Thesis for doctoral degree (Ph.D.)
2021

Molecular biomarkers in melanoma susceptibility and therapy response

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MOLECULAR BIOMARKERS IN MELANOMA SUSCEPTIBILITY AND THERAPY RESPONSE

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Karolinska Institutet

Stockholm 2021

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Published by Karolinska Institutet.
Printed by Universitetsservice US-AB, 2021

ISBN 978-91-8016-407-8
Cover illustration: *Shades*, 2021. By Hai-Yuan Yu, my cousin who inspired me endlessly with his dedication and passion in art.

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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Tuesday, December 21, 2021, 9:00 am

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"Tho' much is taken, much abides; and tho'
We are not now that strength which in old days
Moved earth and heaven, that which we are, we are;
One equal temper of heroic hearts,
Made weak by time and fate, but strong in will
To strive, to seek, to find, and not to yield."
By Alfred Lord Tennyson, *Ulysses*

To my family

POPULAR SCIENCE SUMMARY OF THE THESIS

Melanoma at an early stage can often be cured by surgery. If the disease spreads to other organs, it is one of the most aggressive forms of malignant tumors. The best strategy for improving the clinical outcome of melanoma patients, is to detect it at an early stage. Sweden has implemented a national preventive program that facilitates melanoma early detection by offering genetic screening to individuals of increased risks, mainly based on their family history. These efforts have already led to the discovery of abnormal genetic changes (i.e., mutations or genetic variants) that are either distinct or prevalent in the Swedish population, and are still uncovering new abnormalities such as the genes described in Paper I. During the last decade, the prognosis of advanced-stage melanoma patients has been vastly improved, through the discovery of drugs that block the activity of *BRAF* mutants, present in approximately 50% of cutaneous melanoma (e.g., BRAF inhibitors), and drugs that invigorate patients' own immune system by blocking the molecules of immune suppression (e.g., PD-1 inhibitors). Despite that many patients have long-term benefit from the treatment, a subset of patients unfortunately does not respond or cease to respond to the therapy after a short time (due to the so-called therapy resistance). Therefore, extensive efforts have been put into finding the biomarkers that can predict durable therapy response. As described in paper II, we evaluated the response rate in carriers of gene variants in one of the most prevalent melanoma gene in Sweden and compared it with the non-carriers. Throughout the years of genetic screening, scientists would often discover previously unknown genetic variation. When these genetic variants show clear indication of family clusters or disruption in the function of genes (based on computational algorithms), as is described in paper III, scientists would set out to design experiments by cloning the mutations of interest and study their biological function using cells or animal models. Animal models are only implemented out of absolute necessity and always follow the protocol of careful ethical evaluations. In paper IV, we have used the latest technology to compare the protein expression in a previously validated cell line model of BRAF inhibitor resistance, in search of biomarkers that could be targeted in combination with BRAF inhibitors. Altogether, this thesis includes four studies that examine the prevention and treatment of melanoma by applying a wide range of biotechnologies on patients-derived materials.

ABSTRACT

Melanoma accounts for the majority of the mortality from all types of skin cancers. Five to ten percent of the cases are familial melanoma, yet more than 50% of the melanoma families cannot be explained by established susceptibility genes. By understanding the genetics behind unexplained families may shed lights on melanoma etiology and in turn provide these susceptible individuals with opportunities of more precise medical care. Over the last decade, treatment options for melanoma patients have expanded with the introduction of targeted therapies and immunotherapies. For the melanoma patients with advanced disease whose tumors harbor *BRAF* mutations, targeted therapy (TT) of BRAF-inhibitors (BRAFi) in combination with MEK-inhibitors have shown promising results with improved clinical outcome. However, acquired resistance is common among these patients. Immunotherapy with immune checkpoint inhibitors (ICIs) have also been proven successful in melanoma. Yet a subset of patients shows intrinsic or acquired resistance and benefits little from ICIs. There is an urgent need of predictive biomarkers to identify patients who will benefit from a durable response to TT or ICIs. In this thesis, we first describe a targeted sequencing project in which we performed massive parallel sequencing of a selected 120 genes in the germline DNA of 92 melanoma patients with family history or multiple primary melanomas. We reported a rare nonsense variant in *BRCA2* and showed moderately increased risk for melanoma according to our case-control analysis. Moreover, we identified another rare variant in the *BRIP1*gene that segregated with the disease in one family, but the variant did not seem to alter the expression or subcellular location of BRIP protein. In paper II, we genotyped the *MC1R* gene for 103 melanoma patients receiving ICIs and compared the characteristics and prognosis of those carrying ≥1 *R* alleles and those not. Our data showed that patients with ≥1 *R* alleles had more favorable progression free survival (PFS) after ICIs, but no significant difference in overall survival (OS). Multivariate analyses suggested that the superior PFS in "≥1 *R* alleles" group was attributed to more than the previously anticipated *R* allele-associated pigmentation phenotype. This study was the first to assess the impact of germline *MC1R* variants on the efficacy of ICIs in melanoma. In paper III, we performed whole-exome sequencing on multiple cases from a melanoma-prone family and identified a germline, heterozygous, frameshift variant in *DENND5A* gene. We were able to report two additional rare variants in two separate melanoma-prone families segregating with the disease. Our follow-up functional study discovered that DENND5A was enriched in pigmented tissue. Impaired DENND5A function led to mis-trafficking of melanosomal cargo protein and thereafter reduction of melanin content *in vitro* and in zebrafish. Altogether, paper III uncovered a novel biological function of *DENND5A* in melanoma and linked its loss-of-function variant to melanoma susceptibility. In paper IV, we revisited our previously published quantitative proteome profiling on vemurafenib-induced BRAFi-resistant subline A375VR4 and its parental A375 cell line, and identified the down-regulation of RAB7B in A375VR4. We showed that silencing of RAB7B in A375 cells led to reduced sensitivity towards BRAFi in 3D spheroids and to enhanced cell motility *in vitro* and in zebrafish. We performed RAB7B-immunoprecipitated mass spectrometry (IP-MS) and identified tyrosine kinase SRC as a novel effector of RAB7B. Silencing RAB7B in A375 spheroids led to up-regulated kinase activity reflected by increase of phospho-SRC (Tyr416), which might have contributed to the BRAFi resistance and enhanced cell motility.

LIST OF SCIENTIFIC PAPERS

I. Rainer Tuominen, Pär G Engström, Hildur Helgadottir, Hanna Eriksson, Per Unneberg, Sanela Kjellqvist, **Muyi Yang**, Diana Lindén, Daniel Edsgärd, Johan Hansson, Veronica Höiom. *The role of germline alterations in the DNA damage response genes BRIP1 and BRCA2 in melanoma susceptibility*. Genes Chromosomes Cancer. 2016;55(7):601-11

II. **Muyi Yang**, Marie Jalsenius, Fernanda Costa Svedman, Vitali Grozman, Jasmine Dahlberg, Suzanne Egyházi Brage, Veronica Höiom, Hildur Helgadottir. *Germline MC1R mutation status and efficacy of immune checkpoint inhibitor in patients with advanced melanoma.* Manuscript.

III. **Muyi Yang**, Per Johnsson, Lars Bräutigam, Xiaohong R. Yang, Kim Thrane, Jiwei Gao, Nicholas P. Tobin, Yitian Zhou, Rong Yu, Noemi Nagy, Pär G. Engström, Rainer Tuominen, Hanna Eriksson, Joakim Lundeberg, Margaret A. Tucker, Alisa M. Goldstein, Suzanne Egyházi Brage, Jian Zhao, Yihai Cao, Veronica Höiom. *Novel loss-of-function mutation in DENND5A impedes melanosomal cargo transport and predisposes to familial cutaneous melanoma*. Genetics in Medicine (In press).

IV. **Muyi Yang**, Jiwei Gao, Lars Bräutigam, Yafeng Zhu, Xingkang He, Alireza Azimi, Xiaofang Cao, Yihai Cao, Veronica Höiom, Suzanne Egyházi Brage. *Silencing of RAB7B increases migratory capacity and decreases BRAF inhibitor sensitivity in melanoma cells*. Manuscript.

SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

Soudabeh Rad Pour, Hiromasa Morikawa*, Narsis A Kiani*, **Muyi Yang***, Alireza Azimi, Gowhar Shafi, Mingmei Shang, Roland Baumgartner, Daniel F J Ketelhuth, Muhammad Anas Kamleh, Craig E Wheelock, Andreas Lundqvist, Johan Hansson, Jesper Tegnér. *Exhaustion of CD4+ T-cells mediated by the Kynurenine Pathway in Melanoma*. Sci Rep. 2019 Aug 21;9(1):12150.
*Equal contribution

CONTENTS

LIST OF ABBREVIATIONS

ASIP	Agouti signaling protein
BAP1	BRCA1-Associated Protein 1
BCL2A1	BCL2-related protein A1
BLOC	Biogenesis of lysosome-related organelles complex
CDK4	Cyclin-dependent kinase 4
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CTLA-4	cytotoxic T-Lymphocyte-associated antigen-4
DC	Dendritic cell
GNA11	G Protein Subunit Alpha 11
GNAQ	G Protein Subunit Alpha Q
GAP	GTPase-activating protein
GDF	GDI displacement factor
GDI	GDP dissociation inhibitor
GEF	Guanine nucleotide exchange factor
GGT	Geranylgeranyl transferase
GWAS	Genome-wide association study
HGF	Hepatocyte growth factor
HPS	Hermansky-Pudlak syndrome
ICIs	Immune checkpoint inhibitors
ILVs	intraluminal vesicles
IGF1R	Insulin-like growth factor 1 receptor
IPRES	Innate anti-PD-1 resistance signature
LROs	lysosome-related organelles
MAPK	Mitogen-Activated Protein Kinase
MART1	MART1, melanoma antigen recognized by T cells 1
MBAITs	BAP1-mutated atypical intradermal tumors
MC1R	Melanocortin 1 Receptor
MDSC	Myeloid derived suppressor cell
MiTF	Microphthalmia-associated transcription factor
MLPH	Melanophilin

α-MSH	α-melanocyte stimulating hormone
OCA2	Oculocutaneous albinism II
$p16^{INK4A}$	p16 inhibitor of cyclin-dependent kinase 4
$p14^{ARF}$	p14 alternate reading frame
PD-1	Programmed cell death protein 1
PDX	Patient-derived xenograft
PDGFRβ	platelet-derived growth factor receptor-β
POT1	Protection of telomeres protein 1
PV	Pathogenic variant
REP	Rab escort protein
RPE	Retinal pigment epithelium
RTK	Receptor tyrosine kinase
SNP	Single nucleotide polymorphism
TAM	Tumor-associated macrophage
TCGA	The Cancer Genome Atlas
TERT	Telomerase reverse transcriptase
TYR	Tyrosinase
TYRP1	Tyrosinase-related protein 1
NF1	Neurofibromatosis type 1
UV	Ultraviolet
WGS	Whole-genome sequencing

1 INTRODUCTION

Melanocytes are pigment-producing cells derived from neural crest (1). Melanoma is a malignancy of melanocytes and is a common cancer type worldwide, with several histopathological subtypes and varying incidence rates among different populations. Melanocytes can be found not only in skin, but also in hair follicles, uveal tract of the eye and other part of the body. Cutaneous melanoma is the most common type of melanoma among populations with fair skin, whereas acral and mucosal melanomas are most frequently found in populations from Africa and Asia (2). Melanoma etiology is complex and comprised of multiple risk factors, such as genetic predisposition and ultraviolet (UV)-exposure. Cutaneous melanomas can occur on chronically sun-exposed areas such as head and neck, and then tend to have high mutation burdens. Melanomas arising from intermittent sun-exposed sites, such as the trunk and proximal extremities, are usually present earlier in life with comparatively lower mutation burdens and predominance of *BRAF-V600E* mutation (3). Individuals carrying germline mutations in susceptibility genes, such as Cyclin-dependent kinase inhibitor 2A (*CDKN2A*) and Cyclin-dependent kinase 4 (*CDK4*), are more sensitive to UV exposure and are predisposed to melanoma. Approximately 5-10% of the cases occur in familial context (4), and of these, approximately 50% have an unknown genetic basis (3).

1.1 MELANOMA SUSCEPTIBILITY GENES AND THEIR BIOLOGICAL FUNCTIONS

1.1.1 High-risk genes

Cyclin-Dependent Kinase inhibitor 2A (*CDKN2A*)

CDKN2A gene is localized on chromosome 9p21 with four exons, due to alternative splicing, encoding two distinct tumor suppressor proteins p16 inhibitor of cyclin-dependent kinase 4 ($p16^{INK4A}$, alpha transcript exon 1 alpha) and p14 alternate reading frame ($p14^{ARF}$, beta transcript, exon 1beta) (**Figure 1A**). $p16^{INK4A}$ induces cell cycle arrest (senescence) through inhibiting cyclinD1/CDK4 complex. Once $p16^{INK4A}$ is mutated, cyclinD1/CDK4 complex is no longer inhibited and can phosphorylate Retinoblastoma-associated protein which in turn releases transcription factor E2F favoring cell cycle progression. $p14^{ARF}$ prevents ubiquitylation of p53 through interacting with E3 ubiquitin-protein ligase MDM2. Therefore, when $p14^{ARF}$ is mutated, MDM2 promotes p53 degradation, allowing mutated cells to escape p53-mediated apoptosis. To date, *CDKN2A* is the most common melanoma susceptibility gene with its pathogenic variants (PV) accounting for 20~40% of the melanoma families. Patients with germline pathogenic *CDKN2A* variants are often associated with features such as multiple primary melanomas, higher number of melanoma cases per family, as well as early onset (5, 6). Of note, *CDKN2A* germline PV carriers also have increased risks of developing other cancers than melanoma, such as Pancreatic Cancer (7, 8). Moreover, carriers of *CDKN2A* PVs carriers have significantly worse overall survival in melanoma and non-melanoma cases, with no regard of sex, age, and T classification (according to the 2009 American Joint Committee on Cancer melanoma staging and classification system)(9).

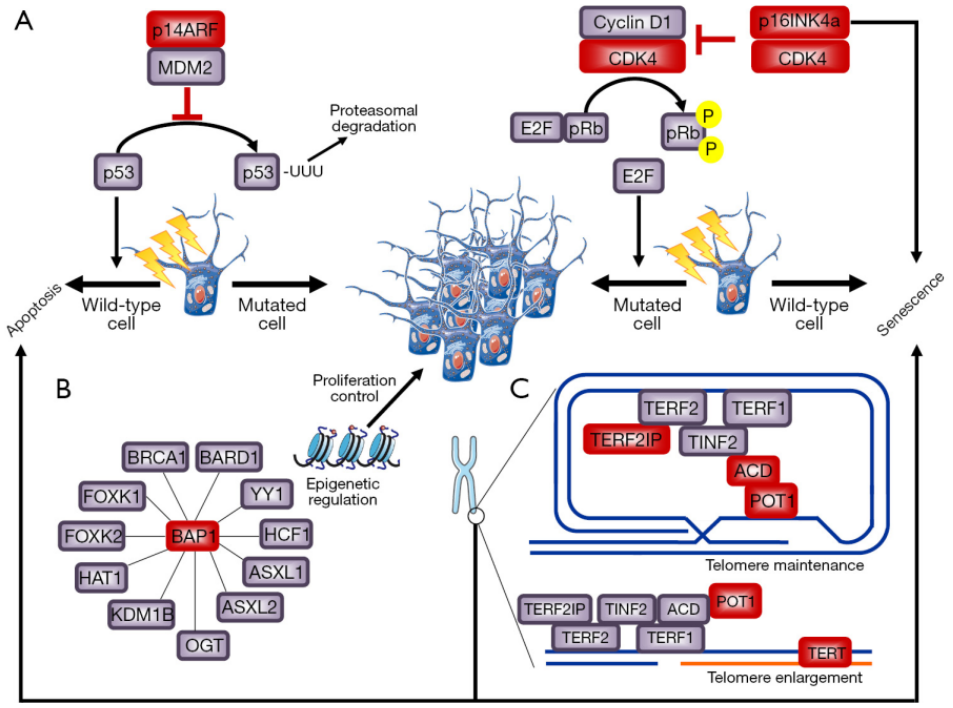


Figure 1. Biological functions of the melanoma susceptibility genes. CDKN2A, cyclin-dependent kinase inhibitor 2A; CDK4, cyclin-dependent kinase 4; BAP1, Breast cancer 1 (BRCA1) associated protein 1; POT1, protection of telomeres 1. Potrony, M. *et al*., Ann Transl Med (2015), reproduced with permission.

Cyclin-Dependent Kinase 4 (*CDK4*)

The second high risk melanoma susceptibility gene that was identified is *CDK4* on chromosome 12q14, impacting the cell cycle progression through G1 phase the same way as $p16^{INK4A}$ mentioned above (**Figure 1A**)(10). Therefore, *CDK4* mutation carriers share similar phenotype as $p16^{INK4A}$ mutated ones. *CDK4* germline PVs have been found in 18 melanoma families worldwide so far at the same loci (codon 24, exon 2), leading to amino acid change from Arginine to either Histidine (R24C) or Cysteine (R24H).

BRCA1-Associated Protein 1 (*BAP1*)

BAP1 gene on chromosome 3p21 is a tumor suppressor regulating transcription via chromatin modeling and the ubiquitin-proteasome system (11). It was initially discovered for its interaction with BRCA1. However, it was later demonstrated that BAP1 does not affect BRCA1 deubiquitylation (12), and suppresses tumor growth in a BRCA1-independent manner (13). Through its interaction with multiple partners primarily in the nucleus (**Figure 1B**), BAP1 protein, as a deubiquitinating enzyme, is involved in a broad spectrum of biological processes such as cell cycle, cell differentiation, cell death and DNA damage response. Thus, families with inherited *BAP1* PVs are often associated with multiple different cancer types, such as

cutaneous and uveal melanoma, meningioma, cholangiocarcinoma, renal cell carcinoma as well as basal cell carcinoma (14-16).

In contrast to the atypical naevi – large size, irregular border, asymmetry color – often seen in *CDKN2A* mutation carriers, the naevi phenotype of *BAP1* PV carriers are quite distinct. Melanocytic *BAP1*-mutated atypical intradermal tumors (MBAITs) are usually with pink to tan papules and nodules, symmetrical shape, and uniform color (17). Identification of these lesions are of vital diagnostic values as they often occur earlier than other BAP1-associated cancers. Of note, loss of BAP1 protein expression, validate by immunohistochemistry staining (IHC), is documented in around 5% of the sporadic cutaneous melanomas (18).

As for uveal melanoma (UM) of which only around 1% is regarded as hereditary, *BAP1* so far is the only high risk susceptibility gene found, with a frequency ranging from 1.6% to 3% depending on cohorts (19). Recent studies suggest that the inherited risk of *BAP1*-related cancers show autosomal dominant pattern. Inactivating mutations in *BAP1*, or negative IHC staining of BAP1, is associated risk of uveal melanoma metastases (20, 21). IHC staining of BAP1 is at times required for BAP1 mutation testing since BAP1 aberrations can be the result of hemizygous exonic deletions, which cannot be detected by sanger sequencing.

Telomere maintenance-related genes

Telomeres play crucial roles in maintaining genome stability and are protected by shelterin protein complex, including TERF1/2, TERF2IP, TINF2, POT1 and ACD (**Figure 1C**). Several disruptive germline variants found in genes encoding these proteins have been linked to increased risks of developing melanoma and other cancers and altogether account for approximately 1% of familial melanomas. For example, through linkage analyses and targeted sequencing, Horn et al. identified a germline mutation in telomerase reverse transcriptase (*TERT*) in one family and a large proportion of somatic mutations in TERT promoter with UV-signature (22). *POT1* (in 7q31 of the genome) encodes a protein that binds to the overhang of single-stranded DNA and therefore prevents its exposure to telomerase. Robles-Espinoza *et al*. reported 4 pedigrees with co-segregating variants in *POT1* gene (23). Shi *et al*. identified a founder mutation in *POT1* in 5 unrelated multi-case melanoma families (24). In addition, out of 510 unexplained melanoma-prone families, Aoude *et al*. discovered 6 families with *ACD* mutations and 4 with mutations in *TERF2IP*, adding further association between telomere dysfunction and melanoma susceptibility (25).

1.1.2 Low to intermediate risk genes

Medium risk genes are referring to variants that confer 2-5 times higher overall risk of melanoma. One medium risk variant alone does not drive carcinogenesis, whereas multiple medium risk variants together render carriers predispose to melanoma.

Melanocortin 1 Receptor (*MC1R*)

MC1R is a highly polymorphic gene on chromosome 16q24. It is a master gene encoding the G-protein coupled receptor on melanocytes needed for binding α-melanocyte stimulating hormone (α-MSH) secreted by neighboring keratinocytes. With fully functional MC1R, it activates downstream of cAMP signaling and the transcription of microphthalmia-associated transcription factor (MITF), which leads to further production of melanin synthesis (**Figure 2**). Matured melanin (pigmented) is to be transported in melanocyte-specific organelles called melanosomes. Melanosomes will then be transported back to the nearby keratinocytes which are closer to the surface of epidermis, serving as a shield protecting the melanocytes beneath from UV radiation. The melanin synthesized in melanosomes is a mixture of eumelanin (with brown-black color) and pheomelanin (with yellow-red color). One of the key factors of the ratio of eumelanin to pheomelanin is tyrosinase activity. Variants in *MC1R*, either affecting a α-MSH binding or cAMP signaling, will lead to insufficient production of tyrosinase (TYR) and higher ratio of pheomelanin, which is inadequate in UV protection. Several *MC1R* variants are defined as *R* variants because of their strong association with red hair phenotype (RHC). *MC1R* variants with lower associations are designated as *r*. Besides RHC, *R* variants carriers are often associated with fair skin, poor tanning ability, and of course, higher risk of developing melanoma. Studies suggest that RHC variants show additive risks (4~6 fold) when individuals carry two *R* alleles (26, 27).

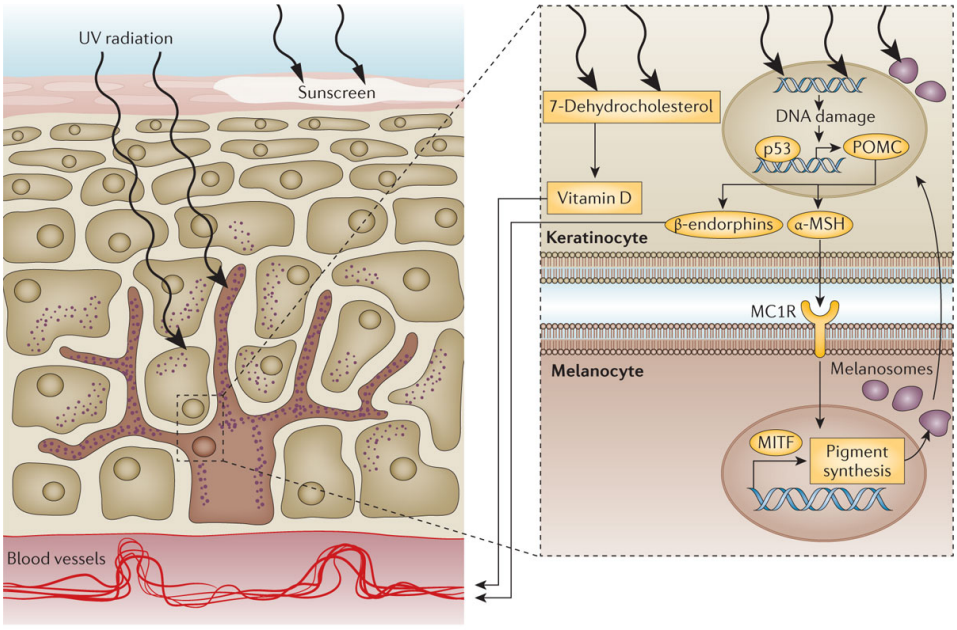


Figure 2. An illustration of UV radiation and pigmentation. POMC, pro-opio-melanocortin; α-MSH, α-melanocyte-stimulating hormone (α-MSH); MC1R, melanocortin 1 receptor; MITF, microphthalmia-associated transcription factor. Schadendorf, D. *et al*. Nat. Rev. Dis. Primers (2015), reproduced with permission.

Besides its role in pigmentation, *MC1R* polymorphisms have been demonstrated to be involved in melanocytic apoptosis and DNA repair through phosphorylating DNA repair proteins (28). Robles-Espinoza et al. found that carriers of ≥1 *R* allele have higher mutational load compared to non-carriers (29). Interestingly, increased variant count was observed in the UV-associated C>T substitutions and also the non-C>T types, the latter of which are in fact the prevalent type in melanoma hotspot mutations (30).

MiTF

MiTF is the master transcriptional factor that regulates a wide range of gene expression in melanocytes and the development of melanoma (31). Functional variant *MiTF* p.E318K is found in 1.6-2.8% of the melanoma patients and 0.6-0.8% in control cohorts (32-34). It decreases the SUMOylation of MiTF and therefore leads to up-regulation of *MiTF* transcription. It confers moderate risk of melanoma and susceptibility of renal cell carcinoma (RCC).

Low-risk genes

In addition to genes listed above, common variants that exist in general population have also been associated with less than 2-fold of increased melanoma risk and therefore counted as low-risk variants (35). These variants are found in genes of a variety of biological processes, such as 1) pigmentation (36): agouti signaling protein (*ASIP*), *TYR*, tyrosinase-related protein 1 (*TYRP1*), oculocutaneous albinism II (*OCA2*), 2) metabolism (37): glutathione transferases-coding genes (*GSTM1*). Families carrying each of these genes alone do not often have more than 2 cases, yet carrying several low or moderate risk variants may result in multiple cases, which also depend on environmental factors such as UV exposure (38).

Latest meta-analysis from genome-wide association studies (GWAS)

In 2020, Landi *et al*. has reported a meta-analysis of a GWAS study performed on 36760 melanomas (67% of which were newly diagnosed) and 375188 controls (96% were newly enrolled)(39). In this report, sixty-eight independent single nucleotide polymorphisms (SNPs), identified from 54 loci, reached statistical significance. These SNPs confer varying risks of melanoma, 1) further supported the importance of pigmentation, telomere maintenance and nevogenesis, 2) and also highlighted the distinction in acral melanoma for lacking pigmentation features. Importantly, this latest analysis has confirmed 19 out of 21 risk loci reported by previous GWAS studies (mainly on cutaneous melanomas of European ancestry)(40-46). Interestingly, in the meta-analysis, Landi *et al*. has also used a transcriptome prediction mapping strategy (TWAS) by compiling data from cell-type-specific *cis*-eQTL, tissue-based cis-eQTL and genotype-tissue expression (GTEx), which has led to the discovery of 5 genes in 4 loci, including *CBWD1*, *ZEP90*, *HEBP1* and *MSC/RP11-383H13.1*.

1.2 COMMON SOMATIC MUTATIONS AND MOLECULAR CLASSIFICATION OF MELANOMA

Melanomas, ninety percent of which are sporadic, carry the highest mutation burden of all tumors with a median number of >10 mutations per megabase (30). The genomic alterations of cutaneous melanoma have clear UV signature and are significantly different from the ones found in mucosal or acral melanomas (47). The latter types show higher percentages of structural aberrations. And in terms of the significantly mutated genes, the majority of the mutations in cutaneous melanoma comes from the component of Mitogen-Activated Protein Kinase (MAPK) cascade.

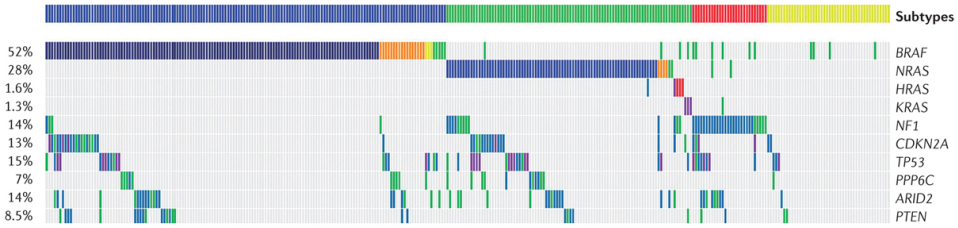


Figure 3. Frequency and overlap of alterations in driver and tumor-suppressor genes associated with melanoma. Originally from Elsevier © The Cancer Genome Atlas Network. *Cell* 161, 1681–1696 (2015). Luke, J. J. *et al*. Nat. Rev. Clin. Oncol. (2017), reproduced with permission. Subtypes: blue, *BRAF* hotspot; green, *RAS* hotspot; red, *NF1* mutant; yellow, triple wildtype (WT).

The Cancer Genome Atlas (TCGA) systematically analyzed 333 cutaneous melanoma samples and proposed to classify cutaneous melanoma into 4 different genomic subgroups, *BRAF*, *RAS* (*N/H/K*), *NF1* and triple-wildtype (**Figure 3**)(48). Six different types of global molecular analysis, covering DNA, RNA and protein level, were performed. Not surprisingly, the *BRAF* subtype was the largest with in total 166 cases (52%), 124 of them were the hotspot V600E mutation. Consistent with previous report, *BRAF* hotspot mutation V600 and K601 showed inverse correlation with *NRAS* mutations (49). RAS subtype came second biggest with 95 cases, and *NF1* was mutated in 14% of all samples. In total, 46 cases, lacking hotspot mutations in *BRAF*, *RAS* (*N/H/K*) or *NF1*, were grouped together as triple-WT subtype. Within the study, samples with more than 60% of C>T transition at dipyrimidine sites or more than 5% of CC>TT transitions were classified as harboring a UV signature. Only around 30% of the tripe-WT cases had UV signature while each of the other three subtypes had more than 90% of cases harboring UV signature. Forty-six out of 49 melanoma tumors with a detected *TP53* mutations had a UV signature. When checking other frequently mutated genes, *TP53* mutations were more common among *BRAF*, *RAS* and *NF1* cases comparing to Triple-WT. *PTEN* mutations were more frequently found in *BRAF* subtype, while *AKT3* amplification and mRNA overexpression were significantly more frequent in the other three subtypes than *BRAF*-mutant cutaneous melanomas.

The proposed classification was recently adopted by Hayward *et al*. in a whole-genome sequencing (WGS) project on 183 melanoma samples that included 35 acral and 8 mucosal melanomas (47). With this high-coverage WGS platform, it allows researchers to examine the

structural changes within the genome and to compare the landscape across different types of melanomas. In this study, *KIT* mutations were prevalent in mucosal and acral melanoma and observed in 10-20% of these subtypes. Fifty-one percent of the non-cutaneous melanomas did not harbor *BRAF*, *NRAS* or *NF1* mutations, therefore belonged to triple-WT subtype. And for the first time, *SF3B1* was identified as a significantly mutated gene in mucosal melanoma. Among all the non-coding mutations, including regulatory and untranslated regions, *TERT* promoter mutations were the most common ones, accounting for 69% of all melanomas and 86% of cutaneous melanomas, while no association was found between them and *BRAF* mutations.

Uveal melanoma (UM) is not the focus of the introduction, it is however important to note that the molecular classification of primary UM is vastly different from cutaneous melanoma. For example, 85% of the uveal melanomas have mutations in either *GNAQ* or *GNA11* genes, while only less than 1% of cutaneous melanomas harbor mutations in one of these two genes (50). Almost half of the UM patients have metastatic lesions, primarily in their livers. Consistent with the finding that *BAP1* alterations were associated with UM metastases (51), *BAP1* mutations were found in more than ninety percent of 32 metastatic UM samples (52).

1.3 MOLECULARLY TARGETED THERAPIES, IMMUNOTHERAPIES AND THERAPY RESISTANCE IN MELANOMA

The landmark TCGA study of cutaneous melanoma, and the whole-genome study on cutaneous, acral and mucosal melanomas provide comprehensive information of the genetic and molecular landscape of melanoma. The proposed types and the revealed interplay between significantly mutated genes and additional molecular aberrations have important implications on personalized therapies, especially for triple-WT cases and for overcoming therapy resistance.

In fact, treatment options for advanced-stage melanoma have evolved rapidly over the last decade, especially for *BRAF*-mutant melanomas, shifting from single-agent BRAF inhibitor to combinational therapy with a BRAF plus a MEK inhibitor. Meanwhile, the advancement of immunotherapy is revolutionizing the melanoma treatment regimens. Checkpoints inhibitors cytotoxic T-Lymphocyte-associated antigen-4 (CTLA-4) and Programmed cell death protein 1 (PD-1) have been particularly successful in melanoma for several reasons, such as the high mutation burden and tumor infiltration of T cells. With all these treatment options, the median overall survival (OS) of advanced-stage melanoma patients has increased from ~9 months to more than two years, with a group of patients still under long-term effect of the treatment. By combining evidence from comprehensive genome-wide studies and clinical trials, clinicians and researchers are exploring the mechanisms behind therapy resistance (BRAFi, BRAFi+MEKi, and immunotherapies), and identifying biomarkers to improve therapy efficacy and to predict patients' prognosis.

1.3.1 Mechanisms of resistance to BRAF inhibitors

Studies suggested that mutated-*BRAF*-activated signaling attenuates upstream receptor tyrosine kinase RTK (such as EGFR, HER2) with a negative feedback loop (**Figure 4**). BRAFi in this case will break such negative feedbacks and in turn unleash the RTK-ERK signaling and RAF dimerization (53-55). In fact, it has been confirmed that the ineffective suppression of the second-generation RAF inhibitors on dimeric RAF are among the common cause of RAF resistance.

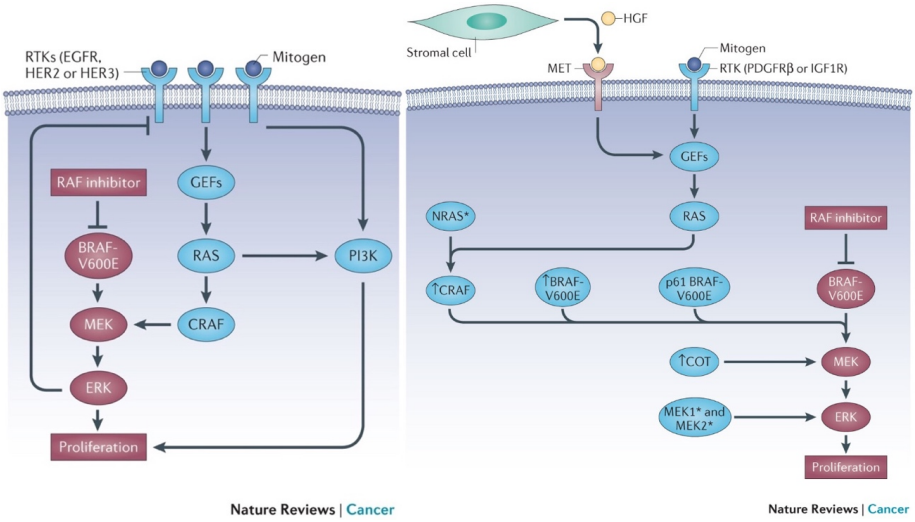


Figure 4. Left: Loss of feedback inhibition and activation of the PI3K pathway mediates resistance to BRAF-V600E inhibition. GEFs, guanine nucleotide exchange factors. Right: Additional mechanisms of resistance to BRAF-V600E inhibition that lead to reactivation of the MAPK pathway. Multiple mechanisms of acquired resistance (shown in blue) to BRAF-V600E inhibitors lead to reactivation of the MEK-ERK pathway (shown in red) in the presence of a RAF inhibitor. RTKs, receptor tyrosine kinases; PDGFRβ, platelet-derived growth factor receptor-β; IGF1R, insulin-like growth factor 1 receptor; MET, also known as hepatocyte growth factor receptor. Holderfield, M. et al., Nat Rev Cancer (2014), reproduced with permission.

There are a number of resistance mechanisms lead to re-activation of the MAPK pathway (**Figure 4**), such as secondary activating mutations in *NRAS* and *MEK* (56-58). Amplification of *BRAF*-V600E, and consequently overexpression and/or dimerization of the mutant protein, have been detected in resistant melanoma tumors (59-61). The splicing variants of *BRAF*-V600E, encoding a BRAF without its RAS-binding domain, have also been found in resistant melanoma tumors and shown to increase dimerization and kinase activity in a RAS-independent manner (62, 63).

It is suggested that ~30% of BRAFi resistance happened in MAPK-independent scenarios. Studies showed that PTEN-PI3K-AKT signaling is activated due to increased expression of platelet-derived growth factor receptor-beta (PDGFR-β) or Insulin-like growth factor 1 receptor (IGF-1R) found in patients-derived specimens (56, 64)., Besides these, stromal cells release growth factors (such as hepatocyte growth factor HGF) or cytokines can contribute to

BRAFi resistance by binding to its RTK (such as MET) on melanoma cell surface and in turn activating parallel PI3K or RAS signaling (56, 65-68). Moreover, activation of HER3 and amplification of MiTF or its downstream effector BCL2-related protein A1 (*BCL2A1*) have also been suggested to confer resistance to BRAFi or BRAFi+MEKi (69-71).

Numerous other RTKs have been demonstrated to mediate BRAFi resistance. EphA2, belonging to one of the largest family of RTKs, is over-expressed to promote cell survival in melanoma and several other cancer types (72-75). It is also over-expressed in trastuzumab-resistant breast cancer cell line and involved in BRAFi resistance partly through phosphorylation at Ser897 (76, 77). It has also been hypothesized that BRAF mutated tumor cells could sustain their cell survival through a diverse pool of RTKs (78), and therefore targeting RTKs is less efficient than the blocking molecules downstream of RTKs, such as MEK (79).

1.3.2 Mechanisms of resistance to immunotherapy

In 2015, CheckMate 067 (phase III) trial showed that anti-PD-1 alone (nivolumab) or in combination with anti-CTLA-4 (Ipilimumab), as the frontline therapy, had more favorable PFS, compared to anti-CTLA-4 alone (80). Based on data from 2016, approximately 25% of metastatic melanoma patients developed acquired resistance to immune checkpoint inhibitors (ICIs) against PD-1 or CTLA-4 (81). Several clinical trials are ongoing aimed at overcoming immunotherapy resistance (82, 83). More importantly, further studies on the mechanisms of immunotherapy resistance will identify biomarkers that distinguish advanced-stage melanoma patients and group them into different frontline therapies.

The reason why melanoma patients do not respond to immunotherapy (primary or adaptive resistance), or they respond initially but relapse (acquired resistance) can be both intrinsic and extrinsic (**Table 1**).

	Mechanism	Examples
tumor cell intrinsic	absence of antigenic proteins	low mutational burden lack of viral antigens lack of cancer-testis antigens overlapping surface proteins
	absence of antigen presentation	deletion in TAP deletion in B2M silenced HLA
	genetic T cell exclusion	MAPK oncogenic signaling stabilized b-catenin mesenchymal transcriptome oncogenic PD-L1 expression
	insensibility to T cells	mutations in interferon gamma pathway signaling
tumor cell extrinsic	absence of T cells	lack of T cells with tumor antigen-specific TCRs
	inhibitory immune checkpoints	VISTA, LAG-3, TIM-3
	immunosuppressive cells	TAMs, Tregs

Table 1. Mechanisms of primary and adaptive resistance to Immunotherapy. Sharma, P. *et al*., Cell (2017), reproduced with permission.

Intrinsic mechanisms within tumor cells include (1) MAPK signaling that produces secreted proteins, such as VEGF and IL-8, which have inhibitory effects on T cell recruitment (84), (2) the stabilization of beta-catenin leading to constitutive Wnt signaling that expels CD103+ dendritic cells (DCs)(85), (3) last but not least, the continuous interferon-gamma signaling that leads to immune escape of cancer cells by tuning down downstream chain receptors JAK1/2 (86, 87). Besides these, a group of genes that are enriched in the non-responders of anti-PD-1 therapy have been identified and named innate anti-PD-1 resistance signature (IPRES)(88). This list of genes is implicated in a variety of biological processes such as stemness and mesenchymal transformation.

Extrinsic factors include other components within the tumor microenvironment than tumors cells, from Tregs, M2 macrophages as well as myeloid derived suppressor cells (MDSCs). Published data in murine models showed that an increased ratio of effector T cells to Tregs is associated with a better response to anti-CTLA-4 therapy (89, 90). Tumor-associated macrophages (TAM) have been reported to directly suppress T cell responses through PD-L1 in hepatocellular carcinoma and B7-H4 in ovarian cancer (91, 92).

So far, most of the studies on immunotherapy response have only allowed retrospective analyses on patients-derived materials. The development of preclinical models with patient-derived xenografts (PDX) in humanized mouse have offered the possibilities of testing and predicting patient-specific responses to ICIs, such as adoptive cell transfer (93) or anti-PD-1 (94).

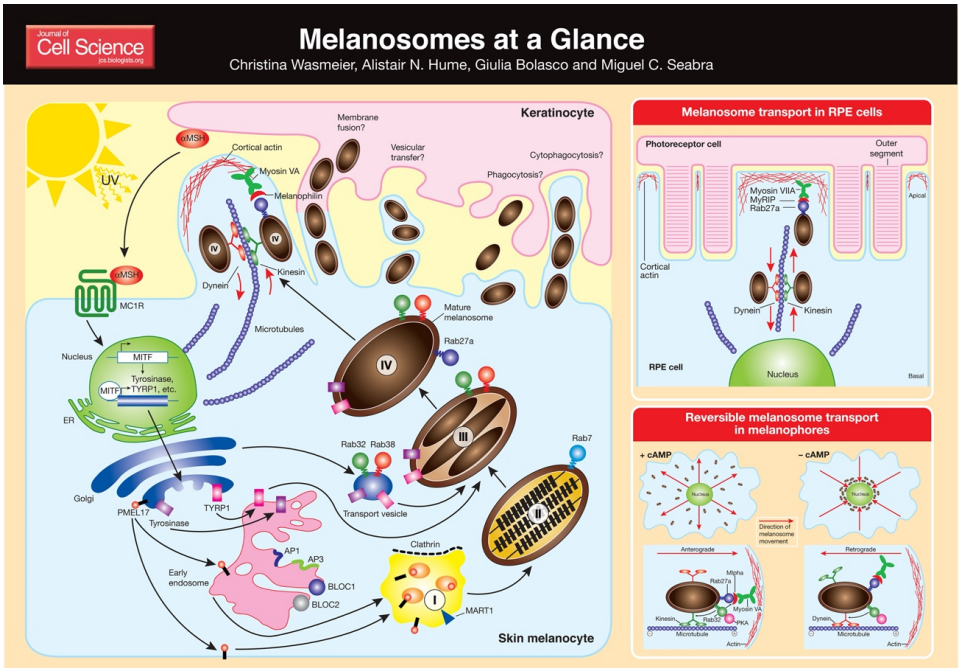
1.4 MELANOSOMES

Melanosomes are endosomal membrane-derived, melanin-containing organelles that specifically exist in the melanocytes of epidermis or retina, iris and ciliary body of the eye (95, 96). They are considered lysosome-related organelles (LROs), the biogenesis of which may resemble other cell type-specific LROs (97). There are typically two types of melanins: eumelanins (black and brown, most common) and pheomelanins (red and yellow)(98), both of which are synthesized through multi-step oxidation and polymerization, and thereafter stored in melanosomes (99). The whole process of melanosome maturation and secretion is an intricate and well-coordinated network, such as the initial segregation from conventional endolysosomal pathway and the signal transduction of melanosomal cargo transport (**Box 1**). Yet, pigmentation defects do occur, most often in hereditary scenarios (100, 101), which shed lights on the biogenesis of this complex network.

1.4.1 Melanosome maturation and secretion process

Melanosome maturation is categorized into 4 stages based on their characteristic morphological features. Stage I/II melanosomes are not pigmented. Stage I melanosomes are derived from early endosomes and show distinct intraluminal vesicles (ILVs). Melanosomes begin to form

fibrils that are visible under electron microscopy during the transition from Stage I to II. After the fibrils are completed in stage II melanosome, enzymatic melanosomal



Box 1. The relationship between melanosomes and endosomes. Abbreviations: αMSH, α-melanocyte-stimulating hormone; AP, adaptor protein; BLOC, biogenesis of lysosome-related organelles complex; cAMP, cyclic adenosine 3',5'-monophosphate; MART1, melanoma antigen recognized by T cells 1; MC1R, melanocortin 1 receptor; MITF, microphthalmia-associated transcription factor; Mlpha, melanophilin (zebrafish); RPE, retinal pigment epithelium; TYRP1, tyrosinase-related protein 1. Wasmeier, C. *et al.*, J Cell Sci (2008), reproduced with permission.

cargo proteins, tyrosinase (TYR) and tyrosinase-related protein 1 (TYRP1) are to be recruited into stage II melanosomes (the route of which are debated) and initiating the melanin synthesis which will gradually mask all structures with pigments by the end of stage IV melanosomes (102-104). Melanosome maturation relies heavily on the correct sorting of a series of integration of structural components and enzymatic activities. Most of these structural components and enzymes are melanocyte-specific. As is shown in **Box 1**, PMEL17 (also known as SILV, gp100, or referred to as PMEL) is transported to early endosomes and stage I melanosome (the exact sorting mechanism is still debated), which signals the start of this parallel route of vesicle trafficking and is thereafter largely responsible for the formation of fibrillar matrix where melanin deposits. Matured (stage IV) melanosomes of melanocytes in epidermis are transported to multiple nearby keratinocytes through their extended dendritic tips (105, 106), which is molecularly coordinated by myosin-5A-RAB27A and melanophilin (MLPH)(107). Of note, the secretion of melanosomes does not occur in retinal pigment epithelial (RPE) cells, where melanin synthesis has been completed after birth and stored intracellularly lifelong. And in contrast to the melanocytes in mammals, pigmented cells in lower vertebrates, called melanophores, can transport pigmented vacuole, from perinuclear

region to plasma membrane, rapidly and reversibly within a matter of 15 to 60 minutes, serving as an ideal model for genetic studies of melanosome dynamics and pigmentation (108). A number of vesicle trafficking proteins that are of importance in melanosomal cargo transport have been identified by genetic evidence from Hermansky-Pudlak syndrome (HPS), such as the adaptor protein AP-3 (109, 110), and three members of the biogenesis of lysosome-related organelles complex (BLOC-1, -2, -3)(111, 112). Mutations in mouse *Rab38* gene or its guanine nucleotide exchange factor *claret* would show HPS-like syndrome or pigment defects in the eyes (101, 113). It is understood that RAB32 is a homologous protein of RAB38 and could appear redundant unless RAB38 function is impaired (114).

1.4.2 Melanosomes and RAB GTPases

Melanosome transport falls into the category of vesicle trafficking, in which the family of RAB GTPases plays a central role through their interaction with various types of effector proteins.

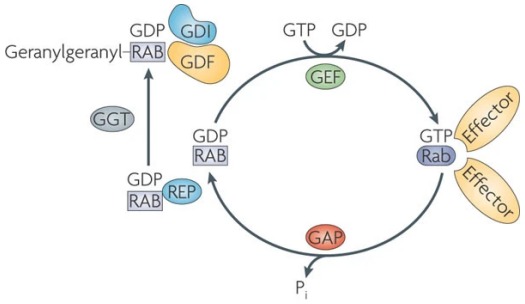


Figure 5. The Rab switch and its circuitry. GEF, guanine nucleotide exchange factor; GAP, GTPase-activating protein; REP, Rab escort protein; GGT, geranylgeranyl transferase; GDI, GDP dissociation inhibitor; GDF, GDI displacement factor.

Briefly, RAB GTPases switch between two states: the GDP-bound inactive form and the GTP-bound active form (**Figure 5**)(115). The switch involves conformational change that replies on the GDP to GTP exchange and is facilitated by relatively specific guanine nucleotide exchange factors (GEFs). While the GTP-bound RAB GTPases actively recruit effector proteins, GTPase-activating proteins (GAPs, often contain Tre-2/Bub2/Cdc16 domain) turn them back to GDP-bound inactive form through hydrolysis of GTP. Newly synthesized RABs are captured by Rab escort proteins (REPs) and then geranylgeranylated with 1-2 carboxy-terminal Cys residues by geranylgeranyl transferase (GGT). Rab GDP dissociation inhibitor (GDI) would recognize the Rabs, stabilizing the GDP-bound conformation, and transport them to specific membranes through interaction with GDI displacement factor (GDF)(116). Hirosaki *et al*. found that over-expression of dominant-negative form of RAB7 led to mis-trafficking of TYRP1 (117). Jordens *et al.* showed that RAB7 is primarily found on early-stage melanosomes where it regulates melanosomes transport along microtubule. Patwardhan *et al.* reported that RAB6 is involved in the cargo transport of melanosomal protein MART1 and tyrosinase-related protein-2 (TYRP2), and that RAB6-knockout mice has pigmentation defects (118).

2 RESEARCH AIMS

The overall aim of the thesis was to identify molecular biomarkers for melanoma susceptibility and therapy response by applying DNA sequencing or proteome profiling on patient-derived materials or *in vitro* models.

Paper I: To identify germline variants that confer moderate to high risk of cutaneous or uveal melanoma through targeted sequencing of a selected 120 candidate genes.

Paper II: To evaluate the significance of inherited *MC1R R* alleles for predicting the efficacy of ICIs in melanoma patients.

Paper III: To search for novel susceptibility genes behind melanoma-prone families; to functionally characterize the impact of unknown variants in melanoma.

Paper IV: To find molecular biomarker behind BRAFi resistance and its associated cytoskeletal changes, through immunoprecipitation-coupled mass spectrometry (IP-MS).

3 METHODOLOGICAL CONSIDERATIONS

Targeted genomic DNA sequencing (HaloPlex)

In paper I, we adopted targeted sequencing and applied it to 92 melanoma patients that were considered with inherited high risks. There were 21084 amplicons designed, covering 98% of the targets, including coding regions, exon-intron borders, and several parts of the untranslated regions (UTR) from 5' and 3'. Comparing to exome sequencing approach which could provide a broad overview of the genomic landscape and allow the researchers to explore unknown causative variants, targeted sequencing had the advantage of much deeper analyses in terms of specific regions of the genome. The targeted sequencing emphasized on finding the novel and low-frequency variants in candidate genes that were to some degree already recognized as deleterious. Those genes might have already been associated with melanoma or other cancer types, or involved in key biological processes. As for this study, targeted sequencing certainly was more appropriate, considering the genomic landscape of melanoma had already been examined by several independent resources.

However, when it came to each individual family, it became difficult to propose whether the identified variant (s) was indeed exclusively disease-causing, not a mere association, or functioned in a polygenic manner. For example, at the start of the project, genes such as *POT1*, *ACD* and *TERF2IP* were not yet identified as melanoma susceptibility genes, therefore not included in the gene panel. We therefore were not able to rule out that our patients were non-carriers of damaging variants in those genes, especially in several families where identified variants were not found in all affected members.

In one family, our pedigree analysis confirmed that a variant in *BRIP1* segregated with the disease, yet it did not change its protein expression. To pursue the impact of such variant, transcriptome profiling on the blood samples from the mutation carrier and his/her healthy relatives might be a plausible next step.

Whole exome sequencing

In paper III, we examined a multi-case melanoma family where previously known susceptibility genes were not present or segregating with the disease, we chose to use whole exome sequencing. In total, 3 melanoma patients and 1 healthy relative from this newly identified melanoma-prone family (with 4 cutaneous melanomas) were sent for whole-exome sequencing. Among a large pool of called variants, we pursued only the rare variants (minor allele frequency<1%) that were predicted to be deleterious. Yet, without functional characterization of candidate variants, it would have been difficult to establish a disease-causative role of the variant. And since it was an exome sequencing project, we could not rule out the possibility that certain intronic variants might have also contributed to the disease.

Patient-derived material

We established Epstein-Barr virus (EBV)-transformed lymphoblastoid cells (LCLs) from peripheral blood sample from one of the mutation carriers/melanoma patients in the family of interest. We subjected them to western blotting and checked the impact of one heterozygous

germline mutation on its coding protein. The method of EBV-LCLs requires fresh blood samples. In theory, it would be more informative if we could establish EBV-LCLs from fresh blood samples of all mutation carriers in the family and 1-2 blood samples from the healthy relatives. RNA or protein expression profiling on these EBV-LCLs would provide us more insightful findings that could explain the multiple melanomas found in the family. Yet this also requires extensive communications between researchers, clinicians, patients, and their healthy family members which can be challenging and regarded as excessive.

We performed spatial transcriptomics on a melanoma lymph node metastasis. The method required fresh frozen sample and enabled multiplexed *in situ* sequencing of up to 3000 transcripts per domain. In the year 2018, when spatial transcriptomics was still at its early developmental stage, the resolution was not as high and each domain represented a spot with a diameter of 100 μm and covered around 5-40 cells (119). And there has been a great effort in improving the resolution to a "single-cell" level. Spatial techniques in DNA and protein have also improved significantly, aiming at a pipeline of *in situ* analyses of DNA, RNA, and protein of the same tissue sample. However, gigantic amount of spatial information, indicating high levels of heterogeneity, raises questions from clinical researchers on how to translate these findings into clinical practice.

Zebrafish experiments

In paper III, we modulated the expression of several genes in zebrafish embryos and observed the changes in the development of pigmentation. Zebrafish is arguably a more suitable *in vivo* model in terms of melanosome dynamics and pigmentation than mammals, owing to the rapid and reversible vesicle transport that could deliver vesicles from perinuclear regions to plasma membrane within 60 minutes (120).

In paper IV, we implanted pre-stained human melanoma cell lines into the perivitelline space (PVS) of zebrafish embryos. Zebrafish embryos were kept in 0.2 mmol/L 1-phenyl-2-thio-urea (PTU) and therefore had delayed development of pigmentation, which facilitated the observation of fluorescent melanoma cells. This served as a perfect model for studying cells' migratory capacity, which recapitulates a chain of metastatic events, including the invasion through the PVS membrane, dissemination through the blood vessel and the relocation to distant secondary site. All zebrafish experiments were conducted within 5 days post-fertilization at the Zebrafish Core facility of the Department of Comparative medicine, Karolinska Institutet.

Mass spectrometry-based proteome profiling

We implemented immunoprecipitation-coupled mass spectrometry (IP-MS) to study the molecular function of protein-of-interest RAB7B. As explained in the Introduction, RAB GTPases undergo conformational changes and therefore have stage-specific binding partners. Our experimental setup included i) RAB7B immunoprecipitation in parental A375 cell line where RAB7B was abundant. And RAB7B immunoprecipitation in vemurafenib-resistant A375VR4 cell line that over-expressed the constitutively active form (ii: Q67L), or constitutively negative form (iii: T22N) of RAB7B. Such setup would enable us to interpret

the data based on particular research aim, e.g. to find the GEF of RAB7B or to find the effector of RAB7B. These three groups of samples were prepared in biological triplicates. Protein lysates were subject to electrophoresis on Bis-Tris gel for separation and stained with coomassie blue for quality control. Mass spectrometry analysis was performed by the Clinical Proteomics Mass Spectrometry facility, Karolinska Institutet/ Karolinska University Hospital/ Science for Life Laboratory. For data analysis, our IP-MS was not a quantitative approach. It offered us sufficient perspectives of potential binding partners of RAB7B. For biological interpretation, since cross-linking was included in our immunoprecipitation protocol, theoretically we could capture the protein complex of RAB7B and multiple binding partners. Therefore, we would preferably focus on the interaction of RAB7B and a network of multiple proteins with previously known interactions, rather than that of RAB7B and a single protein. Another issue on biological interpretation was that, even though we restored RAB7B expression in its vemurafenib-resistant subline A375VR4, the abundancy of the interacting proteins of RAB7B might be significantly different in daughter cell line compared to A375, which could potentially affect the chance of being captured in MS analysis. These factors above had to be taken into consideration in the data analysis of the IP-MS included in paper IV.

4 RESULTS AND DISCUSSION

4.1 PAPER I

In this paper, we performed targeted sequencing of 120 candidate genes on 92 melanoma patients with high risks of melanoma. More specifically about the cohort, we first enrolled 78 cutaneous melanoma patients from 59 melanoma-prone families that were negative for germline mutations in *CDKN2A*, *CDK4* or *MITF* E318K. We added 10 cutaneous melanoma patients without familial melanoma history but with multiple primary melanomas ($n \geq 3$). There were also another 4 uveal melanoma patients included that were non-carriers of germline *CDKN2A*, *CDK4* or *BAP1* mutations. We designed the probes with 98% target coverage of candidate genes, which included known high- and low-penetrance genes in melanoma (and other cancers), as well as genes involved in various biological functions, such as cell cycle regulation, histone methylation, chromatin modification and DNA repair. Identified gene variants were then 1) validated through bidirectional sanger sequencing and 2) analyzed for their co-segregation within each corresponding family, when possible. After aligning to human genome, we got 3477 variants that passed quality control. We followed a series of filtering criteria, such as including only the variants that alter protein sequence, affect splicing, and excluding variants that were common in the general population or not shared between affected relatives (when applicable). We retained 670 novel variants and 598 variants with minor allele frequency (MAF)<1%. Thirty-nine of these variants were predicted functional and only 1 was not validated by sanger sequencing. Meanwhile, twenty-two of these variants were successfully genotyped in a Swedish case-control cohort with 460 melanoma patients and 620 healthy controls. Among these final 22 variants, we identified a rare variant in breast cancer type 2 susceptibility protein (BRCA2)-coding gene (c.9976A>T, p.L3326X, rs11571833) that was found in 2.7% (12/452) of the unrelated melanoma patients and 0.97% (6/618) of controls (odd ratio, OR=2.80, p=0.035) after adjusting for gender. Samples available allowed us to perform pedigree analyses of two families for this variant in *BRCA2*, which turned out to be shared by affected members in one family, suggesting a possible risk-modifying role of this variant. Previously, *BRCA2* rs11571833 has been linked to the predisposition of cancers in several other tissue, including lung, esophageal and breast (121-124). It is considered to contribute with low-to-intermediate risk with a OR of 1.26 to 6.0. Later, the same variant was described in an Australian melanoma family, but it did not co-segregate with the melanoma phenotype in that family (125).

Another rare variant in BRCA1-associated C-terminal helicase 1 gene (*BRIP1*, c.2543G>A, p.R848H; rs374334794) was found in the final 22-variant list and shared by all affected members in a 3-case melanoma family. There were additional two members of the family carriying the variants but had not been diagnosed with melanoma. One of them had adenocarcinoma, while the other was much younger than the average melanoma onsets. Germline variants in BRIP1 have previously been linked to breast and cervical cancer. The BRIP1 protein plays a crucial role in UV radiation-related DNA damage response (126). The gene variant we identified is located in the strongly conserved helicase C-terminal domain, and

predicted to be disease causative by several prediction algorithms (SIFT, PolyPhen-2, MutationAssessor, MutationTaster and FATHMM). In summary, only 19 out of the 39 predicted functional variants were possible to pursue for co-segregation analyses which to a certain degree limited our ability to assess the impact of variants. However, our study showed that targeted sequencing of a pre-defined panel of genes simultaneously is a robust approach for discovering novel and rare susceptibility genes.

4.2 PAPER II

In this paper, we collected the clinical characteristics of 103 patients with unresectable melanoma who had received immune checkpoint inhibitors (ICIs) and compared their therapy responses in relation to their genotypes of the melanocortin 1 receptor (*MC1R*) gene. We collected peripheral blood samples from these patients who received ICI at the Department of Oncology, Karolinska University Hospital between the years 2012 to 2020, from which we prepared genomic DNA. We designed two pairs of primers that covered the genomic position of *MC1R* with certain degree of overlaps between the two pairs, and then performed bidirectional sanger sequencing, using the BigDye™ Terminator v3.1 Cycle Sequencing kit. *MC1R* alleles were called using Mutation Surveyor® and labeled as harboring *R* allele according to well-established consensus (29). Individuals carrying *R* alleles had previously been linked with red-hair, pale skin and freckling, since MC1R mediates melanocyte-stimulating hormone (MSH) signaling which controls the production and characteristics of melanin.

For these patients, we had access to their basic information, such as age and gender. We also had information of their hair color and skin type according to Fitzpatrick classification. More specifically about melanoma, we compiled information of 1) their personal/family history of melanoma, 2) characteristics of the primary melanoma and tumor characteristics at the time of starting ICI treatment, 3) tumor *BRAF* mutation status and 4) plasma lactate dehydrogenase (LDH). In terms of therapies, we also documented previous lines of treatments that patients had received and also noted specifically the chosen ICI (i.e.: anti-CTLA-4, or anti-PD-1).

Within this cohort, 37.9% had at least one *MC1R R* allele, which was comparable to that in previous reports (127, 128). As expected, patients with ≥1 *R* alleles had higher percentage of "skin type" I-II (p=0.020) and "red hair" (p=0.044), compared to patients who had 0 *R* alleles. Intriguingly, in our cohort, patients with ≥1 *R* alleles had more superficial spreading melanoma (SSM) and less "unknown primary tumor". Overall, the characteristics of patients in our cohort were representative of northern European population. Through univariate analysis, we found that patients with SSM or nodular melanoma (NM) had significantly better progression-free survival than other types (hazardous rate HR=0.48, p=0.043). However, only 11 patients had melanomas of other subtypes. Patients with normal LDH had better PFS compared to those with elevated LDH (HR=0.49, p=0.003).

Notably, patients with ≥1 *R* allele had significantly more favorable PFS (HR=0.60, p=0.043). Recently, Robles-Espinoza *et al.* discovered that the germline *MC1R R* alleles were

associated with elevated mutation burden, which is a favorable marker for ICI efficacy (29). More importantly, such association was also seen in non-UV radiation-related substitutions, which suggests a broader impact of MC1R than UV-pigmentation pathway. Consistent with such notion, *MC1R* polymorphisms have been linked to deregulation in cell proliferation, apoptosis, and DNA damage repair (129). Through multivariate analysis, we adjusted comparison for its phenotypic features that were closely associated with pigmentation pathway and retained a HR of 0.56 (p=0.064). When adjusting for the characteristics of primary tumor (site, subtype, T-stage and *BRAF* mutation) the association between favorable PFS and harboring ≥1 *R* allele disappeared. We could in this study, show that carriers of *MC1R R* alleles have a favorable survival compared to those without any *R* alleles, and further studies on germline *MC1R* variants as a prognostic and predictive marker for ICIs are warranted.

4.3 PAPER III

In this paper, we sought to uncover novel melanoma susceptibility genes in a multi-case melanoma-prone family. The family of interest does not share any known predisposition genes that segregate with the disease. We sent the genomic DNA from 3 out of 4 melanoma patients and 1 healthy relative of the family for whole exome sequencing, after which only rare (minor allele frequency<0.1%), heterozygous exonic variants were kept. We evaluated the impact of these variants using at least 4 algorithms and retained 7 candidates (4 missense, 1 splice-site and 2 frameshift). After validating the remaining variants via cross-checking with other databases, we prioritized one of the frameshift variants found in DENN domain-containing 5A (*DENND5A*) gene. DENND5A is also known as RAB6-interacting Protein 1 (RAB6IP1), for its interaction with RAB6, which has been shown to transport golgi-derived cargoes to lysosome-related organelles, including melanosomes (118). Germline homozygous mutations in *DENND5A* have been linked to epileptic encephalopathy and intellectual disability (130, 131). In our case, the heterozygous frameshift variant would lead to a premature stop codon at the 985 amino acid. We found that one melanoma patient (III:1) carrying the variant had significantly lower wild-type DENND5A protein expression, comparing to two unrelated controls. Moreover, we looked through the biobank at Karolinska University Hospital and checked DENND5A protein expression in 10 tumors from different melanoma-prone families through western blotting. We noted that the DENND5A protein expression was suppressed in 3 out of 10 tumors, compared to the frameshift variant carrier (II:1, #355). Through collaboration, we successfully identified two additional families with several members carrying rare *DENND5A* variants of unknown impact, segregating with the disease. The individuals with *DENND5A* variants had skin type II or “fair skin”. Altogether, we proceeded to analyze the loss-of-function in DENND5A and melanoma susceptibility. After applying the previously established gene signatures to The Cancer Genome Atlas (TCGA)(132), we showed that *DENND5A* mRNA expression was significantly higher in “pigmentation” subgroup,

comparing to the group of "normal-like". Through applying the "trendsceek" algorithm to the spatial transcriptomics on a melanoma lymph node metastasis(133), we found that *DENND5A* mRNA was significantly more enriched in the pigmented areas of the tissue, suggesting a role of *DENND5A* in pigmentation. Intriguingly, further analyses on TCGA showed that melanoma tumors (n=15) with the lowest *DENND5A* mRNA expression had a significantly higher proportion of CC>TT substitution, as a part of "UV signature", compared to the ones with the highest *DENND5A* expression (n=15). Through extensive microscopic approaches on investigating DENND5A expression subcellularly, we found that DENND5A co-localized with the melanosomal cargo protein Melan-A (MLANA), Premelanosome protein (PMEL) and its previously known interacting protein Sorting nexin 1 (SNX1). We showed that DENND5A was enriched in the melanosome-enriched fraction of the cell lysates and that it interacted with MLANA, PMEL and SNX1. Through silencing *DENND5A* through small-interfering RNA or CRISPR/Cas9 editing in the pigmented melanoma cells line MNT-1 led to significant reduction in intracellular melanin content and morphological changes, compared to the negative control MNT-1 cells. The dendrites on melanocytes or melanoma cells play important roles in serving as the focal points of secretion of matured melanosomes. Through enrichment of secreted particles, we showed that melanosome cargo protein (PMEL, more specifically) decreased when silencing *DENND5A*, compared to the control MNT-1 cells. Of note, the reduced melanin contents or impaired melanosome function was observed in a zebrafish model, after silencing DENND5A with a morpholino or by introducing a splice-site morpholino that mimics the frameshift variant of interest. We analyzed the changes in melanosomal cargo proteins, using isotope-specific antibodies that recognized different stages of melanosome maturation. We were able to show that silencing or the truncation of DENND5A led to mis-trafficking of melanosomal cargo proteins (PMEL in particular) from stage I melanosome to lysosomal degradation, which was partly due to the disconnection between DENND5A and the core component of retromer - SNX1. Such mechanism has also been described in the dysfunction of other retromer components, such as SNX4, SNX27 and SNX17(134).

4.4 PAPER IV

In this work, we explored molecular factors that may be accountable for BRAF inhibitor resistance. Previously, Azimi *et al*. had repeatedly exposed melanoma BRAFV600 mutated A375 cell line to a gradually increasing doses of BRAF inhibitor (BRAFi) vemurafenib, and thereby established a BRAFi resistant subline A375VR4(77). A375VR4 displayed obvious morphological changes, such as less elongated and more dendrites, compared to its parental cell line. This pair of cell lines had been subject to iTRAQ-labeled in-depth proteome profiling, in search of molecular mechanisms behind the development of BRAFi resistance.

Here in this paper, with IncuCyte live-cell imaging platform, we showed that A375VR4 cells had significantly faster wound healing capacity, which is indicative of a higher migratory capacity. We then revisited the proteome profiling data and generated a list of proteins based on the relative value of $\log_2$-transformed fold change in protein expression of A375VR4 versus A375. We performed Gene Ontology Enrichment Analyses by inputting the pre-ranked list of

254 proteins into the online tool (http://geneontology.org) and noted that on top of the most deregulated biological processes was 'intermediate filament cytoskeleton organization GO:0045104'. These data altogether suggested cytoskeleton changes as a key biological process underlying the development of BRAFi resistance and the accompanying changes in cell migratory capacity. Further analyses of the said list on cellular component highlighted changes in vesicles (GO: 0031982) and focal adhesion (GO: 005925). Intriguingly, among the top in the list was a RAB GTPase RAB7B, which is a lesser known RAB7 protein and once considered to be an isoform of RAB7A(135). We were able to confirm the down-regulation of RAB7B in A375VR4 compared to its parental cell line through western blotting. At the same time, we analyzed RNA sequencing of A375 and another BRAFi (PLX4720)-resistant subline generated from an independent source(136). Interestingly, we found that RAB7B mRNA was down-regulated in its BRAFi-resistant subline compared to parental A375 cells. Moreover, RAB7B mRNA expression showed inverse correlation with RAB7A mRNA in the said RNA sequencing data.

We sought to silence RAB7B with small-interfering RNA in A375 and cultured it as 3D spheroids. Three dimensional-cultured siRAB7B-A375 cells showed less sensitivity towards 0.5μM vemurafenib 72 hours after the exposure, compared to the siCON-A375. Furthermore, we showed that RAB7B was partly accountable for the more migratory phenotype using IncuCyte live-cell imaging and zebrafish model. To further decipher the role of RAB7B in melanoma cells, we performed immunoprecipitation-coupled mass spectrometry (IP-MS) in parental A375 cells, and A375VR4 overexpressing the constitutively active (Q67L) or negative (T22N) form of RAB7B. Through unsupervised analyses with SAINTexpress, we generated Venn diagram, indicating the numbers and names of potential interacting proteins of RAB7B (all supplementary Tables are available through contact with the corresponding author). We highlighted the interacting network around tyrosine kinase SRC and validated this previously unknown interaction between it and RAB7B using IP immunoblotting in A375 and another BRAF wildtype melanoma cell line ESTDAB105. Interestingly, after silencing RAB7B in 3D-cultured A375, we noted an approximately 65% increase in phosphorylation of SRC at Tyr416 which indicates an activation of the enzymic activity. RAB7B has previously been considered to be an isoform of RAB7A, which has been extensively studied. In recent years, researchers reported RAB7B-related biological processes that are distinct from RAB7A. RAB7A has recently been proposed as a melanoma driver throughout melanoma metastatic transformation, which made it interesting to address the role of RAB7B, particularly after the observation of its downregulation in BRAFi resistant subline. RAB GTPases in general make their impact through their interacting proteins (effectors). Identifying the novel interaction of RAB7B-SRC makes SRC inhibitor (or blockage of SRC phosphorylation) an attractive alternative to overcome BRAFi resistance in melanoma.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

In **Paper I**, through targeted sequencing of 120 candidate genes with high coverage, we reported a list of 22 variants found in 17 different genes. Among these, we highlighted a rare nonsense variant found in the *BRCA2* gene that conferred low risk for developing cutaneous malignant melanoma.. More evidence from melanoma genetics settings and functional studies are needed to validate the risk of having this variant. Additionally, we found a rare variant in the *BRIP1* gene that was shared by three melanoma patients in one family, two of which were diagnosed with also other cancers. These data altogether prove that targeted genome sequencing is a robust approach for capturing rare variants conferring increased risk of cancers. But to be able to identify rare variants it needs to be supported by comprehensive information and biomaterials from the patients, their healthy relatives and large cohorts of disease and normal controls.

In **Paper II**, we investigated the occurrence of germline *MC1R R* alleles among 103 melanoma patients who received immune checkpoint inhibitors (ICIs) at Karolinska University Hospital. We found that patients with ≥1 *R* alleles had significantly more favorable progression-free survival compared to those with 0 *R* allele. We showed that the primary tumors of patients with ≥1 *R* alleles had different characteristics which were accountable for the favorable outcome. Our data implied that *R* allele of the *MC1R* gene exerted its impact not only through pigmentation pathway, but also through non-UV radiation-related pathways. Our study is an intriguing report after the latest finding that germline *MC1R* status correlates with mutational burden in melanoma. We have explored the significance of germline *MC1R* as a predictive marker for the efficacy of ICIs and call for a larger scale study that examines such correlation in different populations.

In **Paper III**, we analyzed a melanoma-prone family with unknown genetic background through whole-exome sequencing, and identified a frameshift mutation in *DENND5A* gene segregating with disease. We found rare *DENND5A* variants in two additional melanoma-prone families, segregating with the disease. We provided mechanistic insights of DENND5A's physiological role in pigmentation pathway and linked its dysfunction to melanoma susceptibility.

This study is of significant translational value, yet needs further epidemiological evidence before it could be a part of the melanoma preventive programs. With more than 50% of the melanoma-prone families unexplained, targeted deep sequencing in melanoma-specific pathways, such as pigmentation and DNA damage pathways, could be a plausible approach to unveil novel susceptibility genes.

In **Paper IV**, through in-depth proteome profiling, we found that RAB7B was associated with BRAFi resistance and cell migration in melanoma cells. Furthermore, we identified tyrosine kinase SRC as a novel interacting partner (effector) of RAB7B in melanoma. Next, we plan to evaluate the role of RAB7B in BRAFi in a zebrafish *in vivo* model by implanting RAB7B-modified melanoma cells into zebrafishes and have them exposed to vemurafenib.

Mechanistically speaking, we seek to i) dismantle the interaction of RAB7B-SRC specifically or ii) pharmaceutically block the phosphorylation of SRC and monitor melanoma cells' sensitivity towards BRAFi and their migratory capacity.

RAB GTPases (RABs) are ubiquitously expressed in a wide variety of cells and have long been known to be involved in vesicle trafficking. Albeit RABs are "too many", they show specificity while comparing its role between organs of different origins. Our work so far has focused mainly on the intracellular part of a specific RAB GTPase, however, utilizing fluorescence labeled RABs and exploring their roles extracellularly might provide interesting mechanistic insights and opportunities for drug delivery.

6 ACKNOWLEDGEMENTS

I am deeply grateful to the patients and their families who contributed enormously to the studies included in this thesis.

I would like to express my most sincere gratitude to my main supervisor **Veronica Höiom**, for always being supportive, for encouraging me to explore, to become more independent. For enduring my last-minute changes and submissions. Throughout my PhD, you had to put up with so many *Whys* that just spontaneously popped up in my head. At one point you had to tell me that you don't know how to do magics. But I always know Iron-woman is more amazing!

To my co-supervisors, **Suzanne Egyházi Brage**, for being the most considerate and kind supervisor anyone could ask for. For nurturing a collaborative and stimulating research environment that pushes our group forward. For always making sure that I feel included, cared for. And, very importantly, for sharing a good laugh always. **Jian Zhao**, for showing me your genuine interest in cell biology and your way of approaching a new territory. For your dedicated efforts into the details. For always being able to come up with better ideas overnight and guide me through it. And most of all, for your care in me, not just as a PhD student, but a kid from Jinan. **Johan Hansson**, our group leader, for simply taking a chance on me and offering an opportunity to do my doctoral study in your research group. For always being available when I ask for guidance.

Yihai Cao, thank you for introducing me to the Hansson group, for being a true mentor in every sense. For your broad spectrum of knowledge. I got the chance to sit beside you, listen to your perspectives on study design, on manuscript writing and on every aspect of science and life. Your enthusiasm and persistence are truly inspirational.

Hildur Helgadottir, for your patience in my research pitch and for simply being the perfect researcher/clinician. It certainly is not easy to balance clinical duties, research and life, but you have shown me the way. **Fernanda Costa Svedman**, for your energies, for always willing share what might be the best for me. **Karin Kjulin**, I definitely feel spoiled when having you around. The Christmas dinners you arranged were unparalleled. And to the former members of the Hansson group: **Rainer**, for teaching me the impeccable lab routines, for I don't how many random conversations we had, some of which were remotely about science (shocking). **Alireza**, for your expertise in literally everything about proteins, for the forever proliferating A375VR4 cells and so much more. **Ishani**, for being my PhD buddy but ditched me way earlier than I had anticipated. For the sweet memories we had in Australia together with **Ankit**. I would like to thank **Johan F**, **Diana**, **Marianne**, **Kalle**, **Stefano**, **Eva Månsson Brahme** for the cheerful environment we share in the group. And **Jasmine**, for the reversed supervision I sometimes feel…

To the fantastic coauthors and collaborators: **Lars Bräutigam** for your structured style and highly appreciated expertise in the zebrafish work, **Sussie Lindström** for your kind and timely help, **Per Johnsson**, for your objective opinions and for reading my long emails. To **Nick Tobin** and **Pär Engström**, for your encouraging words and much-needed data analyses. To **Xiaohong Yang** and **Alisa Goldstein**, for your crucial contribution to the project and your valuable comments on our manuscript. **Yafeng Zhu**, **Noemi**, **Yitian**, **Kim Thrane**, **Hanna**

Eriksson, **Lars Hååg**, **Stina**, **Annika**, **Malin** who have all generously contributed to the studies included in this thesis. To **Martin Enge**, I have always been drawn to your ideas which I often cannot grasp at first. **Marco Gerling**, for your generous help and scientific input during the final period of my doctoral study. To **Mats Nilsson**, for kindly sharing your knowledge on the fascinating and colorful "landscape".

To our OnkPat administration, in particular **Erika** and **Hanna,** who helped me every step of the PhD journey. To **Andreas Lundqvist** and **Lars Holmgren**, for taking good care of us PhD students.

To my office roommates, **Huaitao**, for your positive vibes. **Vasilios**, for your constant pursuit of bettering things. **Solrun**, for checking on me even when your experiment did not go as planned. I have no doubt that you will do great in your PhD. **Katja**, for taking care of every aspect of the office. And thank you **Luuk** for rejuvenating that corner of the office.

To my supervisor during my master programme, **Bo Han**, thank you for introducing me to science, and making sure that I get my lab routines right from day 1. I truly appreciate your unreserved support over the years. To **Wanzhong**, for your reassuring smiles and generous support during these years I live in Stockholm.

To my friends, **Christina**, for still going to lunch adventures with me after that major dispute over Benjamin Button (such a sweet movie). **Sander**, for simply so much fun we had and more to come, for offering your perspectives whenever I feel disoriented. **Nicole**, for the fantastic days we had in CCK, I still got quite a few good jokes to tell if you ever show up in the cell lab here. And hej to **Joel**, is it me or is this place smells like up dog (haha I did it)? I appreciate the low profile after your impressive publication but try not to search yourself on pubmed that often. **Jiwei**, for the poker, the basketball, the Asphalt. You are truly the brightest and the kindest. I have always enjoyed chatting with you. Keep going and I am sure your talent will be appreciated wherever you go. **Liang Zhang**, you are one of the most determined people. Thank you for all the fun and I look forward to more basketball sessions with the one and only Sollentuna Ginobili. **Francesca**, for the Duolingo challenges, **Nienke**, for the legendary skiing trips, and **Pablo** my buddy who is the absolute genius in life choices. I am sure in one of those parallel universes, several of our brilliant plans might have worked out…And to **Lilly**, for being so understanding and calm. **Ioannis**, for leading the way with your hard work, and for that "whatever it takes" spirit demonstrated by the "microwave-based multiplexed staining protocol". To **Pedro&Mathilde**, for always cheering me up, and as **Ziqi** used to say "xiexie Peter!). **Yasmin&Felix**, for our overdue hotpot, **Elin**, the handover of that thick blue lab coat was a very significant event I know! To **Ran Ma**, **Xinsong**, **Xianli**, **Yuanyuan**, for helping me to settle in, for giving me lots of suggestions, especially when I first started at CCK. **Xuan Li** and **Ning Wang**, the roller coaster was too wild, never again, thank you. To **Nikos&Robert**, for the festivals in Uppsala**.**

To my lovely group: **Jiawei** (we skip the handshake this time), **Qiaoli**, **Yang**, **Yunhan**, **Jing Wang**, **Yujiao**, **Hao**, **Xin Li**, **Chenhong**. It seems that we could barely agree on anything but still chose to stick with each other. With you guys around, I always feel a sense of belonging. And to the Jägargatan gang: **Gina**, **Sebastian**, **Nathalie**, those 9 m^2 rooms were brutal, living room chats with you guys were the only way to survive.

To the CCK crew, **Susanne Agartz**, for the spontaneous conversations. **Alessandro**, **Mercedes**, **Yingbo**, **Yumeng Mao**, **Yuanjun Ma**, **Xiaonan**, **Limin Ma**, **Lisa**, **Elisabeth**, **Sören**, **Elle**, for the nice atmosphere at CCK.

To **Xu Jing**, **Xingkang**, **Jieyu**, **Qiqiao**, **Jing Wu**, **Jun Gao**, for the heart-warming activities during the holiday times. **Min Guo**, **Shuijie**, **Liyuan**, **Na**, **Weng-Onn**, **Xiangling**, **Liu Yang** for your help when it was most needed. **Zhiqing Fang**, for you way of thinking, **Dawei**, it has been fun to have you around. **Ninni**, for surprisingly very helpful at times…**Yaxuan** for the cell lab karaoke times. To **Tiantian Liu**, **Xiuming Liang**, **Tenghao**, **Ting Zhuang**&**Jian Zhu**, for guiding me with you experience in research. And my sincere gratitude to the Pink group: **Bertrand**, **Patricia** and **Dalel**, and to my colleagues at OnkPat and BioClinicum: **Ziqing**, **Jikai**, **Ying Yang**, **Yachao**, **Le Tong**, **Theo**, **Dimitris**, **Pedram**, **Vicky** (keep the breaking news coming), **Shiyong**, **Sylvya** (people ought to know that you check all the freezer doors every day before you go home), **Sonia** (cue the suspenseful music), **Aishwarya**, **Ewa**, **Sophia**, **Angelos**, **Aarren**, **Steffi**, **Anderson**, **Tom**, **Yumeng Zhang**, **Paula**, **Amineh**, **Chuanyou**, **Jingya Yu**, **Xiaotian**, **Simin**, **Xiaohao**, **Takashi**, **Dominika**, **Yajing**, **Agnes**, **Roger**, **Yinxi**, **Ran Luo**. Thank you all for the friendly every day encounters, for making our research environment into a fun community.

To my Yan-You and **Chunni**. To **Chongyang**, **Xipei**, **Hang er**, **Xiao Zou, Allen** and **Juan**.

To my family, for always trying to provide the best for me. To **my mother**, for pursuing challenging endeavors in your life. Also, I know you won't let me get by without a special mention…

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