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SUSTAINED LIPOGENESIS AS A KEY MEDIATOR OF RESISTANCE TO BRAF-TARGETED THERAPY

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“A person daringly first raised their eyes to brave the fame of Gods and the terror of lightning and thunder, to be the first to break down the barred-locks to the gates of nature ... This is far from an impious road … as it is superstition itself that makes of a child a sacrificial beast.”

- Lucretius, written 2080 years ago

Acknowledgments

Firstly, I would like to thank all patients that selflessly gave their consent in using material from their bodies, and in sometimes undergoing additional procedures in order to give us the chance of better studying their disease. These selfless acts done in the hope of saving others from suffering from their misfortune, and done knowing that it is irrelevant to their misfortune.

This is a remarkable time to be alive. In the few short years that I have been doing research, I have seen marvellous leaps in technologies, in the advent and the ubiquity of DNA and RNA sequencing technologies, in automated live cell imaging and in mass spectrometry based metabolomics and proteomics. These technologies have been instrumental in starting to shine a light in the dark valley that is cancer, in a way that was inconceivable a decade ago. Furthermore it is a privilege to be in research. There are not many areas in this world that are comparatively free of racism, sexism and discrimination, where people get along regardless of beliefs and work towards a common goal that is in essence noble: to reduce human suffering. This freedom is something that we take for granted every day, however it is noteworthy that academia is a good example of how harmony is very real and achievable.

I am immensely grateful to Johan for all of the lessons that he has taught me. If there is one lesson that is etched in my mind it is that of dedication. Although the level of your dedication to research is impossible to reach it sets a very good example. You have taught me that we exist inside a bigger world and that our goals should be larger than just what you can do inside the lab, there is plenty that we could do, and should do to shape the future in highlighting the importance of research. You have taught me to focus and refine my often scattered goals (with no sense of irony!). And that success is a measure of ever-refining and following through with your beliefs. I admire the respect that you have for your team, and that you are reasonable and are willing to change your mind and to invest heavily to explore our ideas. I have learned that in general, this is rather the exception than the rule in academia and in management.

It has been a privilege to have worked together with so many capable and dedicated individuals over the years, and to be able to take away so many lessons. I am grateful to my co-promoter Chris Marine, Chris, it is a privilege to see mastery of someone at the forefront of their field and I am amazed at your ability to digest the key messages and value of research so quickly. It is a privilege to have worked with, and to continue to work with you. Dear Sarah, I have enjoyed working with you, I admire both your unmatchable and intimidating knowledge of metabolism and your ability to have assembled such a wonderful and successful team; I am sure that this is just the beginning given the development of such a wonderful metabolomics cluster within our university. Dear Patrizia, if I had to pick one lesson that I have taken away from you it's that is it possible to be a perfect gentleman and still be immensely successful. I am privileged to have

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List of abbreviations

ACACA	Acetyl-CoA carboxylase alpha
ACLY	ATP citrate lyase
ACP	Acyl-carrier protein
AMPK	AMP-activated protein kinase
ATF6	Activating transcription factor 6
ATO	Arsenic trioxide
cAMP	Cyclic adenosine monophosphate
ccRCC	Clear cell renal cell carcinoma
CD36	Cluster of differentiation protein 36
ChREBP	Carbohydrate-responsive element-binding protein
CPT	Carnitine palmitoyltransferase
EGFR	Epidermal growth factor receptor
ELOVL	Elongation of very long fatty acid
EMT	Epidermal-mesenchymal transition
FADS1	Fatty acid desaturase 1
FADS2	Fatty acid desaturase 2
FASN	Fatty acid synthase
FAT	Fatty acid transporter
GPX4	Glutathione peroxidase 4
HDAC	Histone de-acetylase
HDL	High density lipoprotein
HGFR	Hepatocyte growth factor receptor
HNE	4-hydroxy-2-nonenal
IDL	Intermediate density lipoprotein
INSIG1	Insulin-induced gene 1
INSIG2	Insulin-induced gene 2
IR	Insulin receptor
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
LPL	Lipoprotein Lipase
LXR	Liver-X-receptor
MBTPS1	Membrane bound transcription factor peptidase 1
MBTPS2	Membrane bound transcription factor peptidase 2
MC1R	Melanocyte-Stimulating Hormone Receptor
MDA	Malondialdehyde
MDM	Mouse double minute homolog
MITF	Microphthalmia-Associated Transcription Factor
MUFA	Mono-unsaturated fatty acid
NAC	N-Acetyl Cysteine
NHEM	normal neonatal human epidermal melanocytes

PD-1	Programmed cell death protein 1
PDTX	Patient derived tumour xenograft
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PPARs	Peroxisome proliferator-activated receptors
PUFA	Poly-unsaturated fatty acids
ROS	Reactive oxygen species
RTKs	Receptor tyrosine kinases
RXR	Retinoid X-receptor
SCAP	SREBP cleavage-activating protein
SCARB1	Scavenger Receptor Class B Member 1
SCD	Stearoyl-Coa desaturase
SFA	Saturated fatty acid
SLC7A11	Solute Carrier Family 7, Member 11
SREBP	Sterol-regulatory element binding protein
TAG	Triacylglycerides
TCA	Tricarboxylic acid
TCGA	The Cancer Genome Atlas
ULDL	Ultra-low density lipoprotein
UPR	Unfolded protein response
UV	Ultraviolet
VLCFA	Very long-chain fatty acids
VLDL	Very low density lipoprotein

Preface

A cell is the smallest unit of life and single celled organisms such as bacteria and amoebae are self-sufficient. They can survive if they exist in or move toward the right environment, can get sufficient nutrients and can evade threats. Successful single celled organisms can divide forming identical offsprings, and are thus essentially immortal. Although flexible, the chemistry and the abilities of single celled organisms are very limited. In contrast, more complex beings such as plants and opisthokonts (fungi and animals) are composed of a group of cells, all specialised into distinct tissues with distinct roles, all working in concert in driving a complexity that would otherwise not be possible in simpler organisms.

In us, this complicity allows a high degree of flexibility in maximising the chance of our continued existence and successful reproduction. With our specialised senses such a sight, smell, hearing and touch, we can assess how best to acquire nutrients and avoid dangers. During periods of plenty, our liver can generate fats from the excess nutrients and this can be stored in specialised adipose tissue. In times of nutrient deprivation, these fats can be hydrolysed and released throughout the blood to fuel the function of our other tissues. Our immune system can exclude external threats that would exploit our tissues for their own ends. Taken together, multi-cellularity gives rise to a number of advantages. However, this wondrous piece of work requires a high degree of communication between tissues and a tremendous degree of ordered control over the individual growth, life and death of every cell. The consequences of the dysregulation of this rigid control can be catastrophic.

To a single celled organism, the question of what is self and what is not-self is quintessentially simple. The organism is defined by a membrane that excludes the external universe, what is self is everything inside that membrane. However, this distinction is much more subtle in single celled beings that cooperate. Some single celled organisms exist in colonies. It is established that a virally infected yeast cell can kill itself rather than risking the life of the entire colony[1]. Likewise, the parasitic trypanosomes rely on nutrients from its host to survive. If their numbers becomes too great in the fly, individual parasites kill themselves to save the clonal colony[2]. Whilst this is bad news for some of the single celled organisms, overall, such altruistic behaviour protects the entire colony. The consequences for having selfish members of such colonies could be devastating.

Animals have a much more clearly defined self and not-self. So much so that if you were to cut two simple sea sponges and graft them together, they would reject and kill each other at the border. In an analogous manner, our immune system recognises what is self and will attack external non-self-organisms and organs. Although some organisms and viruses can successfully circumvent this process by employing various ingenious means, the immune system is generally sufficient in excluding external threats.

More subtle threats emerge from what is self, and through dangerous corruptions becomes something that-was-self but is now altogether something very different. A normal cell generally follows external orders and does what it is told; this is hard-wired in its DNA. However, through bad luck[3], exposure to various chemicals, viruses, or by radiation, the hard-set sequence of DNA can be altered and the hard-wired commands that it entails can change. The DNA* of such a cell can be damaged in such a way as to give rise to proteins that take on unusual functions. These new functions may for example dismantle the response to anti-growth signals from the body or downregulate the ability of the immune system to detect such corrupt cells.

There are robust protective mechanisms where the affected cell detects this damage, attempts to repair itself, but failing that, it will kill itself. In this way, the cell protects the whole organism. Some of these transformed cells can be so damaged that these fail-safes fail altogether. In the majority of these cases, such corrupted cells are detected and killed by our ever-vigilant immune system. There are exceptions, in that some of these corruptions in the DNA of these cells give rise to just the right combination of unusual functions as to allow the corrupted cell to selfishly grow, divide, reject orders from the body and effectively become a quasi-living organism living and existing in the body at its expense.

The generation of such a cancer requires several successive steps, so its overall probability is low. This is why the higher the rates of insults, for example the number of cigarettes smoked, more extent of exposure to the sun, and increased ageing all additively contribute to the probability of developing cancer. Of course, this is a matter of probability, whilst reducing risks can certainly contribute in lowering the risk of developing some kinds of cancer, the majority of cases are simply due to bad luck[4].

A successful cancer takes on a number of abilities, the most potent of which is arguably the downregulation of processes that maintain the integrity controlling cell division. Cell division is normally robustly controlled so that the exact division of DNA occurs without fault to the dividing cells. By circumventing this tightly regulated process, cancer cells adopt a high-risk high-gain strategy in gaining the ability to acquire mutations that can have profound new effects. This ability may mean that a majority of issued cancer cells acquire unhelpful or even deleterious mutations in the daughter cells, but it does also give the cancer cell flexibility. If the mutation changes a protein in just the right way, the cancer

* Imagine DNA as a robust library, that is passes down generation to generation, set in gold, and is unchanging. This library contains the instructions for every process that the cell and the entire body is capable of. When it is necessary to start a process, image that a scribe carefully enters the library section, finds and begins to copy the correct book, and then leaves the library to pass on its precise instructions. This process is called transcription where DNA is transcribed to RNA. From here, the scribe assembles a group capable of assembling a machine that will allow the actual work to be done, thereby translating the instructions from the library to actual work. This process is called translation, where proteins are translated from the instructions of the RNA molecule. Proteins are the final effectors in cells, carrying out the final metabolic or structural functions. We can effectively generate more than 100,000 different proteins.

cell may suddenly have gained a new function. In order to illustrate the awesome flexibility that this property gives cancer cells, we can look at the power of evolution.

For example, a few mutations are necessary and sufficient in altering the DNA polymerase enzyme so that it can function in either us, in organisms from the permafrost of Antarctica, to bacteria in a pool of boiling acid. By unlocking the power of evolution, cancers can rapidly develop completely new abilities, and can thereby develop resistances to therapy after therapy. This makes the treatment of disseminated cancers particularly challenging, as they can no longer be removed by simple surgery.

A key and promising strategy in anti-cancer research is to try and find common convergent pathways in cancer; in trying to see if all of these various mutations lead to common properties that cancers need to acquire. Finding and exploiting these bottlenecks is only possible thanks to an array of recent technologies. For the first time in history, technology is allowing us to 1 - precisely look at every single mutation in cancer cells, even in individual cancer cells, 2 – identify general convergent pathways that are activated by global changes to the epigenome, studying RNA and the quantity and post-translational changes in proteins, and 3 – in identifying the amount of effector metabolites that are essential to specific processes in cancers. These emerging technologies hold tremendous promise for the discovery of targetable vulnerabilities in cancer.

Chapter 1: Introduction

1. Cancer

Cancers are diseases that are characterised by the unregulated and proliferative growth of cells. Claiming more that 8 million lives per year, cancer is the second leading cause of death globally, with a global mortality burden nearly 1 in 6. This amounts to an economic cost of about $1.2 \cdot 10^{12}$ USD. Around 33% of cancers are preventable by behavioural or dietary interventions, of which 66% is the result of tobacco use. Cancer causing viral infections account for a further 25% of cancers, with a higher burden in low income countries[5]. As the population ages and global income increases, non-preventable forms of cancer are likely to increase. In addition, whilst some leading behavioural causes such as tobacco use decreases in high income countries, other behaviours such as sun-seeking increase, eventually causing different cancer burdens.

The processes that underlie and power cancer's unregulated and proliferative growth are dizzyingly complex and varied, making its treatment challenging. Processes that are necessary in generating and in sustaining cancer are conceptually characterised in the Hallmarks of Cancer. Classically, these hallmarks include self-sufficiency in sustaining proliferative signalling and in dampening anti-growth signalling, overcoming brakes that limit cell proliferation in essentially obtaining immortality, resisting multiple endogenous cell death mechanisms, inducing angiogenesis to fuel the tumour growth and importantly in activating pathways that allow cancer cells to infiltrate and metastasise to distant sites. More recently, four additional cancer hallmarks are firmly established. Genomic instability, immune stealth, immune evasion, and altered cellular energetics give cancers significant flexibility in rapidly responding to changing needs to therapies (Figure 1).[6]

Cancer can originate in almost any organ or tissue type, with cancers of the skin being the most common types in white populations.

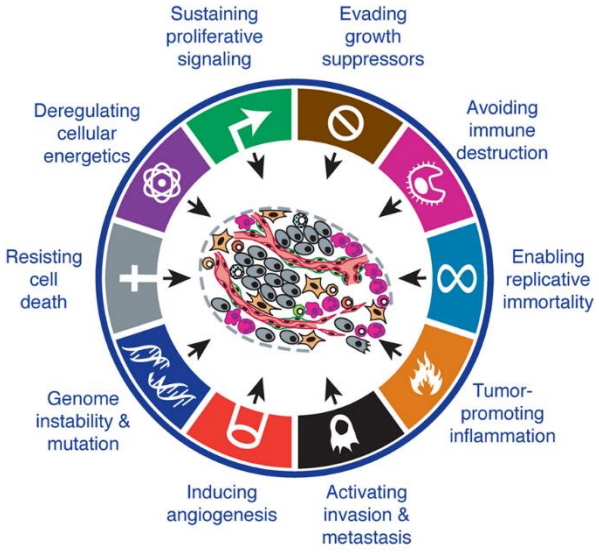


Figure 1. ***Hallmarks of Cancer.*** Cancers are conceptually characterised by multiple Hallmarks that are necessary in sustaining multiple cancer processes.[6] *Retrieved from: Hanahan and Weinberg, 2011*.

2. The skin

The skin is the largest organ which comprises approximately of 1/7th of the human body weight. The skin primarily provides a barrier function in retaining and regulating moisture, in providing sensory feedback from the environment and in keeping out and in reacting to pathogens.

Architecturally, the skin is made of three layers, the epidermis, the dermis and the subcutis. The epidermis is primarily made of keratinocytes which rapidly proliferate in the lower layers of the epidermis and die and harden towards the upper layer of the epidermis. At the base of the epidermis, pigment producing melanocytes surround groups of keratinocytes and secrete melanin, protecting the cells from ultraviolet (UV)-induced DNA damage. Hair follicles are found in the collagen rich dermis and sebaceous glands that secrete waxes, oils and fatty acids which provide a number of protective functions[7]. Melanocytes can also be found at the base of hair follicles. The subcutis is composed of connective tissue and adipocytes, and provides important functions in thermal insulation and in vitamin D production (Figure 2).

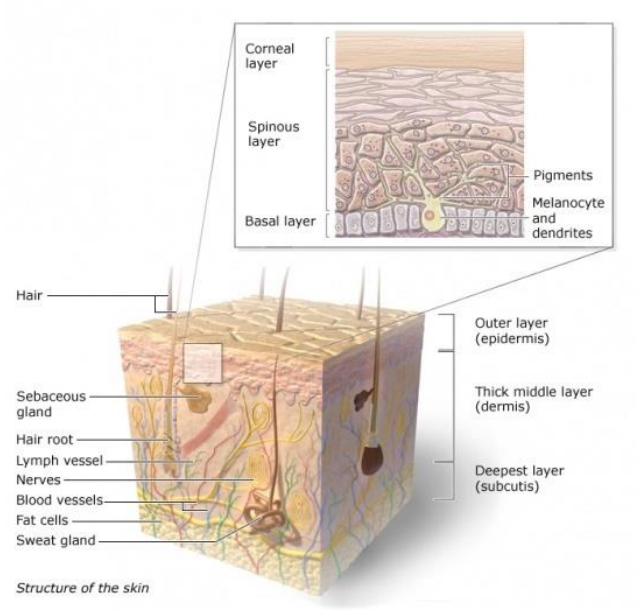


Figure 2. ***Skin architecture.*** The skin is architecturally made of three layers, the epidermis, the dermis and the subcutis[8]. *Retrieved from: Pubmed 2016.*

2.1 Melanin and melanocytes of the skin

Pigments are commonly found in living organisms, ranging from complex plant carotenoids and flavonoids to substances that are also synthesised in animals including anthraquinones, aphins, pterins, tetrapyrroles, ommochromes, papiliochromes and melanins[9]. Dyes including melanins are generally poly-cyclic polymers derived from either linear or cyclic ketides or amino acid precursors. Pigments have a plethora of evolutionary roles, including a role as antioxidant molecules, in courtship and mating rituals, and in camouflage.

Melanins are a group of evolutionary ancient dyes that are ubiquitous in animals and are synthesised from the oxidation and cyclisation of tyrosine. In mammals, these include eumelanin, pheomelanin, and neuromelanin. Eumelanin is the major melanin present in the skin. Pheomelanin is synthesised from tyrosine and cysteine, has a pink colour and is present predominantly in the mucosa. Neuromelanin is synthesised by a specific subset of neurons. High amounts of neuromelanin appears to be uniquely human, whilst neuromelanin is also found in the brains of primates to a lesser extent[10].

In mammals, eumelanin biosynthesis is limited to melanocytes, a specialised type of neural crest-derived skin-resident pigment synthesising cells. Early in embryogenesis, melanocytes migrate along nerves from the neural crest into the epithelial structures that later form the skin[11]. In humans, melanocytes reside predominantly in the basal layer of the dermis and also in hair follicles, whereas in other mammals they only reside in hair follicles[12]. Melanocytes are highly dendritic in architecture, and are in close contact with a group of 30 – 40 keratinocytes in forming epidermal melanin units (Figure 3).

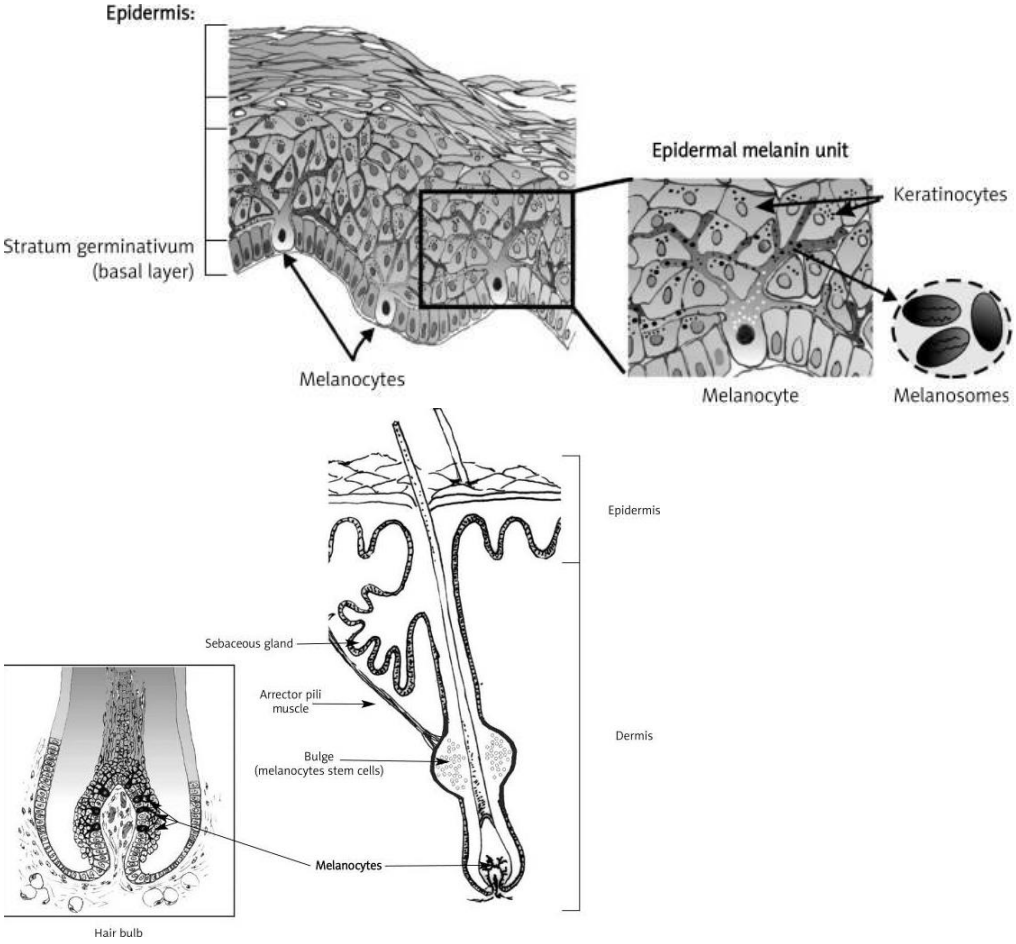


Figure 3. ***Melanocytes in the skin*****.** Melanocytes are found in the basal layer of the epidermis and in hair follicles. In the epidermis, melanocytes surround groups of keratinocytes with dendrites forming epidermal melanocyte units. Melanocytes can also be found at the base of hair follicles, and melanocyte stem cells at the hair bulge[12]. *Retrieved from: Cichorek et al., 2013.*

Melanocytes function predominantly as eumelanin producing cells, and can be identified based on the high expression of melanogenic proteins including tyrosinase, tyrosinase-related proteins 1 and 2 and Microphthalmia-Associated Transcription Factor (MITF). MITF is a transcription factor that is the master regulator of melanogenesis and promotes the expression of melanogenic enzymes. MITF also upregulates anti-oxidant and DNA-repair pathways. MITF acts downstream of receptor tyrosine kinases (RTKs) and is a downstream target of KIT and the Map Kinase pathway[13]. MITF is induced indirectly by UV radiation. UV results in oxidative stress and in DNA damage which results in P53 stabilisation. In keratinocytes, this results in the transcription, processing and export of α–melanocyte-stimulating hormone which binds the melanocyte-Stimulating Hormone Receptor (MC1R) receptor on melanocytes and initiates cyclic adenosine monophosphate (cAMP) signalling, promoting MITF activation.

Melanin is synthesised in specialised lysosome-like organelles called melanosomes where its production is tightly regulated by the melanogenic enzymes tyrosinase, TYRP1 or gp75 and TYRP2[14,15]. This could be in part due to the toxicity of the intermediates in melanin biosynthesis. Melanin in melanosomes is transported by Rab27a, complexed with MLPH and MYO5A, and exported from the cell. Rab27a is a member of the rab superfamily of proteins which are involved in vesicle trafficking and the trafficking of lipids. Melanosomes are taken up into surrounding skin cells, where they have protective roles due to melanin's ability to absorb light (Figure 4).

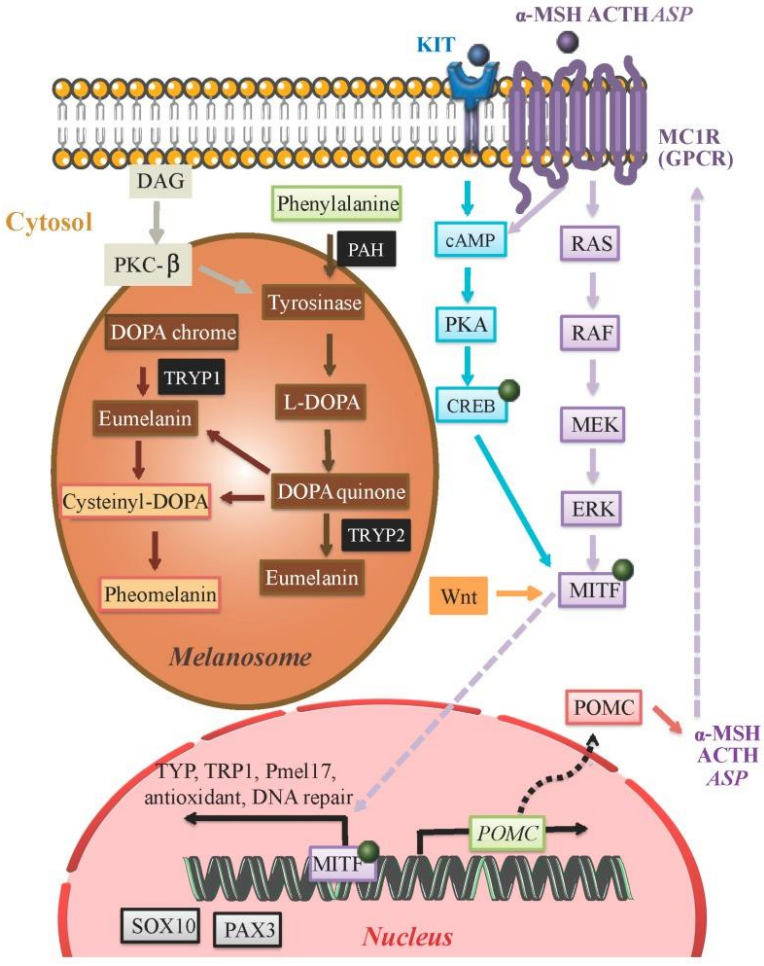


Figure 4. *Regulation of melanogenesis*. Melanin biosynthesis is tightly regulated by a number of external signalling cues converging on the activation MITF. Melanin biosynthesis is specially restricted to melanosomes[16]. *Retrieved from D'Mello et al., 2016.*

In mammals, melanocytes are restricted to the base of the hair follicle. Uniquely, in humans, melanocytes are found in the base of the dermis and not only give hair, but also the skin its pigmentation. This is thought to be a recent evolutionary adaptation which coincided with the loss of body hair in humans. In primates and in the great apes, skin remains pale. Diseases such as albinism give insight as to the function of melanin in humans, as albinism is associated with rapid onset of cancer in sun exposed individuals. Melanin's role is predominantly the protection of DNA from damage by UV radiation. Melanin broadly absorbs both UV and visible light with an absorbance peak of 350 nanometres. High pigmentation also prevents the degradation of several secondary metabolites such as folates and vitamin D[17]. However, UV radiation is required to generate Vitamin D, therefore high pigmentation can interfere with this process. The necessity in generating sufficient Vitamin D could in part explain both the selection pressure towards depigmentation and the prevalence of tanning in human populations that migrated from Africa[17].

In addition to photo-protective roles, melanin is also shown to have anti-oxidant and metal chelating properties[18]. These functions could hint towards the role of neuromelanin in protecting brain cells from oxidative damage through direct anti-oxidant action, and importantly by iron chelation. Neuromelanin is shown to prevent the onset of Parkinson's disease in some models and may function as a negative regulator of neurodegeneration and ferroptosis[19].

2.2 UV-induced DNA damage and melanoma aetiology

DNA is a structurally stable molecule and is a largely inert polymer, however it can be reactive under certain circumstances. Aside from cytotoxic compounds that can cause direct DNA damage by DNA-binding, UV radiation can cause indirect DNA damage. UV radiation can excite olefin functionalities within the pyrimidine nucleotide bases of DNA, and is generally emitted in the form of reforming the double bond and generating heat. Depending on the local structure of DNA, it is possible that two adjacent thymine bases react in a UV induced pseudo-Diels Alder cycloaddition reaction creating a thymine - thymine dimer. Thymine-cytosine and cytosine-thiamine can also occur (Figure 5)[20].

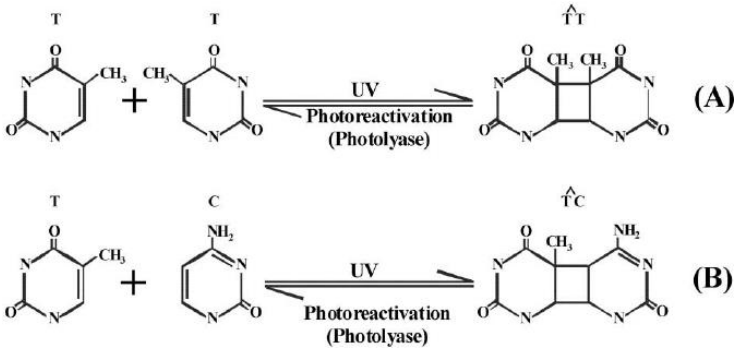


Figure 5. UV induced cycloaddition of pyrimidines[20]. As opposed to 4pi + 4pi thermally allowed cycloaddition reaction that can proceed due to the formation of a pseudo-

aromatic transition state, formation of 2pi + 2pi cycloaddition reactions does not occur. However, under UV excitation, this reaction becomes possible forming a stable 4 membered carbon ring structure. For example, thymine-thymine dimers (A) and thymine-cytosine dimers (B) can occur as shown. *Retrieved from Mouret et al., 2006.*

These errors are generally repaired by the nucleotide excision repair mechanism, however, an inherent bias in C to T transformation results in UV signature mutations in melanoma and in skin cancer, the frequency of which increase with UV exposure (Figure 6)[21,22]. Generally, mutations activating oncogenes rapidly drive cell senescence, thereby forming benign melanocytic nevi[23,24]. Although nevi can form melanoma, most melanoma do not develop from nevi, suggesting additional transformative steps. Although it was previously assumed that any melanocyte can transform into a melanoma, recent evidence suggests that mature pigmented melanocytes are more likely to form melanoma following de-differentiation, pointing towards transformative roles of the stroma[25].

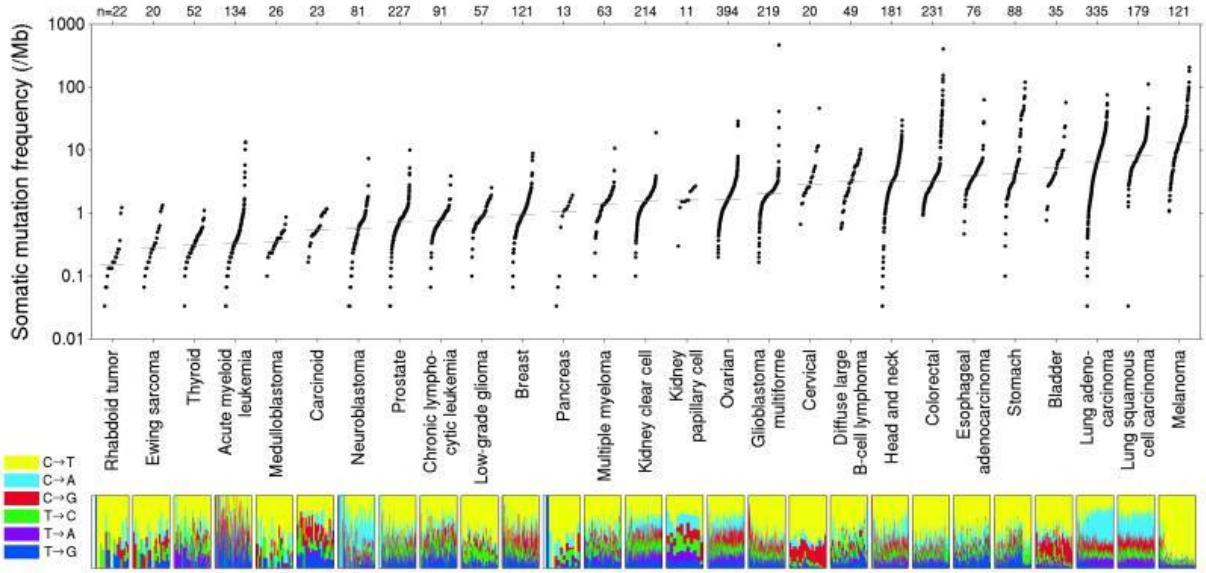


Figure 6. ***Mutational frequency and modality across cancer types*****.** The mutational modality in melanoma is generally a C to T transformation due to the inherent bias in the nucleotide excision repair mechanism. In addition, melanoma has the highest somatic mutation rate in cancers, likely due to the long-term accumulation of UV induced DNA damage[21]. *Retrieved from Lawrence et al. 2013.*

3. Melanoma

3.1 Types of melanoma

Cancers of the skin are amongst the most common malignancies, and can be divided between basal cell carcinoma, squamous cell carcinoma and melanoma. Melanoma is the rarest type of skin cancer, but has the most dismal prognosis. The most common type of melanoma is skin cutaneous melanoma which arises from the skin. In women, there is a higher probability that the primary skin cutaneous melanoma occurs on the arms and legs, whereas in men it is more likely to occur on the head and neck or the torso. This difference is likely caused by the differences in sun exposure of these skin sites. Rarer types of melanoma include desmoplastic melanoma which occurs mainly on chronically sun damaged skin of older patients, and uveal melanoma. In general, melanoma occurs most frequently in light skinned individuals and is much rarer in darker skinned individuals. For example, melanoma incidence across Asian and African populations is 1 in 100,000 people. However, the rate is as high as 60 in 100,000 individuals in Australia and in New Zealand[26]. This is due to the confounding effects of a predominantly light skinned population and increased UV intensity due to a thinner ozone layer. An exception is subungual melanoma which occurs with similar frequency across all races. Furthermore, melanoma incidence is increasing, likely due to changes in behaviour leading to greater sun exposure, especially in at risk populations.

Melanoma has a rapid propensity to metastasize, first to lymph nodes and then to virtually any target organ. There are exceptions, for example in uveal melanoma which predominantly metastasizes to liver.

3.2 The Map Kinase pathway in melanoma

The Map Kinase pathway is also known as the Ras/Raf/MEK/ERK pathway and is quintessentially a proliferative pathway, with additional roles in cytoprotection and differentiation. Extracellular growth factors activate Map Kinase signalling by binding to a number of RTKs, such as the epidermal growth factor receptor (EGFR), hepatocyte growth factor receptor (HGFR) and the insulin receptor (IR), leading to receptor dimerization and the initiation of the signalling cascade. Lipid anchored (through prenylation or palmitoylation) and plasma membrane bound RAS is phosphorylated, and in turn activates RAF proteins which subsequently activate ERK proteins. Phosphorylated ERK can enter the nucleus and activate multiple transcription factors which mediate the effects of the Map Kinase pathway (Figure 7).

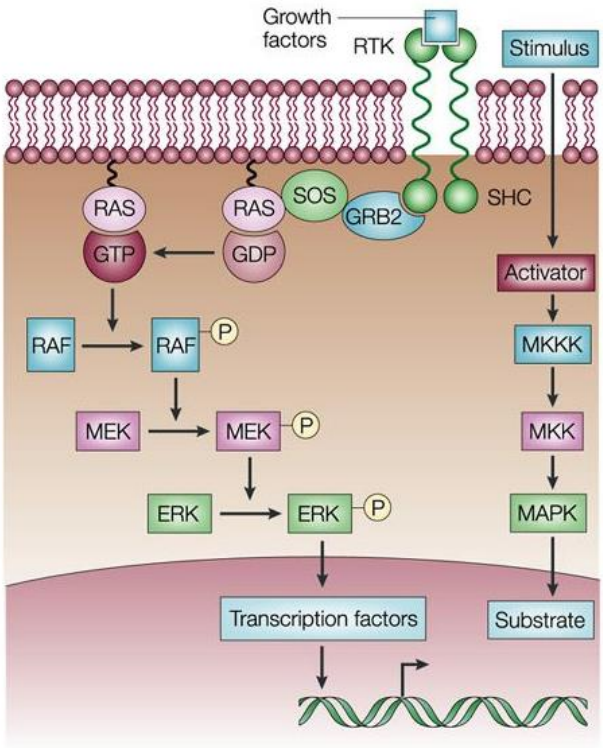


Figure 7. *The Map Kinase pathway*. The Map Kinase pathway converges on the activation of ERK proteins which mediate a plethora of cellular functions including promoting proliferation and cell survival[27]. *Retrieved from Chin 2003.*

As the Map kinase pathway plays a central role in key cellular processes, components of the pathway are often amplified and mutated and form oncogenes in several cancer types. Map Kinase pathway constituents are altered in 25% of all cancers. In addition to low frequency mutations in most cancers, specific mutations in Map Kinase components are common in some cancer types. In general, activating mutations in these kinases results in a conformational shift, 'fixing' the proteins in their active, phosphorylated conformation.

Oncogenic activation of KRAS occurs in 70% of pancreatic cancer. NRAS mutations are frequent in melanoma (20%) and also occur in other cancers, with significant subsets of NRAS mutated testicular, thyroid, AML and colorectal cancers. BRAF mutations occur in over half of all melanoma and thyroid cancers.

Due to the high rate of activating driver mutations in the Map Kinase pathway, targeting the Map Kinase pathways is a strategy in anti-cancer treatment. To this end, multiple Map Kinase pathway inhibitors have been developed and are clinically used or in clinical trials. The first approved Map kinase inhibitors targeted oncogenic BRAF in the context of melanoma[28].

The majority of skin cutaneous melanoma have constitutively high Map Kinase signalling. The most frequent oncogenic mutations found in skin cutaneous melanoma are activating mutations in BRAF (50%), NRAS (20%) and KIT (10%). BRAF and NRAS mutations are mutually exclusive, pointing towards the sufficiency of Map Kinase activation in

melanoma. NF1 which negatively regulates Map Kinase signalling is mutated in 10% of cases. Surprisingly, inactivation mutations or a homozygous loss of TP53 (10%) and PTEN (15%) are infrequent[29]. However, P53 is inactivated in melanoma by increased mouse double minute 2/4 (MDM2/4) homolog signalling[30]. The mutational landscape is different in other forms of melanoma, making effective treatment more challenging.

The most frequent activating mutation in oncogenic BRAF is a thymine to adenine substitution (T1799A) in the gene sequence. This genetic mutation drives a substitution of valine to glutamate (BRAF-V600E) of the gene product. 75% of BRAF mutant melanoma carry the resulting BRAF-V600E mutation[29] and a significantly smaller proportion of patients carry the BRAFV600K mutation. Functionally, both mutant proteins constitutively activate BRAF and functionally equivalent. Wild-type BRAF is activated by phosphorylation by the serine-threonine Ras proteins at T599 and S602. It is therefore likely that a V600E substitution mimics BRAF phosphorylation, thereby driving the constitutive activation of the protein.

3.3 BRAF-targeted and other melanoma treatment

Early melanoma detection has the highest positive effect on prognosis. If detected early, curative surgery is likely. Patients and primary care physicians follow the so called ABCDE rule in early melanoma detection[31]. Histological analysis is required if the suspicious nevus has an asymmetric shape ('A'), an irregular border ('B'), is pigmented ('C'), has a diameter of greater than 6 mm ('D'), or changes in any of the previous ('E'). Treatment of metastatic melanoma is far more challenging. Before the advent of targeted therapies, melanoma response to classical chemotherapy such as carboplatin and dacarbazine was poor and hence the prognosis was dismal.

The discovery that a majority of skin cutaneous melanoma carry an activating mutation in oncogenic BRAF[29,32] led to the generation of drugs that target this oncoprotein. Vemurafenib was the first FDA approved effective anti-Map Kinase targeted therapy and specifically inhibits BRAF-V600E. Vemurafenib elicited a response in nearly half of all BRAF mutant patients when compared to only 5% in classical chemotherapy[28]. However, BRAF inhibitors lead to the paradoxical induction of wild-type BRAF activity and can drive the acquisition of secondary tumours[33]. The combination of co-targeting BRAF and MEK is a clinical standard of care for BRAF mutant melanoma[34]. Approved combinations used include Vemurafenib + Cobimetinib and Dabrafenib + Trametinib, however further compounds are in clinical trials such as the paradox-breaking BRAF inhibitor PLX8394[35]. Unexpectedly, the combination of BRAF/MEK inhibitors is better tolerated than BRAF monotherapy[36]. In spite of this unprecedented effect in melanoma, patients rapidly develop resistance to therapy[37]. Multiple modes of both intrinsic and acquired therapy resistance have been extensively described, and are expanded on below.

In addition to the steady success of targeted therapies in melanoma, immune checkpoint inhibitors targeting programmed cell death protein 1 (PD-1) such as Nivolumab and

Pembrolizumab, and CTLA-4 such as Ipilimumab have further revolutionised treatment. A small subset of melanoma patients treated with Ipilimumab (20%) show a sustained response even after three years[38]. The combination of Nivolumab and Ipilimumab elicited the best response to date[39]. Patients respond to anti-immune checkpoint therapy irrespectively of the driver mutation. It is currently unknown as to why some patients respond and there are no easily accessible biomarkers of response. The best indicator of response to anti-immune checkpoint therapy is tumour lymphocyte infiltration or mutational load. Brisk and T-cell infiltrated melanoma have both a better prognosis and respond better to anti immune checkpoint therapy than non-brisk cases[40]. Furthermore, mutational load is indicative of a good response to immune checkpoint inhibitors, likely due to the increased probability in generating more tumour neo-antigens[41,42].

3.4 Resistance to melanoma targeted therapy

Although combined BRAF and MEK inhibition in BRAF mutant melanoma generally meets with a favourable initial response, a significant minority of patients are intrinsically resistant to BRAF inhibition therapy (20%)[43], and to BRAF/MEK inhibitor therapy (6 %)[34]. The mechanisms of intrinsic resistance to Map Kinase inhibition are poorly understood, however several pathways converge on the upregulation of Akt signalling[44].

Acquired resistance to BRAF/MEK inhibitor therapy occurs in the majority of cases[43]. 90% of patients acquire drug resistance following 5 years of treatment[45], with a median time to disease progression of 12 months[46]. Many mechanisms of therapy resistance have been extensively described in patients and in pre-clinical models, generally leading towards the re-activation of Map Kinase signalling and/or phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) pathways[47]. Furthermore, different mutational events can be selected from the drug resistant clones of the same patients and can even co-occur in the same lesion[48,49].

Several genetic modes of resistance acquisition have been described. BRAF amplification and alternate splicing[50], sometimes concurrent with MEK mutations lead to re-activation of Map Kinase signalling, thus overcoming t BRAF inhibition. This is seen in about 20% of patients[50,51]. Secondary acquisition of an oncogenic NRAS mutation and increased RTK signalling is observed *in vitro* and well as in patients[52].

A number of non-genetic modes of resistance have been described. Even in drug naïve melanoma, increased hypoxia or other stress responses are shown to induce epigenetic remodelling that increases multi-drug resistance by enhancing innate stress response pathways[53]. This phenomenon also occurs in BRAF/MEK treated patients and could in part mediate resistance to therapy[51]. Loss of PTEN and upregulation of PI3K/Akt signalling is commonly seen in *in vitro* and *in vivo* models[54,55]. Increased TGF-beta signalling leads to increased EGFR signalling which can overcome the inhibitory response to BRAF inhibitor treatment[56]. High EGFR signalling in colon cancer could in part explain their poor response

to BRAF inhibitors[57]. Increased RTK signalling has been reported to overcome the inhibitory effects of Map Kinase inhibition[52,58,59].

MITF is downregulated as a target of BRAF upon Map Kinase inhibition, and is often re-activated in therapy resistant melanoma[60–62]. Furthermore, MITF has been shown to mediate therapy response in melanoma[63]. MITF state is in part associated with a phenotypic switch in melanoma and may underlie changes in invasive or proliferative states[64].

3.5 Overcoming resistance to therapy in melanoma

Myriad genetic and non-genetic pathways can cause resistance to targeted therapy in melanoma, making treatment challenging. There is likely high redundancy in signalling pathways that can reactivate resistance to Map Kinase inhibitors. Therefore exploiting targets that are well-downstream of Map Kinase signalling is attractive. To date, several mechanisms of overcoming therapy resistance targeting myriad targets have been described in pre-clinical models.

Since a majority of therapy resistant BRAF mutant melanoma acquire resistance by re-activation of Map Kinase signalling, targeting ERK1/2 could be a good means of overcoming therapy resistance. ERK inhibition has been shown to overcome resistance in several pre-clinical models, especially when combined with PI3K inhibition[65–67]. A major limitation of this approach is the paucity of ERK inhibitors, however, recently several are currently in phase I clinical trials[68,69].

MITF is shown to play key roles in melanoma oncogenicity, and its expression levels closely correlate with a proliferative phenotype. Importantly, MITF expression is reduced upon Map Kinase inhibition in melanoma and is generally restored in therapy resistance. Histone de-acetylase (HDAC) mediated inhibition of MITF inhibits melanoma proliferation[70]. Furthermore, inhibition of MITF by nelfinavir mediated proteasomal degradation both sensitises BRAF and NRAS mutant cells to Map kinase inhibition and overcomes therapy resistance in a melanoma model with a secondary NRAS mutation[71]. However, MITF may be dispensable to melanoma resistance as MITF low cells have been observed in multiple models and MITF low status is linked to an invasive phenotype in melanoma[64]. Indeed a recent study observed that restoration of MITF in drug resistant models was lethal in the context of drug withdrawal[72]. Finally, the low specificity of these drugs does not preclude other MITF independent mechanisms of action. For example, HDAC inhibitors have a plethora of cellular effects, and it has recently been shown that an HDAC inhibitor results in cell death induction by ROS[73]. In addition, nelfinavir is also an inhibitor of MBTPS2 which inhibits both Activating transcription factor 6 (ATF6) and sterol-regulatory element binding protein (SREBP) processing. Both the unfolded protein response (UPR) and lipogenesis (this thesis) are shown to be involved in therapy resistance in melanoma[74,75].

The recent advent of single cell sequencing technologies has allowed the interrogation of melanoma and minimum residual disease models[60,76]. Studying minimum residual disease is an attractive means of dissecting the identity of remaining populations, and thereby targeting them, however the complexity of the remaining populations complicates this approach; often multiple distinct populations are observed. Nevertheless, a recent study shows that during drug tolerance, there is an enrichment of a neural crest like – retinoid X-receptor (RXR) driven minimal residual disease cells, whereby targeting this population increases tumour free survival in pre-clinical patient derived tumour xenograft (PDTX) models[60], pointing towards the promise of this approach.

Drug dosing can of itself be used to delay the acquisition of resistance in tumours in some models. Upon the acquisition of BRAF inhibitor resistance, some tumour models are shown to be drug addicted and have a growth defect if drugs are withdrawn[77]. Furthermore, tumours that were intermittently dosed with drugs had a delayed onset of resistance, in part attributed to the growth defect of resistant clones under drug withdrawal. A further study showed that overcoming therapy addiction requires transcriptional reprograming which is reminiscent of epidermal-mesenchymal transition (EMT). In melanoma, overcoming drug addiction upon drug withdrawal relied on low MITF, and MITF restoration was lethal to these cells[72]. A recent clinical trial re-challenged patients that are resistant to BRAF/MEK therapy and have been off-treatment with BRAF/MEK inhibitors, and astonishingly found a partial response in a minority of patients, pointing towards the promise in this strategy[78]. However, the application of this strategy to promote survival is doubtful due to the heterogeneity of drug resistant phenotypes and may be ineffective in cases of mutational driven drug resistance, by for example and acquired NRAS mutation.

Overcoming or delaying resistance acquisition by exploiting acquired vulnerabilities incurred by drug treatment is an attractive strategy. Several recent studies have identified that upon BRAF/MEK inhibitor treatment in melanoma, drug resistant minimal residual disease cells adopt a low metabolic / ROS sensitive phenotype. Further increasing cellular reactive oxygen species (ROS) by GPX4 or solute Carrier Family 7, Member 11 (SLC7A11) inhibition is shown to be cytotoxic to minimal residual disease cells in these models[73,79,80]. Targeting metabolic dependencies in drug tolerance in this context is a promising strategy, especially as often key metabolic enzymes drive an entire pathway. To this end, we have shown in this thesis that drug resistant melanoma sustain therapy resistance by sustaining lipogenesis, and that inhibition of lipogenesis re-sensitises cells to ROS[75].

4. Lipid metabolism

Lipid metabolism is a subset of metabolism, i.e. the collective group of chemical reactions that occur in living organisms. In order to conceptualise the myriad reactions that occur in living systems they are divided into distinct conceptual subunits, which are by no means independent of each other. To illustrate this point, lipids were originally defined as any cell or tissue extract that preferentially dissolved in octanol and are insoluble in water.

However, in order to rationalise metabolic pathways, lipids are now defined as metabolic products of specific metabolic pathways including the mevalonate pathway, fatty acid pathway and the isoprenoid pathways. Lipids can be further modified by other cellular pathways to form a dizzying multitude of chemical species as diverse as including glycerophospholipids, glycolipids and eicosanoids.

The basic building blocks of many lipids are fatty acids. Fatty acids are aliphatic hydrocarbon chains with a carboxylic head group. They can be derived from the diet or synthesized *de novo*. In mammals *de novo* fatty acid synthesis initially produces the saturated fatty acid (SFA) palmitic acid which can be further desaturated and elongated by other pathways. *De novo* lipogenesis is defined to a limited number of tissues including adipocytes and the liver. Most other tissues are not or poorly lipogenic, and rely on the uptake of lipids from the circulation and from the diet (Figure 8).

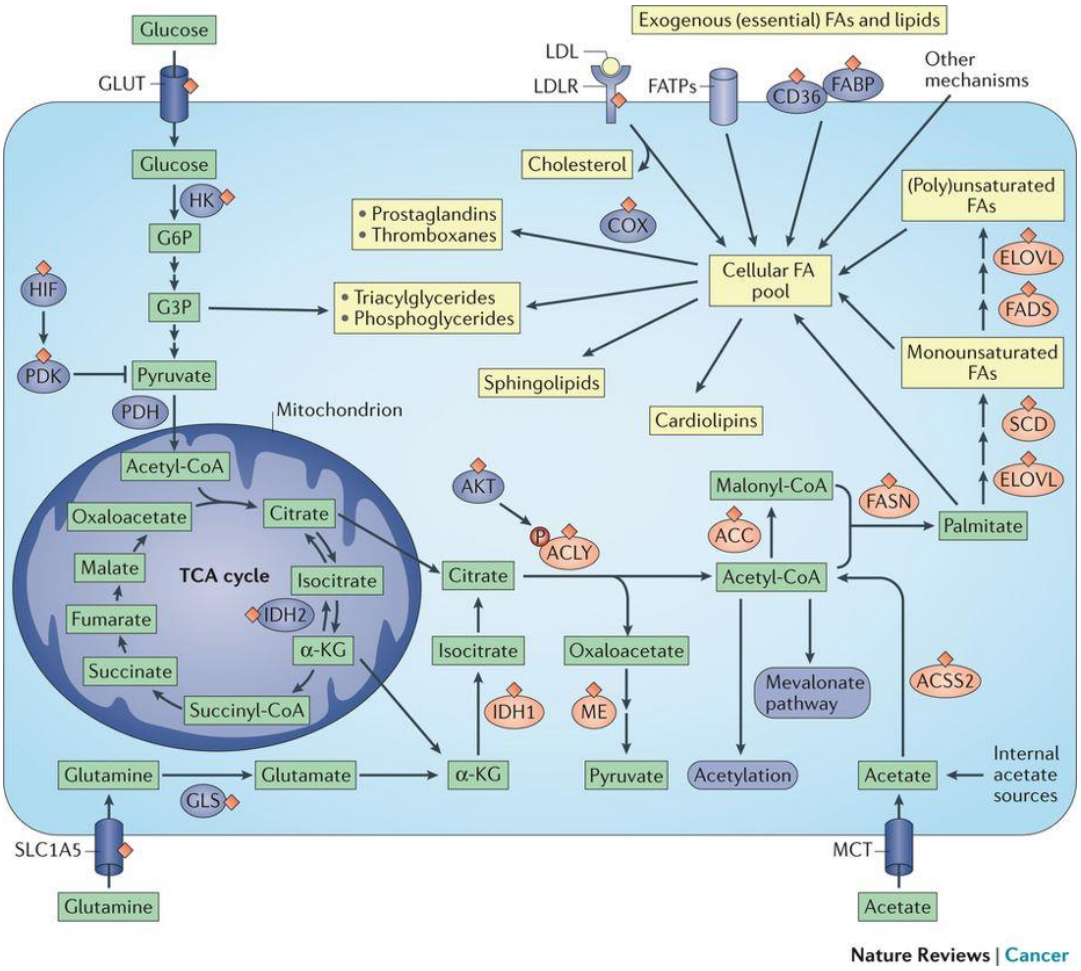


Figure 8. *Cellular lipid metabolism*. Lipogenesis requires glucose, glutamine or acetate generated Acetyl-CoA which is converted to palmitate. Fatty acids and lipids can be taken up extracellularly from the circulation, of which the diet is a major contributor. Fatty acids can be further by desaturases and elongases and used in a plethora of cellular functions, including in forming membrane lipids. *Retrieved from Röhrig and Schulze 2016.*

4.1 Fatty acid biosynthesis

In lipogenic tissues, palmitic acid can be synthesised from citrate. In normoxia, glucose is a major source of citrate, however, in hypoxia, glutamate becomes the major carbon source. Using Co-enzyme-A (CoA) as cofactor, the ATP dependent enzyme ATP citrate lyase (ACLY) both cleaves citrate into Acetyl-CoA and oxaloacetate and shuttles the products into the cytosol. Citrate itself can be derived from the tricarboxylic acid (TCA) cycle or can be derived from the beta-oxidation of fatty acids. Generally, there are low amounts of free acetate in the cell as it is rapidly used as an intermediate in chemical reactions. Oxaloacetate can be converted to pyruvate which generates Nicotinamide adenine dinucleotide phosphate (NADPH). NADPH itself is necessary to give reductive power to drive fatty acid synthesis. Acetyl-CoA carboxylase alpha (ACACA) carboxylates a molecule of Acetyl-CoA. The thioester bonds of Acetyl-CoA and Malonyl-CoA is broken and form thioester bonds with the Acyl-carrier protein (ACP). ACP is a highly conserved enzyme which bears homology to non-ribosomal peptises which highlights its early evolutionary origins. ACP shuttles both Aceto-Acyl-ACP and Malonyl-ACP to the multi-subunit enzyme fatty acid synthase (FASN) where successive rounds of condensation, reduction, dehydration and reduction reactions lead to palmitate synthesis. FASN is a multi-subunit monogenic enzyme in Mammalia but exists as distinct subunits in bacteria and plants. The FASN subunits retain the same function as the individual monomeric subunits in bacteria and plants.

Firstly, FASN drives the spontaneous decarboxylation of Malonyl-ACP which reacts with Acyl-ACP leading to the creation of 3-keto-Acyl-ACP and a loss of CO_2 and an ACP protein. FASN then drives an NADPH dependent reduction of the beta ketone to form 3-hydroxy-Acyl-ACP. FASN then drives the loss of an alpha proton on 3-hydroxy-Acyl-ACP which isomerises to bind with the ketone oxygen generating a tetrahedral intermediate. The subsequent reaction drives the overall loss of the hydroxyl group, thereby forming water as a leaving group, leaving the alpha-beta unsaturated Enoyl-ACP product. A final round of NADPH powered reaction drives the reduction of the olefin to generate butyryl-ACP. In turn, butyryl-ACP reacts with Acyl-ACP as before, in generating a fatty acid that is two carbon units longer. Successive cycles of these reactions leads to the generation of palmitate as the final product of FASN (Figure 9).[81] However, there is increased evidence that FASN may not have as high a fidelity as previously though. Whilst in most tissue, FASN generates palmitate, there is accumulating evidence that in metabolic disease and in cancer, the successive reactions that generate palmitate may prematurely terminate generating myristate, a normally rare fatty acid (unpublished data).

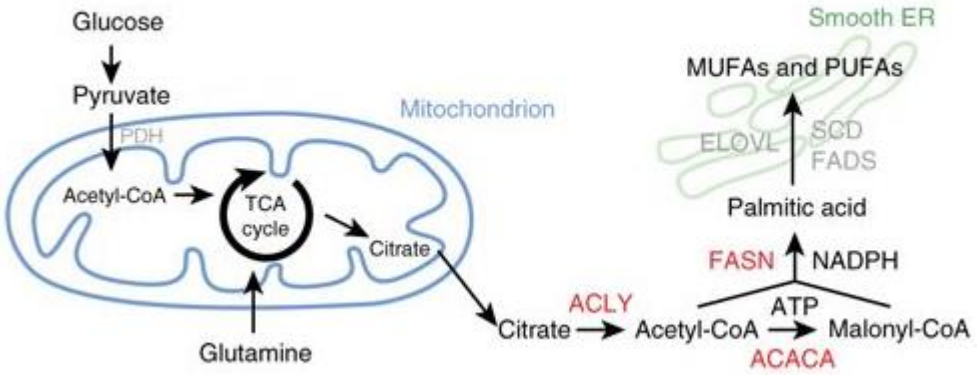


Figure 9. *Lipogenesis*. *De novo* lipogenesis involves the synthesis of palmitic acid from citrate. Palmitic acid can be further remodelled by desaturases and elongases. *Retrieved from Talebi et al. 2018.*

4.2 Fatty acid modification

As multiple studies and our own routine phospholipidic profiling of cells and tissue show, proportionally few of the fatty acid species in a cell comprise of palmitate. This is true even in highly lipogenic cells and tissue. Palmitate generated by FASN is thus rapidly modified into different fatty acid species.

Unsaturation of fatty acids is required to maintain normal membrane properties and functions including homeoviscosity. Palmitate is the major end product of lipogenesis, however an accumulation of saturated palmitate is toxic. Saturated fatty acids can be desaturated by enzymes which require reductive power and dioxygen in driving this energetically unfavourable reaction. For example, SFA can be desaturated by Stearoyl-CoA desaturase (SCD), for example palmitate can generate palmitoleate and stearate can generate oleate, the most common mono-unsaturated fatty acid (MUFA) species. Further desaturation is not possible in mammals as we lack the delta 12 and delta 15 desaturases necessary in generating poly-unsaturated fatty acid (PUFA) species such as linoleate or linolenate, therefore some PUFA species are essential. The enzymes fatty acid desaturase 1 and 2 (FADS1) and (FADS2) can however modify PUFAs at the omega 3 or 6 position generating other PUFA species. For example, FADS1 can generate eicosapentaenoic acid and arachidonic acid.

Non-FASN dependent fatty acid elongation can elongate fatty acids by two carbon units in analogous reactions to FASN. Elongation is driven by seven elongation of very long fatty acid (ELOVL) enzymes which are generally highly promiscuous but some drive specific reactions. For example, ELOVL6 can drive the first step in the elongation of palmitate to stearate.

Several additional metabolic pathways drive complex modifications of fatty acids, generally generating signalling molecules or secondary metabolites required in generating signalling molecules. For example, arachidonic acid can be further modified by lipoxygenases to generate eicosanoids, prostaglandins and leukotrienes, bioactive lipid signalling molecules.

4.3 Lipid uptake

Whereas the mediators of lipogenesis have been surgically delineated and defined, lipid uptake mechanisms remain poorly explored. This is exemplified by the increasing availability of pre-clinical inhibitors of lipogenesis, whereas there is a general lack of lipid uptake inhibitors.

Lipids are sparingly soluble in water, and therefore need to be emulsified in order to be transported in the circulation. Lipids are complexed into a micelle like protein/phospholipid complex called lipoproteins. Lipoprotein complexes can differ in composition of their proteins and be derived from multiple families such as high density lipoprotein (HDL), low density lipoprotein (LDL), intermediate-density lipoprotein (IDL), very low density lipoprotein (VLDL) and chylomicrons which are ultra-low density lipoproteins (ULDL). Different lipoprotein complexes can have different lipid specificities[82].

Lipids can be taken up into the cell through several mechanisms which include: phospholipase or lipase mediated passive diffusion of fatty acids through the plasma membrane, receptor mediated uptake of fatty acids, or by direct endocytosis of lipoprotein complexes.

In cells with a large need for lipid uptake such as muscle cells, there is generally a high expression of Lipoprotein Lipase (LPL). LPL mediates the hydrolysis of fats bound to complexes in the serum facilitating their uptake into the cell[83]. Although in principle, passive diffusion of fatty acids through the plasma membrane is possible, surface receptors are required to increase the rate of lipid uptake[84]. Cluster of differentiation protein 36 (CD36) is a major fatty acid uptake receptor and studies have shown that it plays a key role in sustaining the physiologically necessary rate of lipid uptake into tissues in several models[85,86]. Fatty-acid transporter (FAT) family transporters can also mediate lipid uptake[87]. Several FAT transporters are expressed in tissues.

Receptor mediated endocytosis of extracellular protein complexes by for example low density lipoproteins receptor (LDLR) or scavenger Receptor Class B Member 1 (SCARB1) is a non-selective mode of lipid uptake that can occur independently of fatty acid transporters. Lipoprotein complexes are endocytosed, emulsified in lysosomes and fatty acids are then hydrolysed and made available to the cell[88].

A fourth and more controversial mode of lipid uptake is through intercellular tunnelling actin nanotubes[89,90]. These nanotubes have been shown to transport protein complexes,

organelles and lipid droplets between cells [91], but their contribution to the overall rate of lipid uptake remains unexplored (Figure 10).

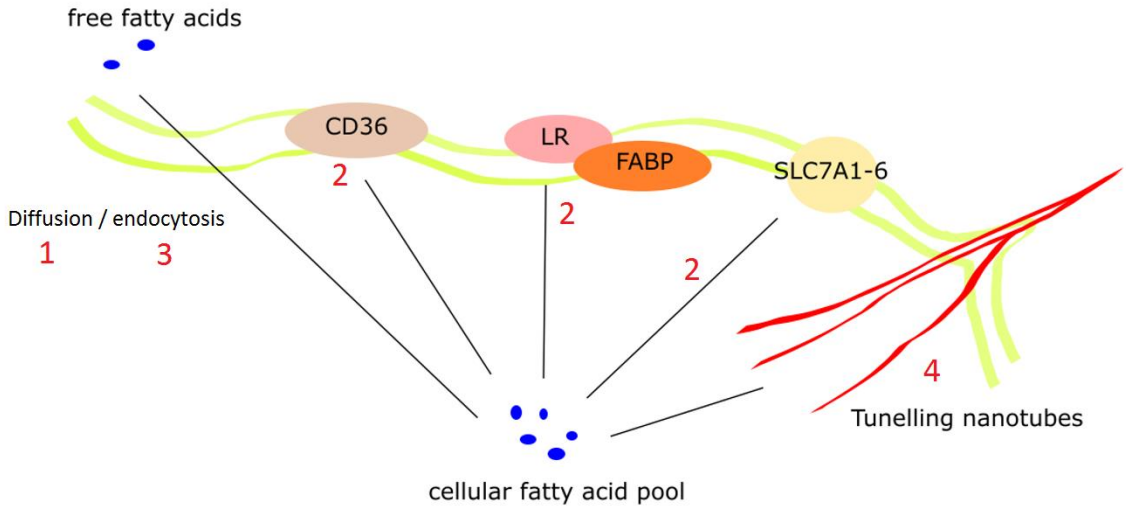


Figure 10. Lipid uptake. Lipids are taken up into cells through multiple mechanisms, including lipase mediated fatty acid diffusion (1), receptor mediated uptake (2) or by endocytosis (3). The role of actin tunnelling nanotubes in lipid uptake has been observed (4) [92]. A*dapted from: Rohrig and Schulze 2016.*

4.4 Biological roles of fatty acids

Fatty acids can be stored as neutral lipid pools by condensing on a glycerol backbone forming triacylglycerides (TAGs). TAGs are generally used as cellular energy stores and form lipid droplets within tissue. Lipids are highly energy dense and their combustion yields twice the energy when compared to carbohydrates. In addition, TAGs are highly hydrophobic and can aggregate forming essentially insoluble cold pools, and can therefore be stored without interfering with cellular processes, as water is needed to solubilise and store carbohydrates making their storage limiting. In this way, specialised tissue such as adipose can store TAGs abundantly. Formation of TAGs can be a dynamic process, and could have additional biological roles. For example, during ROS stress, TAGs have an antioxidant role in neuronal stem cells, as cells remodel membranes by transferring membrane PUFAs into TAG cold pools, thereby reducing membrane lipid peroxidation[93].

In most tissues, fatty acids predominantly form membrane phospholipids. Phospholipids can spontaneously associate with each other in forming either micelle aggregates or larger tail-to-tail bi-layer structures as membrane sheets, or as smaller liposomes. Biological membranes are not homogeneous in terms of phospholipid distributions. Membrane asymmetry in terms of phospholipid composition between the inner and outer leaf of the plasma membrane is sustained by membrane flippases. Furthermore, the composition of membrane lipids differs in different membranes. Cardiolipins are exclusively found in

mitochondria. Ceramides and cholesterol generally increase membrane packing density and are predominantly found in the plasma membrane.

Many lipids form important signalling molecules. For example the negatively charged phosphatidylinositides form a reservoir of signalling lipids that can initiate diverse signalling cascades upon phosphorylation and cleavage. Fatty acid derivatives of arachidonic acid can have diverse roles including modulating immune cell function and mediated cell movement and inflammation.

4.5 Beta-oxidation

Beta-oxidation is the process in which fatty acid chains are progressively oxidised, forming reductive equivalents and acetate. Beta-oxidation is spatially compartmentalised from lipogenesis as it occurs in either the mitochondria or in the peroxisome. Furthermore, the lipogenesis metabolite Malonyl-CoA is shown to inhibit mitochondrial carnitine palmitoyltransferase (CPT) proteins, thereby inhibiting a futile cycle of lipogenesis and beta-oxidation[94,95].

CPT1A, CPT1B CPT1C and CPT2 gene products are required to generate fatty-Acyl-carnitines thereby facilitating fatty acid transport into the mitochondrial matrix where the fatty acid is transferred to ACP and the enzymes required for beta-oxidation are expressed. A chemical reaction that is essentially the reverse of lipogenesis occurs. Briefly, Acyl-CoA-dehydrogenase dehydrogenates the fatty-Acyl chain generating an alpha-beta desaturated thioester and FADH2. Next Enoyl-CoA hydrolyses the molecule generating a beta-Hydroxyl-Acyl-CoA molecule, which is oxidised by 3-Hydroxyacyl-CoA dehydrogenase using NAD+ as a reducing agent, generating a Beta-Ketone-Acyl-CoA and NADH. Finally, beta-ketothiolase hydrolyses the beta ketone generating an Acetyl-CoA molecule and thereby shortening the fatty acid chain by two carbon units. The generated reductive power in the form of NADH can be used directly for oxidative phosphorylation or can be exported and used for cellular reductive power.

Fatty acids with a chain length of greater than 22 carbon units are first contracted by peroxisomal beta-oxidation before being transferred to the mitochondria. Analogous chemical reactions are involved in peroxisomal beta-oxidation, however, here, NADH cannot be used to generate ATP, but can be used to drive the generation of hydrogen peroxide, thereby generating heat, or it be exported to power the cell's reductive reactions.

4.6 Lipid peroxidation

Saturated fatty acids have very little chemistry and are highly unreactive. However, unsaturation, especially poly-unsaturation gives functionality to the carbon chain. Often, PUFA are conjugated, increasing their reactivity to electrophiles, especially towards radicals. ROS such as superoxide and hydrogen peroxide are a major source of biological radicals and can peroxidate PUFAs leading to the generation of labile hydroperoxides by

rapid non-enzymatic reactions. These hydroperoxides can transfer radical species to surrounding PUFAs propagating the chain reaction. The degradation products of PUFAs such as malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE) are toxic and are cytotoxic if they accumulate. A host of hydroperoxidases (predominantly glutathione powered GPX proteins) catalyse the degradation of lipid hydroperoxides species. Likewise, anti-oxidants, especially lipid soluble antioxidants can directly inhibit the peroxidation of lipids. Importantly, lipid peroxidation has recently been shown to be a major driver of a form of programmed necrotic cells death called ferroptosis (Figure 11) [96]. An accumulating body of evidence has shown key roles of ferroptosis in pathology and as an exploitable target in cancer[97].

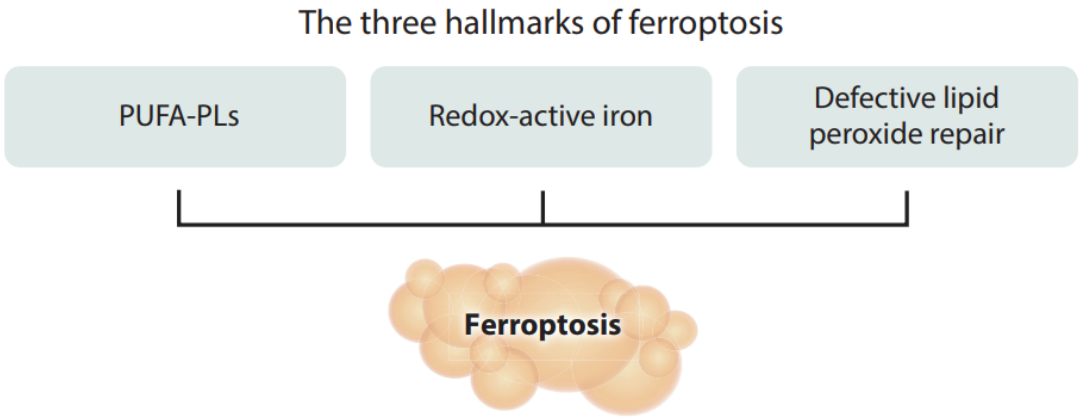


Figure 11. Ferroptosis. Ferroptosis is a form of programmed necrosis that requires peroxidation of (especially) poly-unsaturated phospholipids and reactive iron. Critically depletion of membrane PUFA pools, enhanced antioxidant potential and iron chelation can inhibit this form of programed cell death. Retrieved *from: Dixon and Stockwell 2018.*

We have shown that in cancer cells, membrane lipid saturation by enhancing lipogenesis is protective by depleting cells of poly-unsaturation fatty acid species making them less susceptible to ROS[98]. Moreover, membrane saturation is key feature of therapy resistant melanoma cells, and that, lipogenesis inhibition promotes membrane lipid poly-unsaturation and thereby susceptibility to ROS induction. This is a process that can be rescued by the addition of antioxidants[75].

Importantly, membrane lipid peroxidation is a key requirement of canonical ferroptosis induction, with a number of the key signalling lipid hydro-peroxides recently identified[99]. Recently, a plethora of key publications have shown the importance of membrane lipid peroxidation and interfering with cellular ROS to overcome cancer therapy resistance. In multiple cancer cell types, two reports have shown that drug resistant cancer cells adopt a specific phenotype that renders cells sensitive to glutathione peroxidase 4 (GPX4) inhibition, a key lipoxygenase in detoxifying lipid hydroperoxides[79,80]. However, the tool-box GPX4 inhibitor RSL3 and the SLC7A11 inhibitor Erastin have limited in vivo efficacy.

Recently, Withaferin A was identified as a novel GPX4 and iron accumulation in cells, resulting in two-pronged induction of ferroptosis[100].
In melanoma cells, directing the evolution of cells by inhibiting oncogenic BRAF drives a ROS scavenging phenotype which is sensitive to SLC7A11 inhibition. Using the HDAC inhibitor vorinostat to inhibit SLC7A11 depletes cellular glutathione production and thereby inhibits the emergence of therapy resistant melanoma cells[73].

4.7 Phospholipids

Phospholipids are the major constituents of biological membranes. Phospholipids generally contain two fatty acid chains linked at the sn-1 and sn-2 position of a glycerol backbone through an ester bond (Figure 12). Other rarer linkages such as through an ethoxy or vinyl bonds can occur. The fatty acids themselves are highly variable and generally differ in length and unsaturation degree. The double-bonds are synthesised in the cis configuration due to the stereochemistry of the enzymes, however trans-fatty acids can occur through radical excitation and relaxation of the double bond. Exotic modifications to the fatty acid such as alcohol, ether or epoxide linkage are possible but are comparatively rare. The head group which define the phospholipid class is linked to the sn-3 position on the glycerol backbone through a phosphate linkage and can vary. Phospholipid species include phospohatiditic acid, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylglycerol, cardiolipin, sphingomyelin and lyso-phospholipid species (Figure 13).

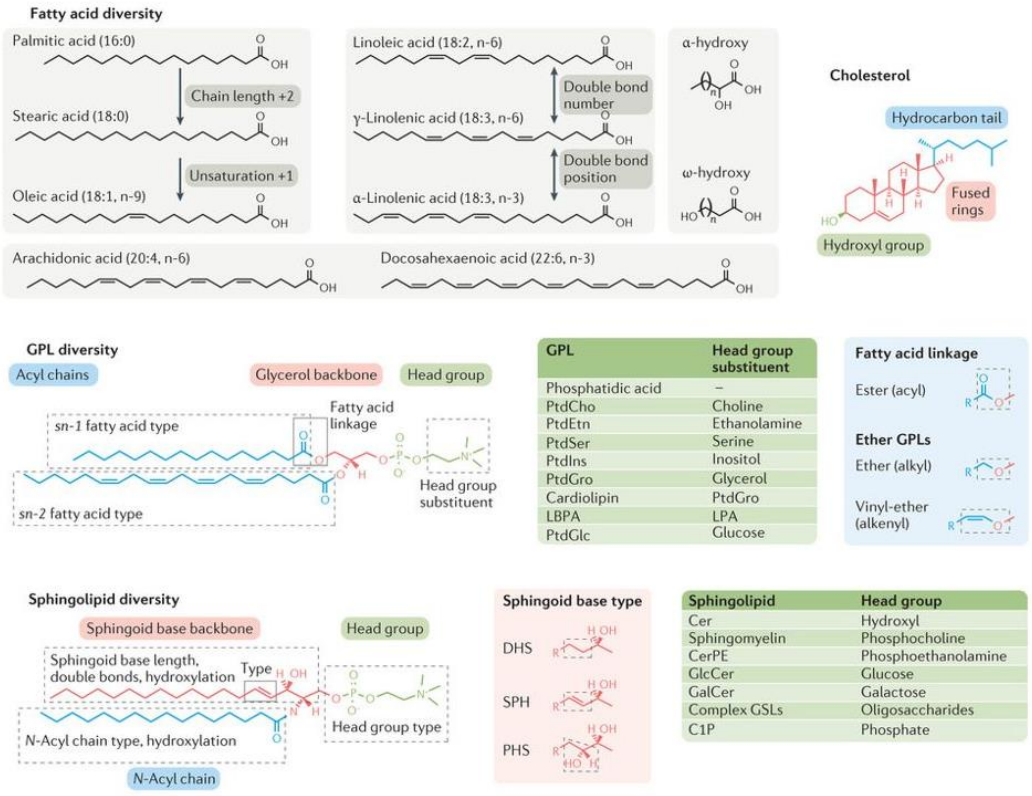
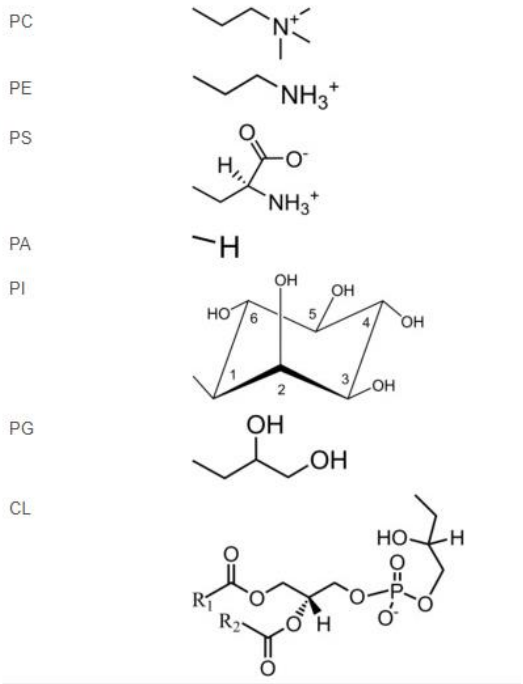


Figure 12. ***The diversity in lipid species*****.** Palmitate is the final product of lipogenesis. Palmitate can be readily modified by elongation by (successive) 2-carbon units and/or by desaturation in forming cis-double-bonds to other fatty acid species, for example to oleic acid. PUFAs are either diet derived or modified forms of diet derived fatty acids and can be further modified to essential signalling lipid precursors such as arachidonic and docosahexaenoic acid. Phospholipids generally contain two fatty-Acyl moieties linked to a glycerol backbone. The head group can vary and define the phospholipid species. Sphingomyelin are generated from the transfer of phospho-choline to ceramides. Cholesterol is an important neutral lipid membrane constituent and can be modified by esterification at the hydroxyl group or could be used to make important signalling hormones[101]. *Retrieved from: Harayama and Riezman, 2018.*



Figure[102] 13. Chemical structure of phospholipid head groups. The chemical structure of the head group structures of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidic acid, phosphatidylinositol, phosphatidyl glycerol and cardiolipin species. *Adapted from Deng et al. 2015.*

4.8 Lipid modifications as modulators of membrane biophysics

Modifications of the composition, saturation degree and length of membrane phospholipid species can greatly increase the diversity of membrane lipid species and has profound implications on membrane biophysics and function. Saturated fatty-Acyl chains of membrane lipids pack tightly based on little steric hindrance and maintain a relatively low level of membrane fluidity due to Van der Waals attraction forces. Cholesterol packs efficiently between phospholipid species thereby increasing packing density and membrane order. Cholesterol can additionally form nanostructures with other lipid species with a high packing density such as ceramides and sphingomyelins in forming membrane rafts which are necessary for enhancing signalling of some proteins (Figure 14). Furthermore, increasing Acyl-chain length of saturated fatty acids further increases membrane packing efficiency. In bacteria, altering membrane lipid species length is a predominant means of homeoviscous adaptation. In animals, this is achieved by altering membrane saturation degree. Mono-unsaturation of membrane lipids generates a cis-olefin in the fatty-Acyl chain of the phospholipid, thereby increasing steric hindrance in associating with surrounding lipid species. Increasing the length of the MUFA species enhances this effect. Likewise, PUFA species decrease membrane order. Altering membrane order by desaturation is a major means of homeoviscous adaptation in animals.

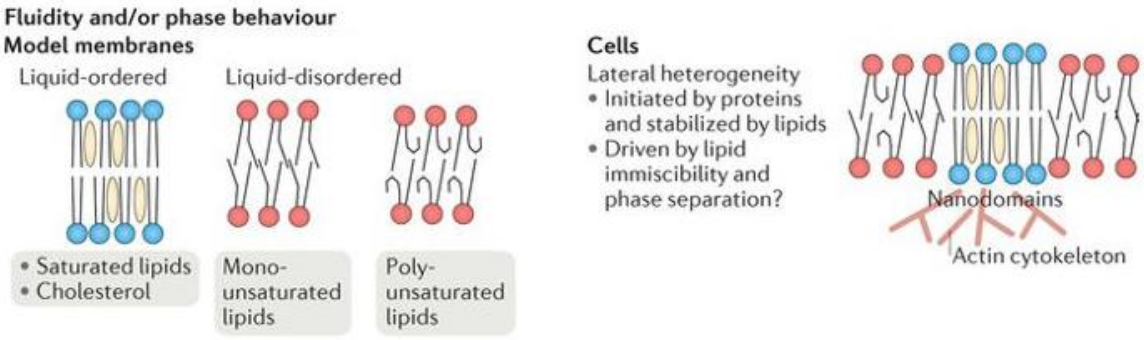


Figure 14. ***Membrane lipid composition affects membrane biophysics*****.** Saturated phospholipid species can pack tightly, and form ordered membranes, cholesterol can enhance packing efficiency. Lipid mono-unsaturation and poly-unsaturation prevents close packing and reduces membrane order. Lipids with high packing density can spontaneously form membrane micro-domains in more fluid surroundings[101]. *Retrieved from: Harayama and Riezman, 2018.*

4.9 Sterol regulatory element binding proteins (SREBP)s as master regulators of lipogenesis

In Mammalia, extracellular signals tightly regulate lipogenesis. Dysregulation of this process is linked to metabolic disease, such as steatosis, cardiovascular disease and cancer [92,103,104]. Hormones such as insulin promote lipogenesis by inducing the activation of a group of membrane bound transcription factors, the SREBP proteins[105]. SREBPs are generally considered as the master regulators[106]. SREBPs exist as two gene products, namely SREBP-1 and SREBP-2 which are of similar but different function. SREBP-1 exists as two well-defined splice variants, SREBP-1a and SREBP-1c[107]. In organisms, SREBP-1c promotes lipogenesis whereas SREBP-1a and SREBP-2 promotes the mevalonate pathway and cholesterol biosynthesis. In cell lines however, SREBP-1a strongly promotes lipogenesis. SREBP-1 also regulates the expression level of genes involved in NADPH synthesis as this metabolite is required to power lipogenesis [108].

SREBPs are unique amongst the basic helix-loop helix class of transcription factors since they are synthesised as inactive ER bound transmembrane precursors.[109] Extensive post translational modification is required to activate SREBP proteins (Figure 15). This process has been exquisitely delineated by the Goldstein and Brown lab[9]. Briefly, newly synthesised SREBPs must bind SREBP cleavage-activating protein (SCAP) in order to be trafficked to the Golgi. If in turn, the SCAP-SREBP complex binds to either Insulin-induced gene 1 or 2 (INSIG1 / INSIG2) the complex is retained in the endoplasmic reticulum (ER), and activation cannot occur. In the Golgi, SREBP is cleaved sequentially by two proteases, membrane bound transcription factor peptidase 1 and 2 (MBTPS1 and MBTPS2), releasing the transcriptionally active head group which contains a nuclear localisation sequence and is therefore trafficked to the nucleus.

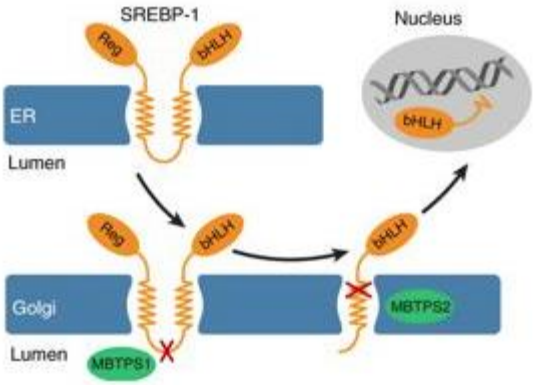


Figure 15. *SREBPs are post-translationally activated*. SREBPs are synthesised as inactive precursors. SREBPs are bound to SCAP and translocated to the Golgi, where they are activated by proteolytic cleavage of the TF head group by MBTPS1 and MBTPS2. *Retrieved from: Talebi et al. 2018.*

SREBP activity can be further modulated at this stage as it is readily targeted for proteasomal degradation. Phosphorylation[110–112], acetylation[113], sumoylation[114] and ubiquitination[111,115] can all further affect SREBP head group stability. SREBP-1 activates a plethora of genes involved in lipogenesis such as ACLY, ACACA an FASN and some genes involved in fatty acid modification such as SCD and ELOVL6.

4.10 Other lipogenic transcription factors

Besides SREBPs, several other transcription factors also play key roles in the regulation of lipid homeostasis. These include peroxisome proliferator-activated receptors (PPARs), and Carbohydrate-responsive element-binding protein (ChREBP). PPAR transcription factors heterodimerise with the RXR and initiate the transcription of genes involved in a host of physiological processes, including oncogenic signalling, cell differentiation and lipid metabolism. PPARs exist as three gene products, PPAR-alpha, PPAR-beta and PPAR-gamma. PPAR-alpha and PPAR-gamma are activated by lipid ligands. Whilst PPAR-alpha is activated by fatty acids PPAR-gamma is activated by eicosanoids including leukotrienes.
The liver-x-receptor (LXR) is conserved soluble nuclear hormone receptor which is structurally related to PPARs and is activated by oxy-sterols. Upon activation, LXR activates an array of lipogenic genes and transcription factors, including SREBPs.
ChREBP is a more promiscuous transcription factor with a host of other cellular roles. ChREBP is involved enzymatically in driving acetylation of histones and cellular proteins[116]. ChREBP also acts as a transcription factor in driving lipogenesis, and ChREBP inhibition has been shown to be sufficient to decrease lipogenesis and reverse insulin resistance in animal models[117].

5. Lipid metabolism in cancer

Metabolic rewiring is a fundamental feature of cancer cells. Cancer cells often upregulate biosynthetic processes, which can in part give the cancer cell flexibility in generating the feed-stocks needed in forming biological molecules needed to sustain proliferation or evade stresses.[6] Specifically, whilst *de novo* lipogenesis is generally low in most organs with the exception of the liver, myriad studies show that lipogenesis is re-activated in cancer cells. Historically, this seminal observation has been made since 1953, where the levels of tumour lipogenesis were measured to be similar to that of the liver[118]. More recently, studies in our lab and others have shown that lipogenesis is high in several cancer models[119–121]. In particular, the over-expression of key enzymatic drivers of lipogenesis such as FASN, ACACA and SCD is observed in multiple cancers[92,122–125]. Specifically, FASN over-expression is observed in several ovarian, prostate, colorectal, lung and bladder cancers[126]. Furthermore, more aggressive forms of breast cancer express FASN more highly[127]. The over-expression of key lipogenic enzymes is controlled by SREBPs, and SREBP activation is sufficient to induce lipogenesis. Accordingly, SREBPs are often highly activated in cancer,[75,128–131] and cancer cells adopt strategies to sustain SREBP signalling[132].

Furthermore, multiple oncogenes and initiating oncogenic events such as Map Kinase activation, myc over-expression and chromosome 8p deletion are associated with enhanced lipogenesis, establishing lipogenesis as fundamental oncogenic transformation event[75,128,133,134]. Taken together, lipogenesis is firmly integrated into the altered metabolic profile of cancers.

As the tumour microenvironment is poorly vascularised, lipids may even be more deficient than soluble metabolites in the surrounding stoma. Therefore, cancer cells face challenges in acquiring sufficient fatty acids to fuel key processes in generating biological membranes which is fundamental to sustaining proliferation. To this end, strategies in overcoming the deficiency in available lipids include 1 - enhancing lipogenesis, or 2 - to further increase the propensity of taking up lipids directly from surrounding tissues. Importantly, in contrast to cancer cells, normal cells, even those with high rates of proliferation are shown to attain their lipids from dietary sources[126,135]. Lipogenesis is limited to a few adult tissues, but remains high in proliferating embryonic tissues, which could suggest that this process is associated with de-differentiation in cancer[136]. Taken together, targeting lipogenesis and its mediators is a promising area of research in cancer. However, unlike nucleotide metabolism which is invariably required to sustain cancer cell proliferation,[137] targeting lipogenesis is context-dependent in cancer. Certain cancers preferentially rely on lipid uptake, or metastasis to lipid rich organs which allow for the uptake of lipids. Other cancer types specifically rely on high rates of beta-oxidation, whilst other cancers may have a more unsaturated or elongated lipid profile when compared to surrounding tissue. This complexity can in part arise from the interplay and differential activation of various lipid metabolic pathways, however this difference can prognostic in highlighting multiple exploitable vulnerabilities in several types of cancer.

5.1 SREBPs in cancer

Several studies implicate increased activity of SREBP proteins as a necessity in increasing lipogenesis in cancer[138–141]. In line with this observation, SREBPs are shown to be regulated by several oncogenes and oncogenic pathways in cancer. The PTEN/PI3K/Akt and mTorC1/2 pathway are directly upstream in SREBP signalling[142–146]. P53 is a negative regulator of SREBP-1 mediated lipogenesis[147,148]. ERK1/2 drive SREBP mediated lipogenesis by direct phosphorylation of SREBP-1[149,150]. Importantly, several tumour suppressor genes negatively regulate SREBP activation. For example, PTEN inhibits SREBP processing through inhibiting Akt signalling. More directly, AMP-activated protein kinase (AMPK) negatively regulates SREBP mediated lipogenesis by direct phosphorylation of SREBPs[151]. More recently, key driving mutations in the Map Kinase pathways are shown to drive SREBP mediated lipogenesis, thereby coupling proliferation to lipogenesis. In particular, these studies, including our own recently published work shows that oncogenic KRAS or BRAF drives SREBP mediated lipogenesis, although the mechanisms behind this process remain obscure[75,128,152,153].

Consistent with the cross-talk of multiple oncogenic signals in cancer, targeting SREBPs has had some success in preclinical models. Recently, the synthesis of a small molecule pre-clinical inhibitor of SCAP, Fatostatin A, and the discovery of the ability of the natural product Betulin to inhibit SREBP processing shows promise as a strategy for directly targeting SREBPs clinically[154,155]. Importantly, these compounds do not show toxicity in mouse models. In colorectal cancer, genetic knock-down of SREBPs inhibited cancer cell colony formation and proliferation rates, this was translatable in *in vivo* xenograft models[129]. In several glioma models, SREBP is shown to be required to support cell growth and proliferation in EGFR dependent tumours[130,156]. SREBP is shown to be required in glioma to drive lipogenesis under hypoxia[130]. This may at first be surprising in that pyruvate entry into the TCA cycle is reduced in hypoxia, and it can expected that the flux from glucose to lipids is reduced. However, it has been shown that in hypoxia, there is a compensatory flux from glutamine to lipids[157–159]. Co-incident with this, a high expression of lipogenic enzymes is observed in hypoxia models which could in part help to increase the flux to lipogenesis[160].

Androgen receptor signalling is a key transformative event in prostate cancer initiation, and the androgen receptor is a regulator of SREBP[161,162]. Multiple studies in prostate cancer have shown that inhibition of SREBP-1 inhibit prostate cancer proliferation and survival[163]. Importantly, in addition to its anti-proliferative effects, inhibiting SREBP-1 by genetic or pharmacological approaches was shown to result in increased oxidative stress in prostate cancer[131]. More recently, SREBP is shown to be critical in supporting the ability of prostate cancer cells to metastasize[128]. Critically, metastatic cells are shown to resist cellular ROS[164]. Taken together, targeting SREBP-1 mediated lipogenesis is a potential approach in anti-neoplastic therapy. The role of SREBP in integrating oncogenic signalling, cell metabolism and oxidative stress points towards a role beyond a simple switch in fulfilling the cellular need in membrane biogenesis.

5.2 Targeting lipogenic enzymes in cancer

Besides targeting upstream lipogenic transcription factors as a means of downregulating lipogenesis, key lipogenic enzymes such as ACLY, ACACA and FASN can be directly targeted. As multiple studies show, these enzymes have critical roles in sustaining cancer cell viability and proliferation. Whilst ACLY can be bypassed in lipogenesis by using alternate carbon sources such as glutamine to generate Acetyl-CoA, ACACA and FASN are required to generate Malonyl-CoA and palmitate respectively. Therefore it is not surprising that ACACA and FASN are the subject of intense pharmacological interest in treating metabolic disease and in cancer. Recently, candidate small molecule inhibitors have been shown to have significant anti-neoplastic effects in several pre-clinical models.

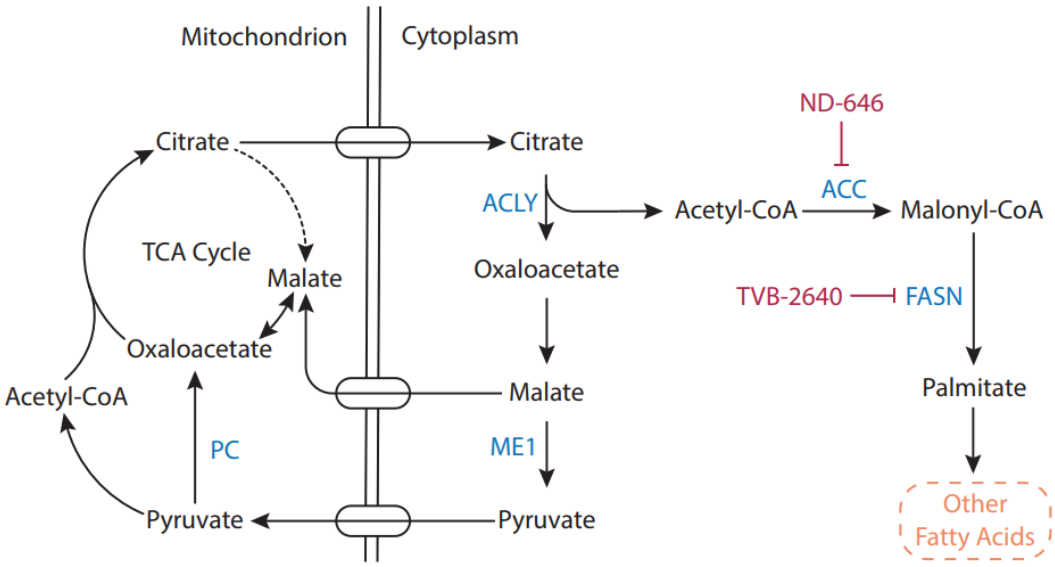
A high rate of glycolysis supports the generation of citrate which is converted by ACLY to Acetyl-CoA. Interfering with this process by using genetic and pharmacological approaches attenuated the rate of tumour growth in multiple proof-of-concept cancer models[165,166]. ACLY is thus shown to be required for lipogenesis and mevalonate biosynthesis in highly glycolytic cancer models of several tumour types. However, the translational impact of this finding remains to be better established. Since hypoxia would uncouple the link between glycolysis and lipogenesis, and that the solid tumour microenvironment is generally hypoxic, the contribution of ACLY to lipogenesis in hypoxia remains to be explored. However, ACLY is clearly required for the survival of multiple cancer models, including in clear cell renal cell carcinoma (ccRCC)[167]. It is conceivable that in well-vascularized cancers such as ccRCC or if coupled with vasculature normalizing treatments, there could be significant translational benefit in inhibiting ACLY in cancer.

ACACA is required to generate Malonyl-CoA from intracellular acetate, which is in turn required as a substrate by FASN to generate palmitate. Interfering with either ACACA or FASN are therefore good strategies to directly inhibit lipogenesis. To this end, significant research efforts are focused on these two targets in anti-cancer anti-metabolic disease therapy, culminating in the recent availability of pre-clinical and in clinical trials drugs targeting these enzymes.

Several early reports from our lab and from others have shown the key importance of ACACA and FASN in sustaining cancer cell proliferation and viability[98,168–171]. The ACACA inhibitors TOFA and Soraphen A have shown cytotoxic effects in several cancer models[172–174]. The FDA approved natural product FASN inhibitor Cerulenin is shown to inhibit multiple cancer processes[127,168,169,172]. The synthetic FASN inhibitor C75 is similarly shown to induce apoptosis in multiple model[175–177], and can cause cell cycle arrest[178–181]. Similar observations are made for the FASN inhibitor orlistat[182,183]. However, the clinical use of these early FASN inhibitors as anti-neoplastic agents is limited due to poor efficacy, systemic side-effects and poor drug tolerance.

Recently, newer disclosed and undisclosed inhibitors of ACACA and FASN have been developed that are bioavailable, significantly more potent and less toxic than the previous

generation of drugs. These new small molecule inhibitors bring lipogenesis inhibition in cancer much closer to the clinic. ND-646 is currently the most promising ACACA inhibitor generated by Nimbus therapeutics and purchased by Gilead. ND-646 has been used in a proof of concept study in inhibiting tumour growth in a genetic model of lung cancer, but has not yet moved on to clinical trials[184]. Several FASN inhibitors have been recently developed or identified, including IPI-9119 which has shown efficacy in pre-clinical prostate models[185]. Currently, only derivatives of TVB-3664 generated by 3V-Biosciences are in clinical trials and have been shown to effectively attenuate tumour growth in preclinical models (Figure 16)[186,187].



Figure[188] **16**. Whilst a number of strategies are possible in inhibiting lipogenesis, the most promising targets are the critical enzymes ACACA and FASN. Currently, the most promising pre-clinical inhibitors for these targets are ND-646 and TVB-2640 respectively. These inhibitors are currently in clinical trials for cancer and anti-viral therapy. The previous generation of ACACA and FASN inhibitors suffers from low efficacy and off target effects. *Retrieved from Vander Heiden et al. 2018.*

6. Research Scope

Although molecular targeted therapies have successfully revolutionised BRAF mutant melanoma treatment, a significant subset of patients remain unresponsive to these therapies; furthermore, the vast majority of patients acquire resistance to Map kinase targeting therapy. Strategies in sensitising melanoma to Map Kinase inhibitors and in overcoming therapy resistance are therefore sorely needed.

Several mechanisms of therapy resistance have been described. Although the majority of resistance mechanisms converge on the reactivation of Map Kinase signalling and/or increased mTOR/AKt signalling, the described mode of resistance acquisition differs in different models. Furthermore, the myriad Map Kinase mTOR/AKt and signalling pathway constituents points towards the redundancy of individual enzymes and therefore the many ways in which these signals could be re-activated.

Due to these limitations, targeting vulnerabilities that are well-downstream of these pathways could firstly highlight resistance mechanisms that are shared between resistant tumours and could highlight new targetable opportunities in overcoming therapy resistance.

Searching for context dependent metabolic vulnerabilities is an attractive strategy in overcoming therapy resistance as myriad studies have shown that cellular metabolism is altered in cancer. Furthermore, metabolic pathways are sometimes driven by one or a few key enzymes, and in this way, targeting such vulnerabilities would limit the cancer cell's ability to develop resistance to this acquired dependency.

Lipid metabolism is of special interest in this context, as 1 – lipogenesis is often upregulated in cancer, 2 – lipogenesis is generally low in normal tissue and 3 – lipogenic pathways are driven by a few key enzymes and 4 - increasingly, a number of clinically used and in clinical trials lipid metabolism inhibitors are available.

To this end, using a panel of Map Kinase inhibition sensitive and resistant melanoma models, we focused on linking Map Kinase inhibition to lipogenesis. In doing so, we show that:

- Map Kinase inhibition inhibits lipogenesis in therapy responsive but not in therapy resistant melanoma models. Furthermore, inhibition of lipogenesis re-sensitises therapy resistant melanoma to therapy
- By mediating membrane-lipid poly-unsaturation, lipogenesis inhibition renders melanoma cells exquisitely sensitive to ROS inducers. Further challenging cancer cells with ROS inducers is cytotoxic to melanoma cells.

Chapter 2: SustainedSREBP-1-dependent lipogenesis as a key mediator of resistance to BRAF-targeted therapy

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7. Abstract

Whereas significant anti-tumour responses are observed in most BRAF-V600E-mutant melanoma patients exposed to MAPK-targeting agents, resistance almost invariably develops. Here, we show that in therapy-responsive cells BRAF inhibition induces downregulation of the processing of Sterol Regulator Element Binding (SREBP-1) and thereby lipogenesis. Irrespective of the escape mechanism, therapy-resistant cells invariably restore this process to promote lipid saturation and protect melanoma from ROS-induced damage and lipid peroxidation. Importantly, pharmacological SREBP-1 inhibition sensitizes BRAF-V600E-mutant therapy-resistant melanoma to BRAF-V600E

inhibitors both *in vitro* and in a pre-clinical PDTX *in vivo* model. Together, these data indicate that targeting SREBP-1-induced lipogenesis may offer a new avenue to overcome acquisition of resistance to BRAF-targeted therapy. This work also provides evidence that targeting vulnerabilities downstream of oncogenic signalling offers new possibilities in overcoming resistance to targeted therapies.

8. Introduction

While targeted approaches are revolutionizing the treatment of cancer, the management of both intrinsic and acquired therapy resistance remains a major limitation. This is exemplified by the unprecedented, but transient, anti-tumour responses seen in patients with BRAF-V600E-mutant malignant melanoma exposed to agents that selectively inhibit oncogenic BRAF[189,190]. Many of these patients show almost complete remission in response to such targeted agents, however, therapy resistance eventually develops in ~80% of all cases[43,45,191].

Many genomic and non-genomic mechanisms have been described, all leading to re-activation of the MAPK- and/or PI3K-signalling pathways[47,192,193]. Moreover, different mutational events can be selected in distinct drug-resistant clones from the same patient[49] and even co-occur within the same lesion[48]. These findings have highlighted the need to improve effectiveness of treatment, by for instance, the co-targeting of other essential cancer vulnerabilities and/or key mediators of MAPK signalling itself.

One of the pathways that is emerging as a central player in multiple oncogenic processes and that functions downstream of a multitude of oncogenic signal transduction pathways is *de novo* lipogenesis. Accordingly, this pathway is specifically activated in many cancers[92,119,126,194], in part through induction of the transcription factor Sterol Regulatory Element Binding Protein (SREBP-1), a master regulator of lipogenesis[130,148,156,161,195,196]. Aberrant activation of the lipogenic pathway in cancer is required for the synthesis of phospholipids, which function as essential building blocks of membranes and that support cell growth and proliferation[171,174]. As this pathway mainly produces saturated and MUFA, an increase in the proportion of these lipids in the cellular membrane composition of cancer cells is often observed[98,197–199]. Importantly, SFA and MUFA are less prone to lipid peroxidation, thereby providing a survival advantage to cancer cells, particularly those exposed to oxidative stress[98].

Here, we show that the lipogenic pathway is a key mediator of oncogenic BRAF and that its constitutive activation, which is mediated by SREBP-1, contributes to therapy resistance. Our findings support the use of SREBP-1 inhibitors in a novel combinatorial approach to overcome resistance to BRAF-V600E-targeted therapy.

9. Results

9.1 *De novo* lipogenesis is inhibited by BRAF-V600E-targeted therapy

As in many cancers, there is evidence that *de novo* lipogenesis is activated in melanoma[200,201]. We reasoned that ectopic MAPK-activation may be one key triggering event of such activation. To test this possibility, we assessed the impact of BRAF inhibition on lipid metabolism. We exposed BRAF-mutant, therapy-sensitive, melanoma cell lines (M249 and A375) to Vemurafenib and profiled their transcriptome by RNA-seq. Ingenuity pathway analysis (IPA) identified fatty acid metabolism as one of the most affected pathways by the treatment (Figure 1a). Consistently, expression of key lipogenic enzymes such as ATP citrate lyase (ACLY), Acetyl-CoA carboxylase-1 (ACACA), and fatty acid synthase (FASN) were consistently decreased (Figure 1c, Supplementary Figure 1a). Alterations in the expression of these enzymes by mutant BRAF inhibition was confirmed by RT-qPCR on an extended panel of therapy-sensitive BRAF-V600E parental and isogenic cell lines that have acquired resistance to Vemurafenib through diverse mechanisms (Supplementary Table 1). These include Raf-kinase flexibility in MAPK signalling and in increased IGF-1R/PI3K signalling (451lu R)[202], enhanced RTK signalling (M229 R and M238 R) and secondary acquisition of oncogenic NRASQ61K (M249 R)[52]. Whereas Vemurafenib decreased the expression of lipogenic enzymes in all sensitive BRAF-mutant cell lines, this was not seen in normal neonatal human epidermal melanocytes (NHEM) and in the therapy-resistant lines (Figure 1c, Supplementary Figure 1b). If anything, the opposite effect was observed in the Vemurafenib-resistant cells. Direct measurement of the overall rate of lipogenesis by assessing 14C-acetate incorporation into lipids confirmed an overall increase in lipogenesis in melanoma cell lines compared to NHEM (Figure 1d). A marked decrease in *de novo* lipogenesis was observed in all BRAF-V600E therapy-sensitive, but not resistant, cell lines upon Vemurafenib exposure. These findings were further corroborated by isotopomer spectral analysis, a method that measures fatty acid biosynthesis rates by measuring the fraction of *de novo* synthesized palmitate. In general, there was a marked decrease in the fraction of *de novo* synthesized palmitate in therapy-sensitive lines. In contrast, Vemurafenib did not cause any decrease in palmitate synthesis in some therapy-resistant cells or induced only a moderate reduction in others (Supplementary Figure 2). We conclude that lipogenesis is sustained in therapy-resistant cells when compared to therapy-sensitive cells upon Vemurafenib treatment. Notably, lipid uptake, cholesterol synthesis rate or cholesterol uptake were not affected in any of the conditions and cell lines, indicating that Vemurafenib predominantly affects *de novo* fatty acid biosynthesis (Supplementary Figure 3a–c).

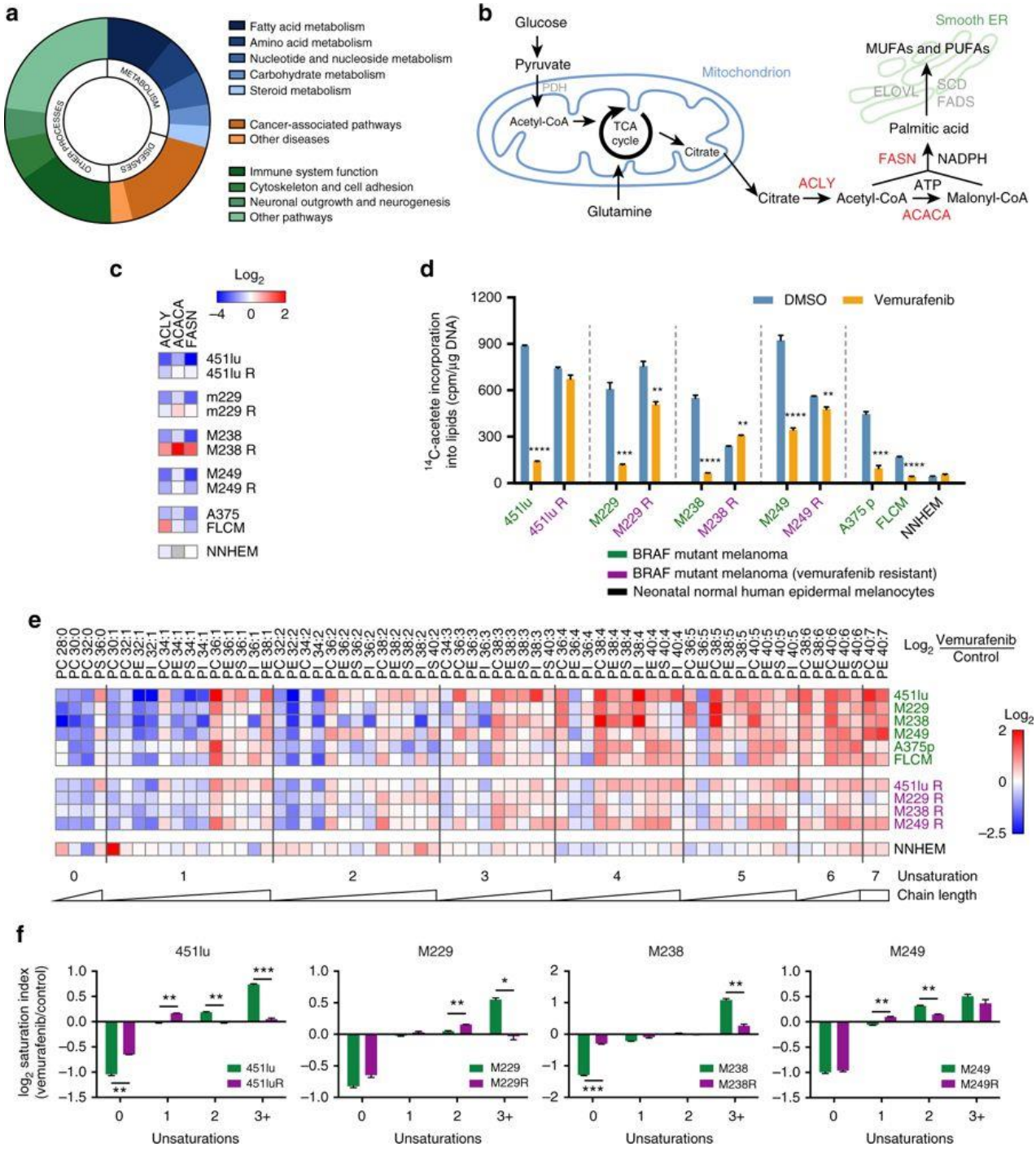


Figure 1. ***De novo lipogenesis is inhibited by Vemurafenib in therapy-sensitive cells, but remains activated in therapy-resistant ones***. a. Significantly altered (fold change of at least 1.5 and $p < 0.05$) pathways as indicated by ingenuity pathway analysis (IPA) of RNA-seq of Vemurafenib (5 µM) treated M249 and A375 cells (n = 3). b. Vemurafenib affects major components of the fatty acid synthesis pathway (fatty acid synthase, ATP citrate lyase and Acetyl-CoA carboxylase). c. The effects of Vemurafenib treatment on the mRNA levels of FASN, ACLY, and ACACA in therapy-sensitive versus resistant BRAF-mutant cell lines and in NHEM (n = 3). d. Vemurafenib or vehicle treated cells were assayed for their

ability to incorporate 14C-acetate into lipids. Data are represented as mean ± s.e.m. The significance was determined with an unpaired t-test and compares Vemurafenib-treated cells to their matching controls (n = 3). (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). e. Heatmap of log2 ratios of the abundance of phospholipid species in Vemurafenib-treated cells over vehicle treated cells. Species are indicated by their total number of fatty acid carbons, followed by a colon and the total number of unsaturations (n = 3). f. log2 ratios of the Vemurafenib-induced changes in saturation index. The saturation index was calculated by summing the species with the same level of unsaturations.

9.2 BRAF-V600E-induced lipid metabolism is mediated by SREBP-1

The selected lipogenic enzymes, the expression of which are downregulated upon oncogenic BRAF inhibition, are well-established transcriptional targets of SREBP-1. We therefore examined whether activity of SREBP-1 itself may be decreased by Vemurafenib. Because SREBP-1 is synthesized as an inactive precursor, which is activated upon proteolytic cleavage[107] (Figure 2a), we used western blot analysis to assess the protein levels of both full-length and mature SREBP-1. Vemurafenib caused a decrease in the levels of the mature form of SREBP-1 in all BRAF-V600E-therapy-sensitive, but not (or less so) in resistant cell lines (Figure 2b). In contrast, the non-processed form was either unaffected or increased. To further substantiate this finding, we exploited the paradoxical activation of the MAPK pathway by Vemurafenib in two NRAS mutant cell lines (M202 and M207)[33,50,203]. Vemurafenib resulted in both an expected increase in the levels of pMEK 1/2 proteins and of mature SREBP-1 (Figure 2c). In addition, over-expression of a BRAF-V600E-encoding plasmid in these BRAF wild-type cells further support the ability of oncogenic BRAF to induce SREBP-1 processing (Figure 2d). RT-qPCR analysis of the transcripts encoding SREBP-1a and SREBP-1c showed a decreased expression upon Vemurafenib exposure in 451lu and A375, but not in the other cell lines (Supplementary Figure 4). As SREBP expression is subject to autoregulation, these findings indicate that Vemurafenib acts, at least in part, at the level of SREBP maturation. Note, however, that more direct transcriptional effects on the regulation of SREBF-1 transcription cannot be fully excluded. To further substantiate these findings, we looked at the effect of Vemurafenib on the cellular distribution of SREBP-1. To this end, we generated a transcriptionally inactive recombinant full-length SREBP-1 construct with an N-terminal HA-tag and a C-terminal myc-tag, allowing visualization of the active and inactive forms of the protein (Figure 2e). Western blotting established that the transgene is processed in a manner that is indistinguishable from endogenous SREBP-1 (Supplementary Figure 5a). Similarly to endogenous mSREBP-1, which localizes to the nucleus, the processed HA-tagged exogenous protein was detected in the nuclei of untreated melanoma cells. In contrast, both HA- and myc-tagged proteins co-localized in the ER–Golgi, indicating that proteolytic activation is halted in Vemurafenib-treated cells (Figure 2f, Supplementary Figure 5b). Taken together, oncogenic BRAF targeting inhibits the processing and activation of SREBP-1 in therapy-sensitive, but not therapy-resistant, melanoma cells and this effect is, by and large, mediated by a post-translational mechanism.

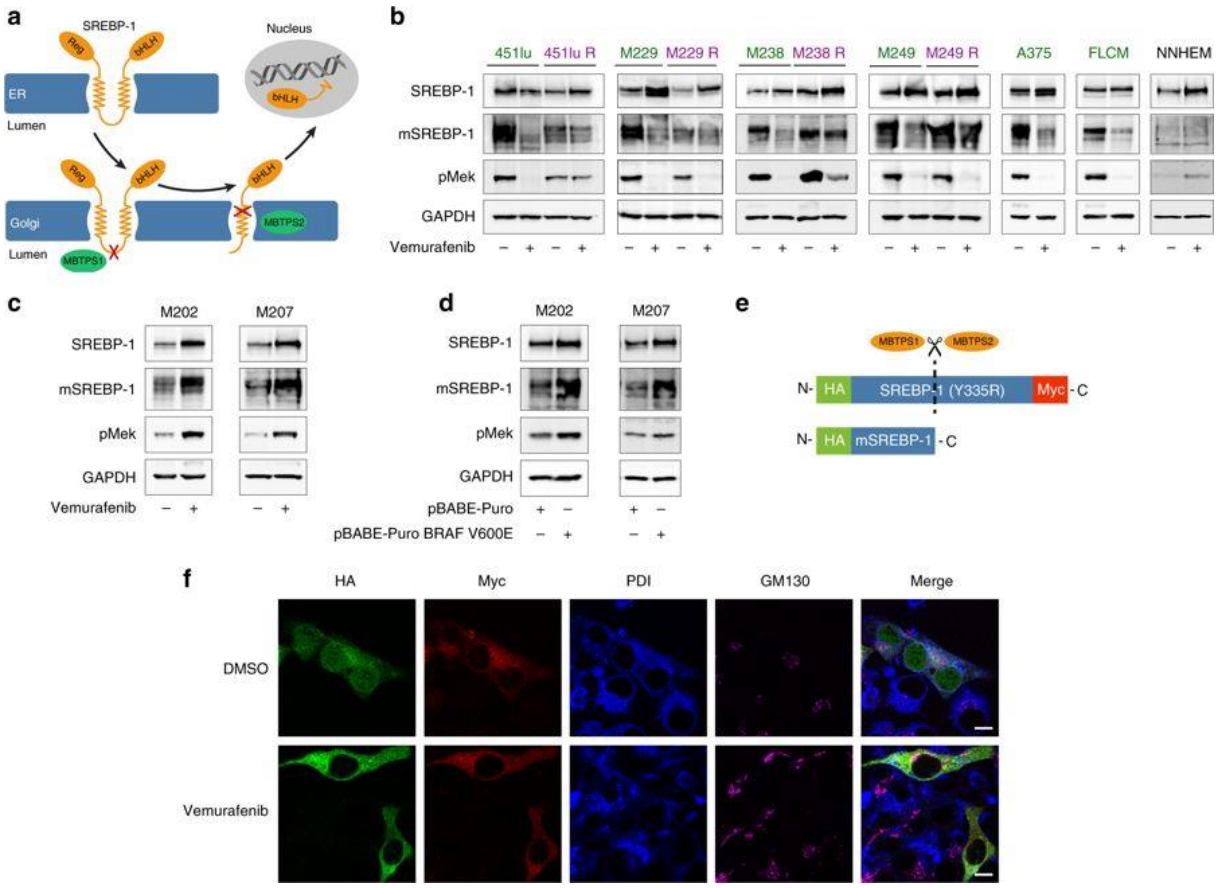


Figure 2. ***Effects of Vemurafenib on lipid metabolism involves SREBP-1*****.** a. Diagram illustrating the processing of SREBP. b.–d. Western analysis of SREBP-1 and pMEK (Ser217/221). GAPDH is used as a loading control. b Western blot analysis for mSREBP levels. c Western blot analysis of BRAF wild-type (but NRAS mutant) melanoma cells M202 and M207 treated with Vemurafenib (5 µM). d. Western blot analysis of BRAF wild-type, NRAS mutant melanoma cells, 72 h after transfection with pBABE-Puro BRAF-V600E. e. Diagram illustrating the HA-SREBP-1(Y335R)-myc construct. f. Confocal analysis of 451lu cells expressing HA-SREBP-1(Y335R)-myc. A PDI antibody was used to mark the ER and GM130 was used for cis-Golgi. Scale bar indicates 25 micrometres.

Interestingly, BRAF inhibition only induced a moderate decrease in mSREBP-1 levels and did not significantly affect lipogenesis in therapy-resistant cells (Figure 2b). Since alternative activation of the ERK pathway is a common contributor to therapy resistance and a known regulator of SREBP[196,204,205], we treated the therapy-resistant cell line 451lu R with the MEK inhibitor Trametinib (Supplementary Figure 6). As expected, these cells maintained high levels of pMEK upon Vemurafenib treatment (Figure 2b). Interestingly, MEK inhibition substantially decreased the levels of mSREBP-1 in these cells (Supplementary Figure 7). Consistently, expression of well-established mSREBP-1 downstream targets, such as ACLY, ACACA, and FASN, was also reduced (Supplementary Figure 8). These findings indicate that re-activation of the ERK pathway contributes to sustained SREBP-1 activity in therapy-resistant melanoma cells.

9.3 Sustained SREBP-1 activity maintains lipogenesis in therapy-resistant cells

To further assess the role of SREBP-1 in the changes in lipid metabolism evoked by Vemurafenib, we inhibited SREBP-1 in 451lu R cells using pharmacological and genetic approaches. We used two small-molecule inhibitors, Betulin and Fatostatin, which inhibit the trafficking of SREBP to the Golgi, and thereby its proteolytic activation through distinct mechanisms[154,155]. Exposure of 451lu and 451lu R cells to these inhibitors induced the expected dose-dependent decrease in the levels of mature SREBP-1; an effect that was more pronounced in the therapy-sensitive cell line (Supplementary Figure 9a). Phospholipidomic analysis revealed that chemical inhibition of SREBP-1 dose-dependently depleted mono-unsaturated and fully saturated phospholipid species and increased membrane poly-unsaturation, partially recapitulating the effect of BRAF inhibition on the therapy-sensitive cell line (Figure 3a). Furthermore, these effects were further enhanced with the addition of Vemurafenib, whereby the phospholipidome of the resistant line under SREBP-1 inhibition and Vemurafenib closely resembled that of the sensitive line in response to Vemurafenib (Figure 3a).

In order to corroborate these data using a genetic approach, we generated a heterozygous and two homozygous SREBF-1 knockout clones from the therapy-resistant cell line 451lu R using CRISPR-Cas9. Both Sanger sequencing and western blot analysis confirmed partial or full SREBF-1 deletion in the heterozygous and homozygous mutant cells, respectively (Supplementary Figure 9b, c)[206]. Similar changes in the lipid membrane composition to the ones observed upon pharmacological inhibition were observed in the heterozygous SREBF-1 KO clone, and to an even greater extent, in homozygous KO clones (Figure 3b). Since inhibition of SREBP-1 activation resulted in membrane lipid changes that closely mimicked the effects of Vemurafenib, we concluded that SREBP-1 is the major mediator of BRAF inhibition-dependent lipid metabolism rewiring.

9.4 Inhibition of SREBP-1 re-sensitizes resistant cells to BRAF targeting therapy

In order to investigate whether Vemurafenib-induced processing of SREBP-1 contributes to its anti-tumour response, we assessed the ability of melanoma cells to grow in both 2D and 3D cultures in response to Vemurafenib upon pharmacological or genetic inactivation of SREBP-1. Vemurafenib potently inhibited cell proliferation of the therapy-sensitive, but not the therapy-resistant line (Figure 3c, Supplementary Figs. 10, 11). Proliferation of the therapy-resistant cells was strongly inhibited upon combined exposure to Vemurafenib and SREBP-1 inhibitors. Similarly, SREBP-1 inhibitors synergized with Vemurafenib in inhibiting the ability of therapy-resistant cells to form colonies (Figure 3d, Supplementary Figure 12). Genetic ablation of SREBF-1 inhibited the rate of cell proliferation in 2D cultures of therapy-resistant cells when compared to the resistant parental cell line. In contrast, inactivation of only one SREBF-1 allele had no effect on cell proliferation (Figure 3e, Supplementary Figs. 13, 14). The rate of proliferation of these cells decreased significantly upon exposure to Vemurafenib. Similarly, these heterozygous SREBF-1 knockout cells were able to form colonies in 3D cultures; an ability that was reduced in the presence of Vemurafenib (Figure 3f, Supplementary Figure 15).

In order to assess whether sustained SREBP-1 processing also mediates therapy resistance to Vemurafenib in BRAF-V600E mutant cells that are endogenously resistant to BRAF targeting therapy, we treated M23340 with Betulin and Fatostatin. Both Betulin and Fatostatin treatment further sensitized the cell line to Vemurafenib (Supplementary Figure 16).

In order to assess whether our results directly reflect the effects of BRAF inhibition and are not off-target effects of Vemurafenib, in parallel we treated 451lu and 451lu R cells with Dabrafenib. Dabrafenib caused a decrease in both 14C-acetate incorporation into lipids and mature SREBP-1 protein levels in 451lu cells, but not in 451lu R cells (Supplementary Figure 17). Furthermore, combined Betulin and Fatostatin treatment with Dabrafenib inhibited proliferation in 451lu R cells, mirroring the effects of Vemurafenib (Supplementary Figure 18).

Since combined BRAF and MEK inhibitor treatment is a standard of care for BRAF-mutant melanoma patients, we further assessed the cell proliferative response to SREBP inhibition in A101D BMR and D10 BMR. These cell lines are resistant to both Dabrafenib and Trametinib (A101D BMR is partially resistant and retains some level of sensitivity). Here we show that SREBP inhibition by either Betulin or Fatostatin sensitized the cells to combined BRAF and MEK inhibition (Supplementary Figs. 19–22).

Taken together, these data indicate that SREBP-1 contributes to the anti-tumour response induced by BRAF inhibition and that SREBP-1 inhibition sensitizes therapy-resistant melanoma cells to MAPK-targeting therapy.

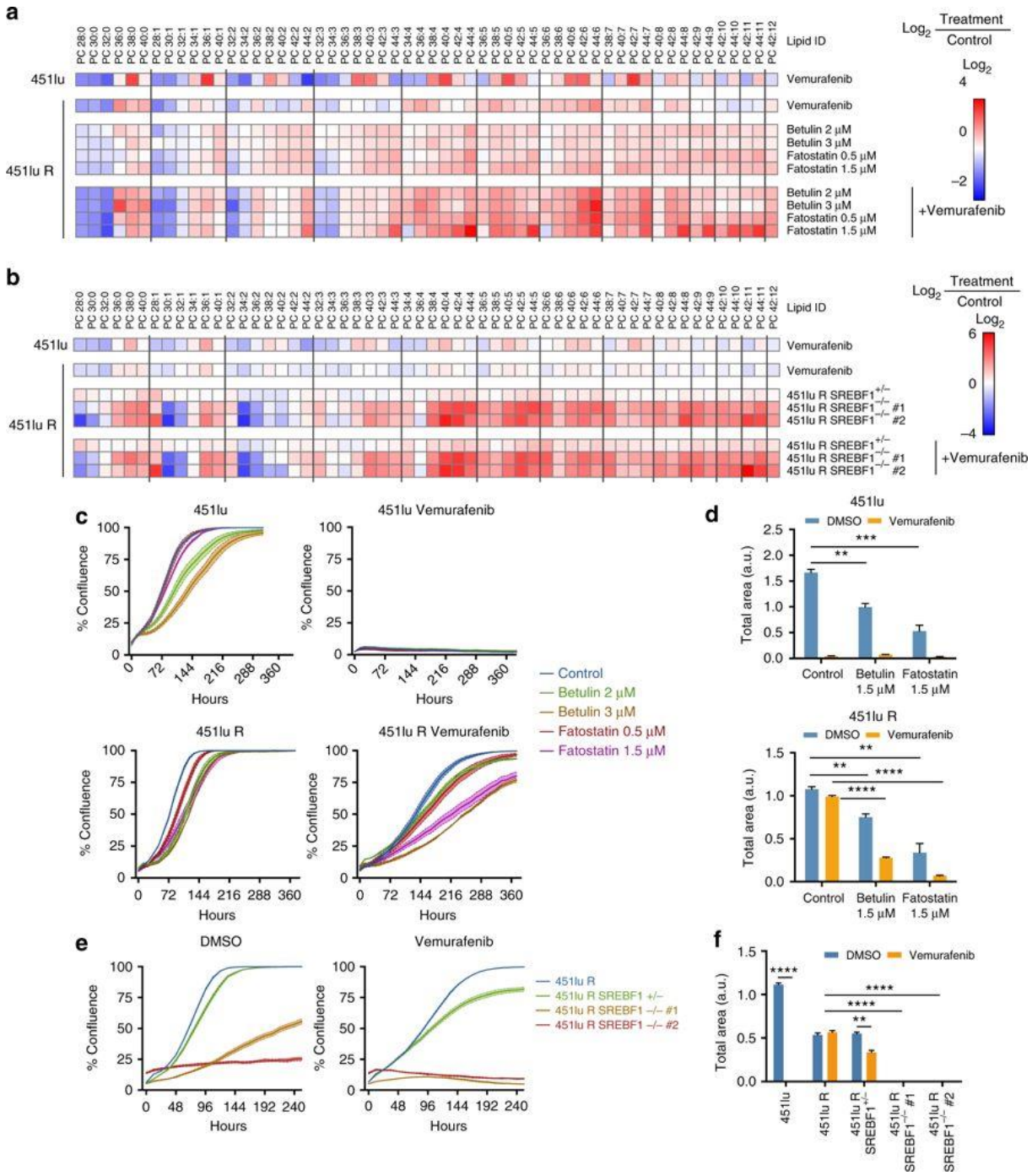


Figure 3. ***SREBP-1 contributes to membrane saturation and therapy resistance***. a. Lipidomic (phosphatidylcholine) profiles of 451lu and 451lu R cells treated with Vemurafenib (5 µM) and SREBP inhibitors Fatostatin and Betulin (n = 3). .b Lipidomics of hetero- and homozygous CRISPR-Cas9 knockouts of SREBF-1 in combination with Vemurafenib treatment (n = 3). c. Proliferation curves of 451lu and 451lu R cells treated with Vemurafenib and Fatostatin or Betulin (phase contrast density measured by the Incucyte system, raw images and bar graph are shown in Supplementary Figs. 10, 11) (n = 4). d. 451lu and 451lu R cells were treated with SREBP inhibitors and Vemurafenib and were assayed for their ability to form colonies in soft agar, raw images and bar graph are shown in Supplementary Figure 12 (n = 3). e. Proliferation curves of 451lu R SREBP KO cells, raw images and bar graph are shown in Supplementary Figs. 13, 14 (n = 4). f. SREBF-

1 KO 451lu R cells were assayed for their ability to form colonies in soft agar in presence or absence of Vemurafenib, raw images and bar graph are shown in Supplementary Figure 15 (n = 3). All data are represented as mean ± s.e.m. (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001)

9.5 SREBP-1 protects Vemurafenib-resistant cells from lipid peroxidation

The findings described above predict that SREBP-1-mediated therapy resistance is a consequence of enhanced membrane lipid saturation and, consequently, decreased lipid peroxidation. To test this hypothesis, we mimicked the effect of SREBP-1 inhibition on the lipid composition of therapy-resistant cells by treatment with the PUFAs linoleic and linolenic acid (PUFA). When combined with Vemurafenib, PUFA addition slightly attenuated the growth of the cultures. Conversely, supplementing cells with a final product of lipogenesis, oleic acid, slightly enhanced proliferation of cells under Vemurafenib treatment. Both of these effects were significantly enhanced upon pharmacological or genetic inactivation of SREBP-1 indicating that membrane lipid saturation contributes to SREBP-1-mediated resistance to BRAF inhibition (Figure 4a, Supplementary Figs. 23, 24).

We have previously reported that membrane saturation protects cancer cells from ROS- and chemotherapy-induced cell death[98]. Here, we observed that both Fatostatin and Vemurafenib increase the levels of mitochondrial ROS independently, and that a combination of the two leads to an additional effect (Figure 4b). In addition, ROS levels were either further enhanced or decreased by addition of PUFAs and oleic acid, respectively. Under combined Vemurafenib and Fatostatin treatment, addition of oleic acid reduced the levels of mitochondrial ROS to levels observed in cells treated with Vemurafenib alone (Figure 4b). ROS has been linked to membrane lipid peroxidation, which results in the accumulation of toxic by-products[207]. Since PUFA are more prone to lipid peroxidation than saturated lipids, we next measured lipid peroxidation by measuring levels of cellular malondialdehyde (MDA), which is a direct by-product of lipid hydro-peroxide degradation. Whereas generally low in melanoma cells, levels of MDA increased upon exposure to Fatostatin and to an even greater extent upon Vemurafenib treatment (Figure 4c). Combined Fatostatin and Vemurafenib treatment resulted in a further increase in MDA levels, which is directly in line with the levels of membrane lipid poly-unsaturation incurred by the treatments (Figs. 3a and 4c). The levels of MDA under combined therapy increased further with addition of exogenous PUFA and decreased to levels seen under Vemurafenib alone upon addition of exogenous oleic acid.

Interestingly, steady state metabolomics analysis of cellular AMP, ADP, and ATP indicated that cellular ATP levels or energy charge are not markedly altered by combined BRAF and SREBP-1 inhibition (Supplementary Figure 25). Metabolomics analysis of NAD, NADH, NADP, NADPH, GSH, and GSSG ratios revealed that combined SREBP-1 and BRAF inhibition significantly lowers the cell antioxidant potential.

To corroborate our findings that SREBP-1 inhibition sensitizes cells to Vemurafenib through lipid peroxidation, we supplemented cells with the antioxidants Alpha-Tocopherol, Ferrostatin, and N-Acetyl-cysteine (NAC). Under combined SREBP-1 and oncogenic BRAF inhibition, addition of antioxidants partially rescued cell proliferation (Figure 4d, Supplementary Figs. 26, 27). To show that lipid poly-unsaturation and lipid peroxidation also plays a role in Vemurafenib response in drug sensitive cells, we treated therapy-sensitive cell lines M229 and 451lu with either Betulin or Fatostatin and found that these compounds further enhance the cytostatic effects of the BRAF-inhibitor (Supplementary Figs. 28, 29). Furthermore, we show that treatment with alpha-tocopherol, in part, rescued the cytostatic effects of Vemurafenib in the M229 cell line, and alpha-tocopherol, NAC and Ferrostatin treatment rescued the proliferation of 451lu cells (Figure 4e and Supplementary Figs. 30, 31). We conclude that SREBP-1 inhibition sensitizes cells to Vemurafenib, at least partly, though alterations of membrane poly-unsaturation and, thereby, lipid peroxidation.

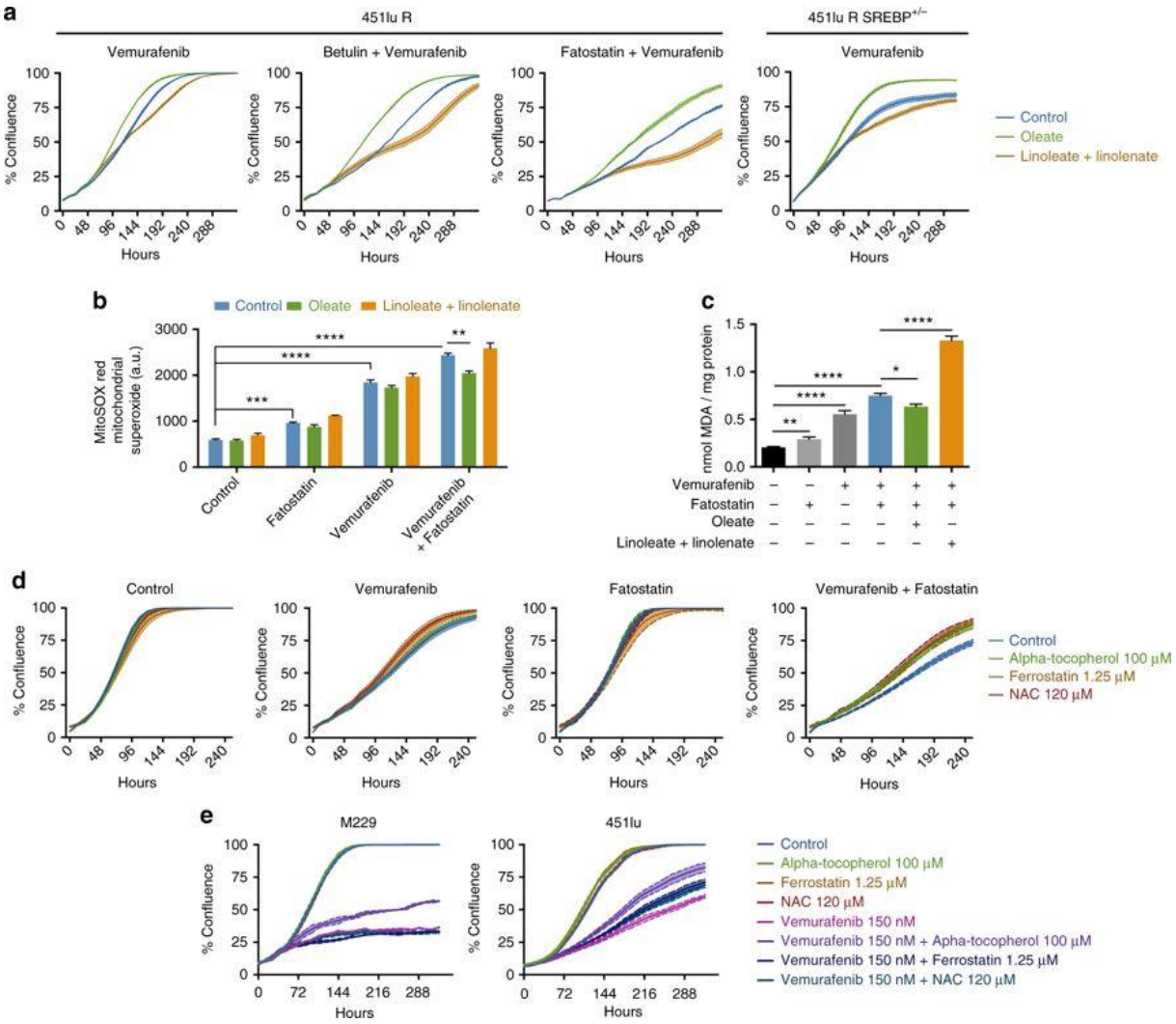


Figure 4. ***Re-sensitization to Vemurafenib involves lipid peroxidation*****.** a. Oleate (20 µM) or a mixture of linoleate (10 µM) + linolenate (10 µM) was added to the culture medium of 451lu R SREBP+/− cells and 451lu R cells treated with either Vemurafenib alone or a combination with Fatostatin or Betulin, raw images and bar graph are shown in

Supplementary Figs. 23, 24 (n = 3). b. Mitochondrial superoxide levels in 451lu R cells treated with Vemurafenib and Fatostatin; medium was supplemented with oleate or linoleate + linolenate (n = 3). c. MDA levels normalized to protein content in 451lu R cells (n = 5). d. Effect of Alpha-Tocopherol, Ferrostatin and NAC supplementation on proliferation curves of 451lu R cells treated with Vemurafenib + Fatostatin, raw images and bar graph are shown in Supplementary Figs. 26, 27 (n = 4). e. Proliferation curves of the therapy-sensitive cell lines M229 and 451lu treated with Vemurafenib, Alpha-Tocopherol, Ferrostatin, and NAC (phase contrast density measured by the Incucyte system) raw images and bar graph are shown in Supplementary Figs. 30, 31 (n = 3). All data are represented as mean ± s.e.m. (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001)

9.6 SREBP-1 inhibition sensitizes melanoma to Vemurafenib *in vivo*

To assess the therapeutic potential of these findings we investigated the impact of SREBP-1 inhibition in an *in vivo* pre-clinical BRAF-V600E-mutant melanoma model. We chose PDTX MEL006, which has been extensively characterized previously and was shown to poorly respond to BRAF inhibitors alone[208,209]. Mouse cohorts were treated blindly with either vehicle, Vemurafenib alone, Fatostatin alone or a combination of Vemurafenib and Fatostatin. Fatostatin treatment alone inhibited tumour growth more potently than Vemurafenib. Importantly, combined Vemurafenib/Fatostatin co-treatment had a greater anti-tumour effect than any of the monotherapy regimens (Figure 5a–c). Phospholipidomic analysis of the various treated melanoma lesions revealed a correlation between the changes in the poly-unsaturation of phospholipids and anti-tumour growth response (Figure 5d), whereby membrane poly-unsaturation was synergistically enhanced by the combination treatment. MDA analysis revealed that whereas Fatostatin or Vemurafenib treatment alone did not significantly increase lipid peroxidation, the combined Vemurafenib/Fatostatin treatment greatly enhanced lipid peroxidation (Figure 5e). In the Mel006 tumour treated with a combination of Dabrafenib and Trametinib, an increase in MDA was found shortly after the start of treatment and, to a lesser extent, after establishment of resistance (Supplementary Figure 32).

Taken together, combined Fatostatin and Vemurafenib therapy enhanced therapy response *in vivo* and increased membrane lipid poly-unsaturation and lipid peroxidation. These data support the concept of a novel combinatorial approach to overcome therapy resistance in BRAF-V600E mutant melanoma models.

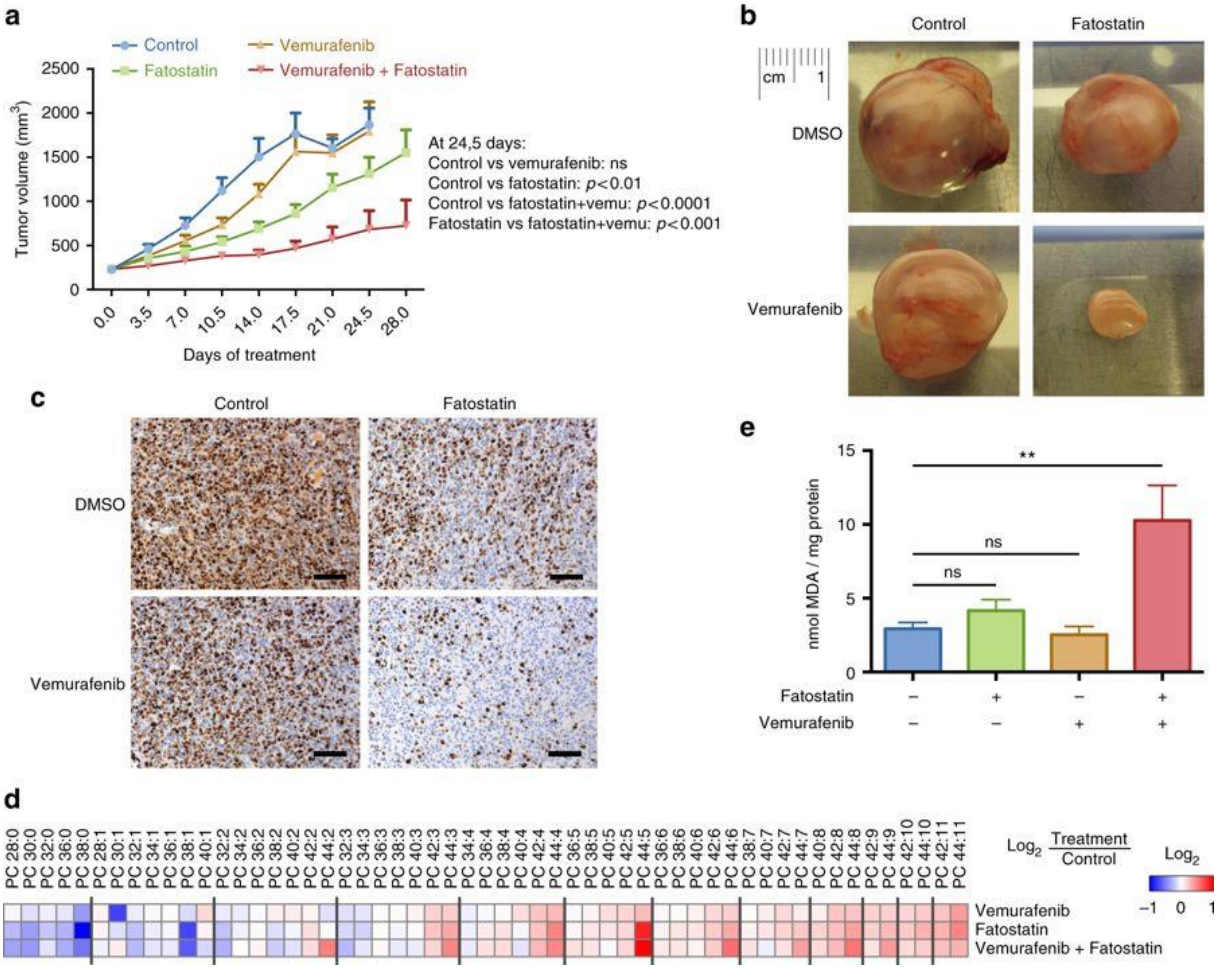


Figure 5. SREBP-1 protects Vemurafenib-resistant cells from lipid peroxidation. BRAF-V600E-mutant PDTX tumours (mel6 model) were transplanted into nude mice (NMRI-Fox1nu) which were blindly administered vehicle (n = 8), Fatostatin (20 mg/kg) (n = 10), Vemurafenib (20 mg/kg) (n = 10) or a combination of Vemurafenib and Fatostatin (n = 10) (daily by oral gavage). a. Tumour size was measured blindly with digital callipers every 3 days. b. The tumour pictures show the tumours that represent the median of their respective cohort. c. Expression of Ki67 protein in PDTX tumours that represent the median of their respective cohort. Scale bars indicate 100 µm. d. Lipidomics of PDTX tumour homogenate. e. MDA quantification of PDTX tumour homogenate. The data represent the average over all mice for each cohort compared to the control. Data are represented as mean ± s.e.m. (**p < 0.01)

10. Discussion

Resistance to targeted therapy represents a major clinical challenge. This is partly a consequence of the fact that most therapeutic targets to date, including BRAF-V600E, act in the proximal part of their signal transduction cascade. This offers multiple opportunities for cancer cells to bypass drug response through for instance the acquisition of mutation(s) that reactivate the pathway downstream (e.g., by MEK mutation). An attractive strategy to overcome therapy resistance is therefore the identification and exploitation of vulnerabilities, which are activated by and act downstream of such

oncogenic pathways. Metabolic pathways are of particular interest in this context as they often rely on a few essential enzymes, are frequently rewired in cancer cells, provide essential survival/adaptive capabilities and can easily be pharmacologically targeted. Here, we identified the lipogenic transcription factor SREBP-1 as a key downstream target of oncogenic BRAF signalling. We have shown that sustained lipogenesis through the maintenance of active SREBP-1 is a key feature of therapy resistance to Vemurafenib in BRAF-mutant melanoma, and that inhibition of SREBP-1 sensitizes melanoma to targeted therapy.

Critically, the addiction of therapy-resistant melanoma cells to SREBP-1 is independent of the mechanisms exploited by the cancer cells to overcome drug response. We observed similar effects in cells that acquired resistance acquisition of through RTK upregulation (M229 R, M238 R), NRAS mutation (M249 R) or enhanced IGF1/PI3K signalling and RAF kinase flexibility (451lu R). In all sensitive BRAF-mutant models, Vemurafenib caused a decrease in lipogenesis and attenuated the processing and thereby the activity of SREBP-1. This was not seen in therapy-resistant models, which all showed high levels of lipogenesis even in the presence of the inhibitor. Together with our observation that pharmacological or genetic inactivation of SREBP-1 in resistant cells attenuates cell proliferation and sensitizes to Vemurafenib (irrespective of the escape mechanism), these findings indicate that SREBP-1-mediated lipogenesis is a central pathway acting downstream of mutant BRAF.

Previous work has shown that SREBP-1 is activated through several mechanisms including, regulation by the PTEN/PI3K/Akt/mTOR pathway[143,196], p53[147,148] modulation of MAPK signalling by KRAS46 and by direct SREBP phosphorylation by Erk1/2[149,150]. Our work identifies mutant BRAF as another key modulator of SREBP-1 processing and function.
Consistent with our findings, SREBP and its downstream targets are highly expressed in many cancers[122]. Importantly there is a growing body of evidence showing that SREBP-1-dependent activation of lipogenesis is required for tumour growth in multiple models, including in prostate cancer[163] and EGFR-dependent glioma[130,156]. SREBP-1 was shown to promote adhesion-independent growth[143] and cell proliferation[132,210–212], including growth factor-independent proliferation[213].

Similarly, proteins involved in the post-translational processing of SREBPs have also been linked to oncogenic potential in multiple models. SCAP modification and inhibition inhibit tumour growth through SREPBs[144,163,214,215]. Consistently, expression levels of SCAP inversely correlate with overall survival in multiple cancers in The Cancer Genome Atlas (TCGA) cohorts. Taken together, these data strongly support a pro-oncogenic role for SREBP processing in multiple cancers.

These data are in line with the well-established necessity of cancer cells to adapt their metabolism to their increased need of building blocks. Activation of SREBP-1 and enhanced ability to generate lipids in a cell-autonomous manner is thought to be required to sustain rapid tumour cell proliferation[104,123]. Earlier findings from our team have shown

that lipogenesis also contributes to resistance to cell death by altering membrane lipid composition and susceptibility to lipid peroxidation[98]. Here we provide evidence that lipogenesis driven by oncogenic BRAF signalling promotes resistance to targeted therapy. In therapy-sensitive cells, inhibition of oncogenic BRAF decreases membrane lipid saturation. Inhibition of SREBP-1 mimics this effect in therapy-resistant cells, especially upon BRAF therapy. We further show that this effect is a consequence of increased cellular ROS and lipid peroxidation. In therapy-resistant cells, Vemurafenib resulted in a substantial increase in mitochondrial ROS and lipid peroxidation. This is consistent with the well-established ability of Vemurafenib to induce ROS production[216,217]. Similarly, although to a lesser extent, SREBP inhibition also increased mitochondrial ROS levels. Combined inhibition of SREBP and oncogenic BRAF further enhanced ROS and lipid peroxidation, which can either be rescued or enhanced by exogenous addition of oleate or PUFA, respectively. Furthermore, addition of the antioxidant NAC results in a rescue of cell proliferation under combined therapy.

Importantly, SREBP inhibition enhanced the efficacy of Vemurafenib in a pre-clinical PDTX model of melanoma, emphasizing the clinical relevance of these findings. Our data support the growing interest in lipogenesis inhibition as a novel antineoplastic strategy and ongoing efforts aimed at identifying new classes of SREBP inhibitors, including those that interfere with the nuclear accumulation of mature SREBP. By showing that SREBP-1 has a key role in the resistance to mutant BRAF-targeted therapy our work identifies an important clinical setting in which such inhibitors may provide clear clinical benefit.

11. Methods

11.1 Cell culture

A375 was obtained from ATCC. FLCM was generated from melanoma derived from BrafCA, Tyr-CreER and Ptenlox4−5 mice. M202, M207, and M233 were gifted by professor A. Ribas. 451 and 451lu R, M229, M229 R, M238, M238 R, M249, and M249 R were gifted by R. Lo. A101D BMR and D10 BMR were gifted by Professor Daniel Peeper. NHEM was obtained from melanocytes derived from the foreskin of a pool of three healthy neonatal donors. The procedure was approved by the ethical committee of the University of Leuven and executed according to Helsinki guidelines.

All cell lines were propagated in DMEM High Glucose (Sigma), supplemented with 10% FBS (Gibco Lot 41F4234K) and 4 mM glutamine (ThermoFisher). A101D BMR and D10 BMR growth media was additionally supplemented with Dabrafenib and Trametinib. NHEM were cultured in Medium 254 (ThermoFisher) supplemented with HMGS (ThermoFisher) and Antibiotic-Antimycotic to 1×(ThermoFisher). 451lu R SREBF-1 KO clones were grown in DMEM High Glucose supplemented with 30% FBS and 4 mM glutamine. All cell cultures were periodically tested for mycoplasma contamination. All experiments were performed in DMEM High Glucose, supplemented with 2% FBS (Gibco Lot 41F4234K) and 4 mM glutamine, except for 13C-glucose metabolite tracer studies, where 4.5 g L−1 13C-glucose

(Cambridge isotope laboratories) was supplemented to DMEM no glucose (ThermoFisher). The following compounds were used at the stated concentrations. NAC (120 µM) from Sigma, Alpha-Tocopherol (100 µM) from Sigma, Ferrostatin (1.25 µM) from Sigma, Vemurafenib (5 µM) from ApexBio, Dabrafenib (2.5 µM) from Selleckchem, Trametinib (0.5 µM) from Selleckchem, Betulin (2 or 3 µM) from Sigma, Fatostatin (0.5 or 1.5 µM) from Tocris Bioscience, oleic acid (20 µM) from Sigma, linoleic acid (10 µM) from Sigma and linolenic acid (10 µM) from Sigma.

11.2 RNA-seq

RNA concentration and purity were determined spectrophotometrically using a Nanodrop ND-1000 (Nanodrop Technologies) and RNA integrity was assessed using a Bioanalyser 2100 (Agilent). Samples were analyzed on an HiSeq2000 (Illumina). The raw sequencing data are publically available and can be accessed at https://www.ncbi.nlm.nih.gov/sra/SRP143504.

11.3 Plasmid transfections

M202 and M207 were transfected by electroporation (Neon Transfection System, ThermoFisher) with either pBabe-puro or pBabe-puro-BRAF-V600E. A375, 451lu, and M249 were transfected by electroporation (Neon Transfection System, ThermoFisher) with pbabe-puro-HA-SREBF-1(Y335R)-myc.

11.4 Construction of knockout cell lines

451lu R cells were transfected (Neon Transfection System, ThermoFisher) with CRISPR-Cas9 plasmid constructs with a guide-RNA targeting human SREBF-1 exon 1 (VectorU6gRNA-Cas9-2A-GFP, target ID: HS0000039707 and HS0000039709) (Sigma). 72 h post-transfection, the top 10% of GFP expressers were sorted by FACS (BD bioscience, ARIA III) into single wells for colony formation. Targeted regions of individual clones were sequenced (Sanger sequencing, LGC) and indels in allelic sequences we genotyped using CRISP-ID (Supplementary Figure 9c)[206].

11.5 14C-acetate incorporation into lipids and 13C-glucose tracing

Cells were grown in 6-well plates up to 80% confluence and were treated with 0.1 µCi acetate-2-14C (55 mCi/mmol; Amersham) for 4 h. After three washes with PBS (Sigma), cells were trypsinized and suspended in 1 mL PBS, followed by sonication. 0.125 mL of lysate was set aside for DNA measurement and 0.7 mL of lysate was mixed with 0.9 mL MeOH solution (MeOH:HCl = 8:1) (Sigma) and 0.8 mL CHCl3 (Sigma). After vortexing the mixture for 30 s followed by centrifugation for 10 min at 2000 × g, the organic fraction was counted for radioactivity (Tri-Carb 2810 TR scintillation counter, PerkinElmer). Mass spectrometry analysis of 13C-glucose incorporation into palmitate and subsequent isotopomer spectral analysis was performed as described previously[218].

11.6 Untargeted metabolomics

Cell extracts were separated on an Acquity HSS T3 UPLC column (Waters Corp, 2.1 mm × 150 mm, 1.8 µm particle size) using an Ultimate 3000 HPLC (Dionex ThermoFisher Scientific Inc). Elution of metabolites was performed using a quaternary solvent system. The data was collected over a mass range of 50–1050 m/z. Data analysis was done using ThermoFisher Scientific Quan software (Xcalibur version 4.0) and manually verified. Further analysis was done using in house software tools.

11.7 Cholesterol amount and uptake quantification

Cholesterol was quantified using Amplex Red cholesterol assay kit (ThermoFisher). Cholesterol uptake was quantified using NBD cholesterol (ThermoFisher).

11.8 Analysis of intact phospholipid species by ESI-MS/MS

0.7 mL of homogenized tissue or cells were mixed with 0.9 mL MeOH:HCl(1 N) (8:1), 0.8 mL CHCl3 and 200 µg mL−1 of the antioxidant 2,6-di-tert-butyl-4-methylphenol (Sigma). The organic fractions were evaporated under vacuum, reconstituted in MeOH/CHCl3/NH4OH (90:10:1.25) and lipid standards were added (Avanti Polar Lipids). Phospholipids were analyzed by electrospray ionization tandem mass spectrometry (ESI-MS/MS) on a hybrid quadrupole linear ion trap mass spectrometer (4000 QTRAP system, AB SCIEX) equipped with a TriVersa NanoMate robotic nanosource (Advion Biosciences) as described[98].

11.9 Immunoblotting analysis

Following ice-cold PBS washes, cells were collected in sample buffer (ThermoFisher) supplemented with DTT (Sigma), sonicated and boiled for 5 min. Equal amounts of protein were loaded onto precast gels (NuPAGE, ThermoFisher), transferred to nitrocellulose membranes, and incubated with antibodies against SREBP-1 (1/1000 dilution) (Active Motif, #39939), phospho-MEK1/2 ser217/221 (1/1000 dilution) (Cell Signalling, #9154); GAPDH (1/20000 dilution) (Cell Signalling, #5174), myc-Tag (1/2000 dilution) (Cell Signalling, #2276), HA-Tag (1/5000 dilution) (Cell Signalling, #3724), phosphor-AKT ser473 (1/1000 dilution) (ThermoFisher, #98H9L8), pS6 ser235 (1/1000 dilution) (Cell Signalling, #2211), and pERK 1/2 (1/1000 dilution) (Cell Signalling, #9101). Full unedited blots are shown in Supplementary Data 1.

11.10 RNA extraction and RT-qPCR

RNA extraction and RT-qPCR were performed as described previously24. Primers used were: FASN: Fw 5′-TCCGAGATTCCATCCTACGC-3′, Rv 5′-GCAGCTGTGACACCTTCAGG-3′, ACLY: Fw 5′-TGTAACAGAGCCAGGAACCC-3′, Rv 5′-CTGTACCCCAGTGGCTGTTT-3′, ACACA: Fw 5′-TGAACTTCACACAGGTAGTCTGCC-3′, Rv 5′-TGGAACACTCGATGGAGTTTCT-3′, SREBP-1a: Fw 5′-GCTGCTGACCGACATCGAA-3′, SREBP-1c: Fw 5′-GGAGCCATGGATTGCACTTT-3′, SREBP-1a/1c Rv 5′- TCAAATAGGCCAGGGAAGTCA-3′, and 18 S as a reference gene: Fw 5′-CGCCGCTAGAGGTGAAATTC-3′, Rv 5′-TTGGCAAATGCTTTCGCTC -3′.

11.11 Confocal microscopy

Cells were transfected (Neon Transfection System, ThermoFisher) with a plasmid coding an HA and myc-tagged transcriptionally inactive SREBP-1 (Y335R) (GenScript). The SREBP cDNA was obtained from pTK-HSV-BP1a[219]. Cells were treated as indicated, fixed and incubated with antibodies against: myc-Tag (1/2000 dilution) (Cell Signalling, #2276) and HA-Tag (1/5000 dilution) (Cell Signalling, #3724), GM130 (1/500 dilution) (BD Biosciences, #610822), and PDI (1/1000 dilution) (ThermoFisher, #MA3-018). Samples were imaged using an Olympus FluoView FV1000 confocal microscope.

11.12 Proliferation assays

Proliferation curves were generated using an IncuCyte ZOOM system (Essen BioScience) on cells seeded of microplates (TPP) based on phase contrast images taken at 2 h intervals for the duration of the experiments. Colony formation in soft agar was performed as described by Franken et al[220]. Except, colonies were stained with Vybrant DyeCycle Green nuclear stain (ThermoFisher), imaged with a Typhoon FLA 9500 laser scanner (GE Healthcare) and quantified in ImageJ.

11.13 Mitochondrial ROS measurement

Mitochondrial ROS was measured using MitoSOX red mitochondrial superoxide indicator (ThermoFisher) according to the manufacturer's instructions. Cells were assayed using a FACS Verse flow cytometer (BD Biosciences).

11.14 Lipid peroxidation assay

Lipid peroxidation was quantified using the MDA assay kit (Sigma) according to manufacturer's instructions with some exceptions. Briefly, cells or tissue were collected in BHT supplemented PBS. Thiobarbituric acid-acetic acid solution was buffered to pH 3.5. Plates were read using an EnSpire Multimode Plate Reader (PerkinElmer).

11.15 Animal experiments

The Mel006 PDTX model was derived from an in-transit metastasis from a patient undergoing surgery as part of standard-of-care melanoma treatment at the University Hospitals Leuven. The patient provided written informed consent and this procedure was approved by the UZ Leuven Medical Ethical Committee (S54185) and carried out in accordance with the principles of the Declaration of Helsinki. All procedures involving animals were carried out in accordance with the guidelines of the IACUC and the Animal Care and Use Ethical Committee (KU Leuven, P038/2015). Fresh tumour tissue carrying the BRAF-V600E mutation was collected in RPMI640 medium with antibiotics, rinsed in PBS and transplanted subcutaneously in the interscapular fat pad of female SCID-beige mice (Taconic). Sedation and analgesia was performed using ketamine, medetomidine and buprenorphine. Upon reaching generation 3, tumour fragments were transplanted into nude mice (NMRI-Fox1nu, Taconic) and when tumour size reached 200 mm3, mice were randomly assigned to a cohort and drugs or vehicles were blindly administered daily

by oral gavage. Vemurafenib and Fatostatin were both administered daily at 20 mg/kg. Fatostatin or vehicle (30% PEG (Sigma) in water (Baxter)) was administered an hour after Vemurafenib or vehicle (2% hydroxypropylcellulose (Sigma) in water (Baxter)). Tumour size was measured blindly with digital callipers (Fowler Sylvac) every 3 days. Mice were sacrificed at 28 days following start of treatment or when tumours reached a volume of 1500 mm3. The investigators were blinded for the evaluation of the results.

11.16 Statistical analysis

The results were analyzed in GraphPad Prism 6.0 h using a t-test. In case of multiple comparisons a correction was applied using the Holm–Sidak method. P-values of <0.05 were considered to be statistically significant. All data presented represent means ± s.e.m.

11.17 Data availability

The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary Information files.

11.18 Supplementary data

Please find all supplementary figures and data on the online version of this manuscript: https://www.nature.com/articles/s41467-018-04664-0

12. Acknowledgements

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13. Contributions

A.T., J.D., and J.V.S. designed the experiments and wrote the paper. A.T., J.D., and F.V. performed the experiments. J.-C.M. provided extensive helpful comments and discussion. A.T. and J.D. analyzed data. J.V.S. oversaw the project and acquired funding. F.R. analyzed and generated the TCGA, RNA-seq data. A.R. established and characterized the Mel006 PDTX model. R.D. executed the phospholipidomics analysis. E.W. allowed use of the mass spectrometer. D.N., K.V.d.E., F.B., and J.V.d.O. generated the mouse IHC slides. K.N., M.V.P., and P.A. extracted the NHEM. J.A.G.D. and S.-M.F. performed and analyzed the stable isotope metabolic flux experiments.

14. Competing interests

The authors declare no competing interests.

Chapter 3: Lipogenesis inhibition sensitises BRAF mutant melanoma to inducers of reactive oxygen species

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1. Summary

One of the key limitations of targeted cancer treatments is the general propensity of cancer cells to escape therapies and to develop resistance, often in spite of a good initial response. It is therefore necessary to find common modalities of resistance in order to enhance or sustain therapy response.

Recently we have shown that SREBP-1-mediated *de novo* lipogenesis plays a key role in therapy resistance in skin cutaneous melanoma and that SREBP-1 inhibition sensitizes cancer cells to therapy. Here we have extended the translational potential of this study by the use of preclinical fatty acid synthase (FASN) inhibitors. Building on our observation that subsequent lipid poly-unsaturation is a key determinant of therapy responsiveness, we have explored the potential of exploiting the susceptibility of poly-unsaturated lipids to reactive oxygen species (ROS) in a novel combinatorial treatment strategy. We show that inhibition of lipogenesis in therapy-resistant BRAF-mutant melanoma models, generates an exquisite vulnerability of cancer cells to inducers of reactive oxygen species (ROS) such as Arsenic trioxide (ATO). These findings provide the basis for a novel combinatorial treatment of therapy-resistant tumours.

2. Introduction

Melanoma is the most aggressive form of skin cancer and arises from melanocytes. Approximately half of all melanoma carry the BRAF-V600E mutation and are generally responsive to BRAF and MEK targeting therapies. In spite of an initial favourable response, therapy resistance rapidly ensues in the majority of cases[43,45,191]. Many genomic and non-genomic mechanisms of resistance have been described, all leading to re-activation of the MAPK- and/or PI3K-signalling pathways[47,192,193]. Moreover, different mutational events can be selected in distinct drug-resistant clones from the same patient[49] and even co-occur within the same lesion[48]. These findings have highlighted the need to improve effectiveness of treatment.

Targeting metabolism is an increasingly attractive avenue of research in exploiting cancer vulnerabilities. Importantly, metabolic pathways are often less degenerate than other signalling pathways. Moreover, specific metabolic enzymes are often required in generate metabolites making escape mechanisms more challenging. Metabolic rewiring is a fundamental hallmark of cancer cells. When compared to normal tissue, cancer cells often upregulate biosynthetic processes, which can in part give the cancer cell flexibility in generating the feed-stocks needed in forming biological molecules needed to sustain proliferation or evade stresses.[6]

Concurrently, *de novo* lipogenesis is upregulated in myriad cancer models,[118–120]. In particular, the over-expression of key enzymatic drivers of lipogenesis such as fatty acid synthase (FASN), Acetyl-CoA carboxylase alpha (ACACA) and stearoyl-Coa desaturase (SCD) is observed in multiple cancers[92,122–124]. Multiple oncogenes and initiating oncogenic events such as Map Kinase, establishing lipogenesis as fundamental oncogenic

transformation event[75,128,133]. Crucially, most normal tissues obtain fatty acids from the circulation[126,135], making lipogenesis a promising and possibly specific anti-neoplastic target. Since upregulation of lipogenesis is an early cancer event, and precedes rapid proliferation of cancer cells,[221] lipogenesis may play other critical roles in cancer biology. Importantly, most *de novo* generated fatty acid are saturated and mono-unsaturated, thereby causing a shift in the membrane profile of lipogenic cancer cells towards membrane saturation[98]. It is well-established that PUFA are sensitive to radical mediated lipid peroxidation. In line with this observation, we have previously shown that membrane lipid saturation renders cancer cells more resistant to reactive oxygen species (ROS), and that cancer cells can be re-sensitised to ROS by interfering with lipogenesis[75,98].

We have recently shown that therapy resistance in BRAF mutant melanoma relies on sustained lipogenesis, and that by interfering with this process, cancer cells are re-sensitised to therapy and are rendered sensitive to ROS[75]. Lipogenesis driven membrane lipid saturation in part underlies this effect.

Here, we show that Map Kinase targeting therapy synergises with lipogenesis inhibition in BRAF-V600E mutant melanoma and drives a cytostatic, membrane lipid poly-unsaturated and ROS sensitive cell phenotype in therapy resistant melanoma. Critically, we show that further ROS induction in these cells is cytotoxic. Taken together, we show a novel treatment strategy in pre-clinical BRAF mutant melanoma models. In conclusion, by combining Map Kinase inhibition with lipogenesis inhibition we confer a new acquired vulnerability to ROS.

3. Results

3.1 ACACA and FASN correlate with poor survival in melanoma and correlate with therapy response

We have previously shown that therapy resistance in BRAF mutant melanoma relies on sustained lipogenesis through SREBP-1 signalling and that BRAF inhibition reduces the expression on several lipogenic enzymes including ACACA and FASN[75]. Here, based on TCGA analysis[29], we show that SREBP-1 signalling correlates to a poor prognosis in melanoma. This correlation is valid for enzymes involved in SREBP processing such as SCAP expression and INSIG (data not shown), but particularly also for lipogenic enzymes, namely ACACA and FASN (Figure 1a-c). Moreover, we show that both ACACA and FASN are highly expressed in melanoma and that their RNA-expression correlates strongly to the amount of protein (data not shown). Importantly, we show that both ACACA and FASN expression are reduced following treatment of Mel006 BRAF-V600E mutant PDTX mice with Dabrafenib and Trametinib, and is increased following the acquisition of therapy resistance (Figure 1d). Taken together, ACACA and FASN expression correlates with poor prognosis in melanoma and their expression is linked to therapy response in tumours.

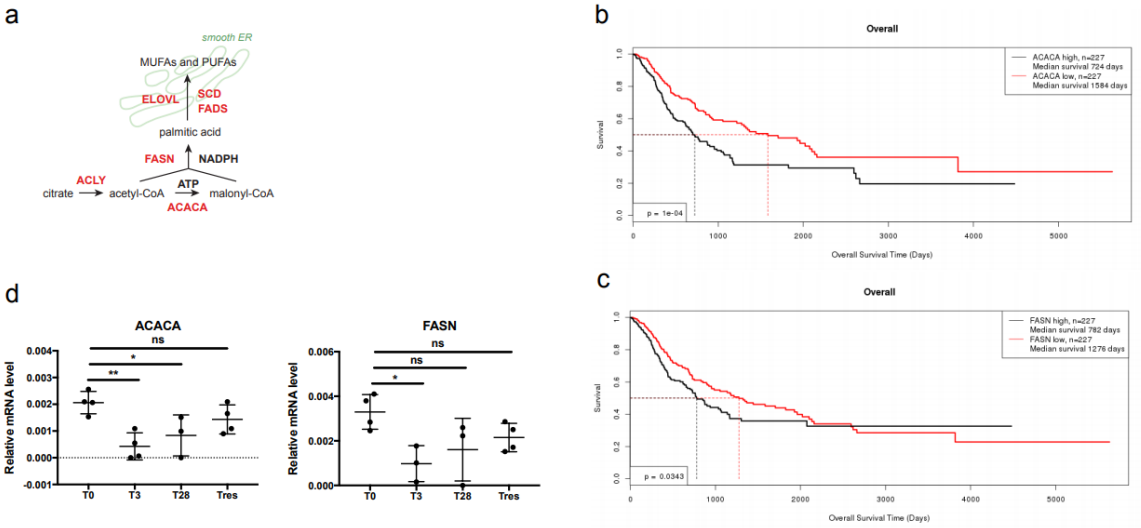


Figure 1. ***Map Kinase inhibition decreases ACACA and FASN expression*****.** a. ACACA and FASN are necessary in driving lipogenesis. b. Patient survival data from the TCGA SKCM cohort of the differential ACACA expression between the top and bottom 50% expressers. c. Patient survival data from the TCGA SKCM cohort of the differential FASN expression between the top and bottom 50% expressers. d. QPCR analysis of ACACA and FASN expression following Dabrafenib + Trametinib treatment in BRAF-V600E mutant Mel006 PDTX melanoma. Tumours were harvested from mice that were treated for 0, 3, 28 days and following the onset of therapy resistance.

3.2 FASN inhibition drives membrane lipid poly-unsaturation by increasing lipid uptake

Using a panel of pre-clinical ACACA and FASN inhibitors, we show that ACACA and FASN inhibition inhibits lipogenesis by reducing ^{14}C acetate incorporation into lipids in the drug resistant cell line 451lu R (Figure 2a). We next show that for each inhibitor, ACACA and FASN inhibition drive membrane lipid poly-unsaturation of phosphatidylcholine species in the cell line, a pattern that is characteristic of lipogenesis inhibition coupled to a compensatory increase in exogenous lipid uptake (Figure 2b and 2c). FASN inhibition drives membrane lipid poly-unsaturation in the drug responsive cell line 451lu as well as the endogenously drug resistant cell line M233 (data not shown). We finally show that exogenous PUFA supplementation mimics the effects of lipogenesis inhibition in promoting membrane lipid poly-unsaturation, and that this synergizes with FASN inhibition to further drive membrane lipid poly-unsaturation (Figure 2d and 2e), implying that lipid uptake is enhanced under conditions of lipogenesis inhibition. Taken together, lipogenesis inhibition drives membrane lipid poly-unsaturation and enhances lipid uptake in melanoma.

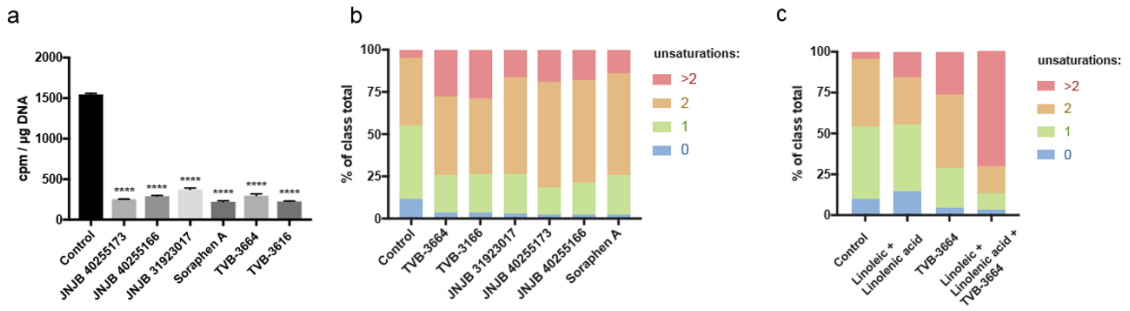


Figure 2. ***Lipogenesis inhibition drives membrane lipid poly-unsaturation*****.** a. 14C radiolabelled acetate incorporation into lipids upon treatment with ACACA and FASN inhibitors in the cell line 451lu R. b. poly-unsaturation index of intact membrane phosphatidylcholine species based on number of fatty acid olefins upon ACACA and FASN inhibition in the cell line 451lu R. c. poly-unsaturation index of intact membrane phosphatidylcholine species based on number of fatty acid olefins upon PUFA supplementation and FASN inhibition in the cell line 451lu R. Data are represented as mean ± s.e.m. The significance was determined with an unpaired *t*-test and compares treated cells to their matching controls ($n = 3$). (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

3.3 Lipogenesis inhibition synergizes with Map Kinase inhibition to inhibit cell proliferation in drug resistant melanoma

We next assessed the effects of lipogenesis inhibition in re-sensitizing Map Kinase inhibitor resistant cells to therapy. We show that cell proliferation is attenuated under combined Dabrafenib/Vemurafenib and TVB-3664 treatment in 451lu R cells, and that this effect is enhanced by exogenous PUFA supplementation, and can furthermore be rescued by the addition of more saturated fatty acids (Figure 3a). We replicate these effects using JNJB-31923017 and Soraphen A as additional lipogenesis inhibitors (Figure 3b). We next repeated these experiments using the endogenously BRAF inhibitor resistant cell line M233, (data not shown). Since combined BRAF / MEK inhibition is the standard of care in BRAF mutant melanoma, we next used the BRAF/MEK inhibitor resistant cell line model D10 BMR. We show that Map Kinase inhibition synergizes with FASN inhibition to attenuate cell proliferation, both in 2D cell culture and in 3D spheroid formation assays (Figure 3c and 3d). Since it is formally possible that FASN inhibition inhibits beta-oxidation by increasing the pool of Malonyl-CoA thereby inhibiting CPT1, we next assessed whether acetate supplementation could attenuate these anti-proliferative effects. However, in contrast to exogenous fatty acid supplementation, acetate supplementation could not rescue cell proliferation (data not shown). In order to show the translational potential of combining FASN inhibition with Map Kinase inhibition, we used the pre-clinical BRAF-V600E mutant melanoma PDTX Mel006 model. We show that FASN inhibition by TVB-3664 treatment alone has no effect on tumour growth. However, whilst Dabrafenib treated mice rapidly acquire resistance to BRAF-inhibiting Dabrafenib treatment, the combination of Dabrafenib and TVB-3664 exerts a synergistic inhibitory effect on tumour growth (Figure 3e). Membrane phospholipidomics of tumour homogenates show that the

combination of Dabrafenib and TVB-3664 treatment significantly drives membrane lipid poly-unsaturation (Figure 3f). Since we have shown *in vitro* that exogenous lipid supplementation potently attenuate the cytostatic effects of lipogenesis and Map Kinase inhibitor treated cells, we included two additional cohorts where mice were fed with an iso-calorific high saturated fat, and high poly-unsaturated fat diet. Dietary interventions had no effects on tumour response (data not shown). TVB-3664 treatment and diet influenced membrane lipid saturation, albeit to a much lesser extent than in the *in vitro* experiments. This is not surprising as changes in membrane lipid profiles of the mouse skin and tumour were subtle (data not shown). This is in itself promising from a translational viewpoint, as diet control is restrictive and difficult to achieve in patients. Taken together, lipogenesis inhibition synergizes with BRAF inhibition to exert a cytostatic effect both *in vitro* and in preclinical models.

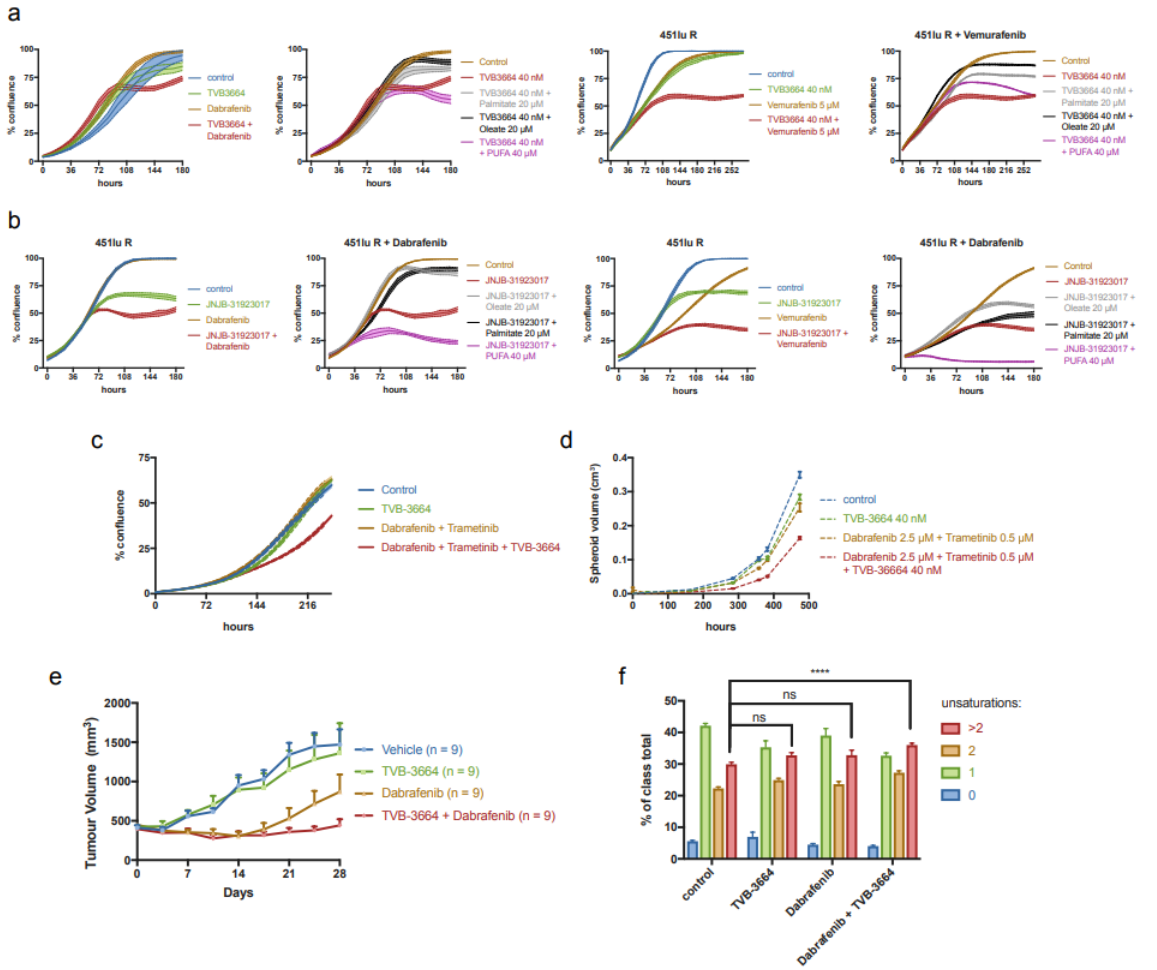


Figure 3. ***Lipogenesis inhibition synergises with Map Kinase inhibition to inhibit melanoma proliferation***. a. 451lu R cells were treated with TVB-3664, Dabrafenib or Vemurafenib and supplemented with lipids. Proliferation was masked and measured in real time using live cell analysis. b. 451lu R cells were treated with JNJB-31923017, Dabrafenib or Vemurafenib and supplemented with lipids. Proliferation was masked and measured in real time using live cell analysis. c. D10 BMR cells were treated with Dabrafenib + Trametinib and TVB-3664. Proliferation was masked and measured in real time using live cell analysis. d. D10 BMR spheroids were treated with Dabrafenib + Trametinib and TVB-3664. Proliferation was masked and measured in real time using live cell analysis. e. BRAF-V600E mutant Mel006 bearing nude mice were treated with vehicle, TVB-3664, Dabrafenib or a combination. Tumours growth was measured using digital callipers. f. phospholipidomics of Mel006 tumours harvested following sacrifice. Data are represented as mean ± s.e.m. The significance was determined with an unpaired *t*-test and compares treated cells to their matching controls ($n = 3$). (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

3.4 BRAF inhibition sensitises cells to inducers of ROS

Recent studies have shown that Map Kinase inhibition infers a new vulnerability in melanoma by directly its evolution towards a ROS sensitive phenotype. In addition, we have shown that sensitivity to the deleterious effects of ROS is linked to membrane lipid

poly-unsaturation. Membrane lipid poly-unsaturation increases the abundance of radical labile lipid species and thereby renders cells more sensitive to the deleterious effects of lipid peroxidation. To this end, we wondered whether promoting membrane lipid poly-unsaturation would enhance the acquired ROS susceptibility to Map Kinase inhibition. Since we have shown that Map Kinase inhibition alone drives membrane lipid poly-unsaturation, we treated 451lu R cells with a number of clinically used or in clinical trials ROS inducers in combination with Dabrafenib. Beta-Lapachone, Menadione, Arsenic trioxide (ATO), Elesclomol and 2-Methoxy-Oestradiol re-sensitise 451lu R cells to Dabrafenib (Figure 4a and 4b), with Arsenic trioxide having the greatest effect. It has previously been reported that therapy response can be enhanced in melanoma cells by treating the tumour with ROS induction by inhibiting GPX4 or SLC7A11[73,79]. However, the effects of antioxidants in overcoming the effects of these targets has been previously described. To this end, we tested the translational potential of combining ATO treatment with Dabrafenib in the mel006 PDTX model, and show that whilst ATO alone has no effect on tumour growth, the combination of ATO and Dabrafenib dramatically shrink tumours (Figure 4c). Importantly, there was no significant change in mouse weight between the cohorts during the course of the study (data not shown), additionally, there was no obvious sign of toxicity in any of the cohorts. Although ATO + Dabrafenib treatment results in the measurable disappearance of the tumour, following 28 days, three mice from this cohort were taken off treatment. Tumours began to rapidly grow once mice were off-treatment (data not shown).

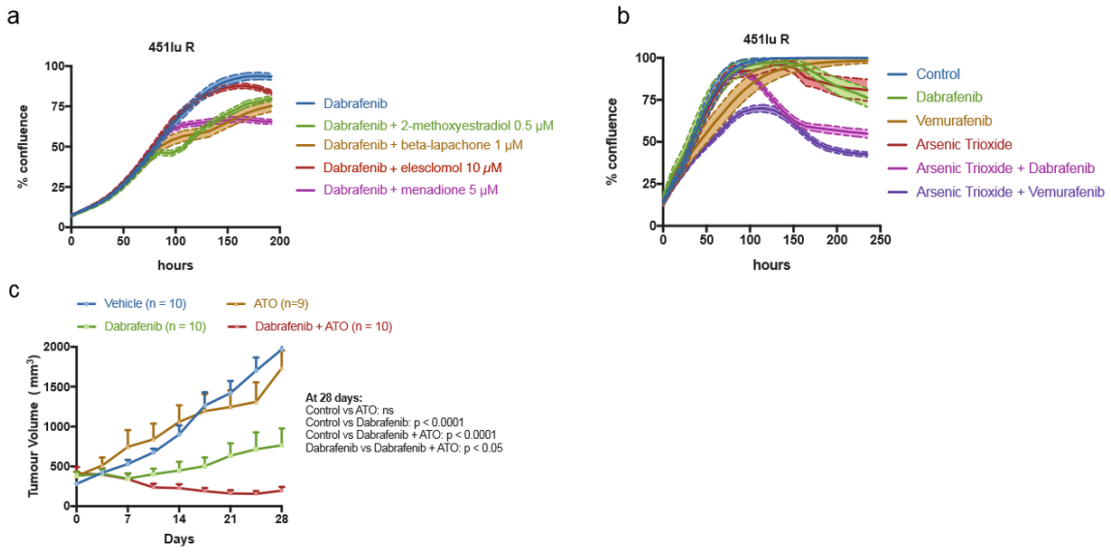


Figure 4. ***Lipogenesis inhibition synergises with Map Kinase inhibition to inhibit melanoma proliferation***. a. 451lu R cells were treated with Dabrafenib or Dabrafenib in combination with the ROS inducers shown. Proliferation was masked and measured in real time using live cell analysis. b. 451lu R cells were treated with Dabrafenib, Vemurafenib or in combination with Arsenic trioxide. Proliferation was masked and measured in real time using live cell analysis. c. D10 BMR cells were treated with Dabrafenib + Trametinib and TVB-3664. Proliferation was masked and measured in real time using live cell analysis. d. BRAF-V600E mutant Mel006 bearing nude mice were

treated with vehicle, ATO, Dabrafenib or a combination. Tumours growth was measured using digital callipers. Data are represented as mean ± s.e.m. The significance was determined with an unpaired *t*-test and compares treated cells to their matching controls ($n = 3$). (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$)

3.5 FASN inhibition directs Map Kinase inhibitor treated melanoma cells towards a ROS sensitive phenotype

Since we have shown that BRAF inhibition sensitises melanoma to Arsenic trioxide, we wondered whether further forcing membrane lipid poly-unsaturation can render cells further sensitive to ROS induction. We show whilst combined BRAF and FASN inhibition had no appreciable effects on cell ATP synthesis in the BRAF inhibitor resistant cell line 451lu R (data not shown), however, mitochondrial superoxide levels were elevated under these conditions (Figure 5a). In order to further investigate this finding, we treated 451lu R cells with BRAF and FASN inhibitors, and following 72 hours of incubation; we supplemented cells with either water or hydrogen peroxide, followed by refreshing media at 250 hours (Figure 5b). This shows that the anti-proliferative effects of combined BRAF and FASN inhibition are primarily cytostatic and reversible. However, importantly, we show that supplementing cells with hydrogen peroxide further attenuates the effect of combined BRAF and FASN inhibition, and critically, is cytotoxic and attenuates the recovery of cell proliferation once media is refreshed. Next we combined BRAF and FASN inhibition in combination with a proof-of-concept ferroptosis inducer, piperazine-erastin, and show that that both FASN inhibition, BRAF inhibition, and especially the combination of both sensitises cells to cysteine uptake inhibition and thereby to ROS induction (data not shown).

Since ROS induction potentiates the combined effects of Map Kinase inhibition and FASN inhibition, we next assessed the ability of our panel of ROS elevating compounds in sensitising cells in these conditions. The sensitising effects of BRAF inhibition were further enhanced by concurrent FASN inhibition combined (data not shown). Importantly, the cytotoxic effects of ATO are further potentiated by combined FASN and Map Kinase inhibition in the cell lines 451lu R and in D10 BMR (Figure 5c and 5d). Critically, this combination results in increased amounts of cleaved caspase 3 activity and cell death in D10 BMR (data not shown). In order to show that membrane lipid saturation degree modulates the sensitivity of cells to ROS induction, we treated D10 BMR cells with exogenous lipids in combination with ATO, wherein saturated fatty acids protect cells from ATO induced cytotoxicity, and PUFA exacerbate it (Figure 5e). We next treated D10 BMR cells with the anti-oxidant alpha-tocopherol in addition to the drug combinations, in order to show that these effects are in part mediated by ROS and can be rescued by anti-oxidant supplementation (Figure 5f). Taken together, lipogenesis inhibition mediated membrane lipid poly-unsaturation sensitises melanoma cells to ROS induction.

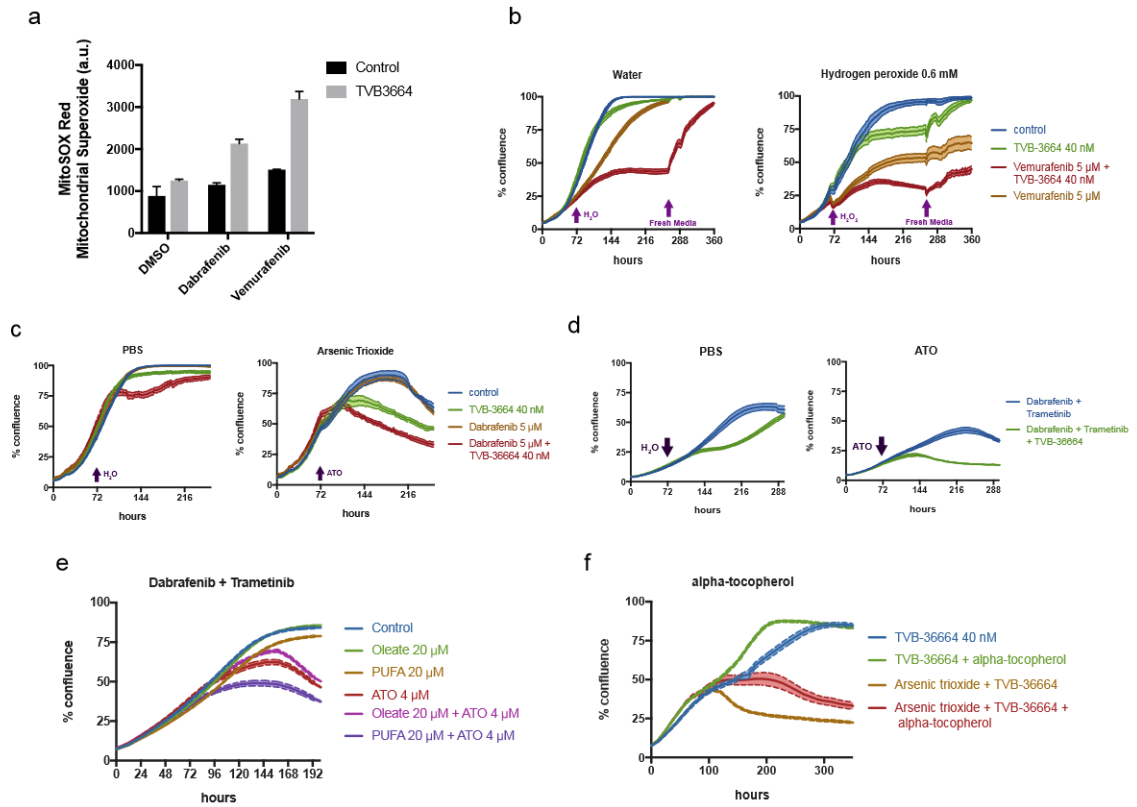


Figure 5. ***Lipogenesis inhibition synergises with Map Kinase to sensitise cells to ROS induction*****.** a. Mitochondrial superoxide levels were measured in the cell line 451lu R upon BRAF and FASN inhibition. b. 451lu R cells were treated with Vemurafenib or TVB-3664, followed by treatment with water or with hydrogen peroxide at the indicated time-points. Proliferation was masked and measured in real time using live cell analysis. c. 451lu R cells were treated with Dabrafenib, TVB-3664 or a combination of both compounds. Proliferation was masked and measured in real time using live cell analysis. d. D10 BMR cells were treated with Dabrafenib + Trametinib and TVB-3664 with and without Arsenic trioxide. Proliferation was masked and measured in real time using live cell analysis. e. D10 BMR cells were treated with Dabrafenib + Trametinib, TVB-3664, Arsenic trioxide and exogenous lipids as indicated. Proliferation was masked and measured in real time using live cell analysis. f. D10 BMR cells were treated with Dabrafenib + Trametinib, TVB-3664, Arsenic trioxide and alpha-tocopherol as indicated. Proliferation was masked and measured in real time using live cell analysis.

4. Discussion

The development of therapy resistance is a major yet common obstacle in cancer treatment. In the case of BRAF mutant melanoma, 20% of patients show intrinsic resistance to targeted therapy whilst over 80% of patients rapidly become therapy resistant. Therefore enhancing therapy response and overcoming therapy resistance by exploiting acquired vulnerabilities in cancer is a promising avenue of research, and one that has yielded several promising leads in pre-clinical multiple cancer models, including melanoma.

We have previously shown that the Map Kinase pathway drives SREBP-1 dependent lipogenesis in melanoma, and that inhibition of SREBP-1 re-sensitises therapy resistant melanoma to Map Kinase inhibition. Here, using two PDTX models, we show that Map Kinase inhibition results in a decrease in the expression of lipogenic genes during therapy response, but not upon the acquisition of therapy resistance. Using in vitro and PDTX models, we show that ACACA and FASN inhibition exert cytostatic effects on therapy resistant melanoma. Importantly, lipogenesis inhibition drives membrane lipid poly-unsaturation in these models. Critically, we show that under combines Map Kinase and lipogenesis inhibition, therapy resistant melanoma acquires an exquisite vulnerability to ROS induction by ATO, which depends on the extent of membrane lipid peroxidation. Taken together, using compounds that are either clinically approved or are close to the clinic, we show a novel strategy in re-sensitising therapy resistant melanoma to Map Kinase inhibitors.

Important to the translational potential of this study, Arsenic trioxide is a clinically used drug and FDA approved, furthermore it is not patentable meaning that the drug can be quickly repurposed. In addition, several other FDA approved or in clinical trials drugs such as elesclomol, menadione or 2-Methoxyestradiol are known ROS inducers and may be important compounds for these applications. Moreover, several ACACA and FASN inhibitors are in pre-clinical and in clinical trials bringing this concept closer to the clinic. Taken together, the strategy of directing cells towards a ROS sensitive phenotype by lipogenesis inhibition and then further inducing ROS can be readily adopted clinically.

A historic criticism of such a combinatorial approach is that of additive toxicity of multiple drugs. However this fear is sometimes unfounded, as in the case of melanoma, where combined BRAF + MEK inhibitors are both more effective and better tolerated than BRAF targeting therapy alone. Furthermore, several pre-clinical reports have shown that the effective doses of multiple combined drugs can be more effective than the maximal tolerated dose of either approved therapy. A clear challenge in approving multiple drug combinations clinically is the large size of patients required for clinical trials, and therefore the exponential increase in the cost. However based on the context dependency of multiple oncogenic and metabolic pathways, evidence points towards the necessity of such an approach.

In line with our finding, recently a number of key publications have shown the importance of membrane lipid peroxidation in overcoming cancer therapy resistance. Two key reports have shown that following treatment with Map Kinase inhibitors, melanoma cells sensitive to lipid hydroperoxides[79,80]. Using a single-cell approach a study shows both drug responsive and drug resistant melanoma are ROS tolerant, and that the evolution of therapy resistance in melanoma relies on a transition between these states through a neural crest like ROS sensitive phenotype which is sensitive to lipid peroxidation.[222] Here, we show that both therapy resistance can be overcome, and that therapy response can

be enhanced by targeting fatty acid biosynthesis, and that critically, this confers a sensitivity of the cancer cells to inducing ROS induction.

Whilst these pre-clinical studies have shown that treating melanoma with ROS inducers can overcome or delay the acquisition of therapy resistance, our hypothesis that this is linked to the extent of membrane lipid poly-unsaturation. Importantly, membrane lipid poly-unsaturation in tuneable by either inhibiting lipogenesis and/or by altering dietary fatty acids.

Specifically, we hypothesise that lipogenesis inhibition synergises with Map Kinase inhibition in order to further render the remaining melanoma tumours cells susceptible to ROS induction by promoting membrane lipid poly-unsaturation.

5. Methods

5.1 Cell culture

A375 was obtained from ATCC. M233 were gifted by professor A. Ribas. 451 and 451lu R, M229, M229 R were gifted by R. Lo. D10 BMR were kindly gifted by Professor Daniel Peeper. All cell lines were propagated in DMEM High Glucose (Sigma), supplemented with 10% FBS (Gibco Lot 41F4234K) and 4 mM glutamine (ThermoFisher). D10 BMR growth media was additionally supplemented with Dabrafenib and Trametinib. All cell cultures were periodically tested for mycoplasma contamination. All experiments were performed in DMEM High Glucose, supplemented with 2% FBS (Gibco Lot 41F4234K) and 4 mM glutamine. The following compounds were used at the stated concentrations. TVB-3664 (40 nM, 3V-Biosicneces and ARSPICHEM), Alpha-tocopherol (100 µM) from Sigma, , Vemurafenib (5 µM) from ApexBio, Dabrafenib (2.5 µM) from Selleckchem, Trametinib (0.5 µM) from Selleckchem, Arsenic trioxide acid (4 µM) from Sigma, oleic acid (20 µM) from Sigma, palmitic acid (20 µM) from Sigma, linoleic acid (20 µM) from Sigma and linolenic acid (20 µM) from Sigma.

5.2 14C-acetate incorporation into lipids

Cells were grown in 6-well plates up to 80% confluence and were treated with 0.1 µCi acetate-2-14C (55 mCi/mmol; Amersham) for 4 h. After three washes with PBS (Sigma), cells were trypsinized and resuspended in 1 mL PBS, followed by sonication. 0.125 mL of lysate was set aside for DNA measurement and 0.7 mL of lysate was mixed with 0.9 mL MeOH solution (MeOH:HCl = 8:1) (Sigma) and 0.8 mL $CHCl_3$ (Sigma). After vortexing the mixture for 30 s followed by centrifugation for 10 min at 2000 × g, the organic fraction was counted for radioactivity (Tri-Carb 2810 TR scintillation counter, PerkinElmer).

5.3 Analysis of intact phospholipid species by ESI-MS/MS

0.7 mL of homogenized tissue or cells were mixed with 0.9 mL MeOH:HCl(1 N) (8:1), 0.8 mL CHCl3 and 200 µg mL−1 of the antioxidant 2,6-di-tert-butyl-4-methylphenol

(Sigma). The organic fractions were evaporated under vacuum, reconstituted in MeOH/CHCl3/NH4OH (90:10:1.25) and lipid standards were added (Avanti Polar Lipids). Phospholipids were analyzed by electrospray ionization tandem mass spectrometry (ESI-MS/MS) on a hybrid quadrupole linear ion trap mass spectrometer (4000 QTRAP system, AB SCIEX) equipped with a TriVersa NanoMate robotic nanosource (Advion Biosciences) as described[75].

5.4 Immunoblotting analysis

Following ice-cold PBS washes, cells were collected in sample buffer (ThermoFisher) supplemented with DTT (Sigma), sonicated and boiled for 5 min. Equal amounts of protein were loaded onto precast gels (NuPAGE, ThermoFisher), transferred to nitrocellulose membranes, and incubated with antibodies against SREBP-1 (1/1000 dilution) (Active Motif, #39939), phospho-MEK1/2 ser217/221 (1/1000 dilution) (Cell Signalling, #9154); GAPDH (1/20000 dilution) (Cell Signalling, #5174).

5.5 Proliferation assays

Proliferation curves were generated using an IncuCyte ZOOM system (Essen BioScience) on cells seeded of microplates (TPP) based on phase contrast images taken at 2 h intervals for the duration of the experiments. Spheroids were formed in ultra-low adhesion round bottom plates (Thermo Fischer).

5.6 Mitochondrial ROS measurement

Mitochondrial ROS was measured using MitoSOX red mitochondrial superoxide indicator (ThermoFisher) according to the manufacturer's instructions. Cells were assayed using a FACS Verse flow cytometer (BD Biosciences).

5.7 Animal experiments

The Mel006 PDTX model was derived from an in-transit metastasis from a patient undergoing surgery as part of standard-of-care melanoma treatment at the University Hospitals Leuven. The patient provided written informed consent and this procedure was approved by the UZ Leuven Medical Ethical Committee (S54185) and carried out in accordance with the principles of the Declaration of Helsinki. All procedures involving animals were carried out in accordance with the guidelines of the IACUC and the Animal Care and Use Ethical Committee (KU Leuven, P038/2015). Fresh tumour tissue carrying the BRAF-V600E mutation was collected in RPMI640 medium with antibiotics, rinsed in PBS and transplanted subcutaneously in the interscapular fat pad of female SCID-beige mice (Taconic). Sedation and analgesia was performed using ketamine, medetomidine and buprenorphine. Upon reaching generation 3, tumour fragments were transplanted into nude mice (NMRI-Fox1nu, Taconic) and when tumour size reached 200 mm3, mice were randomly assigned to a cohort and drugs or vehicles were blindly administered daily by oral gavage. Tumour size was measured blindly with digital callipers (Fowler Sylvac) every 3 days. Mice were sacrificed at 28 days following start of treatment or when

tumours reached a volume of 1500 mm^3. The investigators were blinded for the evaluation of the results.

Statistical analysis

The results were analyzed in GraphPad Prism 6.0 h using a t-test. In case of multiple comparisons a correction was applied using the Holm–Sidak method. P-values of <0.05 were considered to be statistically significant. All data presented represent means ± s.e.m.

6. Acknowledgements

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7. Author Contributions

A.T. and J.V.S. designed the experiments. A.T., J.D., and J.V.S wrote the paper. A.T. and F.V. performed the experiments. V.d.L, X.S., A.T. and S.M. performed *in vivo* experiments. J.-C.M. provided extensive helpful comments and discussion. A.T. and J.D. analysed data. J.V.S. oversaw the project and acquired funding. F.R. analyzed and generated the TCGA, RNA-seq data. A.R. established and characterized the Mel006 PDTX model. R.D. executed the phospholipidomics analysis. E.W. allowed use of the mass spectrometer. D.N. expertly executed the histological staining and analysis. T.R. assessed and generated the pathology reports. E.L. Managed the PDTX facility.

8. Declaration of Interests

The authors declare no competing interests.

Chapter 4: Discussion

1. Therapy resistance in melanoma

In spite of a good initial response, the majority of BRAF mutant melanoma ultimately progress. As BRAF and MEK inhibition are the standard therapy in this disease, it is urgent to find ways in overcoming therapy resistance. To this end, several pre-clinical studies have shown the importance of the secondary acquisition of oncogenic Map Kinase mutations, MITF re-activation and PI3K pathway upregulation as key mediators of therapy resistance (as discussed previously). Inhibition of the PI3K/Akt pathway and of ERK phosphorylation are attractive avenues in overcoming therapy resistance. However, the paucity of clinical inhibitors of EKR phosphorylation limit this approach. Several pre-clinical AKt and PI3K inhibitors combined with BRAF/MEK inhibitors are currently in clinical trials and hold promise[223–225]. However, non-mutational PI3K independent mechanisms of resistance have been observed[52,61]. Additionally, non-mutational ERK independent resistance mechanisms have been observed in BRAF/MEK inhibitor resistant models[72].

In general, the initial response to combined BRAF/MEK inhibition in BRAF mutant melanoma is massive, however a small proportion of cells, i.e. minimal residual disease remains. Since these cells give rise to therapy resistance, identifying key targetable profiles in these remaining populations is a good strategy. To this end, using single cell transcriptomic, two recent studies have identified that minimal residual disease states in melanoma converge on a low metabolic, de-differentiated states[60,222], with one study showing that these cells converge on a phenotype that is sensitive to ferroptosis induction[222]. Recently, several publications have shown that minimal residual states are sensitive to ROS and/or to ferroptosis[79,79,202,226]. In line with these observations, lipogenesis inhibition drives membrane lipid poly-unsaturation by increasing lipid uptake in cells, rendering cells more susceptible to ROS and to ferroptosis. By inhibiting lipogenesis, it is possible to further confer a sensitivity to ROS in minimal residual disease. We aim to show the translational applicability of this strategy in our current work.

It is well-established that melanin can act as an antioxidant[19], however the cytoprotective role of pigmentation remains to be explored in melanoma. Importantly, under Map Kinase inhibition, MITF is downregulated and melanoma is depigmented, this is co-incident with ROS sensitivity. Resistance often converges on re-activating MITF signalling, providing a rationale towards the role of melanin as an important antioxidant in melanoma. Moreover, melanin has iron-binding properties and therefore act as a negative regulator of ferroptosis[19]. Indeed depletion of neuromelanin is implicated in the onset of Parkinson's disease. To this end, the strategy of combined melanogenesis inhibition and ROS induction should be explored. Withaferin A is a good candidate in such a study as it causes ferroptosis by both inhibiting GPX4 and by increasing intracellular iron pools[100].

2. Lipid metabolism in cancer

In the context of systemic cancer treatment, identifying key metabolic dependencies that are well-downstream of oncogenic drivers is a promising strategy. The main reasons are that 1 – oncogenic pathways are often degenerate, leading to myriad possible resistance mechanisms, and that 2 – key metabolic processes are often entirely dependent on one or a few targetable enzymes.

In these studies, we addressed the fundamental problem of overcoming therapy resistance. We investigated the role of lipogenesis in therapy resistance in BRAF mutant melanoma, and focused on overcoming therapy resistance by inhibiting lipogenesis. In our published work, we have shown that 1 - BRAF inhibition inhibits lipogenesis in therapy responsive BRAF mutant melanoma cells but not in their therapy resistant counterparts. 2 – Therapy resistant cells sustain lipogenesis by sustaining active SREBP-1 levels, 3 – and that inhibition of SREBP-1 restores therapy sensitivity both in cells and in a preclinical PDTX melanoma model. 4 – Finally, we have shown that in part, SREBP-1 mediated lipogenesis protects cancer cells from the deleterious effects membrane lipid peroxidation by leading to membrane lipid saturation.[75] taken together, sustained SREBP-1-dependent lipogenesis as a key mediator of resistance to BRAF-targeted therapy.

Importantly, this proof of concept study showed that SREBP-1 mediated lipogenesis sustains therapy resistance in BRAF mutant melanoma, irrespective of the resistance mechanism. However, inhibiting SREBP-1 had a predominantly cytostatic effect *in vitro* and *in vivo*, which could suggest that therapy resistance to this combination can occur further on. Moreover, SREBP-1 is a transcription factor with myriad processes and is a blunt tool for inhibiting lipogenesis. Furthermore, SREBPs are regulated at multiple levels intracellularly. It is feasible that SCAP over-expression, INSIG1/2 expression, or non-classical SREBP processing can overcome Betulin or Fatostatin mediated SREBP inhibition. Therefore, the small molecule inhibitors Betulin and Fatostatin may remain as pre-clinical proof of concept drugs, and may not be adopted clinically. That said, neither drug shows overt toxicity, even at high concentrations, which could mean that lipogenesis inhibition is well-tolerated *in vivo*.

Inhibition of MBTPS1 or MBTPS2 is an alternative approach that would inhibit SREBP or ATF6 activation. Although no clinically used drugs that alter SCAP or INSIG activity are available, it has been recently shown that the FDA approved anti-HIV drug nelfinavir inhibits MBTPS2, and thereby SREBP and ATF6 processing[227]. Nelfinavir treatment in liposarcoma or in androgen resistant prostate cancer attenuate cancer cell survival by inducing lipotoxicity[227,228]. However, nelfinavir has been used with limited success in inhibiting SREBP processing in several models (unpublished data).

At approximately the same time a number of important studies have been published in the field showing that targeting cells by ROS inducers can prevent the onset of therapy resistance.[73,79,222] This has led us towards our current unpublished manuscript, showing

that lipogenesis inhibition sensitises drug resistant BRAF mutant melanoma to reactive oxygen species. In this study, we recapitulate the findings above ACACA and FASN inhibitors. Importantly, these targets are essential in driving lipogenesis as there are no alternative enzymes that can drive this process. Furthermore, inhibitors of both ACACA and FASN are much closer to the clinic, with a derivative of the FASN inhibitor TVB-3664 currently in clinical trials. In the current ongoing study, we show that lipogenesis inhibition in combination with BRAF/MEK inhibition is cytostatic. However, these conditions, cells become susceptible to ROS, in part through enhanced membrane lipid poly-unsaturation. Importantly, we show that by directing melanoma cells towards a lipid uptake dependent phenotype, further enhancing ROS by the drug Arsenic trioxide is cytotoxic. We are in the process of investigating the clinical relevance of this observation in readily translatable, pre-clinical PDTX models before submission of the completed study.

Since the Map Kinase pathway and lipogenic pathways are highly conserved and are often dysregulated across many cancer types, it is possible to extend this approach to other cancers. Indeed, a link between the lipogenic pathway and the Map Kinase pathway has been previously reported[149,150,204], most recently in a prostate cancer model[229]. The contribution of lipogenesis to other cancer types that reply on Map Kinase activation remains to be explored and is a promising avenue of research. To this end, the mechanistic link between the Map Kinase pathway and SREBP processing should be delineated. Identifying the signalling events that lie between the cross-talk of lipogenesis and proliferation can lead to the discovery of new anti-cancer drug targets.

In the context of melanoma, the contribution of lipogenesis in driving BRAF wild-type melanoma should be explored. Although BRAF wild-type, these at least half of remaining 50% of melanoma patients harbour mutations that activate Map Kinase signalling. Although targeted approaches exist for BRAF mutant melanoma, treatment options (aside from immunotherapy) for other subtypes is lacking. The most notable BRAF wild-type melanoma subtype is oncogenic NRAS mutant melanoma. Ras proteins are famously untargetable, moreover, NRAS mutant melanoma are even more aggressive and have a higher propensity to metastasise than BRAF mutant melanoma[230,231]. MEK inhibitor monotherapy has had success in improving the clinical outcomes of NRAS mutant patients in clinical trials, with only binimetinib showing a partial response in a small fraction of NRAS mutant patients[232,233]. This is not surprising as combined BRAF and MEK inhibition are both better tolerated and achieve a larger response in BRAF mutant patients. If Map Kinase activation drives lipogenesis in NRAS mutant patients, exploring the translational effects of combining lipogenesis inhibition with MEK inhibition is tantalising.

Although we have focused on melanoma as a model, the concept of a cross-talk between key oncogenic and metabolic pathways can apply to multiple cancer types. Due to the ready availably of bio-available inhibitors of many metabolic processes, this is a concept that is being more widely explored in the field. Specifically, several observations have linked Map Kinase signalling to lipogenesis and in SREBP activation. Importantly, several cancer types carry driver mutations in Map Kinase pathway enzymes, and thereby have

higher rates of lipogenesis. In contrast to melanoma, many Map Kinase pathway mutant cancers such as lung and pancreatic cancers respond poorly to Map Kinase inhibitors. Therefore, the potential anti-tumour effects of combining Map Kinase inhibition with lipogenesis inhibition should be assessed in these backgrounds. Furthermore, existing several cancer therapies such as anti-androgen therapy in prostate cancer inhibits lipogenesis and results in membrane lipid poly-unsaturation, and could therefore render cancer cells more sensitive to ROS inducers. The combination of using existing anti-cancer therapy that results in membrane lipid poly-unsaturation with ROS inducers may hold therapeutic promise.

3. Strategies in targeting lipid metabolism in cancer

Several pre-clinical reports show the significance of targeting lipogenesis and lipid uptake in cancer. It is formally possible that by inhibiting lipogenesis, tumour cells could enhance exogenous lipid uptake. In highly lipogenic cancers such as melanoma, it is unlikely that the rate of lipid uptake from a lipid efficient microenvironment could compensate for the loss of lipogenesis. Indeed ACACA or FASN inhibition have been shown to have antineoplastic effects as a monotherapy[184,187,234]. Although the previous generation of lipogenesis inhibitors such as orlistat and C75 were only effective at high concentrations and had severe side effects, the recent small molecule inhibitors of ACACA and FASN are much more promising. For example, whereas orlistat is commonly used *in vitro* at a concertation of 100 µM, 40 nM of TVB-3664 is sufficient to fully inhibit *de novo* palmitate biosynthesis.

Since most tissues are low lipogenic, it is not expected that ACACA and FASN inhibition would cause adverse effects on most organs. However, lipogenesis is shown to be important in adult neuronal stem cell function[235]. If the drugs cross the blood-brain barrier, the effects on neuronal toxicity should be carefully studied. Furthermore, lipid metabolism has been shown to be required in several immune cell functions, for example in the generation of memory T cells and in potentiation of macrophages[236,237]. Therefore the effects of lipogenesis inhibition on tumour progression in immune competent models should be carefully addressed.

ACACA and FASN inhibition may not lead to identical effects. In addition to the roles of these enzymes in lipogenesis, the inhibition of either enzyme may have contrasting roles of the levels of Malonyl-CoA. Malonyl-CoA is an important signalling molecule and its accumulation negatively affects CPT1 mediated lipid import into the mitochondria and therefore inhibits beta-oxidation[95]. ACACA inhibition may lead to the depletion of cellular Malonyl-CoA and further enhance beta-oxidation. This could have an added benefit in depleting already limiting lipids in the cancer cells, but may have undesired side effects in for example affecting muscle cell function. FASN inhibition has been shown to result in the accumulation of Malonyl-CoA, and thereby inhibit beta-oxidation in some models. Although beta-oxidation inhibition may mean that more fatty acids are available for membrane biogenesis, importantly, beta-oxidation leads to the generation of reductive

equivalents in the cells. Indeed several studies have shown that beta-oxidation protects cancer cells from the deleterious of ROS in preclinical cancer models[238,239]. One could envision that by blocking FASN, cancer cell susceptibility to ROS may be increased by both enhanced uptake of exogenous (poly-unsaturated) fatty acids and reduction of cellular NADPH pools. In addition to driving lipogenesis, ACACA generated Malonyl-CoA can be used for fatty acid elongation. Membrane lipid elongation is recently implicated as a mediator of cancer progression in some both published and in unpublished work[240]. ACACA inhibition could conceivably be used as a blunt tool in inhibiting membrane lipid elongation.

In contrast to lipogenic cancers, other cancer types are shown to rely predominantly on lipid uptake. For example, CD36 mediated lipid uptake from surround adipose tissue is shown to fuel ovarian cancer metastasis and proliferation,[241] and likewise glioma require FABP3 and FABP7 mediated lipid uptake from the hypoxic and lipid rich neuronal stroma[242]. Lipid uptake could present an important target in cancers that develop in lipid rich microenvironments. Whereas targeting ACACA or FASN can effectively halt lipogenesis, targeting lipid uptake is more challenging as cells can take up exogenous lipids through several mechanisms.

It is well-established in several cancer models that a lipid rich microenvironment drives the propensity of cancer cells to increase lipid uptake, however the mechanisms of lipid release from surrounding adipose or lipid rich tissue is less clear. It is conceivable that local release of pro-inflammatory exosomes or cancer cell secretion of phospholipases can drive this process. Indeed, the role of exosomes in causing inflammation is well-established[243], as is the role of (venom derived) phospholipases in lysing cells[244]. The mechanisms and contributions of such processes in increasing the pool of exogenously available lipids should be explored.

4. Lipid peroxidation as cancer target

Importantly, membrane lipid peroxidation is a key requirement of canonical ferroptosis induction, with a number of the key signalling lipid hydro-peroxides recently identified[99]. This recently identified form of programmed cell death relies on membrane lipid peroxidation. Recently, a plethora of key publications have shown the importance of membrane lipid peroxidation and interfering with cellular ROS to overcome cancer therapy resistance. In multiple cancer cell types, two reports have shown that drug resistant cancer cells adopt a specific phenotype that renders cells sensitive to GPX4 inhibition, a key lipoxygenase in detoxifying lipid hydroperoxides[79,80]. However, the tool-box GPX4 inhibitor RSL3 and the SLC7A11 inhibitor Erastin have limited *in vivo* efficacy. Recently, Withaferin A was identified as a novel compound that both inhibits GPX4 and leads to iron accumulation in cells, resulting in two-pronged induction of ferroptosis[100].

In addition to directly targeting lipogeneses in cancer, targeting vulnerabilities that are conferred by modulating lipogenesis can hold significant promise. Specifically, we have

shown that modulating membrane lipid poly-unsaturation by either lipogenesis inhibition and/or poly-unsaturated fatty acid supplementation can confer sensitivity to ROS inducers. At the same time, several labs are compiling related work showing that proliferation of lipid uptake dependent cancers can be modulated by altering the saturation degree of dietary fatty acids, and that saturated fatty acid supplementation plays a protective role towards ferroptosis whilst PUFA have the opposite effects. In the context of melanoma, treating tumours with GPX4 and SLC7A11 is shown to delay or overcome the acquisition of therapy resistance.[73,79]

However, several lines of evidence show that antioxidant treatment can fully rescue the effects of ferroptosis inducers, and this can point towards the possibility of several escape mechanisms in the cancer cell by increasing the cellular antioxidant potential. Using more direct ROS inducers such as ATO may mitigate this possibility. Although ATO is clearly toxic in a dose-dependent manner, the drug has been used for millennia with some success in pre-scientific medicine and it is approved for treating a rare form of leukaemia. Accordingly, ATO is receiving increased interest in medical research.

Aside from modulating lipogenesis to confer ROS resistance, the spatial distribution of fatty acids can be modulated to protect cells from the deleterious effects of lipid peroxidation. This novel concept has already been shown to play a key protective role in neuronal stem cells[93]. Under hypoxia induced or other ROS stress, these cells remodel the spatial distribution of their lipids by sequestering PUFA from membranes into unreactive TAG 'cold pools'. Importantly, TAGs are distant to the site of ROS production and TAG cold pools are unreactive. It is conceivable that a similar mechanism protects cancer from ROS, or that this is key antioxidant mechanism in lipid uptake dependent cancers are fatty acid uptake is less selective. However, this concept remains to be explored.

5. The emerging world of Immunotherapy in melanoma

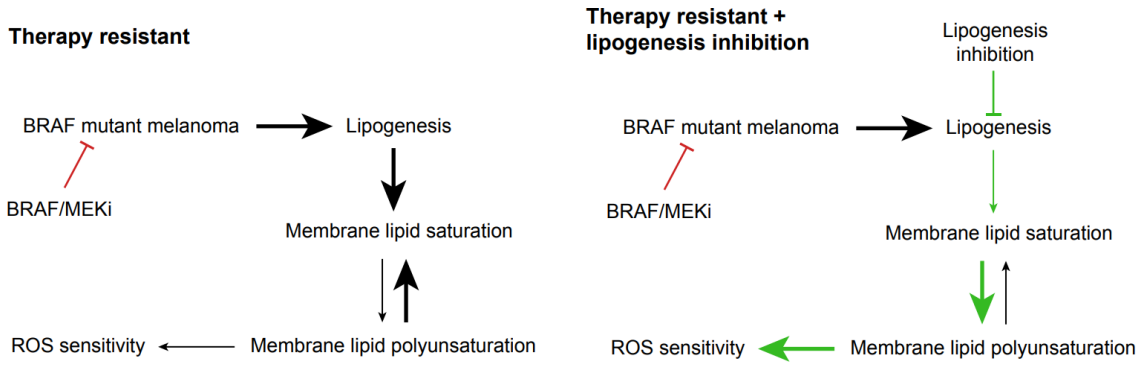
Anti-immune checkpoint PD-1 and CTLA-4 targeting therapies have revolutionised treatment in several cancer types and have led to unprecedented long term responses in a minority of patients[38,39]. This unprecedented long-term response marks the advantage of this approach over targeted therapies. However, whereas mutational background a good indicator of targeted therapy response, it is currently unknown as to why some patients respond and others do not. Another advantage of targeted approaches in melanoma, namely in BRAF/MEK targeting therapy is that tumour regression occurs within days. In contrast, even in patients that respond well to immunotherapy, tumour regression takes much longer. Due to this difference, clearly for advanced disease, a treatment regimen must include targeted therapy. Furthermore, whilst the side effects of targeted therapy, especially of combined BRAF/MEK inhibition is generally manageable, the side-effects of immune checkpoint inhibition can be much more severe.

Due to the unprecedented response of a subset of patients to immune checkpoint inhibitors, there is an urgent need to sensitise tumours to immunotherapy. To this end, a clearer understanding of mediators of immune response in cancer and in fundamental biology are required.

Lipid metabolism is known to affect multiple immune cell functions and fatty acid metabolism has been implicated in modulating immune responses[237]. A study had previously identified oxidized phosphatidylcholine species as substrates for the fatty acid uptake receptor CD36[245]. Importantly, CD36 also acts as a macrophage scavenger receptor. Synthetic oxidized phosphatidylcholine products were shown to be able to act as a ligand for CD36. Since oxidized lipid species are implicated in a number of disease processes, it is possible that they could act as immune ligands. Moreover, since it is well-established that cancer cells have high antioxidant potential and that membrane lipid saturation is a common feature in cancer cells. It is therefore conceivable that this is a mechanism by which cells deplete PUFA from membranes, thereby depleting the presentation of oxidized fatty acid species as an immune ligand. However, the role of CD36 as an immune receptor remains controversial as it has also been implicated in promoting diseases such as atherosclerosis[246].

Lipid metabolism has been shown to be key in regulating immune cell function. For example, immune cells activation is co-incident with high rates of immune cell proliferation, which relies on high rates of anabolic reactions including lipogenesis. In T-cell function, *de novo* lipogenesis has been shown to regulate T-cell differentiation, in that ACACA inhibition promotes T-cell differentiation into anti-inflammatory T-reg cells[247,248]. Moreover, this study showed that unlike lipogenic Th-cells, T-regs rely predominantly rely on lipid uptake. Cholesterol is required for clustering of the T cell receptor and is required in the formation of a T-cell receptor complex[249]. By inhibiting cholesterol binding to the TCR using a natural analogue of cholesterol, cholesterol sulphate, TCR-complex formation and TCR signalling could effectively be inhibited[250]. Negatively charged (acidic) phospholipids have also been shown to be required in forming a TCR-CD3 complex[251].
Although the role of lipids in immune cell and in cancer cell function is established, the role of lipids in modulating the interaction between cancer cells and immune cells remains obscure and is a promising area of research. Since lipogenesis inhibition may affect both cancer cells and immune cell functions, it is important to delineate their contributions to cancer cell and immune cell functions. A major limitation in investigating cancer cell biology, especially in metabolism in cancer is the use of immune compromised *in vivo* models. Whilst these are good models of cancer cell proliferation, they largely neglect immune-cell interactions. Therefore there is a need to complement metabolic studies with immune competent GEMs to assess the importance of such interactions.

Graphical Summary



- Map Kinase pathway signalling promotes lipogenesis thereby promoting membrane lipid saturation
- BRAF inhibition inhibits lipogenesis in therapy responsive BRAF mutant melanoma thereby promoting membrane lipid poly-unsaturation and acquired ROS sensitivity
- Therapy resistant BRAF mutant melanoma sustain lipogenesis upon BRAF inhibition
- Combines BRAF and lipogenesis inhibition renders therapy resistant melanoma exquisitely sensitive to ROS.

Summary

In spite of remarkable improvements in patient treatment, metastatic melanoma remains a devastating disease. Approximately half of all melanoma patients carry activating mutations in BRAF and are treated with Map Kinase targeting therapy. Although the majority of patients initially respond well, most of them ultimately develop resistance. There is therefore a clear clinical need to overcome therapy resistance.

One of the hurdles in this context is the multitude of mechanisms contributing to therapy resistance. These mechanisms involve upregulations of oncogenic and pro-survival signalling pathways, such as activation of NRAS, MITF, PI3K and the AKT pathway. Importantly, these proteins comprise members of degenerate signalling pathways, and this could indicate that their targeting could rapidly lead to compensation mechanisms.

One of the strategies in overcoming therapy resistance involves the identification and targeting of more downstream oncogenic processes that are less redundant. In this context, metabolic enzymes are of particular interest as they act downstream of multiple signalling events, they are less degenerate when compared to signalling proteins and they create unique dependencies in cancer cells.

In this thesis manuscript, we show that fatty acid biosynthesis is such a central player in therapy response of melanoma. Fatty acid synthesis is activated in many tumours and in therapy-responsive melanoma it is downregulated by BRAF targeting therapies. Strikingly, in therapy-resistant melanoma, fatty acid synthesis activity is maintained and contributes to the resistance phenotype. In fact, interfering with fatty acid biosynthesis by inhibiting SREBP-1 activation or by directly inhibiting key lipogenic enzymes such as ACACA and FASN re-sensitises melanoma to therapy, irrespective of the myriad mechanisms of therapy resistance. Mechanistically, lipogenesis inhibition drives membrane lipid poly-unsaturation in cancer cells, thereby conferring sensitivity to reactive oxygen species (ROS). This occurs as poly-unsaturated lipid species are oxygen-labile and their degradation products are toxic. Under combined lipogenesis and Map Kinase inhibition, cells are directed towards a phenotype that is exquisitely sensitive to ROS. This vulnerability can be exploited by co-treatment with ROS inducing agents such as Arsenic trioxide, which in combination with the induced poly-unsaturation, strongly attenuates cell proliferation and induces cell death.

The findings in the thesis manuscript may pave the way towards the development of a novel combination treatment of both therapy-responsive and therapy-resistant melanoma. As lipogenesis is activated in many tumour types and is affected by several anti-cancer therapies inducing membrane lipid poly-unsaturation, these findings may find more broad applications and warrant further investigation in pre-clinical models of both melanoma and other cancer types.

Samenvatting

Ondanks opmerkelijke vorderingen in de kankerbehandeling, blijft metastische melanoma een bedreigende ziekte. Bijna de helft van alle melanomapatiënten is drager van een activativerende mutatie in BRAF en wordt behandeld met Map Kinase gerichte therapie. Hoewel de meerderheid (zo'n 80%) van deze patiënten initieel goed reageert op deze therapie, worden de meeste patiënten uiteindelijk resistent tegen de behandeling. Deze resistentie tegengaan vormt derhalve een belangrijke klinische nood.

Eén van de struikelblokken hierbij is de veelheid aan mechanismen waardoor therapieresistentie kan ontstaan. Verscheidene veranderingen in signaaltransductieprocessen zijn beschreven, waaronder activatie van NRAS, MITF, PI3K en Akt, die leiden tot een alteratieve activatie van stroomafwaartse oncogene processen. De uitgesproken redundantie van deze signaaltransductiewegen, werkt therapieresistentie in de hand en bemoeilijkt de ontwikkeling van behandelingen die een langdurige onderdrukking van de ziekte bewerkstelligen.

Eén van de pistes om therapie-resistentie te omzeilen is het zoeken naar en bestoken van meer stroomafwaarts functionerende eiwitten die minder redundant zijn. Metabole enzymen zijn daarbij bijzonder interessant omdat ze stroomafwaarts werken van vele signaaltransductiewegen, weinig redundant zijn en vaak een unieke afhankelijkheid veroorzaken in kankercellen.

In dit thesismanuscript tonen we aan dat de vetzuursynthese dergelijke centrale speler is in melanoma. Een actieve nieuwsynthese van vetzuren is kenmerkend voor vele kankers en wordt in therapie-gevoelige melanoma aanzienlijk onderdrukt door Map kinase inhibitoren. In therapie-resistente kankers blijkt dit proces onderhouden te blijven en bij te dragen aan de therapieresistentie. Interferentie met de vetzuursynthese via inhibitie van de lipogene transcriptiefactor SREBP- of via directe inhibitie van sleutelenzymen in dit process, zoals Acetyl-CoA carboxylase (ACACA) of vetzuursynthase (FASN), maakt resistente melanomacellen opnieuw gevoelig voor therapie. Dit wordt grotendeels verklaard doordat inhibitie van lipogenese de membraanlipiden van kankercellen meer poly-onverzadigd maakt. Poly-onverzadigde lipiden zijn vatbaarder voor peroxidatie door reactieve zuurstof (ROS), wat schadelijk is voor de kankercellen. We kunnen deze gevoeligheid verder versterken door een gecombineede inhibitie van lipogenese and Map Kinasen, waardoor kankercellen exquisiet gevoelig worden voor ROS. Deze gevoeligheid buiten we verder uit door co-behandeling met ROS-inducerende agentia zoals arseentrioxide, wat in combinatie met de geïnduceerde poly-onverzadiging de groei en overleving van therapie-resistente kankercellen aanzienlijk onderdrukt.

De bevindingen in dit thesismanuscript bieden potentieel voor de ontwikkeling van nieuwe vormen van combinatiebehandeling van zowel therapie-gevoelig als therapie-

resistent melanoom. Aangezien vele andere kankertypes ook lipogeen zijn en verscheidene andere kankerbehandelingen ook een poly-onverzadinging van lipiden veroorzaken, gaat dit concept mogelijks ook op voor meerdere kankertypes. Deze bevindingen zetten dan ook aan tot verder onderzoek in verscheidene pre-klinische modellen.

Author contributions

Please refer to Chapter 2 and Chapter 3 for full author contribution statements

Conflict of interest statement

The authors declare no conflict of interests.

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Publication List

1. (co-)first author publications

Sustained SREBP-1-dependent lipogenesis as a key mediator of resistance to BRAF-targeted therapy
Ali Talebi*, Jonas Dehairs*, Florian Rambow, Aljosja Rogiers, David Nittner, Rita Derua, Frank Vanderhoydonc, Joao A. G. Duarte, Francesca Bosisio, Kathleen Van den Eynde, Kris Nys, Mónica Vara Pérez, Patrizia Agostinis, Etienne Waelkens, Joost Van den Oord, Sarah-Maria Fendt, Jean-Christophe Marine & Johannes V. Swinnen
Nature Communications; 2018.

CRISP-ID: decoding CRISPR mediated indels by Sanger sequencing
Jonas Dehairs*, Ali Talebi*, Yacine Cherifi & Johannes V. Swinnen.
Scientific Reports; 2016.

2. Publications as contributing author

Evidence for an alternative fatty acid desaturation pathway that increases metabolic plasticity in cancer cells
Kim Vriens*, Stefan Christen*, Sweta Parik, Dorien Broekaert, Kazuaki Yoshinaga, Ali Talebi, Jonas Dehairs, Carmen Escalona-Noguero, Roberta Schmieder, Thomas Cornfield, Catriona Charlton, Laura Romero-Perez, Matteo Rossi, Gianmarco Rinaldi, Martin F. Orth, Ruben Boon, Axelle Kerstens, Suet Ying Kwan, Brandon Faubert, Andy Méndez, Charlotte C. Kopitz, Ting Chen, Juan Fernandez-Garcia, Arndt Schmitz, Patrick Steigemann, Mustapha Najimi, Andrea Hägebarth, Jo A. Van Ginderachter, Etienne Sokal, Naohiro Gotoh, Kwok-Kin Wong, Catherine Verfaillie, Rita Derua, Sebastian Munck, Mariia Yuneva, Laura Beretta1 Ralph Deberardinis, Johannes V. Swinnen, Leanne Hodson, David Cassiman, Chris Verslype, Sven Christian, Sylvia Grünewald, Thomas G.P. Grünewald, and Sarah-Maria Fendt
Nature; 2019 (accepted for publication).

Inhibition of glutamine synthetase in monocytes from patients with acute-on-chronic liver failure resuscitates their antibacterial and inflammatory capacity
Hannelie Korf, Johannie du Plessis, Jos van Pelt, Sofie De Groote, David Cassiman, Len Verbeke, Bart Ghesquière, Sarah-Maria Fendt, Matthew J Bird, Ali Talebi, Matthias Van Haele, Rita Feio-Azevedo, Lore Meelberghs, Tania Roskams, Rajeshwar P Mookerjee, Gautam Mehta, Rajiv Jalan, Thierry Gustot, Wim Laleman, Frederik Nevens, Schalk Willem van der Merwe.
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Loss of Chromosome 8p Governs Tumor Progression and Drug Response by Altering Lipid Metabolism
Yanyan Cai, Jonathan Crowther, Tibor Pastor, Layka Abbasi Asbagh, Maria Francesca Baietti, Magdalena De Troyer, Iria Vazquez, Ali Talebi, Fabrizio Renzi, Jonas Dehairs, Johannes V. Swinnen & Anna A. Sablina
Cancer Cell; 2016.