The identification of therapeutic targets in metastatic melanoma

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Abstract

Metastatic melanoma, a cancer historically refractory to chemotherapeutic strategies, has a poor prognosis and accounts for the majority of skin cancer related mortality. Although the recent approval of two new drugs combating this disease, Ipilimumab and Vemurafenib (PLX4032), has demonstrated for the first time in decades an improvement in overall survival; the clinical efficacy of these drugs has been marred by severe adverse immune reactions and acquired drug resistance in patients, respectively. Thus, understanding the etiology of metastatic melanoma will contribute to the improvement of current therapeutic strategies while leading to the development of novel drug approaches.

In order to identify recurrently mutated genes of therapeutic relevance in metastatic melanoma, a panel of stage III local lymph node melanomas were extensively characterised using high-throughput genomic technologies. This led to the identification of mutations in *TFG* in 5% of melanomas from a candidate gene sequencing approach using SNP array analysis, 24% of melanomas with mutations in *MAP3K5* or *MAP3K9* though unbiased whole-exome sequencing strategies, and inactivating mutations in *NF1* in *BRAF*/*NRAS* wild type tumours though pathway analysis. Lastly, this thesis describes the development of a melanoma specific mutation panel that can rapidly identify clinically relevant mutation profiles that could guide effective treatment strategies through a personalised therapeutic approach.

These findings are discussed in respect to a number of important issues raised by this study including the current limitation of next-generation sequencing technology, the difficulty in identifying ‘driver’ mutations critical to the development of melanoma due to high carcinogenic exposure by UV radiation, and the ultimate application of mutation screening in a personalised therapeutic setting. In summary, a number novel genes involved in metastatic melanoma have been identified that may have relevance for current therapeutic strategies in treating this disease.

Key Words

Melanoma, somatic mutation, next-generation sequencing, targeted drug, cancer.

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Abbreviations

AAML	Adult acute myeloid leukaemia
aCGH	Array comparative genomic hybridisation
ACT	Adoptive cell therapy
ADAM	A disintegrin and metalloproteinase
ADAMTS	A disintegrin and metalloproteinase with thrombospondin domains
AML	Acute myeloid leukaemia
BAC	Bacterial artificial chromosome
CAN	Cancer gene
cDNA	Complementary deoxyribonucleic acid
CGH	Comparative Genomic Hybridisation
CLIA	Clinical laboratory improvement amendments
CML	Chronic myeloid leukaemia
COSMIC	Catalogue of somatic mutations in cancer
DNA	Deoxyribonucleic acid
DTIC	Dacarbazine
EGFP	Enhanced green fluorescent protein
GIST	Gastro-intestinal stromal tumour
GPCR	G protein coupled receptor
HMPS	Hereditary mixed polyposis syndrome
HREC	Human Research Ethics Committee
IFN-α	Interferon alpha
IL-2	Interleukin 2
IPA	Ingenuity pathway analysis
JPS	Juvenile polyposis syndrome
JMML	Juvenile myelomonocytic leukaemia
LCL	Lymphoblastoid cell line
LOH	Loss of heterozygosity
MAPK	Mitogen activated protein kinase
MBP	Myelin basic protein
miRNA	Micro ribonucleic acid
MMP	Matrix metalloproteinase
MSI	Microsatellite instabile

MSS	Microsatellite stabile
MSP	Melanoma specific mutation panel
MTIC	5-(3-methyl-2-triazeno)imadazole-4-carboxamide
mTOR	Mammalian target of rapamycin
NGS	Next-generation sequencing
N:S	Non-synonymous to synonymous ratio
PARE	Personalized analysis of rearranged ends
PARP	Poly ADP ribose polymerase
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol-3-kinase
PTP	Protein tyrosine phosphatases
QIMR	Queensland Institute of Medical Research
RNA	Ribonucleic acid
RTK	Receptor tyrosine kinase
RT-PCR	Real-time polymerase chain reaction
siRNA	Short interfering ribonucleic acid
shRNA	Short hairpin ribonucleic acid
SMA	Smooth muscle actin
SNP	Single nucleotide polymorphism
TIL	Tumour infiltrating lymphocyte
TMZ	Temolozolomide
UTR	Untranslated region
UV	Ultraviolet
WES	Whole exome sequencing
WT	Wild-type

Statement of originality

The work contained in this thesis has not been previously submitted to meet the requirements for an award at this or any other higher educational institute. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made. A detailed account preceding each publication stating the contribution of authors is made. All co-authors have provided their consent for the inclusion of the papers in this thesis.

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Ken Dutton-Regester
23rd March 2012

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Now that the PhD is finished, it is time to reintegrate myself back into society! Just after I finish that fellowship application, start writing up that review, start collecting samples, perform that.......

Chapter 1- Introduction

1.1. Significance of metastatic melanoma

Skin cancer (non-melanoma and melanoma) is the most common form of cancer in Australia and represents a significant burden on the healthcare system costing approximately $300 million a year [1]. In 2007, there were 10,342 cases of melanoma in Australia, representing approximately 10% of all cancers diagnosed. As such, Australia has one of the highest incidences of skin cancer in the world [2]. Of particular concern is that the incidence of melanoma is increasing, which, similar to other countries including the United States [3], has more than doubled over the last 20 years [2]. Although all forms of skin cancer can seriously impact health, melanoma accounts for the majority of skin cancer related mortality [4]. A total of 1279 deaths due to melanoma were documented in Australia in 2007 [2].

Melanoma is a malignant skin cancer originating from the unregulated growth of melanocytes, cells responsible for pigmentation in the skin. Although a subset of melanomas have a familial hereditary component, the majority arise through the gradual accumulation of genetic abnormalities caused by carcinogenic exposure of solar ultraviolet (UV) radiation. It is the acquisition of somatic mutations in critical genes controlling a range of important cellular processes that results in the proliferation and dissemination of melanoma throughout the body.

For melanomas that are detected early in pathogenesis, surgical removal at the primary site results in a 95% overall survival rate. However, due to the highly aggressive nature of melanoma, tumours may remain undetected without regular skin examinations until they have metastasized either locally to lymph nodes or distally to other organs. Upon metastasis, surgical excision becomes problematic and survival rates are dramatically reduced, with a median life expectancy of 6 months and only 5% of patients surviving beyond 5 years [4].

The high mortality rate of melanoma is essentially due to the lack of effective treatments, particularly for late stage or disseminated disease. Improvements to existing therapies or

the development of novel drug strategies are required in order to increase overall survival in patients with metastatic melanoma.

1.2. Therapeutic approaches in the treatment of metastatic melanoma

Until recently, treatment regimens for patients with metastatic melanoma have remained bleak with the availability of only a handful of approved therapies which have had minimal impact on overall survival. Traditional chemotherapeutic approaches fail to promote durable response rates, while immunological strategies, such as treatment with interleukin 2 (IL-2) or interferon alpha (IFN-α), result in high grade toxicities limiting their use in the clinic [5].

In 2011, for the first time in 40 years, two new drugs were approved for metastatic melanoma and each has demonstrated a significant improvement in overall survival. These drugs, Ipilimumab and Vemurafenib, have significantly altered the standard of care for patients with metastatic melanoma [6]. The following section describes previous and current strategies that have been implemented in the clinic for the treatment of metastatic melanoma.

1.2.1. Chemotherapeutic strategies

Melanoma has historically been refractive to chemotherapeutic treatments. Although a number of agents have been assessed in clinical trials [7], dacarbazine (DTIC), until recently, has been the standard approved treatment option for patients with advanced (stage IV) melanoma.

DTIC is a cytostatic agent with alkylating properties that inhibits DNA synthesis and promotes growth arrest. Intravenously administered, DTIC is a pro-drug that requires processing within the liver to first release the active compound 5-(3-methyl-1-triazeno)imadazole-4-carboxamide (MTIC). Although complete responses in patients are occasionally observed, clinical trials have demonstrated response rates in only 5-15% of patients, with a median durability of 6 to 12 months [8].

An orally administered pro-drug, Temozolomide (TMZ), also uses MTIC as its active compound, however it does not require liver processing and can be converted

systemically. One advantage of TMZ to DTIC is its ability to cross the blood-brain barrier, potentially enabling it to be efficacious for treating brain metastases. Clinical trials have since shown low objective response rates of brain metastases to TMZ [9], although evidence suggests that it may decrease the incidence of relapse [10]. Regardless of comparable low response rates between TMZ and DTIC [11], alternative strategies to standard chemotherapeutic approaches are required to improve overall rates of survival in patients with metastatic melanoma.

1.2.2. Immunological approaches

Compared to other malignancies, melanoma has long been regarded a forerunner for immunotherapeutic approaches in the treatment of solid cancers. The first immunological drugs approved for the treatment of metastatic melanoma was IFN-α and high dose IL-2 [12, 13]. The use of these cytokines is typically associated with response rates of 10-20%, with approximately 5% of patients exhibiting long-term responses; in some cases, remission of up to 5-10 years [12, 13]. Due to the nature of these treatment regimens in eliciting strong immune reactions, severe adverse side effects are frequently observed. As such, treatment via these modalities is usually limited to those patients who are relatively healthy and have excellent organ capacity, but still require intensive clinical observation during treatment. These drugs can be used in conjunction with chemotherapeutic strategies, however this approach is associated with the risk of increased toxicity with minimal survival benefit [14].

More recently, results from anti-CTLA4 antibodies designed to promote sustained T cell activation have led to the approval of Ipilimumab (Yervoy) in the treatment of metastatic melanoma. Phase III clinical trials of Ipilimumab used as a single agent or in conjunction with DTIC have improved rates of overall survival [15, 16]; however, response is often associated with initial delays in tumour regression. Despite these findings, the use of Ipilimumab can promote severe grade III and IV side effects leading to premature termination of therapy, and on rare occasions, treatment-related mortalities [16].

A number of challenges regarding the clinical management of Ipilimumab remain, and hopefully, with the identification of positive biomarkers of drug response, improvements in the clinical utility of this drug will occur. Investigations into biomarkers are currently in their infancy, however, tumours with active immune microenvironments [17] and those

expressing immune-related genes [18] may indicate favourable responses in patients. Despite the current lack of robust biomarkers, Ipilimumab has quickly been established as the standard treatment for non-*BRAF*-mutated melanoma patients (discussed in more detail below).

1.2.3. Molecularly based targeted therapies

Aside from chemotherapeutic and immunological approaches, molecularly based targeted therapy is a novel drug approach that counteracts the effect of acquired mutations responsible for tumorigenesis. The first of these drugs to demonstrate significant success in cancer was Imatinib mesylate (Gleevac); a tyrosine kinase inhibitor, originally designed to target an oncogene formed by a *BCR-ABL* translocation event observed in chronic myeloid leukaemia (CML). Imatinib is highly effective in CML treatment, with 76% of patients (n=343) showing some form of cytogenic response and 41% with complete remission [19]. The effectiveness of Imatinib in *BCR-ABL* fusion tumours provided a 'proof of principle' for subsequent targeted therapeutics in other malignancies.

The first forays of targeted therapies in melanoma coincided with the seminal finding of somatic oncogenic mutations in *BRAF*, a member of the serine/threonine family of protein kinases, occurring in 66% of melanomas [20]. Mutation of *BRAF* has since been more accurately estimated to occur in ~50% of melanomas, the majority of which are accounted for by a valine to glutamate substitution at coding position 600 (V600E, initially reported as V599E). Mutations such as V600E disrupt the inactive conformation of the kinase domain resulting in constitutive auto-phosphorylation and downstream signalling of the mitogen-activated protein kinase (MAPK) pathway [21].

Functional analysis of mutant *BRAF* showed that exogenously expressing V600E *BRAF* in mice promoted tumorigenicity [22], while conversely, suppression of mutant *BRAF* by siRNA led to cell growth arrest and apoptosis [23, 24]. The development of a small molecular inhibitor of tyrosine kinases, Sorafenib, and successful application in other malignancies, provoked testing the efficacy of such drugs in melanoma [25]. Unfortunately, phase II and phase III clinical trials of Sorafenib in metastatic melanoma did not demonstrate an increase in overall survival compared to standard chemotherapeutic strategies [26, 27].

Further refinement in molecular drug design led to the development of highly selective inhibitors of *BRAF* V600E tumours [28]. One of these, Vemurafenib (also known as PLX4032 or Zelboraf), was the second drug to receive approval in 2011 for use in metastatic melanoma. Vemurafenib results in dramatic rates of initial tumour regression, with patients demonstrating an increase in overall survival at 6 months compared to DTIC [29]. However, long-term response rates have been hindered by tumour acquired drug resistance frequently observed in the majority of patients. A number of mechanisms of tumour resistance to Vemurafenib have been identified [30-34], and it is hoped that through application of this knowledge, together with continued research, long-term response rates and overall survival will be improved (Figure 1.1).

Interestingly, the use of Vemurafenib frequently results in the development of skin lesions, such as squamous cell carcinomas, in patients undergoing therapy [35]. Although not life-threatening when managed by frequent clinical observation and surgical removal, it is a concerning phenomenon pointing towards off-target side effects. Paradoxically, the use of BRAF inhibitors in non *BRAF* mutant tumours results in the activation of the MAPK pathway [36, 37], thus promoting cell proliferation and tumour progression. This finding highlights the critical importance of drug selection based on the presence of a *BRAF* V600E mutation within a patient's tumour, a concept that has now become known as personalised medicine.

The BRAF inhibitor story demonstrates an excellent example of translational research in practice; moving from initial gene discovery to drug design, clinical trials and approval within a timeframe of 10 years. However, not all mutations discovered in tumours will require heavy investments in development. The availability of existing therapeutics, or 'off the shelf' drugs, that have shown efficacy in malignancies with comparable mutation profiles, can be rapidly trialled for treatment of a new cancer type.

An excellent example of this approach in metastatic melanoma is the use of Imatinib in *KIT*-mutated tumours. As mentioned previously, Imatinib is an inhibitor of tyrosine kinases, preventing substrate phosphorylation through competitive inhibition of the ATP-binding domain. In addition to activity in *BCR-ABL* mutant CML, Imatinib is also approved for gastro-intestinal stromal tumours (GIST), a cancer that exhibits oncogenic mutations of *KIT* in approximately 80% of patients [38, 39]. The positive response of Imatinib in GIST, combined with an early observation of *KIT* expression in melanoma, led to clinical trials of

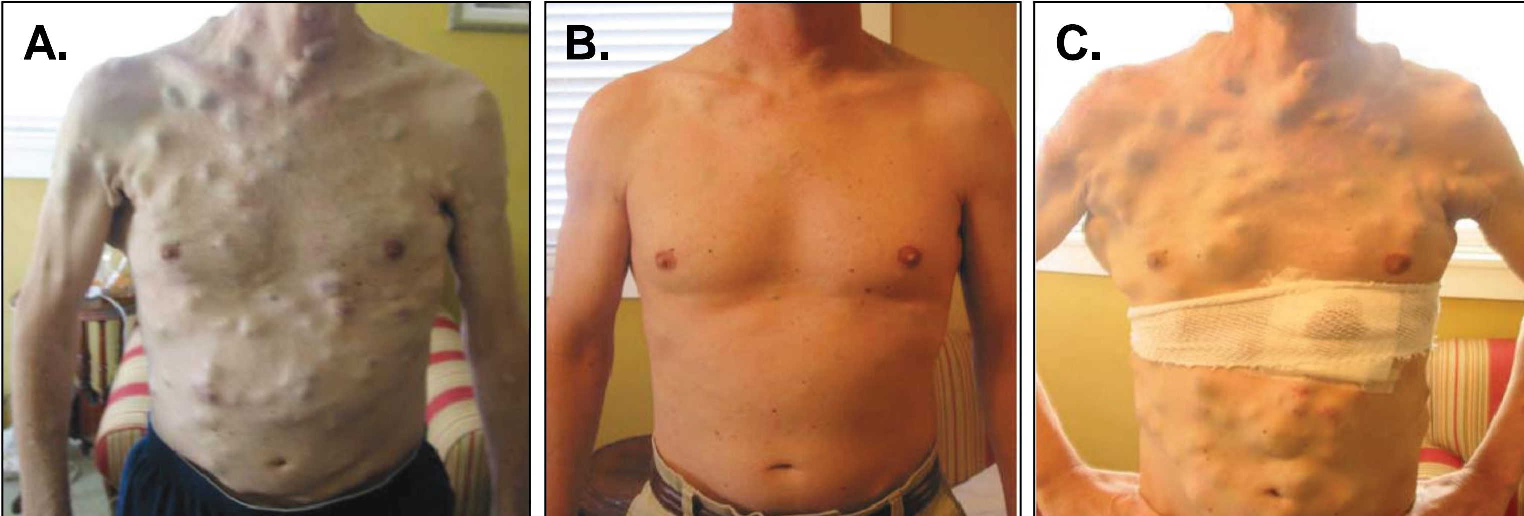


Figure 1.1: The effect of a molecularly targeted drug in metastatic melanoma. A representative depiction of a typical response to Vemurafenib in a patient with subcutaneous metastatic melanoma whose tumours exhibit BRAF V600E mutations. A. Patient before treatment with extensive tumour burden throughout the body. B. 15 weeks post Vemurafenib treatment. Significant regression of tumour mass is observed. C. 23 weeks post treatment. Tumour acquired drug resistance to Vemurafenib frequently occurs in patients. Understanding the mechanisms of drug resistance will hopefully improve the efficacy of these drug strategies. Adapted from Wagle, et al. 2011 (31)

Imatinib in metastatic melanoma. Although overall results of Imatinib lacked efficacy in the treatment of melanoma [40-42], closer analysis of a single patient who responded favorably identified them to harbour a mutation in *KIT*, suggesting putative efficacy of Imatinib in a subset of melanomas.

Interestingly, sequencing analysis has revealed a distinct lack of *KIT* mutation in intermittently sun-exposed cutaneous melanomas but an increased representation of mutation in acral, mucosal and chronically sun-exposed melanomas [43]. As the latter subtypes of melanoma are rare, the poor efficacy in early Imatinib trials was most likely explained by the under-representation of *KIT*-mutated tumours within the studies. Subsequent case reports have since demonstrated major responses to Imatinib in patients with *KIT*-mutated acral and mucosal melanoma [44-47], and more recently, in a phase II clinical trial [48].

1.3. Understanding the genetics of cancer- the identification of driver mutations

The successful development and recent approval of two drugs for metastatic melanoma, in particular the molecular based targeted approach of Vemurafenib, can be attributed to the extensive effort in understanding the genetic aetiology of melanoma. Characterisation of the multitude of genetic alterations promoting the development and progression of melanoma has led to the identification of frequently mutated genes, of which, a proportion are amenable to therapeutic intervention.

The discovery of mutated genes driving the development of cancer has dramatically changed over time with advances in technology. More recently, the advent of next-generation sequencing has allowed unparalleled, unbiased analysis of the cancer genome. This section provides a brief history of large-scale sequencing strategies with particular emphasis on the somatic genetics of metastatic melanoma.

1.3.1. DNA sequencing by capillary electrophoresis

Early investigations into the genetic aetiology of cancer relied strongly on candidate gene approaches for gene discovery. This included the analysis of cancer prone families via

linkage analysis, positional cloning and subsequent *in vitro* transformation assays. Although these candidate gene approaches have the capability to reveal genes implicated in disease, these methods are primarily efficient for the identification of highly mutated genes and less so for those mutated at low frequency. The ability to screen large numbers of genes simultaneously in an unbiased manner was thus highly desirable [49]. It was the completion of the human genome project that provided a catalyst for improving existing sequencing techniques and led to significant increases in throughput capability, improved bioinformatics, and provided infrastructure to perform large scale sequencing efforts.

Initial large scale sequencing efforts first focused on the sequencing of gene families; not surprisingly, members of the kinase family were first to be analysed due to their frequent involvement in cancer and potential to be targeted therapeutically [50]. One of the earliest studies sequenced the entire family of tyrosine kinases, representing 90 genes, in 35 colorectal cancer cell lines [51]. The data suggested that approximately 30% of colorectal cancers have a mutation within the tyrosine kinome, and that potentially, other mutations may reside in alternative kinase families. Another study extensively sequenced the tyrosine kinase family in 254 tumour cell lines of various tissue origins, including 53 melanomas [52]. Interestingly, melanoma had the highest rate of mutation, with nearly all tumours having a mutation within a tyrosine kinase.

Logically, the next progressive step forward involved sequencing of the entire complement of kinases in the human genome; this was completed in breast cancer [53], lung cancer [54] and testicular germ cell tumours [55]. The most comprehensive of these projects sequenced 210 tumours from a variety of cancers including 6 melanomas [56]. Again, melanoma had one of the highest rates of mutation, with approximately 24 somatic kinome mutations per sample tested; this either indicated the importance of kinases in melanomagenesis or that a high rate of mutation may be a feature inherent to melanoma. Regardless, these studies began to highlight the complexities of large scale sequencing efforts, including the difficulty in the identification of critical mutations contributing to the neoplastic process.

Mutations are thought to stochastically accumulate within the genome, a large majority of which are not likely to confer a growth advantage to the cell. This type of mutation, known as a ‘passenger’ mutation event, will also be intrinsically present prior to and gained during clonal expansion of the tumour. In contrast, a small handful of mutations that are

deleterious or oncogenic in nature will be responsible for ‘driving’ the process of tumorigenesis. One of the main challenges faced in analyzing large amounts of data produced from genome-wide studies is determining ‘passenger’ mutations from ‘driver’ mutations [57].

‘Driver’ mutations in genes causally involved in tumorigenesis have been putatively labelled as cancer genes (CAN). A central aim in cancer research has been to identify CAN genes in order to understand the complex process of oncogenesis. ‘A census of human cancer genes’ using existing literature was the first step in cataloguing all CAN genes known to have a causal role in progression of a variety of cancer types [50, 58]. Although originally 291 CAN genes were identified (approximately 1% of the genome), continuing research efforts have now identified a total of 474 CAN genes [58].

Cancer progression can be considered as a gradual accumulation of ‘driver’ mutations in CAN genes. Original estimates based on theoretical models suggested as little as three mutations could be sufficient for tumorigenesis [59, 60]. However, early large scale sequencing efforts utilising traditional Sanger sequencing in colorectal and breast cancer revealed that individual tumours accumulated an average of 90 mutated genes, with a predicted average of 16 genes contributing to the neoplastic process [61]. Although revealing a higher complexity than previously predicted, this study was far from comprehensive as only half the coding region of the genome was sequenced in a low number of tumours (11 of each tumour type).

Subsequent investigations sequenced even larger proportions of coding regions in cancers, further revealing the complexity of the cancer genome. Large scale Sanger sequencing projects assessing close to the entire coding region of the genome (from 18000 to 20000 genes) was completed in breast and colorectal cancer [62], pancreatic cancer [63] and glioblastoma [64]. The extent of these studies revealed large differences in the genes mutated between individual tumours, with few genes frequently being mutated in a large proportion of samples. The majority of mutations in genes occurred in less than 5% of all tumours tested. These 'mutation profiles' presented an interesting dilemma in interpreting the precise roles of mutations promoting tumour progression. Is cancer progression largely driven by a small number of genes frequently mutated in a large proportion of tumours (termed 'mountains' – possibly equivalent to ‘drivers’), or is it the

accumulation of large numbers of infrequent mutations responsible for driving tumour growth (termed 'hills' - possibly equivalent to ‘passengers’), or some combination of both?

To further elucidate the relevance of 'hill' CAN genes in respect to specificity of these mutations occurring between tumour types, one investigation used 27 of these 'hill' CAN genes identified in breast and colorectal cancer [62] to screen for mutations in melanoma, glioblastoma and pancreatic cancer [65]. Only 4 of these CAN genes were shown to be reciprocally mutated in melanoma and pancreatic carcinoma while no similar mutations were identified in glioblastoma. From these results, it was suggested that 'hill' CAN genes are not shared between tumours derived from different tissues and that each cancer type has a distinct mutation profile and genomic landscape.

Although large scale efforts using traditional sequencing technology answered some early yet critical questions in cancer genetics, the associated costs and throughput capabilities of the technology was limiting comprehensive investigations into the cancer genome. The advent of next-generation sequencing platforms provided a viable, unbiased, cost effective solution for high-throughput genetic analysis.

1.3.3. The application of next-generation sequencing technology

Following the completion of the first draft of the human genome in 2001 [66, 67], a number of sequencing platforms were developed using novel chemistries allowing unparalleled data generation compared to DNA sequencing by capillary electrophoresis. These next-generation sequencing technologies, or massively parallel sequencing, allowed the whole genome of an individual to be sequenced for one hundredth the cost of Sanger dideoxy chemistry and in a timeframe of months [68-70]. These sequencing efforts were critical for the establishment of the methodology, alignment programs and bioinformatics required for the data generated from next-generation sequencing platforms.

A seminal paper for cancer genomics was released in late 2008 when an entire acute myeloid leukaemia (AML) genome and its matched normal counterpart were characterised using next-generation sequencing [71]. This was the first glimpse into the genetic architecture of a tumour and provided a methodological template for somatic mutation analysis of cancer genomes. In particular, this project highlighted the necessity for sequencing not only the tumour at high depth, but also a matched normal sample in order

to differentiate between inherited germline variants and mutations acquired during tumorigenesis, albeit at an increased expense.

A total of 10 non-synonymous mutations were identified in the cytogenetically normal AML genome, indicating that somatic mutations in this genome are extremely rare [71]. Of these mutations, two were well known AML-associated mutations while the remaining eight had not been previously documented. Although half of the eight mutations had been linked to genes involved in cancer pathogenesis; none of these genes would have been selected for hypothesis-driven candidate gene studies, thus highlighting the benefits of unbiased cancer genome sequencing analysis.

In 2009, the second AML genome was sequenced, revealing a number of new mutations in genes not previously associated with AML [72]. A total of 64 somatic mutations were identified including 12 non-synonymous mutations. Surprisingly, none of the non-synonymous mutations identified overlapped with the first AML genome; this was an initial indication of the heterogeneity of tumours and suggested that uncovering the complexity of cancer will require the sequencing of numerous cancer genomes. However, genotyping of the 12 non-synonymous mutations in an additional cohort of AML tumours revealed 15 of 187 samples with mutations in *IDH1*, demonstrating the ability of cancer genomics to identify causal drivers of tumorigenesis.

The first catalogue of somatic mutation of a melanoma genome involved the sequencing of a commercially available metastatic melanoma cell line, COLO-829, and its matched lymphoblastoid cell line, COLO-829BL [73]. As this melanoma cell line had been extensively studied, this analysis provided an opportunity to accurately assess the sensitivity of the sequencing method employed. A total of 42 of 48 known mutations within this cell line were detected, indicating a sensitivity of 88%. In all, 292 somatic mutations were found, of which, 187 were non-synonymous; a mutation rate considerably higher than that of AML or the first solid cancer that was sequenced prior (breast cancer) [74].

Interestingly, analysis of the somatic base substitutions in COLO-829 revealed a mutation profile consistent with a UV radiation based carcinogenic signature [75]. The majority of mutations detected were C>T (G>A) transitions with ~70% being CC>TT/GG>AA, this is expected to be caused by DNA damage by UV radiation resulting in the formation of covalent links between two adjacent pyrimidines [76]. A carcinogenic signature of

G>T/C>A transversions has since been identified in small-cell lung cancer through excessive tobacco exposure [77].

To assess the significance of mutations identified in COLO-829 in regards to driver versus passenger events, one approach is to use the non-synonymous to synonymous (N:S) mutation ratio [56, 78]. This statistic implies the assumption that non-synonymous mutations are biologically selected for as these mutations can affect the structure of proteins. As such, higher N:S ratios indicate positive selection overall compared to what is expected by chance. The N:S ratio of the COLO-829 genome was 1.78, not higher than the N:S ratio of 2.5:1 predicted for non-selected passenger mutations; this indicated that the majority of mutations were likely to be passenger mutations not relevant for pathogenesis of melanoma. This observation, in conjunction with high mutation rates in melanoma compared to other malignancies, highlights a potential difficulty in identifying causal genes involved in this disease. One approach to overcome this problem is by analyzing large numbers of tumours to identify frequently mutated genes.

Integrative analysis of RNA-seq and high-resolution chromosomal copy number data was an early approach to comprehensively assess the mutation rate in a large subset of melanomas [79]. Although a number of interesting mutations were identified, this study was limited by the detection of mutations in the most abundant transcripts expressed in melanoma and the lack thereof of a matched normal sample for comparison. An improvement of this method involved the application of whole-exome sequencing (WES) strategies to cancer genomics. This led to the first exome report of 12 metastatic melanomas and their matched normal samples in mid 2011 [80]. Although the N:S ratio was 2.0:1, suggesting the majority of mutations were passenger mutations, a number of interesting genes were identified when the list of mutations was compared between samples. This led to the identification of a recurrent mutation of *TRRAP* in 4% of melanomas, as well as the identification of 25% of melanomas exhibiting mutations in *GRIN2A* (discussed in more detail later). Despite the high burden of mutation in melanoma, this study provided a proof of principle that genes relevant to the pathogenesis of the disease could be detected with small sample sets.

1.4. Genetics of cutaneous metastatic melanoma

Since the identification of *BRAF* mutations in melanoma, studies have identified a number of oncogenes and tumour suppressor genes involved in a variety of pathways, including cell signalling, division and apoptosis. As the introduction of new technologies is making powerful genome-wide scale studies achievable, it is becoming apparent that determining affected pathways, rather than single genes in isolation, will be important in understanding tumorigenesis [81]. This section reviews the well-characterised classical pathways of cutaneous melanoma development in addition to novel emerging pathways revealed by recent sequencing efforts (Figure 1.2).

1.4.1. Classical pathways to melanoma development

1.4.1.1. The mitogen-activated protein kinase (MAPK) pathway

The MAPK pathway regulates cell growth regulation and survival through a series of signalling cascades in response to external stimuli (reviewed extensively in [82]). Under normal physiological conditions, extracellular signals initiate the binding of receptor tyrosine kinases (RTK) to RAS, a membrane-bound GTPase at the cell surface membrane. This process leads to a series of downstream phosphorylation cascades causing stepwise activation of BRAF, MEK1/2 and ERK1/2, and ultimately leads to cell regulation of proliferation, angiogenesis, invasiveness and metastasis. Although ERK activity is tightly regulated in melanocytes, high constitutive activity of the MAPK pathway is frequently observed in melanoma, largely due to the acquisition of oncogenic mutations in members of this pathway [83, 84].

Activation of the MAPK pathway in melanoma is predominantly driven by mutation of *BRAF* (approximately 50% of melanomas), however, some tumours exhibit mutations in *RAS. RAS* mutations have been observed in 10-20% of melanomas [85], the most frequently mutated member of this family being *NRAS*. Notably, mutations of *BRAF* and *NRAS* tend to be mutually exclusive (except for a few rare cases) indicating redundancy in their biological function.

Mutations in *MAP2K1* (MEK1) and *MAP2K2* (MEK2) have also been recently identified in 8% of metastatic melanomas through use of WES [86]. Interestingly, MAP2K1/2 mutations not only cause constitutive activation of the MAPK pathway, but can also be acquired in

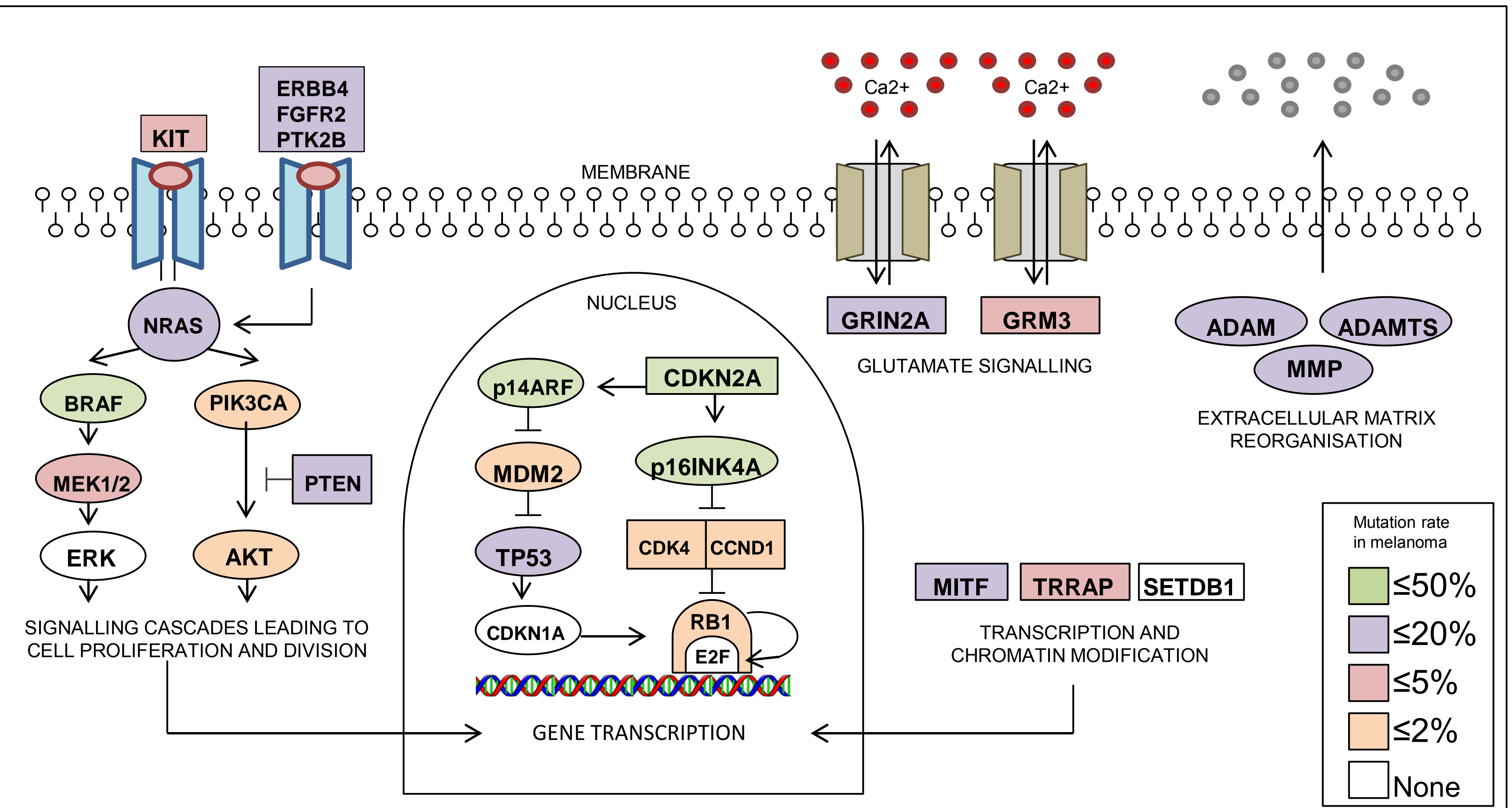


Figure 1.2: Pathways frequently deregulated in cutaneous metastatic melanoma

drug resistant tumours following the use of BRAF inhibitors [30, 31]. Alternatively, mutations in an upstream RTK, such as *KIT* or *ERBB4*, can result in MAPK activation (these are described in other sections of this review).

1.4.1.2. The phosphatidylinisitol-3-kinase (PI3K) pathway

Apart from the MAPK pathway, NRAS also signals through phosphatidylinisitol-3-kinase (PI3K) to activate AKT [87, 88]. AKT interacts with a number of other signalling networks that control a variety of cellular functions including cell survival, proliferation, apoptosis and tumour cell chemo-resistance. A gene known as *PTEN* (phosphatase and tensin homolog, deleted from chromosome 10) negatively regulates this pathway by preventing downstream AKT signalling and controls cell cycle progression.

Activation of the PI3K pathway in melanoma occurs primarily through *NRAS* mutation (~20%); however, mutations can also occur in *PIK3CA* and *AKT*, albeit at low frequencies [89, 90]. In contrast, *PTEN* mutation results in deregulation of the PI3K pathway through the loss of negative regulation of AKT. *PTEN* mutation has been observed at high frequency in melanoma and was originally identified by its frequent deletion in a number of cancers. A variety of mutations including missense and splice site mutations, deletions and insertions in *PTEN* have since been observed in up to 30-50% of melanomas [91, 92]. As mutation of *NRAS* results in the deregulation of both the MAPK and PI3K pathways, mutation of *PTEN* is generally associated with *BRAF* mutant tumours.

1.4.1.3. The Rb pathway

The Rb pathway, responsible for controlling cell cycle division and progression, is another frequently deregulated pathway in melanoma [93]. A key regulator of this pathway is *CDKN2A* (cyclin-dependent kinase inhibitor 2A), a tumour suppressor gene identified in a range of tumours including melanoma [94]. *CDKN2A* encodes two different proteins, p16INK4A and p14ARF through alternative transcription start sites and use of different reading frames. Similar to *PTEN*, deletion of a region of chromosome 9 (where *CDKN2A* is located) was observed in a number of melanomas, indicating the presence of a putative tumour suppressor gene [95]. Deletions of *CDKN2A* have since been observed in up to 50% of melanomas [96].

The *CDKN2A* product p16INK4A negatively regulates cell division by inhibiting kinases CDK4 and CDK6 bound to CCND1. The CCND1-CDK4/6 complex, when not inhibited,

phosphorylates pRb (*RB1*), an active repressor of E2F-mediated gene transcription, allowing transcription of a variety of genes that promote cell cycle division. Besides inactivation of p16INK4A, isolated reports have also identified mutations within *CDK4*, *CDK6, CCND1* and *RB1* that can cause deregulation of this pathway [97-99].

1.4.1.4. The TP53 pathway

CDKN2A, through an alternative reading frame, encodes another tumour suppressor called p14ARF. p14ARF is responsible for the inhibition of MDM2 which in turn regulates the activity of p53 (*TP53*), a well known tumour suppressor gene involved in DNA repair, apoptosis and cell cycle division [100]. One role of p53 is to activate p21 (*CDKN1A*) which, like p16INK4A, prevents the phosphorylation of pRb by binding to CDK2/CCNE1 complexes. Besides inactivation of p14ARF, deregulation of the p53 pathway occurs through mutation or deletion of *TP53* in approximately 20% of melanomas [101, 102].

1.4.2. Emerging pathways of melanoma development

1.4.2.1. Receptor tyrosine kinases and protein phosphatases

RTKs are cell surface receptors that respond to external stimuli and are responsible for the control of a variety of cellular processes. This class of kinase has been extensively studied due to their frequent involvement in tumorigenesis and ability to be targeted for pharmacologic inhibition [50, 103]. Early studies identifying frequent RTK mutation in cancer [53, 104], including KIT mutations in melanoma [105], suggested the possibility of other deregulated RTKs in the development of melanoma.

To investigate this premise, Prickett et al. performed a comprehensive analysis of the tyrosine kinase family in melanoma [106]. A total of 99 non-synonymous mutations were found in 19 protein tyrosine kinases, with the highest frequency occurring in *ERBB4* (19%), *FLT1* (10%) and *PTK2B* (10%). Focusing on *ERBB4*, *in vitro* functional analysis revealed that mutation led to an increase in cell growth and receptor auto-phosphorylation activation but more importantly, cells transfected with *ERBB4* mutation had increased sensitivity to the drug Lapatinib, a FDA approved ERBB pharmacological inhibitor [106]. These results, if confirmed through additional *in vivo* experiments, suggests that *ERBB4* could be a *bona fide* target for existing ERBB inhibitors in this subset of patients.

Analysis of another group of RTKs identified the fibroblast growth factor family as having a putative functional role in melanoma progression. Sequence analysis of *FGFR1-4* in an initial cohort of 47 melanoma cell lines, followed by additional sequencing of *FGFR2* in 66 samples revealed a total of 15 different mutations in *FGFR2* (mutated in ~10% of all samples tested). Additional analysis revealed mutations in 3 of 28 metastatic samples and 5 of 72 primary tumours. However unlike *ERBB4*, bioinformatic analysis and *in vitro* functional assays indicated that the majority of mutations in *FGFR2* result in a loss of receptor activity [107]. Inactivation of protein function presents difficulties in regards to drug development; however, further investigation into the role of FGFR mutations in melanoma is warranted.

Deregulation of protein tyrosine phosphatases (PTP), proteins that co-regulate the activity of tyrosine kinases, have also been identified in melanoma. *PTPRD* was first shown to be lost through homozygous deletion in melanoma [108]. Building on this finding, sequencing of this gene in melanoma revealed 10 mutations in *PTPRD* in 7 of 57 tumours (12%) [109]. Lentiviral transfection assays of mutated forms of *PTPRD* led to a decrease in cell death compared to transfected wild type *PTPRD,* contributing further evidence of a tumour suppressing role for *PTPRD* in melanoma. The entire extent of the role that deregulated PTP plays in melanoma has yet to be determined; it remains to be seen whether these proteins are viable therapeutic targets.

1.4.2.2. G protein coupled receptors and glutamate signalling

The first study using WES analysis in metastatic melanoma provided a glimpse into the melanoma genome and identified a number of novel recurrently mutated genes [80]. Aside from *BRAF*, the most frequently mutated gene found in this discovery screen was *GRIN2A*, which was mutated in ~25% of melanomas. Mutations occurred throughout the entire length of the gene and were most likely inactivating, suggesting that *GRIN2A* acts as a tumour suppressor.

GRIN2A, an N-methyl-D-aspartate (NMDA) receptor, belongs to a class of ionotropic glutamate-gated ion channels. Binding of glutamate to *GRIN2A* allows calcium and potassium to traverse the cell membrane, however, the biological effect of *GRIN2A* mutation and its role in melanoma has yet to be determined. Targeted exon capture paired with next-generation sequencing of the G protein coupled receptor (GPCR) family in melanoma identified mutations in members of a second class of glutamate receptors, the

metabotropic glutamate receptors [110]. This included mutation of *GRM3* and *GRM8* in ~16% and ~9% of melanomas respectively. Biochemical analysis of mutant *GRM3* showed that it caused an increase in anchorage-independent growth and migration *in vitro* and *in vivo*.

Additional evidence for the role of this emerging pathway in melanoma is demonstrated by mutation of *PLCB4*, a downstream effector of GRM signalling [80]. Other members of the GRM family have also been implicated in melanomagenesis; this includes the correlation of *GRM1* expression to hyper-proliferation of mouse melanocytes and increased expression of *GRM1* in human melanoma biopsies compared to melanocytes [111]. Lastly, mutant *GRM3* was shown to increase the activation of MEK, possibly suggesting crosstalk between the MAPK and glutamate pathways. Mutant *GRM3* exposed to AZD6244, a small selective molecular inhibitor of MEK, resulted in greater inhibition and drug sensitivity compared to wild-type *GRM3*, suggesting this may be a viable drug strategy in patients with mutations of the glutamate pathway [111].

1.4.2.3. Extracellular matrix regulation

A number of recent studies have identified frequent mutations in gene families involved in the regulation of the extracellular matrix and may have a role in cell motility, invasion or metastasis. These studies combined have resulted in the emergence of a novel pathway to melanoma development.

Matrix metalloproteinases (MMP) belong to a family of 23 proteolytic enzymes that degrade the extracellular matrix and basement membranes surrounding cells. The role of MMPs in cell invasion, including that of melanoma, has long been identified [112], however, investigations into somatic mutations within this family of proteins has only recently been performed [113]. Mutations were found in 8 MMP genes in 23% of melanomas, of these, *MMP8* and *MMP27* were most frequently mutated. Interestingly, mutant MMP showed a decrease in proteolytic activity but an increase in tumour growth both *in vitro* and *in vivo* [113]. These results suggested that wild type *MMP8* has the ability to inhibit melanoma progression and subsequently has a putative tumour suppressing role.

Another related family, disintegrin-metalloproteinases with thrombospondin domains (ADAMTS), is part of a larger superfamily of zinc-based proteinases called metzincins, to which the MMPs belong. The role of ADAMTS proteins in cancer has not been well

established, however, *ADAMTS15* was shown to be genetically inactivated in colorectal cancer [114]. This prompted mutational analysis of the ADAMTS family in melanoma [115], a study which identified a large fraction of tumours (~37%) harbouring mutations in 11 of the 19 genes comprising the family. Mutant *ADAMTS18*, the most frequently mutated member at ~18%, was shown to be critical for cell migration *in vitro* and caused increased metastases *in vivo*, suggesting an oncogenic role in the proliferative, migratory ability of metastatic melanomas [115].

Mutational analysis of a third family of the metzincins, the disintegrin-and metalloproteinases (ADAM) family, also revealed high rates of mutation in melanoma [116]. The ADAM family is a group of membrane-bound glycoproteins that have a variety of biological roles including cell adhesion, migration and proteolysis. Sequencing of the 19 ADAM family genes revealed 8 genes collectively being mutated in 34% of melanomas, the most frequently occurring in *ADAM7* (~12%) and *ADAM29* (~15%). Functional analysis demonstrated that mutant *ADAM7/29* affected the adhesion capacity to a variety of extracellular matrix proteins and increased migratory abilities.

Although early clinical trials investigating first generation pan-inhibitors of proteolytic activity of MMP yielded disappointing results [117], numerous investigations using novel approaches targeting secretase activity are currently underway [118].

1.4.2.4. Transcriptional and chromatin modification

Micropthalmia-associated transcription factor (*MITF*) is a key regulator of melanocyte development controlling a variety of processes such as pigmentation, apoptosis and cell cycle progression. In an early study using high density SNP arrays to investigate chromosomal copy number change in the NCI60 panel of cell lines, amplifications at a locus on chromosome 3p were identified that defined the melanoma subcluster [119]. Within this region, *MITF* was the only gene that correlated highly between amplification and high transcript expression. Subsequent analysis revealed that 10-20% of melanomas exhibited amplification of *MITF* and that deregulation, in combination with *BRAF* V600E mutation, was capable of melanocyte transformation [119]. As such, somatic alteration of *MITF* by amplification was suggested to define a specific oncogenic subclass based on ‘lineage survival’ or ‘lineage addiction’.

Further analysis of *MITF* revealed that in addition to amplification, somatic mutation also occurs in ~8% of cutaneous melanomas [120]. Additionally, a gene upstream of MITF, *SOX10*, was found to have putative inactivating mutations in a small proportion of melanomas; these mutations occurred in a mutually exclusive pattern, possibly indicating functional redundancy between both events. Both of the aforementioned studies documented a distinct association between *MITF* and *BRAF* mutation and mutual exclusivity to *NRAS* mutation. *MITF* has been shown to act through the TP53 and RB1 pathways [121] and recently was characterised for direct interactions of genes involved in DNA replication, repair, and mitosis [122]. Due to the complexity of *MITF* interactions, additional studies will be required to determine if this critical gene in melanocyte biology can be targeted therapeutically.

Studies have also revealed a number of other genes implicated in melanoma development that are involved in transcriptional control and chromatin modification. WES analysis performed by Wei et al. revealed a recurrent mutation in a novel gene, *TRRAP,* with a role in transcription and DNA repair and complexes with histone acetyltransferases [80]. Mutations in *TRRAP* clustered locally, similar to *BRAF*, *PIK3CA* and *RAS*, suggesting that *TRRAP* may be a new oncogene involved in metastatic melanoma. *TRRAP* mutation occurred in ~4% of melanomas and mutant *TRRAP* was shown to be essential for cell survival and transformation [80].

In further regard to chromosomal copy number alterations, functional screening using a zebrafish melanoma model revealed *SETDB1* in a region of recurrent amplification of human chromosome 1 that can cooperate with *BRAF* (V600E) [123]. *SETDB1* is a histone methyltransferase and contributes to cellular functions involving histone methylation, gene silencing and transcriptional repression. Alternatively, homozygous deletions in a histone deacetylase, *HDAC4*, have also been documented in metastatic melanoma, although the consequences of deletion have not been determined [108]. With increasing WES reports identifying mutations in histone and chromatin modification genes in cancer [124, 125]; it will be interesting to see how this class of mutations contributes to the development of melanoma and whether they are amenable to histone deacetylase inhibition.

1.5. Genetics of non-cutaneous metastatic melanoma

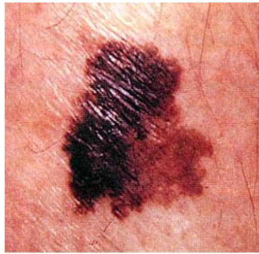
Melanomas of the skin (cutaneous melanoma) account for approximately 90% of all diagnosed melanomas; the remaining melanomas arise within the eye (uveal ~5%), or from mucosal membranes of the body (mucosal ~2%) [126]. A small proportion, although of cutaneous origin, occur in typically non-UV-exposed regions of the body such as the palms or sole of the feet (acral ~5%), and are classified as a distinct subtype of melanoma (Figure 1.3). The common feature between these rare forms of melanoma is that they have distinct genetic alterations or profiles compared to melanomas of cutaneous origin.

In a seminal study in 2005, Curtin et al. assessed chromosomal copy number changes in 126 melanomas from a variety of subtypes, including acral, mucosal, and cutaneous melanoma with or without chronic sun exposure [127]. Interestingly, each of these groups had significantly different sets of chromosomal aberrations and could accurately be categorised based on these profiles. In addition, this study revealed a lack of *BRAF*/*NRAS* mutation in non cutaneous melanomas and was associated with increased copy gains of *CDK4* and *CCND1*. This indicated that the underlying mechanisms behind the development of these tumours are fundamentally different to cutaneous melanoma. This section provides a brief summary of the genetics of non-cutaneous melanoma (Figure 1.4) and the associated approaches to therapy.

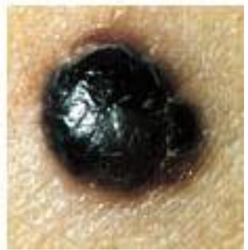
1.5.1. Uveal melanoma

Uveal melanoma arise anywhere within the uveal tract, including the choroid plexus, ciliary body or iris, and account for approximately 5% of all melanomas [126]. Melanomas of the choroid or ciliary body, classed as 'posterior uveal melanomas', represent approximately 90% of all uveal melanomas and are clinically distinct from iris melanomas. Notably, uveal tumours lack mutations in *BRAF* or *NRAS*, this is despite the presence of constitutive activity of the MAPK pathway [128]. The discovery of hypermorphic mutations in *GNAQ* and *GNA11* in dermal hyper-pigmented mice from mutagenesis screens led to sequencing of these genes in melanoma [129]. This study found somatic mutations of *GNAQ* in 83% of blue naevi and 46% of ocular melanoma of the uvea [130]. With remarkable similarity to *BRAF*, mutation of *GNAQ* nearly exclusively occurs in a single coding position (Q209) locking the GTPase in a manner that leads to constitutive activity and downstream signalling of the MAPK pathway.

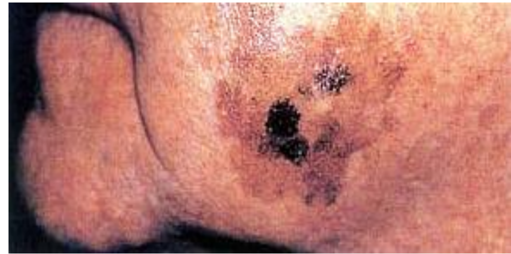
Cutaneous melanoma



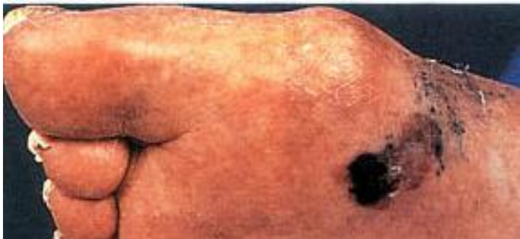
Superficial spreading melanoma (SSM)

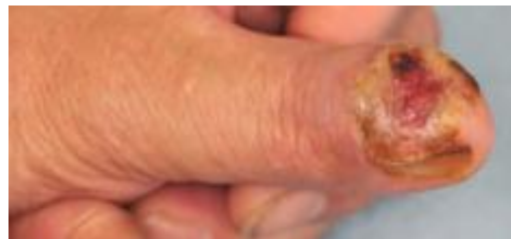


Nodular melanoma (NM)



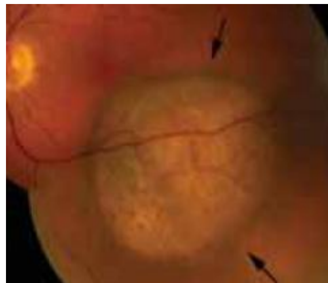
Lentigo maligna melanoma (LMM)

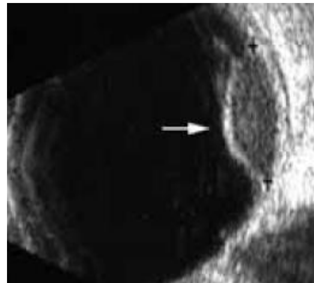




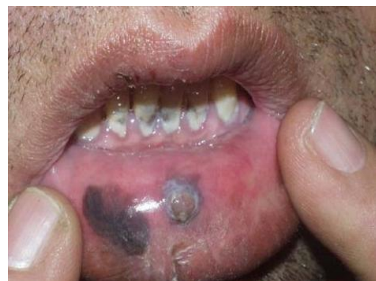
Acral lentigenous melanoma (ALM) occurring on the sole of the feet (left) and on the thumb (right)

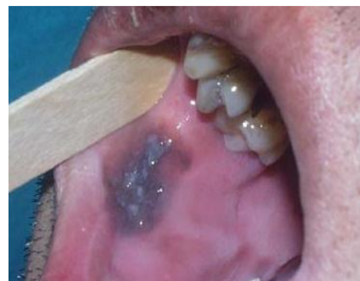
Non-cutaneous melanoma





Fundus photography (left) and ultrasonography (centre) of a choroid uveal melanoma. The picture on the right depicts an iris uveal melanoma.





Mucosal melanoma of the lower lip (left) and inner cheek (right)

Figure 1.3: The different subtypes of melanoma (primary sites shown). Approximately 90% of melanomas are of cutaneous origin; the most frequently diagnosed form being superficial spreading melanoma (SSM). Uveal melanomas (~5%) and mucosal melanomas (~2%) are less frequently diagnosed. Percentages of incidence are indicative of a Caucasian population. Figures adapted from [14, 148-150].

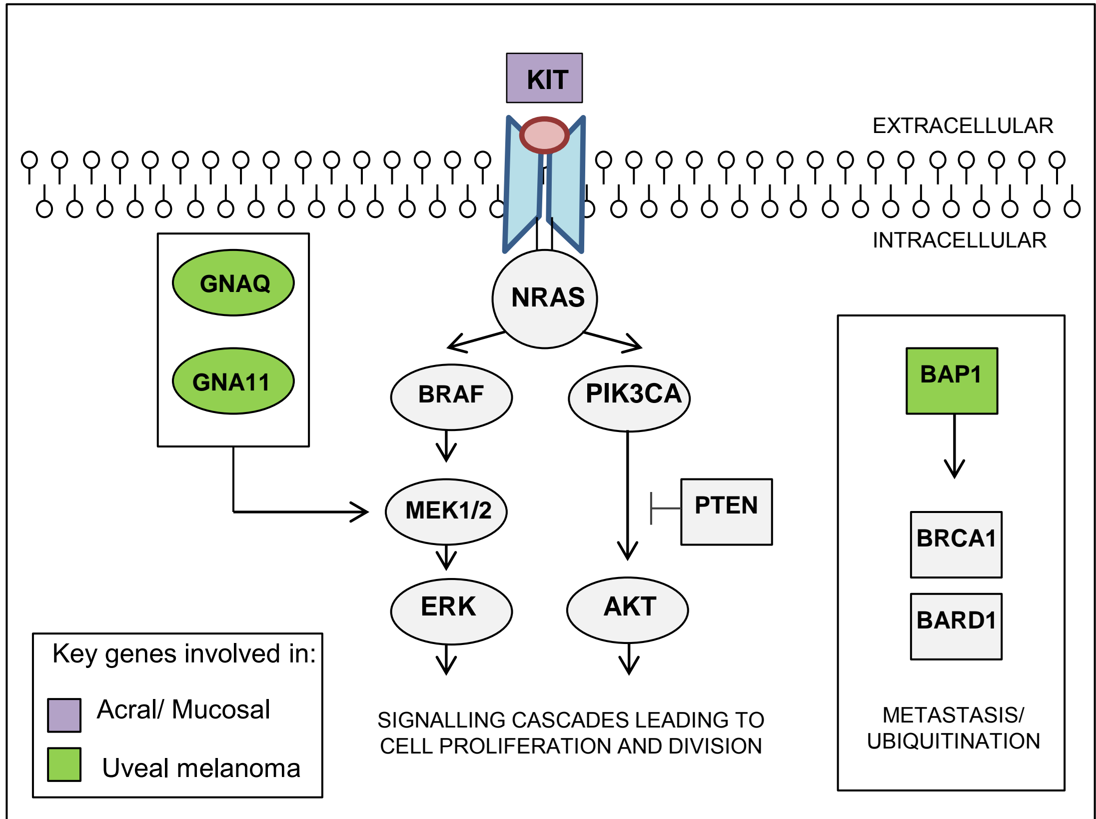


Figure 1.4: Genetics of non-cutaneous melanoma.

A subsequent study that sequenced the highly homologous gene family member *GNA11*, led to the identification of reciprocal mutations in 7% of blue naevi, 32% of primary uveal melanoma and 57% of uveal melanoma metastases [131]. Interestingly, mutation of *GNA11* occurs mutually exclusively to *GNAQ* mutations; together these mutations account for ~85% of uveal melanomas and are unique to this tumour type. As both genes lead to activation of the MAPK pathway, it was proposed that drugs targeting this pathway, such as MEK inhibitors (AZD6244), may represent an effective therapeutic avenue. Currently, clinical trials using this approach in uveal melanomas are underway, however, a recent study revealed only mild sensitivity to AZD6244 of *GNAQ* mutant uveal cell lines *in vitro* [132].

Uveal melanomas can be classed based on their metastatic capability, class 1 (low risk) and class 2 (high risk); and until recently, not much was known about the genetic basis of either class. Although mutations can occur in *GNAQ* and *GNA11*, these appear to be an early event during tumorigenesis (as demonstrated by their presence in benign lesions or blue naevi) and are not correlated with a particular class [131]. Furthermore, transcriptomic and genomic profiling of class 1 and 2 uveal melanomas revealed a striking propensity for chromosome 3 monosomy in class 2 high risk metastases [133]; this suggested that loss of an allele on this chromosome could expose an inactivating mutation on the remaining allele that promotes metastasis.

Exome sequencing of two class 2 tumours and their matched normal counterparts revealed inactivating mutations in *BAP1* (encoding BRCA1 associated protein 1) on chromosome 3p21.1 [134]. Mutation screening of *BAP1* in a larger cohort of tumours revealed mutations in 26 of 31 class 2 (84%) and only 1 of 26 (4%) class 1 uveal melanomas, revealing a strong selection for metastatic tumours [134]. BAP1, a nuclear ubiquitin carboxyterminal hydrolase, appears to have a complex role, with binding domains for the tumour suppressors BRCA1 and BARD1, and can complex with the histone-modifier HCFC1. Although targeting of this gene in a therapeutic sense is challenging, inhibition of RING1 deubiquitinating activity may be a viable approach [134].

1.5.2. Acral and mucosal melanoma

Acral melanomas are rare cutaneous melanomas that occur in non-hairy regions of the skin including the soles of the feet, palms or nail-bed. This form of melanoma is the predominant subtype in non-Caucasians and is associated with a worse prognosis than cutaneous malignant melanoma overall [135]. Due to its location on the body, acral melanoma is typically associated with a non UV exposure profile.

The first glimpse into the genetic architecture of acral melanoma was recently reported through the whole genome sequencing of a chemo-naïve primary acral melanoma and its matched lymph node metastasis [136]. Not surprisingly, the total number of nonsynonymous mutations detected in these tumours was 40, about 10 fold less than that of observed rates of sun exposed cutaneous melanomas (typical range of 200-600). This rate of mutation is also consistent with the observed rates in non-carcinogen exposed cancers such as breast [74] and prostate [79]. Interestingly, the type of mutation was largely typical of a UV based signature with 60% exhibiting C>T (G>A) transitions; however, it was suggested that this might be due to a nonsense mutation in *ERCC5*, a gene responsible for the repair of dipyrimidine lesions. Although this tumour did not have a point mutation in *KIT*, it did exhibit strong amplification of the gene.

Mucosal melanomas are even rarer (~2% of all melanomas) and occur in mucosal membranes of the body including the oral/nasal cavity, anorectal region, urinary tract or genitalia. This subtype is even less characterised than acral melanoma, however, both subtypes share a high propensity for *KIT* mutation; it was shown that 39% of mucosal and 36% of acral melanomas had a mutation in *KIT*, while none were detected in non chronic sun damaged cutaneous melanomas [43]. This is of clinical relevance due to the positive response of Imatinib in *KIT*-mutated GIST [39]. Subsequently, this has prompted similar investigations of Imatinib in *KIT* mutated melanomas including recent phase II trials demonstrating significant activity [48, 137]. Unlike *BRAF*, mutation of *KIT* can occur throughout the entire gene; interestingly, the majority of responders had mutations in exons 11 or 13, suggesting that further selection criteria for drug eligibility may be beneficial.

Trials into other KIT inhibitors are also currently in progress, including the recent completion of a Sunitinib trial of *KIT*-mutated melanoma [138]. It will be interesting to

compare the activity of these drugs, particularly in regard to their efficacy in targeting the variety of mutation events observed in *KIT*. In summary, although these tumours are rare, further genomic characterisation is warranted. This will lead to a deeper understanding of the genetic mechanisms in effect, potentially leading to new therapeutic avenues that may be relevant to other subtypes of melanoma. Access to tumour material and subsequent enrolment in clinical trials will remain a challenge for these rare subtypes of melanoma.

1.6. Rationale of this study

Metastatic melanoma has historically been difficult to treat and is refractive to traditional chemotherapeutic strategies. The approval of two new drugs in 2011, Ipilimumab and Vemurafenib, are the first drugs in 20 years that have improved rates of overall survival in patients. The success of these drugs, particularly the latter, have stemmed from earlier efforts in understanding the complex mechanisms behind the development and progression of metastatic melanoma. Thus, cataloguing the diverse range of mutations in melanoma will ultimately reveal undiscovered targets for which effective therapeutics may be designed, or alternatively, identify mutations that can dictate the efficacy of current drug strategies.

This study aims to extensively characterize a panel of stage III local lymph node metastatic melanoma cell lines using a range of high-throughput technologies, including next generation sequencing, to identify novel genes and therapeutic targets in metastatic melanoma. At the beginning of this thesis, next-generation technology had not been used to assess the melanoma genome and hence, provided the opportunity to thoroughly investigate this cancer at unprecedented depth. By layering data from multiple high-throughput technologies in a complementary manner, the limitations of each technology individually can be overcome. Using this approach, this study will identify numerous mutations that will hopefully be translated into effective therapeutic treatments for patients with metastatic melanoma (Figure 1.5).

The panel of stage III melanoma cell lines was established by the Cancer Immunotherapy Laboratory at the Queensland Institute of Medical Research from treatment naïve tumours collected prior to a clinical trial (see appendix for selection criteria). This panel is a valuable source from a research perspective as the use of cell lines allows the extensive generation of materials required for experimental work, and can also be used for downstream functional analysis.

Although cell lines can acquire genetic mutations as a result of the culturing process, the impacts of this effect have been minimized in this project. Firstly, these cell lines have been established at the earliest passage to reduce the possibility of cell culture acquired mutations. As such, stocks of DNA and RNA, on average, were collected at passage nine. Where available, remaining portions of tumours were retained, stored, and extracted for

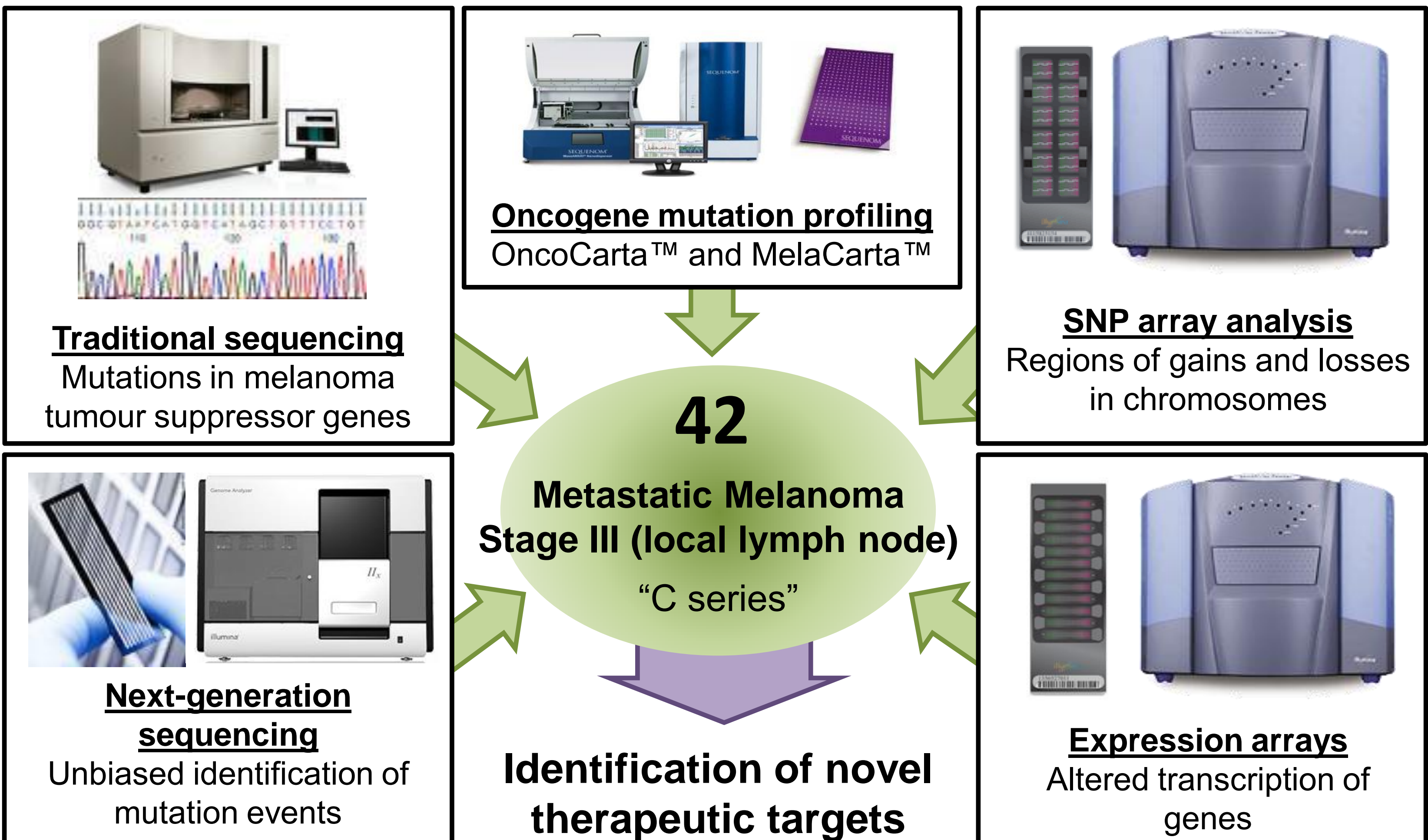


Figure 1.5: Flow diagram depicting the experimental design of this thesis. Screening a variety of high-throughput technologies across a panel of stage III melanomas is used to reveal the genetic mechanisms of tumorigenesis. The goal of this comprehensive analytical approach is to identify novel therapeutic targets in metastatic melanoma.

DNA. Thus, for any mutations identified in the melanoma cell lines, matching tumour DNA can be used to ascertain the presence of the aforementioned mutations within the original tumour to determine the possibility of cell culture acquired artifacts. Early passage lymphoblastoid cell lines (LCL) have also been established for the majority of these melanoma cell lines and represent a matched normal genomic specimen. This enables germline variants to be distinguished from somatically acquired tumour mutations by subsequent screening of cell line mutations in matching LCL.

Tumour specimens are heterogeneous in nature by the presence of contaminating stromal cells; this reduction in purity is important in the context of genetic analysis and can result in the false negative identification of mutations. In contrast, cell lines are typically more homogeneous and can overcome said contamination problems associated with tumour specimen analysis; however, in the same sense, the process of cell culturing can select for and allow outgrowth of specific subpopulations within the tumour and can lead to an under-representation of the true mutational complexity.

In regards to chromosomal copy number analysis using high density SNP arrays, comparison of primary neuroblastoma tumours and matching early-passage cell lines (~7 passages) identified a significant underestimation of LOH and copy loss events in tumours due to stromal contamination; this suggested that low passage cell lines may be superior specimens for genome-wide SNP array studies. [139] The use of next-generation sequencing to identify the overall concordance of single nucleotide mutations in matched tumour versus cell lines has not yet been published. However, unpublished data from our laboratory using whole-exome sequencing in early passage cell lines indicate a high concordance to matched tumours; this suggests that the acquisition of cell culture acquired mutations, at least at low passages, is low.

The melanoma cell lines were established from stage III local lymph node metastases prior to therapy, including chemotherapy. An increase in mutation load or genotoxicity through chemotherapeutic regimens have been observed both in normal somatic cells [140] and tumours [141]. As such, analysis of tumour specimens prior to any systemic treatment alleviates the need for discerning treatment acquired mutations to mutations acquired during tumorigenesis. Reducing the complexity of mutation analysis is particularly important for genetic research into melanoma as this cancer inherently has a high mutation rate due to UV exposure [73].

The panel of stage III metastatic cell lines represents an average clinical distribution of melanoma subtypes (Table 1). The majority of melanomas are of cutaneous origin that presented as superficial spreading or nodular subtypes. No acral, uveal or mucosal melanomas are included in this cohort. For some patients complete clinical information could not be obtained. Unlike systemic disseminated melanoma (stage IV), patients with stage III melanomas present with metastases in local lymph nodes and exhibit 5 year survival rates between 27-70%; this is primarily dependent on the number of metastatic deposits present within the lymph nodes [142]. After lymph node dissection, approximately 30% of patients will be recurrence-free; for the majority of patients however, recurrence can either occur locally at the primary site (~10%), regionally to the sentinel lymph node (12%), or distantly to other parts of the body (44%) [143]. As the majority of melanoma related mortality is accounted through disseminated disease, genetic analysis of the stage III melanomas will identify relevant mutations for patients that can be therapeutically targeted upon clinical presentation.

1.7. Specific aims of this study

Aim 1: To perform an extensive characterisation of a panel of stage III local lymph node metastatic melanoma cell lines.

- **1.1.** To establish cell line banks and extract DNA, RNA, miRNA and protein.
- **1.2.** To carry out mutational analysis of approximately 230 known oncogenic mutations.
- **1.3.** To sequence select tumour suppressor genes known to be involved in melanoma.

Aim 2: Use comparative genomic hybridisation with single nucleotide polymorphism arrays to identify novel genes involved in focal DNA copy number changes.

Aim 3: Use whole exome sequencing on metastatic melanoma cell lines to identify novel frequently mutated genes.

Aim 4: Investigate *BRAF*/*NRAS* WT melanomas using whole-exome sequencing.

Aim 5: Develop a melanoma specific mutation panel to identify clinically relevant “mutation profiles” in metastatic melanoma.

Table 1.1: Clinical Information for the Stage III local lymph node metastatic melanoma cell lines
Age is at removal of metastatic tumour form which cell line is created, N/A Not applicable or available, LCL: Lymphoblastiod Cell Line.

Sample	Age	Gender	Characteristic	Primary Tumor Site	Metastatic Tumor Site	Matched Normal	Tumour
C001	50	F	superficial spreading melanoma	R calf	R calf nodule	LCL	Y
C002	60	M	nodular	R upper leg	R thigh	LCL	Y
C004	42	F	n/a	n/a	L axilla lymph nodes	LCL	Y
C006	35	F	nodular	R lower back	R inguinal lymph nodes	LCL	Y
C011	34	F	superficial spreading melanoma	L upper leg	lung and liver	LCL	Y
C013	71	M	nodular	upper back	L and R axilla lymph nodes	LCL	Y
C017	57	F	cutaneous	R mid back	R axilla lymph nodes	LCL	Y
C021	38	M	superficial spreading melanoma	L upper back	L axilla lymph nodes	LCL	N
C022	45	F	superficial spreading melanoma	R arm	R axillary lymph nodes	Lymphocytes	N
C025	41	M	superficial spreading melanoma	L upper back	Lymph node	LCL	N
C027	61	M	nodular	L upper back	L axilla lymph nodes	LCL	Y
C037	27	F	n/a	n/a	R axilla lymph nodes	LCL	N
C038	33	F	n/a	n/a	n/a	N/A	N
C042	39	M	n/a	n/a	L Axilla	N/A	N
C044	51	F	superficial spreading melanoma	L lower back	L groin	LCL	Y
C045	22	F	superficial spreading melanoma	R shoulder	R axilla lymph nodes	LCL	N
C052	69	M	nodular	mid back	L axilla lymph nodes	LCL	N
C054	52	F	cutaneous	R lower leg	R groin	LCL	Y
C055	80	M	nodular	centre back	R axilla lymph nodes and Lgroin	LCL	Y
C057	35	F	superficial spreading melanoma	L upper back	L axilla lymph nodes	LCL	Y
C058	39	M	n/a	n/a	L axilla lymph nodes	LCL	Y
C060	64	F	superficial spreading melanoma	R upper leg	R groin	LCL	Y
C062	67	M	n/a	n/a	L and R groin	LCL	Y
C065	39	M	superficial spreading melanoma	L lower back	L axilla lymph nodes	LCL	Y
C067	69	M	superficial spreading melanoma	upper back	R axilla lymph nodes	LCL	Y
C071	33	M	superficial spreading melanoma	R upper back	R axilla lymph nodes	LCL	Y
C074	45	F	superficial spreading melanoma	mid back	axillary lymph nodes	LCL	Y
C077	48	M	nodular	mid upper back	L and R axilla lymph nodes	LCL	Y
C078	60	M	superficial spreading melanoma	L lower back	axillary lymph nodes	LCL	Y
C081	54	M	superficial spreading melanoma	R upper leg	L groin	LCL	Y
C083	33	M	superficial spreading melanoma	R neck	neck	LCL	Y
C084	75	M	superficial spreading melanoma	R upper back	R neck	LCL	Y
C086	27	F	n/a	n/a	L Neck	LCL	N
C088	55	F	superficial spreading melanoma	R upper abdomen	R axillary lymph nodes	LCL	Y
C089	50	F	n/a	n/a	L axilla lymph nodes	LCL	Y
C091	53	F	superficial spreading melanoma	L lower leg	groin	LCL	Y
C094	20	F	superficial spreading melanoma	L upper back	L axilla lymph nodes	LCL	Y
C096	45	M	superficial spreading melanoma	R lower leg	groin	LCL	Y
C097	43	F	nodular	L neck	neck	LCL	Y
C100	78	M	nodular	Mid scalp	R neck	LCL	Y
C106	52	M	superficial spreading melanoma	R upper leg	R groin	LCL	Y
C108	47	M	n/a	n/a	L Neck	N/A	N

1.8. Account of research progress linking the research papers

The results presented in this thesis highlight the progressive nature of sequencing technology and its application to cancer genomics and personalised medicine.

Firstly, independent early passage cell line stocks were generated from a bank of cell lines previously established at the Cancer Immunotherapy Laboratory at the Queensland Institute of Medical Research. Sufficient quantities of cells were then harvested for extraction of DNA, RNA, miRNA and protein, which were used for comprehensive genetic analysis. The generation of these stocks relate to Aim 1.1 of the thesis; details of the methods used are found within the materials and methods sections of Chapters 2 to 5. Results of melanoma tumour suppressor gene sequencing (Aim 1.2) and oncogenic mutation profiling (Aim 1.3) are included in Chapters 4 and 5 respectively. The results of Aim 1 collectively contribute to the entire thesis and are hence, apart from here, are not discussed independently.

Chapter 2 describes the use of high density SNP arrays to identify chromosomal copy number changes involving focal amplification and focal homozygous deletions to reveal putative oncogenes and tumour suppressor genes, respectively [144]. Candidate gene sequencing of 5 genes in a larger cohort of samples revealed a putative tumour suppressor gene, *TFG* (TRK Fused Gene), being mutated in approximately 5% of melanomas. Although the function of TFG is not properly understood, it has been shown to activate the MAPK pathway, indicating that mutation of *TFG* may have clinical relevance for drug strategies targeting this pathway.

Although SNP arrays are cost effective, have high-throughput capabilities, and have the ability to identify genes implicated in cancer (as evidenced by the results of Chapter 2), the rapidly decreasing costs and unbiased analysis provided by next-generation sequencing have placed this technology competitively at the forefront of modern cancer research. As such, Chapter 3 describes the use of whole-exome sequencing (WES) in metastatic melanoma cell lines to identify novel genes involved in the aetiology of this disease [145]. The results of this study revealed mutually exclusive mutations of *MAP3K5* and *MAP3K9* in 25% of melanomas, some of which may contribute to chemo-resistance in patients. Furthermore, this finding identifies a new pathway involving apoptosis that may be critical to the development and progression of metastatic melanoma.

Next-generation sequencing has allowed the rapid identification of novel genes involved in cancer; however, it is important to place these findings in context of the molecular pathways they control. One pathway frequently deregulated in melanomas is the MAPK pathway; a pathway activated by mutation of *BRAF* and *NRAS* in 50% and 20% of melanomas, respectively. However, not much is known about the remaining 30% of melanomas that are *BRAF*/*NRAS* wild type (WT). To address this issue, Chapter 4 describes the use of multiple genomic datasets, including WES, to determine if *BRAF*/*NRAS* WT melanomas are distinct molecular entities that may be susceptible to novel drug strategies. In summary, three of the four cell lines screened with WES had nonsense mutations in *NF1*, a negative regulator of *NRAS*, and potentially contributes to the activation of MAPK pathway in these cell lines. In addition, there is evidence to suggest that *BRAF*/*NRAS* WT melanomas with *NF1* mutation may be amenable to mTOR inhibition by rapamycin thus identifying a novel therapeutic strategy for this subset of melanomas.

Lastly, the identification of mutations occurring in melanoma has led to the successful development of molecular based targeted drugs, including the BRAF inhibitor Vemurafenib. However, positive clinical activity of these drugs relies strongly on the presence of mutations or 'mutation profile' of the patient's tumour. In Chapter 5, the development of a high-throughput mutation screening panel for the identification of clinically relevant mutation profiles in melanoma is described [146]. The final panel, consisting of 39 mutations in 20 genes, identified a total of 252 mutations in 271 melanomas including mutations in *BRAF* (n=154, 57%), *NRAS* (n=55, 20%), *CDK4* (n=8, 3%), *PTK2B* (n=7, 2.5%) and *ERBB4* (n=5, 2%). This panel, when used in a clinical research setting, has the potential to rapidly and identify effective treatment strategies using novel or existing molecularly targeted drugs.

The final chapters of this thesis discuss the abovementioned findings in regards to their application and relevance to current therapeutic approaches. Chapter 6 (Discussion) describes the limitations of next-generation sequencing technology in the detection of somatic mutations in melanoma through a perspectives article written for *Pigment Cell and Melanoma Research* [147]. Lastly, Chapter 7 (Conclusion) discusses the role of next-generation sequencing in personalised medicine and how this technology is progressing into a clinical setting; this is explained in relation to the results presented in this thesis.

These results have led future investigations and experiments which are detailed in the last segment of the conclusion.

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Chapter 2

Identification of *TFG* (Trk-Fused Gene) as a putative metastatic melanoma tumour suppressor gene

Ken Dutton-Regester, Lauren G. Aoude, Derek J. Nancarrow, Mitchell S. Stark, Linda O'Connor, Cathy Lanagan, Gulietta M. Pupo, Varsha Tembe, Candace C. Carter, Michael O'Rourke, Richard A. Scolyer, Graham J. Mann, Christopher W. Schmidt, Adrian Herington and Nicholas K. Hayward. Identification of TFG (TRK-Fused Gene) as a Putative Metastatic Melanoma Tumour Suppressor Gene. Genes, Chromosomes and Cancer. 2012. 51(5):452-61.

2.1. Relevance to thesis aims

This chapter addresses Aim 2 of the thesis by identifying novel genes involved in the development of metastatic melanoma using SNP arrays. SNP arrays have previously been utilised to identify changes in chromosome copy gain (amplifications) or loss (homozygous deletions) in metastatic melanoma and these events can be responsible for tumorigenesis. Identifying causal genes within large regions of copy number changes can be difficult to determine, however, regions of focal amplifications or homozygous deletions involving one, or a small number of genes, have the potential to identify putative oncogenes and tumour suppressor genes respectively.

In this report, SNP arrays were used on a panel of 39 stage III metastatic melanoma cell lines to identify unbalanced chromosomal events. This revealed a number of genes previously implicated in the development of melanoma including *CDKN2A*, *CDKN2B*, *PTEN*, *PTRPD* and *TP53* in regions of homozygous deletions and *CCND1*, *MITF*, *MDM2* and *NRAS* in regions of amplification. In order to identify novel genes that may contribute to the pathogenesis of melanoma, a filtering strategy was used to identify genes involved in focal amplification and homozygous deletions. A total of 5 genes within focal homozygous deletions were selected for further screening using traditional Sanger sequencing. One of these genes, *TFG* (Trk-Fused Gene), was shown to be mutated in

approximately 5% of samples and may have clinical relevance to current therapeutic strategies for treating metastatic melanoma.

This study demonstrates the application of SNP arrays for identifying novel genes involved in melanoma tumorigenesis. Although significant decreases in costs associated with next generation sequencing will inevitably see this use of SNP arrays obsolete, the data produced from these arrays will remain valuable as companion data for next generation sequencing analysis. As such, the data produced from this study also contributes to Chapter 5; characterising the events involved in frequently mutated pathways in a panel of stage III metastatic melanomas.

2.2. Contribution of candidate

Initial project design and planning was conducted in association with supervisors. The harvesting of DNA from the C series of melanoma cell lines used in this study was performed with the help of Lauren Aoude. I generated and analysed the SNP array data, sequenced candidate genes and prepared the manuscript.

2.3. Acknowledgment of the contribution of others

The panel of C series stage III metastatic melanoma samples were collected by Michael O'Rourke and cell lines from these samples were established by Cathy Lanagan, Linda O'Connor and Christopher W. Schmidt. Lauren Aoude contributed to the generation of cell line stocks and the subsequent extraction of DNA. SNP array data generation and analysis was compiled under the guidance of Mitchell S. Stark and Derek J. Nancarrow, respectively. An independent cohort of melanoma tumour and cell line DNA was prepared by members of the Melanoma Institute of Australia that included Gulietta M. Pupo, Varsha Tembe, Candace D. Carter, Richard A. Scolyer and Graham Mann. All authors reviewed and supplied comments on the final manuscript prior to publication.

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Chapter 3

Frequent somatic mutations in *MAP3K5* and *MAP3K9* in metastatic melanoma identified by exome sequencing

Mitchell S. Stark*, Susan L. Woods*, Michael G. Gartside*, Vanessa F. Bonazzi*, **Ken Dutton-Regester***, Lauren G. Aoude, Donald Chow, Chris Sereduk, Natalie M. Niemi, Nanyun Tang, Jonathon J. Ellis, Jeffrey Reid, Victoria Zismann, Sonika Tyagi, Donna Muzny, Irene Newsham, YuanQing Wu, Jane M. Palmer, Thomas Pollak, David Youngkin, Bradford R. Brooks, Catherine Lanagan, Christopher W. Schmidt, Bostjan Kobe, Jeffrey P. MacKeigan, Hongwei Yin, Kevin M. Brown, Richard Gibbs, Jeffrey Trent and Nicholas K. Hayward. Frequent somatic mutations in MAP3K5 and MAP3K9 in metastatic melanoma identified by exome sequencing. Nature Genetics, 2012. 44(2): p165-169.
* These authors contributed equally to this work.

3.1. Relevance to thesis aims

The results of this manuscript contribute to Aim 3 of the thesis and describe the use of whole-exome sequencing (WES) in 8 metastatic melanoma cell lines. Advances in next generation sequencing technology have recently enabled unbiased, cost effective and comprehensive mutation analysis. Use of WES in matched tumour and normal DNA has the potential to identify the entire catalogue of mutations occurring within the protein coding region of the human genome. This is of relevance to this thesis as nonsynonymous mutations, those mutations that result in a change to protein sequence, may have significance to, or may be amenable to therapeutic intervention. Furthermore, overlaying WES data from multiple melanomas has the ability to identify genes that are frequently mutated and are thus more likely to be ‘drivers’ of tumorigenesis.

WES was used to sequence 8 matched metastatic melanoma cell lines and lymphoblastoid cell lines to identify somatically mutated genes. A large number of mutations were identified within each melanoma, highlighting the difficulty of identifying putative mutations driving tumour growth compared to benign passenger mutations. Two mutated genes, *MAP3K5* and *MAP3K9*, were selected for further sequencing in a larger series of samples due to their putative significance to current therapeutics. This identified

mutually exclusive mutations in *MAP3K5* and *MAP3K9* in approximately 9% and 15% of samples respectively. Functional analysis of select mutations in *MAP3K5* and *MAP3K9* suggested these mutations were inactivating, resulting in a reduction of kinase activity and downstream signalling of apoptotic pathways. Lastly, the use of siRNA to knockdown MAP3K9 activity led to an increase in cell viability after temozolomide treatment, suggesting that decreased MAP3K9 activity can lead to chemo-resistance in melanoma.

Prior to this publication, there was a single report of whole-genome sequencing on a commercially available melanoma cell line and a single report of WES in 12 metastatic melanoma cell lines. The current report confirmed the high rate of mutation in melanoma as compared to other cancer types, a phenomenon most likely explained by high UV radiation exposure from the sun. This was the first report to identify frequent mutations in the MAP3K family and subsequent reduction of downstream signalling of apoptotic pathways in melanoma. In summary, this report highlights the power of next generation sequencing technology in identifying novel genes responsible for the development and progression of melanoma in a cost effective and high-throughput manner.

3.2. Contribution of candidate

During the second year of my PhD, I visited the Human Genome Sequencing Centre at Baylor College of Medicine in Houston, Texas to undertake next generation sequencing using the Applied Biosystems SOLiD 3 system. A total of 5 matched metastatic melanoma cell lines and lymphoblastoid cell lines were sequenced and analysed, 4 of which contributed to the 8 samples screened by WES in this publication. The other samples in this study were sequenced on the Illumina GA2 platform at the Queensland Institute of Medical Research by Mitchell S. Stark and Michael G. Gartside. I performed confirmation sequencing and follow-up genomic sequencing of *MAP3K5* in a panel of 85 metastatic melanoma cell lines, contributed to the design of Figure 1, and helped devise the potential significance of *MAP3K5* and *MAP3K9* mutation in regards to melanoma development. This project was a large collaborative effort that contained functional analysis that was outside the scope of my thesis. This was performed by colleagues and collaborators described below.

3.3. Acknowledgment of the contribution of others

Experimental study design was devised by Nicholas K. Hayward, Kevin M. Brown, Richard Gibbs, and Jeffrey Trent. Mitchell S. Stark and Michael G. Gartside performed WES using the Illumina GA2 platform. Sonika Tyagi established the bioinformatics pipeline for analysing the WES data obtained using the Illumina GA2 platform. Jeffrey Reid, Donna Muzny, Irene Newsham and YuanQing Wu contributed to the WES data generation and analysis of samples sequenced using the SOLiD 3 platform. Susan L. Woods, Michael G. Gartside, Vanessa F. Bonazzi, Bradford R. Brooks, Donald Chow, Natalie N. Niemi, Jeffrey P. MacKeigan, Thomas Pollak and Hongwei Yin produced and analysed the functional data. Jonathon J. Ellis and Bostjan Kobe performed protein modelling. Donald Chow, Chris Sereduk, Nanyun Tang and Hongwei Yin performed the TMZ and siRNA sensitization studies and RT-PCR. Confirmation sequencing was performed with the help of Mitchell S. Stark, Michael G. Gartside, Lauren G. Aoude, Victoria Zismann and David Youngkin. Christopher W. Schmidt and Catherine Lanagan established the melanoma cell line panel and provided fresh melanoma tumours. Jane M. Palmer extracted and collated clinical records for the melanoma patients. Mitchell S. Stark, Susan L. Woods, Michael G. Gartside, Vanessa F. Bonazzi, Jeffrey P. MacKeigan, Hongwei Yin, Nicholas K. Hayward and I wrote the manuscript. All authors read and approved the final manuscript.

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Chapter 4

Exome sequencing of wild-type *BRAF* and *NRAS* metastatic melanomas reveals novel mutations in known pathways of melanoma development

Ken Dutton-Regester, Lauren G. Aoude, Mitchell S. Stark, Derek J. Nancarrow, Glen M. Boyle, Michael G. Gartside, Vanessa F. Bonazzi, Susan L. Woods, Cathy Lanagan, Linda O'Connor, Michael O'Rourke, Christopher W. Schmidt and Nicholas K. Hayward. Exome sequencing of wild-type BRAF and NRAS metastatic melanomas reveals novel mutations in known pathways of melanoma development- manuscript in preparation.

4.1. Relevance to thesis aims

This manuscript relates to Aim 5 and combines data that has been generated from the entire thesis, including Aim 1. Cancer, including melanoma, can generally be considered a genetic disease; that is a disease caused by the accumulation of mutation events over time. It is frequently observed that these mutations occur in particular molecular pathways, some of which are more frequently mutated in certain cancer types compared to others. One pathway that is frequently mutated in melanoma is the MAPK pathway, which includes *NRAS* and *BRAF*, genes that are mutated in approximately 20% and 50% of melanomas respectively. The remaining 20-30% of melanomas that are *BRAF*/*NRAS* wild type (WT) has not been extensively characterised. As such, it is currently unknown if these melanomas represent distinct molecular subgroups, have mutations in novel genes that activate the MAPK pathway or are susceptible to unique treatment strategies.

Comprehensive genetic analysis including candidate gene sequencing, oncogene mutation profiling, SNP array and expression array analysis were combined to assess the overall pathways affected in a panel of 42 local lymph node metastatic melanoma cell lines. A small subset of cell lines did not exhibit mutations in either *BRAF* or *NRAS*. Unsupervised and supervised clustering of these *BRAF*/*NRAS* WT cell lines did not indicate that these cell lines were unique molecular entities and were likely to have constitutive MAPK activity. To investigate the possibility of mutations occurring in novel genes that could activate the MAPK pathway, whole exome sequencing (WES) was

performed on 4 *BRAF*/*NRAS* WT cell lines (C022, C067, C077 and C084). This revealed a number of interesting mutation events, including 3 of 4 cell lines with nonsense mutations in *NF1*, a negative regulator of the MAPK pathway. *NF1* has previously been shown to be susceptible to mTOR inhibition in leukaemia, possibly providing a novel therapeutic strategy for *BRAF*/*NRAS* WT cell lines with mutations in *NF1*.

The results from this study highlight the benefits of overlaying multiple sets of data to identify genes involved in the development and progression of melanoma. In addition, this study demonstrates that key pathways are frequently mutated in melanoma and that mutations can occur in novel genes upstream or downstream of genes involved in these pathways. Lastly, next generation sequencing can comprehensively identify mutations occurring in tumours and will be a useful tool within the clinic in regards to personalised therapy in the future.

4.2. Contribution of candidate

Initial project design and planning was conducted with supervisors. The harvesting of DNA and RNA from the C series of metastatic melanoma cell lines used in this study was performed with the help of Lauren G. Aoude. I generated and analysed data produced from the SNP and expression arrays with the help of Mitchell S. Stark, Derek J. Nancarrow and Glen M. Boyle. I generated and analysed data from candidate gene sequencing and oncogenic profiling using OncoCarta and MelaCarta mutation panels. WES was performed by Macrogen (Korea); however I performed downstream bioinformatic analysis that also included Ingenuity pathway analysis. I compiled and wrote the manuscript.

4.3. Acknowledgment of contribution of others

The panel of C series stage III metastatic melanoma samples were collected by Michael O'Rourke and cell lines from these samples were established by Cathy Lanagan, Linda O'Connor and Christopher W. Schmidt. Lauren G. Aoude contributed to the generation of cell line stocks and the subsequent extraction of DNA/RNA. SNP array data generation and analysis was compiled under the guidance of Mitchell S. Stark and Derek J. Nancarrow, respectively. Glen M. Boyle assisted with the clustering analysis of the expression array results. Mitchell S. Stark, Michael G. Gartside, Lauren G. Aoude, Vanessa F. Bonazzi and Susan L. Woods contributed to the generation of WES

BRAF/NRAS mutant data mentioned within this report and will assist with validation and functional analysis that will be required for publication. All authors reviewed and supplied comments on the final manuscript.

Exome sequencing of wild-type *BRAF* and *NRAS* metastatic melanomas reveals novel mutations in known pathways of melanoma development

Ken Dutton-Regester, Lauren G. Aoude, Mitchell S. Stark, Derek J. Nancarrow, Glen M. Boyle, Michael G. Gartside, Vanessa F. Bonazzi, Susan L. Woods, Cathy Lanagan, Linda O'Connor, Michael O'Rourke, Christopher W. Schmidt and Nicholas K. Hayward.

ABSTRACT

Recent success with BRAF inhibitors in patients with BRAF V600E mutant metastatic melanoma has shifted extensive research efforts into this molecular subtype of the disease. However, patients with *BRAF*/*NRAS* wild type (WT) tumors, accounting for approximately 30% of patients, have not been extensively characterised. To further investigate the genetic mechanisms behind this subtype of melanoma, comprehensive mutation analysis of 42 stage III metastatic melanoma cell lines was performed using a variety of high-throughput methodologies and subsequent pathway analysis. There was no association between *BRAF*/*NRAS* mutant cell lines and *BRAF*/*NRAS* WT cell lines based on unsupervised and supervised clustering of expression array data. To determine if mutations occurring in novel genes could alternatively explain the activation of the MAPK pathway, Whole-Exome Sequencing (WES) was performed on four *BRAF*/*NRAS* WT cell lines. Putatively inactivating somatic mutations were identified in multiple samples in *NF1*, *PLCE1* and *SGK1* and were mutually exclusive to *BRAF*/*NRAS* mutations. Interestingly, previous experiments have demonstrated these genes are potent negative regulators of the MAPK pathway; hence, mutational inactivation of this class of protein may represent a novel mechanism of MAPK pathway activation in *BRAF*/*NRAS* WT tumors. Lastly, WES of *BRAF*/*NRAS* WT cell lines identified a number of clinically actionable mutations, lending support to the application of next generation sequencing in the choice of treatment for cancer patients.

INTRODUCTION

Patients with late stage or disseminated melanoma, a cancer historically refractory to chemotherapeutic treatment, exhibit high rates of mortality, with a median life expectancy of 6 months. Recently, the approval of two new drugs, Ipilimumab and Vemurafenib

(PLX4032), has improved rates of progression-free and overall survival in patients with metastatic melanoma [1-3].

Ipilimumab, an anti-CTLA4 antibody, is designed to promote durable immune responses in patients by blocking the immune suppressing activity of CTLA4 on the surface of cytotoxic T cells [4]. Although Ipilimumab has demonstrated complete responses in patients and improved overall rates of survival, this drug can promote severe grade III and IV side effects leading to premature termination of therapy, and on rare occasions, treatment-related mortalities [2]. As such, a number of challenges regarding the clinical management of Ipilimumab remain, and hopefully, with the identification of positive biomarkers of drug response, improvements in the clinical utility of this drug will occur.

The other recently approved drug is Vemurafenib, a molecular based targeted drug that counteracts the effect of constitutive kinase signalling produced by mutations at valine coding position 600 in the gene *BRAF*. Mutations of *BRAF* occur in 40-70% of cutaneous melanomas [5], the majority accounting for V600E alterations, which are the target of Vemurafenib [6]. Although Vemurafenib can rapidly reduce tumour mass and progression-free survival, its long-term ability to significantly impact on overall survival has been limited by a multitude of tumour acquired resistance mechanisms [7-12]. Significant research efforts are currently underway in an attempt to reverse this trend, from investigations of the primary resistance mechanism to combination molecular drug therapies targeting the BRAF/MEK/ERK mitogen-activated kinase (MAPK) pathway.

The MAPK pathway is one of the most frequently activated and investigated pathways in the development and progression of melanoma. Aside from approximately 50% of cutaneous melanomas exhibiting somatic mutations in *BRAF*, 20% have mutually exclusive mutations in *NRAS*, a gene upstream of *BRAF* mutation of which can also result in activation of the MAPK pathway [13]. Although *BRAF* and *NRAS* mutations account for up to 70% of melanomas, activation of the MAPK pathway and subsequent ERK phosphorylation is inherent to almost all melanoma tumors [14]. Currently, the remaining 30% of melanomas that are *BRAF*/*NRAS* wild type (WT) have not yet been extensively characterised. This is of interest, as *BRAF*/*NRAS* WT melanomas may represent a unique molecular entity to *BRAF*/*NRAS* mutant melanomas, have mutations in novel genes that activate the MAPK pathway, or be susceptible to different therapeutic strategies.

Whole-Genome Sequencing (WGS) and Whole-Exome Sequencing (WES) strategies have begun to shed light on the complexity of the 'mutational landscape' of cancer, including metastatic melanoma [15-18]. In particular, WES studies have led to the identification of a number of frequently mutated genes involved in melanomagenesis including *GRIN2A* [15], *MAP3K5*/*MAP3K9* [16] and *MAP2K1*/*MAP2K2* [17]. Notably, due to the high percentage of melanomas with *BRAF*/*NRAS* mutations and low number of samples sequenced, *BRAF*/*NRAS* WT melanomas have been under-represented in these WES studies. As such, important questions remain about the 'mutational landscape' of *BRAF*/*NRAS* WT melanomas and warrant unbiased mutation analysis provided by next generation sequencing technology.

In order to address these disparities, comprehensive genetic analysis was performed on a panel of 42 local lymph node metastatic melanoma cell lines to identify a small subset of *BRAF*/*NRAS* WT melanomas. This subgroup of melanomas were then subjected to a variety of analytical methods including WES to determine if mutations in novel genes could be responsible for activation of the MAPK pathway and to potentially identify novel therapeutic targets in these melanomas.

MATERIALS AND METHODS

Cell line and patient tumors

A panel of 42 stage III local lymph node metastatic melanoma cell lines were established with informed consent at the Queensland Institute of Medical Research as previously described [19]. Clinical information of these cell lines is included in Supplementary Table 1. All cell lines with matching Lymphoblastoid Cell Lines (LCL) and available tumour sample were confirmed for authenticity using STR profiling analysis using an AmpFISTR Profiler Plus PCR amplification kit (Life Technologies) and analysed on a 3100 Genetic Analyzer (Life Technologies). Cell lines were cultured in filter-sterilized RPMI1640 supplemented with 10% heat-inactivated foetal bovine serum (55°C for 30 minutes), 100 U ml^{-1} penicillin and 100 µg ml^{-1} streptomycin at 37°C (5% CO^2). Cells were cultured at the lowest number of passages in order to obtain sufficient material for analysis. Samples were collected at an average of nine passages, at which point, cells were trypsinised and cell pellets washed in PBS before DNA or RNA extraction.

DNA/ RNA extraction and cDNA synthesis

Cell line DNA was extracted using QIAamp Blood Maxi Kits (QIAGEN) according to the manufacturer's instructions. For tumor DNA, 20-30 mg of fresh frozen tumor was extracted with QIAamp DNA mini kits (QIAGEN) on-column RNAse digestion. Tissue was pulverised in liquid nitrogen then incubated with ATL buffer (QIAGEN) and Proteinase K for 96 hoursr at 56°C. RNA from cell lines was extracted using RNeasy Mini Plus Kits (QIAGEN) from lysates isolated directly from culture flasks. Complementary DNA (cDNA) for sequencing was synthesized from 5 µg of RNA, random hexamers and superscript reverse transcriptase III (Invitrogen).

Mutation analysis of genes previously implicated in melanoma

Genes previously implicated in the development of melanoma were sequenced using cDNA via traditional capillary electrophoresis. Primer sequences and PCR conditions for *TP53* [20], *PTEN* [21], *RAC1* [22], *MAP3K5* and *MAP3K9* [16] were as previously described. Putative tumor suppressor genes involved in metastatic melanoma identified through candidate gene sequencing, *TFG*, *ACTA2* and *BMPR1A*, were sequenced as detailed [19]. Mutations detected through capillary sequencing were confirmed as somatic using matched LCL and tumor samples, if available, to rule out mutations acquired during the process of cell culturing. Genes mutated in an oncogenic fashion (*BRAF*, *NRAS*, *CDK4*, *ERBB4*, *PIK3CA*, *PTK2B*, *NEK10* and *MET*) were identified using MelaCarta v1.0 (Sequenom, San Diego) and OncoCarta® v1.0 mutation panels on the Sequenom MassArray® platform [19].

SNP array analysis

39 of 42 stage III melanoma cell lines from the C series were analysed for chromosomal copy number gains and losses using Illumina HumanCNV370-Quad v3.0 Sentrix Bead arrays as described previously [23]. Data was analysed using Genome Studio V2009.1 (Illumina) and an R script based on SiDCoN methodology [24]. Strong imbalanced amplification events representing at least 4 or more gains in autosomal chromosome were determined with a Log *R* ratio above 0.4 and a *B*-allele score above 0.58 (Supplementary Table 2). Homozygous deletions were identified using Log *R* ratios below -3 (Supplementary Table 3).

Expression array analysis

Complementary RNA (cRNA) was generated using Illumina TotalPrep RNA Amplification Kits (Ambion) and hybridised for 16 hours onto Illumina HumanHT-12 v3 Sentrix BeadChip arrays. Data was analysed and extracted using Genome Studio V2009.1 (Illumina) and imported into GeneSpring v11.5.1 (Agilent Technologies Inc.). Hierarchical clustering analysis (supervised and unsupervised) was performed using probes filtered by expression of genes using $p \leq 0.05$ in 4 of 42 samples that varied with a standard deviation > 2 using a Pearson Centered Complete linkage algorithm. Supervised clustering was performed on *BRAF* or *NRAS* mutation status using a Mann-Whitney test to see where *BRAF*/*NRAS* WT cell lines were distributed.

Whole-Exome Sequencing (WES)

Cell lines selected for WES were sequenced and analysed by Macrogen (Korea) using Illumina TruSeq sample prep and sequencing chemistry according to Illumina protocols. Briefly, 1 µg of gDNA was fragmented by nebulisation, end repaired and 'A' tail ligated at the 3' end of fragments. Illumina adaptors were then ligated and fragments size selected for 350-400 bp products before amplification and validation using the Agilent Bioanalyzer. DNA libraries were then hybridized to streptavidin exome capture beads, washed, hybridized a second time, eluted and amplified. Enriched libraries were then sequenced-by-synthesis using an Illumina HiSeq 2000 according to Illumina Protocols. Sequencing data was extracted using CASAVA v1.7, aligned to the UCSC HG19 reference genome using BWA and variants detected using the SAMTOOLS suite of bioinformatic tools. A list of novel somatic mutations was generated through removal of variants within dbSNP131, dbSNP132 and HG1000 databases and overlapping variants within matched normal exome data. This list was then filtered using highly stringent criteria described previously [16] to reduce the number of potential false positives within the datasets. Single nucleotide mutations with a quality score above 100, had ≤ 10 reads of which ≤ 20% of reads were of the mutant allele, were deemed as robust mutations. Insertion and deletion variants required quality scores ≤ 300 due to increased rates of false positives inherent to this type of analysis.

Pathway Analysis

Genes mutated in 3 or more samples that were assessed by WES were processed using Ingenuity software to identify genes involved in the MAPK pathway. Gene lists were

analysed for experimentally observed, direct and indirect interactions up to a distance of 2 events to *NRAS*, *BRAF*, *MEK* and *ERK*.

RESULTS AND DISCUSSION

Pathway analysis identifies a subset of cell lines lacking mutations in MAPK pathway

Comprehensive genetic analysis was performed on a panel of 42 local lymph node metastatic melanoma cell lines that included candidate gene sequencing, oncogene profiling, SNP array and expression array analysis. Mutations identified in these cell lines were grouped into biological relevant pathways, with a main focus on the four classical pathways of melanoma development (MAPK, PI3K, TP53 and RB1 pathways). Mutations occurred in *NRAS* and *BRAF* in 21% (9 of 42) and 57% (24 of 42) of cell lines, respectively; this resulted in approximately 80% (33 of 42) of cell lines exhibiting mutations within the MAPK pathway. There was no association between mutual exclusivity of mutations in the TP53 or RB pathways to *BRAF* or *NRAS* status. There was a small subgroup of samples that did not exhibit mutations within the MAPK pathway and were *BRAF*/*NRAS* WT (9 of 42 or ~20%). A summary of the pathways mutated in each cell line of the C series from the 4 main pathways to melanoma development can be seen in Table 1. This data is expanded in Supplementary Table 4 that includes mutations in genes known to directly cause deregulation of pathways as well as genes that hypothetically may deregulate pathways.

BRAF/NRAS WT cell lines do not cluster separately to *BRAF/NRAS* mutant cell lines according to expression array analysis

Currently, *BRAF*/*NRAS* WT melanomas have not been extensively characterised and as such, may be unique molecular entities, have mutations in novel genes that activate the MAPK pathway, or be susceptible to different therapeutic strategies. To determine if *BRAF*/*NRAS* WT melanomas belong to a unique molecular subtype of melanoma, unsupervised and supervised clustering was performed on the C series using expression array data. Unsupervised clustering by probes and samples did not show any significant segregation based on *BRAF*/*NRAS* status or pathway specific mutation status (Figure 1). Supervised clustering based on *NRAS* and *BRAF* mutation status by probes and samples resulted in clear segregation of 2 groups indicative of their respective mutation status, as would be expected (Figure 2). Interestingly, most *BRAF*/*NRAS* WT cell lines clustered

	MAPK Pathway	PI3K Pathway	TP53 Pathway	RB Pathway
Sample	Mutated	Mutated	Mutated	Mutated
C062	*BRAF*	*PTEN*	*P14ARF*	*P16INK4A*
C002	*NRAS*	*NRAS*	*P14ARF*	*P16INK4A*
C097	*BRAF*	*PTEN*	*P14ARF*	*P16INK4A*
C071	*BRAF*	*PTEN*	*P14ARF*	*P16INK4A*
C042	*BRAF*	*PTEN*	*P14ARF*	*P16INK4A*
C055	*BRAF*	*PTEN*	*P14ARF*	*P16INK4A*
C001	*NRAS*	*NRAS*	*TP53*	
C100	*BRAF*	*PIK3CA*	*TP53*	
C083	*NRAS*	*NRAS*	*TP53*	
C044	*BRAF*	*PTEN*	*TP53*	
C038	*BRAF*	*PTEN*		*P16INK4A*
C096	*NRAS*	*NRAS*		*P16INK4A*
C091	*BRAF*	*PTEN*		*P16INK4A*
C054	*NRAS*	*NRAS*		*P16INK4A*
C057	*BRAF*	*PTEN*		*CDK4*
C106	*NRAS*	*NRAS*		
C006	*NRAS*	*NRAS*		
C013	*NRAS*	*NRAS*		
C027	*NRAS*	*NRAS*		
C089	*BRAF*		*P14ARF*	*P16INK4A*
C058	*BRAF*		*P14ARF*	*P16INK4A*
C081	*BRAF*		*P14ARF*	*P16INK4A*
C045	*BRAF*		*TP53*	
C060	*BRAF*		*TP53*	
C074	*BRAF*		*TP53*	
C065	*BRAF*		*MDM2*	
C004	*BRAF*			
C017	*BRAF*			
C088	*BRAF*			
C078	*BRAF*			
C094	*BRAF*			
C108	*BRAF*			
C011	*BRAF*			
C077*	[*BRAF*]	*ERBB4*	*TP53*	
C021			*TP53*	
C084*			*TP53*	
C086			*TP53*	
C052				*CCND1*
C067*				[*RB1*]
C022*				
C025				
C037				

Table 1: Summary of pathways mutated in C series metastatic melanoma cell lines.
Cell lines were analysed for mutations in key genes from 4 frequently deregulated pathways of melanoma development. 8 of 42 samples did not have mutations in the MAPK and PI3K pathway. * Samples that were selected for Whole-Exome Sequencing (WES). Genes in parenthesis indicate mutations detected after WES.

Figure 1: **Unsupervised clustering of the C series of metastatic melanoma cell lines shows no significant sub-grouping associated with mutation status.**

Data generated from Illumina HT12 expression arrays and clustered by probes and samples using GeneSpring X. Cell line name with mutation status is listed below the cluster plot based on *BRAF* (green), *NRAS* (red) or *BRAF*/*NRAS* WT (blue). Four pathways that are frequently deregulated in melanoma are also listed; solid blue squares indicate mutation of the pathway in the associated sample. Metastatic melanoma cell lines did not segregate based on mutation or pathway analysis.

Figure 2: **Supervised clustering based on BRAF and NRAS mutation status.** Data generated from Illumina HT12 expression arrays and clustered by probes and samples using GeneSpring X. Cell line name with mutation status is listed below the cluster plot based on *BRAF* (green), *NRAS* (red) or *BRAF*/*NRAS* WT (blue). Four pathways that are frequently deregulated in melanoma are also listed; solid blue squares indicate mutation of the pathway in the associated sample. Supervised clustering based on *NRAS* or *BRAF* mutation status segregating into 2 distinct entities. Interestingly, *BRAF*/*NRAS* WT cell lines segregated in between *BRAF* and *NRAS* groups but closer to the latter. These results indicated that *BRAF*/*NRAS* WT melanomas are likely to have MAPK pathway activation and this may be due to mutations in novel genes that interact with this paper.

predominately towards the *NRAS* mutant subgroup but adjacent to the *BRAF* mutant subgroup. These results appear to indicate that *BRAF*/*NRAS* WT cell lines are not unique molecular entities and are likely to have activation of the MAPK and PI3K pathways. This result is not surprising as it has been shown that the majority of melanomas exhibit constitutive MAPK activity, including melanomas that are *BRAF*/*NRAS* WT [14]. Based on these findings, *BRAF*/*NRAS* WT melanoma cell lines were analysed using WES to determine if these samples harboured mutations in novel genes interacting with the MAPK pathway. A strategy of the filtering process is demonstrated in Figure 3.

Whole-Exome Sequencing (WES) of BRAF/NRAS WT cell lines

To further investigate the mechanisms of mutations driving non MAPK pathway mutated tumors, four *BRAF* and *NRAS* Wild Type (WT) cell lines and their matching LCLs were subjected to WES using an Illumina Hi-Seq 2000. An average of 76,509,000 total reads of 101 bp length was generated per sample resulting in a median read depth of 50X coverage of targeted regions. Comparison of variants called between WES and SNP array analysis indicated a rate of >99.5% concordance and false negative rate of <0.05%. Strict filtering strategies previously established by our laboratory [16], were applied to the variant lists in order to reduce the rate of false positives without overly inflating the false negative rate. The accuracy of this approach was assessed by the comparison of 8 previously identified mutations against the list of mutations in the filtered WES lists (Supplementary Table 5). Four mutations were identified using the strict filtering criteria, three were present in the unfiltered mutation lists and one mutation was not detected at all. This disparity highlights the difficulties of next-generation sequencing analysis and can be the result of insufficient sequencing coverage, unsuccessful hybridisation of targeted captured regions or excessive stringency of filtering criteria. Although it is possible putative driver mutations may be missed, the strict filtering criteria was applied to the WES data sets to reduce false positives in light of the high rate of mutation inherent to cutaneous melanoma due to UV exposure [25].

A total of 8859 somatic mutations were detected (range per sample: 378-3873) that included 5714 Non-Synonymous (NS) mutations and 3036 Synonymous (S) mutations (Supplementary Table 5 and 6). Consistent with previous melanoma exome reports [15-17], the NS:S ratio was 1.88, not higher than the predicted NS:S ratio of 2.5 for non-selected passenger mutations [26]. A number of mutations occurring in key melanoma-associated genes were identified, including *ADAM29* [27], *ADAMTS18* [28], *GRIN2A* [15],

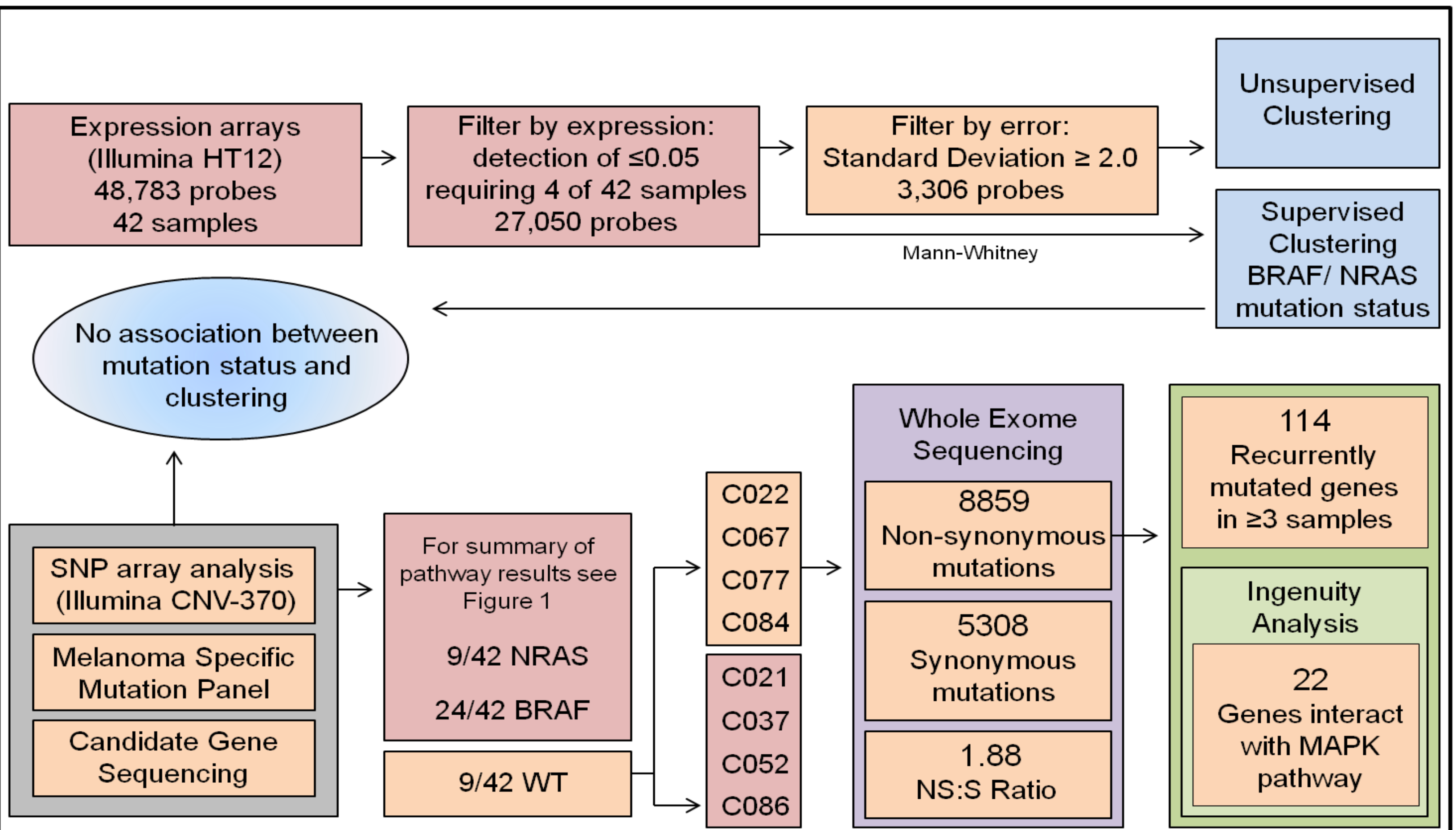


Figure 3: Filtering strategy to identify novel genes of interest in *BRAF*/*NRAS* WT cell lines involved in the MAPK pathway. Supervised and unsupervised clustering analysis of expression array data indicated that *BRAF*/*NRAS* WT cell lines did not segregate separately to *BRAF*/*NRAS* mutant cell lines. To investigate if mutual exclusive mutations occurred in novel genes compared to *BRAF* and *NRAS* mutant cell lines, 4 of 9 *BRAF*/*NRAS* WT cell lines were Whole-Exome Sequenced (WES). A total of 22 genes interacting with the MAPK pathway were identified that were mutated ≥3 samples that were WES.

TRRAP [15], *PTPRD* [29], *MAP3K5* and *MAP3K9* [16] (Supplementary Table 7). Interestingly, mutations were detected in key genes of the MAPK and RB pathways including a *BRAF* S467L mutation in C077 and *RB1* E209K mutation in C067. Although *BRAF* mutation status was assessed using the MelaCarta and OncoCarta mutation panels, these panels only assess the top 26 mutations that are frequently mutated in melanoma. The *BRAF* S467L mutation has only once been previously identified in a Superficial Spreading Melanoma (SSM) [30], highlighting the potential of missing rare variants using the above mentioned mutation panels. *RB1* is rarely mutated in melanoma and hence was not sequenced to assess the mutation status in the C series melanoma cell lines. Regardless, the *RB1* E209K mutation appears to be patient specific and is not present within the Catalogue of Somatic Mutations in Cancer (COSMIC) database [31]. Although not experimentally determined, it is interesting to speculate whether the *BRAF* S467L or *RB1* E209K mutations are responsible for activating or inactivating the MAPK and RB pathways respectively in these cell lines.

Recurrent or 'Hotspot' Mutations

Oncogenic or activating mutations, such as those occurring within *BRAF*, are frequently associated with recurrent or 'hotspot' mutations located within critical functional residues of genes. A total of 13 recurrent mutations were identified occurring in 2 of 4 cell lines used for WES (Supplementary Table 8). Although none of the genes included in this list encode kinases, recurrent mutations in interesting genes included *POLQ* (H2341Y) and *IL7R* (R206X) that were mutated in C077 and C084.

POLQ is a DNA polymerase whose functional *in vivo* role is not clearly understood although it may have a role in base excision repair [32] and double strand break repair [33]. *POLQ* has been implicated in cancer and is over-expressed in colorectal [34] and breast cancer [35] and is associated with poor prognosis and worse clinical outcomes. Ectopic overexpression of POLQ in breast cancer cell lines resulted in defective DNA fork replication causing chromosomal damage suggesting a role in genetic instability [36]. Notably, both C077 and C084 who have *POLQ* H2341Y mutations, exhibit a ~10-fold higher rate of mutation (over 3000 mutations per sample) compared to average rates of mutation in melanoma (300-500 mutations) [15, 16]. This H2341Y mutation occurs within an evolutionary conserved residue of the DNA polymerase motif hence mutation may affect correct protein function resulting in genomic instability in these samples. Genome instability resulting in increased rates of mutation has been demonstrated previously in

Sample ID	Gene	Coding Change	Protein Change	Function
C084	*ALK*	G4591A	G1531S	Receptor Tyrosine Kinase
C084	*ALK*	G4508A	W1503X	Receptor Tyrosine Kinase
C022	*ATF2*	C272T	T91I	Transcription Factor
C077	*ATF6*	C1462T	H488Y	Transcription Factor
C084	*BRCA2*	C2161T	P721S	DNA Damage and Repair
C084	*DICER1*	G1195A	E399K	RNA Processing
C077	*DICER1*	C1835T	P612L	RNA Processing
C077	*DICER1*	C2716T	R906C	RNA Processing
C067	*DOCK2*	G367A	D123N	Actin remodelling and migration
C077	*DOCK2*	G375A	M125I	Actin remodelling and migration
C084	*DOCK2*	C653T	S218F	Actin remodelling and migration
C077	*DROSHA*	T1343G	F448C	RNA Processing
C084	*DROSHA*	C2965T	P989S	RNA Processing
C067	*EGF*	G2941A	D981N	Receptor Tyrosine Kinase
C077	*EGF*	C2654T	S885F	Receptor Tyrosine Kinase
C067	*EGFR*	C584T	P195L	Receptor Tyrosine Kinase
C077	*EGFR*	C2491T	R831C	Receptor Tyrosine Kinase
C077	*EGFR*	C2819T	T940I	Receptor Tyrosine Kinase
C084	*ERBB2*	T2317C	Y773H	Receptor Tyrosine Kinase
C077	*ERBB3*	C2579T	P860L	Receptor Tyrosine Kinase
C084	*ERBB3*	C2867T	P956L	Receptor Tyrosine Kinase
C084	*EZH2*	G1030T	E344X	Histone/ Chromatin Modification
C067	*HDAC9*	G2158A	D720N	Histone/ Chromatin Modification
C077	*HDAC9*	A1085T	K362I	Histone/ Chromatin Modification
C084	*HDAC9*	C2678T	T893I	Histone/ Chromatin Modification
C084	***MAP3K15***	**G748A**	**D250N**	Apoptosis
C084	*MAP3K4*	C2887T	Q963X	Apoptosis
C084	*MAP3K4*	C4646T	S1549L	Apoptosis
C077	*MAP4K3*	C1295T	P432L	Apoptosis
C077	*MAP4K3*	C1702T	P568S	Apoptosis
C077	*MAP4K5*	G1427A	R476Q	Apoptosis
C067	*MAPK10*	C811T	P271S	Apoptosis
C084	***MRAS***	**C91T**	**H31Y**	RAS/ GTPase signalling
C067	*NEK11*	C361T	Q121X	Kinase
C084	*NEK5*	C688T	R230C	Kinase
C077	*NEK5*	C739T	R247X	Kinase
C077	*NF1*	C3163T	Q1055X	RAS signalling
C067	***NF1***	**C3520T**	**Q1174X**	RAS signalling
C077	*NF1*	C4108T	Q1370X	RAS signalling
C084	*NF1*	C7486T	R2496X	RAS signalling
C077	***NOTCH1***	**C2333T**	**T778I**	Growth signalling and Invasion
C067	*NOTCH2*	17_18del	6_6del_fs	Growth signalling and Invasion
C084	*NOTCH2*	C4003T	P1335S	Growth signalling and Invasion
C067	*NOTCH2*	C611T	S204F	Growth signalling and Invasion
C077	*NOTCH3*	G3607A	E1203K	Growth signalling and Invasion
C084	*NOTCH3*	G4414A	E1472K	Growth signalling and Invasion
C084	*NOTCH3*	C3446T	P1149L	Growth signalling and Invasion
C077	***PIK3C2A***	**C4225A**	**L1409I**	Cell Proliferation and survival
C077	*PLCE1*	G4870T	D1624Y	Cell Proliferation and differentiation
C077	*PLCE1*	C1017A	F339L	Cell Proliferation and differentiation
C084	*PLCE1*	G500A	G167E	Cell Proliferation and differentiation
C022	*PLCE1*	C3644T	P1215L	Cell Proliferation and differentiation
C084	*PLCE1*	G3560A	R1187Q	Cell Proliferation and differentiation
C084	*RET*	C2012T	S671L	Receptor Tyrosine Kinase
C084	*RHO*	G364A	E122K	Receptor Tyrosine Kinase
C077	*SETDB1*	C1483T	R495X	Histone/ Chromatin Modification
C077	***SGK1***	**C358T**	**P120S**	Serine/ Threonine Kinase
C084	*SGK1*	C1489T	P497S	Serine/ Threonine Kinase
C067	*SGK1*	A37G	T13A	Serine/ Threonine Kinase
C022	***SMARCA2***	**C92T**	**P31L**	Histone/ Chromatin Modification
C084	*TET1*	G4606A	E1536K	Methylation
C084	*TET2*	G3385A	D1129N	Methylation
C084	*TET2*	G5767A	E1923K	Methylation
C084	*TET2*	G4845A	M1615I	Methylation
C084	***TP63***	**G1517A**	**G506D**	DNA Damage and Repair
C084	***TP63***	**G366A**	**M122I**	DNA Damage and Repair
C077	*TP63*	C1447T	R483X	DNA Damage and Repair
C067	*TP63*	T602C	V201A	DNA Damage and Repair

Table 2: Somatic mutations occurring in genes previously implicated in cancer in *BRAF/NRAS* WT cell lines. Four samples were Whole-Exome Sequenced (WES) to identify novel genes involved in this subset of patients. A number of genes implicated in a range of important cellular processes that have previously been associated with cancer were mutated. Bold indicates homozygous mutations.

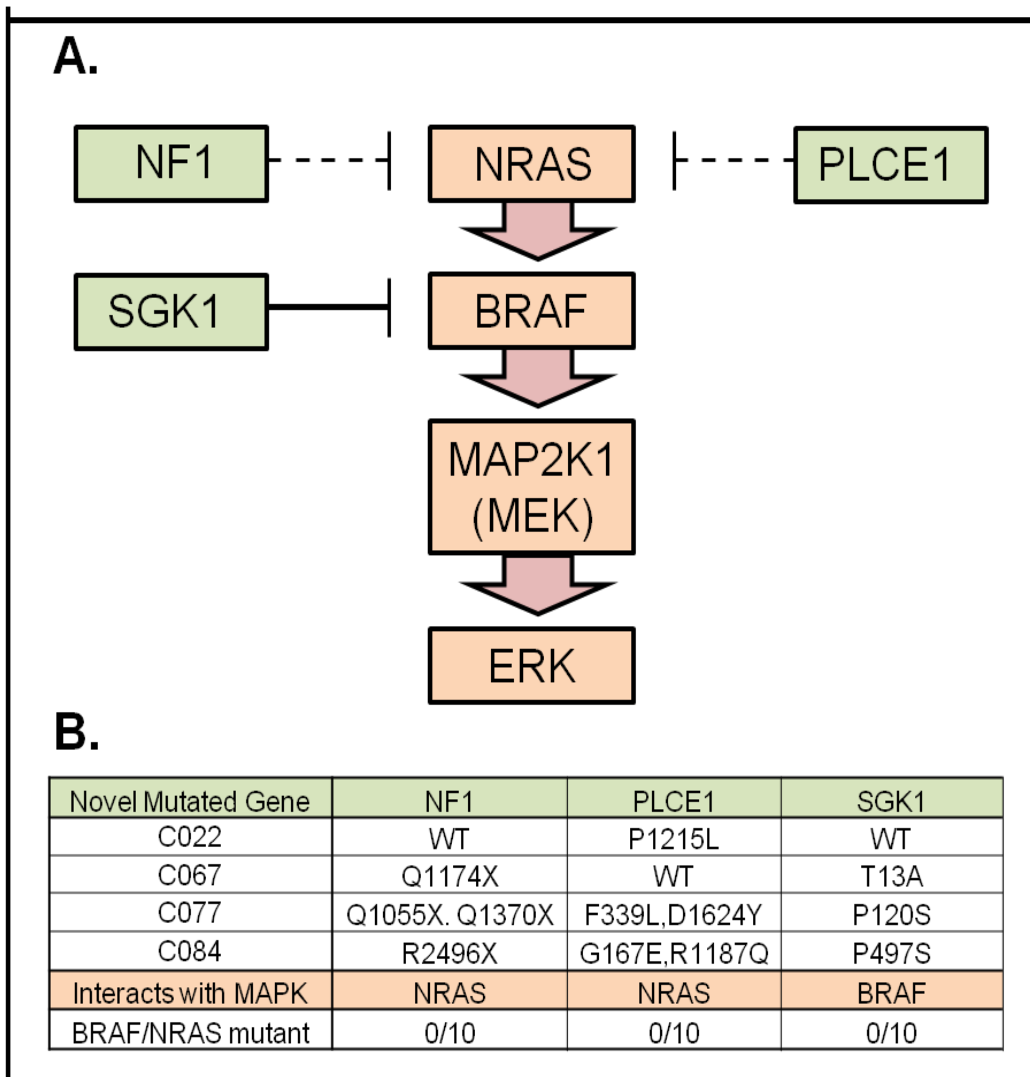
colorectal cancer with 8X more somatic non-synonymous mutations in microsatellite unstable cancers than microsatellite stable cancers [37].

The Interleukin 7 receptor (*IL7R*) has a number of roles in lymphocyte development, immune response, and chromatin modification [38]. Although somatic activating mutations have been identified in *IL7R* in acute lymphoblastic leukaemia [39, 40], the recurrent mutation identified in this study, in contrast, suggests inactivation by premature termination through nonsense mutation. Recently, low transcriptional expression of IL7R has been associated with familial longevity [41] while downregulation of IL7R resulted in decreased histone acetylation ability in thymocytes [42]. Mechanisms of longevity and chromatin modification are often intricately linked to processes of human ageing and cancer [43] and hence warrant further investigation.

Mutations in genes previously implicated in cancer

A number of genes mutated in the WES *BRAF*/*NRAS* WT samples have functional roles with previous associations to the development and progression of cancer (Table 2). This included genes involved in transcription and chromatin modification, apoptosis, cellular proliferation, differentiation, DNA damage repair and RNA processing. C077 and C084 had multiple mutations occurring in *DICER1* and *DROSHA*, key proteins involved in miRNA processing, regulation and maturation. *DICER1* mutations have been recently identified in 30 of 102 (29%) non-epithelial ovarian cancers, however mutation did not abolish functional activity but rather altered RNA processing in a cell context manner [44]. The potential dual-hit inactivation of these genes would suggest the inability of these cell lines to modulate gene expression with miRNA mechanisms produced within the cell.

Frequent mutations also appear in genes involved in histone and chromatin modification including a P31L mutation in *SMARCA2* and 3 of 4 samples harbouring Histone Deacetylase 9 (*HDAC9*) mutations. SNP array analysis has previously revealed homozygous deletions of *SMARCA2* and an alternate histone deacetylase family member of *HDAC4* in metastatic melanoma cell lines [23, 45]. A nonsense mutation was also detected in *EZH2*, a gene with frequent inactivation mutations in acute lymphoblastic leukaemia [46], while multiple mutations were detected in *TET1* and *TET2* which have been linked to myeloid malignancies [47]. Lastly, a nonsense mutation was identified in *SETDB1* in C077; this gene has recently been found to act in an oncogenic fashion in cooperation with *BRAF* V600E mutation [48].



Novel Mutated Gene	NF1	PLCE1	SGK1
C022	WT	P1215L	WT
C067	Q1174X	WT	T13A
C077	Q1055X. Q1370X	F339L,D1624Y	P120S
C084	R2496X	G167E,R1187Q	P497S
Interacts with MAPK	NRAS	NRAS	BRAF
BRAF/NRAS mutant	0/10	0/10	0/10

Figure 4: Novel somatically mutated genes that may be involved in the MAPK pathway in *BRAF*/*NRAS* WT cell lines.

A). Novel mutated genes (*NF1*, *PLCE1* and *SGK1*) in *BRAF*/*NRAS* WT cell lines interacting with the MAPK pathway. Solid connections between genes indicate experimentally observed direct interactions while dotted lines indirect interactions.

B). Mutations status of *NF1*, *PLCE1* and *SGK1* in 4 *BRAF*/*NRAS* WT cell lines that were WES. None of the genes were mutated in 10 *BRAF*/*NRAS* mutant cell lines that have been previously WES (unpublished and published data) indicating mutual exclusivity.

Novel mutated genes potentially regulating MAPK pathway activity

Genes that are recurrently mutated across numerous samples can indicate functional importance and be used as a filtering strategy for identifying genes involved in cancer development. Using this approach, 114 genes were mutated in at least 3 of the 4 cell lines that were screened using WES, of which 6 genes were mutated in all 4 cell lines (Supplementary Table 9). Next generation sequencing approaches can result in the generation of false positive mutations by misalignment caused by technological limitations associated with alignment methodologies and short read lengths. This can be problematic for sequences in repetitive regions, pseudogenes, and members pertaining to gene families sharing conserved regions of sequence. As such, it is likely that the 6 genes mutated in all cell lines are a result of alignment error rather than bona-fide recurrently mutated genes. For this reason we focused on recurrently mutated genes that occurred in 3 of 4 cell lines that were whole exome sequenced.

Since *BRAF*/*NRAS* WT cell lines did not group separately through unsupervised clustering analysis, and in light of constitutive MAPK activity through ERK phosphorylation in this molecular entity [14], Ingenuity Pathway Analysis (IPA) was used to identify novel genes that may be responsible for MAPK pathway activation from the list of recurrently mutated genes. Using search criteria of direct and indirect experimentally observed interactions to RAS/BRAF/MEK/ERK, IPA revealed a total of 22 genes interacting with the MAPK pathway. To filter this list further, we used existing WES data from 10 metastatic melanoma cell lines (published [16] and unpublished) to identify recurrently mutated genes that were mutually exclusive, and not mutated in *BRAF*/*NRAS* mutant melanomas (Supplementary Table 9). This process identified *SGK1*, *PLCE1* and *NF1* that are recurrently mutated genes, mutually exclusive to *BRAF*/*NRAS* mutant melanomas that also interact with members of the MAPK pathway (Figure 4).

Serum/Glucocorticoid Regulated Kinase 1 (*SGK1*) is a serine/threonine kinase implicated in cellular stress responses and cell survival through a variety of molecular pathways [49]. Reports into the mechanism of action of SGK1 are hotly debated with evidence suggesting that either SGK1 activation or inactivation can lead to tumorigenesis; however this disparity is likely to be explained by the context of tissue in question. In regards to SGK1 activation promoting tumorigenesis, SGK1 can activate MDM2-dependent TP53 ubiquitylation [49], can stabilise MDM2 through the mTOR pathway [50], and can directly

interact and activate ERK1/2 and MEK1/2 in hepatocytes [51]. However, the mutations detected in *SGK1* from the *BRAF*/*NRAS* WT cell lines present in a typical tumor suppressor fashion and are spread throughout the entire length of the gene; this suggests that mutations of *SGK1* may rather be inactivating in melanoma but will require functional validation. Supporting this notion, SGK1 has been shown to be a potent negative regulator of BRAF through phosphorylation of Ser 364 [52]. *SGK1* has high constitutive expression across the C series of metastatic melanoma cell lines (probe ILMN-1702487), possibly indicating a negative feedback loop imposed in melanocytes to control the MAPK pathway. Hypothetically, this mechanism could be overridden by either *NRAS* or *BRAF* mutation resulting in auto-phosphorylation, or alternatively, inactivating mutations in *SGK* observed in *BRAF*/*NRAS* WT cell lines.

Phospholipase C, epsilon 1 (*PLCE1*) catalyses the hydrolysis of phosphotidylinositol (PI)-4,5-bisphosphate generating messengers involved in cell growth, differentiation and gene expression [53]. PLCE1 is a putative tumor suppressor gene transcriptionally downregulated in colorectal cancer [54, 55] and is a novel susceptibility locus for esophageal squamous cell carcinoma [56, 57]. The biological function of PLCE1 is complicated due to the various catalytic and binding domains located throughout the protein. Both RasGEF and PI-PLC domains within PLCE1 can activate the MAPK pathway but via different mechanisms [53]. Since both domains can activate the MAPK pathway, a H1144L mutant abolishing PI hydrolysis by the PI-PLC domain in PLCE1 was tested in conjunction with WT PLCE1. Interestingly, both versions promoted MAPK activity, however this effect was increased in H1144L mutant PLCE1 compared to WT suggesting that PI hydrolysis activity, as is present in WT PLCE, can act in a regulatory negative feedback loop. Accordingly, if the mutations observed in *BRAF*/*NRAS* WT cell lines mimicked the effect of the H1144L mutation resulting in decreased PI hydrolysis, these mutations could explain increased activation of RAS and MAPK activity in these cell lines.

Neurofibromin 1 (*NF1*) functions as a negative regulator of RAS via GTPase activity and mutations in this gene are associated with neurofibromatosis 1, a tumour predisposition syndrome [58, 59]. Patients with neurofibromatosis most frequently develop subcutaneous neurofibromas but also exhibit a range of hyper-pigmentary lesions in childhood [60] linking the disease to deregulation of pigmentation pathways [61]. Furthermore, the association between melanoma and neurofibroma originating from neural crest cells suggested that *NF1* may have an important role in melanoma development, but a strong

correlation has remained elusive [62]. A number of clinical case reports have identified the involvement of *NF1* in melanoma [63, 64] but only 0.5%-5% of neurofibromatosis patients are subsequently diagnosed with this tumour.

A number of early reports investigated the mutation of *NF1* in sporadic cutaneous metastatic melanoma [65, 66]. These studies revealed a small proportion of melanoma cell lines had homozygous or hemizygous deletions of *NF1* but did not assess whether point mutations occurred using traditional sequencing approaches. Similarly, analysis of *NF1* in uveal melanoma revealed 18 of 38 (47%) of samples with weak expression of NF1 and identified a homozygous deletion in one sample [67]. Surprisingly, WES of *BRAF*/*NRAS* WT melanomas revealed 3 of 4 cell lines with nonsense mutations in *NF1*, while no mutations were detected in *BRAF*/*NRAS* mutant cell lines. This finding is interesting due to the previously documented role of *NF1* as a negative regulator of RAS and that *NF1* mutation is mutually exclusive to *BRAF* or *NRAS* mutation. Further support of this notion is a high rate of *NF1* loss in desmoplastic melanoma, for which *BRAF* mutations have not been documented [68], compared to common melanomas (10/15 or 67% to 1/20 or 5% respectively) [69].

Mutation of *NF1* has been detected in numerous cancers including ovarian [70], glioblastoma multiforme [71], juvenile myelomonocytic leukaemia (JMML) [72] and Adult Acute Myeloid Leukaemia (AAML) [73]. A number of mutations affecting RAS pathways have been documented in JMML and consistent with our observations, *NF1* mutations are nearly always mutually exclusive to *NRAS* and *KRAS* mutations [72]. More importantly, the recent identification of *NF1* inactivation in AAML demonstrated that NF1 does negatively regulate NRAS, and that essentially, *NF1* inactivation results in a similar phenotype to oncogenic mutant *NRAS* [74]. Furthermore, cell lines with loss of NF1 functional activity displayed a reliance on mammalian target of rapamycin (mTOR) signalling and were sensitive to rapamycin-induced apoptosis. If these results were replicated in *NF1* mutant metastatic melanomas, these findings would have a critical impact for patients with *BRAF*/*NRAS* WT tumors, a clinical entity that currently lacks any targeted treatment strategies.

Potential therapeutic avenues in *BRAF/NRAS* WT cell lines

Performing WES analysis in *BRAF*/*NRAS* WT metastatic melanoma cell lines provides the opportunity to retrospectively investigate mutations within these samples that may be

Cell Line	Gene	Mutation	Therapeutic Avenue	Drug
C021	None	None	Alternative approach	Ipilimumab
C022*	None	None	Alternative approach	Ipilimumab
C025	*TFG*	P199S/ P361L	MEK inhibitor?	GSK112012/ AZD6244
	NEK10	E379K	MEK inhibitor?	GSK112012/ AZD6244
	None	None	Alternative approach	Ipilimumab
C037	None	None	Alternative approach	Ipilimumab
C052	*CCND1*	Amplification	CDK4 inhibitor?	UCN-01
	None	None	Alternative approach	Ipilimumab
C067*	*EGFR*	P195L	ERBB inhibitor	Lapatinib
	MRAS	Amplification	MEK inhibitor/ PI3K inhibitor?	GSK112012/ AZD6244
	NF1	Q1174X	mTOR inhibitor	Rapamycin
C077*	*BRAF*	S467L	MEK inhibitor	GSK112012/ AZD6244
	ERBB4	E452K	ERBB inhibitor	Lapatinib
	EGFR	R831C/ T940I	ERBB inhibitor	Lapatinib
	NF1	Q1055X/ Q1370X	mTOR inhibitor	Rapamycin
C084*	*BRCA2*	P721S	PARP inhibitor	Veliparib (ABT-888)
	ERBB2	Y773H	ERBB inhibitor	Lapatinib
	NF1	R2496X	mTOR inhibitor	Rapamycin
C086	*TFG*	P380L	MEK inhibitor?	GSK112012/ AZD6244
	None	None	Alternative approach	Ipilimumab

Table 3: Potential therapeutic strategies for *BRAF/NRAS* WT patients.
Whole-Exome Sequencing (WES) revealed mutations potentially susceptible to current therapeutic avenues in 3 of 4 patients. The remaining *BRAF/NRAS* WT samples assessed through traditional analytical methods did not reveal definitive therapeutic strategies indicating the potential benefits of next generation sequencing in the treatment of cancer. * Samples that were selected for WES.

susceptible to novel or existing drug therapies. A summary of potentially relevant mutations and appropriate drug strategies are listed in Table 3.

WES revealed mutations in the ERBB family of proteins, including *EGFR*, *ERBB2* and *ERBB4*, in 3 of the 4 samples sequenced. It has been previously documented that approximately 19% of metastatic melanomas have mutations in *ERBB4*, resulting in increased kinase activity and transformation ability of melanoma cell lines *in vitro* [75]. In addition, it was demonstrated that use of a pan-ERBB pharmacological inhibitor, Lapatinib, reduced rates of proliferation while increasing apoptosis in *ERBB4* mutant cell lines compared to counterpart wild-type *ERBB4* cell lines [75]. One of the mutations validated in the *ERBB4* study was E452K, the exact mutation identified in C077 from this study, suggesting that use of Lapatinib may have been an effective treatment strategy in this patient. Similarly, mutation of *EGFR* and *ERBB2* can result in oncogenic activation as frequently observed in lung cancer [76]; however, mutations in *EGFR* and *ERBB2* in this study are either rare or patient specific [31]. It is possible that ERBB inhibitors may represent an effective approach in these patients but would require functional validation.

A number of mutations detected in *BRAF*/*NRAS* WT cell lines may also be susceptible to inhibitors of the MAPK pathway, or more specifically, inhibitors of MEK. As mentioned previously, a rare S467L mutation was detected in *BRAF* using WES that was not detected through oncogenic profiling. Although this mutation is unlikely to be susceptible to current RAF inhibitors such as Vemurafenib [77], this mutation, if responsible for oncogenic activation of the MAPK pathway, may be susceptible to downstream inhibition using MEK inhibitors [78]. Alternatively, mutations were also identified in *TFG* in C025 and C086, a gene that has been shown to activate the MAPK and NFκB pathways [79] and are mutated in 5% of metastatic melanomas [23]. Although speculative without biological validation, it is possible these mutations may also be susceptible to MAPK pathway inhibition.

Another interesting mutation revealed using WES was a *BRCA2* P721S mutation in C084. *BRCA2*, in conjunction with *BRCA1*, is involved in double-strand DNA break repair through homologous recombination and base-excision repair pathways and is frequently mutated in sporadic and familial breast and ovarian cancer [70, 80]. Tumours with *BRCA1/2* mutations have insufficient DNA repair mechanisms and are susceptible to poly ADP ribose polymerase (PARP) inhibition in combination with chemotherapeutic agents, or as a mono-therapeutic approach, and have shown responses in preclinical melanoma xenograft

models [81-83]. Although recent clinical trials of PARP inhibition with temozolomide [84] and dacarbazine [85] have been tolerable, patient responses have been less than enthusiastic with no improvement observed in cohort of chemo-naive melanoma patients. It is interesting to speculate about the possibility of increased efficacy of PARP inhibition in *BRCA1/2* mutant melanoma patients such as observed in C084; albeit realistically, this will be a small population of patients diagnosed with metastatic melanoma due to the low rates of *BRCA1/2* mutation in this disease.

SNP array analysis revealed a small number of amplification events that may be amenable to molecularly targeted therapies. C052 exhibited amplification of *CCND1*, a regulatory subunit of *CDK4/6* subunit that interacts with *RB1* and hypothetically might be susceptible to CDK4 inhibitors [86, 87]. Conversely, C067 harboured an amplification of *MRAS*, a reciprocal member of the RAS family of small GTPases of which *NRAS* is frequently mutated in melanoma. Although not investigated, *MRAS* amplification may be responsible for activation of the MAPK and PI3K pathways for which inhibitors of this pathway may be a rational approach to therapy [88].

Aside from the above mentioned amplifications, comprehensive genetic analysis of candidate gene sequencing, oncogene profiling and SNP array analysis, revealed minimal putative therapeutic targets in *BRAF*/*NRAS* WT cell lines. Of the 6 cell lines that were not screened with WES and hypothetical strategies aside, no conclusive therapeutic strategies were presented from the data that was generated from traditional genetic analysis. This is in contrast to WES of *BRAF*/*NRAS* WT cell lines for which viable therapeutic targets were identified in 3 of 4 cell lines. This notion further supports the viability of next generation sequencing within the clinic as a personalised therapeutic approach, and although a number of important issues in its application remain, steps toward this approach have already begun to be implemented [89]. Lastly, for patients where therapeutic targets are not identified through comprehensive genomic analysis, immunological approaches such as Ipilimumab would most likely represent ideal treatment strategies.

CONCLUSIONS

A comprehensive genetic analysis was performed on a panel of 42 stage III local lymph node metastatic melanoma cell lines in order to reveal deregulated pathways in melanomagenesis. Several mutations occurred in 4 main pathways of melanoma development; however, 9 of 42 cell lines did not demonstrate any known activating

mutation events in the MAPK pathway. These *BRAF*/*NRAS* WT cell lines did not segregate independently to *BRAF*/*NRAS* mutant melanomas upon supervised or unsupervised clustering using expression array data. As nearly all melanomas exhibit constitutive activation of the MAPK pathway, clustering analysis suggested that mutation of novel genes linking into the MAPK pathway may explain the activation of this pathway. As such, WES of 4 *BRAF*/*NRAS* WT cell lines revealed a number of novel mutated genes including *SGK1*, *PLCE1* and *NF1*. Mutations in *NF1* are particularly interesting as they are mutually exclusive to *BRAF*/*NRAS* mutations and may be susceptible to inhibition through rapamycin. Lastly, WES revealed a number of potential therapeutic avenues for *BRAF*/*NRAS* WT melanomas, a distinct molecular entity that currently has no recommended treatment strategy, and further supports the notion of next generation sequencing within a clinical setting. These findings warrant further functional validation and investigation.

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Supplementary Figure 1: Clinical Information and mutation data for metastatic melanoma cell lines

Sample	Exome	SNP	EXP	MSP	CGS	Age	Gender	Characteristic	Primary Tumor Site	Metastatic Tumor Site	Matched Normal	Tumour	STR matched
C001		Y	Y	Y	Y	50	F	superficial spreading melanoma	R calf	R calf nodule	LCL	Y	Y
C002		Y	Y	Y	Y	60	M	nodular	R upper leg	R thigh	LCL	Y	Y
C004		Y	Y	Y	Y	42	F	n/a	n/a	L axilla lymph nodes	LCL	Y	Y
C006		Y	Y	Y	Y	35	F	nodular	R lower back	R inguinal lymph nodes	LCL	Y	Y
C011		Y	Y	Y	Y	34	F	superficial spreading melanoma	L upper leg	lung and liver	LCL	Y	Y
C013		Y	Y	Y	Y	71	M	nodular	upper back	L and R axilla lymph nodes	LCL	Y	Y
C017		Y	Y	Y	Y	57	F	cutaneous	R mid back	R axilla lymph nodes	LCL	Y	Y
C021		Y	Y	Y	Y	38	M	superficial spreading melanoma	L upper back	L axilla lymph nodes	LCL	N	Y
C022	Y	Y	Y	Y	Y	45	F	superficial spreading melanoma	R arm	R axillary lymph nodes	Lymphocytes	N	Y
C025			Y	Y	Y	41	M	superficial spreading melanoma	L upper back	Lymph node	LCL	N	Y
C027		Y	Y	Y	Y	61	M	nodular	L upper back	L axilla lymph nodes	LCL	Y	Y
C037		Y	Y	Y	Y	27	F	n/a	n/a	R axilla lymph nodes	LCL	N	Y
C038		Y	Y	Y	Y	33	F	n/a	n/a	n/a	N/A	N	N/A
C042		Y	Y	Y	Y	39	M	n/a	n/a	L Axilla	N/A	N	N/A
C044		Y	Y	Y	Y	51	F	superficial spreading melanoma	L lower back	L groin	LCL	Y	Y
C045		Y	Y	Y	Y	22	F	superficial spreading melanoma	R shoulder	R axilla lymph nodes	LCL	N	Y
C052		Y	Y	Y	Y	69	M	nodular	mid back	L axilla lymph nodes	LCL	N	Y
C054		Y	Y	Y	Y	52	F	cutaneous	R lower leg	R groin	LCL	Y	Y
C055		Y	Y	Y	Y	80	M	nodular	centre back	R axilla lymph nodes and Lgroin	LCL	Y	Y
C057		Y	Y	Y	Y	35	F	superficial spreading melanoma	L upper back	L axilla lymph nodes	LCL	Y	Y
C058		Y	Y	Y	Y	39	M	n/a	n/a	L axilla lymph nodes	LCL	Y	Y
C060		Y	Y	Y	Y	64	F	superficial spreading melanoma	R upper leg	R groin	LCL	Y	Y
C062		Y	Y	Y	Y	67	M	n/a	n/a	L and R groin	LCL	Y	Y
C065		Y	Y	Y	Y	39	M	superficial spreading melanoma	L lower back	L axilla lymph nodes	LCL	Y	Y
C067	Y	Y	Y	Y	Y	69	M	superficial spreading melanoma	upper back	R axilla lymph nodes	LCL	Y	Y
C071		Y	Y	Y	Y	33	M	superficial spreading melanoma	R upper back	R axilla lymph nodes	LCL	Y	Y
C074			Y	Y	Y	45	F	superficial spreading melanoma	mid back	axillary lymph nodes	LCL	Y	Y
C077	Y	Y	Y	Y	Y	48	M	nodular	mid upper back	L and R axilla lymph nodes	LCL	Y	Y
C078		Y	Y	Y	Y	60	M	superficial spreading melanoma	L lower back	axillary lymph nodes	LCL	Y	Y
C081		Y	Y	Y	Y	54	M	superficial spreading melanoma	R upper leg	L groin	LCL	Y	Y`
C083		Y	Y	Y	Y	33	M	superficial spreading melanoma	R neck	neck	LCL	Y	Y`
C084	Y	Y	Y	Y	Y	75	M	superficial spreading melanoma	R upper back	R neck	LCL	Y	Y
C086		Y	Y	Y	Y	27	F	n/a	n/a	L Neck	LCL	N	Y
C088		Y	Y	Y	Y	55	F	superficial spreading melanoma	R upper abdomen	R axillary lymph nodes	LCL	Y	Y
C089		Y	Y	Y	Y	50	F	n/a	n/a	L axilla lymph nodes	LCL	Y	Y
C091		Y	Y	Y	Y	53	F	superficial spreading melanoma	L lower leg	groin	LCL	Y	Y
C094			Y	Y	Y	20	F	superficial spreading melanoma	L upper back	L axilla lymph nodes	LCL	Y	Y
C096		Y	Y	Y	Y	45	M	superficial spreading melanoma	R lower leg	groin	LCL	Y	Y
C097		Y	Y	Y	Y	43	F	nodular	L neck	neck	LCL	Y	Y
C100		Y	Y	Y	Y	78	M	nodular	Mid scalp	R neck	LCL	Y	Y
C106		Y	Y	Y	Y	52	M	superficial spreading melanoma	R upper leg	R groin	LCL	Y	Y
C108		Y	Y	Y	Y	47	M	n/a	n/a	L Neck	N/A	N	N/A

Legend: Exome: Whole Exome Sequencing
EXP: Expression array
SNP: SNP arrayss
MSP: Melanoma Specific Mutation Panel
CGS: Candidate Gene Sequencing (TP53, PTEN, CDKN2A, TFG, ACTA2, BMPR1A, RAC1)
Age is at removal of metastatic tumour form which cell line is created

Supplementary Figure 1: Clinical Information and mutation data for metastatic melanoma cell lines

Sample	BRAF	NRAS	PTEN	TP53	P16INK4A	P14ARF	MAP3K5	MAP3K9	TFG	MITF	RAC1	PIK3CA	MDM2	PTPRD	CDK4	CCND1	MEK	PTK2B	NEK10	ERBB4	MET	HDAC4	BMPR1A	ACTA2
C001		Q61K		del-257-EDSS																				
C002		Q61K (AMP-L)			HD	HD								HD										
C004	V600E																AMP-L							
C006		Q61L																						
C011	V600E																							
C013		Q61L	G127E																					
C017	V600E																	G414V				HD		
C021				N247K, R248W							P29S													G148E
C022									Q309K															
C025							R256C		P199S/ P361L										E379K					G160I
C027		Q61K																						
C037																								
C038	V600E		H75X		HD			S616F																
C042	V600E		HD		HD	HD																	HD	
C044	V600E		Del Exon 6	HD																				
C045	V600E			A138V						AMP-L														
C052										AMP-L				HD		AMP-L								
C054		Q61K			HD																			
C055	V600E		Del Exon 4		HD	HD			HD															
C057	V600E		Del Exon 6												R24C									
C058	L597S				HD	HD																		
C060	V600E						D408N						AMP											
C062	V600E		HD		HD	HD		W333X																HD
C065	V600E			R247K				S650L																
C067																								
C071	V600E		HD	A119X	HD	HD									R24C									
C074	V600E			V172fs																				
C077				Q100X				S650L												E452K				
C078	V600E																						Y245S	
C081	V600K				HD	HD								HD										
C083		Q61L		C275F							P29S													
C084								G600R, R827Q, P963S																
C086				E286X				D176N, P1020S, P1075S	P380L															
C088	V600K																							
C089	V600E				HD	HD										AMP-L					T1010I			
C091	V600E		Del Exon 5,6		HD		E1096K																	
C094	V600E																							
C096		Q61R			HD			S533Y																
C097	V600E		G165V		HD	HD																		
C100	G469R			R342X								R88Q												
C106		Q61L																						
C108	V600K																							

Supplementary Table 2- Amplifications

List of strong amplification events detected in C series metastatic melanoma cell lines.
Strong amplifications were determined with a Log *R* Ratio above 0.4 and *B*-Alelle above 0.58
Focal amplification involving <5 genes are listed in the "Focal amplification events" column.
Only notable genes of putative functional significance to melanoma/ cancer development are listed within large amplification regions.
* Genes previously implicated in melanoma.

Sample	Chr	Start	End	Length	First SNP	Last SNP	Log *R* Ratio	*B*-Alelle	Large amplification events	Focal amplification events
C002	1	110915677	113343324	2427647	rs923826	rs773433	0.46	0.73	RHOC WNT2B RAP1A	
C002	1	113343324	115484992	2141668	rs773433	rs1286238	0.4	0.99	NRAS	
C002	1	115484992	118140872	2655880	rs1286238	rs1655200	0.46	0.72		
C002	1	118140872	121180077	3039205	rs1655200	rs9730181	0.51	0.63	ADAM30 NOTCH2	
C106	1	225881013	226501018	620005	rs2132367	rs7517088	0.41	0.71		
C106	1	235647229	236440259	793030	rs6673182	rs4623688	0.44	0.71		RYR2 ZP4
C045	3	65454108	71410012	5955904	rs7641222	rs1288699	0.68	0.8	MITF*	
C052	3	65559721	71298189	5738468	rs1524962	rs9845218	0.77	0.89	MITF*	
C052	3	71298189	87562879	16264690	rs9845218	rs9882182	0.66	0.81		
C045	3	71410012	90576572	19166560	rs1288699	rs11711280	0.58	0.72		
C011	3	96538125	97171533	633408	rs2871719	rs9814870	0.54	0.64		AX747417
C067	3	136411407	139497879	3086472	rs6439563	rs12972	0.6	0.83	EPHB1	
C067	3	139497879	152033050	12535171	rs12972	rs6762723	0.43	0.72	ATR MRAS	
C071	4	107563647	112049416	4485769	rs2866904	rs561873	0.81	0.88	EGF	
C071	4	119040239	120467991	1427752	rs4543205	rs7672594	0.66	0.88		
C071	4	121591244	121911349	320105	rs1685593	rs907298	0.61	0.77		PRDM5
C071	4	133396347	134873627	1477280	rs13112575	rs953972	0.44	0.66		PCDH10
C004	5	80564	4350423	4269859	rs2135917	rs7731506	0.43	0.71	TERT	
C096	5	27244464	31167166	3922702	rs11744047	rs4867309	0.57	0.64		CDH9
C096	5	31167166	32715360	1548194	rs4867309	rs2077312	0.51	0.58		
C096	5	33287528	39319510	6031982	rs6891985	rs40119	0.48	0.59	RNASEN	
C081	5	42163691	43551037	1387346	rs982054	rs12520489	0.48	0.7		
C088	5	74204801	76097012	1892211	rs10062244	rs250724	0.46	0.63	RAP1B	
C011	5	164996462	167989258	2992796	rs958994	rs292482	0.47	0.7		PANK3 WWC1 RARS ODZ2
C028	6	15776640	17423817	1647177	rs742208	rs1745074	0.46	0.63		ATXN1 RBM24
C028	6	17423817	23015973	5592156	rs1745074	rs4711037	0.45	0.84	SOX4 ID4	
C028	6	43803542	45203141	1399599	rs9472113	rs9395066	0.44	0.87	VEGFA	
C028	6	45203141	52249471	7046330	rs9395066	rs3765447	0.46	0.83		
C022	6	45490071	45630117	140046	rs1004130	rs13191376	0.41	1		RUNX2
C028	6	54439570	57273588	2834018	rs638540	rs1860652	0.54	0.86	RAB23	

Sample	Chr	Start	End	Length	First SNP	Last SNP	Log *R* Ratio	*B*-Alelle	Large amplification events	Focal amplification events
C004	6	57313879	58183439	869560	rs6459193	rs9396464	0.56	0.77		PRIM2A
C004	6	62021131	63742532	1721401	rs840064	rs4504456	0.95	0.88		G43499 KHDRBS2
C045	6	65261928	66203540	941612	rs9451767	rs12192936	0.47	0.65		EYS
C004	6	65832479	66379302	546823	rs12207169	rs7747445	0.69	0.82		EYS
C045	6	83802778	84943666	1140888	rs12209871	rs6905922	0.52	0.69		
C004	6	154576104	155023331	447227	rs790252	rs6910245	0.49	0.74		CNKSR3 IPCEF1 OPRM1 SCAF8
C091	7	61060840	158812247	97751407	rs35477534	rs1124425	0.48	0.62		
C038	8	47016137	50085228	3069091	rs6558238	rs4873303	0.45	0.7	MCM4	
C038	8	53653712	83778411	30124699	rs2119395	rs17731245	0.45	0.72	RP1/ RAB2A HEY1	
C004	8	60968888	68412982	7444094	rs17342102	rs6472297	0.41	0.71	RAB2A SGK3 CSPP1	
C038	8	120255596	146264218	26008622	rs2081430	rs6599566	0.46	0.73	MYC	
C086	8	121687250	122254596	567346	rs10808513	rs2036539	0.43	0.79		SNTB1
C096	9	23609623	24529632	920009	rs7867438	rs1888109	0.45	0.7		ELAVL2
C096	9	32513737	32665417	151680	rs11795343	rs2117523	0.45	0.76		TOPORS NDUFB6 TAF1L DDX58
C052	11	68167923	73951606	5783683	rs4930585	rs7941941	0.5	0.8	CCND1	
C089	11	68784346	70136496	1352150	rs7940107	rs7119726	0.54	0.82	CCND1	
C089	11	72926633	73987190	1060557	rs11235796	rs10899013	0.55	0.8		
C004	11	76525683	77388489	862806	rs948969	rs921383	0.45	0.75	PAK1	
C052	11	91320958	100443503	9122545	rs2201119	rs11224580	0.67	0.83		
C052	11	100872929	103202627	2329698	rs4403777	rs7104359	0.57	0.79	MMP family	
C060	12	60927818	60984983	57165	rs1907971	rs1389134	0.92	0.97		USP15
C060	12	65920910	65999035	78125	rs12320481	rs10878598	1.02	0.99		CAND1
C060	12	67386217	67990021	603804	rs17126545	rs317667	0.9	0.94		MDM2* CPSF6 CPM NUP
C071	13	60892492	61012972	120480	rs9598274	rs7994441	0.44	0.63		PCDH20
C022	14	24782140	26432781	1650641	rs1461549	rs8018926	0.41	0.58		NOVA1
C060	15	48037645	49457768	1420123	rs2413987	rs2470172	0.5	0.75		
C004	15	62394525	100198883	37804358	rs12102207	rs6598500	0.43	0.72	MAP2K1* NRG4 IGF1R	
C060	15	96479832	98052198	1572366	rs984999	rs4965533	0.68	0.89	IGF1R	
C052	17	54984029	57167530	2183501	rs9908925	rs2159451	0.44	0.72	BRIP1	
C086	X	134552930	154582606	20029676	rs885077	rs557132	0.43	0.63	MAGE family	

Supplementary Table 3 - Homozygous Deletions

List of homozygous deletions detected in C series metastatic melanoma cell lines.
Focal homozygous deletions involving <5 genes are listed in the "Focal Deletions Events" column .
Only notable genes of functional significance to melanoma development are listed within large deletion regions.
* Genes previously implicated in melanoma.
† Candidate genes selected for resequencing in C series- Dutton-Regester *et al.* 2012. *Genes, Chromosomes and Cancer*.

Sample	Chr	Start	End	Length	First SNP	Last SNP	LogR Ratio	Large Deletions Events	Focal Deletions Events
C002	3	60347076	60407332	231811	rs2449250	rs2245556	-6.28		FHIT
C002	3	163993833	164109279	115446	rs206280	rs206276	-6.69		BC073807
C002	4	115354217	115407950	19065	rs9307402	rs4470706	-5.82		
C002	4	181337576	182104506	187043	rs10012888	rs6552500	-6.54		
C002	4	182614622	182798894	280379	rs1517297	rs9312290	-6.21		
C002	4	183023754	183304133	9495	rs40266	rs11167571	-6.07		AK056196 AK094166 MGC45800
C002	6	21188451	21293569	14196	rs1634757	rs9405064	-5.05		CDKAL1
C002	6	31384589	31398785	80364	rs7751252	rs818314	-6.15		HLA-B
C002	6	101701516	101966969	143036	rs9458481	rs6935149	-6.07		GRIK2
C002	6	162544636	162687672	60480	rs4493732	rs2846466	-6		PARKIN PARK2
C002	9	9022872	9822245	630812	rs324541	rs1359176	-5.05		PTPRD*
C002	9	21737803	22590729	298182	rs4345650	rs2383207	-5.72		CDKN2A* CDKN2B* DRMTA1 MTAP
C002	11	83720568	84412643	18250	rs4382935	rs4944603	-6.14		DLG2
C002	12	127620464	128981960	65968	rs9788429	rs1778370	-6.06		
C002	X	31937076	32197713	138047	rs4240095	rs1555256	-4.64		DMD
C011	2	51002576	51132898	241601	rs753302	rs1564976	-6.34		NRXN1
C011	3	163993833	164109279	115446	rs206280	rs206276	-6.5		BC073807
C013	4	115388885	115407950	126583	rs9998128	rs1376539	-6.06		
C013	4	182392020	182579063	273771	rs724658	rs1371215	-6.7		
C013	15	54574406	54608317	87469	rs4227	rs2909430	-6.25		FMN1
C017	2	239779074	240020675	45412	rs9854706	rs4684384	-7.55		HDAC4*
C017	6	79019345	79099709	265453	rs9377263	rs2225912	-5.23		
C021	11	55096252	55217364	121112	rs12800642	rs2868510	-5.15		
C027	19	20385941	20520617	211156	rs2824516	rs154747	-5.88		
C028	1	113952240	114195271	243031	rs11102660	rs1217407	-6.2		MAGI3 PHTF1 PTPN22 RSBN1
C028	3	101866429	102098240	116768	rs3853173	rs206276	-5.73		GPR128 TFG† ABI3BP
C028	3	163993833	164109279	115446	rs206280	rs206276	-6.62		BC073807
C028	8	39350791	39509376	158585	rs10088400	rs7829181	-6.41		tMDC tMDCII
C028	9	16025672	18027759	103298	rs4961729	rs4378062	-6.37		BNC2 SH3GL2 C9ORF39
C028	9	21681392	23641429	852926	rs4636294	rs10811746	-3.25		CDKN2A* CDKN2B* DMRTA1 MTAP
C028	10	10923699	11040345	410512	rs10905883	rs2440119	-6.71		
C028	10	11107078	11517590	446816	rs4933309	rs1188774	-6.07		CUGB2
C037	4	182564832	182838603	184272	rs10027214	rs6841481	-5.74		
C038	3	163993833	164109279	115446	rs206280	rs206276	-6.74		BC073807
C038	9	21943137	21987872	107884	rs10751825	rs7083890	-6.29		CDKN2A* MTAP
C042	3	163993833	164109279	115446	rs206280	rs206276	-6.51		BC073807
C042	9	19642765	32030216	10999404	rs7854782	rs1475562	-6.3	CDKN2A* CDKN2B* DMRTA1 MLLT3	
C042	10	88403292	88673870	1067320	rs17096113	rs2576164	-6.36		OPN4 LDB3 BMPR1A†

Sample	Chr	Start	End	Length	First SNP	Last SNP	LogR Ratio	Large Deletions Events	Focal Deletions Events
C042	10	89250313	90324815	120638	rs2299939	rs2244092	-4.5	PTEN*	
C042	10	90773300	90965187	217838	rs10788636	rs303211	-6.07		CH25H† LIPA
C044	1	236884053	238257010	130322	rs7423296	rs4971724	-4.97		CHRM3
C044	4	180133701	180260284	766930	rs7660942	rs10866243	-5.91		
C044	8	39350791	39509376	158585	rs10088400	rs7829181	-5.47		tMDC tMDCII
C044	17	7431901	7519370	22438	rs2043680	rs16973682	-5.95		SAT2 ATP1B2 TP53* SHBG
C044	X	108067816	108417317						
C052	9	9109327	9740139	45353	rs7034934	rs11792302	-5.88		PTPRD*
C054	9	6282506	6392856	799373	rs172862	rs2761763	-5.66		TPD52L3
C054	9	21892354	21987872	77526	rs4977746	rs3218020	-5.48		CDKN2A* MTAP
C057	3	3276678	3322090	60256	rs2687169	rs478899	-6		
C058	3	163993833	164109279	115446	rs206280	rs206276	-6.33		BC073807
C058	9	21653040	24047376	1960037	rs10811581	rs10966009	-6.28		CDKN2A* CDKN2B* DMRTA1 ELAVL2
C060	1	58176211	58236351	60140	rs1857380	rs1749778	-6.57		DAB1†
C060	3	163993833	164109279	115446	rs206280	rs206276	-6.51		BC073807
C062	9	21807777	22105959	251240	rs885518	rs10116277	-6.07		CDKN2A* CDKN2B* MTAP
C062	10	85850651	86297467	270578	rs2007570	rs7078571	-5.95		PCHD21 RGR GHITM LRCC21
C062	10	89250313	90317633	1074502	rs17096113	rs2477958	-6.15		PTEN* ATAD1 PAPSS2 MINPP1
C062	10	90543031	90781948	191887	rs4934436	rs11594137	-6.75		ACTA2† FAS† STAMBPL1 ANKRD22
C062	10	90934639	91152477	95465	rs17122322	rs10881603	-6.53		CH25H† LIPA IFIT2 IFIT3
C062	10	91228189	91323654	121112	rs12800642	rs2868510	-4.91		SLC16A12
C062	11	55096955	55217364	120409	rs559449	rs2868510	-5.36		
C065	11	55096955	55217364	692075	rs4944483	rs527389	-6.21		
C067	3	163992511	164109279	115446	rs206280	rs206276	-5.95		BC073807
C067	11	55096252	55217364	120409	rs559449	rs2868510	-5.21		
C067	18	36511380	36533818	22438	rs2043680	rs16973682	-5.64		
C071	4	115388885	115407950	19065	rs9307402	rs4470706	-5.83		
C071	5	151493576	151503071	9495	rs40266	rs11167571	-6.14		AK001582
C071	9	11626204	11673392	2002087	rs1887671	rs1360233	-6.36		
C071	9	20866259	22953773	2814579	rs12337907	rs4645624	-5.92	CDKN2A* CDKN2B* DMRTA1	
C071	10	89647130	89767768	60093	rs809367	rs2673813	-5.71		PTEN*
C071	18	36511380	36533818	134676	rs918442	rs7254995	-5.29		
C077	3	163993833	164109279	115446	rs206280	rs206276	-6.51		BC073807
C077	6	162824155	162884635	88740	rs11972861	rs6946957	-6.31		PARK2
C081	9	9206473	9251826	49365	rs3858066	rs16929560	-6.28		PTPRD*
C081	9	9415605	9464970	346262	rs16929713	rs7873669	-5.99		PTPRD*
C081	9	9530141	9876403	47188	rs10809545	rs2821205	-6.31		PTPRD*
C081	9	16686187	16828211	12387451	rs16937678	rs11789732	-6.8		BNC2
C081	9	21820157	22071397	95518	rs756641	rs3218020	-5.84		CDKN2A* CDKN2B* MTAP
C081	10	1962320	2070204	116646	rs11511683	rs62209	-6.11		AK097474
C081	11	85980974	85999224	58627	rs472074	rs2370643	-6.82		ME3
C081	14	40673884	40739852	65968	rs9788429	rs1778370	-5.85		
C081	X	31711148	31962281	260637	rs1293891	rs1475317	-5.33		DMD
C081	X	32177890	32315937	349501	rs1836093	rs588891	-5.68		DMD
C084	3	163993833	164109279	53733	rs13128386	rs4470706	-4.7		BC073807

Sample	Chr	Start	End	Length	First SNP	Last SNP	LogR Ratio	Large Deletions Events	Focal Deletions Events
C084	8	39350791	39509376	124313	rs2281785	rs9987682	-6.72		tMDC tMDCII
C084	21	18148695	18359851	311599	rs2521655	rs5979249	-6.32		CHODL
C086	5	151493576	151503071	105118	rs9350318	rs9465985	-6.14		AK001582
C088	4	115388885	115407950	19065	rs9307402	rs4470706	-6.07		
C089	7	146151167	146239907	158585	rs10088400	rs7829181	-6.01		CNTNAP2
C089	9	2171854	2296167	110350	rs10815402	rs7045097	-6.72		SMARCA2
C089	9	16666601	16769899	142024	rs1888207	rs4995398	-5.71		BNC2
C089	9	20511543	31510947	2087514	rs1986633	rs1463029	-6.44	CDKN2A* CDKN2B* DMRTA1 MLLT3	
C089	11	121208710	121267337	1361496	rs10773554	rs1513196	-6.12		
C091	9	21910346	21987872	77526	rs4977746	rs3218020	-6.4		CDKN2A* MTAP
C091	10	89731786	89791879	238917	rs303499	rs1926189	-6.03		
C096	9	21910346	21987872	44735	rs10757260	rs3218020	-6.86		CDKN2A* CDKN2B* MTAP
C097	8	39350791	39509376	158585	rs10088400	rs7829181	-5.47		tMDC tMDCII
C097	9	21209601	24024180	2394336	rs4512473	rs10966189	-6.31	CDKN2A* CDKN2B* DMRTA1	
C097	14	40673884	40739852	33911	rs13380027	rs703237	-5.4		
C100	4	115388885	115407950	19065	rs9307402	rs4470706	-5.74		
C100	X	9702043	10013642	251133	rs2054233	rs1921371	-5.77		SHROOM2 WWC3
C106	4	115388885	115407950	19065	rs9307402	rs4470706	-5.83		

Supplementary Figure 4: Pathways affected in C series

Mutations occuring within cell lines have been grouped according to biological pathways. Some mutation events putatively regulate pathways, however these events have not been functionally validated (highlighted in cream). Highlighted cells in green represent functionally characterised mutations that can affect downstream signalling.

	MAPK Pathway: NRAS→ BRAF→MEK→ERK			PI3K Pathway: KIT→ NRAS→ PIK3CA (PTEN) → AKT		
Sample	Mutated	Known	Putative	Mutated	Known	Putative
C001	Y	NRAS (Q61K)		Y	NRAS (Q61K)	
C002	Y	NRAS (Q61K/AMP)		Y	NRAS (Q61K/AMP)	
C004	Y	BRAF (V600E)/ MEK AMP		Y		SGK3 (AMP-L) PAK1 (AMP-L) NRG4 (AMP-L)
C006	Y	NRAS (Q61L)		Y	NRAS (Q61L)	
C011	Y	BRAF (V600E)				
C013	Y	NRAS (Q61L)		Y	NRAS (Q61L)/ PTEN (G127E)	
C017	Y	BRAF (V600E)	PTK2B (G414V)	Y		PTK2B (G414V)
C021	Y		RAC1 (P29S)	Y		RAC1 (P29S)
C022	Y		TFG (Q309K)			
C025	Y		TFG (P199S/ P361L)/ NEK10 (E379K)			
C027	Y	NRAS (Q61K)		Y	NRAS (Q61K)	
C037						
C038	Y	BRAF(V600E)		Y	PTEN (H75X)	
C042	Y	BRAF(V600E)		Y	PTEN (HD)	
C044	Y	BRAF(V600E)	CHRM3 (HD)	Y	PTEN (DEL Exon 6)	CHRM3 (HD)
C045	Y	BRAF(V600E)				
C052						
C054	Y	NRAS (Q61K)		Y	NRAS (Q61K)	
C055	Y	BRAF(V600E)	TFG (HD)	Y	PTEN (Del Exon 4)	
C057	Y	BRAF(V600E)		Y	PTEN (DEL Exon 6)	
C058	Y	BRAF (L597S)				
C060	Y	BRAF(V600E)				
C062	Y	BRAF(V600E)		Y	PTEN (HD)	
C065	Y	BRAF(V600E)				
C067	Y		EPHB1 (AMP-L)	Y	NF1 (Q1774X)	MRAS (AMP-L), EGFR (P195L)
C071	Y	BRAF(V600E)	EGF (AMP-L)	Y	PTEN (HD)	
C074	Y	BRAF(V600E)				
C077	Y		BRAF (S467L)/ PTK2B (E577K)	Y	ERBB4 (E452K)/ NF1 (Q1055X)	EGFR (R831C, T9401I)
C078	Y	BRAF(V600E)				
C081	Y	BRAF (V600K)				
C083	Y	NRAS (Q61L)	RAC1 (P29S)	Y	NRAS (Q61L)	RAC1 (P29S)
C084	Y		PTK2B (V833I)	Y	NF1 (R2496X)	
C086	Y		TFG (P380L)			
C088	Y	BRAF (V600K)	RAP1B (AMP-L)	Y		RAP1B (AMP-L)
C089	Y	BRAF(V600E)		Y		MET (T1010I)
C091	Y	BRAF(V600E)		Y	PTEN (Del Exon 6,6)	
C094	Y	BRAF(V600E)				
C096	Y	NRAS (Q61R)		Y	NRAS (Q61R)	
C097	Y	BRAF(V600E)		Y	PTEN (G165V)	
C100	Y	BRAF (G469R)		Y	PIK3CA (R88Q)	
C106	Y	NRAS (Q61L)		Y	NRAS (Q61L)	
C108	Y	BRAF (V600K)				

	TP53 Pathway: p14ARF→MDM2→TP53→CDKN1A			RB Pathway: P16INK4A→CDK4→RB1		
Sample	**Mutated**	**Known**	**Putative**	**Mutated**	**Known**	**Putative**
C001	Y	TP53 (Del257-EDSS)				
C002	Y	P14ARF (HD)		Y	P16INK4A (HD)	
C004	Y		TERT (AMP-L)			
C006						
C011						
C013						
C017						
C021	Y	TP53 (N247K, R248W)				
C022						
C025						
C027						
C037						
C038	Y		MCM4 (AMP-L)/ HEY1 (AMP-L)	Y	P16INK4A (HD)	
C042	Y	P14ARF (HD)		Y	P16INK4A (HD)	
C044	Y	TP53 (HD)				
C045	Y	TP53 (A138V)				
C052	Y		BRIP1 (AMP-L)	Y	CCND1 (AMP-L)	
C054				Y	P16INK4A (HD)	
C055	Y	P14ARF (HD)		Y	P16INK4A (HD)	
C057				Y	CDK4 (R24C)	
C058	Y	P14ARF (HD)		Y	P16INK4A (HD)	
C060	Y	MDM2 (AMP)				
C062	Y	P14ARF (HD)		Y	P16INK4A (HD)	
C065	Y	TP53 (R247K)				
C067	Y		ATR (AMP-L)/ CDKN2AIP (L303X)	Y	RB1 (E209K)	
C071	Y	P14ARF (HD) TP53 (A119X)		Y	P16INK4A (HD)/ CDK4 (R24C)	
C074	Y	TP53 (V172fs)				
C077	Y	TP53 (Q100X)				
C078						
C081	Y	P14ARF (HD)		Y	P16INK4A (HD)	
C083	Y	TP53 (C275F)				
C084	Y	TP53 (W260C)	BRCA2 (P721S)			
C086	Y	TP53 (E286X)				
C088						
C089	Y	P14ARF (HD)		Y	P16INK4A (HD)/ CCND1 (AMP_L)	
C091				Y	P16INK4A (HD)	
C094						
C096	Y		TOPORS (AMP)	Y	P16INK4A (HD)	
C097	Y	P14ARF (HD)		Y	P16INK4A (HD)	
C100	Y	TP53 (R342X)				
C106						
C108						

	Apoptotic Pathway			Transcription/ Chromatin Modification		
Sample	**Mutated**	**Known**	**Putative**	**Mutation**	**Known**	**Putative**
C001						
C002	Y		PTPRD (HD)			
C004	Y		IGFR1 (AMP-L)			
C006						
C011						
C013						
C017				Y	HDAC4 (HD)	
C021						
C022				Y		SMARCA2 (P31L)
C025	Y	MAP3K5 (R256C)				
C027						
C037						
C038	Y	MAP3K9 (S616F)		Y		MYC (AMP-L)
C042						
C044						
C045				Y	MITF (AMP-L)	
C052	Y		PTPRD (HD)	Y	MITF (AMP-L)	
C054						
C055						
C057						
C058						
C060	Y	MAP3K5 (D408N)	IGFR1 (AMP-L)			
C062	Y	MAP3K9 (W333X)	FAS (HD)			
C065	Y	MAP3K9 (S650L)				
C067	Y		MAPK10 (P271S)	Y		HDAC9 (D720N)
C071				Y		PRDM5 (AMP)
C074						
C077	Y	MAP3K9 (S650L)	PTPRD (Multiple)	Y	TRRAP (L422F)	HDAC9 (K263I)
C078						
C081	Y		PTPRD (HD)			
C083						
C084	Y	MAP3K9 (G600R, R827Q, P963S)	PTPRD (Multiple)	Y		EZH2 (E344X)/ HDAC9 (T893I)
C086	Y	MAP3K9 (D176N, P1020S, P1075S)				
C088						
C089				Y		SMARCA2 (HD)
C091	Y	MAP3K5 (E1096K)				
C094						
C096	Y	MAP3K9 (S533Y)		Y		TOPORS (AMP)
C097						
C100						
C106						
C108						

	Microenvironment Reorganisation/ cytoskeleton reorgansiation/ mobiltiy			SMAD Pathway		
Sample	**Mutated**	**Known**	**Putative**	**Mutated**	**Known**	**Putative**
C001						
C002	Y		RHOC (AMP-L)/ ADAM30 (AMP-L)			
C004						
C006						
C011						
C013						
C017						
C021	Y		ACTA2 (G148E)			
C022						
C025	Y		ACTA2 (G160I)			
C027						
C037						
C038						
C042				Y		BMPR1A (HD)
C044						
C045						
C052						
C054						
C055	Y		VEGFA (AMP-L)			
C057						
C058						
C060						
C062	Y		ACTA2 (HD)			
C065						
C067				Y		MEN1 (D362N)
C071						
C074						
C077						
C078				Y		BMPR1A (Y245S)
C081						
C083						
C084						
C086						
C088						
C089						
C091						
C094						
C096						
C097						
C100						
C106						
C108						

	Beta Catenin/ WNT Pathway			RNA splicing and Regulation		
Sample	**Mutated**	**Known**	**Putative**	**Mutated**	**Known**	**Putative**
C001						
C002	Y		NOTCH2 (AMP-L)			
C004						
C006						
C011						
C013						
C017						
C021						
C022						
C025						
C027						
C037						
C038						
C042						
C044						
C045						
C052						
C054						
C055	Y		SOX4 (AMP-L)			
C057						
C058						
C060						
C062						
C065						
C067			NOTCH2 (S204F)			
C071						
C074						
C077				Y		DROSHA/ DICER (mutation)
C078						
C081						
C083						
C084			NOTCH2 (P1335S)	Y		DROSHA/ DICER (mutation)
C086						
C088						
C089						
C091						
C094						
C096				Y		DROSHA (AMP-L)
C097						
C100						
C106						
C108						

Supplementary Table 5- Coverage Statistics of exome sequencing

Sample	Total Reads	Total yield (bp)	Average Read Length (bp)	Mappable Reads	Mappable Yield (bp)	% Mappable Reads (out of total)	On-Target reads	On-Target Yield	% On-Target Reads (mappable)	% On-Target Reads (out of total)	Target Regions (bp)
C022	70,998,058	7,170,803,858	101	63,575,628	5,984,700,420	89.50%	46,379,145	3,586,034,792	73.00%	65.30%	62,085,286
C022-LCL	68,032,178	6,871,249,978	101	59,453,129	5,297,227,123	87.40%	43,586,287	3,201,940,999	73.30%	64.10%	62,085,286
C067	73,407,376	7,414,144,976	101	65,857,998	6,202,023,692	89.70%	47,717,322	3,693,684,521	72.50%	65.00%	62,085,286
C067-LCL	62,511,060	6,313,617,060	101	56,324,082	5,318,554,843	90.10%	41,210,595	3,194,751,103	73.20%	65.90%	62,085,286
C077	103,493,042	10,452,797,242	101	91,946,640	8,666,731,315	88.80%	65,980,700	5,098,996,818	71.80%	63.80%	62,085,286
C077-LCL	61,455,492	6,207,004,692	101	53,400,273	4,741,542,227	86.90%	38,767,216	2,832,717,858	72.60%	63.10%	62,085,286
C084	113,654,126	11,479,066,726	101	101,338,085	9,526,057,650	89.20%	73,533,786	5,672,052,439	72.60%	64.70%	62,085,286
C084-LCL	58,521,330	5,910,654,330	101	51,396,974	4,593,962,144	87.80%	37,762,445	2,779,541,258	73.50%	64.50%	62,085,286

Sample	% Coverage of target Region (more than 1X)	Number of on-target genotypes (more than 1X)	% Coverage of target region (more than 10X)	Number of on-target genotypes (more than 10X)	Median Read Depth of target regions	Median Read Depth of target regions	Number of SNPs	Number of Coding SNPs	Number of Indels	Number of coding indels
C022	93.50%	58,071,121	82.30%	51,111,257	48.0X	57.8X	68,436	20,541	13,313	511
C022-LCL	91.70%	56,930,863	80.30%	49,825,107	43.0X	51.6X	59,972	18,138	11,035	468
C067	93.50%	58,024,995	82.50%	51,239,846	49.0X	59.5X	61,429	17,815	11,860	452
C067-LCL	93.10%	57,775,816	80.90%	50,219,060	43.0X	51.5X	62,789	18,164	12,060	468
C077	93.50%	58,069,453	84.80%	52,620,094	67.0X	82.1X	78,193	24,004	12,680	504
C077-LCL	92.00%	57,099,410	80.10%	49,742,423	39.0X	45.6X	59,731	18,015	11,148	474
C084	93.90%	58,285,670	85.40%	53,041,099	73.0X	91.4X	79,870	24,308	13,349	532
C084-LCL	91.50%	56,801,782	77.90%	48,386,709	37.0X	44.8X	58,402	17,686	10,702	457

Sample	Total Mutations	Nonsynymous	Synonymous	NS:S Ratio	FS Ins	FS Del	NonFS Ins	NonFS Del	Splicing	Stop Gain	Stop Loss
C022	378	243	118	2.06	1	0	0	0	6	10	0
C067	842	508	281	1.81	2	3	0	0	11	37	0
C077	3766	2209	1333	1.66	1	2	0	1	44	176	0
C084	3873	2348	1304	1.80	2	0	0	1	35	181	2

Known mutations occuring within cell lines prior to WES:

	Gene	Mutation	Detected *	Detected ^	QS
C022	TFG	Q309K	No	Yes	55
C067	None	N/A	N/A	N/A	N/A
C077	TP53	Q100X	No	Yes	22
C077	ERBB4	E452K	No	No	N/A
C077	MAP3K9	S650L	Yes	Yes	174
C084	MAP3K9	G600R	Yes	Yes	110
C084	MAP3K9	R827Q	Yes	Yes	146
C084	MAP3K9	P963S	No	Yes	25
C084	TP53	W260C	Yes	Yes	208

* Using standard QS as described in Methods
^ Detected in data set without QS applied

Supplementary Table 7: Genes previously identified to be mutated in melanoma that were identified from WES

Sample	chr	chr_start	chr_end	Gene	Accesion Number	Exon	Coding Change	Protein Change	Region	Mutation Type	ref_base	alt_base	zygosity	quality	depth	alt_depth	%Mutant Read
C084	10	127782630	127782630	ADAM12	NM_021641	exon11	c.C1078T	p.H360Y	exonic	nonsynonymous	G	A	het	176	101	45	45
C067	8	39535036	39535036	ADAM18	NM_014237	exon15	c.C1612T	p.P538S	exonic	nonsynonymous	C	T	het	183	116	55	47
C067	8	39564383	39564383	ADAM18	NM_014237	exon18	c.C1977T	p.S659S	exonic	synonymous	C	T	het	115	88	39	44
C084	8	39468155	39468155	ADAM18	NM_001190956	exon6	c.C452T	p.S151F	exonic	nonsynonymous	C	T	het	137	127	70	55
C077	5	156964958	156964958	ADAM19	NM_033274	exon4	c.C293T	p.S98L	exonic	nonsynonymous	G	A	het	121	119	88	74
C077	8	39645760	39645760	ADAM2	NM_001464	exon9	c.C653T	p.S218L	exonic	nonsynonymous	G	A	het	112	111	48	43
C077	8	39691497	39691497	ADAM2	NM_001464	exon3	c.G154T	p.E52X	exonic	stopgain	C	A	het	157	219	111	51
C084	8	39644506	39644506	ADAM2	NM_001464	exon10	c.G878A	p.G293E	exonic	nonsynonymous	C	T	het	106	128	59	46
C084	8	39645654	39645654	ADAM2	NM_001464	exon9	c.G759A	p.W253X	exonic	stopgain	C	T	hom	164	189	188	99
C022	14	70924671	70924671	ADAM21	NM_003813	exon2	c.C455T	p.A152V	exonic	nonsynonymous	C	T	het	124	213	78	37
C077	8	24187615	24187615	ADAM28	NM_021777	exon11	c.G1090A	p.D364N	exonic	nonsynonymous	G	A	het	128	97	52	54
C077	8	24188816	24188816	ADAM28	NM_021777	exon12	c.G1257A	p.E419E	exonic	synonymous	G	A	het	137	110	67	61
C084	8	24188771	24188771	ADAM28	NM_021777	exon12	c.C1212T	p.I404I	exonic	synonymous	C	T	het	198	130	65	50
C067	4	175898230	175898230	ADAM29	NM_001130705	exon3	c.G1554A	p.E518E	exonic	synonymous	G	A	het	143	53	29	55
C084	4	175897700	175897700	ADAM29	NM_001130705	exon3	c.C1024T	p.H342Y	exonic	nonsynonymous	C	T	het	166	212	108	51
C084	4	175897793	175897793	ADAM29	NM_001130705	exon3	c.G1117A	p.D373N	exonic	nonsynonymous	G	A	het	165	162	67	41
C084	4	175898695	175898695	ADAM29	NM_001130705	exon3	c.A2019T	p.K673N	exonic	nonsynonymous	A	T	het	151	64	38	59
C084	4	175898894	175898894	ADAM29	NM_001130705	exon3	c.C2218T	p.Q740X	exonic	stopgain	C	T	het	149	194	90	46
C084	4	175896778	175896778	ADAM29	NM_001130705	exon3	c.G102A	p.V34V	exonic	synonymous	G	A	het	173	48	25	52
C084	4	175897699	175897699	ADAM29	NM_001130705	exon3	c.C1023T	p.N341N	exonic	synonymous	C	T	het	166	212	108	51
C077	1	120437739	120437739	ADAM30	NM_021794	exon1	c.G1221A	p.V407V	exonic	synonymous	C	T	het	186	129	78	60
C084	1	120437573	120437573	ADAM30	NM_021794	exon1	c.G1387A	p.E463K	exonic	nonsynonymous	C	T	het	172	129	67	52
C084	1	120437208	120437208	ADAM30	NM_021794	exon1	c.C1752T	p.L584L	exonic	synonymous	G	A	het	185	137	66	48
C077	8	39114812	39114812	ADAM32	NM_145004	exon19	c.G2112A	p.R704R	exonic	synonymous	G	A	het	104	194	109	56
C077	8	24324458	24324458	ADAM7	NM_003817	exon6	c.C536T	p.T179I	exonic	nonsynonymous	C	T	het	168	93	43	46
C077	8	24339764	24339764	ADAM7	NM_003817	exon9	c.C815T	p.S272L	exonic	nonsynonymous	C	T	het	110	111	68	61
C084	8	24348379	24348379	ADAM7	NM_003817	exon13	c.G1334A	p.G445E	exonic	nonsynonymous	G	A	het	106	145	69	48
C084	8	24350042	24350042	ADAM7	NM_003817	exon15	c.G1587A	p.M529I	exonic	nonsynonymous	G	A	het	152	205	99	48
C084	8	24358371	24358371	ADAM7	NM_003817	exon19	c.C2071T	p.R691C	exonic	nonsynonymous	C	T	het	124	163	70	43
C084	8	24324357	24324357	ADAM7	NM_003817	exon6	c.G435A	p.V145V	exonic	synonymous	G	A	het	105	248	102	41
C084	8	24350592	24350592	ADAM7	NM_003817	exon16	c.G1692A	p.G564G	exonic	synonymous	G	A	het	135	138	69	50
C084	19	8668655	8668655	ADAMTS10	NM_030957	exon5	c.C549T	p.S183S	exonic	synonymous	G	A	het	154	79	43	54
C077	5	33588852	33588852	ADAMTS12	NM_030955	exon18	c.G2717A	p.R906Q	exonic	nonsynonymous	C	T	het	108	30	13	43
C084	5	33561225	33561225	ADAMTS12	NM_030955	exon20	c.G4032A	p.M1344I	exonic	nonsynonymous	C	T	het	159	66	28	42
C084	5	33615981	33615981	ADAMTS12	NM_030955	exon15	c.G2340A	p.L780L	exonic	synonymous	C	T	het	192	88	45	51
C077	9	136302896	136302896	ADAMTS13	NM_139025	exon13	c.G1463A	p.R488Q	exonic	nonsynonymous	G	A	hom	225	55	54	98
C077	10	72462086	72462086	ADAMTS14	NM_080722	exon3	c.G541A	p.D181N	exonic	nonsynonymous	G	A	het	130	27	14	52
C077	10	72498655	72498655	ADAMTS14	NM_080722	exon11	c.G1657A	p.G553R	exonic	nonsynonymous	G	A	het	107	37	24	65
C077	16	77334280	77334280	ADAMTS18	NM_199355	exon17	c.C2554T	p.P852S	exonic	nonsynonymous	G	A	het	119	51	28	55
C077	16	77398206	77398206	ADAMTS18	NM_199355	exon5	c.G851A	p.R284K	exonic	nonsynonymous	C	T	het	131	68	39	57
C084	16	77369788	77369788	ADAMTS18	NM_199355	exon12	c.G1724A	p.G575D	exonic	nonsynonymous	C	T	het	111	30	15	50
C067	5	128844784	128844784	ADAMTS19	NM_133638	exon3	c.G744A	p.Q248Q	exonic	synonymous	G	A	het	143	33	19	58
C084	5	128864234	128864234	ADAMTS19	NM_133638	exon6	c.C1174T	p.H392Y	exonic	nonsynonymous	C	T	het	155	220	92	42
C084	5	129037121	129037121	ADAMTS19	NM_133638	exon20	c.G2977A	p.G993R	exonic	nonsynonymous	G	A	het	196	149	72	48
C022	12	43771314	43771314	ADAMTS20	NM_025003	exon32	c.G4849A	p.E1617K	exonic	nonsynonymous	C	T	het	195	161	87	54
C077	12	43846407	43846407	ADAMTS20	NM_025003	exon13	c.C1852T	p.R618X	exonic	stopgain	G	A	het	111	121	79	65
C077	12	43925948	43925948	ADAMTS20	NM_025003	exon3	c.G504A	p.M168I	exonic	nonsynonymous	C	T	het	160	125	76	61
C084	12	43771365	43771365	ADAMTS20	NM_025003	exon32	c.T4798G	p.C1600G	exonic;splicing	nonsynonymous	A	C	het	146	79	38	48
C084	12	43826408	43826408	ADAMTS20	NM_025003	exon20	c.C2927T	p.S976F	exonic	nonsynonymous	G	A	het	130	134	70	52
C067	5	64483905	64483905	ADAMTS6	NM_197941	exon22	c.G2848A	p.E950K	exonic	nonsynonymous	C	T	het	108	39	19	49
C077	5	64511194	64511194	ADAMTS6	NM_197941	exon19	c.C2393T	p.S798F	exonic	nonsynonymous	G	A	het	131	96	69	72
C084	5	64483920	64483920	ADAMTS6	NM_197941	exon22	c.A2833G	p.T945A	exonic	nonsynonymous	T	C	het	138	85	36	42
C084	5	64520814	64520814	ADAMTS6	NM_197941	exon17	c.G2128A	p.G710R	exonic	nonsynonymous	C	T	het	142	113	46	41
C084	5	64558732	64558732	ADAMTS6	NM_197941	exon13	c.G1678A	p.G560S	exonic	nonsynonymous	C	T	het	121	60	30	50
C084	5	64511253	64511253	ADAMTS6	NM_197941	exon19	c.G2334A	p.R778R	exonic	synonymous	C	T	het	105	152	86	57
C067	15	79058897	79058897	ADAMTS7	NM_014272	exon19	c.C3356T	p.A1119V	exonic	nonsynonymous	G	A	het	119	13	10	77
C084	3	64601727	64601727	ADAMTS9	NM_182920	exon20	c.G2933A	p.G978D	exonic	nonsynonymous	C	T	het	180	109	52	48
C077	7	140481408	140481408	BRAF	NM_004333	exon11	c.C1400T	p.S467L	exonic	nonsynonymous	G	A	het	124	250	186	74

Sample	chr	chr_start	chr_end	Gene	Accesion Number	Exon	Coding Change	Protein Change	Region	Mutation Type	ref_base	alt_base	zygosity	quality	depth	alt_depth	%Mutant Read
C077	1	240071215	240071215	CHRM3	NM_000740	exon5	c.C464T	p.S155F	exonic	nonsynonymous	C	T	het	159	156	89	57
C077	1	240071328	240071328	CHRM3	NM_000740	exon5	c.T577A	p.W193R	exonic	nonsynonymous	T	A	het	167	140	56	40
C077	1	240071392	240071392	CHRM3	NM_000740	exon5	c.G641A	p.R214K	exonic	nonsynonymous	G	A	het	140	93	49	53
C077	1	240071941	240071941	CHRM3	NM_000740	exon5	c.G1190A	p.G397E	exonic	nonsynonymous	G	A	het	142	55	31	56
C084	1	240072236	240072236	CHRM3	NM_000740	exon5	c.G1485A	p.A495A	exonic	synonymous	G	A	het	126	63	25	40
C067	7	143094674	143094674	EPHA1	NM_005232	exon9	c.G1692A	p.G564G	exonic	synonymous	C	T	het	127	37	24	65
C084	3	89259197	89259197	EPHA3	NM_005233	exon3	c.G341A	p.G114E	exonic	nonsynonymous	G	A	het	110	162	87	54
C084	3	89498379	89498379	EPHA3	NM_005233	exon14	c.G2351A	p.G784E	exonic	nonsynonymous	G	A	het	142	114	50	44
C084	3	89498507	89498507	EPHA3	NM_005233	exon14	c.G2479A	p.E827K	exonic	nonsynonymous	G	A	het	114	77	39	51
C084	2	222347238	222347238	EPHA4	NM_004438	exon5	c.C1152T	p.V384V	exonic	synonymous	G	A	het	137	83	46	55
C022	4	66231763	66231763	EPHA5	NM_004439	exon11	c.G1937A	p.G646E	exonic	nonsynonymous	C	T	het	117	90	44	49
C077	3	97124080	97124080	EPHA6	NM_001080448	exon6	c.G1693A	p.G565R	exonic	nonsynonymous	G	A	het	194	100	67	67
C077	3	96962992	96962992	EPHA6	NM_001080448	exon5	c.G1467A	p.L489L	exonic	synonymous	G	A	het	177	128	59	46
C084	3	96533762	96533762	EPHA6	NM_001080448	exon1	c.G295A	p.E99K	exonic	nonsynonymous	G	A	het	113	72	26	36
C084	3	96706525	96706525	EPHA6	NM_001080448	exon3	c.C802T	p.R268C	exonic	nonsynonymous	C	T	het	121	195	73	37
C084	3	97329676	97329676	EPHA6	NM_001080448	exon13	c.G2552A	p.G851E	exonic	nonsynonymous	G	A	het	140	187	91	49
C084	6	93953145	93953145	EPHA7	NM_004440	exon17	c.G2996A	p.X999X	exonic	synonymous	C	T	het	188	188	103	55
C077	1	23111245	23111245	EPHB2	NM_017449	exon3	c.G487A	p.V163M	exonic	nonsynonymous	G	A	het	130	22	12	55
C084	1	23234629	23234629	EPHB2	NM_017449	exon12	c.G2320A	p.D774N	exonic	nonsynonymous	G	A	het	159	37	24	65
C022	7	100403158	100403158	EPHB4	NM_004444	exon15	c.C2643T	p.P881P	exonic	synonymous	G	A	hom	188	23	22	96
C077	7	100403245	100403245	EPHB4	NM_004444	exon15	c.C2556T	p.L852L	exonic	synonymous	G	A	het	101	18	9	50
C067	7	142563365	142563365	EPHB6	NM_004445	exon8	c.C1082T	p.P361L	exonic	nonsynonymous	C	T	het	160	53	43	81
C084	7	142566864	142566864	EPHB6	NM_004445	exon16	c.G2421A	p.L807L	exonic	synonymous	G	A	het	173	104	44	42
C067	2	212652844	212652844	ERBB4	NM_005235	exon4	c.C462T	p.F154F	exonic	synonymous	G	A	het	105	99	66	67
C077	8	38279355	38279355	FGFR1	NM_001174065	exon8	c.C1035T	p.I345I	exonic	synonymous	G	A	het	186	118	65	55
C022	10	123276903	123276903	FGFR2	NM_001144916	exon5	c.G669A	p.G223G	exonic	synonymous	C	T	het	123	56	46	82
C077	10	123276929	123276929	FGFR2	NM_001144916	exon5	c.C643T	p.R215W	exonic	nonsynonymous	G	A	het	166	113	64	57
C077	10	123279527	123279527	FGFR2	NM_001144916	exon4	c.G560A	p.G187E	exonic	nonsynonymous	C	T	het	115	17	14	82
C077	4	1808954	1808954	FGFR3	NM_001163213	exon18	c.C2392T	p.P798S	exonic	nonsynonymous	C	T	het	107	16	13	81
C084	4	1807647	1807647	FGFR3	NM_001163213	exon13	c.G1822A	p.E608K	exonic	nonsynonymous	G	A	het	104	23	15	65
C067	13	29004259	29004259	FLT1	NM_002019	exon8	c.A1034C	p.E345A	exonic	nonsynonymous	T	G	hom	225	35	35	100
C077	13	28885795	28885795	FLT1	NM_002019	exon27	c.C3567T	p.F1189F	exonic	synonymous	G	A	het	127	153	90	59
C084	13	28980009	28980009	FLT1	NM_002019	exon11	c.G1459A	p.E487K	exonic	nonsynonymous	C	T	het	175	142	76	54
C084	13	28877367	28877367	FLT1	NM_002019	exon30	c.C3954T	p.I1318I	exonic	synonymous	G	A	het	160	40	25	63
C067	5	180047651	180047651	FLT4	NM_182925	exon16	c.C2364T	p.F788F	exonic	synonymous	G	A	het	118	40	23	58
C077	5	180055946	180055946	FLT4	NM_182925	exon8	c.G1039A	p.A347T	exonic	nonsynonymous	C	T	het	161	29	20	69
C077	16	9858347	9858347	GRIN2A	NM_001134408	exon13	c.G3054A	p.V1018V	exonic	synonymous	C	T	het	140	41	17	41
C077	16	9858626	9858626	GRIN2A	NM_001134408	exon13	c.C2775T	p.I925I	exonic	synonymous	G	A	het	117	114	49	43
C084	16	9858373	9858373	GRIN2A	NM_001134408	exon13	c.C3028T	p.P1010S	exonic	nonsynonymous	G	A	het	125	81	36	44
C084	16	9858517	9858517	GRIN2A	NM_001134408	exon13	c.G2884A	p.E962K	exonic	nonsynonymous	C	T	het	156	99	55	56
C084	16	9927967	9927967	GRIN2A	NM_001134408	exon8	c.G1772A	p.G591E	exonic	nonsynonymous	C	T	het	163	126	67	53
C084	16	9892225	9892225	GRIN2A	NM_001134408	exon11	c.C2265T	p.I755I	exonic	synonymous	G	A	het	132	73	34	47
C077	3	51749418	51749418	GRM2	NM_000839	exon4	c.C1629T	p.G543G	exonic	synonymous	C	T	het	194	57	38	67
C084	3	51749694	51749694	GRM2	NM_001130063	exon2	c.G51A	p.R17R	exonic	synonymous	G	A	het	205	79	45	57
C077	7	86394513	86394513	GRM3	NM_000840	exon2	c.G52A	p.G18R	exonic	nonsynonymous	G	A	het	105	163	123	75
C077	7	86468659	86468659	GRM3	NM_000840	exon4	c.C1829T	p.S610L	exonic	nonsynonymous	C	T	het	149	146	107	73
C077	7	86394533	86394533	GRM3	NM_000840	exon2	c.G72A	p.G24G	exonic	synonymous	G	A	het	116	156	111	71
C084	7	86416186	86416186	GRM3	NM_000840	exon3	c.C1078T	p.Q360X	exonic	stopgain	C	T	het	118	94	68	72
C084	7	86468302	86468302	GRM3	NM_000840	exon4	c.C1472T	p.S491L	exonic	nonsynonymous	C	T	het	118	146	47	32
C077	6	34003713	34003713	GRM4	NM_000841	exon8	c.C2174T	p.S725L	exonic	nonsynonymous	G	A	het	144	33	20	61
C084	6	34059731	34059731	GRM4	NM_000841	exon2	c.C665T	p.S222F	exonic	nonsynonymous	G	A	het	109	82	30	37
C084	5	178416406	178416406	GRM6	NM_000843	exon5	c.G1013A	p.G338E	exonic;splicing	nonsynonymous	C	T	het	135	56	26	46
C084	5	178417637	178417637	GRM6	NM_000843	exon4	c.C968T	p.A323V	exonic	nonsynonymous	G	A	het	127	16	12	75
C077	3	7348325	7348325	GRM7	NM_000844	exon4	c.G1019A	p.R340Q	exonic	nonsynonymous	G	A	het	128	53	21	40
C084	3	7620937	7620937	GRM7	NM_000844	exon8	c.G2344A	p.E782K	exonic	nonsynonymous	G	A	het	176	134	63	47
C084	7	126173938	126173938	GRM8	NM_001127323	exon9	c.G1498A	p.E500K	exonic	nonsynonymous	C	T	het	125	121	83	69
C084	4	55561820	55561820	KIT	NM_000222	exon2	c.C210T	p.I70I	exonic	synonymous	C	T	het	142	112	61	54
C084	X	152482624	152482624	MAGEA1	NM_004988	exon3	c.G387A	p.L129L	exonic	synonymous	C	T	hom	225	88	88	100
C067	X	151303797	151303797	MAGEA10	NM_021048	exon4	c.C296T	p.S99F	exonic	nonsynonymous	G	A	hom	133	30	28	93
C084	X	151303126	151303126	MAGEA10	NM_021048	exon4	c.C967T	p.P323S	exonic	nonsynonymous	G	A	hom	205	56	56	100

Sample	chr	chr_start	chr_end	Gene	Accesion Number	Exon	Coding Change	Protein Change	Region	Mutation Type	ref_base	alt_base	zygosity	quality	depth	alt_depth	%Mutant Read
C084	X	151303610	151303610	MAGEA10	NM_021048	exon4	c.G483A	p.L161L	exonic	synonymous	C	T	hom	205	20	20	100
C084	X	151283937	151283937	MAGEA10-MAGEA5	NM_001204811	exon4	c.G76A	p.V26M	exonic	nonsynonymous	C	T	hom	225	34	34	100
C084	X	148797572	148797572	MAGEA11	NM_001011544	exon5	c.A339G	p.Q113Q	exonic	synonymous	A	G	hom	146	26	26	100
C084	X	151900189	151900189	MAGEA12	NM_005367	exon2	c.C612T	p.I204I	exonic	synonymous	G	A	hom	225	51	51	100
C077	X	151935945	151935945	MAGEA3	NM_005362	exon3	c.C222T	p.T74T	exonic	synonymous	G	A	hom	104	50	50	100
C077	X	151092889	151092889	MAGEA4	NM_002362	exon3	c.G753A	p.W251X	exonic	stopgain	G	A	hom	225	103	103	100
C077	X	151869950	151869950	MAGEA6	NM_005363	exon3	c.G640A	p.D214N	exonic	nonsynonymous	G	A	hom	225	59	59	100
C077	X	35820584	35820584	MAGEB16	NM_001099921	exon2	c.G271A	p.E91K	exonic	nonsynonymous	G	A	hom	169	69	69	100
C077	X	26157282	26157282	MAGEB18	NM_173699	exon2	c.C180T	p.I60I	exonic	synonymous	C	T	hom	174	59	58	98
C084	X	30236927	30236927	MAGEB2	NM_002364	exon2	c.C230T	p.S77L	exonic	nonsynonymous	C	T	hom	198	31	31	100
C022	X	30260943	30260943	MAGEB4	NM_002367	exon1	c.G691A	p.D231N	exonic	nonsynonymous	G	A	het	136	172	62	36
C084	X	30260533	30260533	MAGEB4	NM_002367	exon1	c.C281T	p.S94F	exonic	nonsynonymous	C	T	hom	176	92	92	100
C077	X	26212144	26212144	MAGEB6	NM_173523	exon2	c.C181T	p.P61S	exonic	nonsynonymous	C	T	hom	170	30	30	100
C077	X	26212289	26212289	MAGEB6	NM_173523	exon2	c.C326T	p.A109V	exonic	nonsynonymous	C	T	hom	213	47	46	98
C077	X	26212384	26212384	MAGEB6	NM_173523	exon2	c.G421A	p.D141N	exonic	nonsynonymous	G	A	hom	225	106	105	99
C084	X	26212662	26212662	MAGEB6	NM_173523	exon2	c.C699T	p.F233F	exonic	synonymous	C	T	hom	141	22	22	100
C084	X	26213034	26213034	MAGEB6	NM_173523	exon2	c.C1071T	p.F357F	exonic	synonymous	C	T	hom	153	43	43	100
C067	X	140995048	140995048	MAGEC1	NM_005462	exon4	c.G1858A	p.E620K	exonic	nonsynonymous	G	A	hom	185	93	93	100
C067	X	140996006	140996006	MAGEC1	NM_005462	exon4	c.T2816G	p.I939S	exonic	nonsynonymous	T	G	het	104	33	9	27
C077	X	140993449	140993449	MAGEC1	NM_005462	exon4	c.C259T	p.P87S	exonic	nonsynonymous	C	T	hom	209	95	95	100
C077	X	140995658	140995658	MAGEC1	NM_005462	exon4	c.C2468T	p.S823L	exonic	nonsynonymous	C	T	hom	225	62	62	100
C084	X	140994718	140994718	MAGEC1	NM_005462	exon4	c.C1528T	p.P510S	exonic	nonsynonymous	C	T	hom	137	206	204	99
C084	X	140996314	140996314	MAGEC1	NM_005462	exon4	c.G3124A	p.E1042K	exonic	nonsynonymous	G	A	hom	174	35	35	100
C084	X	140995878	140995878	MAGEC1	NM_005462	exon4	c.C2688T	p.S896S	exonic	synonymous	C	T	hom	174	69	69	100
C077	X	140984461	140984461	MAGEC3	NM_177456	exon5	c.C23T	p.P8L	exonic	nonsynonymous	C	T	hom	225	58	58	100
C084	X	140966965	140966965	MAGEC3	NM_138702	exon3	c.G263A	p.W88X	exonic	stopgain	G	A	hom	110	23	23	100
C084	X	51639835	51639835	MAGED1	NM_006986	exon4	c.T1084A	p.W362R	exonic	nonsynonymous	T	A	hom	225	29	29	100
C084	X	75651003	75651003	MAGEE1	NM_020932	exon1	c.C2680T	p.P894S	exonic	nonsynonymous	C	T	hom	201	80	80	100
C077	6	137019647	137019647	MAP3K5	NM_005923	exon4	c.G786A	p.K262K	exonic	synonymous	C	T	hom	145	29	29	100
C077	14	71201180	71201180	MAP3K9	NM_033141	exon10	c.C1949T	p.S650L	exonic	nonsynonymous	G	A	het	174	32	20	63
C084	14	71199648	71199648	MAP3K9	NM_033141	exon12	c.G2480A	p.R827Q	exonic	nonsynonymous	C	T	het	146	84	45	54
C084	14	71205008	71205008	MAP3K9	NM_033141	exon8	c.G1798A	p.G600R	exonic	nonsynonymous	C	T	het	110	58	25	43
C084	14	71197211	71197211	MAP3K9	NM_033141	exon13	c.C3243T	p.L1081L	exonic	synonymous	G	A	het	122	19	14	74
C084	14	71209174	71209174	MAP3K9	NM_033141	exon6	c.C1461T	p.I487I	exonic	synonymous	G	A	het	194	78	40	51
C084	11	102666210	102666210	MMP1	NM_001145938	exon5	c.G556A	p.D186N	exonic	nonsynonymous	C	T	het	204	91	57	63
C084	11	102645936	102645936	MMP10	NM_002425	exon7	c.C1049T	p.T350I	exonic	nonsynonymous	G	A	het	190	181	82	45
C067	14	23311135	23311135	MMP14	NM_004995	exon3	c.C271T	p.P91S	exonic	nonsynonymous	C	T	het	117	95	87	92
C077	14	23313705	23313705	MMP14	NM_004995	exon7	c.C1137T	p.F379F	exonic	synonymous	C	T	het	163	32	24	75
C077	11	5010922	5010922	MMP26	NM_021801	exon2	c.C144T	p.L48L	exonic	synonymous	C	T	hom	196	59	59	100
C084	11	5013262	5013262	MMP26	NM_021801	exon5	c.C664T	p.Q222X	exonic	stopgain	C	T	het	157	111	49	44
C084	11	5013252	5013252	MMP26	NM_021801	exon5	c.C654T	p.H218H	exonic	synonymous	C	T	het	163	107	48	45
C067	11	102567566	102567566	MMP27	NM_022122	exon5	c.G620A	p.G207E	exonic;splicing	nonsynonymous	C	T	hom	171	34	34	100
C084	11	102573778	102573778	MMP27	NM_022122	exon3	c.G413A	p.W138X	exonic	stopgain	C	T	het	147	152	56	37
C077	11	102706926	102706926	MMP3	NM_002422	exon10	c.G1365A	p.Q455Q	exonic	synonymous	C	T	het	173	189	124	66
C084	11	102592240	102592240	MMP8	NM_002424	exon4	c.C514T	p.P172S	exonic	nonsynonymous	G	A	het	143	108	62	57
C084	20	44639879	44639879	MMP9	NM_004994	exon5	c.C747T	p.R249R	exonic	synonymous	C	T	het	105	17	9	53
C077	1	115258716	115258716	NRAS	NM_002524	exon2	c.G66A	p.Q22Q	exonic	synonymous	C	T	het	180	80	39	49
C084	3	178928317	178928317	PIK3CA	NM_006218	exon9	c.C1503T	p.S501S	exonic	synonymous	C	T	hom	206	53	53	100
C077	1	208255772	208255772	PLXNA2	NM_025179	exon11	c.C2380T	p.P794S	exonic	nonsynonymous	G	A	het	126	51	35	69
C077	7	131982905	131982905	PLXNA4	NM_020911	exon4	c.G1448A	p.R483Q	exonic	nonsynonymous	C	T	het	151	80	62	78
C077	7	132193185	132193185	PLXNA4	NM_181775	exon3	c.G268A	p.E90K	exonic	nonsynonymous	C	T	het	159	45	37	82
C077	7	132192499	132192499	PLXNA4	NM_181775	exon3	c.G954A	p.A318A	exonic	synonymous	C	T	het	141	83	63	76
C084	7	131982865	131982865	PLXNA4	NM_020911	exon4	c.C1488T	p.I496I	exonic	synonymous	G	A	het	195	63	42	67
C077	3	48461589	48461589	PLXNB1	NM_002673	exon11	c.C2106T	p.A702A	exonic	synonymous	G	A	het	115	16	10	63
C084	3	48450871	48450871	PLXNB1	NM_002673	exon34	c.T5953C	p.F1985L	exonic	nonsynonymous	A	G	het	129	50	22	44
C084	3	48453990	48453990	PLXNB1	NM_002673	exon26	c.C4894T	p.R1632W	exonic	nonsynonymous	G	A	het	125	44	22	50
C067	22	50716411	50716411	PLXNB2	NM_012401	exon32	c.T4919A	p.F1640Y	exonic	nonsynonymous	A	T	hom	114	12	12	100
C077	8	27296633	27296633	PTK2B	NM_173176	exon19	c.G1729A	p.E577K	exonic	nonsynonymous	G	A	het	201	50	32	64
C084	8	27308569	27308569	PTK2B	NM_173176	exon26	c.G2497A	p.V833I	exonic	nonsynonymous	G	A	het	152	70	27	39
C077	9	8484270	8484270	PTPRD	NM_001040712	exon13	c.C2020T	p.R674C	exonic	nonsynonymous	G	A	hom	225	44	42	95

Sample	chr	chr_start	chr_end	Gene	Accesion Number	Exon	Coding Change	Protein Change	Region	Mutation Type	ref_base	alt_base	zygosity	quality	depth	alt_depth	%Mutant Read
C077	9	8492898	8492898	PTPRD	NM_002839	exon27	c.C2431T	p.R811C	exonic	nonsynonymous	G	A	het	192	70	40	57
C077	9	8633380	8633380	PTPRD	NM_001040712	exon3	c.G289A	p.E97K	exonic	nonsynonymous	C	T	hom	225	83	83	100
C084	9	8341785	8341785	PTPRD	NM_001040712	exon24	c.G3625A	p.E1209K	exonic	nonsynonymous	C	T	het	147	162	95	59
C084	9	8460517	8460517	PTPRD	NM_001040712	exon16	c.C2527T	p.P843S	exonic	nonsynonymous	G	A	het	150	98	58	59
C084	9	8507378	8507378	PTPRD	NM_001040712	exon10	c.C1591T	p.L531F	exonic	nonsynonymous	G	A	het	174	129	56	43
C084	9	8518092	8518092	PTPRD	NM_001040712	exon9	c.C1290T	p.T430T	exonic	synonymous	G	A	het	153	47	23	49
C067	13	48934170	48934170	RB1	NM_000321	exon7	c.G625A	p.E209K	exonic	nonsynonymous	G	A	hom	178	89	89	100
C084	17	7577501	7577501	TP53	NM_001126114	exon7	c.G780T	p.W260C	exonic	nonsynonymous	G	A	hom	208	18	18	100
C077	7	98506499	98506499	TRRAP	NM_003496	exon14	c.C1264T	p.L422F	exonic	nonsynonymous	C	T	het	178	90	55	61
C084	7	98565306	98565306	TRRAP	NM_003496	exon49	c.C7422T	p.I2474I	exonic	synonymous	C	T	het	189	99	65	66

Supplementary Table 8: Recurrent mutations identified in WES

Sample	chr	chr_start	Gene	Coding Change	Protein Change	Region	Mutation Type	ref_base	alt_base	zygosity	quality	depth	alt_depth	%Mutant Read
C022	3	75788376	ZNF717	c.398_399insAA	p.T133fs	exonic	frameshift insertion	-	TT	het	297	15	5	33
C084	3	75788376	ZNF717	c.398_399insAA	p.T133fs	exonic	frameshift insertion	-	TT	het	154	12	3	25
C077	1	152383341	CRNN	c.G217A	p.E73K	exonic	nonsynonymous	C	T	het	116	79	52	66
C084	1	152383341	CRNN	c.G217A	p.E73K	exonic	nonsynonymous	C	T	het	102	85	37	44
C077	11	100221562	CNTN5	c.G2938A	p.G980R	exonic	nonsynonymous	G	A	het	154	75	63	84
C084	11	100221562	CNTN5	c.G2938A	p.G980R	exonic	nonsynonymous	G	A	het	175	87	45	52
C067	12	18891447	CAPZA3	c.G245A	p.R82Q	exonic	nonsynonymous	G	A	het	182	137	68	50
C084	12	18891447	CAPZA3	c.G245A	p.R82Q	exonic	nonsynonymous	G	A	het	174	235	107	46
C067	12	21679895	C12orf39	c.C82T	p.P28S	exonic	nonsynonymous	C	T	het	120	70	35	50
C077	12	21679895	C12orf39	c.C82T	p.P28S	exonic	nonsynonymous	C	T	het	148	109	70	64
C067	15	83791524	TM6SF1	c.G497A	p.R166Q	exonic	nonsynonymous	G	A	het	155	115	58	50
C084	15	83791524	TM6SF1	c.G497A	p.R166Q	exonic	nonsynonymous	G	A	het	165	198	108	55
C022	15	89400951	ACAN	c.G5135A	p.G1712E	exonic	nonsynonymous	G	A	het	107	127	58	46
C077	15	89400951	ACAN	c.G5135A	p.G1712E	exonic	nonsynonymous	G	A	het	125	96	63	66
C077	3	121179028	POLQ	c.C7021T	p.H2341Y	exonic	nonsynonymous	G	A	het	190	68	43	63
C084	3	121179028	POLQ	c.C7021T	p.H2341Y	exonic	nonsynonymous	G	A	het	183	87	42	48
C077	8	70594573	SLCO5A1	c.C1628T	p.S543F	exonic	nonsynonymous	G	A	het	132	124	60	48
C084	8	70594573	SLCO5A1	c.C1628T	p.S543F	exonic	nonsynonymous	G	A	het	121	131	60	46
C077	5	35873660	IL7R	c.C616T	p.R206X	exonic	stopgain	C	T	het	148	135	60	44
C084	5	35873660	IL7R	c.C616T	p.R206X	exonic	stopgain	C	T	het	157	181	85	47
C067	10	100159886	PYROXD2	c.C444T	p.I148I	exonic	synonymous	G	A	het	170	38	21	55
C077	10	100159886	PYROXD2	c.C444T	p.I148I	exonic	synonymous	G	A	het	184	58	42	72
C067	17	28543202	SLC6A4	c.C870T	p.I290I	exonic	synonymous	G	A	hom	215	27	27	100
C077	17	28543202	SLC6A4	c.C870T	p.I290I	exonic	synonymous	G	A	het	138	34	25	74
C077	7	117175322	CFTR	c.C600T	p.F200F	exonic	synonymous	C	T	het	108	79	60	76
C084	7	117175322	CFTR	c.C600T	p.F200F	exonic	synonymous	C	T	het	130	123	79	64

Supplementary Table 9: Genes recurrently mutated in WES
List of genes that are mutated in either 4 samples or 3 samples that were Whole Exome Sequenced (WES)
This list of genes was compared against a set of 10 BRAF/NRAS mutated samples that were also WES
(Stark 2012 Nature Genetics and unpublished data). Genes mutated in 3 samples were analysed using Ingenuity
Direct relationship indicates exprimentally observed interaction, indirect indicates upstream/downstream genes

Genes mutated in all 4 samples

Gene	Accesion Number	Mutated in BRAF/NRAS samples
CSMD1	NM_033225	2
MUC16	NM_024690	3
MUC4	NM_018406	0
MXRA5	NM_015419	4
PKHD1	NM_170724	5
ZNF536	NM_014717	0

Genes mutated in 3 samples

Gene	Accesion Number	Mutated in BRAF/NRAS samples	Ingenuity analysis MAPK pathway	Relationship
A2M	NM_000014	0	RAS/ BRAF/ MEK/ ERK	Indirect
ABCC6	NM_001171	2		
ACAN	NM_001135	0	RAS/ BRAF/MEK/ ERK	Indirect
ACSM2B	NM_001105069	1		
ADAMTS20	NM_025003	2		
ADAMTS6	NM_197941	0		
AKNA	NM_030767	1		
ANK3	NM_020987	0	BRAF/ ERK	Indirect
ANKRD30A	NM_052997	0		
APOB	NM_000384	4	RAS/ BRAF/ ERK	Indirect
ARID1B	NM_017519	1	RAS/ BRAF/ ERK	Indirect
ARMC4	NM_018076	3		
ARPP21	NM_016300	1		
BRWD3	NM_153252	0		
CCDC147	NM_001008723	0		
CHD5	NM_015557	0		
CMYA5	NM_153610	0		
CNGB3	NM_019098	0		
CNTNAP2	NM_014141	1		
CNTNAP3	NM_033655	0		
CNTNAP4	NM_138994	1		
COL21A1	NM_030820	0		
COL3A1	NM_000090	0	RAS	Indirect
COL7A1	NM_000094	1		
CSMD1	NM_033225	2		
CSMD1	NM_033225	2		
CSMD2	NM_052896	2		
CSMD3	NM_052900	2		
CUBN	NM_001081	0		
CXorf30	NM_001098843	0		
DIP2B	NM_173602	0		
DNAH3	NM_017539	5		
DNAH5	NM_001369	9		
DNAH7	NM_018897	1		
DOCK11	NM_144658	0	RAS/ BRAF/ MEK	Indirect
DOCK2	NM_004946	1	RAC	Indirect
DPYD	NM_000110	3		
DSCAM	NM_001389	2		
EGFLAM	NM_001205301	0		
EYS	NM_198283	0		
F8	NM_000132	1		
FAM154A	NM_153707	0		
FBN3	NM_032447	4		
FNDC1	NM_032532	0		
FRAS1	NM_025074	0		

Gene	Accesion Number	Mutated in BRAF/NRAS samples	Ingenuity analysis MAPK pathway	Relationship
GPR98	NM_032119	0		
HDAC9	NM_001204147	1	RAS/ BRAF/MEK/ ERK	Indirect
HEATR7B2	NM_173489	0		
IGSF10	NM_178822	2		
KALRN	NM_001024660	2		
KLHL4	NM_057162	1	ERK	Indirect
LCT	NM_002299	1		
LPA	NM_005577	0	RAS	Indirect
LPHN2	NM_012302	0		
LRP1B	NM_018557	9		
MAGEC1	NM_005462	2		
ME1	NM_002395	0		
MGAM	NM_004668	1		
MOV10L1	NM_001164104	2		
MRGPRX2	NM_054030	0		
MUC16	NM_024690	3		
MUC4	NM_018406	0	RAS/ BRAF/ MEK/ ERK	Indirect
MXRA5	NM_015419	4		
MXRA5	NM_015419	4		
MYH1	NM_005963	1		
MYH7	NM_000257	0		
MYO18B	NM_032608	0		
MYOM2	NM_003970	0		
NCKAP5	NM_207481	0		
NEB	NM_001164507	0		
NF1	NM_000267	0	RAS/ BRAF/ MEK/ ERK	Indirect
NLRP2	NM_001174083	0	RAS/ BRAF/ ERK	Indirect
NLRP9	NM_176820	1		
ODZ1	NM_014253	4		
OR5AR1	NM_001004730	0		
PAPPA2	NM_020318	0		
PCNXL2	NM_014801	0		
PIPOX	NM_016518	0	ERK	Indirect
PKHD1	NM_170724	5		
PLCE1	NM_001165979	0	RAS/ BRAF	Indirect
PLCH1	NM_001130960	0		
PLXDC2	NM_032812	0		
PRICKLE1	NM_001144883	0	RAS/ BRAF/ MEK/ ERK	Indirect
PZP	NM_002864	0		
RAG1	NM_000448	1		
ROS1	NM_002944	0		
SCN10A	NM_006514	3		
SCN4A	NM_000334	0		
SCN8A	NM_014191	0		
SCN9A	NM_002977	0		
SFXN2	NM_178858	0		
SGK1	NM_001143676	0	BRAF	Direct
SH2B1	NM_015503	0	BRAF	Indirect
SIK3	NM_025164	1		
SPEG	NM_005876	0		
SVEP1	NM_153366	0		
SYNE1	NM_033071	4		
TMPRSS11A	NM_182606	0		
TP63	NM_001114979	1	RAS/ BRAF/ ERK	Indirect
TTN	NM_133378	8		
UNC5D	NM_080872	1		
USH2A	NM_206933	4		
YLPM1	NM_019589	0	RAS/ BRAF/ ERK	Indirect
ZFHX4	NM_024721	0		
ZFPM2	NM_012082	0		
ZNF536	NM_014717	0		
ZNF536	NM_014717	0		
ZNF638	NM_014497	0	BRAF	Indirect

Chapter 5

A high throughput panel for identifying clinically-relevant mutation profiles in melanoma

Ken Dutton-Regester, Darryl Irwin, Priscilla Hunt, Lauren G. Aoude, Varsha Tembe, Gulietta M. Pupo, Cathy Lanagan, Candace D. Carter, Linda O'Connor, Michael O'Rourke, Richard A. Scolyer, Graham J. Mann, Christopher W. Schmidt, Adrian Herington, Nicholas K. Hayward. A high throughput panel for identifying clinically-relevant mutation profiles in melanoma. Molecular Cancer Therapeutics. 2012. 11(4):888-897.

5.1. Relevance to thesis aims

This paper describes the development of a melanoma specific panel (MSP) of mutations that can be used for the identification of clinically-relevant mutation profiles in melanoma and addresses Aim 4 of the thesis. Recently, there has been significant success in treating cancers using molecularly based targeted drug strategies. One example is the use of BRAF inhibitor drugs, such as Vemurafenib or GSK2118436, in the treatment of late stage or disseminated melanoma. These drugs are specifically designed to antagonise the proliferative effect in tumours harbouring BRAF mutations with an amino acid substitution of valine at position 600 (V600E, V600K) and have begun to show improvements in overall survival rates. These results have prompted a number of clinical trials using molecularly based targeted approaches against alternative mutation events such as Imatinib in *KIT* mutated metastatic melanomas.

Interestingly, the clinical efficacy of molecularly based targeted drugs relies strongly on the presence of specific mutations within the patient's tumour. For example, use of Vemurafenib in *BRAF* wild type tumours can have the opposite effect and promote tumorigenesis while mutations both upstream and downstream of *BRAF* can render the drug ineffective. As such, the comprehensive identification of these mutations in a rapid fashion is desirable in a clinical setting so that it can inform on choice of modality for patient treatment. This report describes a MSP that can quickly assess the profile of mutations in tumours that could inform effective treatment regimens for melanoma.

A comprehensive literature search and interrogation of the Catalogue of Somatic Mutations in Cancer (COSMIC) database was used to generate a list of potential mutations to be included in the MSP. After filtering this list using strict criteria, a validation stage was performed before a final panel consisting of 46 assays interrogating 39 mutations in 20 genes was designed. In addition to the development of the MSP, this report investigates the occurrence of mutations identified from isolated literature reports in a large cohort of samples. This revealed that a number of mutations previously identified are either rare or patient specific mutation events; the results of which have important implications for future studies utilising next generation sequencing strategies. Lastly, a number of recurrent mutations were identified; reinforcing that these are possible drug targets that may be amenable to therapeutic intervention.

5.2. Contribution of the candidate

Project and experimental design was conducted in association with Nick Hayward and Darryl Irwin. Design of the validation panel and final MSP was performed in conjunction with Darryl Irwin. I performed sample preparation, generated and analysed results from the MSP, and drafted the manuscript.

5.3. Acknowledgment of the contribution of others

The panel of C series stage III metastatic melanoma samples were collected by Michael O'Rourke and cell lines from these samples were established by Cathy Lanagan, Linda O'Connor and Christopher W. Schmidt. Lauren Aoude contributed to the generation of cell line stocks and the subsequent extraction of DNA. An independent series of melanoma tumour and cell line DNA was prepared by members of the Melanoma Institute of Australia that included Gulietta M. Pupo, Varsha Tembe, Candace D. Carter, Richard A. Scolyer and Graham Mann. Darryl Irwin contributed to the design and analysis of the MSP while Priscilla Hunt performed independent replication experiments of the panel to assess accuracy. All authors reviewed and supplied comments on the final manuscript prior to publication.

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Chapter 6

Discussion

Advances in sequencing technology have changed the approach of cancer genomics in the identification of causal genes involved in tumorigenesis. However, as effective as these approaches are, a number of technological limitations still remain. In this section of the discussion, these problems are addressed in the following perspectives article:

Ken Dutton-Regester and Nick Hayward. Reviewing the somatic genetics of melanoma: from current to future analytical approaches. Pigment Cell and Melanoma Research. 2012. 25(2):144-54.

This review discusses a number of limitations associated with cancer genomics using next-generation sequencing analysis, in particular, the difficulty in identifying 'driver' mutation events from the background noise of high mutation rates observed in metastatic melanoma. Although next generation sequencing can be used to identify a number of interesting targets, those that are functionally relevant to the disease are increasingly difficult to identify. In order to overcome this, a number of complementary strategies can be employed that include transcriptome analysis, functional screens, and the combination of multiple high-throughput data sets. In addition, this review discusses areas that have currently been neglected such as the significance of mutations within non-coding regions of the genome.

In the final conclusion chapter, results from this thesis will be discussed in respect to current therapeutic strategies. This includes the putative involvement of *TFG* in the activation of the MAPK pathway, the association of a new apoptotic pathway in the development of melanoma through mutation of *MAP3K5* and *MAP3K9*, and the potential significance of mTOR inhibition in *NF1* mutant *BRAF*/*NRAS* WT melanomas. The decreasing costs and ability of next-generation sequencing to rapidly identify putative drug targets in tumours is rapidly seeing this technology move from research environments into clinical practice. The application of next-generation sequencing in regards to personalised medicine will be discussed and describes the remaining hurdles for the establishment of this technology for routine clinical practice.

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Chapter 7

Conclusion

7.1. Future directions of research

The results presented in this thesis identify a number of new mutations and genes involved in the development and progression of metastatic melanoma (Figure 7.1). These findings, although important in their respective manner, raise a number of questions and research directions that warrant further investigation.

7.1.1. The functional significance of mutations of *TFG* in metastatic melanoma

Chapter 2 describes the identification of a number of putative tumour suppressor genes involved in metastatic melanoma, including the mutation of *TFG* in 5% of melanomas [1]. Although mutations within *TFG* are interesting, experimental evidence is ultimately required to determine the role of these mutations in tumorigenesis.

The biological role of TFG has not been extensively investigated; however, *TFG* has been frequently involved in oncogenic fusion events in a variety of cancers [2-5]. During these events, the 5' end of constitutively expressed *TFG* is fused to the 3' end of a kinase resulting in deregulated signalling activity and increased cell proliferation. Although the functional effect of the oncogenic kinase fusion event has been determined, these studies have neglected to investigate the significance of the carboxyl deletion or disruption of *TFG* and its potential role in tumorigenesis.

It was previously shown that TFG can activate both the MAPK and NF-κB pathways [6]. In regards to the NF-κB pathway, evidence suggests that TFG, through direct binding, is involved in the same high molecular weight complex as NEMO, a member of the IκB complex [7]. The presence of several motifs within the TFG protein, including the presence of a coiled coil domain within the carboxyl end [8], and a Phox and Bem1p (PB1) module responsible for protein interactions and cytoplasmic signalling [9], suggests an important biological role in the binding of TFG to other proteins. Topical data provided in Chapter 2 shows a propensity for mutations to occur at the 3' end of *TFG;* this combined with

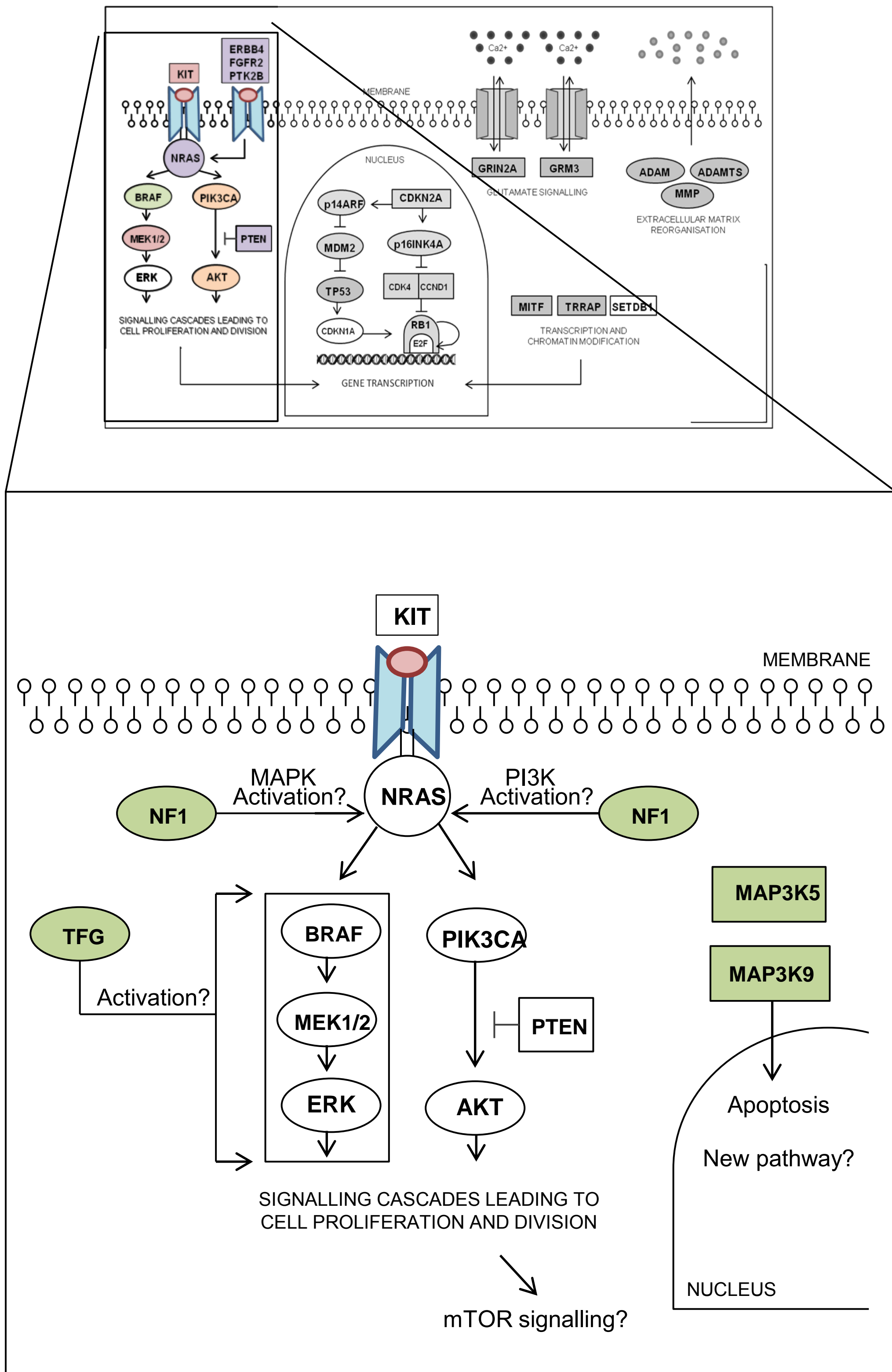

Figure 7.1: New genes mutated in melanoma identified in this thesis. Mutations were identified in TFG and NF1 and may be responsible for the activation of the MAPK pathway. Approximately 25% of melanomas exhibited a mutation in either MAP3K5 or MAP3K9 and may represent a novel apoptotic pathway to melanoma development.

oncogenic fusions resulting in the disruption of the 3' end of *TFG* provides supporting evidence that this region of the gene may be functionally important, hypothetically resulting in differential interactions with cellular proteins. In relation to the NF-κB pathway, mutation of TFG may affect its interaction with NEMO, causing constitutive activation of the pathway (Figure 7.2).

To determine the importance of TFG in tumorigenesis, a number of experiments could be performed. Firstly, in vitro mutagenesis studies that compare wild-type (WT) *TFG* to 3' end deleted or point mutated *TFG* could be used to assess the effect of abrogated protein function in a variety of cellular processes including proliferation, invasion and apoptosis. The aforementioned putative role of TFG in protein interactions could be assessed by the use of co-immunoprecipitation, linked with mass spectrometry analysis or western blot analysis. Abrogated function of the 3' end of TFG may also result in aberrant localisation of TFG and protein interactions within the cell; this could be assessed using immuno-fluorescence experiments. An example of aberrant protein localisation was recently demonstrated by increased nuclear expression of BRMS1 in metastatic melanoma compared to melanocytes, which contributed to increased cell invasion [10].

Point mutations of *TFG* were mutually exclusive to *NRAS* and *BRAF* mutations in metastatic melanoma; this is of relevance to melanomagenesis since this tumour type frequently exhibits constitutive activation of the MAPK pathway. As such, it is possible that mutation of *TFG* may represent an alternative mechanism to activation of the MAPK pathway. Targeted functional analysis focusing on MAPK activation through BRAF, MEK and ERK phosphorylation could be determined through ectopic expression of WT and mutant versions of TFG and western blot analysis in cell lines. If mutation resulted in the activation of these proteins, confirmation of the functional effect on the cell should be tested though a variety of in vitro experiments including proliferation, scratch wound closure and gel invasion assays. Appropriate in vivo studies such as tumorigenicity assays in mice would provide strong evidence towards an important functional role of TFG in melanoma.

Lastly, it is important to note that the overall numbers of melanomas with TFG mutations within this study are relatively low. Sequencing of larger cohorts of melanomas, including *BRAF/NRAS* WT or *BRAF/NRAS* mutant tumours, will be required to determine an

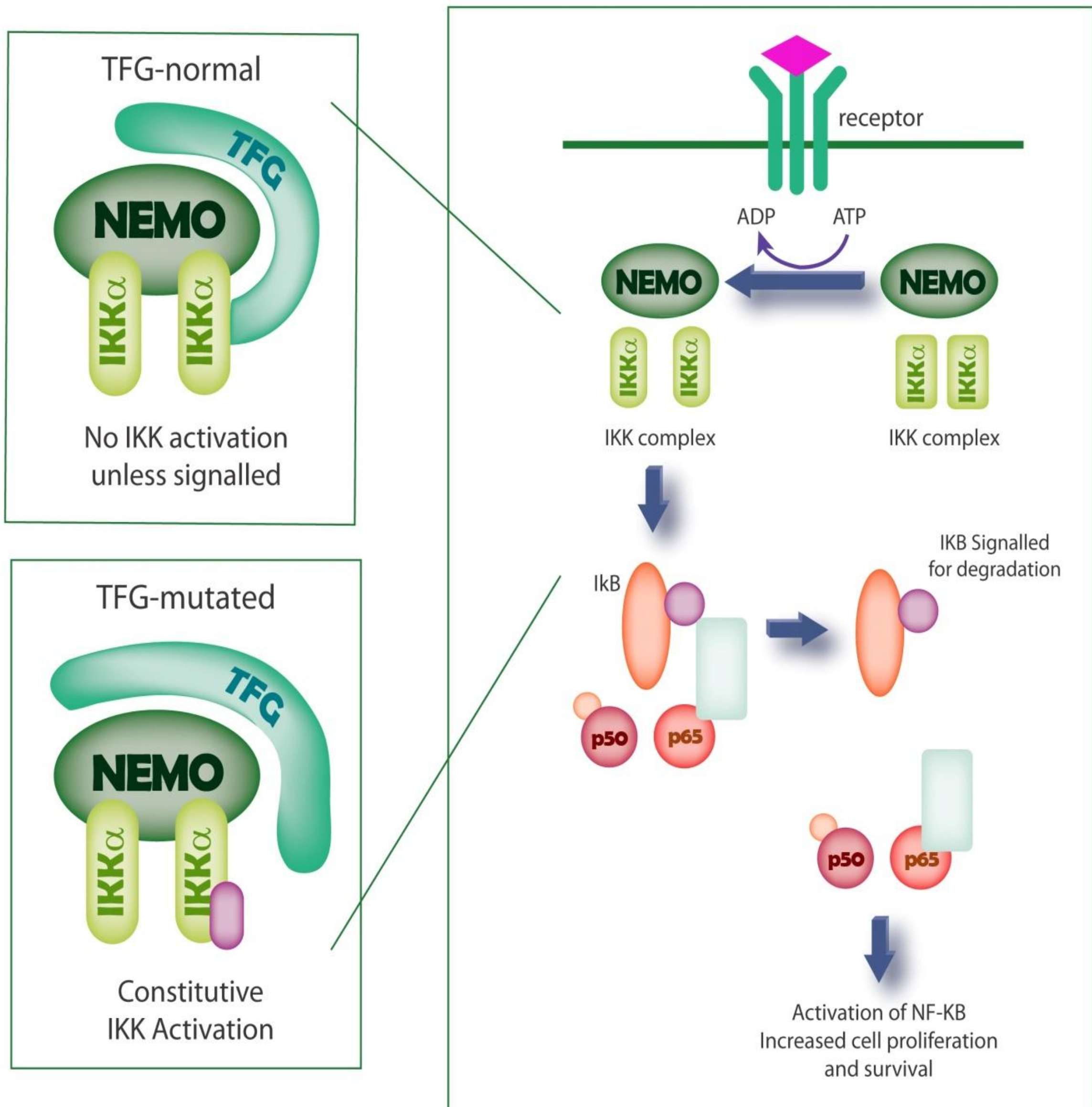

Figure 7.2: Hypothetic model of how mutations within TFG may affect activation of the MAPK pathway. Mutation of TFG, particularly the carboxyl-end of the protein, may change its interaction with other proteins. In this example, mutation of TFG results in a structural alteration of the IKK complex, leading to over-activation of the NF-κB pathway.

accurate frequency of *TFG* mutation and confirm mutual exclusivity to *BRAF/NRAS* mutation.

7.1.2. The therapeutic relevance of *MAP3K5* and *MAP3K9* mutation in melanoma.

In chapter 3, inactivating mutations in *MAP3K5* or *MAP3K9* were identified in 9% and 15% of metastatic melanomas, respectively [11]; these mutations may represent a novel pathway in the development of melanoma (Figure 7.1). This study was part of a large collaboration effort and as such, significant functional analysis was performed to elucidate the biological role of MAP3K mutations. Analysis revealed that *MAP3K5* and *MAP3K9* mutation affects phosphorylation activity of the proteins, downstream signalling, and may contribute to the resistance observed with use of chemotherapy, specifically, the role of *MAP3K9* mutation in Temozolomide resistance.

Although understanding chemo-resistance in melanoma may improve this therapeutic strategy, this approach has recently been superseded by alternative drugs, such as Vemurafenib in patients with *BRAF* mutant tumours [12]. As such, current research has focused on understanding the mechanistic action of molecularly-based targeted drugs in order to attain effective long lasting responses in patients. Since *MAP3K5* and *MAP3K9* have critical roles in the regulation of apoptosis, investigating the effect of mutation in these genes in respect to current molecular based drug strategies is warranted. Mutation of *MAP3K5* and *MAP3K9* may result in intrinsic drug resistance to BRAF or MEK inhibitors, and thus, could predict the efficacy of such therapeutic strategies, acting as a biomarker for the stratification of positive or negative responders. This hypothesis could be determined through a retrospective analysis of the prevalence of *MAP3K5* and *MAP3K9* mutation between patients who responded, to those who did not respond to therapy.

Mutation of *MAP3K5* and *MAP3K9* may be amenable to therapeutic intervention; however, since the mutations within these genes appear inactivating, this presents a difficulty in developing an effective drug strategy. Currently, the majority of drugs that have been approved or are under clinical development, are designed to abrogate the effect of oncogenic mutations that result in a ‘gain of function’. In contrast, the re-establishment of gene function from inactivating mutations is currently not easily within the realms of modern pharmaceutical drug design. However, it is conceivable that intervention through targeting of genes upstream or downstream of the identified mutations may result in

rational drug. This concept is discussed in more detail in 7.2.4. *The identification of targets not directly amenable to therapeutic intervention*.

7.1.3. The functional and therapeutic significance of *NF1* mutation in melanoma.

Whole-exome sequencing strategies in Chapter 4 identified mutually exclusive mutations of *NF1* in *BRAF*/*NRAS* WT melanomas. This finding is particularly exciting as it may represent a new therapeutic avenue for this subset of patients; however, significant research into the role of *NF1* in the development of melanoma still needs to be performed.

Firstly, the frequency of *NF1* mutation needs to be determined in a larger subset of tumours in both *BRAF*/*NRAS* WT and *BRAF*/*NRAS* mutant melanomas. Although analyses of previously published and unpublished melanoma exomes show that *NF1* mutation is mutually exclusive to *BRAF* and *NRAS* mutation, the overall numbers are low. As *NF1* is a large gene consisting of 57 exons, the use of traditional sequencing technology would not be a cost effective approach and would benefit from targeted next-generation sequencing strategies.

Consistent with a dual-hit inactivation model of tumour suppressor genes, tumours from patients with neurofibromatosis, a disease typically associated with abrogated NF1 function, have both copies of the gene disrupted through a variety of mechanisms. Inactivation of *NF1* can occur through the introduction of truncating nonsense mutations, loss of heterozygosity (LOH) or through RNA editing of the *NF1* transcript [13, 14]. Within the results presented in Chapter 4, point mutations of *NF1* were documented in melanoma, of which, all resulted in truncating mutations. Although one patient, C077, had two nonsense mutations in *NF1*, the remaining patients had only one allele affected. Further investigation into chromosomal copy number changes, including LOH, as well as the possibility of RNA editing should be investigated. RNA editing is of particular interest, as potentially, *NF1* may be affected in a larger proportion of *BRAF*/*NRAS* WT melanomas than first indicated by this study.

After determining the extent and frequency of *NF1* mutation in melanoma, the next logical step is to focus on the biological role of abrogated NF1 protein function. In Chapter 4, clustering expression array analysis suggests that *BRAF*/*NRAS* WT melanomas are not distinct molecular entities and are likely to have constitutive activation of the MAPK

pathway; this needs to be conclusively determined through western blot analysis, specifically, through assessment of phosphorylation activity of BRAF, MEK and ERK. As existing literature of NF1 function dictates a significant role in the negative regulation of NRAS [15], it would be expected that upon NF1 inactivation, subsequent increased kinase activity of downstream members of the MAPK and PI3K pathways would occur.

A recent study identified the reliance of mTOR signalling in *NF1* mutant adult acute myeloid leukaemias, which were susceptible to mTOR inhibition by rapamycin [16]. This is a compelling finding with clinical significance for *NF1* mutant *BRAF*/*NRAS* WT melanomas, a subset of tumours that may be amenable to similar therapeutic intervention. mTOR signals downstream of the PI3K pathway; a pathway frequently deregulated in melanoma through mutation of NRAS and PTEN. Although early reports investigating the downregulation of NF1 in melanoma determined no such reliance of mTOR pathway signalling [17], confirmation of these findings should be replicated due to the potential impact that this therapeutic avenue would provide for *BRAF*/*NRAS* WT melanomas.

7.2. Personalised therapeutics- using mutation data within a clinical setting

Recent advances in technology have resulted in an inverse relationship between the associated costs and output capabilities of next-generation sequencing platforms. With this increased capability for the generation of data, it is expected that an avalanche of new genes and mutation events contributing to melanomagenesis will be discovered. In the previous chapter, the limitations of current sequencing technologies in discerning driver mutation events to passenger mutation events were discussed. In contrast, this section will describe the applicability of sequencing technology and mutation detection within a clinical setting, with particular emphasis on the use of this data in personalised therapeutics.

7.2.1. Molecularly-based targeted therapies

Recent advances in molecularly-based targeted drug strategies have begun to show a significant impact on overall survival for patients with metastatic melanoma. Notably, the FDA approval of Vemurafenib in August 2011 was a significant milestone for the melanoma research community and the field of personalised therapeutics. Furthermore, a

number of promising molecular based drug strategies for use in melanoma are currently under investigation or on the horizon; this includes the use of Imatinib or Lapatinib in *KIT* or *ERBB4* mutant melanomas, respectively [18, 19]. However, a common theme between the successful applications of molecularly-based targeted drugs within the clinic, strongly relies on the correct stratification of patients based on their tumour mutation profiles, essentially guiding drug efficacy and/or resistance.

Oncogenic mutation screens, such as the melanoma-specific mutation panel discussed in chapter 5, can be used for the identification of clinically relevant mutation profiles within tumours. These oncogenic mutation panels have a number of advantages compared to alternative methodology platforms and benefit from minimal sample requirements, cost efficacy, and high throughput analysis. The latter is of significance for the successful application of mutation detection within the clinic; delays in implementing treatment regimens can be a critical factor determining patient survival, in particular, for those who have aggressive late stage or disseminated disease.

One consideration that has yet to be comprehensively explored is inter- and intra-heterogeneity of tumour specimens within a patient. This includes differences between the mutational evolution of primary to metastatic tumour sites, variability between multiple metastatic deposits throughout the body, or the spectrum of mutations or subclones present within a given tumour. This was recently addressed through a comprehensive genomic analysis of multiple deposits and tumour sections from biopsies of several patients with renal carcinoma [20]. Interestingly, significant intra-tumour heterogeneity was observed; 63-69% of all somatic variations were not detectable across all tumours and frequent mutant allelic imbalances were observed between tumours. Two main clinical implications arise with the observation of patient tumour heterogeneity. Firstly, singular biopsy analysis, as is routinely performed within the clinic, may be insufficient for estimating the entire spectrum of mutations within a tumour. Secondly, tumour heterogeneity may result in inaccurate diagnoses of effective treatment strategies using molecularly-based drugs. Thus, the unique capabilities of mutation screening panels would allow easy and cost effective analysis of multiple, spatially separated biopsies within a single tumour, and or, testing multiple metastatic deposits.

Despite the advantages, mutation screening panels such as the melanoma-specific mutation panel [21] are limited by their ability to only assess ‘oncogenic’ or single

nucleotide mutation events. Tumorigenesis is a complex interaction of genetic abnormalities contributing to the neoplastic process involving activating oncogenic mutations in combination with inactivating tumour suppressor mutations. The latter, due to the propensity of mutations to occur throughout the entire length of the gene, essentially relies on the use of sequencing technology for the successful identification of all genetic mutations. As such, the use of next-generation sequencing will likely be a desirable platform to comprehensively assess mutation profiles. Indeed, specialist oncology clinics have already implemented routine next-generation sequencing platforms to ascertain therapeutically relevant mutations within individual patient tumours in order to personalise treatments [22].

Although the promise of next-generation sequencing within the clinic seems achievable with existing technology, a number of technical limitations have yet to be solved before it is likely to be widely adopted by oncology clinics (reviewed in more detail [23, 24]). Of utmost importance to the implementation of any methodology within the clinic, not just concerning the concept of next-generation sequencing, is accuracy.

The clinical laboratory improvement amendments (CLIA) certification (or its equivalent) is a regulatory standard to which all clinical laboratory testing must be adhere. Within these guidelines, strict adherence to set protocols is required to uphold consistent accuracy, reliability and timeliness of test results. This is highly significant in a clinical diagnostic cancer setting, as patient survival and prognosis is intimately associated with the rapid adoption of efficacious treatment strategies. Thus, any technology used within this arena will require high accuracy with low rates of false positive and false negative calls. This is problematic for current next-generation sequencing platforms where high-throughput, which is desirable in a research setting, offsets the rate of accuracy. Although excessive coverage increases the rate of accuracy, this in itself poses a number of issues, particularly the additional cost and associated bioinformatic processing time. Platform specific biases must be also considered and is why, if possible, combinations of technologies can significantly improve data quality and output.

Reliability is another critical issue of concern for the implementation of next-generation sequencing platforms. As stated above, strict adherence to protocols is required in order to maintain accuracy and consistency. Due to the rapidly progressive nature of sequencing technology, upgrades to machines or improvements to sequencing chemistry are

consistently being released to increase data output and reduce sequencing costs, sometimes at bi-annual frequency. This is problematic in a CLIA setting due to the investment of time and expenses required for the establishment of standardised workflows and procedures. Other technical considerations include the adoption of automated library preparation to reduce labour intensive procedures and to improve reliability, the standardisation of bioinformatic analysis methods, and the current need for independent platform validation of identified mutations.

These concerns aside, another debate currently exists into what sequencing depth or coverage should be required for use in a clinical setting, specifically, whether to analyse patient samples with whole-genome, exome, or targeted gene sequencing strategies. Regardless, it must be noted that in relation to acquired drug resistance using existing molecular based targeted therapies, genetic testing alone will be insufficient to comprehensively determine all mechanisms of resistance. For example, Vemurafenib resistance in *BRAF* mutant melanomas include acquired mutations in *MEK* and *NRAS*, differential splicing and amplification of *BRAF* and up-regulation of tyrosine kinases such as PDGFRA and COT1 [25-30]. Determining these mechanisms will require multiple platform analysis, and in regards to the latter, non-genetic analytical approaches. Although it is hard to make conclusive predictions due to the regular and rapid advances in the sequencing industry, it is unlikely that widespread adoption of next-generation sequencing within the clinic will occur for at least another 5 years. However, during these interim years, analysis will most likely concentrate on the identification of mutations with known clinical significance to existing molecularly-based targeted drug strategies.

7.2.2. Immunological approaches.

Alongside the recent success of molecularly-based targeted drugs such as Vemurafenib [31], and CTLA4 inhibition strategies with Ipilimumab [32], a number of alternative strategies are currently being investigated. One approach that is showing promising results in patients with metastatic melanoma is adoptive cell therapy (ACT) with use of tumour infiltrating lymphocytes (TIL) [33]. This strategy involves autologous TIL isolation and cultivation *in vitro* with IL2, selection of tumour reactive cultures in matched tumour cell lines, then systemic re-introduction of cultured reactive TILs [34]. Patients will also typically undergo lymphodepletion regimens during cell preparation as this method results in long lasting responses [35]. Using this therapeutic approach, complete responses in 20 of 93

(22%) metastatic melanoma patients were observed, 19 patients of which were alive 3 years post-treatment [36].

Despite these impressive results, a number of limitations to the method have been outlined and it is recognised that TIL therapy will only be effective for 50% of all melanoma patients [36]. Limitations include the requirement of clinical resection of tumour nodules of at least 2 cm in diameter in order to obtain sufficient TIL, and the subsequent isolation of sufficient tumour reactive lymphocytes. Recently, the efficacy of ACT acquiring TILs using ultrasound-guided needle biopsy in 11 patients was performed; although this was a small cohort, 4 patients demonstrated objective clinical responses, highlighting the efficacy of this less invasive, less expensive approach [37]. However, the true clinical benefit of this technique needs to be determined though testing in a larger cohort of patients, and, as mentioned previously, the significance of inter-tumour heterogeneity may need to be determined for this specific application [20].

A promising alternative that circumvents the need for cultivation of TILs from a dissected tumour mass is the genetic engineering of peripheral blood lymphocytes [38]. This strategy can also overcome the difficulty of identifying tumour reactive TILs and involves the manipulation of blood lymphocytes to react to specific antigens presented within the tumour. A seminal paper released in 2006 by Morgan et al., engineered peripheral blood lymphocytes to recognise MAGE-1, a melanoma differentiation antigen, in order to replicate immunogenicity with autologous TILs [38]. Although the response rate with modified blood lymphocytes (2 of 15 patients or 13%) did not achieve the same level of efficacy as the autologous TIL approach (approximately 50%), further research efforts into improvements of the technique may reduce the disparity between these methods.

The promising results of ACT with TILs may benefit from concurrent use of next-generation sequencing technologies within the clinic. It has previously been shown that single point mutations within genes can act as epitopes, such as *NRAS* Q61R in melanoma [39], and elicit strong immunogenic responses in cancer patients. As such, it may be possible to harness the comprehensive identification of mutations in tumours from sequencing technologies to develop highly specific, individualised, engineered TILs from a cocktail of mutation epitopes (reviewed in more detail [40]).

A number of reports have begun to determine the efficacy and details of such an approach; this includes a recent comprehensive investigation of the T cell antigen specificity in human melanoma [41]. In this approach, a comprehensive compilation of all known melanoma-associated antigens were compiled and tested for immunogenicity against 63 TIL cultures from 19 patients. A total of 175 tumour associated antigens that included mutated and over-expressed antigens as well as those involved in differentiation and cancer-testis/onco-foetal origin, resulted in 90 responses against 18 epitopes. Notably, the majority of the responses derived from differentiation antigens and not from mutant epitopes; however, the authors failed to assess the mutation status of the tumours from which the TILs were isolated and this may explain the lack of response with this class of antigen.

Building on this finding, Castle et al. assessed the mutanome of B16F10 murine melanoma cells for its ability to result in an immunogenic response, specifically, in the context of establishing a multi-epitope tumour vaccine [42]. Next-generation sequencing revealed a total of 962 non-synonymous single point mutations, of which, 563 were within genes that were highly expressed. Immunization of mice with long peptides containing 50 of the validated mutations resulted in one third eliciting *in vivo* immunogenic responses (16 of 50); furthermore, 60% of the immunogenic responders showed preferential sensitivity of mutant epitope compared to wild-type sequence. In addition, in vivo mutant peptide immunization conferred tumour control, indicating the efficacy of single amino acid alterations as epitopes in a therapeutic vaccine setting.

These results provide a proof of principle for the potential application of personalised molecularly-engineered TILs that are specific to an individual's tumour mutation profile. However, the application of this strategy has some important considerations and limitations. Firstly, the technology in regards to sequencing and the identification of mutant epitopes eliciting an immune response is currently time consuming and laborious; however, the improvement of sequencing technologies and bioinformatic analyses should reduce the impact of this process. One interesting possibility is the curation of a database containing documented immunogenicity mutant epitopes, observed experimentally or within the clinic, for the rapid identification of targets for therapeutic design. This is exemplified in the aforementioned study where ACTN4, a previously identified epitope [43], was replicated in the mutanome study of B16F10 [42]. Another potential limitation is the loss of expression or clonal selection of mutant epitopes within the tumour, including

evolution selection pressures induced during the course of therapy. Multi-epitope therapeutic design has the potential to overcome this problem, however this approach requires further investigation.

One potential advantage for personalised TIL therapy is that discerning the difference between driver and passenger nonsynonymous mutations should not be necessary as both can elicit immunogenic reactions. This was demonstrated in the B16F10 mutanome study where one of the strongest reactions specific to a mutant epitope was a K739N mutation in KIF18B; this mutation does not localise to any functional or conserved domain and most likely represents a passenger mutation event [42]. If so, this shows promise for immunological therapeutic design as this expands the potential pool of mutant epitopes available; even more so for melanoma where the intrinsic rates of mutation are considerably higher than other cancers due to carcinogenic exposure of solar UVR.

Expanding the suite of treatments available for metastatic melanoma will act positively on rates of overall survival and help overcome issues of therapeutic resistance or tumour remission; any therapy that shows an improvement in overall survival or clinical activity will warrant further investigation. As TIL therapy has already demonstrated robust responses on patients who have undergone multiple refractive therapeutic treatments, including dacarbazine and Ipilimumab [36], first line treatment regimens concurrent with TIL preparation may be an effective strategy for improving overall survival. In this case, if the first line of treatment fails, TIL therapy can be administered rapidly as strong immunogenic personalised TILs will by then have been established; however, this would be at considerable expense but may represent an effective short term strategy until robust drug combination strategies are discovered. The combination of individualised TIL therapy through the identification of tumour specific epitopes using next generation sequencing is an exciting prospect for future treatment of patients with metastatic melanoma, however, this approach requires further research.

7.2.3. Diagnostic and biomarker applications.

Next-generation sequencing technology has demonstrated value in personalised biomarker identification for the clinical management of patients [44]. This study utilised massively-parallel sequencing to identify chromosomal translocation events in a method called 'personalized analysis of rearranged ends' or PARE. In this process, fusion events

in solid cancers were initially identified using PARE, before sensitive digital PCR assays were designed to detect these rearrangements from circulating DNA in patient plasma samples. This approach was highly sensitive, and able to detect re-arrangements at a frequency of 0.001% in sample material also containing normal DNA.

The application of PARE in a series of plasma samples taken throughout the course of a patient's therapy highlighted the potential benefits of this approach in a clinical setting [44]. Levels of the identified re-arrangement detected in circulating DNA from plasma showed a significant decrease after primary resection, an increase after metastatic dissemination, and a decrease after the commencement of chemotherapy; effectively, levels of the detected re-arrangement in plasma closely followed the tumour burden within the patient. As such, PARE could provide an effective and highly sensitive method to determine disease progression following treatment. In the assessment of tumour acquired drug resistance, PARE may detect patient relapse more rapidly than conventional approaches such as computed tomography (CT) scans, however, this has yet to be determined.

Reciprocal to the potential of personalised TIL therapy, the need for discerning driver and passenger translocations is largely negligible; the only requirement is retained tumour expression of the fusion gene throughout the course of treatment. It is interesting to speculate whether point mutations identified through next-generation sequencing may act as superior biomarkers to PARE. Although single base mutations can appear artefactually through the introduction of errors via PCR, simultaneous analysis of multiple mutation events within the tumour may increase accuracy while circumventing the issue of clonal selection or heterogeneity within the tumour. Further research into the application of next-generation sequencing in biomarker identification may have considerable significance for managing patient therapy within the clinic.

7.2.4. The identification of targets not directly amenable to therapeutic intervention

The rate of mutation in melanoma is high; although the majority of these mutations represent passenger events, it is still undetermined how many driver mutations are required for melanomagenesis. A proportion of non-synonymous mutations in genes, such as *BRAF* V600E, are amenable to therapeutic intervention through the design of mutation specific inhibitor strategies, or via high throughput chemical drug screens. However, it has been suggested that in regards to the 'druggability' of proteins within the human genome,

only ~10% of genes can be targeted effectively with traditional pharmaceutical drug design [45, 46]. As such, a large number of mutations identified from large scale cancer genomic studies, even if responsible for driving tumorigenesis, will not be able to be therapeutically targeted directly. This raises an important issue for the application of personalised molecularly-based medicine in a clinical setting, particularly for the subset of patients whose mutation profile does not present with druggable targets.

One strategy to address the abovementioned problems is the use of pathway analysis and requires an understanding of the functional role that mutations play within signalling networks. In this sense, it is theoretically possible to achieve therapeutic success by targeting genes upstream or downstream of the mutant gene in question. An example of this approach has recently been suggested with the inhibition of ERK1/ERK2, proteins downstream of BRAF and MEK in the MAPK pathway, through the use of shRNA [47]. Although for 50% of melanoma patients, the MAPK pathway can be targeted through use of BRAF inhibitors, due primarily to the presence of *BRAF* V600E mutations, patients with *NRAS* mutations, or who are *BRAF*/*NRAS* WT, are currently refractive to this therapeutic approach despite constitutive activation of the MAPK pathway. In vitro inhibition of *ERK1*/*ERK2* in *BRAF* mutant A375 melanoma cells was more effective at promoting apoptosis than BRAF inhibitors, such as PLX4032 [47]. Although the effect of *ERK1*/*ERK2* inhibition on *BRAF* WT melanomas was not assessed, this approach may be an effective strategy in this subset of melanoma patients.

Despite the ability to target downstream or upstream members of biological pathways in an experimental in vitro setting, a number of issues are raised when this concept is considered in an in vivo clinical setting. One approach that has gathered significant interest since their identification is the use of siRNA knockdown strategies to inhibit overactive protein activity, or signalling networks. Although siRNA strategies are effective in vitro cell culture experiments, delivery of the siRNA becomes difficult in vivo as current approaches are ineffective. However, significant research in improving the delivery is currently underway and is beginning to demonstrate clinically actionable results in melanoma [48, 49]. It will be interesting to see how the applications that pathway analysis and inhibitor based strategies will affect treatment strategies within the future; however, an extensive understanding in the biology of the targeted pathways will be required before success with these approaches is achieved.

7.3. Concluding statement

Melanoma is an aggressive cancer that accounts for nearly all skin cancer related mortality; this is largely due to late stage or disseminated melanoma, which historically, has been refractive to traditional chemotherapeutic strategies. However, recent success with molecularly-based targeted drugs in metastatic melanoma, such as Vemurafenib in patients with BRAF V600E mutations, have begun to demonstrate an improvement in overall survival and supports the use of 'personalised medicine' within the clinic. Thus, understanding the genetic mechanisms of metastatic melanoma will ultimately lead to the development of novel drug strategies, while also improving existing therapeutic approaches in treating this disease.

In this thesis, a comprehensive analytical approach has been used to determine the genetic mechanisms of tumorigenesis in a panel of stage III local lymph node metastatic melanomas. Using high-throughput technologies, including whole-exome sequencing strategies, a number of novel genes involved in the development of metastatic melanoma were discovered. This includes the identification a putative tumour suppressor gene *TFG* that is mutated in 5% of melanomas and may activate the MAPK and NF-KB pathways (Chapter 2); the identification of *MAP3K5* and *MAP3K9* mutations in 25% of melanomas involved in cell regulated apoptosis (Chapter 3); and the identification of inactivating mutations of *NF1* in *BRAF*/*NRAS* WT melanomas, which may be susceptible to therapeutic intervention through mTOR inhibition using rapamycin (Chapter 4).

As the numbers of mutation events contributing to melanomagenesis are increasingly identified and their subsequent significance to current or novel drug strategies determined, the utilisation of this information will progressively move from a research setting towards routine clinical applications. For instance, the development of a melanoma specific mutation panel, as described in this thesis (Chapter 5), is one such example of how genetic information could be used to guide efficacious treatment strategies with molecularly-based targeted drugs. However, a number of ethical issues and technical considerations will need to be discussed before the use of mutation screening panels or next generation sequencing platforms can be implemented into routine use within oncology clinics. This aside, a number of specialist clinics are currently embracing this technology and no doubt, will contribute to the development of the standardised practices required for the widespread adoption of this technology.

The advance in sequencing technology over the last decade has without a doubt, changed the approach to cancer genetic research. Consequently, this is an exciting time for both researcher and patient alike, as the adoption of this technology is increasing our understanding of the genetic mechanisms of melanoma development, and as such, will hopefully lead to the improvement of therapeutic outcomes for patients with this disease.

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Appendix

C Series Patient Selection Criteria

Inclusion Criteria:

Prior to surgery, where possible and again one week prior to the first vaccination, the patient must fulfil the following criteria:

1. Histologically confirmed Melanoma of Stage III as determined by tissue sample obtained during surgery
2. ECOG Performance status of 0
3. Normal Haematological parameters
4. Acceptable Liver and Renal function parameters
5. Negative serology for HIV, Hepatitis B and Hepatitis C
6. Willingness to sign and ability to understand an informed consent form. (Both the Patient and parent/guardian for those under 18 years of age.)

Exclusion Criteria:

Patients with any of the following will be excluded from enrolment in this study:

1. Autoimmune Disease or use of immune-suppressive therapy (oral or systemic corticosteroids)
2. Prior chemotherapy or immunotherapy
3. Prior gene therapy, vaccine therapy or cytokine therapy
4. Post-operative complications requiring ongoing medical care that would preclude experimental vaccine therapy.
5. Post-surgical local radiation therapy
6. Unsuccessful vaccine preparation (for example, insufficient tumour cells)
7. Clinically significant active infection
8. Uncontrolled systemic disease or medical problems that would preclude surgery or participation in this study (ie. neurological, cardiovascular, endocrine)
9. History of other malignancies, except for adequately treated and controlled non-melanomatic skin cancer or *in situ* cervical cancer
10. Participation in other investigational new drug trial within 30 days of surgery
11. Pregnancy