Identification and clinical
evaluation of senescence-associated
markers to distinguish melanocytic nevi
from melanomas

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Abstract

Melanoma is a form of cancer that develops in melanocytes. While it represents only 5% of skin malignancies, it is the most aggressive and lethal. Benign proliferation of these cells form the melanocytic nevi. The definitive diagnosis of melanocytic nevi or melanoma lesions is histopathologic. However, it is estimated that a correct diagnosis is established by means of standard skin biopsy in only 83% of the melanocytic lesions; of the remaining cases 8% and 9% are over-interpreted (false positives) and under-interpreted (false negatives), respectively. This underscores the importance of additional diagnostic tests. Since cellular senescence is considered to be a tumor suppressive mechanism, immuno-histochemistry using senescence markers has been suggested for the evaluation of difficult melanocytic lesions; however, the routinely used senescence markers lack the ability to distinguish nevi from melanoma. The general aim of this thesis is therefore to identify novel senescence markers that may aid in melanoma diagnosis.

In study I, we established a cellular model with nevus-mimicking characteristics consisting in primary melanocytes that become senescent. Transcriptomic analysis allowed expanding the set of senescence-associated markers that could distinguish nevi from melanoma and identifying tubulin β -3 as a potential diagnostic marker. Depletion of tubulin β -3 and pretreatment with tubulin destabilizing drugs in melanocytes and melanoma cells induced a senescence-like phenotype *in vitro*. In particular, reduced migration capacity and induction of cell cycle arrest in G2/M phase of the cell cycle was demonstrated.

In study II, a potential inter-cellular signaling pathway between melanoma cells and stromal fibroblasts, that might facilitate melanoma invasion, was investigated. Ultraviolet (UV) radiation was shown, both in melanoma cells and fibroblasts, to promote the release and activation of TGF- β 1 and subsequent increase in expression of the serine protease FAP- α , a protein that plays role in extracellular matrix degradation and therefore facilitates the invasion of melanoma cells. Such mechanism was not functional in senescent melanocytes.

In study III, it was shown that tubulin β -3 immunostaining aids in the diagnosis of nevi and melanomas. The diagnostic criterium was the tubulin β -3 gradient within the melanocytic nevi that was no longer apparent in melanoma. Different patterns of tubulin β -3 immunostaining in melanoma were described, dermoscopy-immunohistochemistry associations were found, specific dermoscopic features highlighted, and the prognostic value of this tubulin β -3 marker was examined. The progression rate in patients whose melanomas had areas with loss of tubulin β -3 was 4 times higher than in patients without this feature, although statistical significance could not be reached ($p=0.06$).

In conclusion, transcriptomic analysis expanded the set of senescence-associated markers that could distinguish nevi from melanoma and identified tubulin β -3 as novel immuno-histochemistry marker shown to have diagnostic and probably prognostic value. From a mechanistical point of view, ultraviolet radiation was shown to promote not only the formation of melanoma but also its progression by increasing a cathepsin-TGF- β 1-FAP- α pathway resulting in extracellular matrix degradation.

Keywords: senescence, nevi, melanoma, melanocytes, fibroblasts, ultraviolet (UV) radiation

Summary in Swedish - Sammanfattning på svenska

Malignt melanom är en form av cancer som utvecklas i melanocyter, de celler i huden som bildar pigmentet melanin. Även om malignt melanom endast utgör 5% av alla hudcancer, är den den mest aggressiva och dödliga. Godartad ansamling av melanocyter bildar en leverfläck eller ett sk nevi. Vid minsta misstanke om melanom, opereras nevet bort och analyseras i mikroskop. Det uppskattas dock att en korrekt diagnos fastställs endast i 83% av de bortopererade lesionerna. Av de återstående fallen är 8% övertolkade och 9% undertolkade. Detta understryker vikten av att förbättra melanomdiagnostik. Melanocyter i nevi anses i motsats till melanom ha avtagande förmåga att föröka sig efter ett visst antal celldelningar på grund av cellåldrande. Påvisande av cellåldrande-markörer skulle därför kunna förbättra diagnostiken av svårbedömda lesioner. De idag routinemässigt använda cellåldrande-markörerna saknar dock förmåga att skilja nevi från melanom. Det övergripande syftet med denna avhandling var därför att försöka identifiera nya cellåldrande-markörer för att förbättra melanom-diagnostiken.

I studie I etablerade vi en cellmodell med nevi-efterliknande egenskaper bestående av melanocyter som åldras. Med genuttryck-analys studerades cellåldrande-markörer som kunde skilja nevi från melanom. Proteinet tubulin β -3 identifierades som en potentiell diagnostisk markör. Minskning av tubulin β -3 och förbehandling med tubulin-destabiliserande läkemedel i melanocyter visade sig inducera ett tillstånd som liknar cellåldrandet.

I studie II undersöktes en signalväg mellan melanomceller och fibroblaster, som kan underlätta melanomcellers förmåga att infiltrera och aktivt förstöra omgivande vävnad. Fibroblaster är en typ av bindvävsceller i läderhuden som tillverkar ämnen som finns mellan celler, s.k. extracellulär matrix. Ultraviolett (UV)-strålning visade sig, både i melanomceller och fibroblaster, främja signalvägen som resulterar i ökat proteinuttryck av FAP- α , ett protein som bidrar till nedbrytning av extracellulär matrix vilket kan underlätta infiltration av melanomceller. En sådan signalväg var inte funktionell i åldrande melanocyter.

I studie III visade vi att proteinet tubulin β -3 är en markör som underlättar diagnostik av nevi respektive melanom. Det diagnostiska kriteriet var tubulin β -3 gradienten som kunde ses i nevi men som ej var synligt i melanom. Olika mönster av tubulin β -3 analys i melanom beskrevs, kliniska associationer hittades och värdet av denna markör för att förutsäga sjukdomens förlopp undersöktes. Melanompatienter, vars melanom hade områden med förlust av tubulin β -3, hade fyra gånger högre progressionstakt av sjukdomen jämfört med patienter utan detta mönster.

Sammanfattningsvis studerade vi cellåldrande-markörer som kunde skilja nevi från melanom och vi identifierade proteinet tubulin β -3 som en markör med diagnostiskt och förmodligen prognostiskt värde. Vi visade att ultraviolett strålning inte bara främjar melanombildning utan också dess progression genom att öka en signalväg mellan melanomceller och fibroblaster som resulterar i extracellulär matrixnedbrytning.

List of papers

The thesis is based on the following papers, which are referred to in the text by their Roman numerals:

- I. **Orfanidis, K.**, Wäster, P., Lundmark, K., Rosdahl, I., and Öllinger, K. (2017). *Evaluation of tubulin β -3 as a novel senescence-associated gene in melanocytic malignant transformation.*
Pigment Cell & Melanoma Research 30, 243–254.
- II. Wäster, P., **Orfanidis, K.**, Eriksson, I., Rosdahl, I., Seifert, O., and Öllinger, K. (2017). *UV radiation promotes melanoma dissemination mediated by the sequential reaction axis of cathepsins–TGF- β 1–FAP- α .*
British Journal of Cancer 117, 535–544.
- III. **Orfanidis, K.***, Lundmark, K.*, Synnerstad, I., Wikström, J.D., Wäster, P., Öllinger, K. (2019). *The diagnostic value of tubulin β -3 immunohistochemistry to discriminate melanocytic nevi from melanomas.*
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Abbreviations

Akt	Protein kinase B
ASIP	Agouti signaling protein
BAP-1	BRAC-associated protein 1
BRAF ^{mut}	BRAF mutant
BRAF ^{non-mut} /RAS ^{non-mut}	BRAF non-mutant/ NRAS non-mutant
CAF	Cancer-associated fibroblast
CDK	Cyclin-Dependent kinase
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CGH	Comparative genomic hybridization
CNA	Copy number alteration
CSD	Chronic sun damage
DAVID	Database for Annotation, Visualization and Integrated Discovery
DDR	DNA damage response
DN	Dysplastic nevi
DPN	Deep penetrating nevus
ECM	Extracellular matrix
ET3	Endothelin 3
EZH2	Enhancer of zeste homolog 2
FAP- α	Fibroblast activation protein alpha
FFPE	Formalin-fixed and paraffin-embedded
FISH	Fluorescence in situ hybridization
GO	Gene Ontology
GSEA	Gene set enrichment analysis
H3K27me3	Histone 3, lysine 27, trimethylated
H3K4me2	Histone 3, lysine 4, dimethylated
HRAS	Harvey RAS viral oncogene homolog
IARC	International Agency for Research on Cancer
IHC	Immunohistochemistry
IMPSG	International Melanoma Pathology Study Group
IPA	Ingenuity Pathway Analysis
KRAS	Kirsten RAS viral oncogene homolog
MAPK	Mitogen-activated protein kinase
MC1R	Melanocortin-1 receptor
MDM2	Mouse double minute 2 homolog
MITF	Microphthalmia-associated transcription factor
MSC	Melanocyte stem cell
mTOR	Mechanistic target of rapamycin
NF1	Neurofibromin 1
NF1 ^{mut}	NF1 mutant
NGS	Next generation sequencing
NRAS	Neuroblastoma RAS viral oncogene homolog
NRAS ^{mut}	NRAS mutant

OIS	Oncogene-induced senescence
PFS	Progression-free survival
PI3K	Phosphatidylinositol 3-kinase
PIP3	Phosphatidylinositol-3,4,5-trisphosphate
POT1	Protection of Telomeres 1
PTEN	Phosphatase and tensin homolog
RB	Retinoblastoma
ROS	Reactive oxygen species
RQ-PCR	Real-time quantitative polymerase chain reaction
SA- β -Gal	Senescence-associated β -galactosidase
SASP	Senescence-associated secretory phenotype
SCP	Schwann cell precursor
SSM	Superficial spreading melanoma
SWI/SNF	Switch/Sucrose non-fermentable chromatin remodeling complex
TERT	Telomerase reverse transcriptase
TET2	Ten-eleven translocase 2
TGF- β 1	Transforming growth factor beta 1
TP53	Tumor protein 53
TP53 ^{mut}	TP53 mutant
TUBB3	Tubulin β -3
UV	Ultraviolet radiation
WHO	World Health Organization
α MSH	α -melanocyte stimulating hormone
5-hmC	5-hydroxymethylcytosine

Introduction

Melanoma

Melanoma is a form of cancer that develops in melanocytes (Figure 1). While it represents 5% of the skin malignancies, it is the most aggressive and lethal (American Cancer Society, 2019). Melanocytes derive from the neural crest and colonize the skin. Benign proliferation of these cells gives rise to melanocytic nevi (Mort et al, 2015). 25-33% of cutaneous melanomas are found in existing nevi, whereas 67-75% arise on normal-looking skin *de novo* (Bevona et al, 2003; Haenssle et al, 2016).

Figure 1. Anatomy of the skin, showing the epidermis, dermis, and subcutaneous tissue. Melanocytes are in the layer of basal cells at the deepest part of the epidermis. Adapted from Wikimedia Commons.

Incidence/Mortality: Across the globe, the estimated age-standardized rates of melanoma incidence and mortality are 3.1 and 0.6 per 100,000 respectively (Ferlay et al, 2018). Its incidence is steadily increasing (Siegel et al, 2019) and varying among populations. The highest age-standardized incidence and mortality rates are reported in Australia/New Zealand (33.6 and 3.4 per 100,000 respectively) followed by Western (18.8 and 1.7 per 100,000), Northern Europe (17.0 and 2 per 100,000) and North America (12.6 and 1.4 per 100,000), whereas the lowest rates are reported in Northern Africa, South-Central and South-Eastern Asia (below 0.5 per 100,000). Sweden is the country with the sixth highest age-standardized incidence of melanoma in the

world (24.7 per 100,000) and the age-standardized mortality rate is 2.5 per 100,000 (Ferlay et al, 2018).

Risk factors: Several risk factors have been linked to melanoma, including heavy exposure to ultraviolet (UV) radiation, family and personal history of melanoma, the presence of atypical, large or numerous (more than 50) nevi and weakened immune system. Sun-sensitive people (e.g. fair skin and hair color) or with history of excessive sun exposure or non-melanoma skin cancer also have an increased risk (Koh et al 1996; Dimitriou et al, 2018). Two distinct biological pathways are thought to lead to development of cutaneous melanoma; a chronic sun exposure pathway by progressive accumulation of sun damage at the melanoma site in people who are sensitive to sun; the other a nevus prone pathway initiated by early sun exposure and promoted by host factors and/or intermittent sun exposure (Armstrong et al, 2017).

UV radiation: UV radiation is part of the electromagnetic spectrum (100 to 400 nm) that reaches the earth from the sun (Seebode et al, 2016). Ultraviolet radiation is the predominant risk factor in melanoma, the majority of which harbor a UV signature mutation. UVA (315 to 400 nm) can reach dermal structures and UVB (280 to 315 nm: higher energy than UVA) reaches the stratum basale. Irradiation by UVA generates reactive oxygen species, which can indirectly cause DNA damage (Venza et al, 2015), whereas UVB interacts directly with DNA and, in sequences where two pyrimidines are adjacent, it might result in the generation of cyclobutane dimers or 6-4 photoproducts (Figure 2) leading mostly to C→T as well as CC→TT tandem mutations (Brash et al, 1997).

Figure 2. Absorption of UV photon energy at UVB wavelength by DNA molecules and subsequent formation of cyclobutane-pyrimidine dimers or 6-pyrimidine-4-pyrimidone photoproducts. Adapted from Seebode et al, 2016.

Nevus origin: Nevi are composed of melanocytes that have lost their dendrites. They commonly begin as flat lesions that over time become elevated. To explain the increase in size, it was suggested that nevi originate from the proliferation of intra-epidermal melanocytes with their subsequent downward migration (Grichnik, 2008). However, this concept has been more recently challenged and the alternative postulates the upward migration of melanocytes into the epidermis (Grichnik, 2008).

Melanoma subtypes: Cutaneous melanoma is traditionally classified in four major subtypes: the superficial spreading, nodular, lentigo maligna and acral lentiginous melanoma. The most common form of melanoma is the superficial spreading melanoma among Caucasians and acral lentiginous melanoma in African-Americans. Less common subtypes include amelanotic melanoma, spitzoid melanoma and desmoplastic melanoma (McGovern, 1970; Dimitriou et al, 2018).

WHO melanoma classification: The World Health Organization (WHO) has recently adopted a multiparametric classification for melanocytic lesions (WHO, 2018) based on three criteria: previous UV-radiation, genomic alterations and cell of origin. The first criterium discriminates two categories: melanoma arising in sun-exposed skin from melanoma in sun-shielded skin or without known previously known UV-radiation exposure. The former category represents melanoma with low degree of cumulative sun damage (CSD) (including superficial spreading melanoma and a subset of nodular melanoma), high-CSD melanoma (including lentigo maligna and a subset of nodular melanoma) and desmoplastic melanoma. The latter category comprises malignant Spitz tumors (Spitz melanoma), acral melanoma, mucosal melanoma, melanoma arising in congenital nevus, melanoma arising in blue nevus and uveal melanoma (WHO, 2018).

Melanoma growth phases: Melanomas typically arise and grow as superficial tumors, remaining such during the radial growth phase, then infiltrate deeply into the dermis in the vertical growth phase (Clark et al, 1969). Significant prognostic features of melanoma are tumor thickness, histologic ulceration and mitotic rate. As tumor thickness increases, survival rates decrease (Gershenwald et al, 2017). Therefore, early detection of melanoma is crucial to improve the prognostic outcome.

Prevention: Melanoma is preventable by minimizing exposure to UV radiation. This can be achieved by avoidance of intense sunlight, sunbathing or the use of indoor tanning, seeking shade, by wearing protective clothing, sunglasses that block UV rays and applying to unprotected skin broad-spectrum sunscreen of at least 30 sun protection factor (American Cancer Society, 2019).

Signs and symptoms: New skin growths, sores that do not heal, or changing skin lesions in size, shape or color are warning signs. The ABCDE rule (Friedman et al, 1985; Abbasi et al, 2004) is devised for primary health care professionals and lay public for detection of melanoma and timely referral to specialists. It stands for Asymmetry (one half of the lesion does not match the other half), Border irregularity (irregular or jagged borders), Color variegation (multiple colors), Diameter (> 6mm) and Evolution (Change).

Early detection: Detection of melanoma by self-examination is common and very important, clinical recognition, though, allows for the earlier detection of melanoma and improved clinical management (Carli et al, 2004, Wolner et al, 2017). The “ugly duckling” sign (Grob & Bonerandi, 1998) in a given individual is the nevus that does not resemble the others; this sign has been used clinically to further help the diagnosis of melanoma. The addition of dermoscopy to the visual inspection improves both the sensitivity (92 vs. 76 %) and specificity (95 vs. 75 %) (Dinnes et al, 2018) of melanoma detection.

Treatment: Most early cutaneous melanomas are treated by removal of the growth and its surrounding normal tissue. Sometimes a sentinel lymph node is biopsied or total body radio-imaging technique is performed to determine the stage. Surgery, chemotherapy and/or radiation therapy have been used to treat melanomas with deep invasion or spreading. In recent years, immunotherapy and targeted drugs have been approved and used in advanced melanoma treatment (American Cancer Society, 2019).

Survival: 84% of melanomas are diagnosed when confined only to the skin, for which the 5-year survival rate is 98%. For regional and distant stage melanomas, the rates drop significantly at 64 and 23 % (American Cancer Society, 2019).

Diagnosis & Conclusion: Microscopic examination of suspected nevi and melanomas is necessary, as the definitive melanoma diagnosis is histopathologic, based on a combination of cytologic and architectural features (WHO, 2018). The pathologic assessment provides diagnostic and prognostic information that influence the clinical management for patients with melanoma. However, it is estimated that a correct diagnosis is established by means of standard skin biopsy in only 83% of the melanocytic lesions; of the remaining cases 8% and 9% are over-interpreted (false positives) and under-interpreted (false negatives), respectively. (Elmore et al, 2017). This underscores the importance of additional diagnostic tests. Immunohistochemistry has been used for the evaluation of difficult melanocytic lesions, however the routinely used immunomarkers lack the ability to distinguish nevi from melanoma (Barnhill et al, 2014).

Ancillary techniques in melanoma diagnosis

Immunohistochemistry

The gold standard for melanoma diagnosis is based on histopathological evaluation. However, over the past decades in dermatopathology practice, a panel of markers have been applied on paraffin-embedded tissue and their immunoreactivities' pattern has helped identifying melanocytes and distinguishing melanocytic lesions from lesions of other origin (Barnhill et al, 2014).

Traditional immunohistochemistry markers:

S-100 family: Antibodies reactive with S-100 proteins exhibit >95% sensitivity in the diagnosis of melanocytic lesions and both nuclear and cytoplasmic staining are seen, though a heterogeneous distribution may be observed. The S-100 protein family include acidic calcium-binding proteins forming homo- and heterodimers, which are expressed in melanocytes, Schwann cells, astrocytes, Langerhans cells, chondrocytes, adipocytes and myoepithelial cells. The staining with antibodies reactive to S100 proteins is of particular value for the diagnosis of spindle cell melanomas (Barnhill et al, 2014).

Melan A: Monoclonal antibodies to Melan-A stain melanocytes in epidermis as well as nevi and melanomas. Almost all primary non-desmoplastic melanomas and most of metastatic melanomas express Melan-A. The immunostaining pattern is membranous and cytoplasmic (Barnhill et al, 2014).

gp100: HMB-45 is a monoclonal antibody which recognizes gp100, a pre-melanosomal glycoprotein. The immunostaining pattern is cytoplasmic, often heterogeneous. The majority of dermal melanocytes do not stain with HMB-45, though blue nevi and the superficial dermal component of nevi are stained. Superficial dermal components of melanomas, which generally contain epithelioid melanoma cells, stain for HMB-45 but desmoplastic and neurotropic melanomas are negative (Barnhill et al, 2014).

Sox 10: Antibodies to Sox 10, a transcription factor that plays role in the development of Schwann cells and melanocytes, show a nuclear staining pattern and has been useful for the diagnosis of spindle cell melanoma and the detection of melanoma metastasis in sentinel lymph node (Barnhill et al, 2014).

Cell cycle proteins: Immunohistochemistry has been also used to identify melanocytes expressing the proliferation marker Ki-67. Ki-67 is a nuclear antigen, expressed in late G1, S, G2 and M phases of the cell cycle but not in early G1 and G0. The product of *CDKN2A* gene, p16, has been also suggested as a potential marker for the distinction of nevi from melanoma, but due to the significant overlap between nevi and melanoma and partial heterogeneous staining, its use is limited (Barnhill et al, 2014).

Novel technologies

Advances of novel technologies have enabled the development of molecular tests to identify genetic and epigenetic alterations. These include comparative genomic hybridization (CGH), fluorescence in situ hybridization (FISH), gene expression profiling of tumors and adhesive patch genomic analysis. These methods might prove significant tools for the diagnosis and prognosis of melanoma. Increasing awareness about their utility and limitations warrants their broad clinical application and use in precision medicine (Lee et al, 2018).

Comparative genomic hybridization: CGH detects chromosomal copy number alterations (CNAs) throughout the genome. DNA from paraffin-embedded sections from lesional and normal tissue is extracted and labeled with different fluorochromes, denatured to a single strand and hybridized to normal chromosomes in metaphase (chromosomal CGH) or microarrays of genomic DNA (array CGH). Fluorescent areas of disproportionate binding indicate copy number gains or losses and this requires fluorescence microscopes and computer software for analysis and comparison of differential signals. The interest of chromosomal CGH is limited for alterations involving small DNA segments (<20 Mb) or areas close to other CNAs. This problem is overcome by array CGH. However, both methods require complex stereoscopic microdissection to avoid inclusion of non-lesional tissue, cannot differentiate homozygous from heterozygous deletions and detect point mutations, balanced translocations or copy number changes that are present in a minor cell population due to tumor heterogeneity. CGH has a 96% sensitivity and 87% specificity to distinguish nevi from melanoma based on at least one genomic aberration (Bastian et al, 2003a). Most melanocytic nevi lack CNAs, with the exception of Spitz nevi that may harbor gain on 11p or loss of chromosome 3. Melanomas show losses on 6q, 8p, 9p and 10q and gains on 1q, 6p, 7, 8q, 17q and 20q (Barnhill et al, 2014).

Fluorescence in situ hybridization: FISH detects chromosomal copy number alterations at targeted genomic loci. Fluorescent, single-stranded DNA probes are hybridized directly on paraffin-embedded sections containing denatured DNA. After processing steps, the alterations are visualized and quantified microscopically. This technique can differentiate homozygous from heterozygous deletions, identify chromosomal translocations and fusion genes and permits detection of alterations in subpopulations of heterogeneous tumors. Yet, it only tests for aberrations in the targeted, predetermined areas, and polyploidy can lead to false-positive results. Its greatest utility has been in assessing the homozygous 9p21 deletion (*CDKN2A* locus) on atypical Spitz tumors (Barnhill et al, 2014).

Gene expression profiling: Quantitative gene expression profiling technologies have become available as adjunctive diagnostic tests. Based on the transcriptomic profiles of biopsied or tape-stripped tissue samples on a panel of genes several tests have been developed, a diagnostic ancillary test to distinguish nevi from melanoma, a test to assess metastasis risk of melanomas, particularly with negative sentinel lymph node biopsy, and a clinical decision tool to aid the decision for biopsy of equivocal melanocytic lesions (Lee et al, 2019).

Molecular analysis and next-generation sequencing: Molecular analysis, such as Sanger sequencing and real-time quantitative polymerase chain reaction (RQ-PCR), have been employed for detection of cancer somatic alterations. They are performed for each gene each time and therefore need a high turn-around time. The development of next-generation sequencing (NGS) technologies enabled the massive analysis of millions of DNA segments in a single assay with improved sensitivity in detection of mutations, applicable even for formalin-fixed and paraffin-embedded (FFPE) specimens. They are increasingly performed routinely, allowing a more personalized approach to match subsets of patients to the most appropriate clinical management (Bustos et al, 2017).

Novel immuno-histochemical markers: The molecular characterization of melanocytic lesions has led to novel immunohistochemical probes against genetic and epigenetic targets, allowing the rapid and inexpensive use of biomarkers. Antibodies to the histone deubiquitinase BRAC-associated protein 1 (BAP-1), have shown that the nuclear labeling of BAP-1 is lost in large epithelioid melanocytes in a subset of Spitz tumors (Wiesner et al, 2012). Immuno-

histochemistry using a highly sensitive and specific monoclonal antibody to BRAF V600E serves as an initial screening test for the detection of BRAF V600E mutation and eventual prediction of therapeutic response to targeted therapies in advanced melanoma. However, genetic analysis may be needed in equivocal staining cases (Anwar et al, 2016). Antibodies to PD-1 and PD-L1, proteins expressed on T-cells and tumor cells, respectively, that show a membranous staining pattern help identify eventual responders to immunotherapy (Sunshine et al, 2017).

Novel immuno-histochemical epigenetic markers: Immunohistochemical expression patterns that may reflect epigenetic modifications have been also used. Immunohistochemical expression patterns of bivalent histone modifications, including the repressive histone 3, lysine 27, trimethylated (H3K27me3) and the activating histone 3, lysine 4, dimethylated (H3K4me2) have demonstrated increased staining at the invasive front of vertical growth phase melanomas and decreased staining in metastatic as compared to primary melanomas. In another study, all spindle cell melanomas showed positive staining for H3K27me3 suggesting that it may help distinguish malignant peripheral sheath tumors from melanoma (Schaefer et al 2015). Antibodies to the enhancer of zeste homolog (EZH2), the catalytic subunit of Polycomb-repressive complex 2 which is essential in stem cell self-renewal, showed stronger staining in melanoma cells than in benign nevus cells. (Kampilafkos et al, 2015). Loss of 5-hydroxymethylcytosine (5-hmC), which is the key intermediate of ten-eleven translocase 2 (TET2)- mediated DNA demethylation, has been shown in melanomas, distinguishing them from nevi (Lee et al, 2018).

Conclusion: The ongoing technical progress have led to the discovery of novel biomarkers that may potentiate the diagnosis and prognosis of melanocytic lesions. In this study, we combined the identification of novel markers at the RNA level using transcriptomic followed by the confirmation at the protein level using immunohistochemistry.

Cellular senescence

Hallmarks of cancer: Cancer has been a field of intense research during the past decades. Although there are distinct cancer types and subtypes, it is believed that underlying organizing principles are shared by all forms of cancer. The seminal paper by Hanahan and Weinberg published in 2000, with its update in 2011, propose that cancer cells have acquired eight functional capabilities that allow them to survive, proliferate and disseminate (Hanahan and Weinberg, 2000 & 2011). Cancer cells are thought to be self-sufficient in growth signals, evade growth-suppressors and cell death, have a limitless replicative potential, induce and sustain the growth of blood vessels, have the potential to disseminate from the primary tumor to local and distant sites, have abnormal metabolic pathways and evade the immune system. They have acquired these capabilities by the development of genomic instability and the inflammatory state that promotes tumor progression (Figure 3).

Figure 3. Hallmarks of cancer. Modified from Hanahan and Weinberg, 2011.

Replicative senescence: Replicative senescence was first described 60 years ago by Hayflick (Hayflick et al, 1961 and 1965). After explant of primary cells from human tissues the multiplication of normal human cells is limited to 60-70 doublings. The induction of this intrinsic, cell-autonomous program is termed replicative senescence. Even though they have lost

their proliferative capacity, the senescent cells are viable and metabolically active (Kuilman et al, 2010). During the phase of rapid proliferation, the telomeres are not completely replicated by the DNA polymerase leading to a critical minimal length of the telomeres after several cycle divisions, which triggers a DNA damage response (DDR). The phosphorylated form of the histone variant H2AX, γ -H2AX, DDR proteins and the activation of DNA damage kinases ATM and ATR characterize this response. The latter subsequently activate CHK1 and CHK2 kinases and several cell cycle proteins including p53 (D'Adda di Fagagna, 2003). The cells can then repair their damage under a transient proliferation arrest state although apoptosis or senescence is induced if the DNA damage exceeds a certain threshold. Replicative senescence is also linked to the p16^{INK4a}/pRB pathway. Cells escaping from replicative senescence undergo telomeric crisis, which eventually results in chromosomal instability and death (Campisi et al, 2013, Kuilman et al, 2010).

Premature cellular senescence: Cells *in vitro* can also become senescent prior to telomere shortening. The artificial concentrations of nutrients and growth factors, physiological oxygen conditions and the absence of neighboring cells and extracellular matrix could induce a stress in the explanted cells in culture, which induces senescence. Strong mitogenic signals can also induce senescence, termed oncogene-induced (OIS). Certain oncogenes, especially mitogen-activated protein kinase (MAPK) pathway components, as *RAS* and *BRAF* are well-studied examples. It seems that OIS mechanisms are not universal across cell types. Some cause DNA damage and persistent DDR signaling, but they eventually engage the p53/p21 and/or p16^{INK4a}/pRB pathways. Loss of tumor suppressors, such as *PTEN* and *NF1*, can also have the same effect. Epigenetic perturbations, for example global chromatin relaxation, from broad-acting histone deacetylase inhibitors, could induce senescence by activating p16^{INK4a} (Campisi et al, 2013, Kuilman et al, 2010).

Cyclin-dependent kinase inhibitors and cell cycle arrest: The p16 and p53 pathways play a major role in human cell senescence. The classically defined cell cycle arrest in G1 phase is a result of accumulation of cyclin-dependent kinase (CDK) inhibitors, thereby preventing DNA replication (Figure 4). Cyclin-dependent kinase inhibitor 2A (*CDKN2A*) gene encodes for two tumor suppressor proteins through alternative splicing: protein p16^{INK4A}, a CDK4/CDK6 inhibitor that activates Retinoblastoma family of proteins, which, in turn, inactivate E2F, the

family of master transcription factors for S-phase, and p14^{ARF}, which inhibits the p53 negative regulator mouse double minute 2 homolog (MDM2) and therefore activates p53. TP53 codes for p53, which, through targeting the cyclin-dependent kinase (CDK) inhibitor p21, can inhibit CDK1, CDK2, CDK4 and CDK6 (Salama et al, 2014). Interestingly, senescence could be also induced during a prolonged G2 arrest mediated by p21 (Gire and Dulić, 2015) and G1 arrest can follow a mitosis skip after the transient activation of p53 at G2 leading to tetraploid G1 cells (Johmura et al, 2014). *CDKN2A* and *TP53* are the most commonly defective genes in human cancer (Ben-Porath and Weinberg, 2005) underlying the importance of senescence in tumor suppression.

Figure 4. Cell cycle and associated cyclin-dependent kinases/cyclin complexes. In G₁ phase cyclin D partners with CDK4 or CDK6 to promote cell cycle. In S phase, cyclin A partners with CDK2. In G₂ phase, cyclin A partners with CDK1. Adapted from García-Reyes et al, 2018.

Senescence markers: Senescent cells are characterized by their long-term exit from cell cycle, though this ability cannot be solely used for their identification. Morphological changes occur and senescent cells have been typically described as large and flattened with vacuolization. It is believed that, due to this enlargement, there is a compensatory activation of lysosomal enzymes, including β -galactosidase, whose activity can be measured at the suboptimal pH 6 and increased staining for β -galactosidase is observed. Multiple markers, including markers for DNA damage

(increased p53 and γ -H2AX), lack of proliferation marker Ki67 and cell cycle regulation proteins (high p16^{INK4A}) have been necessary for identification of senescent cells *in vivo* even if they are not exclusive to this state. Moreover, senescent cells often show altered chromatin structures, as punctate staining patterns of DNA dyes, termed senescence-associated heterochromatic foci. The changes in transcriptomes of senescent cells result in the senescence-associated secretory phenotype, which comprises of proinflammatory cytokines, chemokines, growth factors and proteases (Schosserer et al, 2017; Kuilman et al, 2010).

Senescence *in vivo*: The extent to which replicative senescence takes place *in vivo* is debated since many cell types do not exhaust their replicative potential and experimental evidence is lacking (Schosserer et al, 2017). Senescence *in vivo* is believed to be stress-induced, mainly due to various endogenous and exogenous stimuli as reactive oxygen species, radiation, cytotoxic compounds, oncogene activation and tumor suppressor loss (Campisi et al, 2013).

Senescence in cancer: Senescence has been traditionally viewed as a protective mechanism against malignant transformation (Sager, 1991; Mooi et al, 2006). However, growing evidence show that senescent cells could also generate a pro-tumorigenic microenvironment especially in older age. Senescent cells are shown to be liable to genetic and epigenetic instability and the senescence-associated secretory phenotype (SASP) could directly transform neighboring cells and destroy the extracellular matrix (Schosserer et al, 2017).

Conclusion: Taken together, the senescent cells are difficult to identify, because, on one hand, their phenotype is heterogeneous and dynamic and, on the other hand, their morphological and molecular features are also present in other cellular states (Lee et al, 2019). Currently, the use of multiple markers in the same sample is the method to identify senescent cells (Sharpless et al, 2015). Omics techniques could help the discovery of novel senescence-associated markers (Hernandez-Segura et al, 2018). In this study, transcriptomic analysis was chosen to identify markers associated to melanocytic senescence.

Melanocyte development

Studies in mouse, chicken and zebrafish have provided important insights on melanocyte development and differentiation, melanocyte stem cells and the role of the melanocyte lineage in melanoma (Mort et al, 2015).

Neural crest cells: Melanocytic nevi and melanomas originate from melanocytes. These cells are derived from the neural crest (Figure 5), a transient structure of migratory cells of neuroectodermal origin unique to vertebrates. Neural crest cells, under certain spatiotemporal control, adopt various cellular fates including melanocytes, peripheral neurons, glial cells, craniofacial bone and cartilage, adipose tissue, cardiac smooth muscle cells and secretory adrenal cells. The dorsolateral path is restricted to melanoblasts, the melanocytes' precursors, which populate somite-derived dermis (Mort et al, 2015; Vandamme and Berx, 2019). There is evidence that the ventral pathway, classically seen as the migration route for neurogenic cell populations (glial cells and peripheral sensory neurons), also gives rise to Schwann cell precursors (SCP), which after detachment from growing nerves innervating the lateral plate mesoderm-derived dermis, can differentiate to melanoblasts (Adameyko et al, 2009; Furlan et al, 2019).

Figure 5. Model for avian neural crest migration. The dorsolateral and ventral migration route of neural crest cells. *No* notochord (from paraxial mesoderm), *Nt* neural tube, *MSA* migratory staging area, *S* sclerotome, *DM* dermomyotome (somite from paraxial mesoderm), *LPM* lateral plate mesoderm, *SCP* Schwann cell precursor. Adapted from: Vandamme et al, 2019.

Melanoblasts: The microphthalmia-associated transcription factor (MITF) is a master gene regulator of the melanocyte lineage and the first expressed melanocyte-specific gene in melanoblasts. Transmigration from dermis to epidermis through the basement membrane is driven by c-KIT. The melanoblasts then proliferate and differentiate massively, a process driven also by c-KIT. Dermal melanoblasts show an asymmetric division pattern with one daughter cell being competent to transmigrate to epidermis and the other slow-cycling in the dermis, a process dependent on endothelin-3 (ET3) signaling. The epidermal melanoblasts are distributed throughout the epidermis and cluster in the developing hair follicles segregating into distinct melanocytic populations: the hair follicle melanocytes localizing in the lower part of hair follicle where they differentiate to mature melanocytes responsible for hair pigmentation, the melanocyte stem cells within the hair follicle bulge and the interfollicular epidermal melanocytes that contribute to skin pigmentation. (Furlan et al, 2019; Vandamme and Berx, 2019; Sommer et al, 2011).

Nerve-derived melanocytes: Beside the skin, melanocytes also inhabit other body sites, such as heart (Levin et al, 2009; Yajima and Larue, 2008), inner ear (Steel and Barkway, 1989) and the central nervous system meninges (Goldgeier et al, 1984). In addition, 5% of melanomas have unknown primary site (Hussein, 2008), although some data suggest that they could also derive from the ventral pathway for neurogenic cell populations (Adameyko et al, 2009; Furlan et al, 2019).

Melanocyte stem cells: KIT is required for the survival of the melanocytes, but a KIT-independent cell population resides in the hair follicle, which is identified as melanocyte stem cells (MSCs). They are undifferentiated cells, located within the bulge region of the hair follicle and in a quiescent state until activated in the following anagen phase of hair cycle (Nishimura et al, 2011). A gradual depletion in hair follicle MSCs occurs during hair greying in humans in ageing and after irreparable DNA damage, such as by ionizing radiation. This is caused by differentiation of MSCs into mature melanocytes in the niche, a distinct process from the pathways triggering apoptosis or senescence (Nishimura et al, 2005; Inomata et al, 2009). MSCs have the potential to directly migrate to the epidermis in a MC1R-dependent process to provide melanocytes during wound repair or following UVB-radiation (Chou et al, 2013).

In addition, there is evidence that MSCs could reside in extrafollicular locations. It is shown that dermal stem cells might give rise to extra-follicular epidermal melanocytes (Li et al, 2010). Also, the secretory lower portion of the sweat glands could provide the epidermis with differentiated melanocytes. This niche can also maintain melanoma precursor cells explaining the characteristic dermoscopic parallel ridge pattern in acral melanomas (Okamoto et al, 2014).

Epidermal melanocytes: In postnatal skin, the melanocytes reside within the basal layer of the epidermis in a density of 1,500 cells per square millimeter of human epidermis dividing less than twice a year. UV radiation induces DNA damage to keratinocytes and in a p53-dependent manner α -melanocyte stimulating hormone (α MSH) is secreted, which subsequently binds to the melanocortin 1 receptor (MC1R) on melanocytes inducing melanin synthesis. Melanin is then delivered to keratinocytes to protect their nucleus from UV radiation (Shain et al, 2016).

Melanocytic nevi

Models of nevogenesis: Melanocytic nevi are benign growth of melanocytes. On the one hand, a tumor progression model of melanoma formation describes an initiating mutation event in melanocytes, which undergo proliferation and eventually stabilize in nevi but continue growing in melanomas (Ross et al, 2011). This classical model is based on the epidermal melanocyte and suggests the malignant transformation to melanoma. On the other hand, the dermal precursor model postulates that melanocyte precursors from the neural crest mature upwards along peripheral nerves towards epidermis and, after acquisition of mutations, specific growth patterns are favored. Due to co-existent growth controlling pathways, growth would cease in benign nevi and continue in melanomas following this model. A third model suggests that some nevi might have a dual origin: a component resulting from intraepidermal melanocytes that would migrate downward into the dermis and a component from schwannian cells that would migrate upward (Masson, 1951).

A dual concept of nevogenesis is proposed based on the clinical, epidemiological, dermoscopic, histopathologic and genetic data (Zalaudek et al, 2007); via the endogenous pathway, nevi with a globular or structureless dermoscopic pattern arise during childhood and are thought to derive from dermal melanocytes that acquire the appearance of intradermal nevus. Via the exogenous pathway, nevi with a reticular dermoscopic pattern arise mostly during adult life and are thought to derive from epidermal melanocytes as a result of exposure to UV radiation.

Germline mutations and nevi: The number of nevi and their size are influenced by the germline genotype and significant nevus and melanoma susceptibility loci have been described. High-penetrance alleles, which have low population frequencies, are associated with large nevi and increased melanoma risk and include *CDKN2A*, cyclin-dependent kinase 4 (*CDK4*), telomerase reverse transcriptase (*TERT*) and protection of telomeres 1 (*POT1*). Low-penetrance alleles, which are more frequent, are associated with lighter skin type, decreased tanning ability and smaller melanoma risk. They include pigmentation genes, mostly *SLC45A2*, tyrosinase, *MC1R*, *OCA2* and agouti signaling protein (*ASIP*). Germline variants in these genes probably affect the mutation burden of cells by reducing the UV protection of keratinocytes. For example, melanocortin-1 receptor (*MC1R*) polymorphisms are associated to the incidence of *BRAF*^{V600E}

mutations via an increased pheomelanin production that triggers ROS production after UV exposure (Shain et al, 2016).

Somatic mutations in nevi: Benign melanocytic nevi typically harbor single mutations. Acquired nevi usually harbor a single *BRAF*^{V600E} mutation, which is fully clonal (Shain et al, 2016). Congenital and some acquired nevi harbor an *NRAS* mutation and Spitz nevi harbor an *HRAS* mutation or a kinase fusion of *ALK*, *BRAF*, *ROS1*, *NTRK1*, *NTRK3*, *MET* or *RET*. Blue nevi harbor a *GNAQ* or *GNAI1* mutation (WHO, 2018).

Mutations in components of MAPK pathway have been described in both melanocytic nevi and melanomas and are known to promote cellular proliferation. Studies show that 79% of acquired nevi harbor the *BRAF*^{V600E} mutation and body areas of intermittent sun exposure show more frequently *BRAF* mutations than areas of chronic sun damage (CSD) (Ross et al, 2011). The age distribution of nevi with this mutation arises during the first decades of life and peaks in non-CSD melanomas two to three decades later dropping off after the sixth decade (Shain et al, 2016). *BRAF*^{V600E} hot-spot results from a T→A transversion, which is, though, not a common UV signature and, as it has been proposed, it could involve UVA radiation generating reactive oxygen species (ROS).

Histology of nevi: Nevi can be junctional, compound and dermal depending on the localization of the melanocytes. Two histological patterns have been observed; the lentiginous pattern comprising of an increased number of melanocytes arranged as individual units within the stratum basale (simple lentigo) in combination with small nests of melanocytes (lentiginous melanocytic nevus) and the nested pattern with predominant nests of melanocytes at the dermo/epidermal junction. With increasing distance from the epidermis, melanocytes in the dermis diminish in cell size and pigmentation; in the upper dermis, large epithelioid type A nevus cells are arranged in nests, then small lymphocyte-like type B cells are observed and in the deeper dermis spindled type C cells show signs of Schwannian differentiation with less nesting (WHO, 2018).

Growth in nevi: Existing evidence supports that the constituent melanocytes of nevi retain the ability to grow. A small fraction of explanted melanocytes from nevi are capable of proliferation

for one or a few passages (Soo et al, 2011). In histological sections, it has been shown that 4-19% of common nevi can be mitotically active, the mitoses occur mostly within the upper half of the dermis and they are usually devoid of copy number abnormalities (Gerami, 2009; Jensen et al, 2007; Glatz et al, 2010). Proliferation markers, as Ki-67, have been shown to stain positive for less than 1% of the nevus cell population, though significantly less than in melanoma (Soyer et al, 1989; Rudolph et al, 1997). Studies of UV-irradiated nevi have shown that there is an increase in the number of Ki-67 stained melanocytes as response to a single UV irradiation, though a slight p53 increased staining was observed too (Rudolph et al, 1998; Tronnier et al, 1997). In clinical practice, it has been observed that nevi can recur after incomplete removal (Kornberg et al, 1975). New nevi may occur in pregnancy and the preexisting can enlarge. They more likely have dermal mitoses and there is a trend toward increased Ki-67 proliferation index (Chan et al, 2010; Martins-Costa et al, 2019). The sudden occurrence of new nevi has been reported in association with blistering diseases, immunosuppressive therapy, chemotherapy and immunodeficiency (Burian et al, 2019). Dermoscopic surveillance of nevi show that 31-69% of nevi may change over time (Braun et al, 1998; Kittler et al, 2000; Haenssle et al, 2010).

Involution in nevi: On the other hand, it is observed that nevi can involute and different clinical patterns have been described (Terushkin et al, 2010). Histologically, a dense infiltrate of helper (CD4+), cytotoxic (CD8+) T cells and macrophages fills the dermis between the nevus cell nests, degenerating or apoptotic nevus cells with pyknotic nuclei and hypereosinophilic cytoplasm may be identifiable and gradually the dermal nevus cells are replaced by fibrous tissue or adipocyte suggesting a specific cell-mediated immune response (Barnhill et al, 2014). This could be linked to the fact that senescent cells secrete inflammation-related factors, as interleukin (IL) - 6, which could amplify the activation of the inflammatory network, including IL-8. (Kuilman et al, 2008). Interestingly, it was shown that pre-malignant senescent cells are subject to immune-mediated clearance, which depends on an intact CD4+ T-cell-mediated adaptive immune response, establishing senescence surveillance as an important component against tumorigenesis (Kang et al, 2011).

Senescence in nevi: Common acquired nevi arise in the first decades of life and have a tendency to regress with age (Schäfer et al, 2006; Zalaudek et al, 2011). Most remain unchanged for years and seem to have entered in a growth arrested state. Nevi show a mosaic pattern of p16

immunopositivity irrespective of the BRAF mutational status, without significant upregulation of p53 and p21, and induce the SA- β -Gal activity (Michaloglou et al, 2005). They show negative immunohistochemical staining for the proliferation marker Ki-67, (Michaloglou et al, 2005) and show prominent nucleoli, large cell and nuclear size and sometimes multinucleacy (Bennett et al, 2016).

Taking into account that a common acquired nevus can often contain more than 10^6 cells, it is estimated that 20 or more cell doublings are required to produce them. This indicates that the oncogenic mutation initially promotes proliferation (Bennett et al, 2016) followed by growth arrest. It could be argued that nevi have undergone replicative senescence triggered by telomere attrition as a result of the initial proliferation of melanocytes (Bastian, 2003b). Taken into consideration that telomere length decreases with age (Slagboom et al, 1994), this would implicate that a person's age when a nevus is initiated is related to the doublings the nevus cells undergo. Indeed, this fits well with the clinical observation that congenital nevi can become very large, in contrast to nevi acquired later in life, which do not exceed a few centimeters (Bastian, 2003b). Furthermore, there is epidemiological evidence that telomere length is associated with high nevus counts and larger nevus size, suggesting a delayed melanocytic senescence *in vivo* (Bataille et al, 2007), which could give time for further mutations to occur increasing the risk of malignancy. Also, telomere FISH (fluorescent in situ hybridization) on congenital, common and Spitz nevus sections showed no differences in telomere fluorescence when comparing to surrounding cells and control skin, in contrast to melanoma samples, which showed significantly less telomere fluorescence (Michaloglou et al 2005, Miracco et al, 2002). On the other hand, replicative senescence is latent in nature and critical telomere shortening occurs after approximately 60 population doublings; though, even if all constituent melanocytes proliferate equally, this exceeds the expected occurring doublings in a nevus (Bastian et al, 2014). Therefore, nevi do not seem to suffer from telomere attrition arguing in favor of an oncogene-driven senescent (OIS) state rather than the induction of replicative senescence (Michaloglou et al, 2005).

G1 arrest may not be the only senescence state in nevi. The presence of multinucleated melanocytes (Bennett et al, 2002) in addition to the usually observed features of flattened cell shape, increased cell size and β -Gal staining may be evidence of a non-G0 senescence

mechanism (Ross et al, 2011). Such phenotype would be induced during a prolonged G2 arrest mediated by p21 (Gire and Dulić, 2015) and G1 arrest following mitosis skip after the transient activation of p53 at G2 (Johmura et al, 2014).

Intermediate melanocytic lesions

In 1978, a phenotype of distinctive melanocytic nevi was first described among patients from melanoma families, termed B-K mole syndrome (Clark et al, 1978) and familial atypical multiple-mole syndrome (Lynch et al, 1978). It soon included patients with sporadic melanoma, as well as without melanoma (Elder et al, 1980). Since then, numerous studies on these nevi have been published (Duffy et al, 2012) and the question whether they represent premalignant lesions that may progress to melanoma remains (Barnhill et al, 2010). Their broader significance lies on their relation to melanoma as morphological simulants, biomarkers of increased risk and potential precursors of melanoma.

WHO definition: According to the latest WHO classification (WHO, 2018), dysplastic nevi (DN) are defined as melanocytic nevi which are clinically atypical and histologically characterized by architectural disorder and cytological atypia, always involving the junctional component. They can occur de novo or in association with common or congenital pattern dermal nevi. In regard to the clinical-microscopic morphology and genomic aspects, they are considered intermediate, between common acquired nevi and radial-growth-phase melanoma (WHO, 2018).

Clinical morphology: Atypical nevus is a term frequently used clinically to raise suspicion of nevi likely to have dysplasia. According to the International Agency for Research on Cancer (IARC), in order to identify atypical nevi, a macular component should be present in at least one part of the lesion and at least three of the five following features: not-well-defined border, 5 mm or more in size, variegated color, uneven contour and erythema (Gandini et al, 2004).

Epidemiology: Most epidemiologic studies have based on clinical examination without histologic evaluation (Duffy et al, 2012). However, there is a poor concordance between the use of this clinical term and the histologic diagnosis of DN (Gandini et al, 2004). Therefore, the prevalence of DN is unknown. They are described to develop in adolescence and decrease with increasing age partly because of involution and a cohort effect, i.e. larger nevus numbers in younger persons (Halpern et al, 1993).

Histopathology: The diagnostic criteria of DN have been developed and validated by the International Melanoma Pathology Study Group (IMPSG) (Shors et al, 2006; Xiong et al, 2014; WHO, 2018). DN are bigger than 4 mm in fixed sections and histologically characterized of architectural disorder and cytological atypia (Elder et al, 2010). The architectural disorder is characterized of irregular, dyscohesive nests of intraepidermal melanocytes and increased number of non-nested junctional melanocytes. The presence of cytological atypia is categorized depending on nuclear features, i.e. nuclear size vs. resting basal cells, chromatism, variation in nuclear size, nuclear shape and nucleoli (WHO, 2018). The consensus meeting Working Group has recently recommended to abandon the term mildly dysplastic nevus, which is re-classified as lentiginous nevus and, instead, the use of low- (previously moderate) and high- (previously severe) grade dysplasia is proposed (WHO, 2018).

Genomic aspects of DN: Similar to common nevi, 60-80% of DN harbor activating mutations in *BRAF* (Pollock et al, 2002; Uribe et al, 2006; Wu et al, 2007), rarely (ca 5%) *NRAS* (Papp et al, 2003 & 2005), and ca 60-70% of common and dysplastic nevi retain expression of *PTEN* without significant differences (Tsao et al, 2003; Singh et al, 2007). It appears that DN have higher rates of staining with proliferation markers, though lower than melanoma, and staining of apoptosis-related markers is similar to common nevi as shown in different immunohistochemistry studies (Duffy et al, 2012). DN seem to rarely have heterozygous *CDKN2A* loss, but the number of nevi tested is small to conclude if their incidence is increased in DN compared to common nevi (Lee et al, 1997; Papp et al, 2003; Wang et al, 2005). Genetic alterations in *TP53* gene were also found in very few samples of dysplastic nevi (Levin et al, 1995; Lee et al, 1997; Papp et al, 2003). DN express p16 protein, in a patchy pattern and the staining is cytoplasmic, which could indicate a dysfunctional protein (Gray-Schopfer et al, 2006). A proportion of DN show only a few areas of p53 staining, nuclear or nuclear and cytoplasmic, often without p21. For comparison, p16 staining is nuclear as well as cytoplasmic in common nevi, while p53 and p21 are not stained. These observations could argue that DN have areas still proliferating than in senescence (Gray-Schopfer et al, 2006). The apoptotic cells are few in dysplastic nevi and their number does not decrease in melanoma, instead proliferation increases in melanoma (Gorgoulis et al, 2005). It is reported that melanocytic nevi, including DN, have a significantly lower mutation load than melanoma and a UV-associated mutational signature is verified (Melamed et

al, 2017). The correlation of the genetic alterations with the grading of dysplasia remains unknown (WHO, 2018).

DN as morphological simulants: Most studies show good intra-observer but poor inter-observer reproducibility of DN diagnosis due to overlapping histologic features of common nevi and DN, difficulty in grading DN and classifying severe dysplasia from melanoma (Duffy et al, 2012). The intra-observer reproducibility in the histopathological assessment of nevi with moderate on one hand and severe dysplasia or melanoma in situ on the other hand were only 35% and 60% respectively; the lowest in a series of different types of benign and malignant melanocytic lesions (Elmore et al, 2017).

DN as biomarkers of increased melanoma risk: The presence of DN is associated with 4-15-fold increased risk of sporadic melanoma and more frequently of the superficial spreading compared to nodular type (Duffy et al, 2012). In a study (Shors et al, 2006), mild dysplasia was not associated with increased melanoma risk, whereas moderate or severe dysplasia was associated with 4-fold increased risk. Though a follow-up study on the same database (Xiong et al, 2014), revealed that the diameter of the clinically atypical lesion itself was associated with melanoma suggesting that diameter is a stronger predictor than grading of dysplasia. Atypical nevi are distributed on intermittently sun-exposed body areas, mostly the back, and melanomas on these areas are associated to the presence of atypical nevi (Chiarugi et al, 2015). Though, no germline susceptibility loci unique to DN have been confirmed (Goldstein et al, 2015).

DN as potential melanoma precursors: The role of DN as potential precursors to melanoma lacks direct evidence, since it is not possible to identify a DN without histologic evaluation and a long-term monitoring in case of biopsy is precluded. In addition, there are no ideal models to study malignant transformation of a nevus to melanoma (Duffy et al, 2012). It is estimated that the lifetime risk for malignant transformation of a nevus on a 20-year-old-individual is 1 to 3,000 for men and 1 to 10,000 for women (Tsao et al, 2003). 30% of melanomas are reported to be histologically associated with nevi (Marks et al, 1990; Bevona et al, 2003; Shitara et al, 2014). Of these, 77% had common acquired, mostly intradermal type, and 23% congenital nevi, while 57% were non-dysplastic and 43% dysplastic nevi (Pampena et al, 2017) concluding that DN do not seem to be more associated to melanomas than common nevi. On the other hand, there is

heterogeneity in nevi-associated melanomas, which are mostly non-CSD type, with SSM much more frequent than nodular (Marks et al, 1990), whereas CSD melanomas do not have associated nevi (Shain et al, 2016). In addition, potential errors in reporting of nevus-associated melanomas could derive as a result of collision events of a melanoma with an adjacent nevus or the predominance of nevus remnants by the melanoma cells.

Genetic evolution as a possible model of melanomagenesis: A seminal paper provided insight of a non-obligate genetic evolution of melanoma by sequencing distinct areas of benign, intermediate and melanoma sites from melanomas with histologically distinct precursors (Shain et al, 2015). All benign sites harbored *BRAF*^{V600E} mutation, which was the only apparent pathogenic mutation. The intermediate sites harbored *NRAS* or other *BRAF* mutations as well as additional oncogenic alterations. *TERT* promoter mutations were the earliest secondary alterations, already in 77% of intermediate lesions and melanomas in situ. In these lesions, heterozygous loss of *CDKN2A* were commonly observed. As they evolved at later stages of progression, melanocytic neoplasms became polyclonal leading to tumor heterogeneity. Biallelic loss of *CDKN2A* was exclusive to invasive melanomas. Mutations in *SWI/SNF* chromatin remodeling genes were predominant in invasive melanomas, while losses of *PTEN* and *TP53* were uncommon and occurred in even thicker, invasive melanomas. Copy-number alterations were rare in benign sites, occasional in intermediate lesions and melanomas in situ and prevalent in invasive melanomas affecting larger genomic areas. The burden of point mutations was increasing with each histologic stage and UV signature was observed to occur in all stages implicating its role in both melanoma development and progression.

Consequently, two distinct evolutionary trajectories were observed; melanomas with *BRAF*^{V600E} associated with benign nevi and melanomas with *NRAS* or *BRAF*^{non-V600E} mutations associated with intermediate lesions or melanomas in situ, probably reflecting the differences in non-CSD and CSD melanomas respectively. Furthermore, it seems unclear why *TERT* promoter mutations would undergo so early positive selection in regard to the small number (several hundred thousand) of cells in these lesions and it could implicate a possible attrition of the constituent cells rather than a senescent state. This study identified intermediate lesions with distinctive histological features that harbor more than one pathogenic genetic alterations indicating that they

are biologically distinct (Shain et al, 2015). There are though insufficient data on the extent of non-melanoma-adjacent dysplastic nevi with similar genetic alterations (WHO, 2018).

Genetic characterization of other intermediate nevi: The genetic characterization of melanocytic neoplasms is expanding even in uncommon variants. In general, additional mutations to melanocytic nevus but less than melanomas have been observed in deep penetrating nevus (DPN), pigmented epithelioid melanocytomas and BAP1- inactivated tumors, all of which can be part of combined nevi. Wiesner et al. described, some combined nevi with a spitzoid component having loss of BAP1 expression with concomitant *BRAF*^{V600E} mutations (Wiesner et al, 2012). DPN show a combined activation of MAPK and WNT pathways. Additional alterations, as *TERT* promoter mutations and loss of *CDKN2A*, are shown in melanomas that arose from DPN (Yeh et al, 2017). Different genetic alterations have been also found in pigmented epithelioid melanocytomas (Cohen et al, 2017). A proposed term by the Working Group for these lesions is melanocytomas (WHO, 2018). The current evidence has been, though, too limited to provide insight into these lesions' true biologic potential.

Dilemmas: The genetic profiling of melanocytic lesions challenges the classification systems of benignity and malignancy and suggests a biologically intermediate category with different phenotypic and genetic profiles. Approaches to aid the diagnosis of difficult melanocytic lesions could eliminate the lesions that currently are classified in the intermediate category.

Molecular landscape in melanoma

The NGS approaches in melanocytic neoplasms have expanded the current knowledge from the clinical and histological features to the molecular alterations, offering insights into their biological implications (Palmieri et al, 2018). The results from whole exome and whole genome sequencing studies in melanomas are available (Berger et al, 2012; Hodis et al, 2012; Krauthammer et al, 2012; TCGA, 2015; Hayward et al, 2017).

UV signature: It is estimated that 75% of genomic sequence variations are C→T substitutions and <5% CC→TT, as a result of the mutagenic UV-radiation. This makes melanoma the cancer with the highest mutational burden (Palmieri et al, 2018). More than 90% of the *BRAF*^{mut} and *NRAS*^{mut} melanomas harbor a UV signature (TCGA, 2015) and UV-radiation is thought to predominate during the stages of melanoma development and nevus initiation, since even *BRAF* and *RAS* could be attributed to UVA/UVB damage (Hodis et al, 2012).

Chromosomal instability: Even though random DNA copy number changes are observed within nevus cells, clonal chromosomal aberrations appear at the melanoma stage (Bastian et al, 2003a). Chromosomal aberrations are significantly higher in acral and mucosal melanomas in comparison to CSD/non-CSD melanomas (Curtin et al, 2005). It is suggested that chromosomal instability predominates in the progression of invasive melanoma (Shain et al, 2015).

***BRAF* mutations:** *BRAF* encodes for a serine/threonine protein kinase, critical in the regulation of the mitogen-activated protein kinase (MAPK) pathway. It is activated by RAS protein and in turn activates MEK1-2 kinases, which activate ERK1-2. *BRAF* mutations, which were first characterized in 2002, are reported in 50-66% of melanomas (Davies et al, 2002; Palmieri et al, 2018) and at lower frequency in various cancers. They are all within the kinase domain, with V600E (previously reported as V599E) mutation being the most frequent (90%). A few additional mutations are at codon 600 and more rarely in other codons within the kinase region. This single substitution of valine (V) by the acidic glutamic acid (E) at amino acid 600 mimics phosphorylation and hence activates the kinase (Davies et al, 2002). Like melanomas, 82% of nevi harbor the activating *BRAF*^{V600E} mutation (Pollock et al, 2002). Therefore, the mutation of *BRAF* gene and activation of the MAP kinase pathway is a crucial step to promote melanocytic

proliferation. Yet, it is insufficient for melanoma development. *BRAF*^{V600E} mutations in melanoma are more frequently identified in younger patients and sites with intermittent sun exposure (Palmieri et al, 2018); these non-CSD melanomas can be associated with nevi. In contrast, high-CSD melanomas have other *BRAF* mutations, activating *NRAS* or *KIT* mutations or loss of function in *NF1*, they occur in older patients and are not associated with nevi (WHO, 2018). *BRAF*^{mut} melanomas have more commonly *PTEN* and *CDKN2A* alterations in comparison to *RAS*^{mut} and *BRAF*^{non-mut}/*RAS*^{non-mut} (Palmieri et al, 2018). It controversially seems that *BRAF* mutation has no prognostic significance on progression free survival, but it has on the overall survival after melanoma progression (Long et al, 2011).

Figure 6. The Mitogen-activated protein (MAP) Kinase and Phosphatidylinositol 3' kinase (PI3K) pathways. Adapted from Curtin et al, 2005.

RAS mutations: The RAS family of G-regulatory proteins act through the MAPK and PI3K pathways activating the cytoplasmic proteins RAF and PI3K respectively (Figure 6). They comprise of the Neuroblastoma *RAS* viral oncogene homolog (*NRAS*), the Harvey *RAS* viral oncogene homolog (*HRAS*) and the Kirsten *RAS* viral oncogene homolog (*KRAS*). *NRAS* mutations are the second (20%-28%) most common mutations in melanoma after *BRAF* in mutually exclusive fashion (Hodis et al, 2012), whereas *HRAS* (1.5%) and *KRAS* (1%) are observed much less (Palmieri et al, 2018). *NRAS* mutations have also been described in congenital melanocytic nevi (Kinsler et al, 2013). The mutations are mostly (90%) found in codon Q61; a 10% of the mutations are at codons G12 or G13. This mutation alone is also

insufficient for melanoma development (Shain et al, 2015). There are similar frequencies in both CSD and non-CSD melanomas (Palmieri et al, 2018).

***BRAF*^{non-mut}/*RAS*^{non-mut} melanoma:** Samples with wild-type *BRAF* and *NRAS* have not activated the pathway upstream of phosphorylated ERK, as suggested by its reduced immunohistochemical expression in comparison to the mutated forms. Though, *CCND1* amplification is more common (12.5%) in *BRAF*^{non-mut}/*RAS*^{non-mut} in CSD-melanoma compared to *BRAF*^{mut} (1.5%) and *RAS*^{mut} (5.2%) (Lee et al, 2015). Cyclin-dependent kinase 4 (CDK4) is a serine-threonine kinase which gets activated after binding to cyclin D1 and governs the G1/S checkpoint. Its amplifications are significantly more frequent in acral and mucosal melanomas than other cutaneous melanomas. None of the samples with *CDK4* amplifications had mutations in *BRAF*, *NRAS* or *CCND1* amplifications in a study (Curtin et al, 2005) implicating *CCND1* and *CDK4* as independent oncogenes in melanoma.

Nevi, as well as melanomas, commonly activate MAPK signaling. The coexistence of *BRAF* and *RAS* mutations are not frequent and when they occur (<5% of melanomas) the one component is non-activating. Therefore, it is suggested that cutaneous melanomas are classified as *BRAF*^{mut}, *RAS*^{mut} and *BRAF*^{non-mut}/*RAS*^{non-mut} (Palmieri et al, 2018). The intensity of MAPK signaling is, though, increased with the progression to melanoma. It seems that activating mutations in MAPK signaling are present from the start of melanocytic neoplasia, but this activation escalates from premalignant to more advanced stages of melanoma (Shain et al, 2018).

***NFI* mutations:** Neurofibromin 1 (*NFI*) is a tumor suppressor gene encoding for GTPase-activating protein, which helps convert RAS-GTP to the inactive RAS-guanosine diphosphate. Mutation in *NFI*, resulting in its inactivation, is the third most common mutation in melanoma and accounts for 14% of melanomas (TCGA, 2015; Palmieri et al 2018). Hence, the majority of melanomas have the *BRAF*, *NRAS* and *NFI* mutations affecting the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) signaling pathways. There is an evident UV signature in *NFI*^{mut} melanomas, which also have higher mutational burden and are associated to increased resistance to BRAF and MEK inhibitors. There are *NFI* mutations in *BRAF*^{mut} and *RAS*^{mut} melanomas (Palmieri et al 2018).

***KIT* mutations:** *KIT* proto-oncogene (*KIT*) encodes a transmembrane receptor tyrosine kinase, which binds to the stem cell factor, forms a dimer, autophosphorylates and consequently activates proteins in signaling pathways. 2.2% of *KIT* mutations and 3.1% of copy number increases, mostly in *BRAF*^{non-mut}/*RAS*^{non-mut}, are observed and result in induction of MAPK and PI3K pathways (Palmieri et al, 2018). They occur in 39% in melanomas of the mucosa, 36% of acral skin, 28% of chronically sun damaged skin and none of skin without chronic sun damage. It is observed that these melanoma types usually have a lentiginous growth pattern (Curtin et al, 2006).

***CDKN2A* mutations:** Cyclin-dependent kinase inhibitor 2A (*CDKN2A*) gene encodes for two tumor suppressor proteins through alternative splicing: protein p16^{INK4A}, a CDK4/CDK6 inhibitor that activates retinoblastoma (RB), and p14^{ARF}, which inhibits the p53 negative regulator mouse double minute 2 homolog (MDM2) and therefore activates p53. *CDKN2A* alterations are distributed in high frequency (40-70%) across all *BRAF*, *NRAS*, *NF1* and triple-WT melanomas (TCGA, 2015; Palmieri et al, 2018). Bi-allelic inactivation of *CDKN2A* are very frequent in invasive melanoma and negative reactivity for p16^{INK4A} correlates with invasive melanoma (Shain et al, 2015). *CDKN2A* has mutations in 13.6% of cases, mostly in *RAS*^{mut} melanomas, and deletions in 41.4% of cases mostly in *BRAF*^{mut} melanomas. In addition, there is family history of melanoma in 10% of melanoma patients and 20-40% of those carry germline *CDKN2A* mutations affecting the p16 but not p14 protein (Bastian et al, 2016). 2-3% of familial melanoma patients have *CDK4* germline mutations in the p16 binding domain and similarly there are *CDK4* mutations that disable p14 (Bastian et al, 2014).

p16^{INK4A} expression is significantly decreased in melanomas compared to nevi as a result of the bi-allelic inactivation of *CDKN2A* in invasive melanoma, but even in the absence of genetic alterations suggesting also epigenetic changes (Shain et al, 2018). Melanocytic nevi do not seem to have somatic alterations in *CDKN2A* gene, though melanoma in situ and intermediate nevi are typically heterozygous. This implicates the impairment of cell cycle regulation at the transition to invasive melanoma.

***PTEN* mutations:** Phosphatase and tensin homolog (*PTEN*) encodes for a phosphatidylinositol-3,4,5-trisphosphate (PIP3) 3-phosphatase which dephosphorylates PIP3 resulting in the

biphosphate product PIP2 and inhibition of the phosphatidylinositol 3-kinase (PI3K) pathway. 14% of melanomas consist of both disabling *PTEN* mutations (7.9%) and focal deletions (18.2%) (TCGA, 2015; Palmieri et al, 2018) upregulating the activity of protein kinase B (Akt), antiapoptotic pathways and growth via the mechanistic target of rapamycin (mTOR). *PTEN* alterations are more frequent in thicker primary melanomas and melanoma metastases (Shain et al, 2015). *BRAF* mutant melanomas have less *PTEN* gene copies than *NRAS* mutant melanomas (which can independently activate the PI3K pathway) resulting in expression of phosphorylated protein kinase B (Akt) in both groups (Curtin et al, 2005).

***TP53* mutations:** Tumor protein 53 (*TP53*) disabling mutations occur in 17-19% of melanomas (Hodis et al, 2012) and are found in 10-16% of *BRAF*^{mut}, 17-20% in *RAS*^{mut} and 19.1% in *BRAF*^{non-mut}/*RAS*^{non-mut} (TCGA, 2015; Palmieri et al, 2018). Most melanomas with *TP53* mutation do not have a concurrent mutation in *CDKN2A* implicating that, due to the frequent deletion of *CDKN2A* locus, the genetic pressure to mutate *TP53* is reduced (Hodis et al, 2012). The *TP53*^{mut} melanomas have high mutation counts and C→T transitions. *TP53* mutations are less frequent in primary than metastatic melanoma (Shain et al, 2015). p53 is, thus, accumulated in inactive form in later stages of melanoma progression. Overexpression of this protein is not observed in benign or dysplastic nevi in contrast to melanomas, especially metastatic, which show the highest p53 expression (Lassam et al, 1993). Therefore, p53 pathway defects may account more as antiapoptotic than antisenesence changes (Bennett et al, 2015). Interestingly, sensitization of *BRAF*^{mut} melanomas to BRAF inhibitors can occur after restoration of the intracellular p53 levels (Krayem et al, 2016).

Thick melanomas have more frequently mutations in p53 or PI3K pathways than earlier melanomas. This implicates that these mutations undergo positive selection later in the progression of melanoma (Shain et al, 2018).

***TERT* mutations:** Telomerase reverse transcriptase (*TERT*) provides instructions for the making of telomerase, which counteracts the shortening of telomeres. Mutually exclusive *TERT* promoter mutations, cysteine-to-threonine at codon 228 (C228T) and at codon 250 (C250T), consistent with an ultraviolet signature, are identified in 70-85% of *BRAF*^{mut} and *NRAS*^{mut} melanomas and, thus, usually co-exist with MAPK pathway activation (TCGA, 2015). *TERT*

promoter mutations occur in 77% of melanomas in situ and intermediate lesions adjacent to primary melanomas (Shain et al, 2015).

Telomerase starts becoming evident in intermediate neoplasms and is present in most melanomas. Sequentially, it seems that it follows MAPK activation (Shain et al, 2018).

Conclusion: NGS approaches have revealed a logical sequence of signaling pathways' perturbations that could exist among melanocytic nevi, intermediate lesions and melanoma. MAPK signaling pathway is activated and induces the formation of melanocytic nevus, which seems to be constrained by cell-cycle regulation. Heterozygous *CDKN2A* loss becomes present in intermediate lesions and melanoma in situ overriding the G1/S checkpoint. Telomerase is upregulated and invasive melanomas show biallelic inactivation of *CDKN2A* impairing even further the cell-cycle regulation and the accumulation of more mutations lead to escalation of MAPK pathway signaling and perturbations of p53 and PI3K pathways.

Aim of thesis

The **general aim** of this thesis was to identify novel senescence markers that distinguish melanocytic nevi from melanoma in order to improve the diagnosis of melanoma.

The **specific aims** of the included studies I, II & III were:

- I. To identify senescence-associated markers that distinguish melanocytic nevi from melanoma. Furthermore, to construct a cellular model system of melanocyte senescence, to mechanistically study the role of the selected tubulin β -3 marker and to test its clinical applicability.
- II. To identify signaling pathways that contribute to initiation and progression of melanoma. Specifically, to investigate the relationship between cathepsins, TGF- β 1 and FAP- α after ultraviolet exposure.
- III. To evaluate if tubulin β -3 staining gradient has diagnostic value in distinguishing nevi from melanoma. Furthermore, to describe the patterns of tubulin β -3 immunostaining in melanoma, to find the dermoscopy-immunohistochemistry associations and to examine the prognostic value of this marker.

Materials and Methods

Ethical principles (Study I, II & III)

Approval was obtained by the Ethical Review Board in Linköping, Sweden (Ethical permission; Dnr 2013-31431 and 2014/408-31). The experiments were performed according to the ethical principles in accordance with the Declaration of Helsinki.

Cell cultures (Study I & II)

Pure melanocytic primary cultures were established, as previously described (Larsson et al, 2005), from skin tissues obtained from Caucasian donors (0-3 years of age, parental written informed consent) after foreskin circumcisions. The melanocytes were cultured at 37°C in a humidified atmosphere of 5% CO₂ in air in Medium 199 with supplements. The experiments were performed between passages 2 and 10. The cultures of young melanocytes were obtained 3 days after isolation and seeding, and the cultures of senescent melanocytes were isolated when the cells were flattened and non-proliferative, i.e. a senescent-like morphology, after a minimum of 30 days.

The melanoma cell lines used were: WM115, WM793, WM278, and FM55P (provided by Prof Meenhard Herlyn, Wistar Institute, Philadelphia, PA, USA). The fibroblasts and melanoma cells were cultured in DMEM with supplements (10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin).

When indicated, prior to the experiments, vinblastine (100 nM, 24 h, stock in DMSO) and colchicine (10 µM, 30 min, stock in DMSO) microtubule-destabilizing drugs were used. For inhibition of the respective proteins, neutralizing anti-TGF-β1 (200 ng/ml; Santa Cruz, CA, USA), anti-FAP-α (20.6 µg/ml; Santa Cruz, CA, USA), anti-cathepsin D (42 µg/ml; Athens Research and Technology Inc., Athens, GA, USA) antibodies, inhibitors of cathepsin B (CA-074 Me; 1 µM; Calbiochem, San Diego, CA, US), cathepsin K (inhibitor II; 10 µM; Calbiochem, San Diego, CA, US) were used. Neutralization of lysosomal acidity was performed by NH₄Cl (10 mM, 30 min, Sigma, St Louis, MO, USA). Blocking of FAP-α activity was done by addition

of Gly-PhP(OPh)₂ (H₂N-Gly-Pro diphenylphosphonate; 100 µM; Gilmore et al, 2006). Recombinant TGF-β1 (10 ng/ml, Sigma) was used when indicated.

Reconstructed skin and *ex vivo* skin (Study II)

Reconstructed skin consisting of a fibroblast/collagen matrix in the bottom covered with a layer of melanocytes and keratinocytes was prepared as previously described (Li et al, 2011) allowing to form an epidermis-like layer with cornified keratinocytes. Excess skin from breast reduction plastic surgery was used as *ex vivo* skin. 4 mm punch biopsies were taken and placed in inserts with reconstructed skin medium II (Li et al, 2011).

The samples were UV-radiated, fixed, embedded in paraffin and immunohistochemically stained using a monoclonal anti-mouse primary antibody anti-FAP-α (1:100, sc65398, Santa Cruz Biotechnology) and polyclonal anti-rabbit primary antibody anti-tyrosinase (1:50, ab175997, Abcam, Cambridge, UK).

UV radiation (Study II)

Philips TL20W/12 tubes (spectral range 280–370 nm) for UVB radiation (main output 305–320 nm) and Medisun 2000-L tube (Dr Gröbel UV-Elektronik GmbH, Ettlingen, Germany; 340–400 nm) with Schott WG 305 cutoff filter (Mainz, Germany) for UVA radiation were used. The used radiation doses of UVA 6 J/cm² (80 mW/cm²) and UVB 60 mJ/cm² (1.44 mW/cm²) were sublethal (Larsson et al, 2005). Exposure was performed in phosphate buffered saline (w/o NaHCO₃). Non-irradiated controls (sham) were analyzed in parallel.

β-Galactosidase assays (Study I)

For β-Galactosidase activity, cell lysis was performed in 250 mM Tris, 0.1% Triton X-100, and 1 mM Pefabloc supplemented with 4-MU β-D-galactopyranoside diluted in 0.2 M sodium citrate buffer, pH 4.5. Incubation of the lysate for 40 min at 37°C in the dark followed. Stop solution of 1 M glycine-NaOH buffer was then added and the fluorescence intensity was measured (λ_{ex}355/λ_{em}460 nm). The activity was correlated to the total amount of protein.

For β -Galactosidase staining, as described before (Dimri et al., 1995), cells were fixed in formaldehyde and incubated with fresh senescence-associated β -Galactosidase stain solution at 37°C. Images were taken using an Olympus BX51 Microscope (Olympus, Tokyo, Japan).

Whole human genome microarray analysis (Study I &II)

Total RNA was extracted from young and senescent melanocyte cultures from four different donors 3 and 30 days after seeding, respectively (TRIzol; Sigma, St Louis, MO, USA). An Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) was used to control its quality. At the Bioinformatics and Expression Analysis Core Facility (Karolinska Institute, Stockholm, Sweden), the Affymetrix Human Genome Microarray (WT GeneTitan ST1.1; Plate type, HuGene-1_1-st-v1-l6; Array type, HuGene-1_1-st-v1) was used according to the manufacturer's protocols (Affymetrix Inc., Santa Clara, CA, USA) for labeling and hybridization.

The generated raw CEL files were processed using the Agilent Genespring GX 13 software package (Agilent Technologies, Santa Clara, CA, USA). For exon-level data, the summarization method RMA16 was used (Bolstad et al., 2003), baseline transformation was performed to the median of all samples and entities were filtered based on their signal intensity values between the 20th and 100th percentiles in at least one sample. Quality-assessment of the data, before and after normalization, was performed through the inspection of hierarchical clustering trees and principal component analysis (PCA) plots. For the gene-level data, normalization was based on a quantile algorithm (Bolstad et al., 2003) and baseline transformation was performed to the median of all samples. Statistical analysis to determine whether any observed differences in the data were significant was performed using unpaired t-test, the p-value computation was asymptotic and Benjamini Hochberg FDR method was used for multiple testing correction (Huang et al., 2009a). Entities that satisfied a fold change cut-off of at least ± 2 and corrected p-value ≤ 0.01 were included in further analyses. The data were deposited in the Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/e.bibl.liu.se/geo/>) with accession number GSE83922. The gene IDs were obtained through conversion of the EntrezGene IDs to official gene symbols.

Hierarchical clustering and heat map using Euclidean distance with Ward's linkage rule for the 351 differentially expressed genes was generated using heatmap.2 package in R software (version 3.3.1, <https://www.r-project.org>).

A public dataset, previously submitted by the scientific community (Talantov et al., 2005) in the Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) with the GSE3189 identifier, was loaded into the Agilent GeneSpring GX 13 software to acquire the expression dataset txt file. The used dataset comprised of gene expression profiles derived from the total RNA isolated from 45 primary melanoma and 18 benign skin nevi as analyzed on an Affymetrix Hu133A microarray containing 22,000 probe sets.

Gene ontology, network generation and upstream regulator analysis (Study I & II)

The Database for Annotation, Visualization and Integrated Discovery (DAVID, version 6.7, <http://david.ncifcrf.gov>) was used to find cluster of genes that showed significant functional annotation enrichment. The summarized version of Biological Processes in the Gene Ontology (<http://geneontology.org/>), GOTERM_BP_FAT, was used. To test its significance, DAVID used a hypergeometric test-based method to test the null hypothesis that the enrichment of an annotation is purely by chance. The test is measured by the p-value and Benjamini correction is performed for multiple test error (Huang et al., 2009a, b).

Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Qiagen, Hilden, Germany, <http://www.ingenuity.com/>) was used for network generation and upstream regulator analysis. The latter was used to predict which transcriptional regulators were involved for the observed gene expression changes. Two statistical measures were computed for each transcription regulator, the overlap p-value, measuring whether a statistically significant overlap between the dataset genes and the genes regulated by a transcription regulator by using Fisher's Exact Test, and the activation z-score, that determines whether an upstream transcription regulator has significantly more "activated" ($z > 0$) predictions than "inhibited" ($z < 0$) predictions testing the hypothesis that predictions are random with equal probability.

The IPA network generation analysis transformed a list of genes into a set of relevant networks using the list of the user selected genes (Focus Genes) and the Global Molecular Network, which is composed of thousands of genes and gene products that interact with each other. The IPA algorithm proceeded in several steps. Firstly, the selected genes were sorted in order of their inter-connectedness, measured by the number of triangles that contain a gene. Then, the networks were assembled using decreasingly connected genes from the sorted data until the maximum network size was reached. A metric called “specific connectivity” was used to pick the most connected gene to the growing network presuming that highly inter-connected networks are of biological importance. Additional genes from the IPA database were added to the networks to fill areas in order to merge smaller to larger networks and to grow networks by adding genes to the periphery of the network. The final step was calculation of p-scores ($p\text{-score} = -\log_{10}(p\text{-value})$) used to rank networks. They derived from p-values, the probability of finding f (f the number of Focus Genes in a network) or more Focus Genes in a set of n genes (n the number of genes in the network) randomly selected from the Global Molecular Network, using Fisher’s exact test.

Lysosome-associated genes that are up- and downregulated in young melanocytes and melanomas were identified from the intersection of the common differentially expressed genes (adjusted p-value below 0.05 and minimum fold change 1.5) in the public dataset of melanoma vs. nevi (Talantov et al., 2005) and the experimental system of young vs. senescent melanocytes with the lysosomal gene list. The derived gene set of interest (including cathepsins, TGF- β 1, FAP- α , lysosome-associated genes) were further used as focus genes and generated into relevant networks using the Global Molecular Network via the IPA network generation analysis.

Gene set enrichment analysis (Study I)

Gene set enrichment analysis (GSEA, version 2.2.0) was used to determine if the members of a defined set of genes (S) was randomly distributed throughout a ranked list of genes (L), ordered according to their differential expression between two classes (phenotypes) or primarily found at the top or bottom of this list (Figure 7) assuming that the sets related to this class distinction would show the latter distribution (Subramanian et al., 2005).

Figure 7. Overview of gene set enrichment analysis. (A) Expression data set sorted by correlation with phenotype, the heat map, and the location of genes from a set S within the sorted list L. (B) Running sum plot for S in the data set. Adapted from Subramanian et al., 2005.

The GSEA method comprised of three steps. Firstly, the enrichment score was calculated. It is a running sum statistic calculated by walking down the list L, increasing it when a gene of the list L is in set S and decreasing it when it is not in S. The maximum sum over the whole list L is the enrichment score. This corresponds to a weighted Kolmogorov-Smirnov-like statistic. Then, the significance level is estimated by permuting the phenotype labels for a large number of permutations and re-computing the enrichment score of the gene set for the permuted data, generating a null distribution for the enrichment score. The p-value of the observed enrichment score is calculated relative to this null distribution. At last, a correction for multiple hypothesis testing was made by normalizing the enrichment score for each gene set to account for the size of the gene set (normalized enrichment score, NES) and by calculating the false discovery rate to each NES, which is the estimated probability that a set with a given NES represents a false positive finding.

The genes in the gene set S, that are in the ranked list L at or before the point where the running sum reaches its maximum deviation from zero, are the core members of the gene set and define the leading-edge subset.

The hallmark gene sets represent well-defined biological states or processes. They are coherently expressed signatures generated by aggregating many gene sets from a collection of annotated

gene sets for use with GSEA software, the Molecular Signatures Database (MSigDB v4.0, collections C1 to C6).

Using GSEA, the Hallmark collection of gene sets was used to identify the common gene sets of the experimental system (young versus senescent melanocytes) and the public dataset (melanoma versus nevi) (Talantov et al., 2005). Also, the user-derived datasets of up- and down-regulated genes in the experimental system were used for enrichment analysis to the ranked list of genes of the public dataset (ordered according to their differential expression between the two phenotypic classes of nevi and melanomas). The results with false discovery rates ≤ 0.25 were considered significant.

Western blot and dot blot analysis (Study I & II)

Cell from cultures were used for immunoblot (Appelqvist et al., 2011). In study I, monoclonal mouse antibodies against p16 and p53 (Santa Cruz, CA, USA), monoclonal rabbit antibody against tubulin β -3 (Novus Biologicals, Littleton, CO, USA), polyclonal rabbit antibodies against Bid (BD Pharmingen, San Jose, CA, USA) and Ki-67 (Abcam, Cambridge, UK) followed by the corresponding horseradish peroxidase (DAKO, Solna, Sweden)-conjugated secondary antibody. As an internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Biogenesis, Poole, UK) was used. Image lab software (Version 4.1; Bio-Rad, Watford, UK) was used for densitometric quantification of the bands.

In study II, monoclonal primary antibodies against FAP- α (1:1000, Santa Cruz Biotechnology) and TGF- β 1 (1:1000, sc52893; Santa Cruz Biotechnology) followed by the corresponding HRP-conjugated secondary antibodies. GAPDH (1:50000, NB300–328, Novus Biologicals, Littleton, CO, USA) was the loading control. Gel-Pro Analyzer 3.1 (Media Cybernetics, Rockville, MO, USA) was used for densitometric quantification.

Collected and concentrated supernatants using Amicon Ultra-4 Centrifugal filter (5 kDa; Millipore Corporation, Billerica, MA, USA) at $4000 \times g$ for 30 min at 4 °C were used to analyze secreted proteins in the medium.

Nascent protein assay (Study II)

Pre-incubation of cells in methionine- and serum-free media for 60 min was applied, followed by incubation in 25 μ M Click-iT AHA (L-azidohomoalaine, C10102, Molecular Probes) with or without addition of recombinant TGF- β 1 (10 ng/ml) for 4 h, 37 °C. The samples were then lysed, sonicated and centrifuged at 15 000 g for 5 min at 4 °C. Click-IT ligation using Biotin conjugate and precipitation followed according to the manufacturer's protocol (B10184, B10185, Molecular Probes, Waltham, MA, USA). According to Pierce Classic IP Kit manual (26146, Thermo Scientific), further process of the samples was held for immunoprecipitation of biotin using Pierce Protein Streptavidin beads (20357, Thermo Scientific, Waltham, MA, USA).

Gene silencing (Study I & II)

5 nM *TUBB3* siRNA (SI02636655, SI02780883; Qiagen, Germantown, MD, USA) and 6 μ l HiPerFect Transfection Reagent was applied for siRNA transfection for 24 h. *FAP* siRNA sequences (SI00064050, SI00064057, 1 μ g of each; Qiagen, Germantown, MD, USA) and 6 μ l RNAiFect (Qiagen, Germantown, MD, USA) was applied. The transfection conditions were determined using the negative control, Alexa Fluor 555-labeled non-silencing siRNA, with a scrambled sequence and without homology to mammalian genes (AATTCTCCGAACGTGTCACGT, Qiagen). Lamin A/C siRNA (SI03650332, Qiagen) was the positive control, as recommended by the manufacturer.

Cell migration, proliferation and invasion assays (Study I & II)

Cultrex® 96-well migration assay (Trevigen Inc., Gaithersburg, MD, USA) and scratch assay were used to determine migration in study I and II, respectively. In the former, cells (5×10^4 cells/well) were grown in uncoated top invasion chambers with 8 μ m pores and the migrated cells to the bottom chamber were dissociated and fluorescence-labeled after addition of Calcein-AM. The fluorescence was analysed at λ_{ex} 485/ λ_{em} 520 nm using a VICTOR X3 multilabel plate counter (PerkinElmer, Waltham, MA, USA). Data were correlated to standard curves. Control and experimental replicates were performed in triplicate. For study II, the cell monolayers were scraped in a straight line creating a scratch with a p200 pipet tip (Liang et al, 2007). The cells were washed with PBS and fresh culture medium was added. The cell migration was determined

by following during 24 h the closure of the scratch and capturing images. The percentage of cells that invaded the scratch during 24 h was used as the outcome.

Cell proliferation in cultured cells was measured 24 h after seeding using the WST-1 proliferation reagent (1:10; Roche Diagnostics GmbH, Mannheim, Germany). This method is based on the cleavage of the tetrazolium salt WST-1 to formazan by cellular mitochondrial dehydrogenases considering that an increase in the number of cells results in increased activity of mitochondrial dehydrogenases and subsequently in the amount of formed formazan dye. Formazan dye was quantified by measuring the absorbance at 450 nm using VICTOR X3. The reference wavelength was 650 nm to determine the background absorbance from the medium. Nuclear morphology of at least 200 nuclei in each sample was assessed in fixed cells stained with 4',6-diamidino-2-phenylindole (DAPI; Thermo Fischer, Paisley, UK).

For investigating cell invasion, the Cultrex® 96-well basement membrane extract (BME) cell invasion assay was used. The cells (5×10^4 cells/well) were grown in the top chambers coated with 0.5% BME solution consisting of laminin, collagen IV, entactin, and heparin sulfate proteoglycans. Collection of PBS from irradiated cultures and 1:1 dilution with 2X culture medium was used as conditioned medium from UV irradiated cells. The invaded cells in the bottom chamber were dissociated and fluorescence-labeled after addition of Calcein-AM. The fluorescence was analysed at $\lambda_{\text{ex}}485/\lambda_{\text{em}}520$ nm using a VICTOR X3 multilabel plate counter (PerkinElmer, Waltham, MA, USA). Data were correlated to standard curves. The results were presented as the percentage of invading cells relative to the total number of cells. In the time-dependent invasion assays of co-cultures of fibroblasts and melanoma cells, the cells were pre-stained with Celltracker Green CMFDA ($\lambda_{\text{ex}} 490/\lambda_{\text{em}} 520$ nm) or Celltracker Red CMTPX ($\lambda_{\text{ex}} 580/\lambda_{\text{em}} 600$ nm) supplemented in medium (5 or 10 mol/l, respectively; Invitrogen Molecular Probes, Carlsbad, CA, USA) at 37 °C for 45 min.

Xenograft tumor model (Study II)

The metastatic ability was assessed by quantification of distally disseminated tumor cells after irradiation of zebrafish embryos with UVA (20J/cm²) and UVB (200 mJ/cm²) and incubation for 3 days at 28 °C in the presence of anti-FAP- α (1:50, Santa Cruz Biotechnology). The melanoma

cells were pre-labeled with Dil dye and microinjected (~100–500 cells per embryo) in the perivitelline space of 2 days post-fertilization zebrafish embryos that were anesthetized and dechorionated. The zebrafish embryos with fluorescent cells outside the perivitelline space were discarded.

Immunohistochemistry and image analysis (Study I & III)

Formalin-fixed paraffin-embedded tissue was used for routine immunohistochemistry. Serial sections of 5 µm were obtained, deparaffinized, antigen retrieved in PT-link and antibody stained with anti-tubulin β-3 (1:100), Melan-A (Dako; 1:50) or Ki-67 (1:500). A polymer linked to alkaline phosphatase (MACH2) was applied and Vulcan Fast Red was used for visualization (both from Histolab, Gothenburg, Sweden). The slides were blinded, scanned in Aperio Scanner and viewed on the Imagescope software (Leica, Wetzlar, Germany) and in Olympus BX46.

In study I, the staining pattern of tubulin β-3 was determined by the assessors independently. Image analysis of staining intensities was performed on blinded images using randomly selected same number of melanocytic areas (300 × 200 µm) from the superficial and deep intradermal part of each lesion (of similar melanocytic content in paired selections). The staining intensities were determined using the color deconvolution algorithm (Ruifrok and Johnston, 2001; Ruifrok et al., 2003) on magnified (400x) images using the IMAGEJ program (<http://imagej.nih.gov/e.bibl.liu.se/ij/>). The red chromophore density from luminosity histograms was calculated using the slightly modified formula, $\frac{\Sigma (255 - \text{value}) \times \text{Count}}{\text{Area (pixels}^2\text{)}}$, as previously described (Amber, 2015; Billings et al., 2015). The stained lesions with no or only superficial intradermal melanocytic component were excluded from this analysis.

In study III, the presence of tubulin β-3 gradient (diffuse staining in the superficial part of the intradermal melanocytic component that fades to a faint staining with variable loss of reactivity in the deeper intradermal parts) and increasing staining intensity at the invasive front were determined by 3 assessors independently and blinded using light microscopy. A consensus was reached for every stained sample. The melanoma samples were further categorized in diffuse (the melanocytes demonstrate the same staining intensity), varying intensity, focal loss (max 10

melanocytes unstained in high power field) and partial loss (more than 10 melanocytes unstained in high power field).

Immunocytochemistry (Study II)

The cells were fixed in 4% paraformaldehyde for 20 min at 4 °C and then incubated in the buffer containing PBS (without $\text{Ca}^{2+}/\text{Mg}^{2+}$), 0.1% saponin, 5% fetal bovine serum (Appelqvist et al, 2011) to permeabilize the cells' membranes. Incubation with the primary antibodies followed: monoclonal anti-mouse FAP- α (1:50, Santa Cruz Biotechnology) and lactate dehydrogenase (LDH, 1:100, ab52488, Abcam). The secondary antibody used was Alexa Fluor-488 or -594 conjugated antibody (Molecular Probes, Eugene, OR, USA). Using a confocal microscope (LSM700, Zeiss, Heidelberg, Germany), quantification of fibroblast activation protein- α positivity in 200 cells from randomly selected areas was performed. Negative controls (incubated without primary antibody) did not stain.

Cell cycle analysis (Study I)

The assay was performed as previously described (Vindelöv et al., 1983). The cells were washed in PBS, trypsinized, pelleted and resuspended in citrate buffer (250 mM sucrose, 40 mM trisodium citrate dihydrate, and 5% DMSO, pH 7.6). After digestion in 0.03% trypsin, nuclei were isolated. Staining with 0.37 mM propidium iodide and 2 mM spermine tetrahydrochloride was performed and the cells were subjected to flow cytometry (Gallios flow cytometer; Beckman Coulter Inc., Fullerton, CA, USA). Data from 5000 cells were obtained, in each experiment and the histograms of DNA content were analyzed using the KALUZA software (Beckman Coulter Inc.).

Subcellular fractionation (Study II)

The subcellular fractionation buffer (10 mM KCl, 2 mM MgCl_2 , 1 mM EDTA, 1 mM EGTA, 1 M DTT, PI cocktail (III), Hepes pH 7.4) was used to lyse the cells on ice and passed through a 27-gauge needle. Centrifugation at $720 \times g$ for 5 min to pellet nuclei followed. Centrifugation $10\,000 \times g$, 4 °C for 5 min of the supernatant to pellet mitochondria was performed, and the

supernatant was further centrifuged at $100\,000 \times g$, $4\text{ }^{\circ}\text{C}$ for 1 h, to collect the cytosol. Through a 25-gauge needle, the remaining pellet containing the membrane fraction was passed and re-centrifuged at $100\,000 \times g$, $4\text{ }^{\circ}\text{C}$ for 1 h, followed by resuspension in fractionation buffer. Using Amicon Ultra 3 K, $4000 \times g$, $4\text{ }^{\circ}\text{C}$ for 40 min, the concentration of cytosolic fraction was performed.

Patients and melanocytic lesions (Study I & III)

In study I, sections of formalin-fixed and paraffin-embedded (FFPE) specimens were provided by the Department of Pathology, County Hospital Ryhov, Jönköping, Sweden; five benign melanocytic nevi, five dysplastic melanocytic nevi, five melanomas in situ, five primary malignant melanomas stadium pT1a, and five primary malignant melanomas stadium pT2a-pT4a. Clinical data were not available and the specimens were blinded for diagnostic re-evaluation of tumor type, thickness, Clark level, and presence of ulceration by an experienced pathologist. The grade of sun-induced damage and skin structure were evaluated to identify eventual facial and acral lesions.

In study III, sections of formalin-fixed and paraffin-embedded melanocytic nevi and primary cutaneous melanomas, 36 for each diagnosis, were provided by the Department of Pathology, Linköping University Hospital, Linköping, Sweden as obtained from 60 patients who underwent diagnostic excision at Linköping University Hospital from January 2013 to May 2017. Inclusion criteria were: thickness of the intradermal melanocytic component greater or equal to 1 mm, localization other than acral areas and no signs of chronic sun damage. The used clinical data included: age, sex, skin type, hair color, eye color, total number of melanocytic nevi, personal and family history of melanoma, lesion site, lesion size, histopathological diagnosis and parameters from the histopathology reports, sentinel node biopsy outcome, standard initial TNM staging defined according to the 2009 guidelines of AJCC/ UICC (update 2011), record of disease progression or death and derived overall and progression-free survival. 5 melanoma and 3 nevus samples were excluded as they did not meet the inclusion criteria in the sections after staining. Among the excluded nevus samples, 1 had absence of dermal melanocytic component, 1 was torn and 1 categorized with and without gradient. Among the excluded melanoma samples, 2 had absence of dermal melanocytic component, 2 were classified as nevi and 1 was

downgraded to lesion of unknown malignant potential. Finally, 31 melanomas and 33 nevi were included in the immunohistochemical study. These samples came from 55 patients categorized into the nevus patient group (23 patients) and the melanoma group (32 patients).

Dermoscopic assessment (Study III)

Among the included samples, 49 (16 melanomas and 33 nevi) had available macroscopic and dermoscopic images. They were blinded and independently assessed by 2 assessors using the following parameters (Kittler et al, 2016; Braun & Marghoob, 2018): suspected diagnosis, dermoscopic symmetry, characterization and presence of one or more dermoscopic patterns, lines, pseudopods, circles, clods, dots, structureless areas, vessels, ulceration common nevus patterns, special consideration nevus patterns, melanoma patterns and melanoma specific structures. A form was used to assist the dermoscopic assessments (Appendix). A consensus was reached in case of differences.

Statistical analysis (Study I, II & III)

In study I, the microarray analysis was performed using unpaired t-test, the p-value computation was asymptotic and Benjamini-Hochberg FDR method was selected for multiple testing correction (Huang et al., 2009a). Differentially expressed genes that satisfied a fold change cut-off of at least ± 2 and corrected p-value ≤ 0.01 were included in further analyses. DAVID used a hypergeometric test-based method and Benjamini-Hochberg correction was performed for multiple test error. IPA upstream regulator and network analyses used Fisher's Exact Test as described. GSEA used a permutation test procedure to evaluate the significance of enrichments; false discovery rate ≤ 0.25 was used as the thresholds. For the image analysis, student's t test for independent samples was used. For comparisons between groups in the tubulin β -3 inhibition studies, one-way ANOVA and Dunnett post hoc analyses were used.

In study II, for comparisons between groups, one-way or two-way ANOVA, followed by Dunnett's or Tukey's multiple comparison post-test, respectively, were used via GraphPad Prism 6 Software (Version 6.05, La Jolla, CA, USA). p-values ≤ 0.05 were considered significant.

In study III, unpaired t-test, Mann-Whitney and Fisher's exact test were used to determine the significance of difference. Z-test was performed to compare the column proportions and Bonferroni method to adjust the p-values. Statistically significant differences were indicated as p-values ≤ 0.05 . Survival over time was estimated using the Kaplan-Meier method and survival differences were tested using the log-rank test. Progression-free survival (PFS) was determined as the time from the excision of the lesion to the first disease progression event or death. Statistical tests were performed using GraphPad Prism 7 software (GraphPad Software, San Diego, California, USA) and SPSS (IBM SPSS Statistics, Version 25. Armonk, NY, USA). To determine the effects of different predictors, a logistic regression analysis was performed using SPSS (IBM SPSS Statistics, Version 25. Armonk, NY, USA). In cases of complete separation data patterns, the Firth bias-correction was used (Heinze et al, 2002). The interrater reliability analysis using the Kappa statistic was performed to determine consistency among raters (IHC, dermoscopy). A kappa value of 0.81-1.00 indicates almost perfect agreement, 0.61- 0.8 indicates substantial agreement, 0.41-0.60 indicates moderate agreement, 0.21-0.40 indicates fair agreement, 0.00-0.20 slight and <0 poor agreement (Landis & Koch, 1977).

Results

Summary study I

Aims: The specific aims of this study were to identify senescence-associated markers that distinguish melanocytic nevi from melanoma using bioinformatics and to mechanistically study their role in tumor proliferation and invasion.

Figure 8. Cellular model system

Cellular model system of melanocytic senescence: We constructed a senescence model system which consisted of cultured human melanocytes. Cells were harvested at two time points, after 3 days and after a minimum of 30 days when they had stopped dividing (Figure 8). The senescent cells were identified as enlarged cells with flattened morphology and blue beta galactosidase staining, in stark contrast to the young melanocytes that were beta galactosidase-negative. In order to find more markers that characterized our model system, we performed Western blots on the different cell extracts (four paired samples of young and senescent melanocyte cultured cells); the senescence markers p16, p53, the pro-apoptotic protein Bid and the proliferation marker Ki67, which revealed an increase in protein expression of p16, p53 and a decrease in Bid and Ki-67 in the senescent samples compared to the young ones.

Microarray analysis: RNA extraction was performed from the young and senescent melanocytes to look for differences in gene expression. The transcriptomic analysis identified 351 differentially expressed genes, of those 139 up- and 212 down-regulated in the senescent samples. Using the DAVID database we found that the most significantly enriched gene ontology category for the de-regulated genes was "cell cycle". Network analysis using Ingenuity Pathway Analysis depicted a top-scored cancer related network, with *TUBB3* as the most inter-

connected gene. *TUBB3* encodes tubulin β -3 protein, which is specific to neuroectodermal-derived cell types, including melanocytes. Tubulin β -3 was, therefore, selected for further immuno-blotting in four paired samples of young and senescent melanocyte cells which revealed a clear reduction in protein expression in the senescent samples.

Gene set enrichment analysis: To further investigate whether the list of genes obtained from the model system informed on the transformation of melanocytic nevi to melanomas, we used gene set enrichment analysis using as phenotypes melanomas vs. nevi, based on a previously published profiling of 45 primary melanomas and 18 benign nevi (Talantov et al, 2005), and as gene sets the up- and downregulated genes in young vs. senescent melanocytes. In this analysis (Figure 9), the gene sets allowed a clear distinction between the melanoma and nevi samples implying that senescent cells are similar to nevi cells. The leading-edge subset consisted of 58 genes and among them *TUBB3*, which also appeared in network Ingenuity analysis. Therefore, we chose *TUBB3* for further studies.

Figure 9. Diagram of the process used in gene set enrichment analysis

Tubulin β -3 immunostaining: To evaluate the possible clinical applicability of tubulin β -3 expression as a marker of melanocytes in a senescent state, we stained samples of benign and malignant melanocytic lesions from patients. In benign, as well as in dysplastic nevi, a staining

gradient of tubulin β -3 was observed, showing strong diffuse staining in the superficial part of the intradermal melanocytic component that faded to a faint staining in the deeper intradermal parts. On the contrary, melanomas showed strong staining in both the superficial and deep parts. Image analysis of the staining intensities of the superficial and deep intradermal melanocytic components in both benign and dysplastic melanocytic nevi revealed significant loss of staining intensity, whereas no gradient was found in primary malignant melanomas.

Tubulin β -3 functional studies: To examine the impact of tubulin β -3 on cell growth and migration, *TUBB3* was knocked-down in young melanocytes using siRNA, which resulted in the reduction of tubulin β -3 protein. Cultures depleted of tubulin β -3 or pretreated with the tubulin destabilizing drugs colchicine and vinblastine exhibited significantly reduced proliferation and migration capacity. Similarly, the four melanoma cell lines exhibited reduced proliferation and migration capacity.

To study the effect of *TUBB3* depletion on the cell cycle, flow cytometry cell cycle analysis using propidium iodide DNA staining was performed and showed that cells accumulated in the G2/M phase. To examine if depletion of tubulin β -3 induced senescence features, the activity of β -Gal was studied, which showed increased β -galactosidase activity. The senescent-like state was further confirmed by increased expression of p16 protein decreased expression of Bid protein.

Summary study II

Identification of *FAP* in the cellular model system: In the senescence model system described above we found differences in gene expression between young and senescent melanocytes. In particular, *FAP* was upregulated in the young compared to senescent melanocytes. Immunoblotting of FAP- α protein in young and senescent cell extracts, revealed a clear reduction in protein expression in the senescent samples. Furthermore, *FAP* was among the core genes that contributed to the significant enrichment of the senescent phenotype in nevi versus melanomas.

Aims: The invasion of melanoma cells is promoted and guided by activated stromal fibroblasts, and this process is dependent on TGF- β 1 signalling and cathepsins that degrade the basement membrane and the extracellular matrix (ECM) proteins. It has been previously established: 1) that growth and invasion of melanoma cells depends on the secretion of the lysosomal cathepsins B and D (Eding et al, 2015), 2) that UV-exposed melanoma cells produce several soluble factors including TGF- β 1 (Wäster et al, 2011), 3) that UV radiation up-regulates the expression of FAP- α in melanocytes, melanoma cells and fibroblasts (Wäster et al, 2011), and 4) that FAP- α has serine protease and collagenase activity and, thus, facilitates ECM degradation (O'Brien and O'Connor, 2008). The aim of this study was to identify signaling pathways that contribute to the UV-mediated initiation and progression of melanoma. In particular, main objectives were to study the relationship between cathepsins, TGF- β 1 and FAP- α , to understand the mechanism leading to the up-regulation of FAP- α and to investigate the interaction between fibroblasts and melanoma cells.

FAP- α induction by UV-radiation: The subcellular distribution of FAP- α was studied and, while it was not located on the plasma membrane of non-irradiated cultured human melanocytes, 4 hours after UVA or UVB radiation, FAP- α protein levels were increased and found at the plasma membrane. No soluble form or release of FAP- α was detected in the supernatants in non-irradiated melanocytes or following UV exposure. FAP- α levels returned at control levels 24 hours after UV radiation. In artificial skin and *ex vivo* skin, we showed that both UVA and UVB clearly induced FAP- α expression in melanocytes whereas the surrounding keratinocytes were unaffected.

Relationships between FAP, TGFB1 and cathepsins: We also observed up-regulation of *TGFB1* and a number of cathepsin transcripts in young melanocytes and melanomas. As we were interested to understand how FAP, TGFB1 and cathepsins could be biologically related we generated a network using Ingenuity Pathway Analysis software, which showed that *TGFB1* was the most interconnected gene and suggested an interaction of TGFB1 with FAP and cathepsins.

TGF- β 1 secretion: To determine the release of TGF- β 1, immunoblotting was performed in different cell types. TGF- β 1 was found spontaneously secreted from melanoma cells and enhanced after UVR, as well as secreted after UVR, from young and senescent melanocytes, but not from keratinocytes or fibroblasts.

Figure 10. Conditioned media from melanoma cells, irradiated with UVA or UVB and when indicated treated with anti-TGF- β 1, were added to the corresponding non-irradiated cultures.

Relationship between TGF- β 1 secretion and FAP- α expression: To study this relationship conditioned media from melanoma cells irradiated with UVA or UVB were added to the corresponding non-irradiated melanoma cell or fibroblast cultures (Figure 10). This treatment stimulated the expression of FAP- α and increased the migration and invasion capacity of fibroblasts and melanoma cells. By adding anti-TGF- β 1 antibodies or recombinant TGF- β 1, we demonstrated that these changes were TGF- β 1-dependent. The increase in FAP- α was shown to be due to *de novo* synthesis of the protein controlled by TGF- β 1. It is noticeable that no significant effect on UVA- or UVB-irradiated senescent melanocyte migration or invasion was detected after 24 hours or 1 week after irradiation. However, increased migration and invasion of senescent melanocytes was detected 1 week after addition of recombinant TGF- β 1. Invasion

assays were performed in co-cultures of melanoma cells and fibroblasts, which showed that the fibroblasts penetrated the basement membrane extract several hours before the melanoma cells and this activity was significantly stimulated by both UVA and UVB radiation.

Figure 11. Conditioned media from melanoma cells, when indicated pretreated with cathepsin inhibitors and irradiated with UVA or UVB before added to fibroblasts.

Cathepsins control TGF- β 1 secretion: To assess if release and activation of TGF- β 1 is cathepsin dependent, inhibition of the cysteine cathepsins B using Ca-074Me, cathepsin K using inhibitor II, the aspartic cathepsin D using anti-cathepsin D antibodies and lysosomal pH increase using NH₄Cl was performed and shown to reduce the release of TGF- β 1 from all four melanoma cell lines tested. Conditioned media from melanoma cells pretreated with cathepsin inhibitors and irradiated before addition to fibroblasts (Figure 11) caused diminished invasion as compared to controls.

Figure 12. Conditioned media from irradiated with UVA or UVB melanoma cells were added to the siRNA treated melanoma or fibroblast cells.

Effects of FAP- α : To examine if FAP- α is the primary actor during TGF- β 1 stimulated invasion, depletion of FAP- α in melanoma cells or fibroblasts using two different siRNA sequences was used and resulted in reduced FAP- α expression. Conditioned media from irradiated cultures were added to siRNA-treated cells (Figure 12), which resulted in decreased invasion capacity of the FAP- α depleted cells. Similarly, invasion capacity decreased when inhibiting FAP- α with Gly-PhP(OPh) $_2$ or anti-FAP- α antibodies.

Figure 13. Melanoma cells labeled *in vitro* with Dil dye were injected in the perivitelline space of zebra fish embryos and following UV radiation, with or without addition of anti-FAP- α , the melanoma cells were allowed to invade for 48 h. Modified from Feng & Martin, 2015.

Invasion experiments were also performed using zebra fish embryos in a xenograft tumor model (Figure 13). Melanoma cells (FM55P; 100-500 cells) labeled *in vitro* with Dil dye were injected in the perivitelline space of zebra fish embryos and following UV radiation, with or without addition of anti-FAP- α , they were allowed to invade for 48 h. These experiments showed that UV radiation significantly increased the number of disseminated melanoma cells to the posterior part of zebra fish embryos in a FAP- α dependent manner.

Summary study III

Aims: The main objective of this study was to evaluate if tubulin β -3 staining gradient has diagnostic value in distinguishing nevi from melanoma. In addition, we aimed at describing the patterns of tubulin β -3 immunostaining in melanoma, at finding the dermoscopy-immunohistochemistry associations and at examining the prognostic value of this marker.

Baseline characteristics: There were no differences in patients' sex, skin type, hair color, family and personal history of melanoma between the melanoma and the nevi groups. However, melanoma patients as compared to nevi ones were older and included a larger proportion of individuals with blue eyes or showing 10-19 nevi. The samples of nevi and melanomas did not differ in localization, whereas melanoma samples were larger in diameter and thickness. The nevi in the study were 15 common, 4 congenital, 2 mild, 4 mild-moderate, 5 moderate dysplastic and 3 clonal nevi; all were compound or intradermal. The melanomas in the study were 16 superficial spreading melanomas, 5 nodular, 4 nevoid and 6 non-specified. Dermoscopically, nevi and melanomas had more than one patterns, structureless areas predominated in each group and dermoscopic asymmetry was a common characteristic in melanoma samples. Ulceration, polymorphous vessels, linear serpentine vessels, negative network, milky red areas and black structureless areas were more frequently observed in melanomas compared to nevi samples.

Immunohistochemical outcomes: Tubulin β -3 staining gradient was defined as diffuse staining in the superficial part of the intradermal melanocytic component that fades to a faint staining with variable loss of reactivity in the deeper intradermal parts. 29 out of 33 nevi showed the gradient, while none of 31 melanomas had this pattern. Gradient was therefore significantly more frequent in nevi than melanomas, and a logistic regression model indicated that this parameter could predict the diagnosis of melanoma. Furthermore, 12 melanomas showed diffuse and 19 varying intensity staining. Loss of tubulin β -3 staining within the melanoma areas was observed focally in 4 melanomas and partially in 11 melanoma samples. In addition, 15 melanomas showed an increasing staining intensity at the invasive front of the tumor in the dermis.

Dermoscopic-immunohistochemical associations: The dermoscopic assessment of a skin lesion as nevus could predict the tubulin β -3 staining gradient. The particular dermoscopic features that were significantly more frequent among lesions without gradient than with gradient were linear serpentine vessels, polymorphous vessels, ulceration, negative network, black structureless areas and milky red areas, whereas brown thin reticular lines were more frequent in lesions with gradient. Interestingly, melanomas with increasing staining in the invasive front showed more frequently grey structure-less areas.

Prognostic outcomes: All the patients in the nevus group had a progression-free survival whereas 61.6% of patients in the melanoma group were progression-free. The progression rate among melanoma patients, whose melanomas had loss of tubulin β -3, was 4 times higher than without this feature. However, this difference did not reach statistical significance ($p=0.06$).

Discussion

Discussion study I

In this study, we developed a cellular model of primary melanocytes that become senescent; this model shows nevus-mimicking characteristics. Transcriptomic analysis expanded the set of senescence-associated markers that could distinguish nevi from melanoma. Among those, tubulin β -3 was selected to be investigated further. Depletion of tubulin β -3 or pretreatment with tubulin destabilizing drugs in primary melanocytes and melanoma cells induced a senescence-like phenotype *in vitro*. In particular, reduced proliferation and migration capacity as well as induction of cell cycle arrest in G2/M phase was achieved. Also, immunostaining with tubulin β -3 in a small number of melanocytic nevi and primary melanomas was of possible diagnostic value.

Methodological considerations

There are several limitations regarding the relevance of a monoculture system as a nevus-mimicking model. First, the epidermal melanocytes in this model could be different from the nevus cells, which are speculated to derive from epidermal melanocytes or stem cells (Grichnik et al, 2008). Second, factors such as UV-radiation, anatomic localization, patient characteristics, tissue microenvironment, interacting immune system as well as different types of nevi are not typified in this model. Third, the mechanisms of senescence induction in nevi and in the studied model are not necessarily the same (Kuilman et al, 2010). Fourth, the markers of senescent cells *in vivo* may differ from the *in vitro* state (Sharpless et al, 2015). In spite of these limitations, our model culture system has several advantages: it is simple to construct, convenient to work with, inexpensive, and most importantly, it includes only melanocytes, being free of other cell types that would influence the RNA and protein expression. In addition, the use of one publicly available dataset of transcriptomic profiles of nevi and melanomas (Talantov et al, 2005) is arguable since the majority of these primary melanomas represented early stage of the disease with thicknesses <4 mm. Access to more information on this material as well as for the characterization of samples' mutations was not available.

Importance of the findings

Most melanomas are believed to develop *de novo* (Saida, 2019) and some arise by malignant transformation of nevus cells (Sagebiel, 1993; Tsao et al, 2003). Irrespective of the origin, growth-promoting signaling pathways that bypass or truly escape the growth-arrested state are hypothesized to differentiate melanoma from nevi. Taking into account that the established senescence markers cannot distinguish nevi from melanomas (Tran et al, 2012), the transcriptomic analysis of melanocytes that become senescent expands the set of senescence-associated markers that might distinguish nevi from melanoma.

Among the differentially expressed genes, it was considered relevant to further investigate tubulin β -3 using functional assays and immunohistochemical staining on clinical samples based on several major findings: (i) network analysis by IPA showed *TUBB3* in one of the top scored cancer related networks, (ii) in this network *TUBB3* appeared as the most interconnected gene predicting a main biological role, and (iii) using gene set enrichment analysis on nevi and melanoma samples, *TUBB3* was in the leading edge subset that significantly contributed in the enrichment of the downregulated-in-senescence gene set in melanomas. The relevance of this marker in melanocytic senescence was further considered as (v) *TUBB3* encodes for the neuron-specific subtype of β -tubulin, (vi) microtubules are one of the main components of cytoskeleton that are involved in key cellular events, as cell movement and cell division (Kavallaris et al, 2010) and (vii) a potential diagnostic role is implicated by its expression in a broad spectrum of cancers (Person et al, 2017).

Depletion of tubulin β -3 induced a senescence-like phenotype *in vitro* and the accumulation of cells in G2/M phase, which is different from the reported induction of senescence in G1 arrested cells. However, the relevance of our finding is supported by (i) reports that oxidative stress-induced and replicative senescence can be triggered in the G2 phase (Chen et al, 2000; Gire and Dulić, 2015), although observations that G2/M checkpoint is not as robust as the G1/S one (Gire and Dulić, 2015) and (ii) indications that G1 arrest may not be the only senescence state in nevi (Bennett et al, 2002; Ross et al, 2011).

Discussion study II

In this study, a UV-promoted intercellular signaling pathway between melanoma cells and stromal fibroblasts was shown to facilitate melanoma invasion. The signaling pathway consisted of cathepsins that stimulated the release and activation of TGF- β 1, which subsequently led to increased expression of FAP- α both in melanoma cells and fibroblasts. FAP- α , being a serine protease may contribute to extracellular matrix degradation. In invasion assays with co-cultures of fibroblasts and melanoma cells, we showed that the fibroblasts invaded the basement membrane earlier than the melanoma cells and, facilitated therefore the invasion of melanoma cells. This UV-promoted signaling pathway of cathepsins - TGF- β 1 - FAP- α , as observed in melanoma, seems not functional in senescent melanocytes.

Methodological considerations

The signaling pathway of cathepsins - TGF- β 1 - FAP- α was mainly investigated in monoculture models using conditioned media, while the time dependence for invasion was studied in co-cultures of melanoma cells and fibroblasts. The advantage in using such systems is that the cells are studied in a controlled manner without the influence of other cells that might otherwise influence the RNA and protein expression. However, (i) melanoma cells *in vivo* do not grow in isolation but in a continuous interaction with the stroma and other cells and are oriented in a three-dimensional space, (ii) the normal fibroblasts used in the study, in mono- and co-cultures with melanoma cells, do not necessarily reflect the biology of the cancer-associated fibroblasts that exist in the tumor stroma and (iii) conditioned media may include in addition to TGF- β 1 other growth factors that contributed to the studied sequential axis and are yet to be determined.

Importance of the findings

This study indicates a UV-promoted intercellular signaling pathway between melanoma cells and stromal fibroblasts, which facilitates melanoma invasion. The findings are in accordance with the existing reported evidence: (i) it has been shown that UV radiation impairs TGF- β signaling in human skin fibroblasts and contributes to a switch in the feature of fibroblasts from extracellular matrix synthesis to extracellular matrix degradation (He et al, 2014); (ii), a bidirectional cross-

talk between tumor cells and cancer-associated fibroblasts (CAFs) has been reported, with cancer cells releasing factors that promote fibroblasts to secrete tumor-promoting chemokines, which then signal malignant cells to promote their proliferative, migratory, and invasive properties and also affect the tumor microenvironment (Mishra et al, 2011).; (iii) it has been suggested that in melanoma (Yin et al, 2012) and in squamous cell carcinoma (Gaggioli et al, 2007), cancer cells follow tracks of extracellular matrix deposited by fibroblast leading edges.

In addition, we showed that recombinant TGF- β 1 stimulated the migration and invasion of senescent melanocytes, while UV radiation alone did not, even though TGF- β 1 was released by both young and senescent melanocytes after UV-radiation. This implies that the involvement of the studied signaling pathway of cathepsins - TGF- β 1 - FAP- α is compromised in nevi, thus, keeping nevi in senescence. The study of this compromising effect may elucidate the mechanisms that keep nevi in senescence and help melanoma bypass or truly escape this state.

Another important implication from this study is that UV radiation promotes not only the formation of melanoma but also its progression. This is further supported by current evidence that melanoma metastases often have mutations in TP53, which are UV-signature mutations, while these are not as common in primary melanomas (Shain et al, 2015).

Discussion study III

In this study, tubulin β -3 immunostaining and specifically the absence of tubulin β -3 gradient is shown to aid in melanoma diagnosis. A potential prognostic role of this marker is further suggested as patients having melanomas with areas of tubulin β -3 loss have a worse, although not significant, prognosis. Furthermore, specific dermoscopic features are associated to tubulin β -3 immunostaining patterns.

Methodological considerations

There are several considerations regarding the immunohistochemical study of tubulin β -3 on nevi and melanoma samples. First, moderate agreement (κ -value=0.543) was noticed in the evaluation of staining gradient among the 3 assessors indicating that the assessment of this parameter could be easily performed. Second, the sensitivity of this test (probability of absence of gradient in a melanoma sample) was estimated to be 100%, the specificity (probability of gradient in a nevus) 88% and the diagnostic accuracy (the proportion of correctly classified nevi and melanomas among all samples) 94%. However, erroneous conclusion may be drawn due to the small sample size of the study that might have introduced a complete separation problem (perfect predictions for all melanomas as 0 out of 31 melanomas had the staining gradient pattern).

In addition, the dermoscopic features that distinguished nevi from melanomas in addition to the tubulin staining gradient could be also easily assessed clinically. The dermoscopic feature with the best level of agreement among the assessors was the presence of lines (κ -value=0.836). The distinguished dermoscopic features that showed a moderate inter-observer agreement were ulceration (κ -value=0.563), milky red areas (κ -value=0.458), black structureless areas (κ -value=0.489), linear serpentine vessels (κ -value=0.502) and brown thin reticular lines (κ -value=0.755). The distinguished dermoscopic features that showed a fair inter-observer agreement were number of patterns (κ -value=0.301), structureless areas (κ -value=0.36), dermoscopic asymmetry (κ -value=0.31), polymorphous vessels (κ -value=0.365), negative network (κ -value=0.31) and grey structureless areas (κ -value=0.327).

Importance of the findings

In this study, the diagnostic and potentially prognostic role of tubulin β -3 staining in melanoma was shown. This is further supported by reported findings that: (i) tubulin β -3 is expressed in a broad spectrum of cancers including melanoma (Person et al, 2017) and (ii) tubulin β -3 decreased expression in melanoma is associated with worse prognosis (Shimizu et al, 2016; Akasaka et al, 2009).

The staining patterns in nevi and melanomas could have important implications. First, our observations of tubulin β -3 staining gradient in nevi, in combination with: (i) the morphological (Barnhill, 2014; Lund and Stobbe, 1949) and (ii) antigen-expressing resemblance (Aso et al, 1988) of nevus cells of deeper dermis with Schwann cells, as well as the current knowledge of (iii) lack of tubulin β -3 expression in Schwann cells (Zeisel et al, 2018) and (iv) evidence for melanocytes that originate from Schwann cell precursors (Adameyko et al, 2009), could support the notion that some nevi might have a dual origin: a component resulting from intraepidermal melanocytes that would migrate downward into the dermis and a component resulting from schwann cells that would migrate upward (Masson, 1951). Second, our observations of worse prognosis for melanomas with areas of tubulin β -3 loss, in combination with (i) previous reports that the loss of tubulin β -3 expression in melanoma samples was associated with poor prognosis (Shimizu et al, 2016; Akasaka et al, 2009) and (ii) our findings in study I that depletion of tubulin β -3 induced a senescence-like phenotype of young melanocytes and melanoma cells *in vitro* (Orfanidis et al, 2017) suggest that poor prognosis in melanoma with loss of tubulin β -3 may be related to the induction of growth arrest in melanoma cells; however such detrimental effect of senescence in cancer biology is debatable (Schosserer et al, 2017).

Conclusions

The main conclusions in this thesis are as follows:

- *TUBB3*, which encodes the microtubule protein tubulin β -3, shows a decreased expression in senescent melanocytes and nevi.
- Depletion of tubulin β -3 or pretreatment with tubulin destabilizing drugs, in melanocytes and melanoma cells, induces a senescence-like phenotype *in vitro*. In particular, reduced proliferation and migration capacity as well as induction of cell cycle arrest in G2/M phase is achieved.
- A UV-promoted intercellular signaling pathway between melanoma cells and stromal fibroblasts that facilitates melanoma invasion is identified: Cathepsins stimulate the release and activation of TGF- β 1 and subsequent this leads to increase in expression of FAP- α both in melanoma cells and fibroblasts, which, being a serine protease, contributes to extracellular matrix degradation.
- In invasion assays, the fibroblasts invade the basement membrane earlier than the melanoma cells, which may imply that they facilitate the invasion of melanoma cells.
- UV radiation promotes not only the formation of melanoma but also its progression.
- Tubulin β -3 immunostaining aids in melanoma diagnosis. In particular, absence of tubulin β -3 staining gradient characterizes melanomas and presence of the gradient is noted in nevi.
- A potential prognostic role of this marker is further suggested as patients having melanomas with areas of tubulin β -3 loss have a worse, yet not statistically significant, prognosis.

Future Perspectives

A number of interesting questions have come up with these studies concerning the formation of nevi and melanoma, the induction of senescence, the role of ultraviolet radiation, the use of tubulin β -3 as a prognostic marker and potential aspects of melanoma treatment.

It is a fact that there is still no definite answer to the origin of nevus and melanoma cells. Was Masson right 70 years ago? More evidence is needed to show if nevus cells, probably those in the deeper dermis, originate from Schwann cell precursors or even if Schwannian differentiation occurs. This would also provide insight in the formation of melanoma. Omics comparison among Schwann cells, Schwann cell precursors and nevus cells of deeper dermis could examine their similarities.

In addition, direct evidence on *in vivo* senescence is needed to clarify if all nevus cells are senescent in a nevus, if this concerns G1 or G2 arrested cells, and to elucidate the mechanisms of senescence induction in nevi. It will be very interesting to investigate if melanomas have indeed senescent areas, if they are then made of G1 or G2-arrested cells and if these further promote melanoma progression in primary or metastatic sites - a finding which would be in absolute opposition to the traditional notion that senescence is only a tumor suppressor mechanism. It would be interesting also to answer if chemotherapy induces senescence in melanoma and if this would be a mechanism for induction of chemotherapy resistance.

That ultraviolet radiation promotes not only the formation of melanoma but also its progression needs to be further investigated as this could have implications in melanoma treatment and tertiary prevention. Besides, the signaling pathway of cathepsins - TGF- β 1 - FAP- α , compromised in senescent melanocytes, but UV-promoted in melanoma, could be further studied to elucidate the mechanisms that keep nevi in senescence and help melanoma bypass or truly escape this state.

The importance of tubulin β -3 as a diagnostic marker that helps in clinical praxis the melanoma diagnosis is apparent, especially when such easily applicable markers are missing. Its potential prognostic role would further broaden its use. A larger number of melanoma samples from patients with and without progression will be necessary to assert this point.

With regard to therapeutics, CAF-targeted therapies and optimization of FAP- α targeting, in combination with immunotherapy could increase the elimination of melanoma and metastasis. On the other hand, it is of great importance to investigate if senolytic drugs would help against the formation of nevi and, especially, if they have a position in melanoma treatment, which would open the door to a new class of drugs against melanoma.

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*“As you set out for Ithaka
hope your road is a long one,
full of adventure, full of discovery.
Laistrygonians, Cyclops,
angry Poseidon—don’t be afraid of them
you’ll never find things like that on your way
as long as you keep your thoughts raised high”*
(excerpt from Ithaka, C.Cavafy)

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Appendix

Dermatoscopic assessment of lesion nr: _____
Assessor: _____
Date: _____

Dermatoscopic characteristics		
One pattern	No	Yes
More than one pattern	No	Yes
Lines	No	Yes
		Describe (incl. colors):
Pseudopods	No	Yes
		Describe (incl. colors):
Circles	No	Yes
		Describe (incl. colors):
Clods	No	Yes
		Describe (incl. colors):
Dots	No	Yes
		Describe (incl. colors):
Structureless	No	Yes
		Describe (incl. colors):
Vessels	No	Yes
	Vessel structures	Vessel arrangements
		Dots
		Clods
		Linear straight
		Linear looped
		Linear curved
		Linear serpentine
		Linear helical
		Linear coiled
Ulceration	No	Yes
Other	No	Yes
		Describe (incl. colors):
Dermatoscopic symmetry	No	Yes

Form used to assist the dermoscopic assessment in Study III

Choose the image(s) that correspond to the patterns/structures of the lesion in study

Common nevi patterns

2)

Nevus patterns requiring special consideration

Melanoma patterns

Melanoma on sun damaged skin

Melanoma specific structures

Regression structures

Atypical vascular patterns

Diagnosis:

The images are designed by: Ralph P. Braun & Ashfaq A. Marghoob © for www.dermoscopedia.org

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