# Genetic dependencies in hereditary and sporadic melanoma

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# 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.

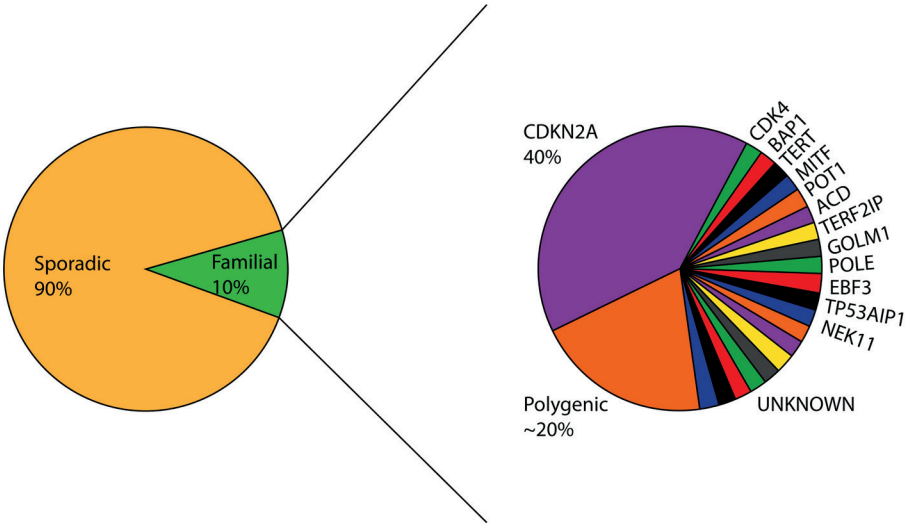


**Figure 1 Updated diagram of candidate high penetrance melanoma susceptibility genes.** The diagram is adapted using data from chapter 2 to show the small contribution of the identification of *NEK11* as a candidate high penetrance melanoma susceptibility gene

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.

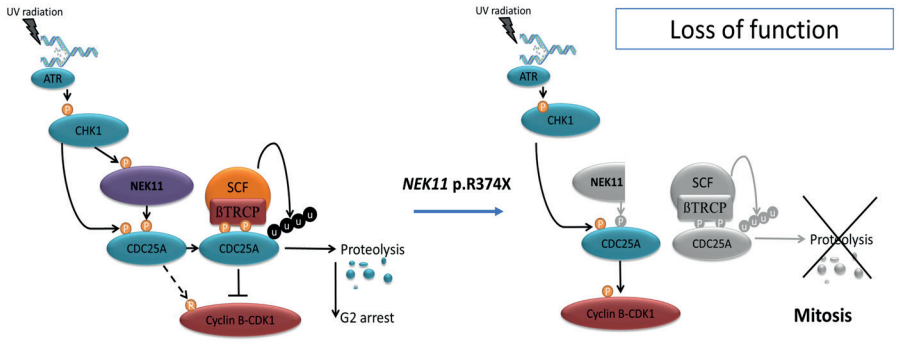


**Figure 2 Proposed model of NEK11 truncating variant function in melanoma development.** NEK11 is an essential component of the G2/M checkpoint arrest pathway. Following DNA damage, CHK1 is activated through ATR kinases, leading to phosphorylation and activation of NEK11 which in turn leads to degradation 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.

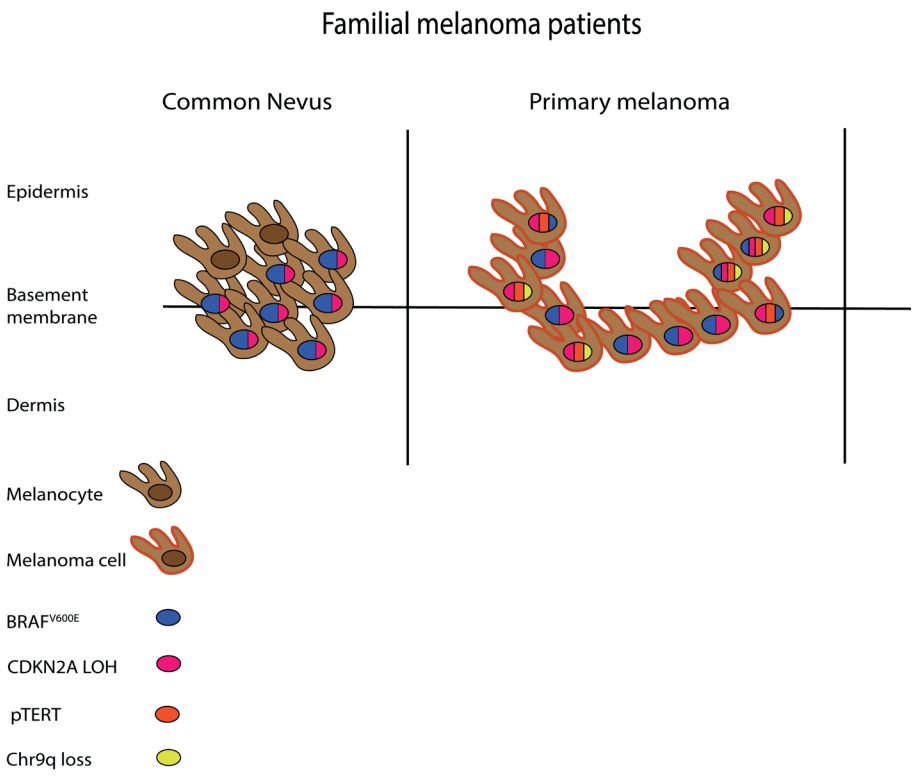


**Figure 3 Proposed model of genetic evolution of melanocytic neoplasia in familial melanoma patients with the *p16-Leiden* mutation.** Proposed order of genetic events based on data from chapter 4. Bi-allelic *CDKN2A* inactivation was found in subclones of common nevus cells that were already affected 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

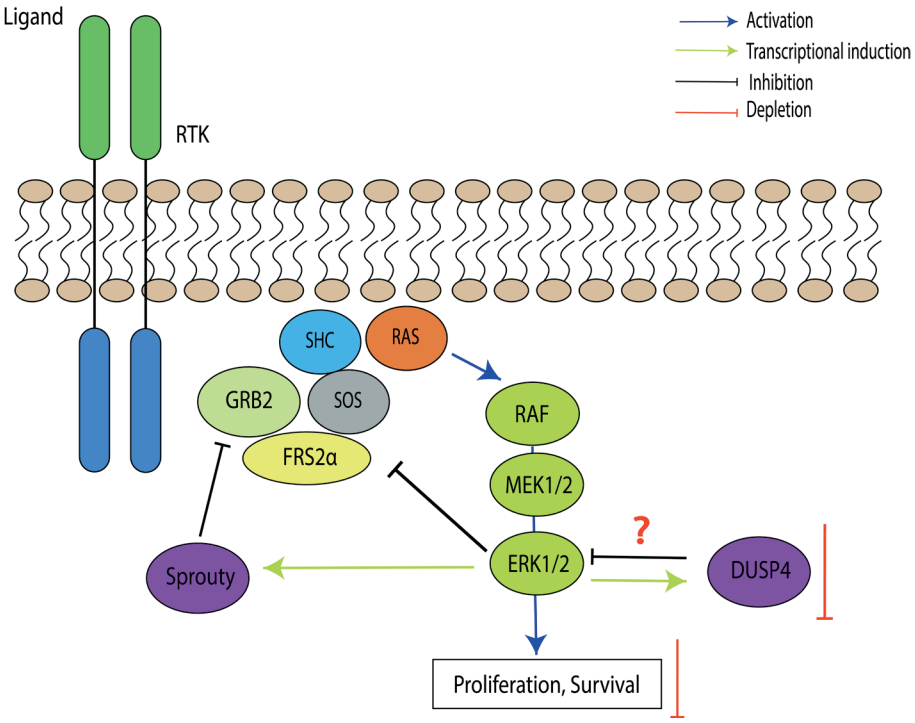


**Figure 4 Depletion of MAPK-pathway negative feedback loop contributes to loss of viability in melanoma.** Adapted model of negative feedback regulation of the MAPK pathway [63] . In chapter 5 we analyzed CRISPR-Cas9 screen data and identified *DUSP4* and *PPP2R2A* as significant fitness genes in melanoma. 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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