Genetic dependencies in hereditary and sporadic melanoma

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Dedicated to my dear family and,
in remembrance of my dearest supporter
Andreas Theodorou

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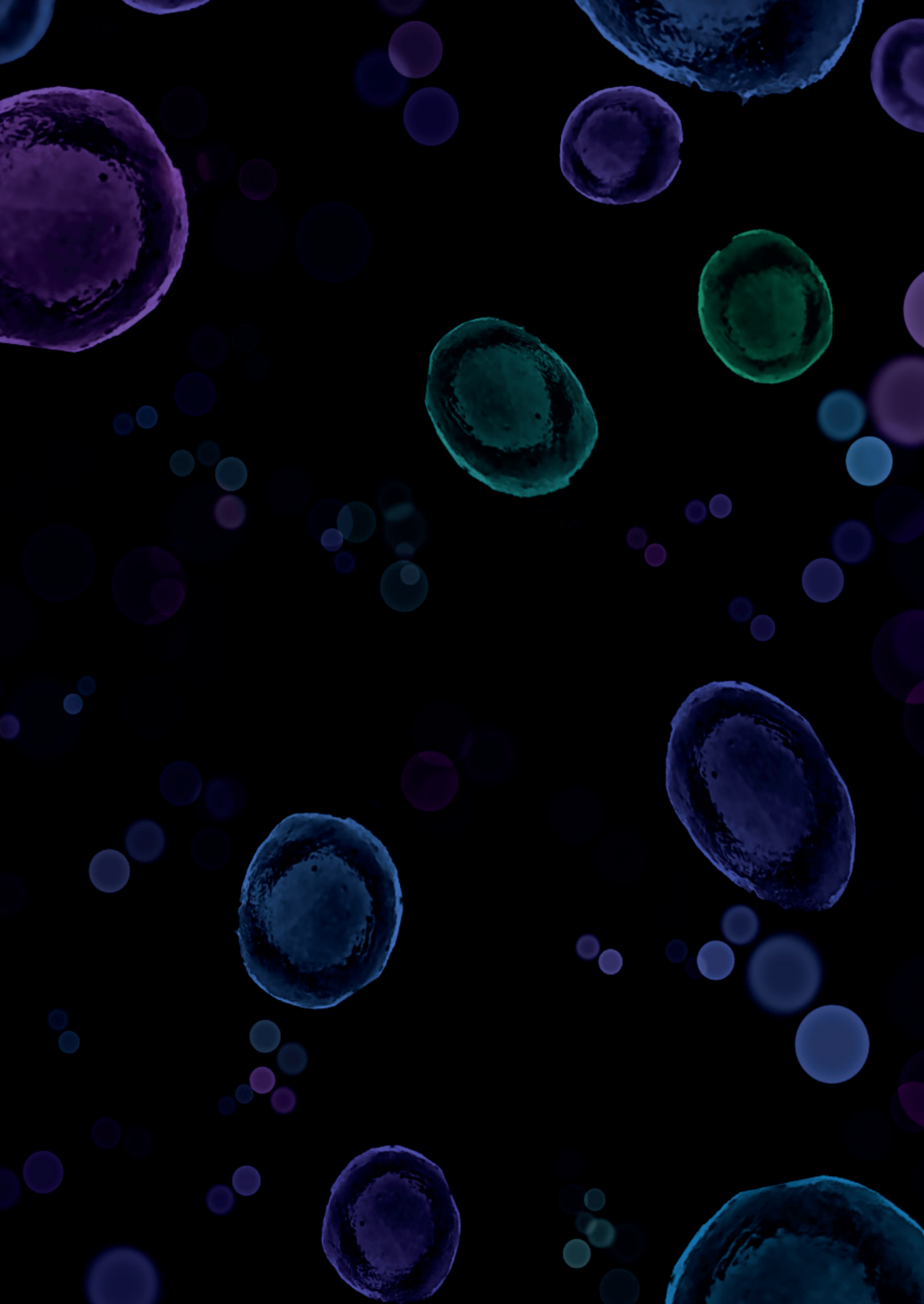

Chapter 1

General Introduction

CUTANEOUS MALIGNANT MELANOMA PREFACE

The word 'melanoma', according to Hippocrates back in the fifth century BC, originates from the ancient Greek adjective 'μέλᾱς', meaning 'black' and the suffix 'ώμᾱ' referring to a tumor; although it was first described as a disease entity by René Theophile Hyacinthe Laënnec in 1812 [8].

Cutaneous Melanoma (CM) develops from malignant transformation of melanocytes, the pigment producing cells residing in our skin. CM is one of the deadliest types of skin cancer due to its high metastatic propensity. Although considerable efforts have been employed to effectively eliminate the disease, incidence rates of CM are increasing considerably worldwide. Approximately 232,100 CM cases are diagnosed and about 55,500 deaths are reported annually [9]. The incidence and mortality rates of CM vary per geographic location although the highest incidence rates are reported for Caucasian populations due to fair skin color [10].

Specifically in the Netherlands, melanoma of the skin is the 5th most common cancer type with 6,189 cases reported in 2017 as well as 796 deaths according to the Dutch Cancer Registry [11, 12]. Most melanoma cases are diagnosed early, at a localized stage and are reported with a two-year survival rate of 96% [11]. Survival of metastatic melanoma however, remains poor in spite of introduction of novel immune and targeted therapies [13].

ENVIRONMENTAL RISK FACTORS AND CLASSIFICATION

To better understand CM development we may consider melanoma as a multi-factorial disease arising from an interplay of genetic and environmental risk factors. An important environmental risk factor for melanoma development is strong intermittent exposure to ultraviolet (UV) radiation and sunburn at a young age [14]. Artificial exposure to UV radiation through tanning bed sessions for cosmetic purposes may also be associated with an increased risk for CM development [15, 16]. UV radiation mainly causes genetic alterations in the skin through direct DNA damage, mainly formation of pyrimidine dimers, resulting in mutations that may drive malignant transformation of expanding keratinocytes and melanocytes [17]. At the molecular level, UV increases skin pigmentation through stimulation of the melanocortin 1 receptor (MC1R) on the surface of melanocytes by its ligand α-melanocyte-stimulating (α-MSH). This mediates production of melanin, the main defense mechanism against UV radiation-induced damage [18, 19]. Germline variants in the *MC1R* gene are associated with fair skin and these individuals have a lower capacity of activating *MC1R*, associated with increased susceptibility to melanoma [20, 21].

CM may be classified into two types depending on UV-exposure duration and genetic signatures; The chronically affected sun-damaged areas such as head and neck and non-chronically affected sun damaged areas such as the trunk, legs and arms (CSD and non-CSD respectively) [3, 22, 23]. The most common non-CSD subtypes are superficial spreading melanoma (SSM) and nodular melanoma (NM) whereas lentigo maligna melanoma (LMM) is a common CSD subtype [24, 25]. An un-common sub-type of melanoma in Caucasian populations is Acral Lentiginous Melanoma (ALM), with only 5% reported cases. ALM is a frequent subtype of melanoma in Asian, African and Hispanic populations [26].

MUTATIONAL SIGNATURE OF CM

Melanocytes are neural crest-derived cells which not only migrate to the skin but also different parts of the body such as the eyes and mucosal areas during development. [27, 28]. These melanocytes can give rise to different types of melanoma, including mucosal and uveal in addition to cutaneous melanoma.

Primary CMs are not only found *de novo* but can develop from precursor lesions such as a common melanocytic nevus, an atypical melanocytic nevus or a lentigo maligna (Figure 1). Approximately 30% of CMs are derived from a common melanocytic nevus, although the percentage in high-risk individuals is higher, reaching 50% [29, 30].

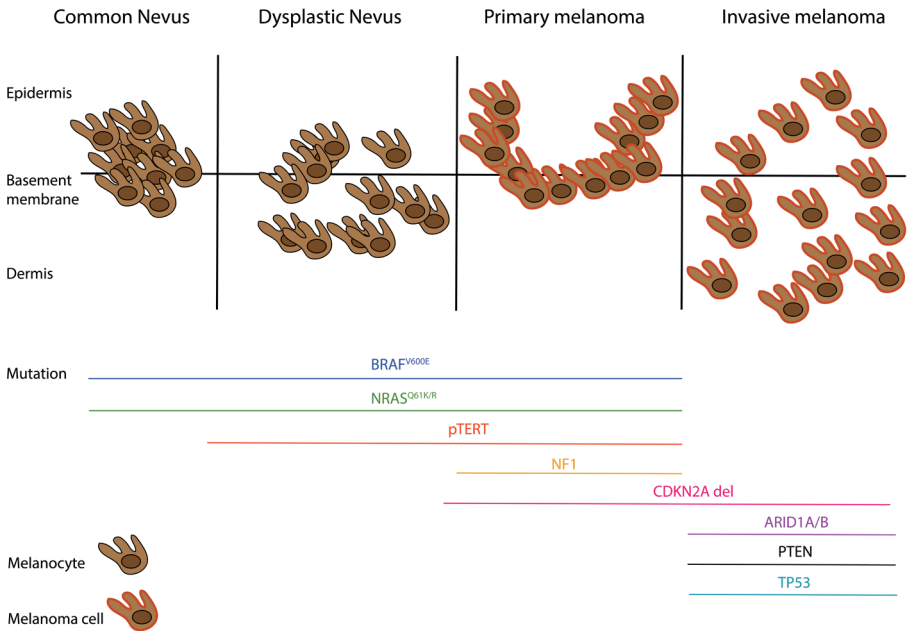

Figure 1 Genetic evolution of cutaneous melanoma. Simplistic model of genetic evolution of cutaneous melanoma (CM) and the underlying common genetic alterations describing precursor lesions (common nevus), intermediate lesions (dysplastic nevus), primary and invasive melanoma. Source data were adapted from the following resources: [1-4].

A complex network of events contributes to CM development and considerable efforts have been employed to enhance our understanding of the different molecular pathways involved. The most frequently hyperactivated signaling pathway in melanoma development is the mitogen-activated protein kinase (MAPK) pathway that mediates transcription of proliferative genes and cell growth. Several oncogenes and tumor suppressor genes are involved in melanoma pathogenesis. The three most common MAPK-activating mutations in two oncogenes are *BRAF*^{V600E} and *NRAS*$^{Q61K/R}$ known to exist in a mutually exclusive pattern. Following these two oncogenes, *NF1*, seems to be the third most commonly mutated tumor suppressor gene and negative regulator of the MAPK-pathway according to whole exome sequencing (WES) analysis and functional validations (Figure 1) [31]. Also, *KIT* is a driver oncogene activating MAPK signaling in a small percentage of melanomas [32, 33].

Collectively, based on the significantly mutated oncogenic driver genes in melanoma, genomic classification reveals four sub-types and these include the BRAF subtype (presence of *BRAF* hotspot mutations), RAS Subtype (presence of *RAS* hotspot mutations), NF1 subtype (presence of *NF1* loss-of-function mutations) and a triple wild-type (WT) subtype that lacks hot-spot *BRAF, NRAS* or *NF1* mutations [34].

Starting from the common melanocytic nevus phase, recent studies provide evidence for clonality of *BRAF*^{V600E} mutation (Figure 1) [2]. A distinct feature that distinguishes benign nevi from melanomas is that nevi eventually stabilize and undergo cellular senescence. Activation of senescence pathways in benign nevi prevents further cell growth. The G1/S checkpoint pathway appears to be the main mediator of senescence in nevi [35]. The concept of oncogene activation in nevus cells that does not result in tumor formation, is known as Oncogene-Induced Senescence. Benign nevi in the current instance enter a permanent cell-cycle arrest following the first *BRAF* mutation [36]. Several lines of evidence show that the immune system plays a role in regulating the apoptotic potential of benign nevi [37, 38]. A clinical study demonstrated a three-fold increased risk of malignant melanoma development in immunosuppressed transplant recipients compared to matched controls, suggesting a role of the immune system in preventing progression into a melanoma [39].

The atypical or dysplastic nevus is a genetically intermediate melanocytic lesion that may be difficult to distinguish from a malignant melanoma [40]. In contrast to benign nevi, those melanocytic lesions not only have a single activating mutation in *BRAF* but multiple driver mutations such as *NRAS*, *TERT* promoter (p*TERT*) mutations and also heterozygous alterations for tumor suppressor genes such as *CDKN2A* (Figure 1) [2]. Collectively, these data suggest that dysplastic nevi are indeed a distinct entity from benign nevi and melanomas based on their genetic make-up. It is worth noting that individuals with increased numbers of dysplastic nevi are also at increased risk of developing melanoma [41].

Regulation of telomerase activity and telomere length has been a contributing factor not only for underlying features of intermediate and primary melanoma lesions but also in determining melanoma risk. The telomerase reverse transcriptase (*TERT*) gene encodes for a ribonucleoprotein that regulates telomere length and cell integrity [42-45]. The wild-type p*TERT* contains binding sites for c-Myc (E-Box), SP1, and ETS transcription factors [46]. Genetic and transcriptomic data suggest that increased telomere length is associated with higher melanoma risk and is correlated with disturbed homeostasis of telomere regulation [47]. The majority of *pTERT* mutations are found at two hotspots, in a mutually exclusive pattern, at −124 bp (c.1-124C>T) and -146bp (c.1-146C>T) upstream from the ATG start site. These mutations create ETS/TCF transcription factor binding motifs causing increased *TERT* expression [48]. Upregulation of *TERT* is correlated with presence of mutations in the promoter region and is mainly observed in primary and invasive stages of melanoma but not in the benign nevus phase [4].

The primary stage of melanoma requires additional genetic alterations and these are mainly centered around the impairment of G1/S checkpoint pathway resulting in senescence escape of melanocytic cells [4]. Specifically, loss of *CDKN2A* mainly by deletions, is a significant contributing factor leading to loss of $p16^{INK4A}$ expression in melanomas [22]. Some novel driver genes identified for CM by application of WGS *include DDX3X, RASA2, PPP6C, RAC1* or *RB1* all found to be specific for CM but not acral or mucosal melanomas [49]. Loss of *Phosphatase and tensin homolog (PTEN)*, a key tumor suppressor gene regulating cell growth, is critical in facilitating melanoma development through deregulation of the PI3K/AKT/mTOR pathway, reduction of apoptosis and promotion of cell survival [50]. In addition, deregulation of p53-dependent apoptotic pathways and mutations within *tumor protein 53* (TP53) are correlated to a more advanced progressed state and metastatic melanoma behavior (Figure 1) [4].

Collectively, improved knowledge on the molecular pathways enhanced the identification of novel biomarkers to improve CM diagnosis and treatment, although there is still more to be uncovered.

PATHWAYS TO CM TREATMENT

BRAF, is a protein kinase and key regulator of the MAPK signaling pathway that is mutated in about 50% of CMs but also in other types of cancer including colorectal, leukemia and thyroid [51]. About 90% of mutations within *BRAF* are specific to position V600E, a gain of function mutation leading to a constitutively active state of BRAF and hyperphosphorylation of MEK thereby stimulating cancer cell growth [52].

Vemurafenib, dabrafenib and encorafenib are FDA-approved BRAF inhibitors that initially presented promising results in melanoma targeted therapy through inhibition of hyperactivation of MAPK signaling and suppression of tumor growth [53-55]. Nevertheless, combinatory treatment using a MEK inhibitor, trametinib, cobimetinib and binimetinib, against downstream components of MAPK pathway, showed delay in the onset of resistance and improved overall survival (OS) in phase 3 clinical trials [56-59]. Even though tumor reduction was observed in more than 50% of $BRAF^{V600E}$ mutated patients, in the majority of cases there was development of tumor resistance within 4-9 months after treatment through re-activation of MAPK pathway [60-63]. Still, there is little clinical evidence about guidance for the best targeted treatment of metastatic melanoma with limited toxic events and no relapse development [64].

Since targeted therapy through *BRAF* inhibition can only be applied in about 50% of patients, immunotherapies can provide effective treatment with long-term responses independent of the mutational status of patients [65]. This has been successful through the development of antibodies against immune checkpoints such as ipilimumab, a monoclonal antibody against cytotoxic T-lymphocyte antigen 4 (CTLA-4) which downregulates immune responses [66]. In addition, nivolumab and pembrolizumab target the programmed cell death protein 1 (PD-1), a T cell inflammatory activity suppressor, and showed improved OS in patients with progressed melanoma. The combination of CTLA-4 and PD-1 inhibitors have been proven superior to monotherapy in patients with PD-L1 negative tumors [67-69]. Even though there has been success in targeting the immune system, still future studies are required to determine the optimal conditions and combinations but also possibly new targets to further improve the outcome of patients with metastatic melanoma [70].

INSIGHT INTO GENETIC SUSCEPTIBILITY- WHAT IS KNOWN SO FAR

A family history of melanoma has a significant role in determining an individual's risk of developing the disease. About 10-12% of reported CM cases occur in familial kindreds (Figure 2), therefore, familial (or hereditary) melanoma is arbitrarily defined by the clustering of at least two or more melanomas in first degree relatives [71]. High-penetrance genes have low population frequency and a higher impact on cancer development while, low-penetrance genes have high population frequencies but with a reduced effect size [72]. Several methods have been employed to identify high-penetrance genes that may predispose to familial melanoma.

Starting with genetic linkage analysis back in 1992 using DNA markers, scientists uncovered the first hint of chromosome 9p21 to be critically important in familial predisposition to melanoma [73]. Follow-up studies aiming to zoom into the chromosomal area of interest, uncovered *cyclin-dependent kinase Inhibitor 2A* (*CDKN2A)* as the first melanoma predisposition gene [74, 75]. A year later, a specific founder mutation was identified in Dutch-kindreds, a 19bp deletion in exon 2, known as the *p16^{INK4A}-Leiden* mutation (c.225_243del, p.(A76Cfs*64)) [76]. *CDKN2A* is the most common high-penetrance melanoma susceptibility gene known today, not only in The Netherlands (70%) but also world-wide (40%) (Figure 2) [77].

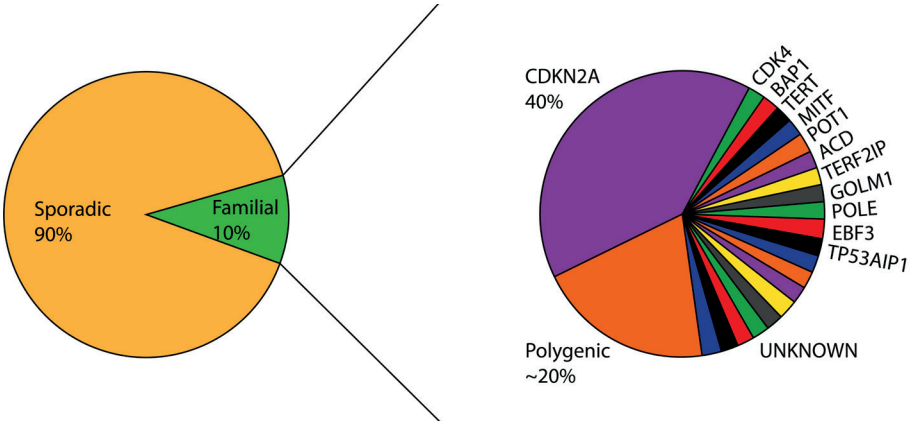

Figure 2 Summary of currently identified candidate high-penetrance melanoma susceptibility genes. The first pie chart on the left represents two settings of CM where 90% of cases are found in the general population (sporadic) and 10% report a family history of melanoma (familial). Zooming into the familial setting, several high-penetrance melanoma susceptibility genes have been identified thus far with *CDKN2A* accounting for most families (40%) and several other candidate genes, each responsible for about <1% of families. There is also a proportion of polygenic risk factors, effect of medium and low penetrance genes (20%) but also environmental risk factors. There is still a proportion of unknown genetic variability in the occurrence of hereditary melanoma.

Carriers of germline mutations in melanoma predisposition genes, such as *CDKN2A*, that may also present with increased number of atypical moles (known as dysplastic nevi) could be designated as Familial Atypical Multiple Melanoma Syndrome (FAMMM syndrome) patients [78]. FAMMM syndrome patients with germline *CDKN2A* mutations have a 70% risk of developing melanoma with the first sign of disease appearing at a relatively young age (mean <45 years) [79, 80]. Germline *CDKN2A* mutation carriers have an additional life-time risk of 15-20% to develop pancreatic ductal adenocarcinoma (pancreatic cancer; PC) [81-83]. Interestingly, clinical studies suggest variability in occurrence of melanoma and PC within families indicating that modifying factors may contribute to the risk of developing these two tumor types in patients with/without germline *CDKN2A* mutations [81, 84]. An example is the genetic variation in the *MC1R* gene, found to modify the risk of developing melanoma in *CDKN2A*-mutated families [85, 86]. Determination of genetic risk factors that modulate the risk of PC and melanoma in *CDKN2A*-mutated families, would therefore allow for a better identification of patients at increased risk that might benefit from personalized clinical management.

CDKN2A is located on chromosome 9p21.3 and encodes for two distinct proteins that are translated in alternate reading frames (ARFs) from alternatively spliced transcripts, therefore consist of different amino acid sequences (Figure 3). The α transcript encodes for $p16^{INK4A}$, a tumor suppressor protein that mediates G1 arrest by inhibiting the phosphorylation of Cyclin-D1-CDK4/6 complex [87, 88]. The alternative β transcript encodes for $p14^{ARF}$ which is also a tumor suppressor protein that inhibits MDM2-mediated ubiquitination thereby promoting p53-dependent apoptotic pathways (Figure 3) [89-92]. The *$p16^{INK4A}$-Leiden* mutation specifically causes a reading frameshift resulting in truncated $p16^{INK4A}$ protein which loses its capacity to bind to CDK4/CDK6 complex and a $p14^{ARF}$ fusion protein that seems to retain functionality (Figure 3) [7].

The implementation of mouse models to generate knock-out (KO) mice for both $p16^{INK4A}$ and $p14^{ARF}$ via conventional gene-targeting approaches has also been successful in providing evidence for cancer development including fibrosarcoma and lymphoma [93, 94].

Combination of mutant *HRAS* and *CDKN2A* KO led to CM development in mice that also showed loss-of-heterozygosity (LOH) for the remaining WT allele [94, 95]. In addition, simultaneous inactivation of *CDKN2A* and *Stk11* (*Lkb1*) loss in $BRAF^{V600E}$ mutant melanocytes induced mTORC1 and mTORC2/AKT activation leading to rapid melanoma formation in mice [96]. More recently, application of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) technology to induce genomic modifications, provided evidence that loss of $p16^{INK4A}$ protein mediates invasive behavior of melanoma cells *in-vivo* due to deregulation of the BRN2 transcription factor [97].

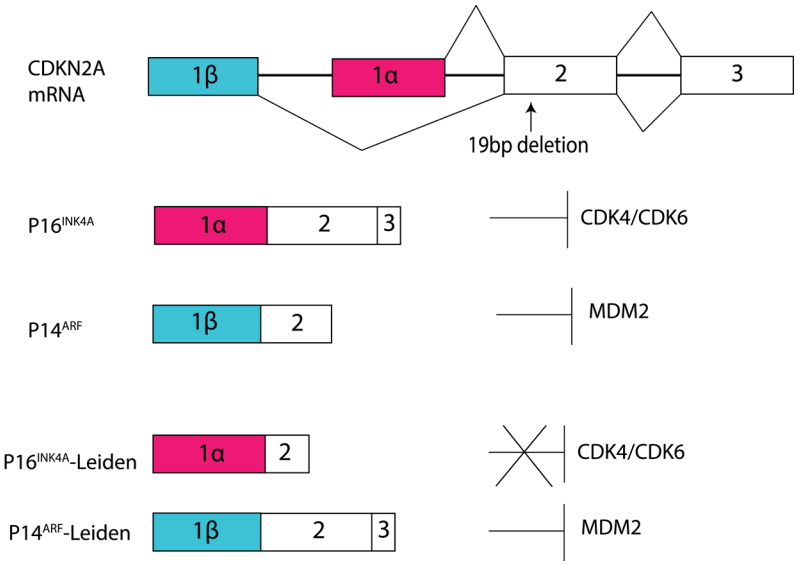

Figure 3 Schematic representation of *CDKN2A* exons and coding proteins. *CDKN2A* is located on chromosome 9p21 and encodes for two distinct tumor suppressor proteins. The 19-bp deletion of *p16^{INK4A}-Leiden* mutation is located in exon 2. The α transcript encodes p16^{INK4A} that regulates G1/S cell cycle arrest by inhibiting the CDK4/6 complex. The β transcript encodes p14^{ARF} which is involved in p53-related apoptotic pathways by inhibiting MDM2. The resulting p16^{INK4A}-Leiden truncated protein disrupts G1/S cell cycle arrest by losing the binding capacity to CDK4/CDK6 complex. The resulting p14^{ARF}-Leiden fusion protein seems to remain functional. Source data were adapted from the following resources [5-7].

Interestingly, *CDKN2A* function is lost in hereditary melanoma, but also in sporadic melanoma, commonly through deletion [22]. p16^{INK4A} expression was significantly reduced in melanomas when compared to nevi according to transcriptomic data, suggesting that p16^{INK4A} is the predominant tumor suppressor protein acting at the transition stage to invasive melanoma [4]. Bi-allelic inactivation of *CDKN2A* is mainly observed in progressed stages of the disease but about 40% of sporadic melanoma cases already carry a somatic mutation, chromosomal deletion and promoter hypermethylation in *CDKN2A* [34, 49]. Collectively, these data suggest a significant effect of both germline and somatic mutations of *CDKN2A* in melanoma development.

Following *CDKN2A*, *Cyclin-dependent kinase 4 (CDK4)* is the second high penetrance melanoma susceptibility gene identified through a candidate gene sequencing approach [98]. *CDK4* is less frequently mutated than *CDKN2A*, since it has only been reported in a total of 18 melanoma families up to date according to follow-up studies [99-102]. Besides human studies, *in-vivo* experiments with mice carrying the germline *CDK4* mutation, p.R24C, revealed susceptibility to melanoma development after exposure to carcinogen treatment [103].

Due to the limitation of genetic linkage and candidate gene screening to discover additional novel high penetrance melanoma susceptibility genes, it was not until 2011, that the development of new genomic sequencing technologies were implemented to discover novel genes. Germline mutations in the tumor suppressor gene, *BRCA1-associated protein 1* (*BAP1*), were identified in two families with atypical melanocytic tumors by application of sequencing technology [104, 105]. Somatic loss of the WT allele was detected in the tumors of patients. In addition, *BAP1* loss increased the predisposition for other tumor types including mesothelioma, renal cell carcinoma and basal cell carcinoma [106-109]. Overall, germline mutations in *BAP1* account for a small percentage of melanoma families.

Moreover, the *microphthalmia-associated transcription factor* gene (*MITF*) is the most well-known medium penetrance melanoma susceptibility gene. *MITF* regulates melanocyte development and differentiation, and was the first gene to be identified by next-generation sequencing (NGS) technology in melanoma susceptibility [110, 111]. The *MITF* p.E318K germline mutation alters MITF transcriptional activity through abrogation of a sumoylation motif. Germline mutation carriers have also been associated with increased risk for renal cell carcinoma and PC [112].

The application of more advanced methods such as WES analysis had a significant effect on identifying high penetrance genes for melanoma. The initial variants identified were members of the telomerase and shelterin complex including genes that protect chromosomal ends. In 2013 a variant was found within the promoter of *telomerase reverse transcriptase* gene (*TERT*). The mutation, -57bp from the translation-start site, segregated with disease in a 14-case melanoma family and functionally created a binding motif for ETS/TCF transcription factor leading to increased *TERT* expression [48]. In concordance, the *pTERT* mutation that was detected in a single melanoma-prone family, (G>A) at −246 bp upstream from the ATG start site, was previously associated with low telomerase activity in patients with non–small cell lung cancer [113]. Two-carriers with germline mutations in *TERT* developed several types of cancer, including ovarian cancer (at 27 years), melanoma (at 20 years), renal cell carcinoma, bladder cancer, breast cancer and finally lung cancer [48]. Collectively these data suggest that *TERT* constitutes an additional high penetrance gene for familial melanoma that is also mutated in sporadic cases.

Moreover, application of whole-genome sequencing (WGS), WES and targeted sequencing identified loss-of-function mutations in the *protection of telomeres 1* (*POT1*) gene in melanoma families from the UK and Australia [114]. Six families were found positive for novel *adrenocortical dysplasia homologue* (*ACD*) mutations and four families were positive for *telomeric repeat binding factor 2 interacting protein* (*TER2IP*) variants including segregating nonsense mutations for both genes by screening 510 melanoma families [115]. Collectively these data suggest that

dysregulation of telomeres is an important contributing-pathway in a proportion of high-risk families CM families.

The most recent application of WES identified additional rare variants within the *golgi membrane protein 1* (*GOLM1*) gene, *EBF Transcription Factor 3* (*EBF3*) gene, *DNA Polymerase Epsilon* (*POLE*) gene and *Tumor Protein P53 Regulated Apoptosis Inducing Protein* (*TP53AIP1*) gene, although the effect size and functional significance of these variants still requires clarification by future studies [116-119].

To summarize, with *CDKN2A* mutations accounting for about 40% of variation in familial clustering of CM world-wide, and rare mutations in *CDK4, MITF, BAP1, TERT, POT1, ACD, TERF2IP, GOLM1, EBF3, POLE* and *TP53AIP1* responsible for up to 10% of variation, there is still about 50% unexplained remaining germline variation (Figure 2). The intensive clinical follow-up data in families with proven germline mutations may reduce the number of melanoma cases. Nevertheless, the possibility for an effect of polygenic risk factors such as multiple medium and low-penetrance genes, including *MITF, MC1R, SLC45A2, ARNT* and others cannot be excluded for these families. The shared environmental exposures of affected family members could also be a contributor to melanoma development (Figure 2) [120].

Combined, other rare high-penetrance genes are very likely to exist and application of WES and WGS analysis provide the best resource in clarifying the unknown genes. The identification of alterations within the regulatory region of *TERT* suggests that WGS analysis is a promising tool in uncovering variation within the non-coding and regulatory region of our genome. Collectively, the identification of novel high penetrance melanoma susceptibility genes is still essential in order to improve genetic testing and counselling in hereditary melanoma patients.

IDENTIFICATION OF CANCER DEPENDENCIES AS NEW THERAPEUTIC TARGETS

In addition to predisposition genes and driver genes, a third class of genes is relevant for the biology and treatment of cancer and these are dependence/fitness/essential genes [121].

Application of large-scale pharmacogenomic screens across different panels of cancer cell lines provides a possible solution in un-revealing novel fitness genes as possible biomarkers for therapy [122]. Moreover, the recent advancement of CRISPR-Cas9 screening technology may provide a precise method in determining novel biomarkers with high precision and less false-positive targets when compared to previously used screens through short-hairpin RNAs (shRNAs) [123, 124]. The CRISPR-Cas9 technique consists of using a single-guide RNA (sgRNA) molecule to bind to complementary DNA sequences, which simultaneously recruits the endonuclease Cas9 to introduce double-stranded breaks in the target DNA. The resulting double-stranded break is then repaired, allowing modification or removal of specific DNA bases. The mechanism of repair usually involves non-homologous end joining, an error prone pathway that results in generation of indels within the gene [125, 126].

Cancer *in vitro* systems are now being investigated using pooled CRISPR-Cas9 screens that employ genome-scale libraries consisting of thousands of sgRNAs. Data from these systems can be used to identify and prioritize new cancer therapeutic targets firstly by infecting tumor cell lines of interest and secondly by measuring the endpoint sgRNA abundance to identify depleted or enriched genes from the screen (usually 14-21 days after infection) (Figure 4) [127-129].

A recent study aiming to identify targets which when knocked-out confer resistance to melanoma immunotherapy, showed that Apelin Receptor (APLNR) was a modulator of interferon-γ responses in tumors by application of CRISPR-Cas9 positive selection screening [130]. On a similar note, negative selection screens have a general goal in identifying genes which when lost have an effect on cell proliferation and therefore are essential for cell fitness [127].

Genes may influence the fitness of melanoma cells either because they encode proteins involved in essential cellular processes, or they are required for viability specifically of cells of the melanocytic lineage. Still, the identification of context-specific fitness genes to therapeutically target for maximal clinical benefit of melanoma patients remains a challenge [129]. Collectively, these data suggest that application of CRISPR-Cas9 screening technology may provide a precise method in determining novel vulnerabilities for melanoma targeted therapy.

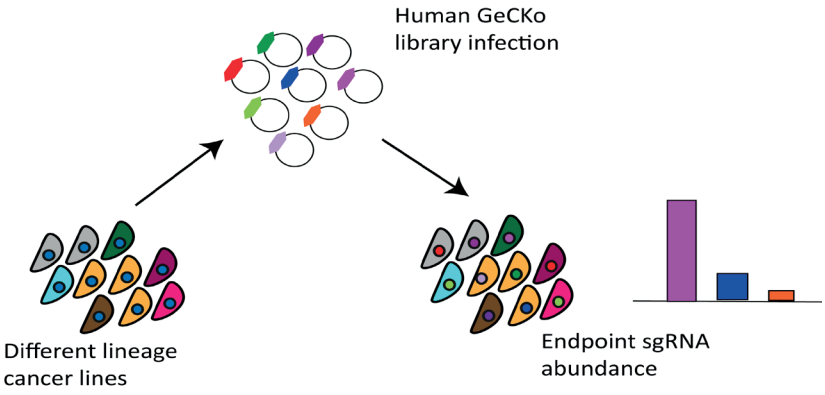

Figure 4 Schematic overview of a CRISPR-Cas9 knockout screen. The cell-lines of interest (different cancer lineage) are infected by the human library of sgRNAs knocking out all known genes (Genome Scale CRISPR Knock-Out library). The sgRNA abundance is read using Next Generation Sequencing technology at days 0 and 14 (or 21) after infection. Those sgRNAs that are depleted compared to the initial sgRNA abundance depict genes essential for cell growth whereas sgRNAs that are enriched indicate genes that may serve as possible tumor suppressors.

OBJECTIVES

This thesis sought to investigate different aspects of CM biology, mostly linked to genetic dependencies in hereditary and sporadic melanoma. Specifically, herein we have employed state of the art technologies such as WES, digital PCR (dPCR) and CRISPR-Cas9 genetic engineering to unravel the complexity of genetic events in hereditary and sporadic melanoma. Specific objectives include:

a) Identification and validation of novel high penetrance melanoma susceptibility genes.
b) Identification of genetic modifiers predicting the risk of melanoma and PC in *p16^{INK4A}-Leiden* mutation carriers.
c) Timing of *CDKN2A* loss-of-heterozygosity in melanocytic tumors of *p16^{INK4A}-Leiden* mutation carriers.
d) Determination of genetic dependencies in melanoma by analyzing and processing CRISPR-Cas9 screening technology data.

To elucidate the genetic basis of familial melanoma and discover novel high penetrance melanoma susceptibility genes, in **chapter 2**, we applied WES in a Dutch melanoma family. The results of WES analysis were also validated in available patient's tissues and functionally verified *in-vitro* using cancer cell lines. This work is absolutely essential to improve genetic testing and counselling of familial melanoma kindreds since about 50% of genetic variation underlying genetic variability remains unknown.

Identification of genetic risk factors, other than a germline *CDKN2A* mutation, responsible for PC and melanoma risk in *CDKN2A*-mutated families has been a challenge. In **chapter 3**, we sought to investigate a variable genomic region (SNP) within *TERT/CLPTM1L* high-cancer risk locus as a modifying genetic risk factor for PC and melanoma in *p16^{INK4A}-Leiden* mutation carriers.

Even though scientific studies provide evidence for *CDKN2A* bi-allelic loss to be an important event in the transition to invasive melanoma in sporadic cases, there is little or no evidence known about inactivation of this tumor suppressor gene in the progression stages of hereditary melanoma. Therefore, in **chapter 4**, we sought to investigate *CDKN2A* inactivation through LOH by applying dPCR in FAMMM syndrome patients, carrying a germline *CDKN2A* mutation. Utilization of dPCR assays allows for numerous applications such as quantitative detection of mutant cell fraction in a population of ad-mixed cells, LOH and quantification of T-cells in tumors (Figure 5) [131-133]. In cases where quantification of the actual mutation of interest is challenging, dPCR technology may be applied to target a common polymorphic region (SNP) that is linked to the specific mutation site. This could

be depicted by the high probability that the SNP-allele linked to the mutation, will end up in the same droplet as the mutant-allele (Figure 5). Application of SNP-based dPCR technology in melanocytic neoplasms of FAMMM syndrome patients allowed for absolute quantification of allelic imbalance within *CDKN2A*, indicative of LOH. We also attempted to deduce the order of genetic events via quantifying cells with *BRAF*^{V600E} mutation, p*TERT* mutations and chromosome 9q LOH.

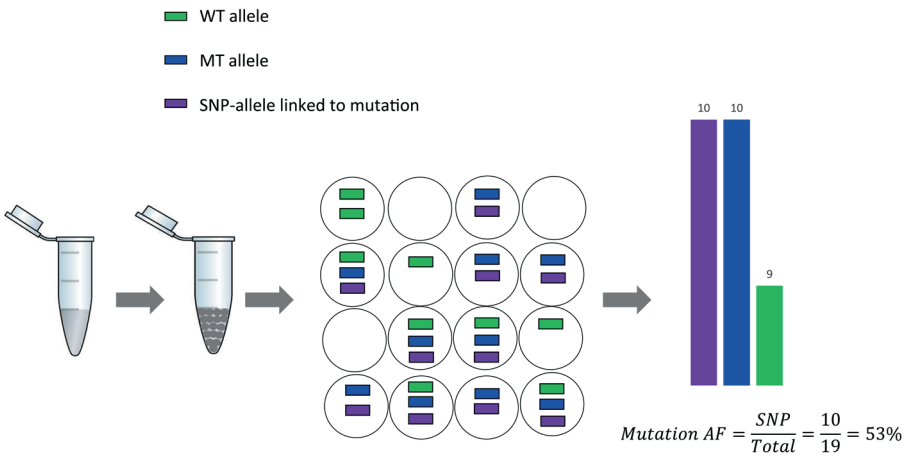

Figure 5 Diagram of SNP-based digital PCR (dPCR) analysis. The sample of interest is partitioned into 20,000 droplets which are then detected for the specific target of interest. The homozygous genotype for a wild-type (WT) sequence is depicted by a droplet positive for the green target only. The heterozygous genotype is depicted by positivity for the WT-allele (green), the mutant (MT) allele (blue) and the SNP-allele (purple) that is linked to the mutation. The homozygous genotype for the mutation is depicted by positivity for the MT allele (blue) and the SNP-allele (purple). In cases where the mutation is not the direct target of amplification, targeting of the SNP allele linked to the mutant allele allows for direct quantification of the mutation allele frequency (AF) and therefore loss-of-heterozygosity (LOH). In the example shown, mutation AF was calculated by dividing the SNP-allele counts (10) over the total allele-counts (19). The mutation AF was 53% depicting LOH in this example (>50%).

Finally, resistance to *BRAF* inhibitors warrants screening for identification of novel pathways to melanoma treatment. The application of CRISPR-Cas9 screening technology is nowadays the leading tool in revealing novel genetic vulnerabilities in cancer. Therefore, in **chapter 5** we performed comparative analysis using bioinformatic tools to study CRISPR knockout (KO) screening data and identify novel fitness genes in melanoma.

Collectively, through application of novel genomic techniques in this thesis, we hope to have explored in detail the genetic dependencies in familial and sporadic melanoma.

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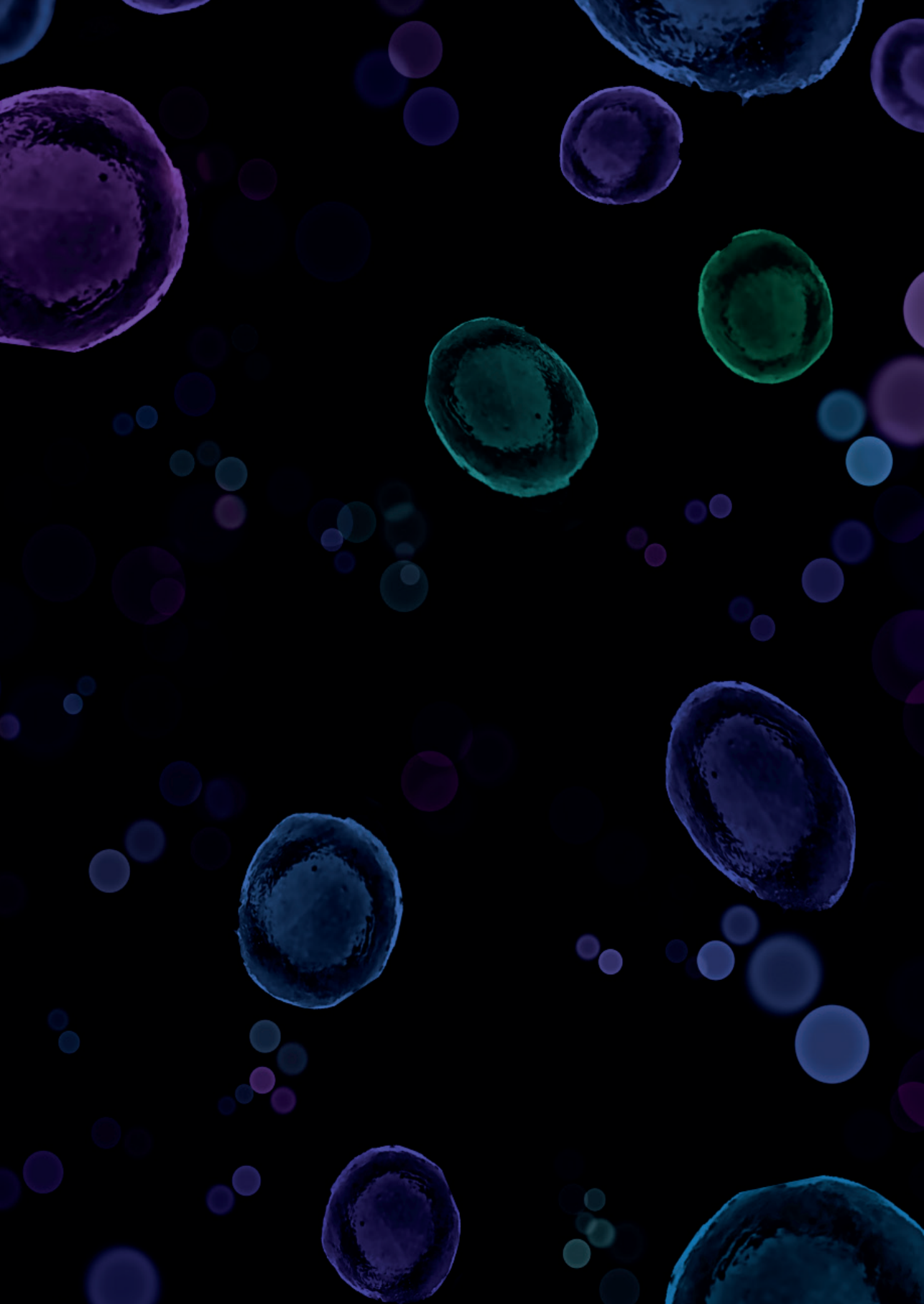

Chapter 2

NEK11 as a candidate high-penetrance melanoma susceptibility gene

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ABSTRACT

A proportion of patients diagnosed with cutaneous melanoma reports a positive family history. Inherited variants in *CDKN2A* and several other genes have been shown to predispose to melanoma; however, the genetic basis of familial melanoma remains unknown in most cases. The objective of this study was to provide insight into the genetic basis of familial melanoma. In order to identify novel melanoma susceptibility genes, whole exome sequencing (WES) analysis was applied in a Dutch family with melanoma. The causality of a candidate variant was characterized by performing co-segregation analysis in five affected family members using patient-derived tissues and digital PCR analysis to accurately quantify mutant allele frequency. Functional in-vitro studies were performed to assess the pathogenicity of the candidate variant. Application of WES identified a rare, nonsense variant in the *NEK11* gene (c.1120C>T, p.Arg374Ter), co-segregating in all five affected members of a Dutch family. NEK11 (NIMA-Related Kinase 11) is involved in the DNA damage response, enforcing the G2/M cell cycle checkpoint. In a melanoma from a variant carrier, somatic loss of the wildtype allele of this putative tumor suppressor gene was demonstrated. Functional analyses showed that the *NEK11* p.Arg374Ter mutation results in strongly reduced expression of the truncated protein caused by proteasomal degradation. The *NEK11* p.Arg374Ter variant identified in this family leads to loss-of-function through protein instability. Collectively these findings support *NEK11* as a melanoma susceptibility gene.

INTRODUCTION

Cutaneous melanoma is an aggressive type of skin cancer resulting from malignant transformation of melanocytes. Incidences of melanoma continue to rise steadily, with more than 230,000 cases diagnosed each year worldwide, accompanied by 55,000 deaths [1]. Ten percent of cases are found in people with familial predisposition, i.e. families with at least two first degree relatives with melanoma [2].

Several high-penetrance melanoma susceptibility genes have been identified and account for approximately 40% of melanoma families [3, 4]. The majority of these families are affected by germline mutations in *CDKN2A* [5], a key cell-cycle checkpoint regulator and first reported high-penetrance melanoma susceptibility gene [6-8]. Following *CDKN2A*, germline mutations in other genes have been linked to familial predisposition to melanoma; these include *CDK4* [9] and *BAP1* [10, 11]. Bi-allelic inactivation has been reported in tumor tissues with germline variants in *BAP1* including mesothelioma, uveal melanoma and cutaneous melanoma [12, 13] suggesting genetic analysis is an informative approach for discovering melanoma-predisposition genes. Considering the discovery of germline *MITF* variants, functional analysis revealed mutant *MITF* to have increased transcriptional activity, migration and invasion in melanoma cell lines [14, 15] suggesting that not only genetic analysis in patient's tissues but also functional validation adds to the current value of mutation screening. Application of Whole Exome Sequencing (WES) analysis has been successful in identifying rare variants including *TERT* promoter [16], *POT1* [17] *TERF2IP* and *ACD* [18], *GOLM1* [19], *EBF3* [20] and *POLE* [21] as candidate high-penetrance melanoma susceptibility genes. Still, the genetic basis of over half of melanoma families remains unknown, impairing genetic testing and counselling in families with predisposition to melanoma [22, 23]. Here, *NEK11* gene was identified by WES in a Dutch family with melanoma and characterized as a potential novel high-penetrance melanoma-susceptibility gene.

METHODS

Whole exome sequencing (WES)

Study population and ethics approval

WES was carried out in blood-derived DNA samples of two members of a Dutch familial melanoma family. Study approval was obtained by the ethics committee of Leiden University Medical Center (LUMC, P00.117).

Sequencing analysis and bioinformatics

Sequencing was performed on Hiseq2000 platform with TruSeq Exome Enrichment kit. Paired-end reads of 110 bp were generated with mean coverage of 40X. The Burrows-Wheeler aligner was used for mapping sequencing reads to the reference UCSC human genome. SNVs were detected using samtools/bcftools. Indels were detected with Pindel and annotated to dbSNP144 using ANNOVAR. Variants altering the coding sequence were selected excluding those that were present at a frequency of 0.0005% or higher in the Kaviar (Known VARiants) control population database, including 162 million variants from human genomes of datasets such as ExAc and 1000Gs [24].

Selection, validation and interpretation of variants

Variants identified were assessed to identify pathogenic or potentially pathogenic variants in ClinVar. Variants were then filtered using *in-silico* prediction algorithms to show if an alteration affects protein function. Details about criteria for interpretation of variants has been reported previously [25]. These included exonic, frameshift, non-synonymous SNVs, splicing and stop-gain SNVs. We then focused on segregating mutations between all family members and functional significance. Co-segregation of *NEK11* p.Arg374Ter mutation was confirmed using Sanger sequencing of germline DNA from family members 1, 4 ,5, 7 and 12 (Supplemental Table 1) (Macrogen, Amsterdam, The Netherlands).

Genotyping and LOH analysis

DNA was extracted from primary melanoma FFPE tissue of a *NEK11* p.Arg374Ter mutation carrier (family member 5) using the QIAamp DNA FFPE Tissue kit (QIAGEN, Venlo, The Netherlands) according to manufacturer's instructions. DNA extraction of normal and tumor tissue was obtained through micro-dissection/ punch biopsy by the pathology department of LUMC, The Netherlands. Genomic primer sets were used to amplify the region of interest containing the mutation (*NEK11* c.1120C>T) and a common SNP (rs4974475, chr3:130882827, MAF 17%), located at 2 kb upstream from the *NEK11* mutation site to verify LOH (Supplemental Table 1). PCR products were cleaned-up using a PCR clean-up protocol (Bio-Rad, Hercules, California, USA) and sequenced by Sanger sequencing analysis-long run

(Macrogen, Amsterdam, The Netherlands). Chromatograms were then analyzed by Chromas Technelysium DNA Sequencing Software (Technelysium Pty Ltd, South Brisbane, Australia).

Digital PCR analysis (dPCR)

Mutation detection assays specific for dPCR (Bio-Rad, Hercules, California, USA) describe the incorporation of both wild-type and mutated targets in a single dPCR mix. In this case, the assay was designed for the detection of *NEK11* p.Arg374Ter mutation. Detailed protocol of mutation detection dPCR assay has been described previously [26] and dPCR sequence information is provided in Supplemental Table 1. QuantaSoft software (Bio-Rad, Hercules, California, United States) was used to analyze the data by calculating the concentration of the amplified dPCR product (copies/µl) [26]. The wild-type (WT) allele frequency was calculated by dividing the WT allele counts over the total allele counts and the mutant (MT) allele frequency was calculated by dividing MT allele counts over the total allele counts.

Cell culturing and maintenance

U2OS (human osteosarcoma tumor cell line) and FM6 (human cutaneous malignant melanoma cell line) cells were maintained in DMEM medium supplemented with penicillin (100 I.U./mL)/streptomycin (100 µg/mL) and 10% Fetal Bovine Serum (FBS) and glutamax 100x (Thermo Fisher Scientific, Waltham, Massachusetts, United States). All cells were grown in a humidified incubator at 37°C and 5% CO_2 and routinely sub-cultured when reaching 95% confluency.

Plasmid construction and introduction of NEK11 p.Arg374Ter mutation

NEK11-FL (full-length isoform [27]) was expressed from a plasmid construct containing WT *NEK11* cDNA, fused with N-terminal FLAG-epitope tag (Kind gift from professor Andrew Fry, University of Leicester, UK) for expression in U2OS and FM6 cells [27]. Site-directed mutagenesis was applied to introduce *NEK11* p.Arg374Ter mutation in flag-tagged *NEK11* expression vectors. Primer-sets were designed specifically targeting the mutation site of *NEK11* exonic sequence (Supplemental Table 1). Thermal cycling was performed to introduce the mutation consisting of 1-minute denaturation at 95°C, followed by 10 cycles of 1-minute steps at 95°C, 63°C and 68°C. The PCR product was then digested with *DpnI* enzyme and transformed into Top10 bacteria to produce inducible vectors for functional experiments. Sequences of both *NEK11* WT and MT expression vectors were confirmed by Sanger Sequencing analysis long-run (Macrogen, Amsterdam, The Netherlands) (Supplemental Table 1).

Lentivirus production

NEK11 WT and MT cDNAs were re-cloned into a lentiviral backbone containing the neomycin resistance gene. Lentiviral stocks were produced by transfections into HEK-293T cells as described previously [28] but calcium phosphate was replaced with polyethylenimine (PEI) in the transfection mix. Virus was quantified by antigen capture enzyme-linked immunosorbent assay (ELISA) measuring HIV p24 levels (ZeptoMetrix Corp., New York, NY, USA).

Transient transfections

U2OS cells were harvested and seeded in appropriate growth medium in 6-well plates ($0.5x10^5$ cells/ml) and 60-mm dishes ($1.8x10^5$cells /ml). The DNA mix was prepared as follows: 0.8µg *pLV-NEO-NEK11-WT, pLV-NEO-NEK11-MT* and *pLV-NEO-empty* lentiviral vectors (see lentivirus production section), 0.1µg *Tomato-Red,* 300ng of *GFP* expression vector and 0.2µg *pSuper.* The PEI mix was prepared as follows: 3:1 PEI (3 parts of PEI to 1 part of DNA concentration) diluted in Gibco™ Opti-MEM™ Reduced Serum medium (Thermo Fisher Scientific, Waltham, Massachusetts, United States). PEI mix was added slowly to DNA mix followed by a short vortex. The mixture was kept at RT for 20 minutes and then added dropwise to U2OS cells. Growth medium was replaced 16 hours after transfection and U2OS cells were further incubated for another 24 hours.

Lentiviral transductions

Fresh culture media were prepared with viral supernatants supplemented with 8 µg/ml polybrene (Sigma-Aldrich, St. Louis, Missouri, United States). FM6 cells were seeded in 6-well plates at a density of $2x10^5$ cells/well. FM6 cells were incubated with virus-containing medium overnight, after which the cells were refed with fresh medium containing G418 (Sigma-Aldrich, St. Louis, Missouri, United States) to produce stable cell lines expressing *NEK11* WT and MT by using neomycin as selection marker.

RNA isolation, cDNA synthesis and gene expression analysis

Lymphocytic RNA of a *NEK11* p.Arg374Ter carrier (family member 18) and a non-relative spouse was isolated using the RNeasy micro kit from QIAGEN (Venlo, The Netherlands). RNA was isolated from FM6 cells using the SV total RNA isolation kit (Promega, Fitchburg, WI, USA). First strand cDNA synthesis was carried out using the iScript™ c-DNA synthesis kit (Bio-Rad, Hercules, California, USA) and Sanger Sequencing analysis (LGTC, LUMC, The Netherlands) was used to detect presence of *NEK11* WT and MT alleles (Primer sequences shown at Supplemental Table 1). *NEK11* gene expression was confirmed using SYBR® green based quantitative PCR on CFX384 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, California, USA) (Supplemental Table 1). Gene expression results were analyzed using Bio-Rad CFX Manager 3.1 Software (Hercules, California, USA) and corrected relative to

reference gene expression (*CAPNS1* and *SRPR*) as well as transfection efficiency target *Tomato-Red* (Supplemental Table 1).

Immunofluorescence staining

Transfected U2OS cells on cover slips were washed twice in phosphate-buffered saline (PBS) solution and fixed with 4% paraformaldehyde (PFA) for 10 minutes. Cover slips with U2OS cells were then incubated for 10 minutes in PBS/0.2%Triton-X100 (permeabilization) and pre-incubated for 30 minutes in PBS/0.05% Tween-20 containing 5% normal goat serum (NGS). Subsequently, U2OS cells were incubated with monoclonal anti-flag M2 Catalog Number F1804 (Sigma-Aldrich, St. Louis, Missouri, United States) diluted in PBS/Tween/NGS (1:500) for 60 minutes at room temperature followed by washing three times in PBS/Tween for a total of 15 minutes. The secondary antibody, anti-Mouse IgG-Cy2 (#115-225-146, Jackson ImmunoResearch Laboratories, Cambridge, UK) was diluted in PBS/Tween/NGS (1:100) and added at room temperature in the dark. U2OS cells were finally washed for 10 minutes with PBS/Tween and coverslips were placed on slides for analysis on a Leica DMRA fluorescent microscope (Nijmegen, The Netherlands).

Drug Treatments

MG132 proteasome inhibitor (Sigma-Aldrich, St. Louis, Missouri, United States) was added to U2OS and FM6 cells at a final concentration of 20 μM for 5-6 hours before RNA and protein isolation procedures. The cycloheximide (CHX), translation inhibitor, was added at a concentration of 50μg/ml (Sigma-Aldrich, St. Louis, Missouri, United States) to FM6 stable-cell lines expressing *NEK11* WT and *NEK11* MT as a time-course treatment of 0, 1, 2 and 4 hours.

Protein isolation and Western Immunoblotting analysis

U2OS and FM6 cells were washed twice in ice-cold PBS and incubated on ice for 10 minutes in Giordano buffer (50mM Tris-HCl pH7.4, 250mM NaCl, 0.1% Triton X-100 and 5mM EDTA; supplemented with phosphatase and protease inhibitors). Lysates were collected by scraping and centrifuged at max speed for 10 minutes and protein concentration was determined using the Bradford method. Western Blot procedure was followed as described previously [29]. NEK11 protein was detected by the Anti-Flag antibody (1:1000) (Sigma-Aldrich, St. Louis, Missouri, United States) and the controls were detected by Anti-USP7 (1:1000) (Bethyl Laboratories, Biomol, Montgomery, Texas, USA), Anti-GAPDH (1:1000) (Sigma-Aldrich, St. Louis, Missouri, United States) and Anti-P53 (1:1000) (Santa Cruz Biotechnology, Heidelberg, Germany). After transient expression, protein levels were determined using the Odyssey machine (LI-COR, Lincoln, Nebraska, United States) and analyzed using Odyssey software according to manufacturer's instructions. Secondary antibodies used were IRDye® 800CW Goat anti-Mouse IgG (H + L), 0.1 mg (1:5000) and IRDye® 680LT Goat anti-Rabbit IgG (H + L), 0.1 mg (1:5000)

(LI-COR, Lincoln, NE, United States). Due to low protein expression in stably-transduced FM6 cells, the ChemiDoc Imaging System was used to detect proteins with increased sensitivity and specificity. The bands were analyzed with Image lab software (Bio-Rad, Hercules, California, USA) according to manufacturer's instructions. Protein-expression quantification was performed relative to unaffected expression of controls (GAPDH, USP7).

Statistical Analysis

All data were analyzed by calculating the mean and standard deviation (SD) and graphs were obtained in GraphPad Prism version 7 (GraphPad software, San Diego, CA, USA). ANOVA and multiple comparisons were applied to detect statistically significant differences between expression patterns of three independent experiments (n=3). Statistical significance was reached when $p<0.05$.

RESULTS

WES analysis and identification of NEK11 p.Arg374Ter

A Dutch melanoma family presented four melanoma cases and one uveal melanoma case with prostate cancer. The diagnosis of melanoma in five members in multiple generations strongly suggests an autosomal dominant mode of inheritance (Figure 1) and members were negative for mutations in established melanoma susceptibility genes (*CDKN2A, BAP1, POT1, TERT, TERF2IP, ACD, MITF, GOLM1, EBF3*). A WES analysis was carried out on DNA from blood cells of two affected members (Figure 1). Among a total of 19 rare, co-segregating non-synonymous variants that met our criteria, 17 were missense mutations, probably damaging, predicted deleterious [30, 31]. These variants however, are not plausible candidate melanoma susceptibility genes (Table 1) since there is no evidence supporting a strong tumorigenic effect based on published literature. Interestingly, two were nonsense stop-gain single nucleotide variants (SNVs) (Table 1). One candidate stop-gain variant was p.Arg66Ter in *ZNF192,* a gene possibly regulating transcription. However, no implications in cancer have been mentioned in published literature. In contrast, the other candidate was a truncating variant (p.Arg374Ter) in the never in mitosis-gene A (NIMA)-related kinase 11 (*NEK11*). This family of proteins functions in different aspects of cell cycle regulation, although the in-depth role of NIMA-related kinases remains to be uncovered [32]. *NEK11* has been reported to be somatically mutated in different types of cancer, including lung, breast, prostate and melanoma [33]. The frequency of NEK11 mutations was >5% in melanomas [34, 35] suggesting a plausible candidate in melanoma development.

Sanger sequencing confirmed *NEK11* p.Arg374Ter to co-segregate within four cutaneous melanoma cases and one uveal melanoma case in this Dutch melanoma family (Figure 1). Family members 13 and 16 were also found to be *NEK11* p.Arg374Ter carriers although with no clinical presentation of cancer/melanoma. Considering the current age of these individuals and absence of the melanoma phenotype, the possibility for non-penetrance is very likely (Figure 1). Somatic loss of the wildtype (WT) allele was detected in primary cutaneous melanoma tissue of a mutation carrier (Figure 2A-C) and confirmed by the highly sensitive and quantitative method, digital PCR (dPCR) [36] whereby, a higher fraction of *NEK11* p.Arg374Ter mutant (MT) than *NEK11* WT allele was detected in melanoma tissue when compared to normal tissue micro dissected from the same biopsy sample (Figure 2D). Furthermore, examination of a common SNP (rs4974475, chr3:130882827, MAF 17%), showed loss of this variant in the melanoma tissue of a *NEK11* p.Arg374Ter carrier, suggesting LOH over a longer genetic region (Supplemental Figure 1). Collectively, these data suggest a potential loss-of-function (LOF) mutagenic effect of *NEK11* p.Arg374Ter.

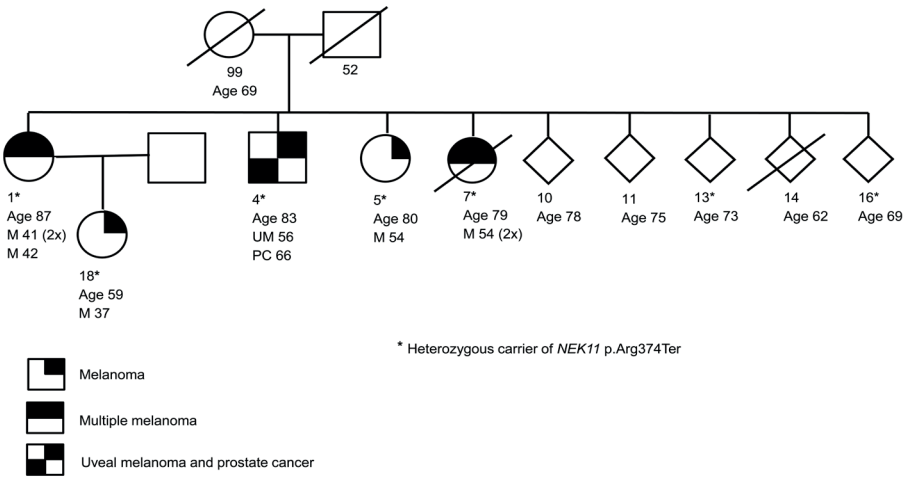

Figure 1 Co-segregation of *NEK11* p.Arg374Ter in a Dutch melanoma family. Whole-exome sequencing was carried out for family members 1 and 4. Co-segregation of *NEK11* p.Arg374Ter was confirmed by analyzing germline DNA from all family members. Current age and age at death of deceased individuals (those reported) are indicated. Age at diagnosis of each tumor type is noted in affected family members (M = melanoma, UM= uveal melanoma, PC= prostate cancer).

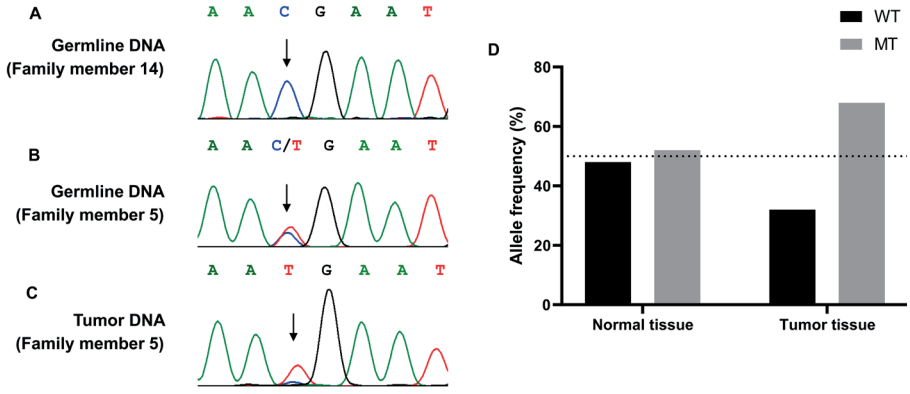

Figure 2 LOH analysis of a *NEK11* p.Arg374Ter mutation carrier. Chromatogram showing DNA sequence of **A)** healthy family member (14), **B)** a *NEK11* p.Arg374Ter carrier (family member 5) and **C)** tumor of a *NEK11* p.Arg374Ter carrier (family member 5). Arrows indicate the *NEK11* p.Arg374Ter mutation site. **D)** dPCR *NEK11* mutation assay showing the *NEK11* wildtype (WT) and *NEK11* p.Arg374Ter (MT) allele frequency detected in normal and tumor tissue from FFPE derived DNA of family member 5.

Table 1 Summary of Whole-exome sequencing (WES) analysis and identification of segregating novel predicted damaging/deleterious variants in a Dutch melanoma family.

Gene	Change	Ch	Ref	Alt	Type[a]	SIFT	Polyphen	MAF[b]
GPATCH3	p.Gly131Arg	1	C	T	missense	deleterious	probably_ damaging	0.00006168
ATPIF1	p.Arg94His	1	G	A	missense	deleterious	probably_ damaging	0.00004406
KALRN	p.Ser1629Cys	3	C	G	missense	deleterious	probably_ damaging	0.00002641
NEK11	p.Arg374Ter	3	C	T	stop-gained			0.00002641
ZNF192	p.Arg66Ter	6	C	T	stop-gained			0.000008791
GPR111	p.Ser168Arg	6	T	A	missense	deleterious	probably_ damaging	0.00006486
GPAM	p.Pro403Thr	10	G	T	missense	deleterious	probably_ damaging	0.000008794
NELL1	p.Val755Met	11	G	A	missense	deleterious	probably_ damaging	0.0002261
KRT77	p.Asp316Asn	12	C	T	missense	deleterious	probably_ damaging	0.00001502
OAS2	p.Tyr269Cys	12	A	G	missense	deleterious	probably_ damaging	-
DNAJC3	p.Arg346Gln	13	G	A	missense	deleterious	probably_ damaging	0.0001553
TEP1	p.Arg1386Trp	14	G	A	missense	deleterious	possibly_ damaging	0.001266
PLCB2	p.Arg253Trp	15	G	A	missense	deleterious	probably_ damaging	0.001074
DNASE1	p.Ala168Val	16	C	T	missense	deleterious	possibly_ damaging	0.001402
BFAR	p.Phe53Ile	16	T	A	missense	deleterious	possibly_ damaging	-
ITGB4	p.Arg556Cys	17	C	T	missense	deleterious	possibly_ damaging	0.0006357
PSG7	p.Trp67Ser	19	C	G	missense	deleterious	probably_ damaging	0.0001473
GPR50	p.Gly93Ala	X	G	C	missense	deleterious	possibly_ damaging	0.00001240
GABRQ	p.Arg254Cys	X	C	T	missense	deleterious	probably_ damaging	0.00007713

a Variants characterized by ExAC/gnomAD/Genome of The Netherlands/Ensembl databases
b MAF in European (Non-Finnish) population

Functional analysis of NEK11 p.Arg374Ter

Following genetic characterization of *NEK11* p.Arg374Ter as a potential LOF mutation, functional analyses were performed to investigate the effects of this truncating mutation on the expression level of *NEK11* mRNA and NEK11 protein. Upon transient transfection of *NEK11* expressing plasmids in osteosarcoma tumor cell line U2OS the expression of *NEK11* MT mRNA was lower than *NEK11* WT mRNA, although the difference was statistically not significant (Supplemental Figure 2). U2OS cell-line provided the ideal conditions for functional analysis since it is an easily transfectable, fast-growing cell line and has been previously used to functionally characterize NEK11 [27, 37, 38]. *NEK11* MT mRNA expression was detected in lymphocyte mRNA of a mutation carrier by Sanger sequencing and dPCR analyses (Figure 3A-C) suggesting that the premature stop codon does not result in significant transcript degradation by nonsense-mediated mRNA decay (NMD). Combined with the finding that the mutant *NEK11* mRNA was detected in U2OS cells (Supplemental Figure 2), these results indicate no significant effect of the mutation on *NEK11* mRNA expression levels.

Introduction of p.Arg374Ter mutation in the *NEK11* expression vector, resulted in synthesis of a truncated NEK11 protein lacking the whole C-terminal PEST-like domain as well as part of the coiled-coil motifs (Supplemental Figure 3A). The coiled-coil region regulates protein activation suggesting that loss or absence of these motifs would affect protein function [39]. The truncated protein runs at approximately 45 kDa, which reasonably fits with the size of 373 amino acids (Supplemental Figure 3A). Immunoblot analysis of protein lysates made from transfected U2OS cells showed that the level of the truncated protein was 3-fold lower than NEK11 WT expression ($p<0.005$; Figure 4A and B) when corrected for mRNA expression. Treatment with the proteasome inhibitor MG132 increased NEK11 protein level, particularly of the truncated product (~ 2 fold). Still, the difference between NEK11 WT and MT protein levels in lysates of MG132-treated U2OS cells is significant ($p<0.005$). Collectively, statistically significant lower protein expression was correlated with the *NEK11* p.Arg374Ter mutation.

Since a distinct subcellular localization of NEK11-FL (645 amino acids) and NEK11-S (450 amino acids) has been reported [27], which might affect the protein expression level, the subcellular localization of the Flag-tagged NEK11 MT was investigated in comparison to Flag-tagged NEK11-FL. Interestingly, both proteins were mainly localized in the nucleus of U2OS cells (Supplemental Figure 3B), in contrast to the earlier publication. However, in that publication GFP-tagged constructs were used. Indeed, using the same GFP-tagged constructs the subcellular localization of NEK11-FL and NEK11-S was as reported; the GFP-NEK11 MT protein localized in the nucleus, similar to GFP-NEK11-S (data not shown).

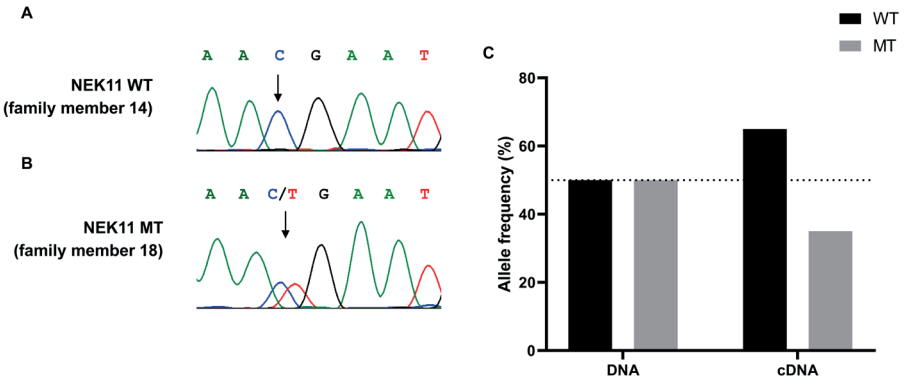

Figure 3 *NEK11* wildtype and *NEK11* p.Arg374Ter mRNA analysis. Chromatogram showing sequence from cDNA of **A)** healthy family member 14, and **B)** a *NEK11* p.Arg374Ter carrier (family member 18) Arrows indicate the *NEK11* p.Arg374Ter mutation site. **C)** Allele frequency of *NEK11* wildtype (WT) and *NEK11* p.Arg374Ter (MT) detected by dPCR *NEK11* mutation assay using DNA and cDNA from family member 18.

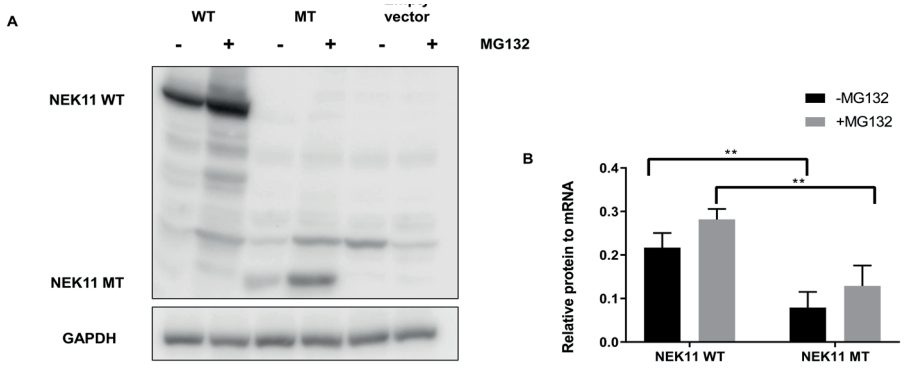

Figure 4 Expression of *NEK11* wildtype and p.Arg374Ter in U2OS cells. A) Lysates of U2OS cells transiently transfected with *NEK11* wildtype (WT) , *NEK11* p.Arg374Ter (MT) and *pLV-empty* expression plasmids were either untreated or treated with MG132. *NEK11* was detected with anti-Flag antibody. GAPDH was determined as a loading control. **B)** Expression was calculated relative to GAPDH for each independent experiment and corrected for mRNA expression of *NEK11*. Data shown represent mean expression from 3 independent experiments. Error bars represent Standard Deviation (SD). Statistical significance is shown as *p<0.05, **p<0.005.

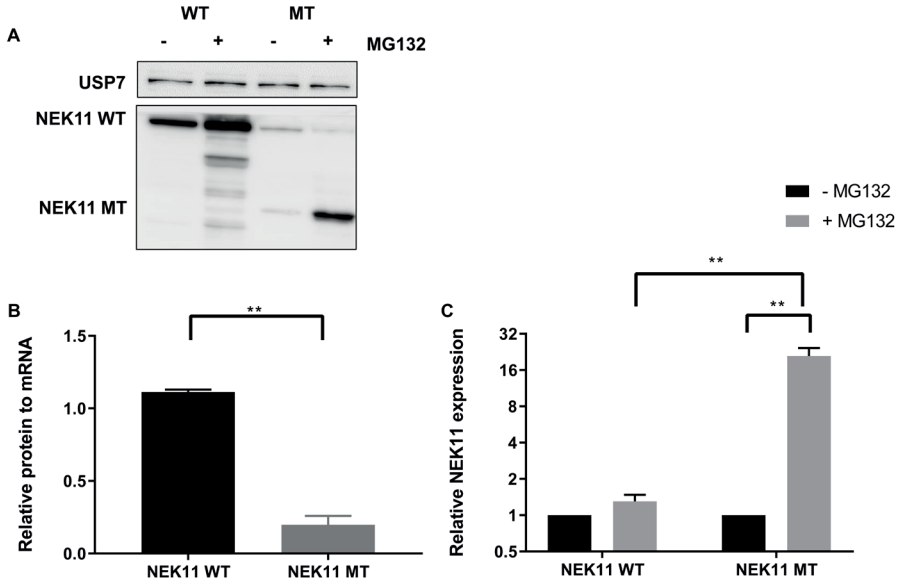

Figure 5 NEK11 protein expression and quantification in stably-transduced FM6 cells. A) Western blot analysis of cell lysates extracted from stably transduced FM6 cells, either untreated or treated with MG132. NEK11 was detected using anti-Flag antibody. USP7 was detected as a loading control. **B)** Protein expression quantifications. Expression was calculated relative to USP7 for each independent experiment and corrected for mRNA expression. **C)** Effect of MG132 on NEK11 wildtype (WT) and p.Arg374Ter (MT) expression. NEK11 WT and MT expression was set to 1 and the log10 relative expression to USP7 is shown. Unpaired t-test was performed for statistical significance. Experiments performed in duplicates. Error bars represent Standard Deviation (SD). Statistical significance is shown as *p<0.05, **p<0.005.

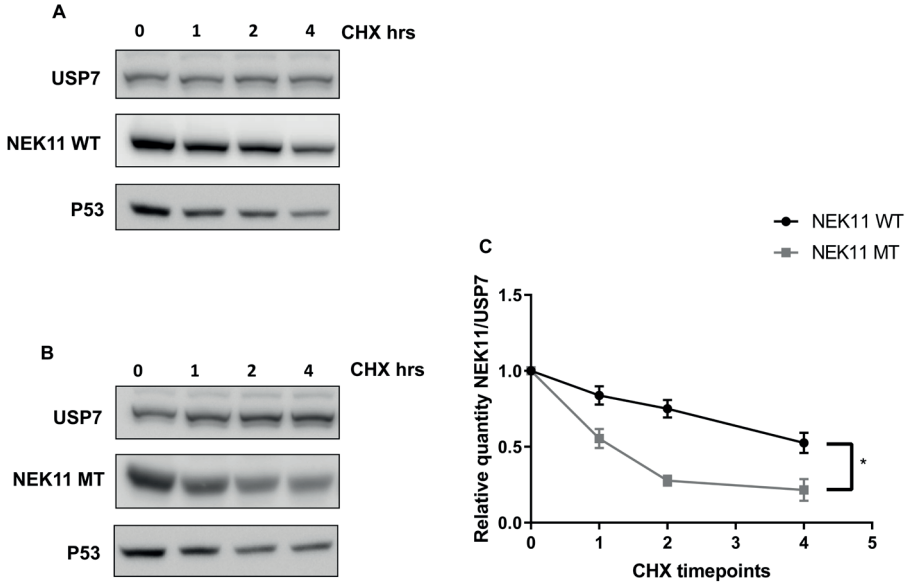

Figure 6 NEK11 protein half-life analysis in FM6 cells using cycloheximide (CHX) treatments. Time-course CHX treatments of FM6 cells expressing **A)** NEK11 wildtype (WT) and **B)** NEK11 p.Arg374Ter (MT). USP7 was detected as a loading control and P53 as a positive control. **C)** Quantification of NEK11 WT and MT corrected for USP7 expression over different time-points of CHX treatments. Error bars represent Standard Deviation (SD). Pearson R squared correlation value was 0.82. Statistical significance is shown as *p<0.05, **p<0.005, ***p<0.0005.

Since transient overexpression yields very high expression levels which might partly mask normal regulation of NEK11 protein expression, a putative difference in protein expression of the NEK11 WT and the NEK11 MT was studied in more detail in stably-transduced, disease-relevant FM6 cutaneous melanoma cells. Strikingly, NEK11 MT protein expression was hardly detectable with 6-fold difference compared to NEK11 WT when corrected to mRNA levels (p=0.0024) (Figure 5A and B). Furthermore, treating FM6 cells with MG132 strongly increased NEK11 MT protein level (Figure 5C), while the effect of MG132 on NEK11 WT levels was much less pronounced, suggesting that the truncated NEK11 protein is prone to faster protein degradation. To examine protein half-life of the NEK11 WT and MT proteins, we decided to treat these FM6 cells with the protein translation inhibitor cycloheximide (CHX) and harvest at different time-points. The NEK11 WT protein appeared to be a stably expressed protein with half-life of approximately 4 hours, in contrast to the NEK11 MT protein showing a half-life of approximately 1 hour in FM6 cells (Figure 6A-C). Collectively, we provide evidence that the *NEK11* p.Arg374Ter mutation leads to the synthesis of a truncated protein with a very short half-life, suggesting a LOF mutation, supporting a tumor-suppressive role for *NEK11* in familial melanoma.

DISCUSSION

Here, a novel nonsense protein truncating variant in *NEK11* p.Arg374Ter was identified as a possible familial melanoma predisposition mutation in a Dutch family. The possibility of any other potentially damaging variants found by WES in this family to either be causal or contributing to the melanoma-risk of this family was considered. Since not enough scientific evidence was available to support a contributing role, these variants were not investigated further.

NEK11 has been initially characterized as a DNA-damage response kinase with two isoforms, the full-length isoform consisting of 645 residues (NEK11-FL) and the short isoform consisting of 470 residues (NEK11-S) [37]. A regulatory effect during IR-induced G2/M cell-cycle arrest has been described, i.e. NEK11 was shown to be involved in phosphorylation of CDC25A triggering its degradation and ultimate blocking of progression into mitosis [27, 38, 40]. *NEK11* has been described to (de) regulate G2/M cell-cycle arrest in colorectal carcinoma and low expression was observed at late-advanced stages of the disease [27, 40]. Furthermore, decreased *NEK11* mRNA levels have also been associated with drug resistance in ovarian cancer cells [41] supporting that *NEK11* may prevent metastatic progression in ovarian cancer. Collectively, these results point towards a putative tumor suppressive role of *NEK11*.

NEK11 expression follows a cell-cycle dependent manner with a peak at G2/M phase [38] and mRNA expression is found in the brain, uterus and lungs with moderate expression in melanoma (median expression = 6) [42, 43]. No significant difference in expression between benign nevi and melanomas can be observed [34], however, cutaneous melanoma patients with higher *NEK11* expression have slightly improved survival, although this association is not statistically significant [44]. Moreover, NEK11 has been suggested to play a role in the G1/S checkpoint in association with NEK2, however, the exact mechanism remains unknown [39, 45]. Therefore, these data suggest that *NEK11* could be regarded an interesting target to validate as a high-penetrance melanoma susceptibility gene.

Genetic analysis confirmed LOH in the melanoma tissue of a mutation carrier. Expression of *NEK11* MT and *NEK11* WT allele is detected in lymphocytic RNA indicating that the mutant transcript is not degraded by NMD, confirmed by mRNA expression analysis in transfected U2OS cells.

The oncogenicity of the *NEK11* p.Arg374Ter mutation could be caused by two possible scenarios. First, a gain-of-function (GOF) mutation, as the non-catalytic C-terminal domain was shown to have an auto-inhibitory effect on protein function [45], thus, loss of this domain could activate the kinase activity. Alternatively, the mutation might lead to the synthesis of a non-functional truncated protein, e.g.

by loss of coiled-coil domain motifs (Supplemental Figure 3A). Here, we provide data strongly suggesting a LOF of the *NEK11* p.Arg374Ter mutation. Collectively our results implicate that the truncated NEK11 protein has a very short half-life, implying that the mutant protein is not significantly expressed in cells and reflects a loss-of-function. Since loss of NEK11 abrogates the G2/M cell cycle arrest upon DNA damaging agents and can induce apoptosis [25], it is very well possible that LOF results in genomic instability with the possible selection of cells with increased survival and proliferation, stimulating the acquirement of additional mutations and the development into a tumor.

Unfortunately, our analyses of *NEK11* p.Arg374Ter mutation were restricted by the limited availability of relevant (tumor) tissue, as we only had access to melanoma tissue and lymphocytic RNA from one affected family member. Analysis of tumor tissue from more affected family members could strengthen the case for *NEK11* as a novel melanoma-susceptibility gene. Moreover, the *NEK11* p.Arg374Ter mutation had 14 submissions in dbSNP, although frequency of the alternate allele was extremely low (0-0.00003) and was not found in the Genome of The Netherlands (GoNL) database [30, 46, 47]. In a recent study, >300.000 UK WES/WGS non-melanoma data sets were analyzed for non-synonymous protein truncating variants (PTVs) [48], however, no mutations were identified in *NEK11*, further strengthening *NEK11* to be a novel but rare melanoma-susceptibility gene and p.Arg374Ter as a potential pathogenic mutation.

As to why this family is predisposed to develop melanoma and not a different tumor type, we cannot conclude based on data from a single family. The increased risk of only one or a few tumor types is common in monogenic tumor predisposition syndromes [49]. Furthermore, the absence of any *NEK11* mutation in 488 Dutch familial melanoma cases [50] warrants screening for *NEK11* mutations in melanoma families worldwide in order to confirm the importance of *NEK11* as a melanoma-susceptibility gene.

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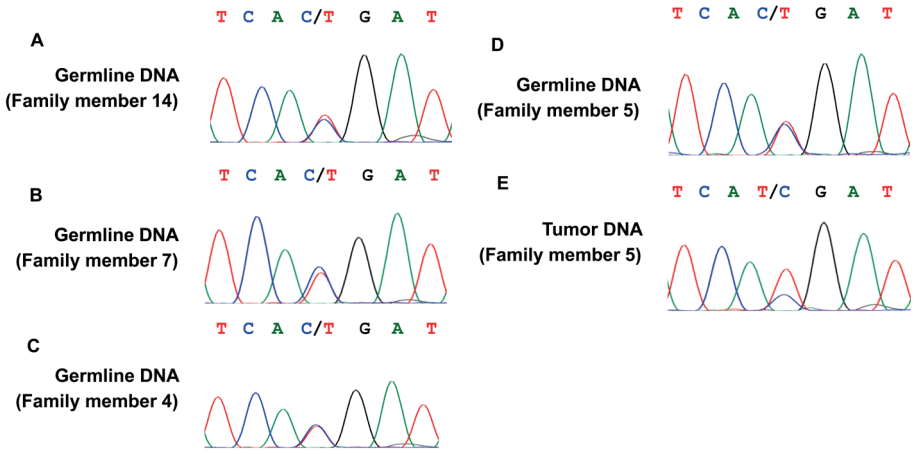
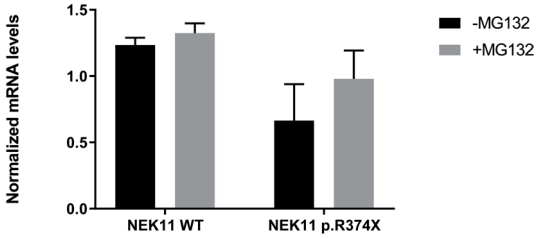
SUPPLEMENTAL MATERIAL

Supplemental Table 1 Sequences of primer combinations

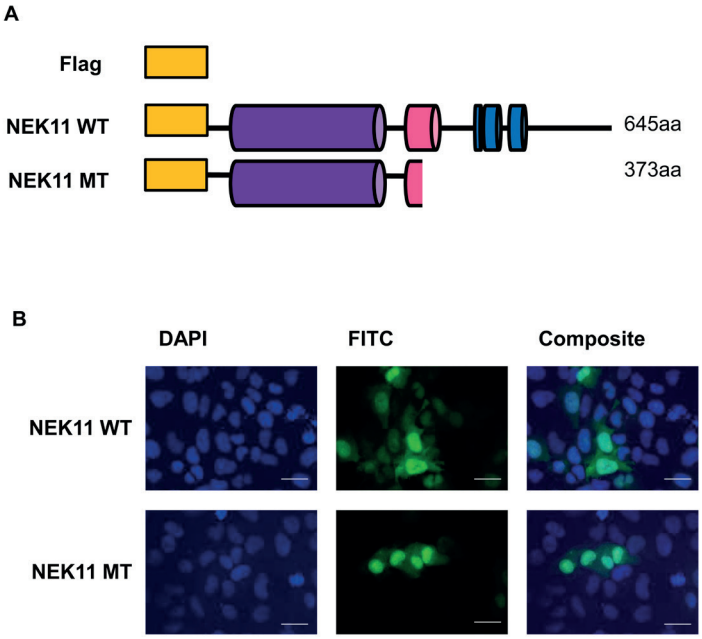
Primer Name	Sequence 5'- 3'
NEK11-seq_exon12long_F	TGCACCACATTCACCTTGAT
NEK11-seq_exon12long_R	ATGCATTCAGTGCCAATTCA
NEK11-seq_exon12_F	CCAGTCAGTCTGTTGAAATAAGGA
NEK11-seq_exon12_R	TGTTTTCTCCCTTGTCCCTTT
MNEK11_2F	GAAGAAAATAGCAAATGAATGCAAGAATTGAG
MNEK11_2R	CTCAATTCTTGCATTCATTTGCTATTTTCTTC
NEK11_exon3-4_F	CAAGAAAGCCAAACGAGGAG
NEK11_exon3-4_R	CCAGCTTGGAGAGGAGTTGG
NEK11_ex2-3_F	AGCCAAACGAGGAGAGGAA
NEK11_ex2-3_R	CATTTGGATTTAGTTCTCCAACA
NEK11_L_ex17-18_F	CCCAGGACCACCAATTTTC
NEK11_L_ex17-18_R	GAGTAGGTTCTTTTCCCATCGT
NEK11_exon11-13_inclMT_short_F	GAAAGCCAGGAAGCTGAAAA
NEK11_exon11-13_inclMT_short_R	TTGCTCCTCCTTTTCTTCCA
NEK11_exon11-12_inclMT_short_F	GAGAAAGCCAGGAAGCTGAA
NEK11_exon11-12_inclMT_short_R	AGCTGCTGAAAGTTCCGAGA
NEO_F	ATTCGGCTATGACTGGGCAC
NEO_R	TTCAGTGACAACGTCGAGCA
TomatoRed_F	TTCATGTACGGCTCCAAGGC
TomatoRed_R	TTGTAGATCAGCGTGCCGTC
CAPNS1_F	ATGGTTTTGGCATTGACACATG
CAPNS1_R	GCTTGCCTGTGGTGTCGC
SRPR_F	CATTGCTTTTGCACGTAACCAA
SRPR_R	ATTGTCTTGCATGCGGCC
NEK11_SNP_rs4974475_F	GAGACAGAAAGAACCTGAACACA
NEK11_SNP_rs4974475_R	CTGCTCACAATGTACTTTCATGGA
dPCR assay ID (WT/R374X) **dHsaMDS101412638**	TCTACATTCACTTTAAATTTACAGAAAGATTGTGGAAGAAAAATATG AAGAAAATAGCAAA[C/T]GAATGCAAGAATTGAGATCTCGGAACTT TCAGCAGCTGAGTGTTGATGTACTCCATGTAAG



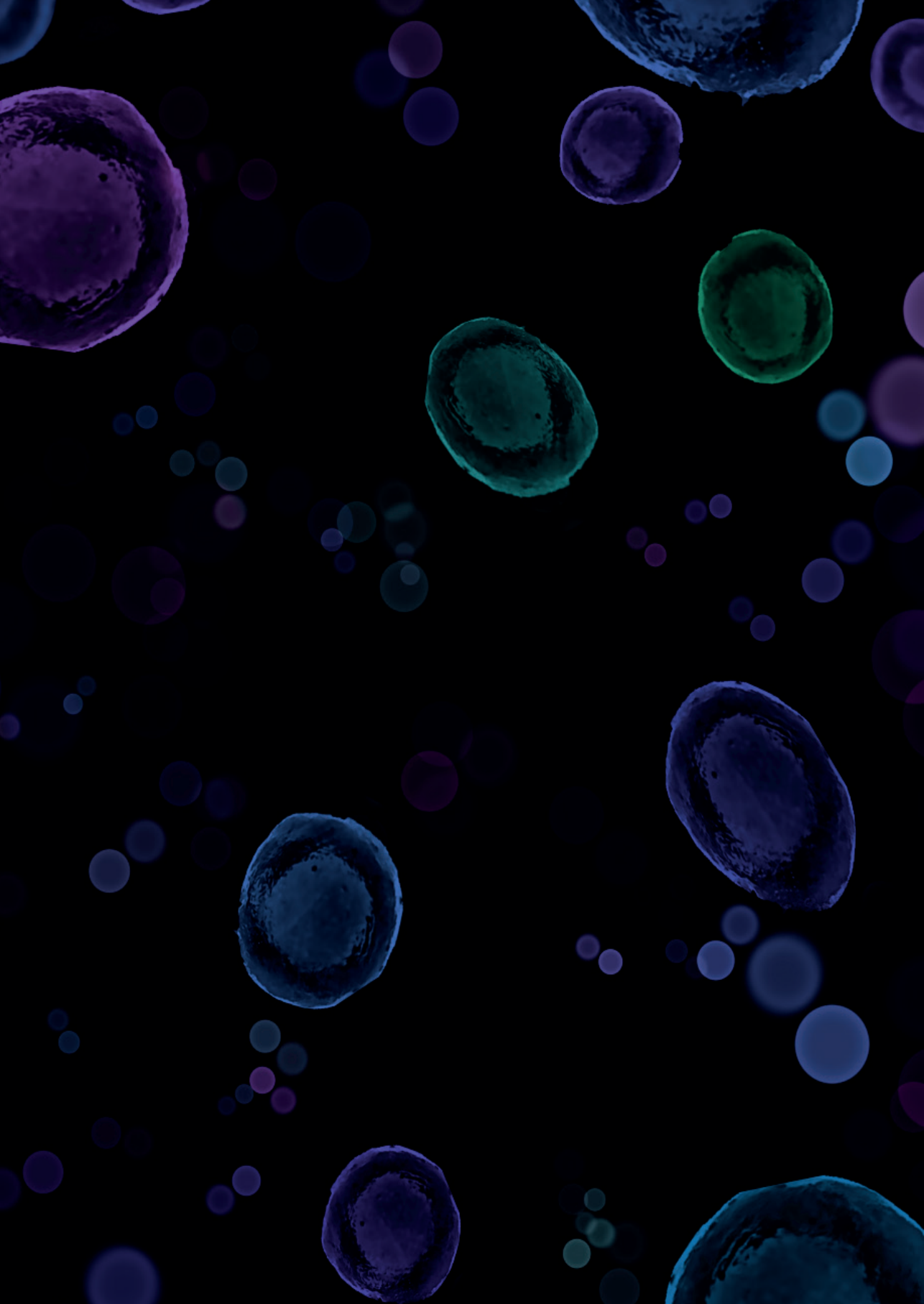
Supplemental Figure 1 Genotyping of a common SNP (rs4974475) in a Dutch melanoma family. Chromatogram showing DNA sequence and variant presence [T] in germline DNA of **A)** healthy family member 14 and in germline DNA of *NEK11* p.Arg374Ter mutation carriers **B)** family member 7, **C)** family member 4 and **D)** family member 5. **E)** Loss of the normal allele [C] was detected in tumor DNA of family member 5.



Supplemental Figure 2 Relative mRNA expression data in transiently transfected U2OS cells. Relative mRNA levels normalized to *CAPNS1* and *SRPR* reference genes and subsequently corrected to Tomato Red to correct for transfection efficiency. Data shown represent mean expression from 3 independent experiments. Error bars show Standard Error of the Mean (SEM). Statistical significance is shown as *p<0.05, **p<0.005, ***p<0.0005.



Supplemental Figure 3 Subcellular localization of NEK11 wildtype and p.Arg374Ter proteins. A) Schematic representation of Flag-tagged NEK11 expression vectors, with the wildtype (WT) construct consisting of 645 amino acids. In purple the N-terminal catalytic domain is shown, in pink is the coiled-coil domain and in blue the C-terminal PEST-like domain. Following site-directed mutagenesis the resulting NEK11 p.Arg374Ter (MT) protein consists 373 amino acids, with the whole C-terminal PEST-like domain and part of coiled-coil domain is lost [27] **B)** Immunofluorescence staining of NEK11 WT and NEK11 MT protein localization in U2OS cells (scale bars represent 10 µm).



Chapter 3

Assessing a single SNP located at *TERT/CLPTM1L* multi-cancer risk region as a genetic modifier for risk of pancreatic cancer and melanoma in Dutch *CDKN2A* mutation carriers

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Christodoulou, E., Visser, M., Potjer, T.P, van der Stoep, N., Rodríguez-Girondo, M., van Doorn, R., Gruis, N.

ABSTRACT

Carriers of pathogenic variants in *CDKN2A* have a 70% life-time risk of developing melanoma and 15-20% risk of developing pancreatic cancer (PC). In the Netherlands, a 19-bp deletion in exon 2 of *CDKN2A* (*p16-Leiden* mutation) accounts for most hereditary melanoma cases. Clinical experience suggests variability in occurrence of melanoma and PC in *p16-Leiden* families. Thereby, the risk of developing cancer could be modified by both environmental and genetic contributors, suggesting that identification of genetic modifiers could improve patients' surveillance. In a recent genome-wide association study (GWAS), rs36115365-C was found to significantly modify risk of PC and melanoma in the European population. This SNP is located on chr5p15.33 and has allele-specific regulatory activities on *TERT* expression. Herein, we investigated the modifying capacities of rs36115365-C on PC and melanoma in a cohort of 283 *p16-Leiden* carriers including 29 diagnosed with PC, 171 diagnosed with melanoma, 21 diagnosed with both PC and melanoma and 62 with neither PC nor melanoma. In contrast to previously reported findings, we did not find a significant association of PC risk with risk variant presence as determined by Generalized Estimating Equations (GEE) modelling. Interestingly, carrier-ship of the risk variant had a significant protective effect for melanoma (OR -0.703 [95% CI -1.201-0.205], p = 0.006); however, the observed association was no longer significant after exclusion of probands to assess possible influence of ascertainment. Collectively, genetic modifiers for the prediction of PC and melanoma risk in *p16-Leiden* carriers remain to be determined.

INTRODUCTION

CDKN2A is the major high-risk susceptibility gene identified thus far for familial melanoma [1]. In the Netherlands, the most common cause of familial melanoma is a *CDKN2A* founder mutation which is a deletion of 19bp in exon 2 (c.225_243del, p.(A76Cfs*64); RefSeq NM_000077.4) also known as the *p16-Leiden* mutation resulting in inactivation of tumor-suppressive properties of p16(INK4a) [2]. Carriers have a lifetime risk of 70% to develop melanoma [3], and a life-time risk of 15-20% to develop Pancreatic Cancer (PC) [4-6].

PC is a highly aggressive cancer subtype with very poor prognosis resulting in a 5-year survival rate of less than 5% [7]. It is therefore one of the leading causes of cancer-related deaths worldwide [8] suggesting there is much to gain by early detection of PC at a stage when surgical removal is still curative [9]. Carriers of the *p16-Leiden* mutation are advised to undergo screening yearly for PC using MRI from the age of 45 [6].

Clinical studies of *p16-Leiden* mutated families have shown variability in occurrence of melanoma and PC among families suggesting contribution of modifying factors to cancer risk [4]. For example, genetic risk factors such as *MC1R*, were found to modify risk of developing melanoma in *p16-Leiden* positive families significantly [10, 11]. Therefore, the variable occurrence of PC in those families might also be explained by modifying genetic risk factors other than the *p16-Leiden* mutation. Determination of those factors would allow for a better identification of patients at increased risk that might benefit from personalized clinical management.

In an attempt to identify genetic factors that modulate the risk of pancreatic cancer in *p16-Leiden* carriers, Potjer *et al.*, analyzed seven SNPs associated with PC risk in the general population in this cohort of carriers and found no significant association [12]. Recently a risk variant, rs36115365-C, was identified to be significantly correlated with PC risk in the European population [13]. This SNP is located at a multi-cancer risk locus on chr5p15.33 and was found to have allele-specific regulatory activities on *TERT* expression, mutations of which have been associated with melanoma risk [14]. These data suggest that variation within rs36115365 (G,C) could contribute to cancer development. Indeed, carriers of the minor C-allele are at increased risk of pancreatic cancer (RR=1.2). Remarkably at the same time, carriers of this C-allele are at diminished risk of developing melanoma [13]. Several risk variants have been reported to be associated with a small but important protective effect against melanoma in sporadic melanoma such as variants in *GSTM1* and *GSTT1* [15] and polymorphisms in the Vitamin D receptor gene [16]. These findings collectively suggest that identification of genetic modifiers in *p16-Leiden* carriers could be used to estimate the risk of developing PC and melanoma more accurately. This study therefore investigates and verifies the risk impact of the reported SNP variant, rs36115365-C in a Dutch *p16-Leiden* positive patient cohort.

METHODS

Cohort description

The study population included only confirmed *p16-Leiden* carriers of which DNA samples were available from the Laboratory for Diagnostic Genome Analysis (LDGA) of Leiden University Medical Center (LUMC). Subject-specific clinical information was collected between 1998 and the 1st of January, 2015. Cases of PC were individuals carrying the *p16-Leiden* mutation who were diagnosed with primary exocrine PC. Similarly, cases of melanoma were *p16-Leiden* carriers who were diagnosed with cutaneous (multiple) melanoma. Detailed medical record data on the study population has been reported previously [12]. Approval of this study was obtained from the ethics committee of Leiden University Medical Center (LUMC #P14.148) [12].

In total, 419 *p16-Leiden* carriers were available for inclusion in the current study. The comparative analysis was formulated by filtering for carriers diagnosed with PC, carriers diagnosed with melanoma, and a group who did neither develop PC nor melanoma but were older than 55 years of age. Subsequently, a master cohort of 283 *p16-Leiden* carriers from a total of 121 *p16-Leiden* families were included. These consisted of 29 carriers with PC (median age 47 years), 171 with melanoma (median age 60), 21 with both PC and melanoma (median age 60) and 62 with neither PC nor melanoma (median age 71) (Table 1).

Genotyping and statistical analysis

Genotyping analysis was carried out using the rhAmp-SNP Genotyping Assay (Integrated DNA Technologies (IDT), Leuven, Belgium). Bi-allelic discrimination was achieved by incorporation of two forward primers specifically targeting the allele of interest (rs36115365-C). The genotyping procedure was performed according to manufacturer's instructions. The reference allele was labelled with FAM reporter dye and the alternate allele with Yakima Yellow (YY) reporter dye which were both detected on CFX384 Touch Real-Time PCR Detection System (Bio-Rad, Veenendaal, The Netherlands) with excitation sources and emission filters for respective wavelengths. The minor allele frequency (MAF) was calculated based on Hardy-Weinberg law. For statistical analysis, a generalized linear model with logit link was used to assess the association between alternate-allele presence and risk for PC and melanoma development. The binary dependent variable was either PC or melanoma and SNP variant was the explanatory indicator variable. Regression coefficients and 95% Confidence Intervals (CI) were calculated. *P*-values of <0.05 were considered statistically significant.

Due to nature of this family-based study, individuals are not independent, they are clustered. In order to account for this feature, a Generalized Estimating Equations (GEE) procedure was used to fit the model. The GEE procedure allows to deal with clustered data. Since the specific correlation structure of this data is difficult to estimate due to the small sample size, an independence working correlation structure was assumed. To avoid the impact of possible misspecification of the model in the confidence intervals and p-values, robust estimates of the standard errors were obtained using a sandwich estimator [17]. All statistical analyses were performed using IBM SPSS Statistics 23.

RESULTS

The risk impact of rs36115365-C on PC was calculated by comparing a total of 50 *p16-Leiden* carriers who developed PC (median age 50) to 143 controls (median age 74) consisting of 62 carriers who did not develop PC and 81 carriers who developed melanoma but were older than 55 years of age (sub-cohort characteristics, Table 2). Similarly, the risk impact of rs36115365-C on melanoma was calculated by comparing a total of 192 *p16-Leiden* carriers who developed melanoma (median age 60) to 73 controls (median age 71) consisting of 62 carriers who did not develop melanoma and 11 carriers who developed PC but were older than 55 years of age (sub-cohort characteristics, Table 3). The latter group in both analyses was treated as a control since it consisted of *p16-Leiden* carriers older than 55 years of age with a subsequent reduced risk of developing melanoma or pancreatic carcinoma in the future.

MAF of the risk variant rs36115365-C for different comparison groups, Beta values and 95% CI were calculated (Table 4). No significant association was found for risk variant presence and PC risk (Table 4). Interestingly, a significant negative association was observed for risk variant carriers and melanoma development suggesting a protective effect of rs36115365-C for melanoma in *p16-Leiden* carriers (OR=-0.703, 95% CI (-1.201,-0.205), *p-value*=0.006) (Table 4). To assess possible influence of ascertainment in the sample cohort, the association with melanoma was further explored by excluding probands. This resulted in a decreased cohort size of 69 families consisting of 158 *p16-Leiden* carriers, 85 of whom had developed melanoma. The statistical significant association did not remain in that case for risk variant carriers (GEE model -0.453,95% CI (-1.051,0.145), p-value = 0.138).

Table 1 Master cohort characteristics of *p16-Leiden* carriers

	***p16-Leiden* carriers (N=283)**			
	Pancreatic cancer (N=29)*	**Melanoma (N=171)***	**Pancreatic cancer and melanoma (N=21)**	**Non-melanoma, non-pancreatic cancer (N=62)***
Median age (yrs)	47 (21-72)	60 (27-93)	60 (42-78)	71 (55-86)
Gender (M:F)	9:20	71:100	8:13	26:36
Multiple melanoma	-	71/171 (42%)	6/21 (29%)	-
Patients diagnosed with other cancer	3	33	6	24

* p16-Leiden carriers who developed either pancreatic cancer or melanoma or neither of the two and were older than 55 years of age served as controls in comparative analysis, see tables 2 and 3

Table 2 Sub-cohort characteristics of *p16-Leiden* carriers with/without pancreatic cancer

	Pancreatic cancer cases (N=50)	**Non-Pancreatic cancer controls (N=143)**
Median age (yrs)	50 (21-78)	74 (55-93)
Gender (M/F)	17/33	60/83
Medical history of melanoma	21	81
Multiple melanoma	6/21 (29%)	37/81 (46%)
Patients diagnosed with other cancer	9	51

Table 3 Sub-cohort characteristics of *p16-Leiden* carriers with/without melanoma

	Melanoma cases (N=192)	**Without melanoma controls (N=73)**
Median age (yrs)	60 (27-93)	71 (55-86)
Gender (M/F)	79/113	28/45
Medical history of pancreatic cancer	21	11
Multiple melanoma	77/192 (40%)	-
Patients diagnosed with other cancer	54	24

Table 4 Association of rs36115365-C presence with PC and melanoma in *p16 –Leiden* carriers

Condition	**MAF rs36115365 (G,C)**		**Allelic OR**	**95% CI**		**p-value**
	Cases	Controls				
Pancreatic cancer	0.23	0.23	-0.027	-0.804	0.750	0.946
Melanoma	0.21	0.29	-0.703	-1.201	-0.205	0.006

DISCUSSION

Identification of genetic modifiers for PC and melanoma risk in *p16-Leiden* carriers could possibly explain the variability of cancer occurrence within *p16-Leiden* positive families and ultimately favor individualized surveillance and clinical management of those patients [4, 12]. Herein, we sought to estimate the risk of developing PC and melanoma more accurately in carriers of the pathogenic variant *p16-Leiden*. This was tested by determining whether a previously published associated risk variant for PC and melanoma, rs36115365-C [13], could explain modified risk in a homogeneous population of *p16-Leiden* carriers.

The MAF of rs36115365-C in the general European (Non-Finnish) population is 0.18 [18] and in the Netherlands specifically, it is 0.20 [19] indicating a common variant with high chances of detection. In this case, the second most-common allele (C) was detected with MAFs ranging from 0.21-0.29 in 283 *p16-Leiden* carriers, slightly higher than in the general population. A limitation of this study however is the small cohort size that could limit possibilities of detecting statistical associations. Moreover, selecting subjects older than 55 years of age not only reduced the control group size but also the possibility of developing PC or melanoma in the future was not fully excluded. A GEE statistical procedure was applied as it is appropriated for studying family-based associations [17, 20]. There was no significant association between rs36115365-C presence and PC-risk in *p16-Leiden* carriers.

Several efforts in scientific literature focused on identifying genetic modifiers of PC risk in *CDKN2A*-mutation carriers. Yang et al., applied Whole Exome Sequencing (WES) in 66 PC patients with/without *CDKN2A* mutation. The combined data from five research groups, including 13 pancreatic *CDKN2A* mutated (*p16-Leiden*) cases from the Netherlands identified 35 variants in PC-related genes. Nominally significant associations were obtained for mismatch repair genes (*MLH1, MSH2, MSH6, PMS2*) in all PC patients, however, variants in *ATM, CPA1,* and *PMS2* were only observed in *CDKN2A* wild-type PC patients. Further, nine *CDKN2A* mutated and four *CDKN2A* wild-type PC patients had rare potentially deleterious variants in multiple PC-related genes. These results therefore suggest that a subset of PC patients may have increased risk because of germline mutations in multiple PC-related genes [21]. Nonetheless, *p16-Leiden* carriers described in the study by Yang were not included in the current study. In addition, the same group showed that sequencing analysis of *PALB2*, another high susceptibility gene for PC, did not reveal any deleterious mutations in PC patients from *CDKN2A* mutated families [22]. Potjer et al., who studied the same cohort of *p16-Leiden* carriers, as in the current study did not identify an association of seven PC-related SNPs with PC risk [12]. Therefore, consideration of other genetic modifiers yet unknown could be an additional explanation of the variability in occurrence of PC within *p16-Leiden* families.

Moreover, epidemiological studies suggest that non-genetic factors may also contribute to PC development specifically in *p16-Leiden* carriers, with the most significant one being smoking [23] as well as alcohol use and obesity in the general population [24]. Collectively these data suggest that rs36115365-C risk variant could not be used to estimate the risk of PC in *p16-Leiden* mutation carriers more accurately unlike in the European population published previously [13].

Modifier genes for melanoma have been well described in literature for *CDKN2A* mutation carriers [10, 11, 25, 26]. The variant rs36115365-C, previously published to be negatively correlated with melanoma risk [13] had a significantly negative association with melanoma development in this study. This effect did not remain however when excluding probands from the analysis suggesting that ascertainment of melanoma cases influenced the results.

CONCLUSION

Here, no significant association was found between rs36115365-C presence and risk of PC development in *p16-Leiden* carriers in contrast to previous published literature in the European population. Reversely, a statistically significant protective effect was determined for melanoma risk in the same cohort of *p16-Leiden* carriers, an effect that lost significance when excluding melanoma probands. Collectively, genotyping and statistical data suggest that genetic modifiers for the prediction of PC and melanoma in *p16-Leiden* carriers remain to be determined.

ACKNOWLEDGEMENTS

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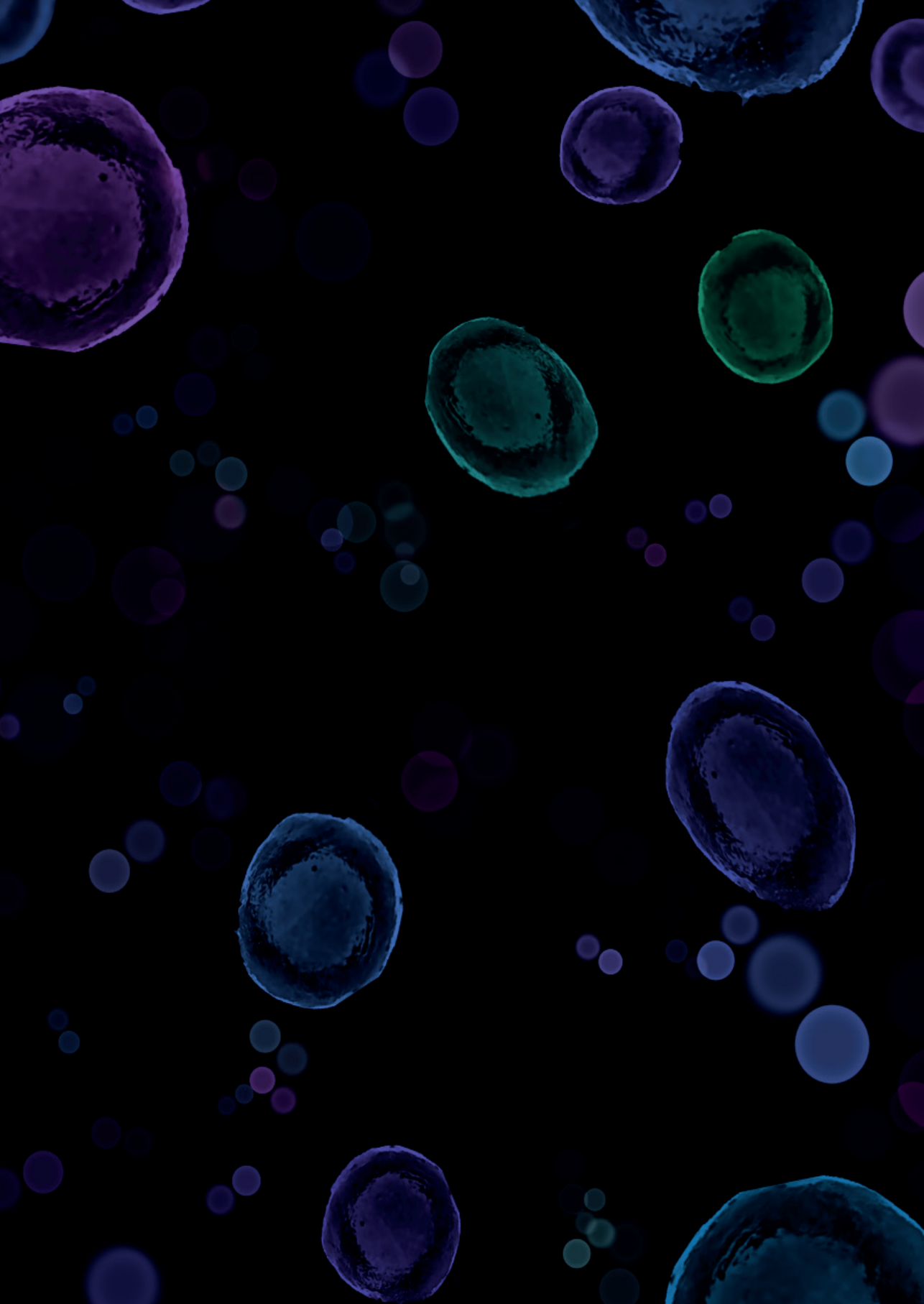

Chapter 4

Loss of wild-type *CDKN2A* is an early event in the development of melanoma in FAMMM syndrome

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ABSTRACT

The development of melanoma involves a sequence of genetic and epigenetic alterations. *CDKN2A* is a key tumor suppressor gene that is commonly inactivated in invasive melanoma, but not in benign precursor lesions. Heterozygous germline mutations in *CDKN2A* cause hereditary melanoma, also termed Familial Atypical Multiple Mole Melanoma (FAMMM) syndrome. The objective of this study was to investigate *CDKN2A* loss-of-heterozygosity (LOH) in melanocytic neoplasms of FAMMM syndrome patients. Here we applied digital PCR methodology for absolute quantification of allelic imbalance of SNPs at the *CDKN2A* locus. Allelic imbalance consistent with *CDKN2A* LOH was observed in 9/14 (64%) primary melanomas. Remarkably, *CDKN2A* LOH was present in 7/13 (54%) common melanocytic nevi with no histopathological atypia. Digital PCR provided insight into tumor heterogeneity and the order of genetic events by quantification of *CDKN2A* LOH relative to $BRAF^{V600E}$, *TERT* promoter mutation and chromosome 9q loss. In nevi, a subclonal fraction of cells demonstrated *CDKN2A* LOH and this genetic event occurred subsequent to *BRAF* mutation. *TERT* promoter mutation and loss at chromosome 9q were observed later in melanoma development of FAMMM syndrome patients. In FAMMM syndrome patients *CDKN2A* inactivation can occur at an earlier stage of genomic evolution of melanocytic neoplasia.

INTRODUCTION

Approximately 10% of patients diagnosed with melanoma report a positive family history of this aggressive cutaneous malignancy. Familial or hereditary melanoma is arbitrarily defined as the occurrence of three or more cases of melanoma within a family [1]. At least a third of melanoma families are caused by germline heterozygous mutations of the *CDKN2A* gene [2]. Hereditary melanoma due to germline *CDKN2A* mutation is designated familial atypical multiple mole melanoma syndrome (FAMMM syndrome) [3]. *CDKN2A*-mutation carriers have an estimated 70% lifetime risk of developing melanoma and are at increased risk for pancreatic cancer, head and neck cancer as well as other tumor types [4, 5]. This dominant high penetrance melanoma susceptibility gene, encodes for two tumor suppressor proteins that are translated in alternate reading frames from the alpha and beta transcript [6]. The larger α transcript encodes for $p16^{INK4A}$, a protein that mediates G1 arrest by inhibiting the phosphorylation of Cyclin-D1-CDK4/6 complex [7, 8]. The smaller β transcript encodes for $p14^{ARF}$ which inhibits MDM2, thereby promoting p53 activity [9, 10].

In carriers of germline *CDKN2A* mutations, the wild-type allele is functionally inactivated in melanoma by a second somatic event, commonly through deletion [11]. The importance of *CDKN2A* as a tumor suppressor gene is underscored by the high frequency of somatic mutation, chromosomal deletion and promoter hypermethylation, estimated at 40%, in sporadic melanoma [12, 13]. In the Netherlands, a specific founder mutation, a 19-bp deletion in exon 2 of the *CDKN2A* gene (c.225_243del, p.(A76Cfs*64)) known as the *p16-Leiden* mutation, is the most frequent cause of hereditary melanoma [14]. Loss of $p16^{INK4A}$ function, that occurs in hereditary as well as sporadic melanoma, not only disrupts the G1 cell cycle checkpoint, but also promotes invasive behavior of melanoma cells due to deregulation of the BRN2 transcription factor [15].

The most frequent de-regulated pathway in melanocytic transformation is the mitogen-activated protein kinase (MAPK) pathway [16]. The two most common mutated genes are *BRAF* and *NRAS* occurring in a mutually exclusive pattern. In melanomas of germline *CDKN2A* mutation carriers *BRAF* and *NRAS* mutations were reported in 43% and 11% respectively [17]. In addition, the genetic landscape of sporadic melanomas frequently involves upregulation of telomerase reverse transcriptase (*TERT*) expression through promoter mutation in primary melanoma [16]. *TERT* promoter mutations create ETS/TCF transcription factor binding motifs that increase *TERT* gene expression. These mutations can be found in both *BRAF* and *NRAS* mutant cases with the majority occurring at two hotspots [18, 19]. In sporadic melanoma, somatic mutations typically sequentially induce MAPK pathway activation (*BRAF, NRAS*), upregulation of telomerase

(*TERT*) and disruption of the G1/S cell cycle checkpoint (*CDKN2A*) in addition to many other pathogenic alterations [16]. Bi-allelic loss of the *CDKN2A* locus is the most common genetic alteration distinguishing melanocytic nevi from invasive melanomas [19]. *CDKN2A* LOH has been previously demonstrated in primary and metastatic melanomas of FAMMM syndrome patients [20, 21]. The timing of wild-type *CDKN2A* inactivation in the development and progression of hereditary melanoma due to germline *CDKN2A* mutation remains to be resolved.

To evaluate the occurrence and timing of somatic events in melanoma-genesis for FAMMM syndrome patients, we developed an innovative digital PCR (dPCR) method allowing accurate quantification of somatic wild-type *CDKN2A* allele loss in patients' tumors including melanomas and common melanocytic nevi. Moreover, dPCR was used to quantify presence of $BRAF^{V600E}$ mutation and the two most frequent *TERT* promoter mutations.

METHODS

Study population

This study was performed on a cohort of 20 heterozygous and one homozygous carrier of a germline inactivating *CDKN2A* mutation (*p16-Leiden*). Blood DNA from *CDKN2A* mutation carriers was available for analysis. Tumor DNA was derived from 18 melanoma and 17 common melanocytic nevi (not matched from the same lesion) formalin-fixed paraffin-embedded (FFPE) tissue samples from *CDKN2A* mutation carriers and extracted by macrodissection with the QIAamp DNA Micro Kit (Qiagen) or with the Maxwell 16 FFPE Plus LEV DNA Purification kit (Promega, Leiden, The Netherlands). Due to quality control measurements, the analysis was restricted to 14 melanoma and 13 common melanocytic nevi. The pathological diagnosis of all lesions was made by two melanoma pathologists independently. The common melanocytic nevi did not show morphologically distinct nevus cell subsets.

Digital PCR (dPCR) analysis

A digital PCR assay was designed targeting a common tri-nucleotide single nucleotide polymorphism (SNP) within the intronic region of *CDKN2A*, rs2811708, located 2kb upstream the 19-bp deletion (*p16-Leiden* mutation) site (G/A/T, chr9:21973422, rs2811708-[T] AF in the Dutch population is 26%, [19]) (Table 1). To validate the assay performance and confirm the copy number amplification within *CDKN2A* region, a different intronic SNP, rs3731257, located 4kb downstream the *p16-Leiden* mutation site was amplified by dPCR (G/A, chr9:21966221, rs3731257-A, AF in the Dutch population is 26% [19]) (Table 1, Figure 1). To control for allelic imbalance and loss in 9p21, the 9q region was targeted by amplifying a SNP, rs4745670, located within *GNAQ* intronic region (T/A, chr9:80423139, rs4745670-[A] AF in the Dutch population is 70% [19]) (Table 1). This SNP-based digital PCR approach follows the design guidelines of a mutation specific digital PCR reaction as described previously [52].

Sanger Sequencing analysis long-run (BaseClear, Leiden, The Netherlands) (Table 1) was performed to validate primer-combinations of the different SNP-assays and chromatograms were analyzed using Chromas software (Technelysium, South Brisbane, Australia). The two most frequent *TERT* promoter mutations (c.1-146C>T and c.1-124C>T) and the *BRAF*^{V600E} mutation were examined using mutation detection dPCR assays predesigned by Bio-Rad (Hercules, California, United States). Raw digital PCR results were acquired using *QuantaSoft* (version 1.7.4, Bio-Rad Laboratories) and imported in an online digital PCR management and analysis application *Roodcom WebAnalysis* (version 1.9.4, available via https://webanalysis.roodcom.nl). The fractional abundance (%) of the alteration of interest (*CDKN2A* LOH, *TERT* promoter, 9q LOH and *BRAF*^{V600E}) was calculated

by dividing the mutant allele counts over the total allele counts. The mutant allele fraction was determined and multiplied by two to obtain the mutant cell fraction:

$$\%\ cells\ with\ mutation = 2 \cdot \frac{[mutation]}{[mutation] + [wildtype]}$$

Allelic SNP imbalance was analyzed using in-house developed digital PCR assays (Table 1). Assuming the allele linked to the *p16-Leiden* deletion remains stable, the copy number value (average number of total *CDKN2A* alleles per cell) was calculated as follows:

$$CNV = 1 + \frac{[var_{unlinked}]}{[var_{linked}]}$$

A copy number significantly lower than 2 was interpreted as being the sum of normal cells (CNV=2) and cells with LOH (CNV=1). The presence of LOH was therefore determined as follows:

$$\%\ cells\ with\ LOH = (2 - CNV) \cdot 100\%$$

For the 9q SNP (rs4745670), the variant with the highest concentration was assumed to be stable and the fraction of cells with LOH was determined as above.

Table 1 Primer/Probe combinations used for digital PCR and sanger sequencing analyses

Primer/Probe name	Sequence 5'to 3'
CDKN2A_rs2811708_F	ACCAATGTAGTTAGGATTCTAAGCCA
CDKN2A_rs2811708_R	AGGAAAAAGAAAAGTGGATAGTTTTGA
variant_rs2811_probe	TGTTCTTCTCCCTTCTCCATTA-FAM
normal_rs2811_probe	TGTTCTTCTCCCGTCTCCATTA-HEX
CDKN2A_rs3731257_F	TTGGTTCAGCACTCACTTGG
CDKN2A_rs3731257_R	AGCAAAAGCTGCCAGAATTG
variant_rs3731257_probe	AAACTTTCTTATTGTTTCCCAGAG-FAM
normal_rs3731257_probe	AAACTTTCTTACTGTTTCCCAGAG-HEX
9q_rs4745670_F	AAGAGCTTTCTGAAAGGGGAA
9q_rs4745670_R	AGCCCTGCATTTGTCTTCCT
variant_rs4745670_probe	CATCTTAGAATCATCAGCATTCACC-FAM
normal_rs4745670_probe	CATCTTAGAAACATCAGCATTCACC-HEX

RESULTS

A Single Nucleotide Polymorphism (SNP)-based digital PCR approach to detect allelic imbalance in FAMMM syndrome patients

Detection of a 19 bp-deletion (*p16-Leiden* mutation) in degraded DNA derived from formalin-fixed paraffin embedded (FFPE) tissue samples turned out to be troublesome, also due to the size difference between wild-type and mutant *CDKN2A* target that caused an amplification bias (Supplemental Figure 1). Therefore, an approach was developed for accurate copy number determination through SNP-specific dPCR technology [22]. The SNP rs2811708 located in intron 1 of the *CDKN2A* gene and 2kb upstream of the *p16-Leiden* mutation had a MAF of 26% in the Dutch population (Figure 1A) [23]. A SNP located 4kb downstream the *p16-Leiden* mutation, rs3731257, was included to confirm the presence of allelic imbalance within 9p21 and had a MAF in the Dutch population of 26%, (Figure 1A) [23]. To investigate the presence of deletions across chromosome 9 or gross genomic instability in melanomas, a SNP at chromosome 9q (rs4745670) located in an intron of the *GNAQ* gene was included with MAF in the Dutch population of 70% (Figure 1A) [23].

To distinguish the different genotypes of common SNPs located within the 9p21 locus (rs2811708 and rs3731257) and rs4745670 located at 9q, capillary sequencing analysis was performed on blood DNA of *p16-Leiden* mutation carriers (Figure 1B). The minor variant allele rs2811708-[T], was found to be linked to this pathogenic germline variant (Figure 1C). This was concluded from analyzing all available *p16-Leiden* carriers and specifically from homozygous carriers of the *p16-Leiden* allele who were also homozygous for rs2811708-[T] (Figure 1B and C). For the confirmative SNP, rs3731257-[C] was present in all *p16-Leiden* carriers and homozygous carriers of the *p16-Leiden* allele were also homozygous for rs3731257-[C] (Figure 1B). This shows that the normal allele [C] was linked to the *p16-Leiden* mutation (Figure 1C).

Since we confirmed linkage of the different SNPs to the *p16-Leiden* mutation and included a common SNP on the 9q arm as a control, this SNP-based digital PCR approach allowed for quantification of *CDKN2A* allelic imbalance and loss in tissues of FAMMM syndrome patients.

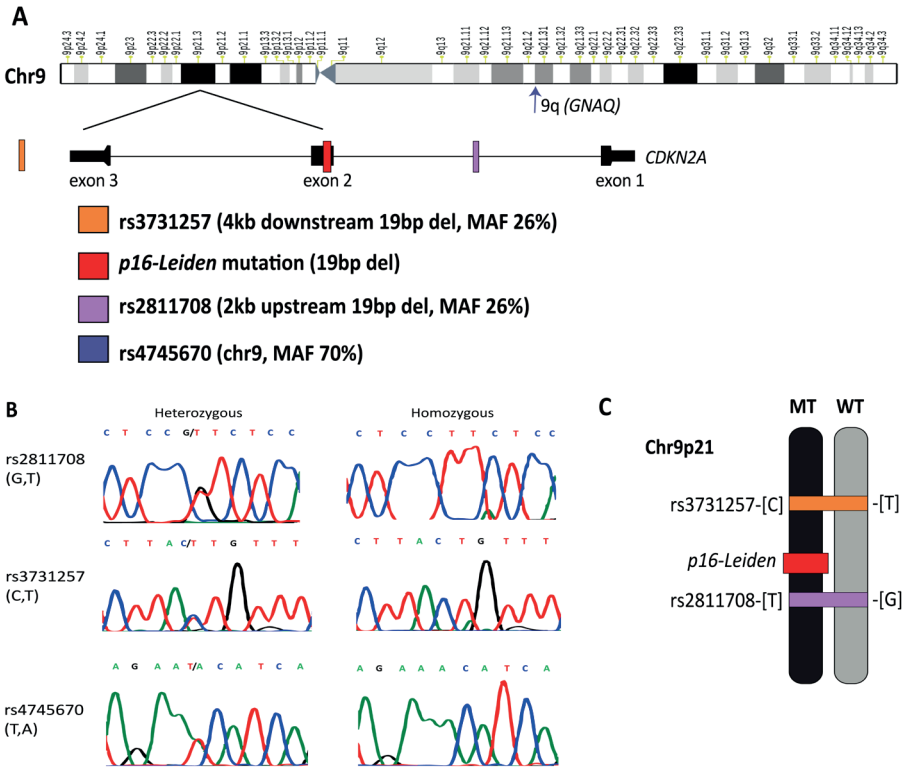

Figure 1 SNP-specific dPCR analysis schematic A) Schematic of the genomic location used to design the digital PCR (dPCR) assays amplifying three common SNPs within chromosome 9: one located in intron 1 of *CDKN2A*, 2kb upstream the *p16-Leiden* mutation (rs2811708) and a second SNP located within the intronic region of 9p21 (rs3731257) at 4kb downstream the *p16-Leiden* mutation. A third SNP was located within the intronic region of 9q (rs4745670) and was used as a control. **B)** Example of genotype from a heterozygous individual for rs2811708 [G/T], rs3731257 [C/T] and rs4745670 [T/A] and a homozygous *p16-Leiden* carrier who was also homozygous for rs2811708-[T], rs3731257-[C] and rs4745670-[A]. **C)** Schematic of linkage between rs3731257-[C] and rs2811708-[T] allele to the *p16-Leiden* mutation (WT- wild-type, MT-mutant).

Loss-of-heterozygosity (LOH) of the CDKN2A locus in melanomas from FAMMM syndrome patients

After having confirmed the specificity of our digital PCR assays, we analyzed melanoma lesions from *CDKN2A* mutation carriers to study allelic imbalance (Figure 2A, Supplemental table 1). The pathological diagnosis of all lesions was made by two melanoma pathologists independently. Absolute quantification showed that in blood DNA of a *CDKN2A* mutation carrier there was 50% fractional abundance of an upstream SNP adjacent to the *CDKN2A* locus (rs2811708) consistent with heterozygosity (Figure 2A). Loss of wild-type *CDKN2A* allele was detected in 9/14 (64%) melanomas of FAMMM syndrome patients by the upstream SNP (rs2811708) (Figure 2). Additional mutation analysis in melanomas showed that 7/14 (50%) tested positive for a $BRAF^{V600E}$

mutation and 6/14 (43%) tested positive for a *TERT* promoter mutation (Figure 2B). Allelic imbalance within 9p21 locus was validated by the fractional abundance of the downstream SNP (rs3731257) in informative (heterozygous for SNP) cases (Figure 3A). These data show wild-type *CDKN2A* LOH as a common event in patients with familial melanoma, leading to the bi-allelic inactivation of *CDKN2A*.

In order to investigate loss at chromosome 9, we quantified a common SNP on the 9q arm (rs4745670). We found that 5/14 (36%) of melanoma had a significant imbalance in amplification (Supplemental figure 2A, Figure 2B) indicating additional deletions across chromosome 9, an event that has been reported previously in sporadic and familial melanomas [24]. Since we found wild-type *CDKN2A* loss to be an early event, in a similar proportion of cells as the *BRAF*^{V600E} mutation, we investigated the frequency of *CDKN2A* allelic imbalance in common melanocytic nevi.

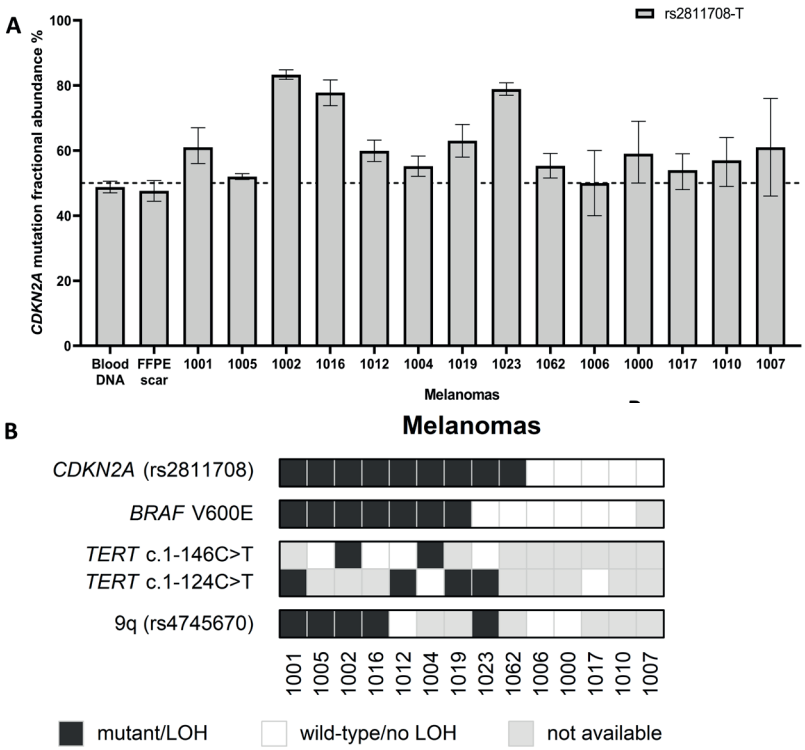

Figure 2 Absolute quantification of genetic events in melanoma lesions from FAMMM syndrome patients. A) Absolute quantification of rs2811708-[T] fractional abundance in blood DNA and primary melanoma lesions from *p16-Leiden* mutation carriers. Error bars represent the 95% confidence intervals (CIs) based on Poisson statistics **B)** Summary of *CDKN2A* loss-of-heterozygosity (LOH) (rs2811708), *BRAF*^{V600E} mutation, 9q LOH (rs4745670) and p*TERT* mutation (c.1-146C>T or c.1-124C>T) in melanomas of FAMMM syndrome patients (n=14). Color legend = positive (mutant or LOH confirmed), white = negative (wild-type or no LOH), grey = not available (not informative, low DNA, low number of droplets or not analyzed).

CDKN2A LOH in common melanocytic nevi from FAMMM syndrome patients

Given the high absolute abundance of loss of wild-type *CDKN2A* in melanomas, we analyzed allelic imbalance in common melanocytic nevi from FAMMM syndrome patients (Supplemental table 1). It is well established that the *CDKN2A* locus remains intact in nevi, whereas homozygous deletion is a common event in sporadic melanomas and is the main cause of inactivation of this tumor suppressor gene [19]. We chose to analyze common, benign nevi without clinical or histopathological atypia, because dysplastic nevi as intermediate lesions can be difficult to distinguish from early stage melanoma.

CDKN2A LOH has been previously shown at primary melanoma and metastasis stage of FAMMM syndrome patients [20, 21]. Remarkably, in this study loss of the wild-type *CDKN2A* allele was detected in 7/13 (54%) common melanocytic nevi from FAMMM

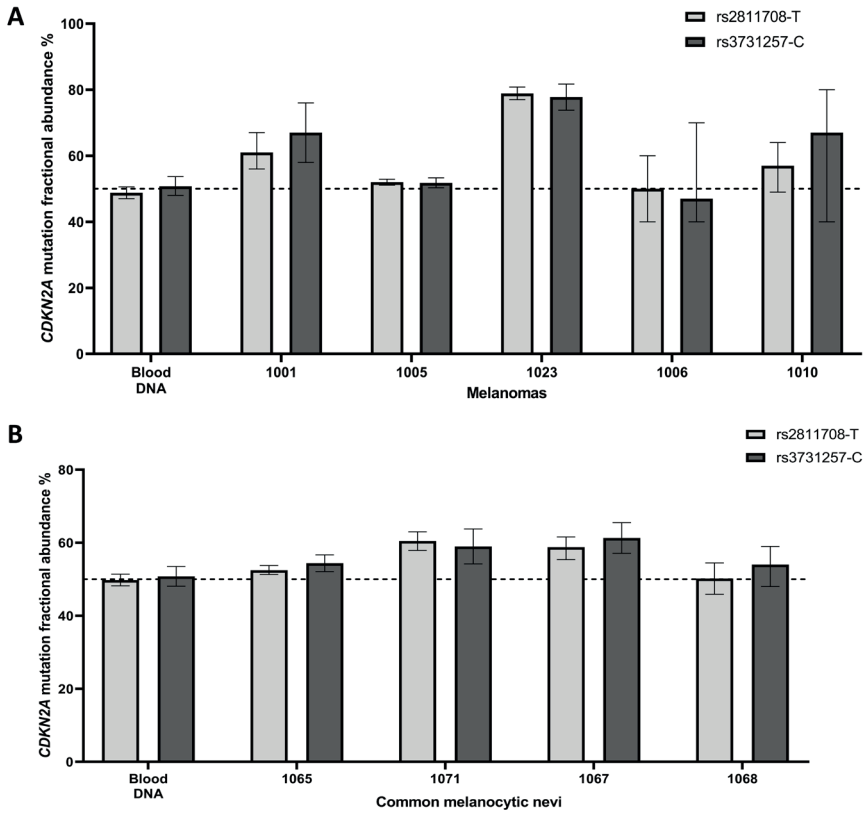

Figure 3 Validation of allelic imbalance within 9p21 locus in melanomas and common melanocytic nevi. Absolute quantification of rs2811708-[T] and rs3731257-[C] fractional abundance in blood DNA and **A)** Primary melanoma lesions **B)** Common melanocytic nevi

syndrome patients, based on absolute quantification of rs2811708 allelic imbalance (Figure 4A). In addition, 8/13 (62%) common nevi tested positive for *BRAF*^{V600E} mutation and five *BRAF* mutant nevi showed significant *CDKN2A* LOH (Figure 4B). As for the melanoma samples, in all informative nevus samples, allelic imbalance of rs3731257 located downstream of *CDKN2A* confirmed loss of the entire *CDKN2A* locus (Figure 3B). We did not detect allelic imbalance at 9q (*GNAQ* locus) in any of the nine tested nevi (Supplemental figure 2B, Figure 4B). The identification of subclonal *CDKN2A* allelic imbalance in common melanocytic nevi instigated histopathological re-evaluation of the lesions which confirmed absence of cytonuclear or tissue architectural atypia (Supplemental figure 3). The clinical and pathological characteristics do not distinguish nevi with *CDKN2A* LOH and nevi without LOH (Supplemental Table 2). Collectively, these data demonstrate bi-allelic loss of *CDKN2A* already at the common melanocytic nevus stage in *CDKN2A* mutation carriers.

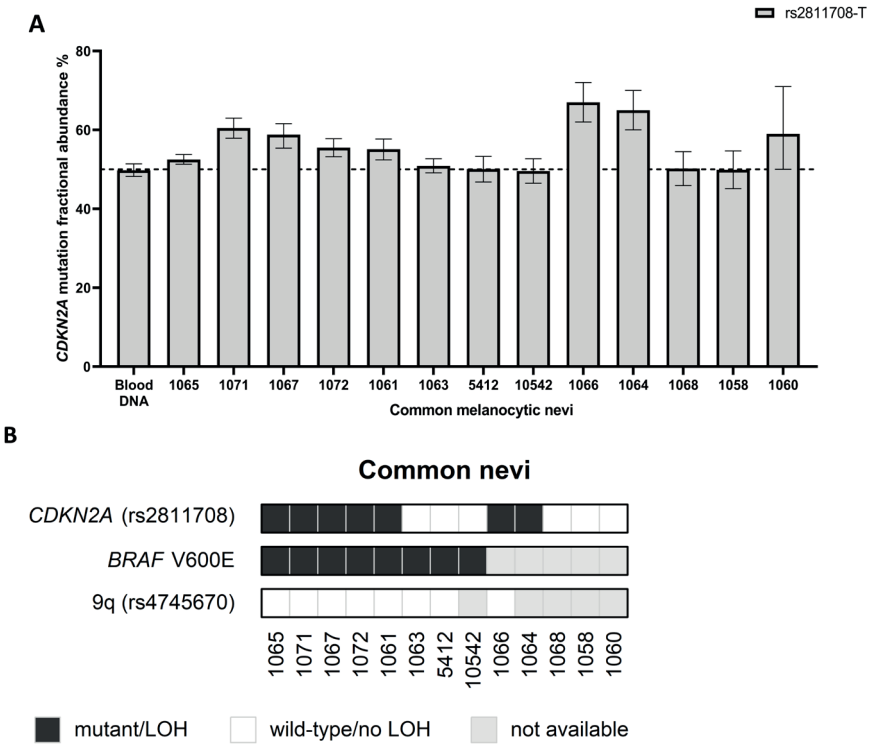

Figure 4 Absolute quantification of genetic events in common melanocytic nevi from FAMMM syndrome patients. A) Absolute quantification of rs2811708-[T] fractional abundance in blood DNA and common melanocytic nevi from *p16-Leiden* mutation carriers. Error bars represent the 95% CIs based on Poisson statistics **B)** Summary of *CDKN2A* LOH (rs2811708), *BRAF*^{V600E} mutation and 9q LOH (rs4745670) in common melanocytic nevi from FAMMM syndrome patients (n=13). Color legend = positive (mutant or LOH confirmed), white = negative (wild-type or no LOH), grey = not available (low DNA, low number of droplets, not analyzed).

Genetic events in nevi and melanomas of FAMMM syndrome patients

Based on combined absolute quantification of mutations and losses, we attempted to deduce the order of genetic events in melanoma and common melanocytic nevi from patients with FAMMM syndrome. In *BRAF* mutant melanocytic nevi, *CDKN2A* LOH was detected in a significantly smaller proportion of the cells than the *BRAF*^{V600E} mutation (Figure 5A, Supplemental Table 1). This suggests that *BRAF* mutation occurs prior to *CDKN2A* LOH. We found no deletions at chromosome 9q in the nevi.

The *BRAF* mutant melanoma biopsy samples contained between 21% and 87% of cells harboring a *BRAF*^{V600E} mutation, due to different proportions of admixed resident and infiltrating cells. Copy number alterations for *BRAF* and *TERT* were observed in a single melanoma lesion that was positive for *CDKN2A* LOH (Supplementary Table 1). The cell fraction harboring *BRAF*^{V600E} mutation, *CDKN2A* LOH and *TERT* mutation was similar in all investigated samples, precluding determination of the order of these ubiquitous genetic events (Figure 5B, Supplemental Table 1). In five melanoma samples, allelic imbalance at 9q (rs4745670) was found; in one case (1023), the fractional abundance of this SNP was significantly lower than that of the *CDKN2A* SNPs (Figure 5B), showing that loss of chromosome 9q occurs in a subclone of cells that already were affected by loss of wild-type *CDKN2A*.

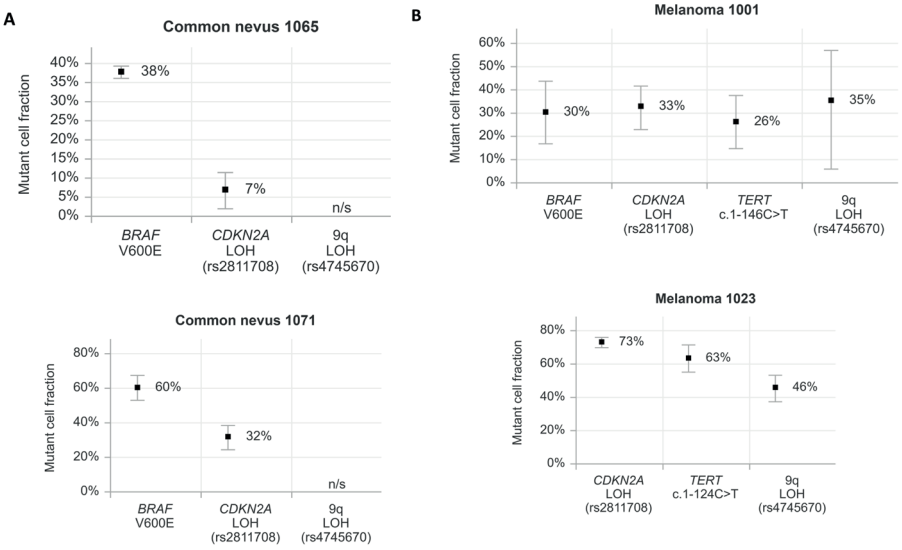

Figure 5 Summary of genetic events in common melanocytic nevi and primary melanomas. All values represent estimates on the fraction of cells having a certain alteration, relative to the complete sample (all cells). This is either a mutation of *BRAF*^{V600E} and p*TERT* (c.1-146C>T or c.1-124C>T) or loss-of-heterozygosity (LOH) for *CDKN2A* (rs2811708) and 9q (rs4745670) **A)** In common melanocytic nevus samples 1065 and 1071 **B)** In primary melanoma samples 1001 and 1023. Error bars represent 95% CIs based on Poisson statistics.

DISCUSSION

The genetic evolution of melanoma is characterized by key oncogenic drivers mainly involving the *BRAF, NRAS, TERT, CDKN2A* and *PTEN* genes [15]. Bi-allelic inactivation of *CDKN2A* is a common event in sporadic melanomas, with deletion being the main genetic mechanism [19]. Studies in primary human melanocytes have also revealed that engineering of *CDKN2A* deletions confer migratory and invasive phenotypes [15]. Homozygous deletion of *CDKN2A* has been reported in a small proportion of sporadic dysplastic nevi but never in common melanocytic nevi [15, 25]. In familial melanoma, *CDKN2A* LOH has been previously reported in primary and metastatic lesions of germline *CDKN2A* mutation carriers [20, 21].

Digital PCR assays targeting SNPs in close proximity to the *p16-Leiden* mutation site were developed to provide precise quantification of allelic imbalance in melanoma and common melanocytic nevus FFPE tissue samples to study LOH of the *CDKN2A* gene. In all informative cases, the fractional abundance of the SNP, located downstream of *CDKN2A*, confirmed the allelic imbalance detected using the SNP located upstream this locus.

Loss of the *CDKN2A* wild-type allele was observed in 9 of 14 (64%) melanomas in FAMMM syndrome. Bi-allelic *CDKN2A* inactivation may be more prevalent, as intragenic mutation and promoter hypermethylation may result in functional inactivation in addition to LOH that was investigated here. The dPCR method optimized for this study is specifically able to detect gene variants at a single locus, allowing quantification of allelic imbalances and hotspot mutations. It is not suited to simultaneously quantify various mutations distributed over a gene such as *PTEN*. Moreover, our analyses are restricted by small amounts of available DNA from the dissected melanocytic lesions. For many samples we have insufficient DNA to perform new analysis on a gene, in addition to *CDKN2A*, *BRAF* and *TERT*.

Remarkably, *CDKN2A* LOH was detected in 7 of 13 (54%) common melanocytic nevi of FAMMM syndrome patients. In melanoma, allelic imbalance at the *CDKN2A* locus occurred in all cells carrying a $BRAF^{V600E}$ mutation. By contrast, in common nevi, loss of the wild-type *CDKN2A* allele occurred in a subclone of cells that had acquired $BRAF^{V600E}$ mutation at an earlier stage of its development. Subclonal loss of the wild-type *CDKN2A* allele in melanocytic nevi was not associated with histopathological alterations; the lesions showed no tissue architectural or cytonuclear atypia. It is plausible that nevi containing subclones with bi-allelic inactivation of *CDKN2A* might be at higher risk to develop into melanoma. *TERT* promoter mutations could be detected in a subset of melanoma samples of FAMMM syndrome patients, but were not found in the few melanocytic nevi that were investigated.

In a proportion of melanocytic nevi of patients with *BAP1* tumor predisposition syndrome, immunohistochemical analysis shows loss of *BAP1* expression, commonly in a regional part of the lesion [26]. These *BAP1*-deficient melanocytic nevi, also termed melanocytic *BAP1*-associated intradermal tumors (MBAITs) or BAP-omas usually show clinical and histopathological atypia [26]. Due to the fact that $p16^{INK4A}$ is not uniformly expressed in nevi and truncated $p16^{INK4A}$ protein encoded by mutant *CDKN2A* is recognized by most antibodies, at the same level as $p16^{INK4A}$ wild-type protein, confirmation of $p16^{INK4A}$ -loss was not possible at the protein level.

This study presents for the first time, *CDKN2A* LOH as an early event in melanoma evolution of FAMMM syndrome patients, relative to other driver events (*BRAF, TERT*). Our quantitative data suggest that chromosome 9 disruption in hereditary melanoma consists of two steps; firstly by focal deletion of the *CDKN2A* locus and secondly by a deletion of chromosome 9 in melanomas. In conclusion, the absolute quantification of allelic imbalance using digital PCR has wider applications in determining the genomic evolution of melanoma and other tumor types. In contrast to sporadic melanoma, *CDKN2A* LOH may occur in a subclone of melanocytes that have acquired a mutation in *BRAF* during nevogenesis. We speculate that subclones of nevus cells with *CDKN2A* LOH are prone to progress to melanoma.

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SUPPLEMENTAL MATERIAL

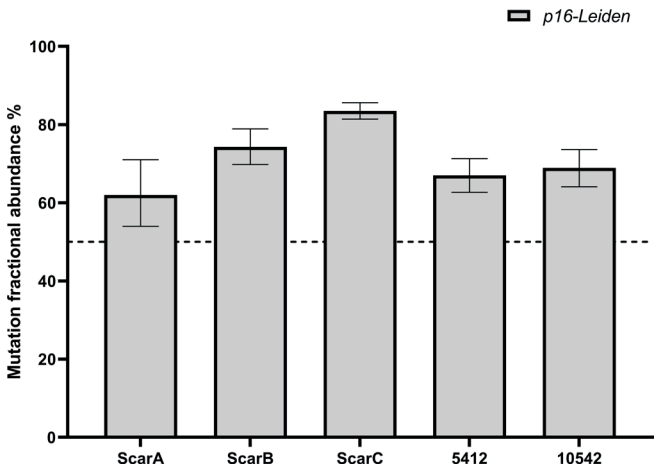
Supplemental Table 1 Summary of estimates on the fraction of cells having a certain alteration, relative to the complete sample (all cells). These include *BRAF*^{V600E} mutation, *CDKN2A* LOH (rs2811708), *CDKN2A* LOH (rs3731257), 9q LOH (rs4745670) and p*TERT* mutation (c.1-146C>T or c.1-124C>T). Benign nevi (n=13) and primary melanomas (n=14) (wild-type- WT, not significant- n/s, Not informative or homozygous for SNP- n/i, Not reliably measurable i.e <=5 droplets positive- n/m, copy number amplification- CNA).

Group	Clinical no	BRAF V600E	rs2811	rs3731	rs4745	TERT C250T	TERT C228T
SCAR	4049	WT	n/s	n/i	n/s	n/a	n/a
SCAR	3749	WT	n/s	n/i	n/i	n/a	n/a
BENIGN NEVUS	1063	28%	n/s	n/i	n/s	WT	n/a
BENIGN NEVUS	1065	38%	7%	16%	n/s	n/a	n/a
BENIGN NEVUS	1072	44%	20%	n/i	n/s	WT	n/a
BENIGN NEVUS	1061	47%	18%	n/i	n/s	n/a	n/a
BENIGN NEVUS	1071	60%	32%	31%	n/s	WT	n/a
BENIGN NEVUS	10542	12%	n/s	n/i	n/i	n/a	n/a
BENIGN NEVUS	1067	42%	29%	37%	n/s	WT	n/a
BENIGN NEVUS	1068	n/m	n/s	n/s	n/m	n/a	n/a
BENIGN NEVUS	1058	n/m	n/s	n/i	n/m	WT	n/a
BENIGN NEVUS	1060	n/m	n/s	n/m	n/m	n/a	n/a
BENIGN NEVUS	1066	n/m	51%	n/i	n/s	n/a	n/a
BENIGN NEVUS	1064	n/m	46%	n/i	n/i	n/a	n/a
BENIGN NEVUS	5412	9%	n/s	n/i	n/s	n/a	n/a
MELANOMA	1002	120% (CNA)	80%	n/i	72%	156% (CNA)	n/a
MELANOMA	1004	21%	20%	n/i	n/i	28%	WT
MELANOMA	1001	30%	33%	51%	35%	n/m	26%
MELANOMA	1012	31%	35%	n/i	n/s	WT	34%
MELANOMA	1005	36%	9%	7%	12%	WT	n/a
MELANOMA	1019	36%	42%	n/i	n/i	n/m	26%
MELANOMA	1016	87%	71%	n/i	55%	WT	n/m
MELANOMA	1007	n/m	n/s	n/m	n/m	n/m	n/m
MELANOMA	1017	WT	n/s	n/i	n/i	n/a	WT
MELANOMA	1006	WT	n/s	n/m	n/s	n/m	n/m
MELANOMA	1000	WT	n/s	n/m	n/s	n/m	n/m
MELANOMA	1010	WT	n/s	n/m	n/i	n/m	n/m
MELANOMA	1023	WT	73%	71%	46%	WT	63%
MELANOMA	1062	WT	14%	n/i	n/i	n/a	n/a

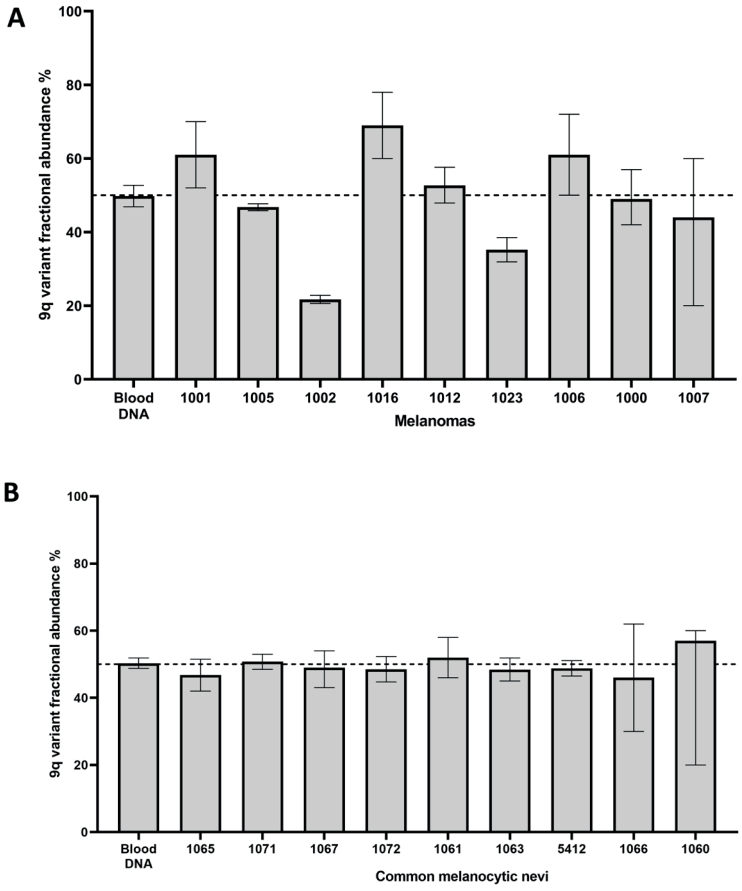
Supplemental Table 2 Clinical and pathological characteristics of benign nevi (BN) (n=13) and melanomas (M) (n=14)

Group (BN/M)	Clinical no	Age	Gender	Diameter (BN)/ Breslow's depth (M)	Anatomical location	Histological type
BN	1063	50	female	6mm	lower back	dermal nevus. No dysplasia.
BN	1065	38	male	7mm	abdomen	compound nevus. No dysplasia.
BN	1072	47	male	7mm	back	junction nevus. No dysplasia.
BN	1061	35	female	6mm	scalp	dermal nevus. No dysplasia.
BN	1071	47	female	7mm	abdomen	compound nevus. No dysplasia.
BN	10542	54	male	6mm	lower back	compound nevus. No dysplasia.
BN	1067	37	female	7mm	flank	junction nevus. No dysplasia.
BN	1068	43	male	8mm	scalp	dermal nevus. No dysplasia.
BN	1058	54	male	6mm	back	compound nevus. No dysplasia.
BN	1060	85	female	9mm	chest	junction nevus. No dysplasia.
BN	1066	55	female	6mm	chest	junction nevus. No dysplasia.
BN	1064	70	female	7mm	upper leg	junction nevus. No dysplasia.
BN	5412	47	male	7mm	back	nevus with junction activity. No dysplasia.
M	1002	43	male	1.4mm	Lower back	nodular melanoma
M	1004	66	female	0.7mm	Upper arm	superficial spreading melanoma
M	1001	58	male	1.0mm	Chest	superficial spreading melanoma
M	1012	45	male	1.4mm	Head	nodular melanoma
M	1005	37	male	0.9mm	Shoulder	superficial spreading melanoma
M	1019	58	female	0.8mm	Upper leg	superficial spreading melanoma
M	1016	52	female	1.2mm	Abdomen	superficial spreading melanoma

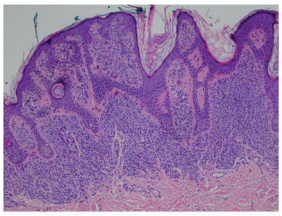
M	1007	57	female	0.8mm	Elbow	superficial spreading melanoma
M	1017	71	male	0.6mm	Upper arm	superficial spreading melanoma
M	1006	35	female	0.8mm	Lower back	superficial spreading melanoma
M	1000	51	female	0.8mm	Shoulder	superficial spreading melanoma
M	1010	44	male	0.8mm	Scalp	superficial spreading melanoma
M	1023	48	female	2.6mm	Lower leg	nodular melanoma
M	1062	69	female	3mm	upper arm	superficial spreading melanoma



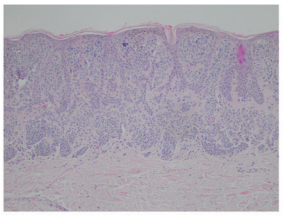
Supplemental figure 1 Absolute quantification of the *p16-Leiden* mutation in scar and common nevi from FAMMM syndrome patients. Fractional abundance of *p16-Leiden* mutation in three scar tissue samples and common nevi 5412 and 10542 from FAMMM syndrome patients. The fractional abundance was significantly higher than 50% in all benign lesions suggesting that this technique is not informative to precisely quantify the mutant cell fraction. Error bars represent 95% CIs based on Poisson statistics. Forward primer: CTGCTGCTGCTCCACG Reverse primer: ACCAGCGTGTCCAGGAAG *p16-Leiden* probe: ACTGCGCCCGACCCGT wild-type probe: ACTCTCACCCGACCCGTG.



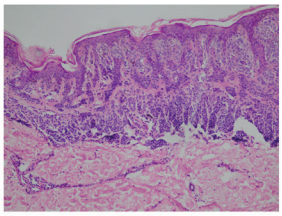
Supplemental Figure 2 Absolute quantification of rs4745670-[A] in melanomas and common melanocytic nevi from FAMMM syndrome patients. Absolute quantification of a copy number control SNP, rs4745670, located within the intronic region of 9q and fractional abundance of [A] allele in informative (heterozygous for SNP) **A)** Primary melanoma lesions and **B)** Common melanocytic nevi. Error bars represent 95% CIs based on Poisson statistics.



Common nevus 1065

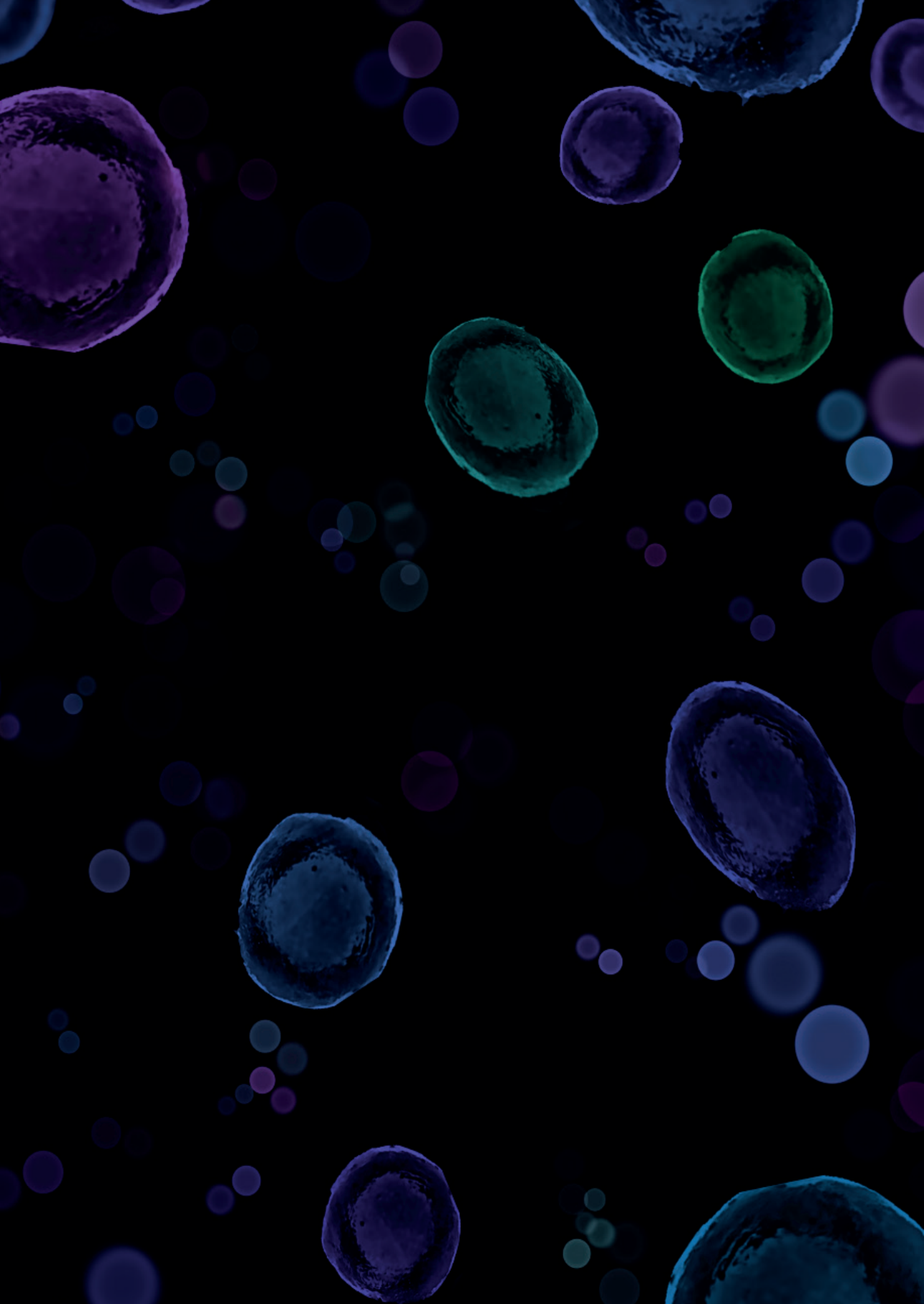


Common nevus 1071



Common nevus 1067

Supplemental Figure 3 Histological examination of common melanocytic nevi from *p16-Leiden* mutation carriers. H&E staining of common melanocytic nevi (1065, 1071 and 1067) from *p16-Leiden* mutation carriers that showed loss of wild-type *CDKN2A* allele (magnification= 100x).



Chapter 5

Analysis of CRISPR-Cas9 screens identify genetic dependencies in melanoma

(Under review)

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ABSTRACT

Targeting the MAPK signaling pathway has transformed the treatment of metastatic melanoma. CRISPR-Cas9 viability screens provide a genome-wide approach to uncover novel genetic dependencies. Here we analyzed recently reported CRISPR-Cas9 screens comparing data from 28 melanoma cell lines and 313 cell lines of other tumor types in order to identify fitness genes related to melanoma. We found an average of 1,494 fitness genes in each melanoma cell line. We identified 33 genes inactivation of which specifically reduced the fitness of melanoma. This set of tumor type-specific genes includes established melanoma fitness genes as well as many genes that have not previously been associated with melanoma growth. Several genes encode proteins that can be targeted using available inhibitors. We verified that genetic inactivation of *DUSP4* and *PPP2R2A* reduces the proliferation of melanoma cells. *DUSP4* encodes an inhibitor of ERK, suggesting that further activation of MAPK signaling activity through its loss is selectively deleterious to melanoma cells. Collectively, these data present a resource of genetic dependencies in melanoma that may be explored as potential therapeutic targets.

INTRODUCTION

Early-stage cutaneous melanoma can be effectively cured by surgical removal, but once metastasized patient prognosis is poor [1]. Increased signaling activity of the mitogen-activated protein kinase (MAPK) pathway is a hallmark of melanoma and can be attributed to mutations in the *BRAF*, *NRAS*, *KIT* or *NF1* genes. These oncogenic mutations commonly occur in the early stages of melanoma development [1, 2]. In patients with *BRAF*-mutant metastatic melanoma, targeted therapy using BRAF and MEK inhibitors can lead to significant tumor regression [3]. Almost invariably melanoma cells acquire resistance to these targeted treatments and disease relapse occurs. Consequently, there is a need to identify additional genetic dependencies that might serve as therapeutic targets in metastatic melanoma.

The application of genome-wide screens in human cancer cell lines has the potential to identify genetic dependencies that may be targeted therapeutically [4, 5]. Genome editing with CRISPR-Cas9 technology has improved the identification of genetic dependencies due to its high precision and limited off-target effects. In CRISPR-Cas9 dropout screens a population of cells is transduced with a pooled sgRNA library and following culture, selective depletion of sgRNAs is measured to identify genes associated with a growth disadvantage or lethal phenotype, designated as fitness genes [6, 7]. Core fitness genes are involved in essential cellular processes that human cells depend on for survival and proliferation. In addition, context-dependent fitness genes are distinguished in that they may be specific for cell lineage or genotype. Recently, genome-wide CRISPR-Cas9 screens have been performed in a range of cancer cell lines, yielding cancer-specific fitness genes [8-13]. Importantly, the majority of cancer-specific fitness genes were found to be limited to only one or two tumor types [13]. Specific genetic dependencies in cancer cells may constitute targetable therapeutic vulnerabilities. The objective of this study was to identify novel genetic dependencies that may serve as potential therapeutic targets in melanoma cells through analysis of CRISPR-Cas9 screen data. Among the 33 fitness genes that we define in melanoma, there are multiple genes that have not previously been associated with melanoma growth, including inhibitors of MAPK signaling activity, that may potentially serve as therapeutic targets for melanoma.

MATERIAL AND METHODS

CRISPR-Cas9 screen data

The generation of CRISPR-Cas9 screen data at Broad Institute, Cambridge Massachusetts, available online at https://depmap.org/ceres/, was described previously [14]. Briefly, 341 tumor cell lines including 28 melanoma lines were engineered to express Cas9 and subsequently screened using the human Avana4 library composed of 70,086 sgRNAs, targeting 17,670 genes (4sgRNAs per gene) and 995 non-targeting control sgRNAs [15]. Cancer cell lines were transduced at a multiplicity of infection (MOI) of 0.3 to ensure that each cell expresses only one sgRNA. Genomic DNA was purified from transduced cells cultured under puromycin selection at day 1 and day 21 for next generation sequencing. The cell lines included in the screens expressed Cas9 and the sgRNA library used targeted the protein-coding genome [14].

Comparative analysis to determine melanoma fitness genes

The CRISPRcleanR package was applied to process the CRISPR-Cas9-derived essentiality profiles and correct for copy number amplifications [16]. CRISPR-Cas9 screen data were analyzed using an R implementation of the BAGEL (Bayesian Analysis of Gene Essentiality) algorithm, generating a scaled Bayesian Factor (BF) score per gene [13, 17]. A 5% False Discovery Rate (FDR) cut-off was applied. The mutation annotation for each melanoma cell-line was derived from the cell-line encyclopedia (CCLE) [18]. Gene-level BFs were computed by calculating the average of the BFs across sgRNAs targeting a gene. This algorithm uses reference sets of predefined essential and non-essential genes. Each gene was assigned a scaled BF computed by subtracting the BF at the 5% FDR threshold (obtained from classifying reference essential/non-essential genes using BF rankings) from the original BF. Those genes with a statistically significant depletion at 5% FDR had a scaled BF above zero. Fitness genes were determined by comparing the average drop out of sgRNAs targeting the same gene, to that of reference essential and non-essential genes [13, 17]. Scaled BFs were binarized to 0 (scaled BF <0) and 1 (>0). A Fisher's Exact test was performed on a two-way contingency table of fitness and non-fitness genes with binarized scaled BF scores from melanoma and the other tumor cell lines with the resulting p-values corrected for multiple testing using the Benjamini-Hochberg procedure (adjusted p-value <0.01). Gene expression data for the 28 melanoma cell lines were available for analysis from the CCLE data portal. The datasets are available at https://data.broadinstitute.org/ccle/CCLE_RNAseq_081117.rpkm.gct [18]. Protein interaction networks and pathway enrichment analyses were performed using STRING and Enrichr [19, 20].

Cell culture, Cas9 and sgRNA lentiviral transduction for validation

Human melanoma cell lines used for functional follow-up experiments were cultured in DMEM supplemented with 10% Fetal Bovine Serum (FBS), penicillin (100 I.U./mL)/ streptomycin (100 µg/mL) and Glutamax (Thermo Fisher Scientific, Waltham, MA). A375, IGR1 and WM983B melanoma cell lines were obtained from ATCC/Rockland (Manassas, VA/Gilbertsville, PA) and HEK293T cells were available from lab stocks (Chemical and Cell biology, LUMC). Cell lines were STR profiled, tested negative for mycoplasma and cultured in a humidified incubator at 37°C and 5% CO_2.

Lentivirus for stable Cas9-expressing cell lines was produced by transfecting pKLV2-EF1a-Cas9Bsd-W (Addgene #68343) into HEK293T cells together with packaging vectors (psPax2 and pMD2.G). For gene inactivation experiments, two sgRNA sequences per gene of interest were cloned into a plasmid DNA vector (U6-sgRNA-PGKpuro-2A-BFP) from the Sanger CRISPR-Cas9 genome-wide arrayed sgRNA library (Supplemental Table 1) containing the puromycin-resistance gene for selection [21]. The sgRNAs used for validations differed from those used in the Broad Avana4 library to provide orthogonal validation. Lentivirus stocks were produced following transfections into HEK293T cells using polyethylenimine (PEI) [22]. Viral titers were determined by antigen capture ELISA measuring HIV p24 (ZeptoMetrix Corp., New York, NY, USA). Following lentiviral transduction with the Cas9-expression vector to reach MOI of 3 (100% infection efficiency) in A375, IGR1 and WM983B cells, the Cas9-editing efficiency was tested [13]. Capillary sequencing analysis was performed using the human U6 promoter forward primer (GACTATCATATGCTTACCGT) to align the sgRNA sequence to the backbone vector LV04 (Sigma Aldrich, St. Louis, MO) and ensure that the sgRNA sequence was correct. For lentiviral transductions with sgRNA-expression vectors, 10^5 cells were seeded in 6-well plates or $2.5x10^5$ cells in 6 cm dishes. Cells were transduced next day in 2 ml or 5 ml culture medium supplemented with 8 µg/ml polybrene to reach MOI of 3. Cells were incubated overnight at 37°C and cultured for 4 days in fresh medium supplemented with blasticidin-S (5µg/ml) and puromycin (2µg/ml) for selection and further analysis.

Immuno-blotting analysis

A375-Cas9, IGR1-Cas9 and WM983B-Cas9 cells were transduced with lentiviral sgRNA expression vectors and cell lysates were prepared for immunoblot analysis as described previously [23]. Antibodies used for detection included anti-DUSP4 (1:1000), anti-PPP2R2A (1:1000), anti-BRAF(1:1000) (Cell Signaling Technology, Danvers, MA), and anti-Vinculin (1:1000, clone hVIN-1, Sigma-Aldrich, St. Louis, MO) as a loading control. Secondary anti-mouse and anti-rabbit antibodies were used at 1:10,000 (Jackson ImmunoResearch Europe, Ely, UK).

Colony formation assays

After puromycin selection, following transduction with sgRNA expression vectors, the A375-Cas9, IGR1-Cas9 and WM983B-Cas9 cells were trypsinized, counted and seeded in triplicate in 12-well plates; 500 cells/ml (A375), 2000 cells/ml (IGR1) and 3000 cells/ml (WM983B) for colony formation assays. Cells were fixed for 5-10 minutes in 4% paraformaldehyde (PFA) and stained for 30 minutes with crystal violet (0.05%) when control wells transduced with negative control sgRNAs targeting *SSX3* reached 80% confluency. Scanned images of the wells were obtained before solubilization of retained dye with 100% methanol to measure absorbance at OD540.

Cell confluency assay

IGR1-Cas9 and A375-Cas9 cells were seeded in 96-well plates to monitor cell confluency using the IncuCyte live-cell analysis system (Essen BioScience, Ann Arbor, MI). For this assay, cells were seeded at a density of 50 cells/well (A375) and 200 cells/well (IGR1) in 6 replicates and scanned images of each well were taken every 12 hours. Data were analyzed using IncuCyte software and confluency was calculated over 6 days (A375) and 8 days (IGR1) and normalized to the confluency on day 1 to correct for seeding variation. Colony formation and cell confluency assays were performed in biological duplicates and combined data were analyzed using GraphPad Prism version 8 (GraphPad software, San Diego, CA). A 2-way ANOVA and Bonferroni's multiple comparisons test were performed to detect statistically significant differences in cell confluency (p-value<0.01).

RESULTS

CRISPR-Cas9 screen analysis for fitness genes in melanoma

To identify genes that are specifically required for viability of melanoma cells, data available from CRISPR-Cas9 screens performed at the Broad Institute (Cambridge, Massachusetts) were extensively processed and analyzed. In these genome-wide CRISPR-Cas9 dropout screens the depletion of sgRNAs from a genome-wide library was measured in populations of Cas9-expressing tumor cells following 21 days of culture after transduction [14]. Fitness genes were determined by comparing the average dropout of sgRNAs targeting the same gene to the profile of reference essential and non-essential genes using a supervised approach called BAGEL [13, 17]. Of all targeted genes 4,423 (25%) negatively affected the fitness of one or more melanoma cell lines. We found an average of 1,494 fitness genes in each of the 28 melanoma cell lines. There were 1,396 genes that reduced the fitness in half or more of the melanoma cell lines, a number similar to what was reported for other cancer types [13]. Our analysis yielded 193 genes that reduced fitness in all melanoma cell lines, 175 of which (91%) had been found to be core fitness genes in the haploid cell line HAP1 and are therefore not specific to melanoma (Supplemental Table 2) [24].

To identify tumor type-specific genetic dependencies for melanoma, we performed comparative analysis of the scaled BFs representing fitness effects for each gene in the 28 melanoma cell lines and in the 313 cell lines from 18 other tumor types analyzed in parallel in the CRISPR-Cas9 screens [13, 14]. CRISPR-mediated inactivation of 33 genes was significantly associated with reduced fitness in melanoma when compared to tumor cell lines from other lineages after multiple testing correction (Figure 1A). The average median FPKM value of 31 genes was 14,606 (average Q1= 9,997 and average Q3= 20,103) with the exception of a few (Supplemental Figure 1). All *BRAF*-mutant melanoma cell lines showed reduced survival upon targeting of *BRAF*. The screen identified melanoma fitness genes having an effect size similar to *BRAF*, including *CHMP4B*, *FERMT2*, and *DUSP4*, supporting their potential as therapeutic targets (Figure 2). Of the 28 melanoma cell lines, 22 harbored *BRAF* V600 mutations, 4 harbored oncogenic *NRAS* mutations, one was *NF1* mutant (in the absence of *BRAF* or *NRAS* mutations) and the remaining was triple-wildtype. There were no *KIT* mutations in any of the tested melanoma cell lines (Figure 2). This set of fitness genes related to melanoma included melanocyte-specific transcription factors such as *MITF* and *SOX10*, established as fitness genes for cells of the melanocytic lineage (Figure 1B, Supplemental Table 3) [25, 26]. A second cluster of fitness genes related to melanoma consisted of components of the MAPK signaling pathway, such as *BRAF* and *MAPK1* (Supplemental Table 3). The finding of these established fitness genes in this gene set confirms the sensitivity of the CRISPR-Cas9 screen analysis. A third group of genes, to which *MDM2* belongs, is involved in regulating p53 activity (Supplemental Table 3). For several genes, a role in melanoma progression has been reported, *FERMT2* for instance has been found to impact melanoma metastasis [27].

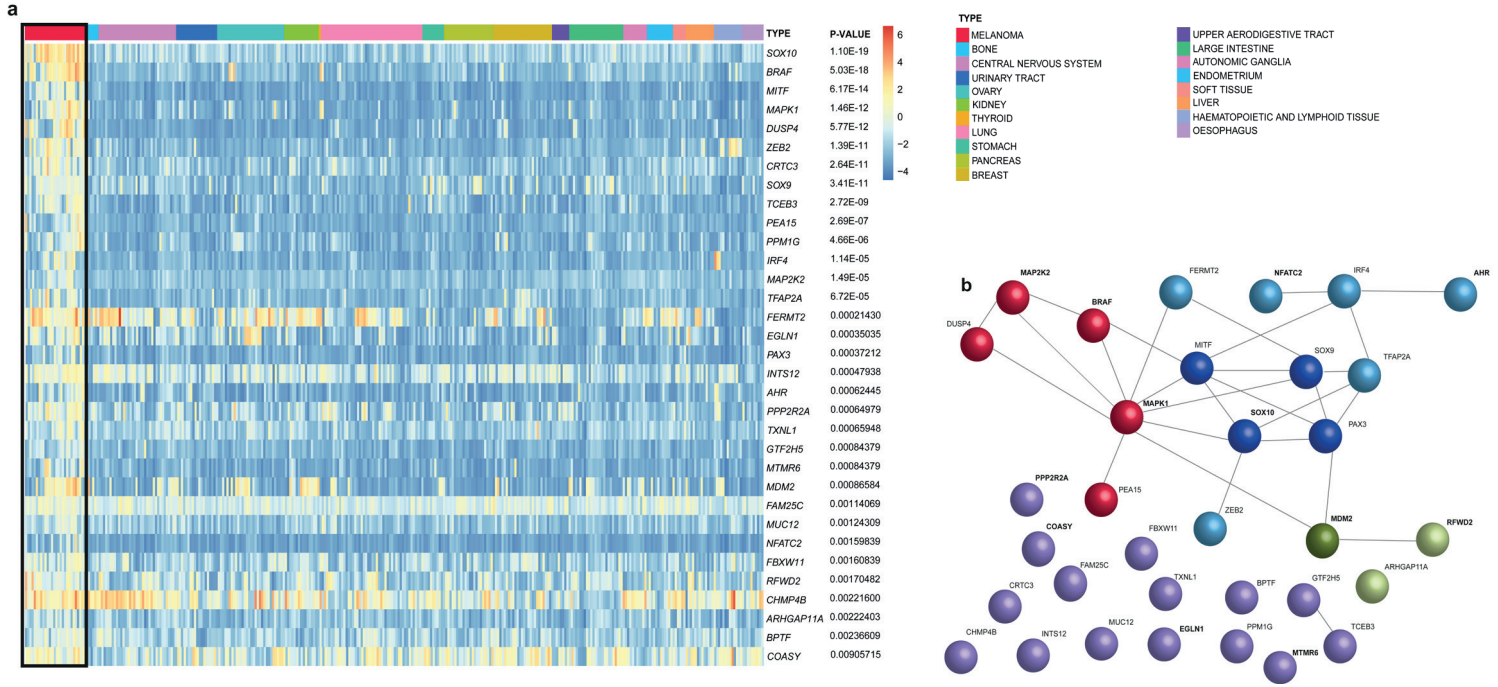

Figure 1 Identification of genetic dependencies in cutaneous melanoma cells a) Heatmap representation of genes significantly under-represented in melanoma cell lines indicated in a box. The scale bar represents scaled Bayesian factors (BFs) within each screen, calculated by subtracting the BF value corresponding to the threshold guaranteeing a 5% FDR obtained when classifying prior known essential/non-essential genes based on their BF's rank [13]. Red color indicates genes that are likely to be fitness genes and, therefore, have a positive scaled BF. Blue color indicates genes less likely to be important for cell fitness with a negative scaled BF. Tumor types are shown and clustered. Genes were ranked according to the noted Fisher exact test adjusted p-values. **b)** String protein interaction network for 33 significant fitness genes in melanoma cell lines. Color coding: Red- proteins involved in the MAPK-signaling pathway; blue- melanocyte-lineage specific proteins; green- p53-regulatory pathway components; purple- unknown interaction network, miscellaneous function; dark- core protein components; light- regulatory components. For proteins indicated in bold pharmacological inhibitors are currently available.

Figure 2 Significant fitness genes in 28 melanoma cell lines. Heatmap representation of fitness genes specifically important in cutaneous melanoma cell lines and indication of established driver mutations (*BRAF, NRAS, NF1*) for each cell line. Supervised clustering was performed on columns to distinguish clusters of *BRAF, NRAS, NF1* mutant and wildtype cell lines. The genes are ranked according to fitness effect that is denoted by the scaled BF score within the set of 28 melanoma cell lines. Genes that are high on the heatmap had a more positive scaled BF score (red) indicating that are more likely to be important for fitness whereas, genes that are low on the heatmap (blue) had a more negative scaled BF score indicating that are less likely to be important for fitness.

Moreover, activation of *AHR* was recently reported to promote resistance to BRAF-inhibitors in melanoma [28]. In addition, a set of fitness genes related to melanoma with undefined roles in melanocyte biology or melanoma pathogenesis was identified, such as *MTMR6* and *CRTC3*. Pharmacological compounds are available that may halt melanoma growth for the products of 12 of the identified fitness genes related to melanoma, including AHR and MDM2 (Supplemental Table 3).

Negative regulators of MAPK signaling are essential in melanoma

Remarkably, the identified MAPK signaling genes encoded not only activating but also inhibitory components such as *DUSP4* and *PEA15*. DUSP4 dephosphorylates ERK1/2, in addition to p38 and JNK (Supplemental Table 4) [29]. While PEA15 is a negative regulator of MAPK signaling that acts by sequestering ERK1/2 in the cytoplasm [30]. The protein phosphatase PPP2R2A has pleiotropic functions, including regulation of ERK1/2 and AKT phosphorylation and is involved in double strand DNA repair (Supplemental Table 4) [31-34]. As melanoma is characterized by activation of MAPK signaling due to mutations in *BRAF, NRAS, NF1* and *KIT* genes, this suggests that further hyperactivation of MAPK signaling through loss of inhibitors of this signaling pathway may be deleterious to melanoma cells. Based on their melanoma specificity (p-value), effect size (scaled BF score) and gene function we proceeded with functional *in vitro* studies of two genes encoding for proteins with inhibitory effect on MAPK signaling activity, *DUSP4* and *PPP2R2A*. *DUSP4* was a significant fitness gene in 16 melanoma cell-lines including one *NRAS* mutant and *PPP2R2A* significantly affected fitness of 16 melanoma cell-lines, including one *NRAS* mutant and one *NF1* mutant (Figure 2). *DUSP4* and *PPP2R2A* were homogenously expressed among the 28 melanoma cell-lines and transcript levels did not provide an explanation for the difference in sensitivity to inactivation– this might imply post-translational factor influence on the function of these genes.

Figure 3 Validation of *PPP2R2A* and *DUSP4* knockout on proliferation of IGR1 and A375 cutaneous melanoma cell lines. a) Immunoblot analysis of BRAF, PPP2R2A and DUSP4 protein expression 4 days post-transduction with sgRNA expressing lentiviruses in IGR1 and A375 cells. Expression of vinculin was investigated as a loading control. **b)** Crystal violet images of a control depletion (*SSX3*), *BRAF* and two independent sgRNAs for *PPP2R2A* and *DUSP4* in IGR1 and A375 cells. **c)** Comparison of two independent techniques, live cell imaging in 96-well plates and cell viability assay in 12-well plates for different knockout IGR1 and A375 lines. A 2-way ANOVA and Bonferroni's multiple comparisons test was performed between the two techniques (*$p<0.01$). Error bars represent SE. **d)** Graphical representation of cell proliferation up to 8-days post seeding of IGR1 cells and 6-days post seeding of A375 cells. sgCtrl represents depletion of *SSX3*, *BRAF* depletion was used as a positive control and 2 independent sgRNAs were used for *PPP2R2A* and *DUSP4*. The normalized confluency (%) of IGR1 and A375 cells was corrected based on day 1 measurements. A 2-way ANOVA and Bonferroni's multiple comparisons test was performed between the control and all other lines (*$p<0.01$).

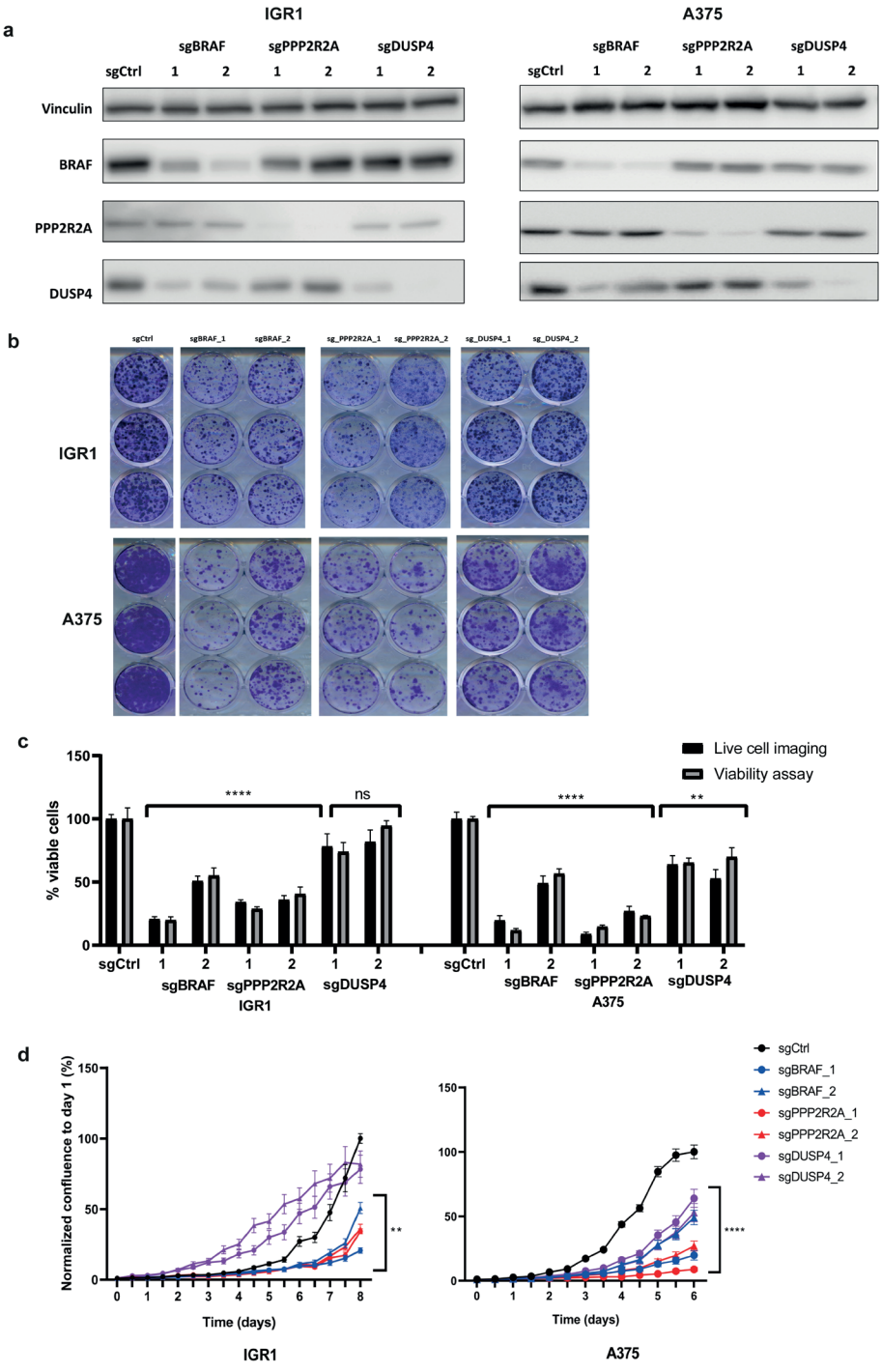
a
IGR1
A375
sgBRAF
sgPPP2R2A
sgDUSP4
sgCtrl
1
2
Vinculin
BRAF
PPP2R2A
DUSP4
b
IGR1
A375
sgCtrl
sgBRAF_1
sgBRAF_2
sg_PPP2R2A_1
sg_PPP2R2A_2
sg_DUSP4_1
sg_DUSP4_2
c
Live cell imaging
Viability assay
% viable cells
****
ns
**
d
Normalized confluence to day 1 (%)
Time (days)
sgCtrl
sgBRAF_1
sgBRAF_2
sgPPP2R2A_1
sgPPP2R2A_2
sgDUSP4_1
sgDUSP4_2

Figure 3

We used CRISPR-mediated inactivation of the non-fitness gene *SSX3* as a negative control and as the cell lines used for validation were *BRAF*-mutant, inactivation of *BRAF* was used as a positive control. First, we analyzed the effects of depletion of the candidate genes in the *BRAF*-mutant melanoma cell line WM983B, which was included in the CRISPR-Cas9 screen. Proteins encoded by the target genes *BRAF*, *PPP2R2A* and *DUSP4* were significantly depleted by CRISPR-Cas9-mediated inactivation using two independent sgRNAs, as was confirmed by immunoblot analysis four days after transduction (Supplemental Figure 2A). The sgRNAs against *BRAF* also led to a decrease in DUSP4 protein levels, in accordance with regulation of DUSP4 by MAPK signaling as part of a negative feedback loop [29]. Upon CRISPR-mediated inactivation of *DUSP4* and *PPP2R2A*, a significant effect on WM983B cell viability was observed as measured using the crystal violet assay (Supplemental Figure 2B). Loss of proliferation caused by *DUSP4*-mediated depletion was similar to *BRAF*-mediated depletion in WM983B cells (Supplemental Figure 2C). The effects of inactivation of *PPP2R2A* using both sgRNAs was slightly less pronounced in this cell line. Combined, these data suggest that genetic depletion of *DUSP4* and *PPP2R2A* has a significant effect on the proliferation of WM983B cells.

Next, we examined the effects of *PPP2R2A* and *DUSP4* depletion in two additional melanoma cell lines, both harboring *BRAF* mutations. Whereas the IGR1 melanoma cell line was also included in the CRISPR-Cas9 screens, the A375 melanoma cell line was not. Immunoblot analysis confirmed depletion of *PPP2R2A* and *DUSP4* upon sgRNA transduction (Figure 3A). We could verify that *PPP2R2A* inactivation affects the viability of IGR1 cells 8 days after seeding according to the colony formation assay (Figure 3B), in accordance with results from CRISPR-Cas9 screen analysis. When monitoring cell confluency over time we observed decreased proliferation upon *PPP2R2A* inactivation in IGR1 cells, in line with the colony formation data (Figure 3C and D). As for the independent A375 cell line, significant loss of viability was confirmed upon inactivation of *DUSP4* and *PPP2R2A* through the colony formation assay (Figure 3B and C) and by normalized cell confluency over time (Figure 3D).

Loss of proliferation upon CRISPR-Cas9-medicated inactivation of *PPP2R2A* was stronger when compared to *DUSP4* depletion using both sgRNAs in A375 cells. One sgRNA targeting *BRAF* was more effective than the other in reducing cell viability and proliferation in all tested *BRAF*-mutant cell lines (Figure 3). Further studies are needed to unravel the nature and mechanism of the effects underlying these genetic dependencies. Decreased protein levels of DUSP4 and PPP2RA were detected in all melanoma cell lines by immunoblot analysis up to 10 days after transduction (Supplemental figure 3) confirming the effects of *DUSP4* and *PPP2RA* on cellular fitness in melanoma cell lines. This suggests dependency of genes encoding inhibitors of oncogenic kinome signaling for proliferation.

DISCUSSION

Here, we present the results of analysis of genome-wide CRISPR-Cas9 screens aimed at identifying genes that melanoma cells depend on for fitness. The comparative analysis of the effects on fitness of gene inactivation in melanoma cells and other cancer cell lines uncovered a set of 33 fitness genes related to melanoma. We verified the fitness effects of *PPP2R2A* and *DUSP4* in multiple melanoma cell lines. Genetic inactivation of several fitness genes had a similar effect on melanoma cell proliferation as *BRAF* inactivation.

Strengths of this study are the sensitivity and robustness of the CRISPR-Cas9 screening methodology by targeting the full human genome using an average of 4 sgRNAs per gene and data analysis through normalization of copy-number-associated effects, as well as comparative analysis of genetic dependencies in 28 melanoma cell lines with those of 313 other tumor cell lines. Evaluation of CRISPR-Cas9 screens in human melanocytes would have allowed further delineation of genes that are essential in melanoma from lineage-specific fitness genes, but such data are not yet available. As our identified hits include the melanocytic lineage transcription factors *MITF* and *SOX10*, it is probable that some of the other genes affect melanocyte fitness as well. Eleven of the identified 33 genes have been previously identified as fitness genes in haploid, CML-derived HAP1 cells (*BRAF, MAPK1, ZEB2, TCEB3, FERMT2, PPP2R2A, BPTF CHMP4B, INTS12, FBXW11* and *COASY*) [24]. Inactivation of these genes is significantly more detrimental to melanoma cells than to other tumor cell types, but they are likely to be involved in essential cellular processes [24]. The composition of the fitness gene set related to melanoma may be determined in large part by dependencies associated with mutant *BRAF*. In a previous study, gene dependency associations with *BRAF* mutation have been identified in 16 cancer types [8]. Mutant *BRAF* was present in nine of 146 of cancer cell lines and this oncogenic mutation was found to be associated with dependency on 50 genes in these cancer types. Many of the identified fitness genes such as *NFATC2* and *EGLN1* have an as yet undetermined role in melanoma biology. For 12 proteins encoded by genes identified as essential in the present study pharmacological compounds are available, implying that certain existing drugs might be efficacious in the treatment of melanoma.

We demonstrate genetic dependency on multiple MAPK signaling components in melanoma. Interestingly, these are not only activators but also inhibitors of MAPK signaling, such as DUSP4, and PEA15. In accordance with our findings, it has been reported previously that ERK1 and ERK2 overexpression results in cell death in *BRAF* and *NRAS* mutant melanoma cells [35]. Our results support the notion that further activation of MAPK signaling through loss of these inhibitors in melanoma cells that already harbor activating *BRAF* or *NRAS* mutations is detrimental, a

hypothesis that will need to be explored with further experiments. Most melanoma cell lines included in the CRISPR-Cas9 screens carried the *BRAF* mutation, but screen data suggested that some *NRAS*-mutant melanoma cells might also be sensitive to inactivation of *DUSP4* and *PPP2R2A*. Targeting these proteins may provide an alternative approach to treatment of metastatic melanoma, particularly after relapse from immunotherapy. It would be interesting to investigate whether *BRAF*-mutant melanoma cells that have acquired resistance to BRAF and MEK inhibitors remain sensitive to inhibition of *PPP2R2A* and *DUSP4*. Acquisition of resistance to BRAF inhibitors commonly involves reactivation of MAPK signaling. Discontinuation of BRAF inhibitor treatment in resistant melanoma cells results in hyperactivated MAPK signaling which may be detrimental to these cells [36, 37]. *DUSP4* depletion was recently reported to diminish the negative effects on melanoma cell viability induced by MEK inhibitors through increasing MAPK activity [38]. We hypothesize that once melanoma cells have acquired resistance to treatment with BRAF and MEK inhibitors by upregulation of MAPK activity they will become more sensitive to *DUSP4* inhibition. Targeting negative regulators of MAPK signaling inhibitors such as DUSP4 and PEA15 could further activate MAPK signaling and may therefore be particularly effective in eliminating melanoma cells that have acquired resistance to BRAF and MEK inhibition. Alternate treatment with BRAF inhibitors and inhibitors targeting negative regulators of MAPK signaling could constitute an effective treatment strategy. The lack of specific pharmacological inhibitors for DUSP4 and PEA15 currently limits the possibility to determine those effects.

Genetic inactivation of *DUSP4* significantly reduced proliferation in a melanoma cell line that was included in the CRISPR-Cas9 screens (WM983B) as well as an independent melanoma cell line (A375). This suggests that *DUSP4* could be an effective target across a larger panel of melanoma cell lines. Consistent with findings from the CRISPR-Cas9 screens, inactivation of *DUSP4* did not induce a growth disadvantage in the IGR1 cell line, showing heterogeneity between different melanoma cell lines with respect to fitness effects. In addition not only *BRAF* mutant but some *NRAS* mutant melanoma cell lines were sensitive to inhibition of *DUSP4* and *PPP2R2A*. *PPP2R2A* was confirmed to be a fitness gene in the two cell lines included in the initial CRISPR-Cas9 screens, IGR1 and WM983B as well as in the independent A375 cell line. PPP2R2A is a PP2A regulatory subunit that has been reported to inhibit MAPK signaling by dephosphorylating ERK [39], but also to promote MAPK signaling by regulating RAF and KSR [40, 41]. The PP2A complex has broader cellular functions, including regulation of oxidative stress signaling and DNA repair response [32, 33, 42, 43]. DUSP4 regulates phosphorylation of p38, JNK and other proteins in addition to ERK [44]. We have not investigated whether the fitness effects of DUSP4 and PPP2R2A are strictly dependent on their function as inhibitors of oncogenic kinome signaling.

The generation of effective treatment strategies in melanoma remains a challenge. Here, we present an analysis of CRISPR-Cas9 screen data aimed at melanoma cell lines, identifying 33 genes that specifically affect the fitness in this tumor type. *In vitro* experiments in human melanoma cell lines confirmed that inactivation of *DUSP4* and *PPP2R2A* results in decreased cell proliferation. Collectively, these data present a resource of genetic dependencies in melanoma that may be explored as potential therapeutic targets.

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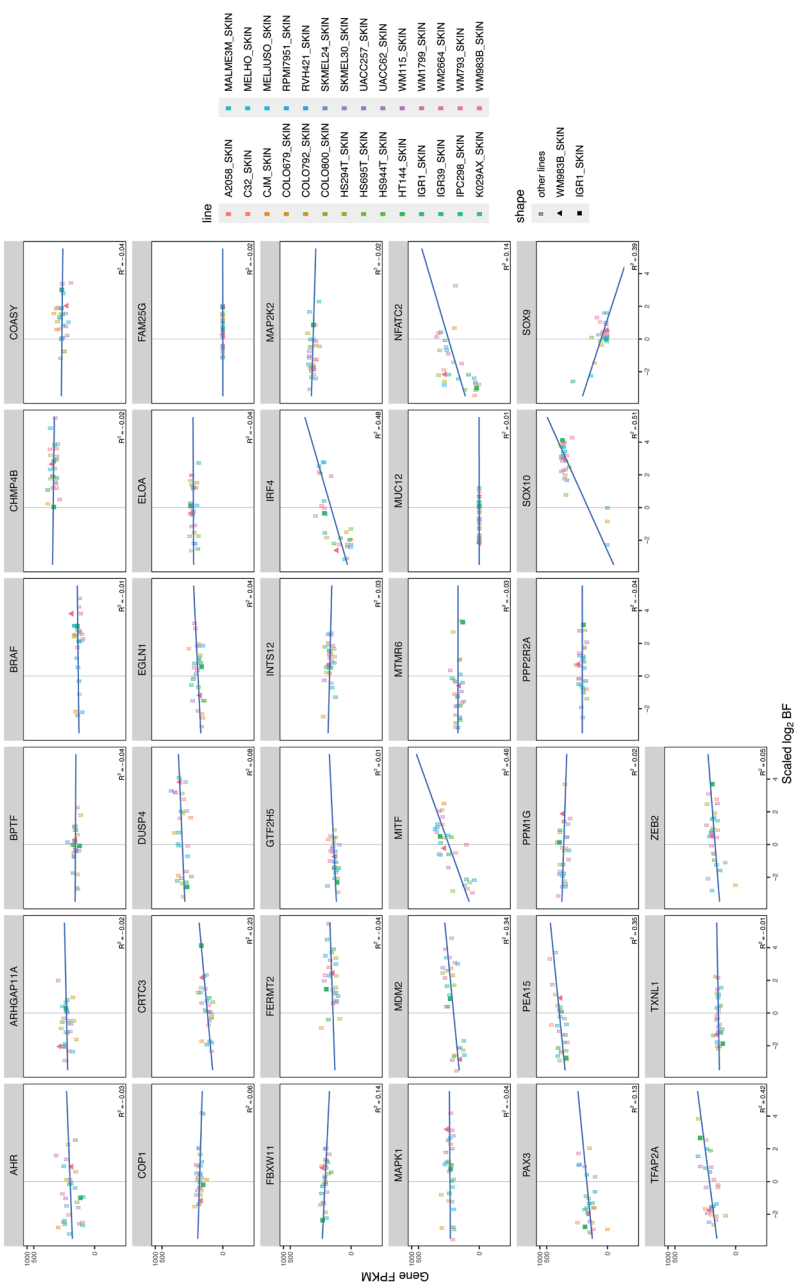
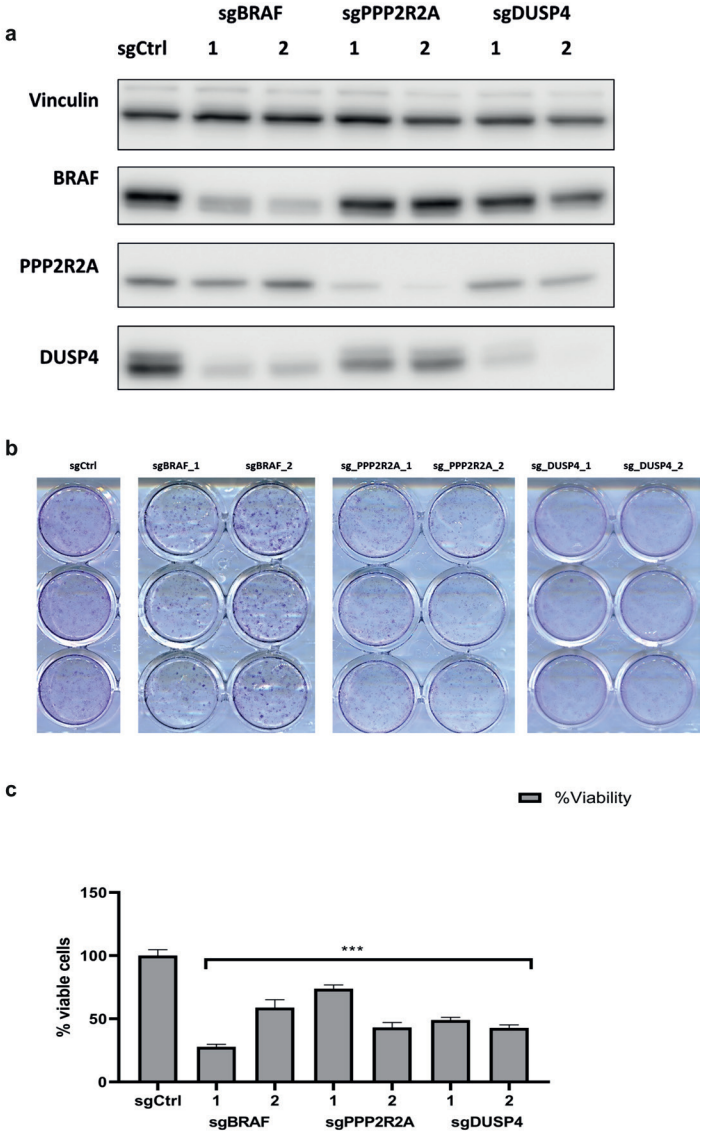
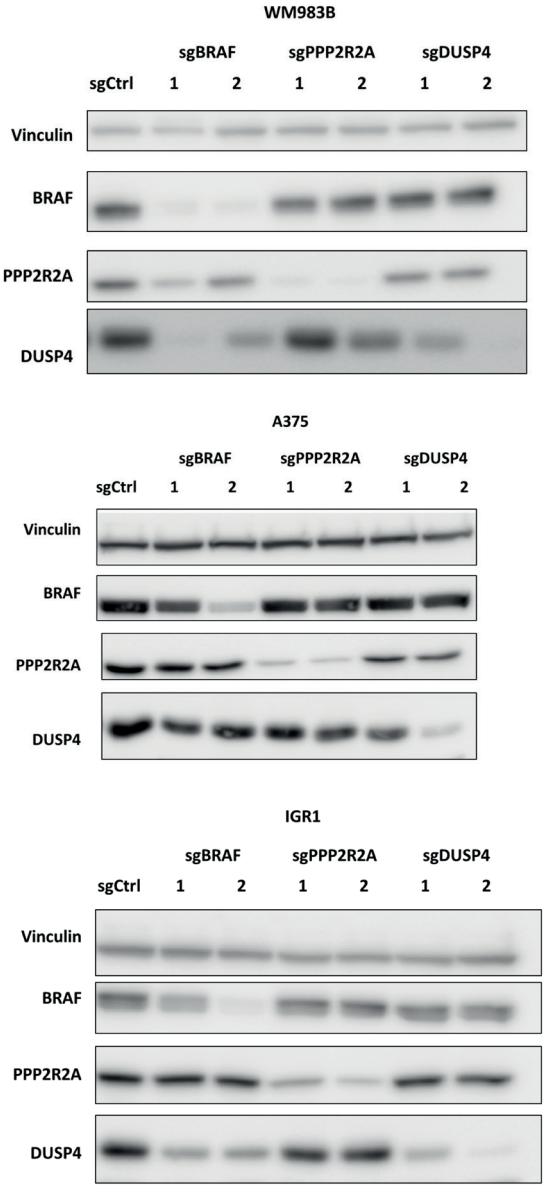


Supplemental Figure 1 Expression of 33 significant fitness genes in melanoma cell lines. The scaled Bayesian factor score is plotted against the FPKM (expression) score for each gene using data from CCLE. The FPKM is shown on a "pseudo-log" axis. The blue line is a simple linear correlation model with the adjusted R-squared value shown. The vertical grey line shows the fitness switch; the left-hand genes are non-fitness, but to the right are most probably fitness genes. All "SKIN" cell lines are melanoma cell lines.



Supplemental Figure 2 Depletion of *PPP2R2A* and *DUSP4* has a significant effect on WM983B proliferation. a) Immunoblot analysis of BRAF, PPP2R2A and DUSP4 protein expression 4 days post-transduction with sgRNA expressing lentiviruses. Expression of vinculin was investigated as a loading control. **b)** Crystal violet images of a control depletion (*SSX3*), *BRAF* and two independent sgRNAs for *PPP2R2A* and *DUSP4*. **c)** Graphical representation of cell viability in WM983B cells 10-days post seeding. sgCtrl represents depletion of *SSX3*, *BRAF* depletion was used as a positive control and 2 independent sgRNAs were used for *PPP2R2A* and *DUSP4*. Experiments were performed in two biological replicates and in Figures 3-5 we show results of a representative experiment. A 2-way ANOVA and Bonferroni's multiple comparisons test was performed between the control and all other lines (*p<0.01). Error bars represent SE.



Supplemental Figure 3 Immuno-blot analysis of tested melanoma cell lines. Immuno-blot analysis of BRAF, PPP2R2A and DUSP4 protein expression 10 days post-transduction in all tested melanoma cell lines (A375, IGR1, WM983B). Expression of vinculin was investigated as a loading control.

Supplemental Table 1 Sequences of gRNAs used for single gene validation analysis.

Name	Sequence
sgCtrl_1	5'- CACCGCGCATGAAAGATGGGAGGAGT-3'
sgCtrl_2	5'-CACCGTCTTTCATGCGTAATAAACGT-3'
sgBRAF_1	5'-CACCGCAAATGATTAAGTTGACACGT-3'
sgBRAF_2	5'-CACCGTGCTTTCTTTAGACTGTCTGT-3'
sgPPP2R2A_1	5'-CACCGGATCCATAGGCCTAAAGACGT-3'
sgPPP2R2A_2	5'-CACCGGCAAATATTCTTCGTGGACGT-3'
sgDUSP4_1	5'-CACCGCAGAATTCTGGGTACTCGGGT-3'
sgDUSP4_2	5'-CACCGGGGGGTGGGATGGCTGCCAGT-3'

Supplemental Table 2 List of melanoma-related fitness genes (SKIN) in haploid cells (Blomen et al., 2015)

FITNESS_SKIN	FITNESS_HAPLOID	FITNESS_SKIN	FITNESS_HAPLOID
PSMD14	YES	RPLP0	YES
RPS3A	YES	KPNB1	YES
RPS2	YES	HSPA5	YES
RPL29	YES	EIF3E	YES
SAP30BP	YES	RPP21	YES
PFDN2	YES	CDC27	YES
RPLP2	YES	MMS22L	YES
RPS3	YES	TSR2	YES
RPL18A	YES	SMC2	YES
NDC80	YES	DHPS	YES
RPL31	YES	ARMC7	YES
SNRPF	YES	FBL	YES
DPH3	NO	RPS20	YES
TOMM40	YES	ORC6	YES
PSMA5	YES	CDC123	YES
CCT3	YES	YRDC	YES
SBDS	YES	RPL32	YES
ALG13	YES	RPL27	YES
OSGEP	YES	LSM2	YES
RPL17	YES	RGPD8	NO
PSMC3	YES	RGPD6	NO
CDC5L	YES	ESPL1	NO
PSMA1	YES	DUT	YES
KRT8	NO	STRAP	YES

Continuing: Supplemental Table 2

FITNESS_SKIN	FITNESS_HAPLOID
DHX15	YES
H3F3A	NO
EEF2KMT	NO
RPL13	YES
PRPF38A	YES
RPL37	YES
DDX56	YES
NUP43	YES
EIF5	YES
RNPC3	YES
EIF2S3	YES
RNGTT	YES
WDR43	YES
CHAF1B	YES
SF3A2	YES
TARS	YES
ATP6V0B	YES
PRPF38B	YES
DBR1	YES
PSMD4	YES
SARS	YES
EIF3F	NO
RPL27A	YES
ZRSR2	YES
SRSF7	YES
DNAJC17	YES
RPL21	YES
GINS3	YES
HNRNPC	YES
WBP11	YES
RAN	YES
SFPQ	YES
ATP6V1A	YES
MARS	YES
RPL18	YES
CDC16	YES

FITNESS_SKIN	FITNESS_HAPLOID
TRMT112	NO
COPB1	YES
EIF3A	YES
PSMA6	NO
FAM86C1	NO
PSMA3	YES
PAK1IP1	YES
PSMD7	YES
RPSA	YES
SMC4	YES
PSMA7	YES
CDK7	YES
COPB2	YES
SYS1	YES
RPL13A	YES
GPN3	YES
RPL9	YES
SNRPB	YES
DYNC1H1	YES
MED6	YES
ELP5	YES
PSMB3	YES
NPM1	YES
WDR75	YES
DTYMK	YES
ZNF718	NO
SNAPC1	YES
ATP6V1G1	YES
RPL23	YES
NCAPG	YES
RPS6	YES
RPS8	YES
EIF4A1	YES
CHMP4B	YES
MED30	YES
GTPBP4	YES

Continuing: Supplemental Table 2

FITNESS_SKIN	FITNESS_HAPLOID
ATP6V0C	YES
THOC2	YES
OR56A5	NO
PSMB5	YES
ATP6V1B2	YES
RPS27A	YES
SRSF2	YES
SMU1	YES
ETF1	YES
RPS13	YES
RPL15	YES
RPS28	YES
RBM17	YES
CPSF3L	YES
PSMG4	YES
RPS11	YES
THAP1	YES
TTC27	YES
MCM2	YES
MPHOSPH10	YES
EEF2	YES
SF3A1	YES
SF1	YES
DDB1	YES
VPS25	YES
WDR82	YES
PSMB6	YES
PPIA	NO
NCBP2	YES
PSMD3	YES
EEF1A1	YES
GINS2	YES
NHLRC2	YES
MYC	YES
RPL11	YES
DUX4	NO

FITNESS_SKIN	FITNESS_HAPLOID
POLR2L	YES
RPL10A	YES
FARSB	YES
CHEK1	YES
RPL26	YES
LUC7L3	YES
SLU7	YES
RPS9	YES
CDT1	YES
KIF11	YES
RPS19	YES
RPL12	YES
RPL5	YES
SRSF3	YES
UTP4	NO
RSL24D1	YES
CTCF	YES
RPL23A	YES
CHERP	YES
RRM2	YES
SUPT5H	YES
NOL10	YES
CDC7	YES
ALG2	YES
DAD1	YES
CDC23	YES
DDX49	YES
GPKOW	YES
ANKRD18B	NO
PRELID1	YES
DCTN6	YES
BUB3	YES
NEDD1	YES
TXN	YES
EIF2S1	YES
LSM8	NO
HAUS7	YES

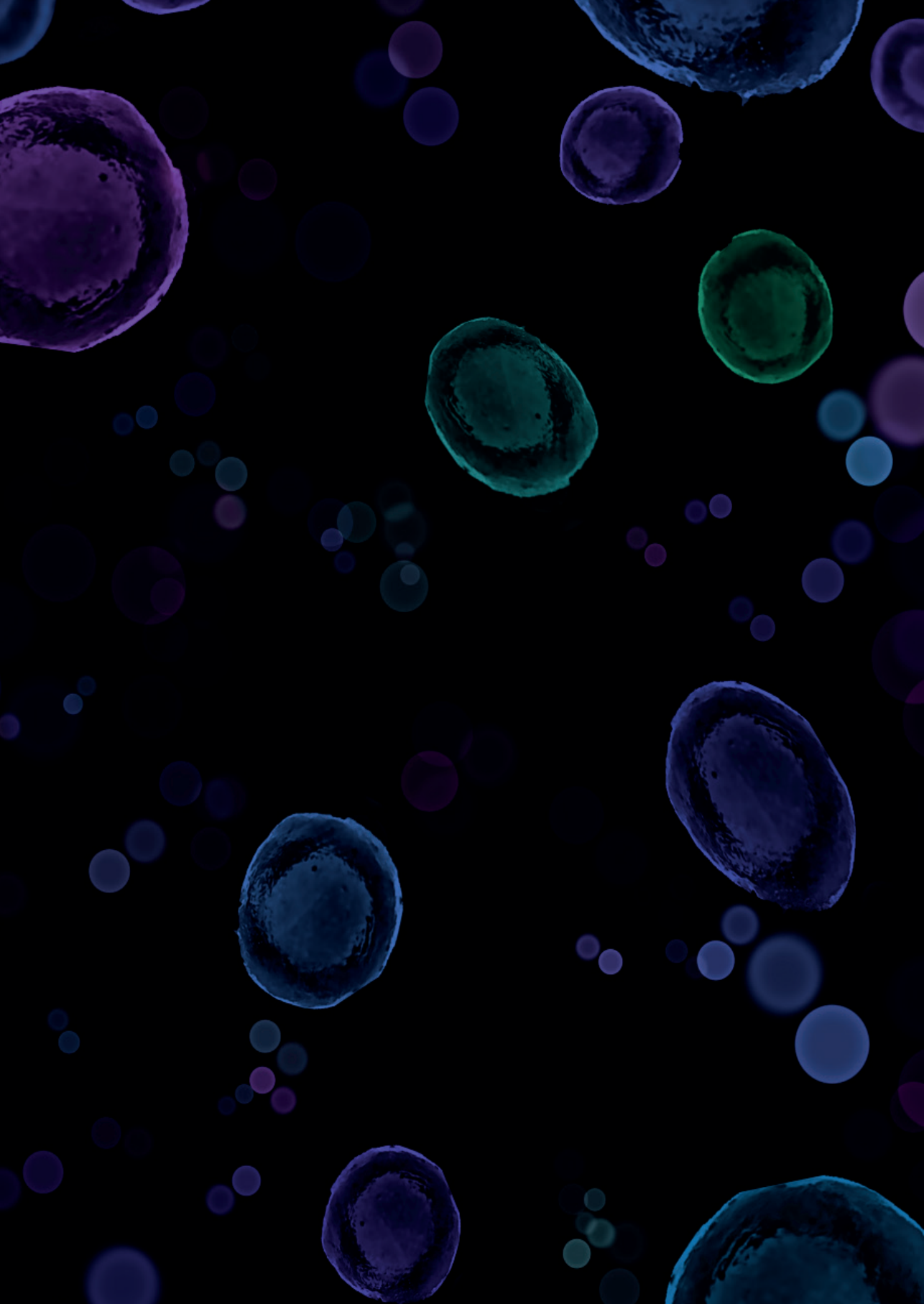
Supplemental Table 3 Description of 33 significant fitness genes in melanoma

GENE	DESCRIPTION	FUNCTION	CANCER GENE CENSUS (YES/NO)	CHEMICAL INHIBITOR (YES/NO)
TCEB3	Elongin A	Increases the RNA polymerase II transcription elongation	NO	NO
ARHGAP11A	Rho GTPase Activating Protein 11A	Regulates p53-dependent cell-cycle arrest and apoptosis	NO	NO
MITF	Melanocyte Inducing Transcription Factor	Transcription factor that regulates the expression of genes with essential roles in cell differentiation, proliferation and survival	YES	NO
NFATC2	Nuclear Factor Of Activated T Cells 2	Inducible expression of cytokine genes in T-cells	YES	YES
GTF2H5	General Transcription Factor IIH Subunit 5	General and transcription-coupled nucleotide excision repair (NER) of damaged DNA	NO	NO
SOX10	SRY-Box Transcription Factor 10	Involved in the regulation of embryonic development and in the determination of the cell fate.	NO	YES
DUSP4	Dual Specificity Phosphatase 4	Negative regulation of members of the mitogen-activated protein (MAP) kinase superfamily	NO	NO
TFAP2A	Transcription Factor AP-2 Alpha	Transcription factor that binds the consensus sequence 5'-GCCNNNGGC-3'	NO	NO
PPP2R2A	Protein Phosphatase 2 Regulatory Subunit Balpha	Ser/Thr phosphatase, implicated in the negative control of cell growth and division	NO	YES
MDM2	MDM2 Proto-Oncogene	E3 ubiquitin ligase, promotes tumor formation by targeting P53	YES	YES
FBXW11	F-Box And WD Repeat Domain Containing 11	Phosphorylation-dependent ubiquitination.	NO	NO
PEA15	Proliferation And Apoptosis Adaptor Protein 15	Negative regulator of apoptosis	NO	NO
PAX3	Paired Box 3	Transcription factor that may regulate cell proliferation, migration and apoptosis.	YES	NO
MUC12	Mucin 12, Cell Surface Associated	Integral membrane glycoprotein, involved in epithelial cell protection	NO	NO
MAPK1	Mitogen-Activated Protein Kinase 1	Essential component of the MAP kinase signal transduction pathway	YES	YES
FERMT2	Fermitin Family Member 2	Scaffolding protein that enhances integrin activation	NO	NO
BRAF	B-Raf Proto-Oncogene, Serine/Threonine Kinase	Activator of the MAP kinase signal transduction pathway	YES	YES

RFWD2	COP1 E3 Ubiquitin Ligase	Mediates ubiquitination and subsequent proteasomal degradation of target proteins	NO	YES
ZEB2	Zinc Finger E-Box Binding Homeobox 2	DNA-binding transcriptional repressor that interacts with activated SMADs	NO	NO
COASY	Coenzyme A Synthase	Coenzyme A (CoA), Important role in metabolic pathways	NO	YES
MTMR6	Myotubularin Related Protein 6	Phosphatase that acts on lipids with a phosphoinositol headgroup	NO	YES
PPM1G	Protein Phosphatase, Mg2+/Mn2+ Dependent 1G	Member of the PP2C family of Ser/Thr protein phosphatases, negative regulator of cell stress response pathways	NO	NO
IRF4	Interferon Regulatory Factor 4	IRF family of transcription factors, negatively regulates Toll-like-receptor (TLR) signaling	YES	NO
CHMP4B	Charged Multivesicular Body Protein 4B	Member of the chromatin-modifying protein/charged multivesicular body protein (CHMP) protein family, probable core component of the endosomal sorting	NO	NO
INTS12	Integrator Complex Subunit 12	Mediates 3-prime end processing of small nuclear RNAs U1	NO	NO
FAM25C	Family With Sequence Similarity 25 Member C	Protein coding gene, unknown interaction network	NO	NO
AHR	Aryl Hydrocarbon Receptor	Ligand-activated transcriptional activator Involved in the regulation of biological responses to planar aromatic hydrocarbons.	NO	YES
TXNL1	Thioredoxin Like 1	Protein coding gene, unknown interaction network	NO	NO
CRTC3	CREB Regulated Transcription Coactivator 3	Acts as a coactivator, in the SIK/TORC signaling pathway	YES	NO
MAP2K2	Mitogen-Activated Protein Kinase Kinase 2	Dual specificity protein kinase, activates MAPK1/ERK2 and MAPK2/ERK3	YES	YES
SOX9	SRY-Box Transcription Factor 9	Transcriptional regulator that plays a role in chondrocytes differentiation and skeletal development	NO	NO
EGLN1	Egl-9 Family Hypoxia Inducible Factor 1	Catalyzes the post-translational formation of 4-hydroxyproline in hypoxia-inducible factor (HIF) alpha proteins, cellular oxygen sensor	NO	YES
BPTF	Bromodomain PHD Finger Transcription Factor	Histone-binding component of NURF (nucleosome-remodeling factor)	NO	NO

Supplemental Table 4 Pathway enrichment analysis of genetic dependencies in melanoma

Term	Overlap	P-value	Adjusted P-value	Odds Ratio	Combined Score	Genes
positive regulation of transcription from RNA polymerase II promoter (GO:0045944)	9/848	1.108360646136E-5	0.005655964377232007	6.064690026954177	69.1983766376923	TFAP2A;ZEB2;CRTC3;IRF4; NFATC2;PAX3;AHR;SOX9
regulation of transcription, DNA-templated (GO:0006355)	12/1598	9.540546380690903E-6	0.005409489797851742	4.291078133381012	49.60469072713027	TFAP2A;MAP2K2;FBXW11; IRF4;MDM2;NFATC2;PAX3; AHR;SOX9;SOX10;BPTF
MAPK cascade (GO:0000165)	6/278	7.904331344981627E-6	0.005041975356680155	12.332990750256938	144.88920465225755	MAP2K2;FBXW11;PEA15; MAPK1;BRAF;SOX9
ERK1 and ERK2 cascade (GO:0070371)	3/21	6.389593665716892E-6	0.004658013782307614	81.63265306122449	976.3950923009298	MAP2K2;MAPK1;SOX9
regulation of transcription from RNA polymerase II promoter (GO:0006357)	12/1478	4.267357920718731E-6	0.003629387911571281	4.639474192924801	57.364851387563085	TFAP2A;ZEB2;CRTC3;IRF4; MDM2;NFATC2;PAX3;AHR; SOX9;SOX10;BPTF
dephosphorylation (GO:0016311)	5/124	2.3626164315747644E-6	0.003014107912581506	23.04147465437788	298.5193754743466	DUSP4;FBXW11;PPP2R2A; MTMR6;PPM1G
protein dephosphorylation (GO:0006470)	5/125	2.4579805682085504E-6	0.0025086149679136465	22.857142857142858	295.2267531972836	DUSP4;FBXW11;PPP2R2A; MTMR6;PPM1G
positive regulation of gene expression (GO:0010628)	13/771	2.528819425209641E-10	1.2904565526844798E-6	9.634982397628312	212.9147887818892	TFAP2A;MAP2K2;FBXW11; NFATC2;PAX3;BRAF;AHR; SOX10;IRF4;MAPK1;SOX9; BPTF
positive regulation of nucleic acid-templated transcription (GO:1903508)	11/502	5.406513574818329E-10	1.3794719386148967E-6	12.521343198634035	267.18350750458654	TFAP2A;MAP2K2;FBXW11; IRF4;NFATC2;PAX3;AHR; SOX9;SOX10;BPTF
positive regulation of transcription, DNA-templated (GO:0045893)	13/1120	2.3107557866707725E-8	3.930595593126984E-5	6.63265306122449	116.62264245061016	TFAP2A;CRTC3;MAP2K2; FBXW11;NFATC2;PAX3;AHR; SOX10;ZEB2;IRF4;SOX9;BPTF



Chapter 6

General Discussion

BRIEF SYNOPSIS

The studies in this thesis explored several aspects of genetic dependencies in the development of familial and sporadic melanoma. *CDKN2A* is the most common high-penetrance susceptibility gene responsible for up to 40% of melanoma families worldwide. Interestingly, more than half of germline variation in familial predisposition to melanoma remains to be determined. To identify novel high-penetrance melanoma susceptibility genes we applied Whole Exome Sequencing (WES) and co-segregation analysis in a Dutch melanoma family. We identified *NEK11* as a candidate high-penetrance melanoma susceptibility gene and performed functional characterization in cancer cell lines to show loss-of-function (**chapter 2**). Our additional focus of investigation was a specific cohort of familial melanoma patients carrying a *CDKN2A* founder mutation, a 19-bp deletion known as the *p16-Leiden* mutation. Due to the variability in occurrence of pancreatic cancer (PC) and melanoma within familial melanoma families, we sought to examine genetic modifiers predicting the risk of PC and melanoma (**chapter 3**). In this specific cohort of familial melanoma patients, the timing of *CDKN2A* wild-type allele loss in melanoma development is unknown. We have applied a customized SNP-based digital PCR (dPCR) methodology to precisely quantify *CDKN2A* allelic imbalance depicting loss-of-heterozygosity (LOH) and attempted to deduce the order of genetic events based on absolute quantification of mutations and losses (*CDKN2A* LOH, $BRAF^{V600E}$, *TERT* promoter, chromosome 9q LOH) (**chapter 4**). Finally, in addition to high-penetrance genes in familial melanoma, there are genes that are important fitness factors for cancer cell growth and may provide insight into the biology and progression of sporadic melanoma. The application of screening technologies has been successful in identifying genetic dependencies that could possibly be implemented as therapeutic targets in cancer. We have therefore analyzed Clustered Regular Interspaced Short Palindromic Repeats (CRISPR-Cas9) screening data to identify fitness genes in melanoma and used *in-vitro* systems to validate our findings (**chapter 5**). Combined, we hope to have uncovered novel genetic dependencies that could be used in the targeted treatment of sporadic as well as familial melanoma.

NEK11 AS A NOVEL HIGH PENETRANCE MELANOMA SUSCEPTIBILITY GENE

Even though *CDKN2A* is responsible for melanoma predisposition in a large subset of familial kindreds, the underlying genetic cause in approximately 50% of families remains unknown [1, 2]. Identification of high-penetrance genes is important, since germline mutation carriers can be enrolled in targeted cancer surveillance programs. The implementation of WES and WGS analyses has been instrumental in the identification of novel germline variants causing predisposition to melanoma [3-7]. In **chapter 2**, application of WES analysis in a Dutch melanoma family, identified a nonsense variant in the checkpoint regulatory gene, *NEK11* (p.R374X) co-segregating among affected family members. We showed LOH in a melanoma tissue sample of a *NEK11* mutation carrier and expressed mutant NEK11 to study protein levels and function in cancer cell-lines. We demonstrated reduced levels of the truncated NEK11 protein caused by proteasomal degradation, suggesting loss-of-function through protein instability [8]. Combined, genetic and functional analysis of *NEK11* p.R374X suggest a candidate high penetrance melanoma susceptibility gene.

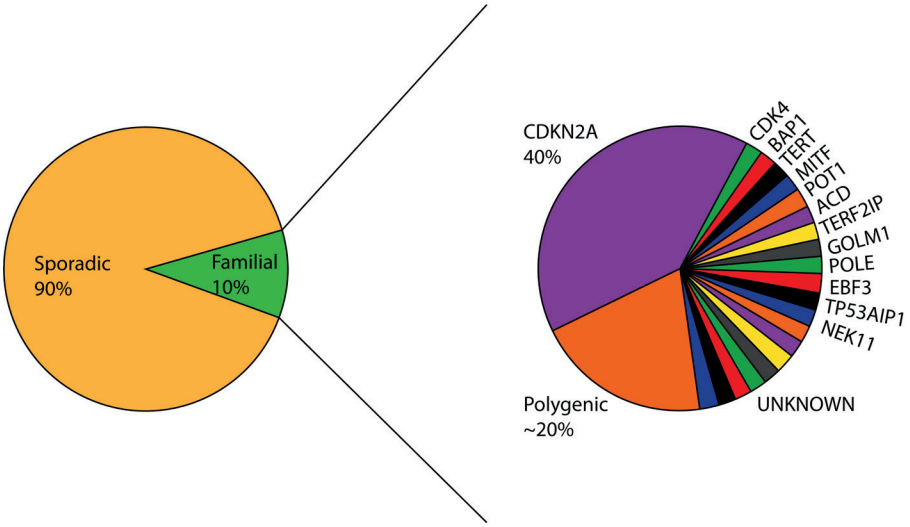


Figure 1 Updated diagram of candidate high penetrance melanoma susceptibility genes. The diagram is adapted using data from chapter 2 to show the small contribution of the identification of *NEK11* as a candidate high penetrance melanoma susceptibility gene

Since, NEK11 emerged in a single Dutch-melanoma family it may only add to the current knowledge of germline variation within a small subset of melanoma families (Figure 1). The data might still provide valuable information for geneticists to include NEK11 in gene panel testing in order to identify more individuals at increased risk. In a recent study, >300.000 UK WES data sets from healthy volunteers were analyzed for non-synonymous protein truncating variants (PTVs) and no mutations were identified in NEK11, further supporting an extremely rare variant [9]. Moreover the NEK11 locus is not frequently deleted in melanoma according to TCGA data suggesting that the truncating mutation identified here is a rare event. This is supported by the absence of any NEK11 mutation in a recent multi-gene panel test of 488 Dutch familial melanoma cases [10]. Variants in the NEK11 gene, or perhaps other components of the pathways it is involved with, should be examined in large cohorts of familial melanoma cases, that are not explained by other established melanoma susceptibility genes to confirm the effect size of this variant [11].

NEK11 truncating variant expression and melanoma development

The functional validation and mechanism of tumor development caused by PTVs is equally important to the initial implementation of sequencing technologies and analysis [12, 13]. This information can assist in improving mutation screening and personalized medicine in high risk patients. Our functional validation analysis demonstrated loss-of-function of the NEK11 truncating variant through protein instability.

To investigate the role of NEK11 on cell proliferation and sensitivity to (UV) irradiation we attempted to knock-down NEK11 in melanoma and human osteocarcoma cells with shRNAs expressed from lentiviral vectors. However, 5 out of 5 different shRNAs were not effective, therefore this approach was stopped. Ideally, in the instance of a successful *NEK11* knock-down system, a zebrafish model would be an informative method to test if knocking-out *NEK11* in a *BRAF* and/or *TP53* mutant background accelerates melanoma formation.

It has been shown that NEK11 is important for DNA damage induced (i.e. IR irradiation and irinotecan treatment) G2/M checkpoint arrest (Figure 2) [14-16]. It is possible, that NEK11 loss, caused by the loss-of-function mutation p.R374X, can result in genomic instability which might lead to the selection of cells with increased and uncontrolled proliferation (Figure 2). Therefore, mutation carriers might be more predisposed to melanoma than to other 'internal' cancer types because of more exposure to UV light. We did not see any differences between the two transfection conditions (NEK11 wild-type and mutant) even when exposing cancer cells to UV radiation. Since the NEK11 p.R374X protein is only expressed at very low levels, one would not expect an effect of expressing this construct in cells. Our results indicate that the p.R374X mutation in NEK11, resulting in truncation and destabilization of the protein is a loss-of-function mutation, which will result in reduced DNA damage-induced cell cycle checkpoints.

Based on the restriction of WES analysis to investigate only the coding part of the genome, the next step towards a better understanding of predisposition to melanoma is the employment of whole-genome sequencing (WGS) technology [17]. The identification of germline variants within the promoter region of *TERT* suggests that there is more to uncover from the non-coding part of our genome. Moreover, hereditary epigenetic alteration through DNA methylation is an additional mechanism regulating gene expression and silencing that deserves further investigation in future studies [18].

Collectively, our data present *NEK11* as a very good candidate high penetrance melanoma susceptibility gene. Further investigation in more families world-wide is required to prove the significance of this candidate gene. Ultimately, in the near future, *NEK11* may be added in clinical genetic testing high-risk melanoma families in order to improve patients' surveillance.

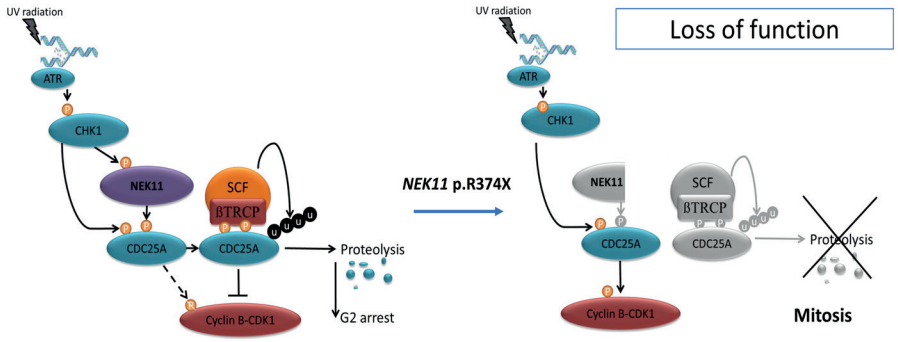


Figure 2 Proposed model of NEK11 truncating variant function in melanoma development. NEK11 is an essential component of the G2/M checkpoint arrest pathway. Following DNA damage, CHK1 is activated through ATR kinases, leading to phosphorylation and activation of NEK11 which in turn leads to degradation of CDC25A and G2 arrest. The p.R374X mutation encodes for a truncated NEK11 protein. NEK11 loss would make a cell more prone to accumulate UV-induced DNA damage by stimulating CDC25A activation and cell cycle progression into mitosis (Figure is adapted from [15]).

GENETIC DEPENDENCIES IN HEREDITARY MELANOMA CAUSED BY *CDKN2A* (*P16-LEIDEN*) MUTATION

Since the discovery of *CDKN2A* as the first high-penetrance melanoma susceptibility gene, a plethora of scientific literature reported on the effect size of *CDKN2A* in familial predisposition to melanoma [19-21]. This dominant high penetrance melanoma susceptibility gene encodes for $p16^{INK4A}$ and $p14^{ARF}$ tumor suppressor proteins regulating the G1/S cell cycle checkpoint and p53-dependent pathways respectively [22]. Germline *CDKN2A* mutation carriers have approximately 70% risk of developing melanoma and an absolute risk of about 15-20% to develop PC [23-25]. A recent study has also shown that these melanoma-prone families should be screened at an early age for additional types of cancer other than melanoma and PC [26, 27]. Moreover, *CDKN2A* not only has a significant causative effect on familial predisposition to melanoma but is also a key tumor suppressor gene acting in the transition stage of invasive melanoma. Bi-allelic loss of *CDKN2A* distinguishes precursor lesions from invasive melanoma in sporadic cases [28]. We attempted to identify genetic modifiers predicting the risk of cancer in familial melanoma patients and investigated the timing of wild-type *CDKN2A* inactivation in the development and progression of hereditary melanoma.

Genetic modifiers predicting the risk of PC and melanoma in CDKN2A mutation carriers

Clinical studies have shown variability in occurrence of PC and melanoma within *CDKN2A*-mutated families, suggesting that modifying factors have a significant role in determining the risk of developing these cancers [23]. The most well-known genetic modifier for melanoma development in *CDKN2A* mutation carriers is *MC1R* [29, 30]. Despite the significant effort in scientific literature to identify genetic modifiers for PC development in families there are still no definitive correlations identified [31, 32].

In **chapter 3** we tested a variable genomic region within the *TERT/CLPTM1L* multi-cancer risk locus that has been significantly correlated to PC risk in the general population [33]. Remarkably at the same time, carriers of the variant allele are at diminished risk of developing melanoma. In the current study, we applied SNP-genotyping through the rhAMP-SNP-genotyping assay that uses reporter dyes suitable for a real-time PCR format and provide a quick and efficient method to genotype multiple samples simultaneously. Unfortunately, we did not find any significant association of the variant allele presence with PC risk in *p16-Leiden* carriers. A significant protective effect was observed for melanoma, similar to the general population, although the observed association was no longer significant after exclusion of probands to assess possible influence of ascertainment [34]. Combined, we did not find a significant association of the variant allele presence with PC or melanoma risk in *p16-Leiden* carriers. The low statistical power of our

study might be a limiting factor in identifying a significant effect but we cannot exclude the possibility that other PC or melanoma-associated SNPs that were not genotyped in this study might modify cancer risk in familial melanoma patients.

A study that used next-generation sequencing data to examine multiple high-risk PC-related genes in melanoma-prone families, identified nominal correlation with variants of mismatch repair genes (*MLH1*, *MSH2*, *MSH6*, *PMS2*), however, there were no loss-of-function mutations identified and only a subset of alterations was classified as potentially deleterious [32]. It has also been reported that environmental risk factors such as smoking, significantly modifies the risk of PC development in *p16-Leiden* mutation carriers [27]. The possibility of a combination of genetic and/or environmental risk factors of PC and melanoma in familial melanoma patients can therefore not be excluded.

Combined, these data suggest that although there is variability in cancer occurrence in familial melanoma patients, we still cannot precisely predict patients at increased risk using a strong genetic marker other than a germline *CDKN2A* mutation. We may also speculate that genetic and/or environmental modifiers predicting the risk of PC and melanoma in the general population are distinct from modifiers in familial melanoma patients that are already predisposed to developing cancer. Remarkably, the reported modifier *MC1R* gene variants modify melanoma risk also in sporadic melanoma suggesting there may also be unknown common variants for familial and sporadic melanoma [13, 35]. Still, more scientific data and effort are required to uncover novel genetic and environmental modifiers that predict PC and melanoma risk in familial melanoma patients and specifically in *p16-Leiden* mutation carriers. Identification of these risk modifiers would ultimately allow clinical geneticists to come forward with a personal risk score in affected families and provide a more patient tailored surveillance program.

CDKN2A LOH is an early event in familial melanoma patients with the p16-Leiden mutation

The genetic evolution of melanocytic neoplasia in sporadic cases has been reviewed intensively with *CDKN2A* loss being shown as a significant factor in invasive melanoma stages [28, 36-38]. Bi-allelic *CDKN2A* inactivation has been reported in a small subset of dysplastic nevi in the general population but never in common melanocytic nevi [39]. Nevertheless, studies have shown *CDKN2A* LOH at the primary melanoma and metastasis stage in germline *CDKN2A* mutation carriers [40, 41]. The timing however, of *CDKN2A* wild-type allele loss in familial melanoma patients is unknown.

Our general aim in **chapter 4** was to investigate the timing of *CDKN2A* LOH in melanocytic lesions of familial melanoma patients carrying the *p16-Leiden* mutation. The application of digital PCR (dPCR) technology provided a breakthrough of absolute

quantification of allelic imbalance and mutations in tumors [42]. We provided absolute quantification data of allelic imbalance through a customized SNP-based dPCR analysis indicative of LOH [43]. This method was efficient and highly informative since our efforts to use the 19bp deletion of *p16-Leiden* as a target of amplification in Formalin Fixed Paraffin Embedded (FFPE) tissue material turned out to be troublesome due to the size difference of wild-type and mutant *CDKN2A* amplicon.

We showed for the first time, subclonal loss of wild-type *CDKN2A* in a subset of common melanocytic nevi with absence of cytonuclear or tissue architectural atypia. We further demonstrated that a higher cell fraction was affected by *CDKN2A* LOH in primary melanomas than *CDKN2A* LOH in nevi [43]. The quantitative conclusions could be drawn from extensive analysis of sensitive dPCR data. In addition, we attempted to deduce the order of genetic events in melanocytic neoplasia of familial melanoma patients through absolute quantification of the presence of $BRAF^{V600E}$ mutation, *TERT* promoter (p*TERT*) mutation and chromosome 9q loss. In nevi, we demonstrated that *CDKN2A* LOH occurred after the driver $BRAF^{V600E}$ mutation in subclones of cells, we found no mutation in p*TERT* and no disruption of chromosome 9q. In melanomas however, we showed that CDKN2A LOH was clonal to $BRAF^{V600E}$ in the tested lesions. There was also presence of p*TERT* mutation in melanomas and chromosome 9q loss (Figure 3). These data suggest genomic instability in melanomas, by additional deletions on the longer arm of chromosome 9 by using a single intronic marker within *GNAQ*. Deletions across chromosome 9 have been reported in familial and sporadic melanomas previously [44]. Frequent somatic mutations within *GNAQ* have been mainly reported for ocular/uveal melanoma but not for cutaneous melanomas [45, 46]. To investigate further the extent of deletions within chromosome 9 in familial melanoma patients, additional markers across chromosome 9q should be investigated. Still, our findings indicate that the loss of chromosome 9q could be regarded as an additional step in melanoma development.

The subclonal bi-allelic inactivation of *CDKN2A* in common nevi of *p16-Leiden* mutation carriers resembles the development of *BAP1*-inactivated melanocytic tumors in patients with *BAP1*-tumor predisposition syndrome [47]. Due to the fact that $p16^{INK4A}$ is not uniformly expressed in nevi and truncated $p16^{INK4A}$ protein encoded by mutant *CDKN2A* is recognized by most antibodies at the same level as $p16^{INK4A}$ wild-type protein, confirmation of $p16^{INK4A}$ loss was not possible at the protein level. In addition, we may also underestimate the functional inactivation of *CDKN2A* in familial melanoma since intragenic mutation and promoter hypermethylation of *CDKN2A* were not investigated in the current study.

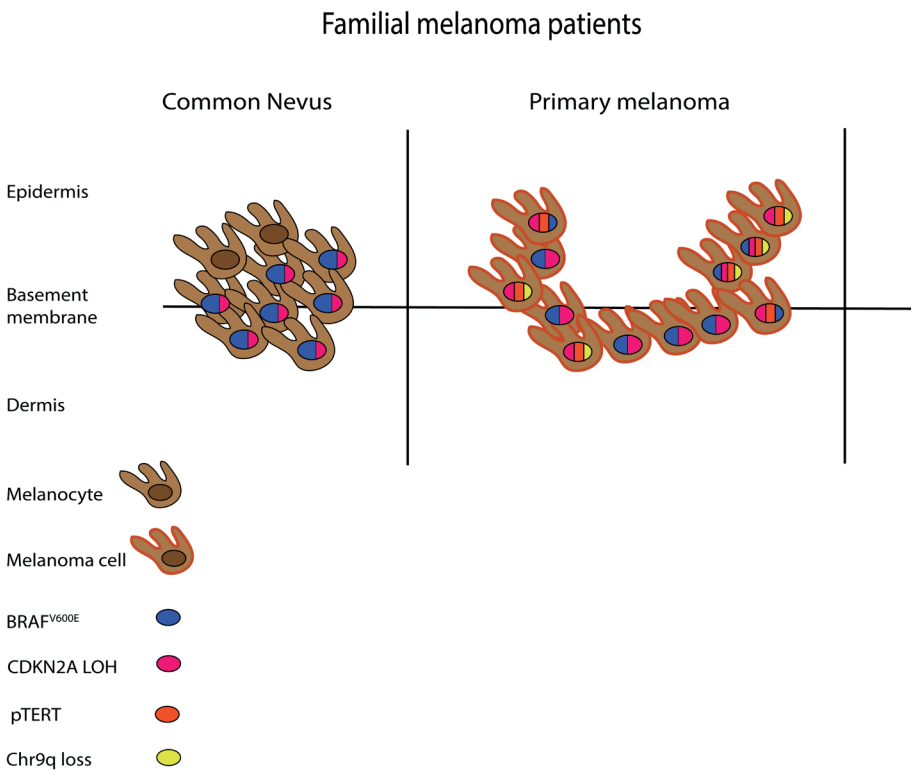


Figure 3 Proposed model of genetic evolution of melanocytic neoplasia in familial melanoma patients with the *p16-Leiden* mutation. Proposed order of genetic events based on data from chapter 4. Bi-allelic *CDKN2A* inactivation was found in subclones of common nevus cells that were already affected by the initial *BRAF*^{V600E} mutation. In melanomas, we found clonality of *BRAF*^{V600E} with *CDKN2A* loss and presence of *TERT* promoter mutations (pTERT), similar to sporadic melanoma and additional loss of chromosome 9q in sub-clones of cells. The dysplastic nevus and invasive melanoma stage requires investigation.

Our results revealed a distinct order of genetic events in familial melanoma from the genetic evolution of sporadic melanoma, precisely involving bi-allelic loss of *CDKN2A* in early precursor lesions of common nevi (Figure 3). The intermediate stage of dysplastic nevus and invasive stage of melanoma requires further investigation to have a complete picture about the order of genetic events in familial melanoma. This however, was not the initial scope of our analysis; we chose not to include dysplastic nevi due to the difficulty to distinguish from early-stage melanoma. Invasive melanoma lesions were not available for analysis from familial melanoma patients with the *p16-Leiden* mutation. With regards to *TERT* promoter mutation, in our study it was only found in primary melanomas although the possibility that it is present in precursor lesions cannot be excluded [38, 48]. The additional somatic mutations found in sporadic melanoma development such as *NRAS, NF1, PTEN* and *TP53* were not investigated here. Our analysis

was restricted to *CDKN2A*, *BRAF* and *TERT* due to limited DNA available from FFPE tissue. Fresh-frozen material could be ideal in this type of investigation although our SNP-based dPCR technique was effective and informative in FFPE material.

Combined, we showed that the highly quantitative and robust application of dPCR could be used to deduce the order of genetic events in melanoma and possibly for other tumor types in future studies through quantification of allelic imbalance. Although bi-allelic *CDKN2A* loss cannot distinguish common nevi from melanomas in this specific cohort of familial melanoma patients, our data suggest that presence of *pTERT* mutation and 9q loss could serve as diagnostic markers distinguishing melanomas from common nevi in *CDKN2A* mutation carriers. We may also speculate that subclones of nevi with bi-allelic *CDKN2A* loss are prone to progress to melanoma.

IDENTIFICATION OF FITNESS GENES IN MELANOMA

In previous chapters we have studied intensively the genetic dependencies in familial melanoma. In a collaboration with the Welcome Trust Sanger Institute (Hinxton, United Kingdom) we have explored the genetic dependencies in sporadic melanoma development. Even though high-penetrance genes may explain the germline variation in familial cases, in sporadic cases there is a different set of important genetic dependencies, known as fitness genes, that have an effect on cancer cell growth and could potentially be used as therapeutic targets [49]. Despite the significant effort in developing novel treatment strategies for melanoma, most advanced cases show relapse upon treatment [50, 51].The application of CRISPR-Cas9 screening technology has been a successful tool in identifying novel targets of therapy by high precision and limited off-target effects compared to RNA interference and previously used methodology [52-54].

In **chapter 5**, we aimed to analyze and process available CRISPR-Cas9 screening data to identify novel fitness or essential genes in melanoma that may provide possible alternative pathways to melanoma treatment. In this instance, we have analyzed fitness scores known as scaled Bayesian factors available from a negative selection screen performed by the Broad Institute [55]. The purpose of negative selection screens is to identify targets with a stimulatory effect on cell growth and survival [56]. Our data and analysis was based on scaled Bayesian factors from a total of 342 cancer cell-lines, 28 of which were melanoma cell-lines. To identify targets specific for the melanoma sub-group we compared the scaled Bayesian factors of melanoma to the other cancer types including breast, lung, central nervous system, prostate and others. A more positive scaled Bayesian factor indicates higher confidence that a given gene's knock-out causes a decrease in fitness but does not necessarily depict the severity of the phenotype [57]. Our analysis resulted in 33 genes that were significantly depleted in the melanoma cell lines but not in the other cancer types. Those were centered around three melanocytic/melanoma-specific clusters of genes confirming the specificity of our analysis. One cluster involved the known fitness genes for melanocytic lineage such as *MITF* and *SOX10*. A second group was centered around p53 responses to DNA damage with fitness genes such as *MDM2*. Thirdly, melanoma-specific essential genes encoding for MAPK signaling pathway components such as *BRAF* and *MAPK1* were among the most significant hits further supporting the sensitivity of our CRISPR-Cas9 screening data. Among the significant hits, we did not only find known fitness genes for melanoma but also genes reported in general cancer-related pathways such as *FERMT2* and *AHR* [50, 58]. Remarkably, inhibitory components of the MAPK pathway were identified to be significant fitness genes in melanoma such as *DUSP4*, *PPP2R2A* and *PEA15* [59-62]. Combined, our analysis of available CRISPR-Cas9 screening data provided a robust and sensitive output specific for melanoma dependencies.

Depletion of the MAPK-pathway negative feedback loop contributes to loss of viability in melanoma

To validate our comprehensive analysis of CRISPR-Cas9 screening data we performed genetic depletion *in vitro*, using two cell lines that were part of the initial screen and one independent melanoma cell line. From our list of 33 significant genes, we selected those with the highest effect size (*p*-value, scaled Bayesian factor) but also functional significance and pathway enrichment. We tested the effect on cell viability upon genetic depletion using two independent sgRNAs and specifically focused on two candidate genes that encode for components of the MAPK pathway negative feedback loop, *PPP2R2A* and *DUSP4*.

The MAPK pathway is subjected to a number of negative feedback loops. These include direct phosphorylation of upstream components such as MEK1/2, RAF, SOS by ERK1/2 but also feedback regulators that inhibit ERK1/2 such as DUSP4 and Sprouty proteins [63]. PPP2R2A has pleiotropic functions including regulation of ERK1/2 levels but also DNA repair response [61, 64, 65]. Our results showed that genetic depletion of *PPP2R2A* and *DUSP4* had a pronounced effect on cell viability in melanoma cell lines. Combined, these data show that the CRISPR-Cas9 screen data could be validated through decreased cell viability caused by depletion of regulators of the MAPK pathway negative feedback loop (Figure 4). We also uncover *PPP2R2A* and *DUSP4* as novel genetic dependencies suggesting that our list of 33 significant fitness genes is valid not only according to bioinformatic data and analyses but also due to functional assays.

Genetic inhibition, using sgRNAs is a strong and precise method to ensure efficient knock-out of a target of interest with low off-target effects and may provide novel genetic vulnerabilities in melanoma [53]. The exact mechanism of depletion however, requires further investigation to provide evidence on functional significance, i.e through the effect on regulation of downstream targets (ERK1/2 is a downstream target for DUSP4) (Figure 4). An important remark is to test the effect of *PPP2R2A* and *DUSP4* depletion on a *BRAF* inhibitor resistant background to confirm the significance of candidate hits as novel alternative therapeutic targets.

The results of this CRISPR-Cas9 screen analysis may be applied in different mutation backgrounds including *NRAS* and *BRAF*. Interestingly, *PPP2R2A* and *DUSP4* were significant fitness genes in two *NRAS* mutant cell lines that were included in the CRISPR-Cas9 screen, 21 *BRAF* mutant and one *NF1* mutant. This suggests that *PPP2R2A* and *DUSP4* may be fitness genes in an *NRAS*-mutant background although loss of viability has been confirmed only in *BRAF* mutant cell lines. Our effort to perform additional analysis for identifying fitness genes specific for an *NRAS* mutant background was restricted by the low statistical power of the different groups, therefore we could not make statistically significant

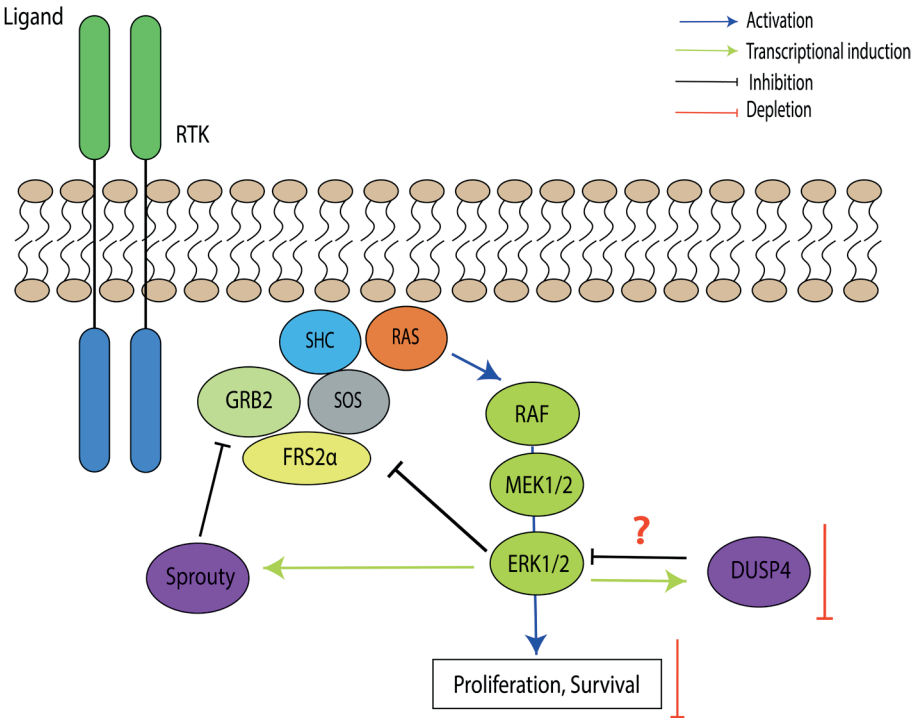


Figure 4 Depletion of MAPK-pathway negative feedback loop contributes to loss of viability in melanoma. Adapted model of negative feedback regulation of the MAPK pathway [63] . In chapter 5 we analyzed CRISPR-Cas9 screen data and identified *DUSP4* and *PPP2R2A* as significant fitness genes in melanoma. Depletion of *DUSP4* and *PPP2R2A* resulted in significant loss of cell viability although the mechanism of depletion has not been studied yet. Here, we show a proposed example of the mechanism of depletion of the MAPK-pathway negative feedback loop components such as DUSP4, that may result in hyperactivation of ERK1/2 and contribute to loss of viability in melanoma cells.

conclusions. The inclusion of more melanoma cell-lines, preferably *NRAS* mutant, since these tumors have high metastatic and resistance rates, would ultimately confirm the importance of candidate fitness genes identified here in patients showing resistance to MEK inhibitor therapy. A recent study using genome-wide CRISPR screen data identified *FBXO42* to be involved in resistance towards MEK inhibition in *NRAS* mutant melanoma [66]. Moreover, the comparison of CRISPR-Cas9 screening data of fitness genes between melanoma and melanocytes, instead of cancer cell lines from different tissue types, would enable us to eliminate lineage-specific dependencies. Future studies should also be implementing mouse models to test whether knocking out essential genes mediates tumor reduction *in-vivo* with limited side-effects. Combined, our extensive analysis and validation of CRISPR-Cas9 screen data uncovered two negative regulators of the MAPK pathway, PPP2R2A and DUSP4 as novel dependencies in melanoma although the mechanism of depletion requires further investigation.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

This thesis aimed at uncovering novel genetic dependencies in familial and sporadic melanoma. Due to the high percentage (50%) of un-explained germline variation in familial predisposition to melanoma, we sought to identify novel high penetrance melanoma susceptibility genes. In **chapter 2** we applied WES analysis in a Dutch family with melanoma and uncovered a novel germline nonsense variant in the checkpoint regulatory gene *NEK11*. We confirmed LOH in the melanoma tumor of a mutation carrier and represent a potential loss-of-function mutation through protein instability. Future studies should aim in confirming the importance of *NEK11* as a candidate high-penetrance melanoma susceptibility gene in melanoma families world-wide and ultimately include *NEK11* in clinical genetic testing to improve patients' surveillance. In **chapter 3** we searched for genetic modifiers predicting the risk of PC and melanoma in *p16-Leiden* carriers and found no significant association of a multi-cancer risk locus within *TERT/CLPTM1L* and PC development. A significant negative association was observed with melanoma development, however there was an influence of ascertainment in our sample group and statistical significance was lost. Combined, these data suggest that genetic modifiers predicting the risk of PC and melanoma in familial melanoma patients with the *p16-Leiden* mutation remain to be determined. This information is important for melanoma and PC-prone *p16-Leiden* families to precisely predict individual's personal risk of cancer development and possibly come forward with more patient tailored surveillance programs. Moreover, in the same cohort of familial melanoma patients, we investigated the sequence of *CDKN2A* inactivation events using FFPE-derived melanocytic lesions. In **chapter 4**, we show for the first time *CDKN2A* LOH as an early event in common melanocytic nevi via the application of SNP-based dPCR technology to precisely quantify allelic imbalance. We further show that while in nevi *CDKN2A* LOH occurs after the driver $BRAF^{V600E}$ mutation, in melanomas there is clonality between *CDKN2A* LOH with $BRAF^{V600E}$ mutation. Additional genetic alterations including presence of p*TERT* mutation and chromosome 9q loss were found only in melanoma lesions suggesting that these events could serve as markers for diagnosing melanoma in the tested cohort of familial melanoma patients. Remarkably, our data support that the sequence of *CDKN2A* inactivation in familial melanoma is distinct from melanocytic neoplasia in sporadic cases. The possibility of complete chromosome 9 loss in melanomas also deserves further investigation to better understand the sequence of events in the genomic evolution of hereditary melanoma. Nevi with bi-allelic *CDKN2A* inactivation in familial melanoma patients may be more prone to progress into melanomas. Our data suggest that *p16-Leiden* carriers may undergo stricter surveillance programs starting from their benign nevi and not even atypical or dysplastic nevi. Our quantitative data of allelic imbalance may have a wider application in determining the genomic evolution of melanoma

but also of other tumor types in future studies. Lastly, in **chapter 5** we explicitly focused on the identification of novel genetic vulnerabilities in melanoma development through the analysis of genome-wide CRISPR-Cas9 screening technology. Our comprehensive analysis identified 33 significant fitness genes specific for melanoma and not for other cancer types. Interestingly, among those hits we have identified regulators of the MAPK pathway negative feedback loop. Functional validation analysis in melanoma cell lines confirmed that genetic depletion of *PPP2R2A* and *DUSP4* induced a pronounced effect on melanoma cell viability suggesting that CRISPR-Cas9 screening technology and data may uncover novel fitness genes in melanoma. The identification of MAPK pathway negative feedback loop as a novel vulnerability in melanoma has a major clinical implication as a potential therapeutic target. Future studies are needed to validate further the precise mechanism of these genetic vulnerabilities in suppressing metastatic melanoma growth and possibly limiting tumor relapse.

Combined, we have investigated thoroughly the genetic dependencies in familial and sporadic melanoma development and propose future studies that may ultimately improve clinical management and surveillance of melanoma patients.

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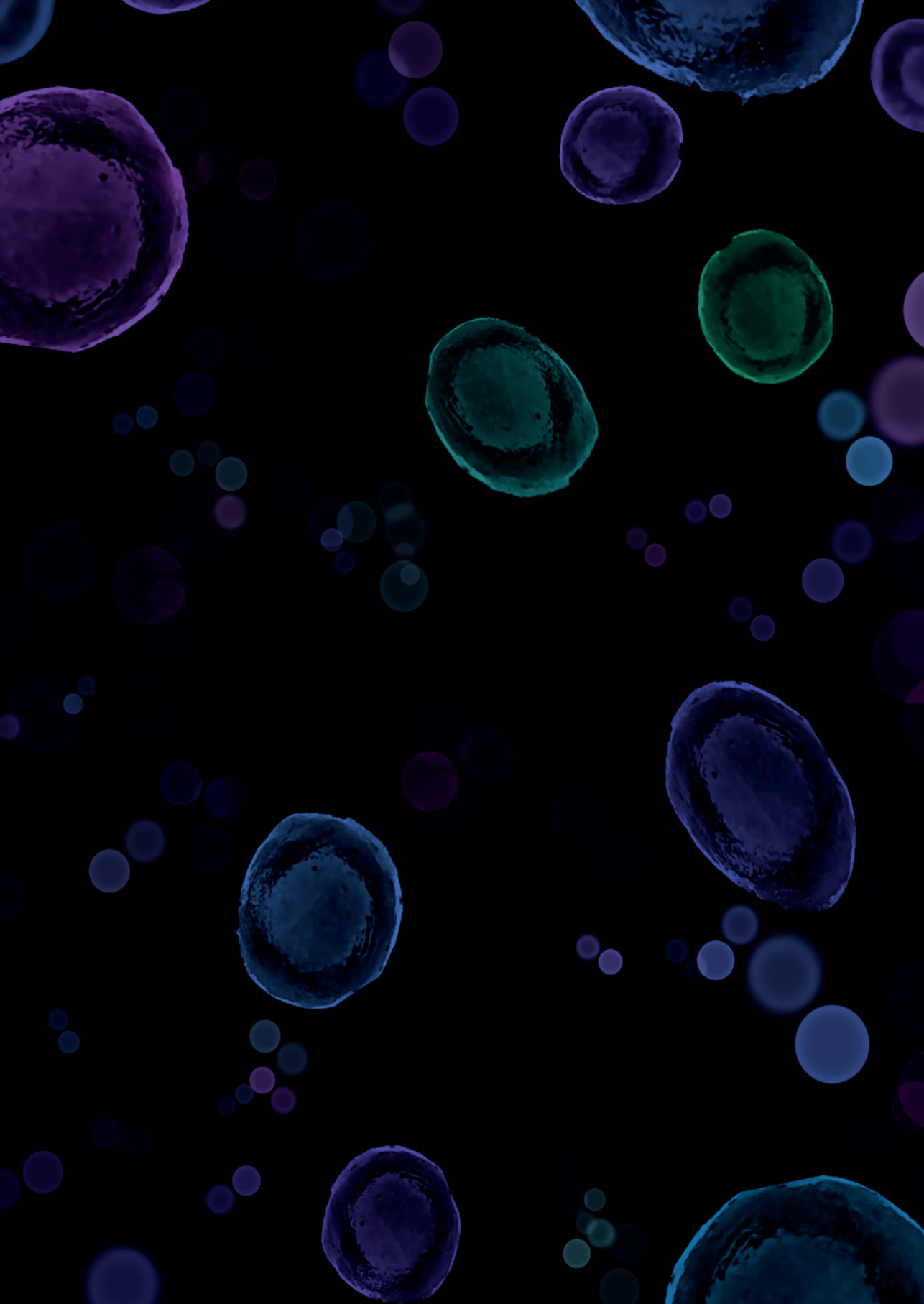

APPENDIX

Nederlandse samenvatting

Cutaan melanoom (CM) ontwikkelt zich door maligne ontaarding van melanocyten, de pigment-producerende cellen van de huid. CM is een van de dodelijkste vormen van huidkanker vanwege het vermogen van tumorcellen om uit te zaaien. Er worden naar schatting wereldwijd 232.100 gevallen per jaar gediagnosticeerd en er zijn ongeveer 55 500 sterfgevallen. Hoofdstuk 1 van dit proefschrift geeft een algemene introductie over de verschillende aspecten van genetische predispositie bij familiair melanoom en verkregen genetische veranderingen die ten grondslag liggen aan de ontwikkeling van melanoom.

Het voorkomen van melanoom bij familieleden is geassocieerd met verhoogd individueel risico op het ontwikkelen van deze vorm van huidkanker. Bij ongeveer 10% van de patiënten met melanoom zijn ook familieleden aangedaan. Er wordt van familiair (of erfelijk) melanoom gesproken bij voorkomen van tenminste drie gevallen van melanoom in een familie waarvan twee of meer bij eerstegraads verwanten. In de jaren 90 werden erfelijke veranderingen in het *CDKN2A* gen geïdentificeerd als oorzaak van familiale clustering van melanoom. In Nederlandse families met melanoom betreft het doorgaans een specifieke 'founder' mutatie in *CDKN2A* , de *p16-Leiden* mutatie. Sinds de identificatie van *CDKN2A* zijn andere genen gerapporteerd als melanoom risico genen als oorzaak van familiair voorkomen, waaronder *CDK4, BAP1, MITF, TERT, POT1, ACD, TERF2IP, GOLM1, EBF3, POLE* en *TP53AIP1*. Erfelijke veranderingen in *CDKN2A* en deze andere genen verklaren tezamen minder dan de helft van de familiair melanoom gevallen; in meer dan 50% van de patienten met familiair melanoom is de oorzaak onbekend.

In hoofdstuk 2 hebben we getracht de genetische basis van familiair melanoom te verhelderen door een erfelijke verandering in het *NEK11* gene te onderzoeken. Deze erfelijke verandering was ontdekt bij alle door melanoom aangedane leden van een Nederlandse familie door toepassing van volledige exome sequencing (WES) -analyse. We toonden somatisch verlies van het wildtype allel (LOH) in het melanoom van een *NEK11*-mutatiedrager aan. Met behulp van experimenten met gekweekte cellen konden we aantonen dat de erfelijke verandering in het *NEK11* gen, dat een rol speelt bij regulatie van de celcyclus, instabiliteit van het NEK11 eiwit waarvoor dit gen codeert veroorzaakt. De erfelijke verandering in het *NEK11* gen leidt tot functieverlies en dit ondersteunt de hypothese dat erfelijke veranderingen in het *NEK11* gen een oorzaak kunnen zijn van verhoogd risico op melanoom.

CDKN2A is het meest voorkomende melanoom risico gen en belangrijkste oorzaak van familiair melanoom. Dragers met erfelijke *CDKN2A* mutaties hebben een risico van 70% om gedurende het leven melanoom te ontwikkelen en daarbij een risico van 15-20% op het ontwikkelen van alvleesklierkanker (pancreascarcinoom, PC).

Interessant is dat klinische studies variabiliteit suggereren in het voorkomen van melanoom en PC binnen families, hetgeen suggereert dat modificerende factoren kunnen bijdragen aan het risico van het ontwikkelen van deze twee tumortypen bij dragers van *CDKN2A* mutaties. Bepaling van erfelijke factoren die het risico op PC en melanoom in families met *CDKN2A* mutatie moduleren zou daarom een betere identificatie mogelijk maken van patiënten met een extra verhoogd risico die baat zouden kunnen hebben bij gepersonaliseerde screening en preventie. In hoofdstuk 3 hebben we getracht erfelijke factoren te identificeren die verantwoordelijk zijn voor het PC-risico in *p16-Leidse* families. We testten een Single Nucleotide Polymorphism (SNP) gelegen in een multi-kanker risico locus, *TERT / CLPTM1L*, en vonden geen significant verband met het PC risico. Met betrekking tot melanoom vonden we wel een significant beschermend effect van de SNP, hoewel er een invloed was op de vaststelling in onze testgroep van patiënten en na correctie was de waargenomen associatie niet langer significant. Onze studie geeft aan dat meer onderzoek nodig is om erfelijke factoren die het risico op PC en melanoom modificeren bij dragers van *CDKN2A* mutaties te bepalen.

Hoewel wetenschappelijke studies hebben aangetoond dat verlies van het tweede wildtype allel bij patiënten met familiair melanoom ten gevolge van een erfelijke mutatie in het *CDKN2A* gen essentieel is voor de ontwikkeling van invasief melanoom is het niet bekend in welke fase in de ontwikkeling van melanocyt, moedervlek (nevus) naar melanoom dit optreedt. In hoofdstuk 4 hebben we *CDKN2A*-inactivatie onderzocht door verlies van heterozygositeit (LOH) bij melanocytaire nevi en melanoom van patiënten met een erfelijke CDKN2A mutatie. Toepassing van gevoelige digitale PCR (dPCR)-technologie maakte nauwkeurige kwantificering van het *CDKN2A* gen mogelijk en toonde aan dat in een aantal melanocytaire nevi het wildtype *CDKN2A*-allel verloren ging in subklonen van cellen. Dit bracht ons tot de conclusie dat bij familiair melanoom bi-allelische inactivatie van het *CDKN2A* gen al in een vroeg, pre-maligne stadium kan optreden. Dit is anders dan wat bekend is over de genomische evolutie van sporadisch (niet-familiair) melanoom. De kwantitatieve gegevens van dPCR-analyse van *CDKN2A, BRAF, TERT* en een locus op chromosoom 9q gaven ook inzicht in de volgorde waarin deze genetische verandering tijdens de vorming van melanoom optreden. Onze resultaten tonen aan dat *BRAF* mutatie optreedt vóór *CDKN2A*-verlies in het nevus stadium en verlies op chromosoom 9q later bij de ontwikkeling van melanoom. Het is aannemelijk dat melanocytaire nevi die subklonen bevatten van cellen met *CDKN2A* LOH een hoger risico hebben om zich te ontwikkelen tot melanoom.

Naast genen geassocieerd met risico om melanoom te ontwikkelen en genen die een oorzakelijke rol spelen bij de vorming van melanoom kan een andere klasse van melanoom fitness genen onderscheiden worden. Deze genen relevant voor de biologie en behandeling kunnen worden gebruikt als doelwit voor therapie en

vormen in zekere zin de Achilleshiel van tumorcellen. Identificatie is vooral van groot van belang om nieuwe aanknopingspunten voor therapie te ontwikkelen. Tegen veel van de huidige therapieen voor uitgezaaid melanoom worden deze tumoren namelijk resistent. In hoofdstuk 5 beschrijven we analyses, verricht in samenwerking met het Wellcome Trust Sanger Institute (VK), op functionele genoom-wijde CRISPR-Cas9 screen resultaten gericht op het vinden van melanoom fitness genen en daarmee kwetsbaarheden voor therapie. Eerder gerapporteerde fitnessgenen voor melanoom, zoals *SOX10*, *BRAF* en *MITF* behoorden tot de door ons geïdentificeerde genen; dit suggereert dat de resultaten van deze screen analyse specifiek zijn voor melanoom. Opvallend was dat inactivatie van genen die coderen voor eiwitten die het MAPK signaaltransductiepad remmen, zoals *DUSP4* en *PPP2R2A*, de celdeling van melanoomcellen blokkeerden. Dit kan erop wijzen dat deze fosfatase eiwitten geschikte doelen voor therapie van melanoom zouden kunnen zijn.

In hoofdstuk 6 bespreken we de bevindingen van dit proefschrift, beperkingen en suggereren toekomstige richtingen. Gezamenlijk hopen we door toepassing van nieuwe genomische technieken een stap vooruit te hebben gezet in het begrijpen van verschillende genetische factoren betrokken bij het ontstaan van familiair en sporadisch melanoom.

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I cannot conclude without taking a moment to appreciate my mother, father and brother - I know how sorely you've missed me throughout the years and despite what it costs you, you are always giving me strength to carry on in the pursuit of my dreams. Your inexhaustible support lends me wings to rise ever higher. Andreas, my love, it is with great pleasure I leave you last. The miles laid between us forged the strongest of bonds. You have been my incentive and encouragement to perseverance, and success. Your patience, joyful aura and ingenuity has ardently brightened my life and made this journey a phenomenal blessing- A delightful adventure that would never be the same without you.

To you all, and for all whom I have not mentioned by name (you know who you are!): My enduring wish is that your future endeavors leave you feeling the gratitude and pride I feel towards you and I really hope to have enriched your lives as you have mine!

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Christodoulou, E., Visser, M., Potjer, T., van der Stoep, N., Rodríguez-Girondo, M., van Doorn, R. and Gruis, N., 2019. Assessing a single SNP located at TERT/CLPTM1L multi-cancer risk region as a genetic modifier for risk of pancreatic cancer and melanoma in Dutch CDKN2A mutation carriers. *Familial Cancer*, **18**(4), pp.439-444.

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Curriculum Vitae

Eirini Christodoulou has a Greek-Cypriot nationality and was born in 1993. She has always been eager to learn, participate in academic and social activities and always with one motive: her passion for science. Her journey in science and scientific research began with her entry at Swansea University (UK) in 2011 as an undergraduate in biosciences. A significant lifetime experience was her election as a president of Swansea University's dance society with more than 400 members during her final year in Swansea in 2014. Her aim was to organize as many charity events as possible and this was achieved by participating in the Children in Need week, whereby the society offered donations to the Children Charity of Wales as well as the end of year show which was dedicated to the Cyprus Anticancer Society, a charity organization dear to Eirini and her mother.

Her enthusiasm and passion for cancer research was then taken to the next level by completing a postgraduate master's in cancer research and molecular biomedicine in 2015 at the University of Manchester (UK). As a high-achieving individual, intrigued by academia, she has always been willing to challenge herself further. The opportunity introduced itself by being selected for the European Horizon 2020 grant of Marie Skłodowska Curie Early Training Network (ETN) in 2016, which enabled her to then pursue a PhD. She was selected among hundreds of applicants around the world to be a PhD candidate based in Leiden University Medical Centre (LUMC) in the Netherlands as a Marie Skłodowska Curie fellow of the MelGen training network, focusing on genetic dependencies in hereditary and sporadic melanoma. Through this intense program, she had the opportunity to undertake her secondment at the Wellcome Trust Sanger Institute in Cambridge (UK) whereby she focused on exploring genetic vulnerabilities in melanoma.

Eirini is now accepted as a post-doctoral research fellow in the division of genomic medicine in the department of pathology & laboratory medicine at the Children's Hospital of Los Angeles (CHLA), a position funded by the Keck School of medicine of the University of Southern California (USC). Her research will be focused on the identification of genes responsible for the development of pediatric solid tumors.

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Appendix | Curriculum Vitae