK pathway inhibits MITF-mediated PGC1α transcription, a protein involved in mitochondrial biogenesis and ROS inhibition (Haq et al., 2013). Thus, BRAF inhibitor treatment has been associated with an increase in MITF and PGC1α-mediated induction of mitochondrial biogenesis and oxidative phosphorylation sustained by higher ATP levels when PGC1α is strongly induced by MITF. Accordingly, a drop in ATP has been observed in cells, which do not express MITF (Haq et al., 2013). The $BRAF^{V600E}$ positive cell lines we used in our study express intermediate levels of MITF (Margue et al., 2013). Taken together, different metabolic switches might be used depending on varying basal MITF levels in different melanoma cell lines: intermediate levels of MITF control proliferation, which relies on aerobic glycolysis; low or high MITF levels are associated with de-differentiation or differentiation-mediated senescence and utilize oxidative phosphorylation (Ahn et al., 2017).

Nevertheless, with the appearance of BRAF inhibitor resistance and the reactivation of MAPK signaling, the effects induced by BRAF inhibition are reversed. Drug resistance in melanoma has been associated with restored glycolysis (Parmenter et al., 2014) but also with increased glutamine dependency (Baenke et al., 2016). These findings can help to develop new therapeutic strategies to eradicate melanomas that become resistant to BRAF inhibitors.

As previously mentioned, in BRAF inhibitor resistance, the MAPK pathway is reactivated by secondary mutations. In the third manuscript (Cesi et al, in preparation), we identified the overexpression of ALK as a possible mechanism driving acquired resistance in melanoma cells. Aberrant ALK is known to activate both the MAPK and the PI3K/AKT pathway (Hallberg and Palmer, 2013) and therefore glycolysis, the pentose phosphate and antioxidant-defense genes are reactivated. It is likely that the acquisition of secondary mutations upon BRAF inhibition may be involved in metabolic rewiring. Further insights into the connection between drug resistance and metabolic reprogramming might help the identification of novel combination partners for use with BRAF and BRAF/MEK inhibitors to be used in patients who relapse on first-line targeted therapies.

5.2 Acidification of tumour microenvironment

Another interesting aspect in the context of metabolic reprogramming that we did not explore in this project but it is important to consider is the acidification of the tumour microenvironment. Metabolic alterations common in cancer cells such as upregulation of glycolysis, glutaminolysis and pentose phosphate pathway promotes an acidic milieu producing more lactate and CO_2 (Böhme and Bosserhoff, 2016). Acidic pH triggers oncogenic signalling and enhances the metastatic potential of cancer cells. Furthermore, acidosis has been associated with poor clinical prognosis (Dhup et al., 2012). Peppicelli et al. showed that acidity triggers an EMT program and increases invasiveness of melanoma cells which, in turn, potentiates migration capacity and development of lung metastasis into immunodeficient cells grown in standard pH (Peppicelli et al., 2014). Robey et al. reported that the administration of bicarbonate to a mouse model of metastatic breast cancer reduces cell dissemination, providing evidence that increasing tumour pH to a physiological level is crucial to abrogate progression to metastasis (Robey et al., 2009). In fact, acidosis can induce the expression or the secretion of proteolytic enzymes leading to the degradation of the extracellular matrix, which is a crucial step for migration and invasion of cancer cells (Kato et al., 2005; Rofstad et al., 2006) and it can also promote angiogenesis triggering the expression of VEGF (Fukumura et al., 2001). Furthermore, an acidic tumour microenvironment has been shown to contribute to drug resistance by stimulating the activity of drug efflux pumps (Thews et al., 2011) and reducing the uptake of weak base drugs (Mahoney et al., 2003).

In this context, we additionally plan to test the absorbency of BRAF inhibitors in an acidic versus basic medium. In acidic media, the basic vemurafenib might get protonated and be poorly absorbed by cells and this simple fact might also play a role in melanoma drug resistance. The management of tumour pH would not only increase drug efficacy but also reduce metastasis formation. Pathways leading to acidification often produce NADPH, which is important in the antioxidant defense. Targeting those pathways will inhibit or reduce ROS defense and consequently extracellular acidification.

To further add complexity to the metabolic reprogramming of tumour cells, a metabolic symbiosis among different tumour cell subpopulations, generating and using lactate, may also take place and contribute to tumour cell dissemination (Sonveaux et al., 2008). Numerous studies have shown that cancer progression is highly dependent on cancer-associated fibroblasts (CAFs), the major cellular stromal component in tumours. CAFs are not tumourigenic themselves, but are important in promoting tumour growth and metastasis. Whitaker-Menezes and colleagues identified a cross-talk between CAFs and breast cancer cells in which CAFs undergo aerobic glycolysis and produce lactate, which is utilized as a metabolic substrate by adjacent cancer cells (Whitaker-Menezes et al., 2011).

Overall, a better understanding of cancer metabolic reprogramming is certainly needed. Exploiting the unique features of tumour metabolism for cancer treatment, is a promising direction in cancer research. We are confident that the presented data together with the suggested follow-up experiments, will improve our understanding of metabolic rewiring in melanoma and may aid the development of novel melanoma-targeting therapeutic approaches.

5.3 Mechanisms of drug resistance

Despite the recent success stories of drugs targeting the MAPK signaling pathway and immunotherapy in melanoma, the majority of patients with metastatic disease still undergo disease progression after initial tumour shrinkage indicating a gradual development of therapy resistance. We and many others have identified mechanisms of resistance in melanoma involving various kinases (e.g. cKit, EGFR, COT, RAS and other receptor tyrosine kinases) most often resulting in the reconstitution of the drug-inhibited MAPK or AKT signalling pathways (Winder and Virós, 2017). Ongoing clinical studies worldwide currently evaluate different inhibitors or inhibiting antibodies alone or in combination in advanced stage melanoma patients to delay the onset of drug resistance. Moreover, additional and new treatments should be administered as soon as patients show signs of drug resistance, side effects or lack of measurable effects.

It has been shown that EVs contribute to the dissemination of the resistant phenotype through different mechanisms (Sousa et al., 2015). Understanding how often and how efficiently EV transfer occurs *in vivo* remains a technical challenge and it is still an open question as to the functional consequences of EV cargo in recipient cells.

The role of ALK in drug resistance

In the third manuscript (chapter 4.3, Cesi et al., in prep), we identified the overexpression of ALK as a potential novel mechanism responsible for drug resistance in melanoma. Interestingly, ALK was also found packaged and functional in EVs shed by drug resistant cells suggesting that ALK might be implicated in the acquisition of drug resistance traits in sensitive cells. The best described mediators of drug resistance transported by EVs are mostly miRNAs and drug efflux pumps (Bach et al., 2017). We describe here for the first time a kinase-mediated horizontal transfer of drug resistance.

In this context, we plan to broaden the scope of this study by performing *in vivo* experiments and by validating these findings in patient samples. In particular, we will stably transduce different ALK-negative cell lines with viral particles containing the truncated form of ALK. Next, we will test both *in vitro* and *in vivo* the ability of ALK to confer resistance to BRAF inhibitors but also its susceptibility to the combination of ALK and BRAF inhibitors. Additionally, in collaboration with our clinical partners, we will analyze the presence of ALK in FFPE samples from resistant melanoma patients.

Taken together, the oncogenic kinase ALK, which is shuttled between cells by EVs appears to play a critical role in drug resistance.

5.4 Biomarkers of drug resistance

The term **biomarker** has been defined by the National Institute of Health Biomarkers Definition Working Group as any molecule which can be "*objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention*"(Biomarkers Definitions Working Group, 2001). The ideal biomarker should be easily obtained with minimum discomfort, sensitive, specific and highly reproducible among clinical laboratories (Strimbu and Tavel, 2010). In reality, a very large number of candidate biomarkers fail to reach the clinic because they lack sensitivity and specificity (Diamandis, 2012). Also, in the context of melanoma, several biomarkers have been suggested for disease monitoring. However, the majority have not been incorporated into clinical routine. The levels of lactate dehydrogenase (LDH) and/or S100B (calcium-binding protein B) are currently still used in clinics to monitor disease progression despite they both have low specificity (Verykiou et al., 2014).

The relative 5-year survival rate for stage II patients fluctuates between 80% (stage IIA) and 50% (stage IIC) (Balch et al., 2009). These differences in survival strongly suggest that in some patients metastatic spread has occurred and could not be detected by conventional methods (Stark, 2017). Although tremendous global efforts have gone into identifying biomarkers of therapy response or drug resistance, the search is still ongoing. In the increasing body of new therapies recently approved by the FDA, as well as many more under development for the treatment of melanoma, there is an urgent need of highly sensitive and reliable biomarkers to indicate the stage of disease and upcoming resistance to the applied treatment. In this context, it has been shown that **circulating tumour DNA (ctDNA)**, which derives from cancer cells undergoing apoptosis or necrosis, allowed the assessment of therapy response in breast and colon cancer (Garcia-Murillas et al., 2015; Murtaza et al., 2013). In plasma samples collected from advanced stage melanoma patients, Girotti et al. showed that ctDNA generally revealed the disease course earlier than imaging, and that it was more accurate at predicting responses than serum LDH (Girotti et al., 2016).

Furthermore, in recent years, **miRNAs** have been studied as diagnostic biomarkers in several malignancies (Fattore et al., 2017). The underlying idea of measuring miRNAs in the circulation is that cancer might induce changes in the levels of secretion so that different amounts and profiles of miRNAs in circulation might be informative of the disease state or response to treatment. The correlation between miRNA signatures and the responses of specific therapies derives from the observation that miRNAs can also be involved in chemoresistance (Lei et al., 2013; Mosakhani et al., 2012). Whether these small non-coding RNA molecules might be used to predict responses to various cancer treatments is still an open question. Our group has previously shown that circulating miRNAs only change significantly at late stages of melanoma progression compared to controls. This observation has serious implications for miRNA biomarker studies in cancer and questions their utility as early diagnostic marker for melanoma (Margue et al., 2015).

Another interesting source of biomarkers can be found in EVs. The **EV cargo** represents a snapshot of the parental cell at the time of release and can change depending on the status of the cell (Cesi et al., 2016). This observation suggests that EV content is dynamic and mirrors the events in the parental cells, providing opportunities and challenges in the evaluation of EVs as biomarkers. The collection and analysis of EV content from blood could be considered as a “liquid biopsy”, thus avoiding performing surgical procedures to sample tissue for biopsies (Figure 23). As an example, circulating EVs from glioblastoma patients contained EGFRvIII mRNA, which can be accurately measured for glioblastoma diagnosis (Skog et al., 2008). Similarly, miRNA-21 and miRNA-1246 were significantly elevated in EVs isolated from plasma of patients with breast cancer compared to controls (Hannafon et al., 2016). Although this new field of EV-based biomarkers is promising, lack

of standard protocols to isolate a pure EV population, heterogeneity of EV populations but also of blood samples and the amount of recovery represent major obstacles for their clinical use. Despite these challenges, in order to elevate the EV research to the next level for EV utilization for cancer diagnosis, appropriate combined platforms including next generation sequencing of EV RNAs and DNAs, proteomic analysis of EV surface proteins, and immune-affinity capturing techniques have to be developed.

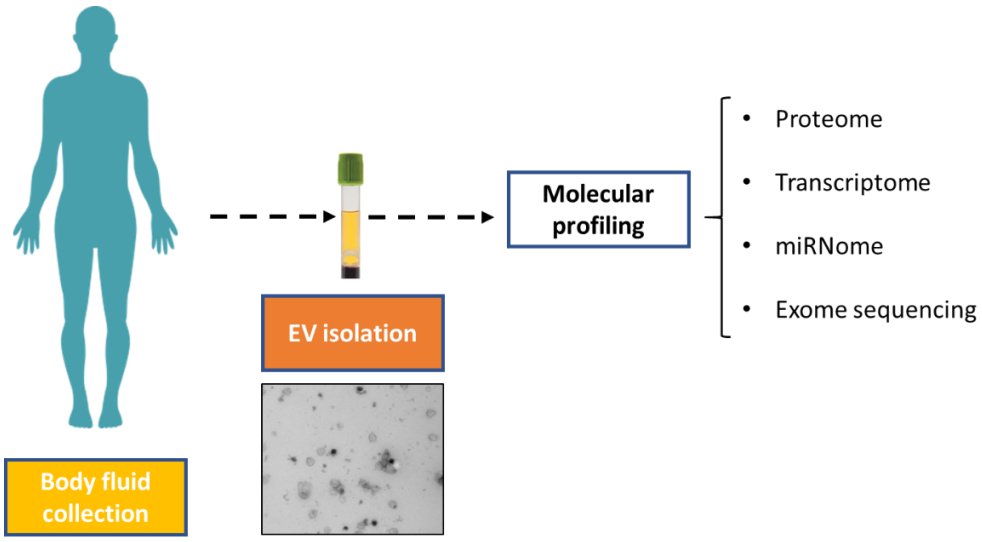

Figure 23. Extracellular vesicles as potential sources for molecular profiling.
EVs circulate in body fluids and they can be recovered and profiled. Profiling of proteins, miRNAs and nucleic acids may reveal signatures of drug resistance or disease progression.

In search of further resistance mechanisms and corresponding biomarkers, we have profiled miRNome and transcriptome changes in melanoma cells developing resistance to BRAF kinase inhibitors and found several miRNAs (such as miR-509-3p, miR-708-5p) and new candidate genes (such as PCSK2) likely to be involved in drug resistance (Kozar et al., 2017). The identified candidates might also be considered as prognostic and/or diagnostic resistance biomarkers in melanoma drug resistance. In fact, while the majority of miRNAs are found intracellularly, a significant number of miRNAs as well as proteins have been observed outside of cells, in various body fluids and packaged in extracellular vesicles (Bach et al., 2017). Interestingly, the upregulated miR-509 and PCSK2 identified in the resistant cell lines in our study (Kozar et al., 2017), have also been identified in resistant EVs isolated from the resistant A375X1 clone (Cesi et al., in prep. and additional data on page 113) suggesting their potential as biomarkers of drug resistance.

In this context, we are currently in the process of collecting plasma samples from patients before and after the development of drug resistance to targeted therapies and we plan to validate these findings in patient samples. If promising biomarkers can be identified around the point of emerging drug resistance, we envisage to enroll in an ISO-certified biomarker validation program at the IBBL (Biobank Luxembourg).

5.5 Towards novel strategies for clinical treatment of melanoma patients

In the last decade, melanoma treatment has dramatically improved. Patients affected by stage II or III melanoma were initially treated with interferon-alpha (INF-α) adjuvant therapy. Despite the high toxicity, INF-α showed modest efficacy (Tsao et al., 2004). For patients with late stage disease, chemotherapeutic drugs (such as dacarbazine and cisplatin), radiotherapy as well as interleukin-2 have been approved by the FDA for the treatment of metastatic disease (Tsao et al., 2004) and are still used in some patients.

Oncology research is currently moving towards a new era of precision medicine where therapeutic approaches are based on the individual genomic landscape as well as on environmental and lifestyle factors. Precision medicine has been fueled by the enormous progress in high-throughput sequencing methods, which enable clinicians to detect rapidly and by now affordably genetic aberrations. However, differentiating mutations that do not have any impact on cell growth and survival from those that do, is still a challenge (Collins et al., 2017). In order to be classified as an oncogene, more than 20% of the recorded mutations in that particular gene have to be at recurrent positions and have to be missense (Vogelstein et al., 2013). Accordingly, for a tumour suppressor gene, >20% of the recorded mutations in the gene have to be inactivating. Following this rule, 71 tumour suppressor genes and 54 oncogenes have been identified (Vogelstein et al., 2013). To date, several oncogenes have been successfully targeted with inhibitors like Vemurafenib for the treatment of $BRAF^{V600}$-positive melanoma, Crizotinib for ALK-positive lung cancer or Gefitinib for EGFR-mutant cancers. Nevertheless, large scale sequencing efforts have also revealed that some tumours including melanoma, are characterized by a tremendous genomic variability, which leads to subclonal aberrations with inter- and intratumour heterogeneity, making therapeutic strategies difficult (Alexandrov et al., 2013; Collins et al., 2017). In the context of melanoma, tumour heterogeneity is certainly considered a key factor that might lead to treatment failure or to mixed responses (Ahn et al., 2017). Over the past few years, the implementation of accurate screening programs together with major advances in melanoma treatment have vastly improved the outcome for advanced stage patients. A frequently observed scenario in melanoma patients treated with

kinase inhibitors is an initial tumour regression followed by drug resistance, which is mostly due to subpopulations of cells able to survive drug toxicity and to rapidly acquire new mutations (Ahn et al., 2017). Even when the treatment leads to complete remission, patients can relapse later and become irresponsive to the therapy suggesting the presence of quiescent, slow-cycling melanoma cells, which were drug-tolerant from the beginning (Tolk et al., 2015). Shaffer et al. recently showed that melanoma cells display a tremendous transcriptional variability at single cell level with a small percentage of cells characterized by higher levels of resistance markers and by a drug-tolerant phenotype (Shaffer et al., 2017). Current treatments aim to hit the bulk of a tumour but recent studies indicate that the slow-cycling cell populations are still aggressive and invasive (Webster et al., 2015). As previously mentioned, the standard care for BRAF-positive patients has shifted from BRAF inhibitors used as monotherapy to a combination therapy with BRAF and MEK inhibitors (Garbe et al., 2016). Additionally, immunotherapy has moved from cytokine-based treatment to antibody-mediated blockade of immune checkpoints. However, given the short-lived responses and side effects, new combination therapies are currently being explored to further increase clinical benefits in a more personalized manner. Ongoing clinical trials are testing, for instance, a triple therapy with BRAF, MEK and PD1 or PD-L1 inhibitors administered concomitantly or consecutively (Luke et al., 2017). Phase I studies have shown so far that this triple combination is well tolerated and effective (Eroglu and Ribas, 2016).

Many more combinations or new drugs are currently being investigated in clinical studies worldwide (https://clinicaltrials.gov) including autophagy inhibitors and histone deacetylase inhibitors (Booth et al., 2017; Ndoye and Weeraratna, 2016). Although this might eventually lead to the discovery of better drug combinations or treatment regimens, the prior knowledge of the molecular background of each patient or the identification of specific biomarkers for therapy responses would facilitate a more personalized therapy with higher chances for long term remission (Long et al., 2016). In this context, an extensive area of cancer research is focusing on the identification of neoantigens, peptide epitopes bound to MHC complexes exposed on the cell surface of malignant cells to selectively enhance T cell reactivity (Schumacher and Schreiber, 2015). This would allow the development of novel therapeutic approaches such as personalized vaccines that could broaden the number of patients responding to immunotherapies (Wang and Wang, 2017).

As previously stated, **cancer metabolism** has become an area of intensive research, which aims to identify altered metabolic pathways that could be targeted to slow down cell growth or to induce apoptosis of cancer cells, saving normal cells. Two populations of cells are often described in a tumour: slow-cycling cells, which appear senescent and prone to develop drug resistance (Roesch

et al., 2013) and which mostly rely on oxidative phosphorylation whereas proliferating melanoma cells utilize glycolytic pathways. The existence of a such heterogeneity within the metabolic pathways of different cancer types, or cancers of different tissues, or even cancer cells within the same tumour, makes the identification of a precise cancer metabolic signature very difficult (Rahman and Hasan, 2015). Nevertheless, drugs that might specifically inhibit key metabolic steps associated with tumour growth are currently at various stages of development. These include drugs that inhibit enzymes involved in glucose uptake and glycolytic pathways, amino acid, fatty acid and nucleotide biosynthesis as well as signaling pathways that modulate cancer metabolism such as TLN-232 (pyruvate kinase inhibitor) or ADI-PEG-20 (inhibitor of arginine synthesis). Although these drugs are not FDA approved for clinical use in melanoma, several are in phase I or II clinical trials (Rahman and Hasan, 2015).

The development of these new strategies is targeted at prolonging clinical benefits and delaying drug resistance. When drug resistance appears, the simplest approach currently used in clinics is to change a patient's therapeutic regimen to another, or to add another compound to the regimen without knowing the real drug resistance drivers. Indeed, repeated biopsies to study genomic alterations after therapies would be invasive and difficult to obtain (Girotti et al., 2016). Moreover, the molecular profile of a tumour evolves dynamically over time (Siravegna et al., 2017). Again, the identification of circulating biomarkers, which would allow for an early assessment of drug resistance might represent a significant leap towards more precise therapeutic approaches. Of note, blood samples contain cell-free DNA, cells and vesicles that can originate from different tissues, including cancers. The possibility of using blood as liquid biopsy to study the molecular landscape of a tumour has attracted remarkable interest and many studies have already illustrated the potential of this method to determine the genomic profile of cancer patients, to monitor treatment responses, to quantify minimal residual disease, clonal evolution and to assess the emergence of therapy resistance (Siravegna et al., 2017). However, before this is becoming routine clinical practice, more sensitive, accurate and affordable methods need to be developed.

In future, further advances in sequencing approaches able to detect low-frequency events occurring before and during treatment together with advances in the field of liquid biopsy, will enable the identification of resistance at earlier timepoints. Consequently, a better guidance will be available towards the choice of a more accurate second-line treatment option, which is crucial to improve patient outcomes in melanoma care.

In summary, this PhD project provides new findings in the field of melanoma biology and treatment. In the first part of this project (Cesi et al., 2017), we mostly focused on drug sensitive melanoma

cells. We described adaptive metabolic alterations in response to BRAF inhibitors and using a PDK inhibitor, we reverted those alterations to induce oxidative stress and arrest cell growth. In the second, third and last part, we focused on melanoma drug resistance and on potential biomarkers. Heterogeneous changes on the miRNome and transcriptome were identified in drug-resistant versus drug-sensitive cells and several interesting candidates will be followed up (Kozar et al., 2017). Finally, we could show that EVs released from drug-resistant cells can propagate resistant traits to sensitive cells by transferring ALK. Therefore, we suggest a new drug combination for ALK and $BRAF^{V600}$ positive cells and the use of EVs to track resistance mechanisms (Cesi et al, in prep). Combining these different approaches and data, we hope to further contribute to improved clinical options for clinical care of melanoma patients.

Chapter 6 References

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