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Evaluation of tubulin β -3 as a novel senescence-associated gene in melanocytic malignant transformation

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Summary

Malignant melanoma might develop from melanocytic nevi in which the growth-arrested state has been broken. We analyzed the gene expression of young and senescent human melanocytes in culture and compared the gene expression data with a dataset from nevi and melanomas. A concordant altered gene expression was identified in 84 genes when comparing the growth-arrested samples with proliferating samples. TUBB3, which encodes the microtubule protein tubulin β -3, showed a decreased expression in senescent melanocytes and nevi and was selected for further studies. Depletion of tubulin β -3 caused accumulation of cells in the G2/M phase and decreased proliferation and migration. Immunohistochemical assessment of tubulin β -3 in benign lesions revealed strong staining in the superficial part of the intradermal components, which faded with depth. In contrast, primary melanomas exhibited staining without gradient in a disordered pattern and strong staining of the invasive front. Our results describe an approach to find clinically useful diagnostic biomarkers to more precisely identify cutaneous malignant melanoma and present tubulin β -3 as a candidate marker.

Significance

The development and progression of a melanocytic nevus into a malignant melanoma is characterized by alteration of gene expression and growth promoting signaling to break the growth-arrested state. By combining the gene expression profiles from skin lesions and cell culture models, we identify candidate biomarkers that distinguish malignant melanoma from benign melanocytic lesions. Tubulin β -3 may have a diagnostic impact in melanoma.

Running title: Tubulin β -3 expression in nevi and melanomas

Keywords: senescence, nevus, melanoma, microarray, tubulin β -3

Introduction

The incidence of malignant melanoma shows a constant increase, particularly in the Caucasian population, worldwide during the past decades (Holmes, 2014; Nikolaou and Stratigos, 2014). The major risk factors are exposure to ultraviolet radiation, high nevus counts, large nevus size and genetic predisposition (Marks, 2000; Rosendahl et al., 2015). Although the mechanism of transformation is incompletely characterized, histological examinations have demonstrated that melanocytic nevi precursors can be identified in between 26 and 58% of cutaneous melanomas (Sagebiel, 1993; Tsao et al., 2003).

The origin and developmental process of nevi is not yet fully defined, although recent data supports a dermal origin (Grichnik et al., 2014). Nevi demonstrate progressive changes in histologic appearance throughout life (Lund and Stobbe, 1949), which could be referred to as maturation. Eventually intraepidermal proliferation of the melanocytes will cease. The nevus becomes entirely intradermal with reduced proliferative activity and finally undergoes progressive involution. Importantly, the features that are believed to distinguish benign nevi from melanoma are noted within the senescence pathways that signal growth arrests (Ross et al., 2011). Cellular senescence can be triggered by extensive loss of telomeric sequences due to replicative shortening (d'Adda di Fagagna et al., 2003) or by constitutive activation of oncogenes or loss of tumor suppressors (Courtois-Cox et al., 2008; Serrano et al., 1997). The cellular senescent state is controlled by multiple mechanisms, including global chromatin and epigenetic modification, DNA damage response and alteration of secretory pathways and autophagy (Campisi, 2013). Several molecular effectors of senescence have been identified, such as the tumor suppressors p53, p16^{INK4a} and Rb (Michaloglou et al., 2005). Melanomas and nevi share growth-promoting mutations, e.g., in BRAF or NRAS, implying that nevi are growth arrested by the activation of oncogene-induced senescence pathways (Ross et al., 2011; Souroullas and Sharpless, 2015). In nevi harboring the most common BRAF mutation, V600E, the induction of both p16^{INK4a} and senescence-associated acidic β -galactosidase (β -Gal) activity can be demonstrated (Michaloglou et al., 2005). Noteworthy, telomere shortening has also been observed in melanocytic nevi, indicating that the growth arrest might be induced by mixed mechanisms (Bandyopadhyay et al., 2001). Taken together, these observations suggest that nevi are mainly growth restricted by the senescence machinery as a defense mechanism against malignant transformation.

Histopathologic examination is the golden standard for diagnosing melanocytic neoplasms. Despite well-defined criteria for diagnosis and classification, the differentiation between melanocytic nevi and

melanomas is challenging and controversial in many cases due to overlapping morphologic features. As presented in several comparative studies, the diagnoses of certain melanocytic tumors show variability and discordance between experienced pathologists (Farmer et al., 1996; Lodha et al., 2008). Immunohistochemistry-based molecular markers are routinely assessed for different cancers; however, none of the protein-based studies in primary melanoma have advanced into clinical practice (Weiss et al., 2015). Given the clinical and histopathological heterogeneity of primary melanomas, new protein markers are needed to better identify patients at risk to develop an advanced disease.

In this study, we constructed a cellular model system of melanocyte senescence, performed a microarray analysis and combined these results with the gene expression arrays analyses extracted from a public database of nevi and malignant melanomas. Novel protein biomarkers that could distinguish senescent cells from proliferating were identified and the clinical applicability of tubulin β -3 was tested.

Results

Identification of differentially expressed genes in young and senescent melanocyte cultures

Human melanocytes were cultured and samples withdrawn after 3 days (young melanocytes) and after a minimum of 30 days (senescent melanocytes) when the cells had stopped dividing. Senescent melanocytes were identified as enlarged cells with flattened morphology and positive β -Gal staining (Figure 1A). β -Gal activity was increased (Figure 1B), and the protein levels of p16^{INK4a} and p53 were elevated in the senescent samples, whereas the pro-apoptotic protein Bid and the cell proliferation marker Ki-67 were decreased (Figure 1C). To identify unique genetic signatures differently expressed in senescent and young melanocytes, an unsupervised mRNA analysis was performed on samples from 4 donors. Hierarchical clustering indicated a good segregation between the young and senescent melanocyte cultures (Figure 1D). Furthermore, the first principal component showed clear segregation between the groups (Figure 1E).

In total, we identified 351 differentially expressed genes (fold change ≤ -2 or $\geq +2$, p -value ≤ 0.01) when comparing young and senescent melanocytes. These genes included 139 up-regulated and 212 down-regulated genes in the senescent melanocytic samples compared with young melanocytic samples (Supplementary tables S1 and S2). Several of the altered genes were functionally linked to the senescent state. Down-regulated genes were enriched with cell cycle-related GO terms, such as M phase, cell cycle and cell cycle phase, whereas up-regulated genes were enriched with the GO terms cell cycle arrest and intracellular signaling cascade (Table 1). The list of differentially expressed genes was transformed into a set of relevant networks using Ingenuity Pathway Analysis (IPA). Nineteen associated networks were generated, of which the category “Cancer, Endocrine System Disorders, Hematological Disease” scored second top (Supplementary table S3) and was considered of interest. This network showed TUBB3 as the most inter-connected gene (Figure 2A). The identification of potential upstream regulators revealed a strong activation of CDKN2A (encoding the p16^{INK4a}) and TP53 and inhibition of TGFB1 and E2F among others in the senescence model system (Figure 2B). Interestingly, in this analysis, TUBB3 was regulated by TP53 and TGFB1. TUBB3 encodes the class 3 beta-tubulin (tubulin β -3) protein, which is specific for neuroectodermal-derived cell types, such as melanocytes. Considering the results presented in Figures 2A and B and the fact that tubulin β -3 suits the concept of a diagnostic marker of melanoma in the skin, since it is not expressed in keratinocytes, we selected TUBB3 as an interesting candidate gene for further studies. To verify the microarray analysis, tubulin β -3 was immuno-blotted in four coupled samples of young and senescent melanocyte cultures, which revealed a clear reduction in protein expression in the senescent samples (Figure 2C).

Relationship between the senescent melanocyte model system and gene expression in nevi and melanoma

To investigate whether the model system harbors information relevant for transformation of melanocytic nevi to melanomas, we compared the transcriptomes of young and senescent melanocytes to a previously published data set consisting of 45 primary melanomas and 18 benign skin nevi (Talantov et al., 2005) using gene set enrichment analysis (GSEA). Six common hallmarks shared between young vs senescent melanocytes and melanomas vs benign nevi were identified. Specifically, E2F targets, mTORC1 signaling and MYC targets were enriched both in young melanocytes and melanomas. In addition, four common hallmarks, including the p53 pathway, were enriched in both senescent melanocytes and nevi (Supplementary figure S1). Thus, there are indications that genes found in the model system of senescence also play a role in benign nevi. We employed our *a priori* defined senescence sets of up- and down-regulated genes to determine whether they were randomly distributed throughout the ranked gene list of the clinical dataset and showed strong significant enrichment ($p < 0.01$) in nevi and melanomas, respectively (Figures 3A and C). Thus this model system is applicable to search for new markers to distinguish between nevi and melanomas. The leading edge subset of the senescent gene sets of up- and down-regulated genes consisted of 26 and 58 genes, respectively (Figures 3B and D). Among these 58 genes, TUBB3 was found and since it also appeared as a hub in the IPA (Figure 2A), it was selected for staining of melanocytic lesions.

Expression of tubulin β -3 in human melanocytic lesions

To evaluate the possible clinical applicability of tubulin β -3 expression as a marker of melanocytes in a senescent state, we stained formalin-fixed paraffin-embedded samples of benign and malignant melanocytic lesions from patients (Table 2). In melanocytes, tubulin β -3 expression was detected in the cytoplasm, and its distribution was similar to Melan-A staining (Supplementary figure S2A). Intra-epidermal melanocytes and the junctional component of both benign and malignant lesions exhibited strong tubulin β -3 expression. In benign acquired and congenital melanocytic lesions, 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. Figure 4A presents a gross overview of the lesion and the insets illustrate the staining appearance of the superficial and deep parts in detail. A similar staining pattern was observed in dysplastic nevi (Figure 4B). Melanoma in situ and thin primary melanomas (≤ 1 mm) stained strongly for tubulin β -3 (Supplementary figures S2B and

C). Thick melanomas (>1 mm) showed strong staining in both the superficial and deep parts (Figure 4C). Interestingly, three out of five thick melanomas (> 2.0 mm) exhibited variable staining in a disordered pattern with focal loss of tubulin β -3 (Supplementary figure S2D). Noteworthy, strong staining was noted in the invasive front of the tumor (Supplementary figure S2D). 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 in benign lesions, whereas no gradient was found in primary malignant melanomas (Figure 4D-F). Given that melanomas in situ and thin primary melanomas (≤ 1 mm) lack the deep intradermal melanocytic component, they were not subjected to image analysis.

Depletion of tubulin β -3 prevents the migration of melanoma cells

To verify the impact of tubulin β -3 on cell growth and migration, tubulin β -3 was depleted in four young melanocytes and the melanoma cell lines WM115, WM793, WM278, and FM55P using siRNA, which reduced the protein expression (Figures 5A, B and Supplementary figure S3). Proliferation was estimated during a 24-h period. Cultures depleted of tubulin β -3 or pretreated with the tubulin destabilizing drugs colchicine and vinblastine exhibited significantly reduced proliferation (Figures 5C and D). Cell cycle analysis revealed that upon depletion of tubulin β -3, the cells increasingly accumulated in the G2/M phase in both primary melanocytes and melanoma cell lines (Figures 5E and F and Supplemental figure S4). Similarly, the migration was reduced in cultures treated with tubulin inhibitors and after the depletion of tubulin β -3 (Figures 5G and H). To determine if depletion of tubulin β -3 was accompanied by senescence like cell appearance, the activity of the senescent marker β -Gal was studied. As presented in Figures 6A and B, β -Gal activity was enhanced 2-fold and 3.7-fold in melanocytes and melanoma cells, respectively. The senescent state was further confirmed by augmented expression of p16 (Figure 6C and D). However, no increase in the number of apoptotic cells, as detected by DNA content in the cell cycle analysis (Supplementary figure S4) and by analysis of nuclear morphology could be found. In addition, decreased levels of the pro-apoptotic protein Bid was detected in both the melanocyte and the melanoma cell lines after depletion of tubulin β -3 (Figure 6C and D).

Discussion

In our model system, growth-arrested melanocytes displayed several well-documented characteristics of senescence, including enlarged and flat morphology, β -Gal activity, and altered expression of p53, p16 and Ki-67. Moreover, we also detected protection against apoptotic cell death since the pro-apoptotic BH3-only protein Bid was down-regulated in senescent cultures. Telomere shortening and the activation of oncogenes are among the central inducers of senescence (Kuilman et al., 2010; Serrano et al., 1997). However, various stress conditions due to in vitro cultivation, such as production of reactive oxygen species (ROS) and growth factor starvation, might also induce a premature senescence state (Kuilman et al., 2010). The gene expression profiles of our material indicated up-regulation in the senescent vs young melanocytes of several manifested senescence markers (reviewed in Campisi and d'Adda di Fagagna, 2007), including CDKN2A (encoding p16) and TP53, and the down-regulation of MKI67 (encoding Ki-67), reflecting reduced proliferation. However, it was recently demonstrated that none of the above mentioned proteins nor the established senescence markers PML and β -Gal could be used to distinguish nevi from melanomas (Tran et al., 2012). Thus, gene expression profiles might identify novel candidate proteins for diagnostic use.

Consistent with recent findings, we revealed that growth promoting signaling pathways, such as E2F targets (Park et al., 2006), mTORC1 signaling (Damsky et al., 2015) and MYC targets (Leikam et al., 2014), are enriched both in young melanocytes and melanomas. Furthermore, a concordant gene expression difference as derived from the senescence model system, is found between melanomas and nevi. The contributing markers are, thus, possible candidate markers to distinguish between benign and malignant melanocytic lesions. The neuron-specific subtype of β -tubulin encoded by TUBB3 was identified among the leading edge subset of the up-regulated genes in young melanocytes and melanoma. According to the IPA of highly inter-connected networks annotated as related to cancer, TUBB3 was identified as the most connected gene and was therefore selected to study in melanocytic lesions.

Melanocytic lesion analysis revealed a tubulin β -3 staining gradient in the intradermal component of benign and dysplastic melanocytic lesions, and this was not observed in melanomas. The results suggest that analysis of the tubulin β -3 staining pattern in the intradermal melanocytic component might have diagnostic impact and should be further verified. In previous investigations of clinical material, tubulin β -3 expression was associated with the ascending histological grade of the malignancy (Kavallaris, 2010). Patients with non-small cell lung cancer showed survival advantages if the tumor was negative for tubulin

β -3 (Levallet et al., 2012). In contrast, loss of tubulin β -3 expression was related with poor prognosis in acral lentiginous melanoma (Akasaka et al., 2009). However, this type of melanoma is rare in Caucasians and was not present in our material. A recent publication by Shimizu et al. also suggests that decreased expression of tubulin β -3 is associated with unfavorable prognosis in melanoma (Shimizu et al., 2016), but the tumor type was not specified. According to the results presented in Figure 3, a few tumors show gene expression similar to that of nevi indicating the heterogeneity of melanomas. However, it could also reflect the diagnostic difficulties associated with the histopathological analysis. Consequently, the comparison of the results obtained here and found in the literature suggests that tumor and skin type have great impact on the expression and the prognostic value of tubulin β -3 in malignant melanoma.

In anticancer treatment, tubulins are targeted with Vinca alkaloids and taxanes, and tubulin β -3 expression is associated with resistance to the above mentioned drugs in a wide range of tumor types (Kavallaris, 2010). In cell culture experiments, tubulin β -3 protein expression correlated with resistance to paclitaxel in malignant melanoma cell lines (Akasaka et al., 2009). Tubulins constitute microtubules, which are essential for cell division including segregation of the chromosomes during mitosis (Vicente and Wordeman, 2015). Accordingly we found growth arrest and G2/M phase accumulation in tubulin β -3 depleted cells. Previously oxidative stress induced senescence also show G2/M phase accumulation (Chen et al., 2000). Thus, we demonstrate that depletion of tubulin β -3 leads to a senescence state, specified by the markers p16 and β -Gal and also manifested as reduced proliferation and migration. Our results are in concordance with previous findings that B16/F10 melanoma cells exposed to the microtubule toxin JG-03-14 exhibited senescence characteristics after treatment (Biggers et al., 2013).

In conclusion, we developed a melanocytic senescence model system to identify genes that impact cellular proliferative activity. The combination of microarray analyses from the cell culture model and clinical samples revealed novel biomarkers that could define senescence-related processes and improve the diagnostic accuracy of melanocytic lesions. Histopathologic evaluation of tubulin β -3 as a biomarker suggested that the staining intensity in the deep intradermal component might offer guidance in diagnosing melanocytic lesions.

Materials and Methods

Cell culture and conditions

Melanocytes were obtained from Caucasian donors (0-3 years of age) by means of foreskin circumcisions, and pure cultures were established as previously described (Andersson et al., 2001). All experiments were performed according to the ethical principles of the Helsinki declaration and were approved by the Ethical Review Board in Linköping, Sweden. The melanocytes were cultured in Medium 199 with supplements and incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. The experiments were conducted between passages 2 and 10. The first sample (young) was obtained 3 days after isolation/seeding, and the second sample (senescent) was isolated after a minimum of 30 days when the cells exhibited senescent-like morphology, i.e., flattened and non-proliferative.

Four primary melanoma cell lines were used: WM115, WM793, WM278 and FM55P (provided by Prof Meenhard Herlyn, Wistar Institute, Philadelphia, USA). The melanoma cells were cultivated in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. The microtubule destabilizing drugs vinblastine (100 nM, 24 h, stock in DMSO) and colchicine (10 µM, 30 min, stock in DMSO) were used prior to experiments when indicated. Controls for DMSO showed no interference with the experiments.

β-Galactosidase assays

β-Gal staining was performed as described before (Dimri et al., 1995). Images were captured using an Olympus BX51 Microscope (Olympus, Tokyo, Japan). For β-Gal activity, cells were lysed 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. The lysate was incubated for 40 min at 37°C in the dark before stop solution of 1 M glycine-NaOH buffer was added, and the fluorescence was analyzed ($\lambda_{\text{ex}}355/\lambda_{\text{em}}460$ nm). The activity was correlated to the total amount of protein.

Whole human genome microarray analysis

Microarray analysis was performed on melanocytes from four different donors. After isolation of total RNA (TRIzol, Sigma), the quality was controlled using an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). Labeling and hybridization were performed using the Affymetrix Human Genome Microarray (WT GeneTitan ST1.1; Plate type, HuGene-1_1-st-v1-16; Array type, HuGene-1_1-st-v1) according to the manufacturer's protocols (Affymetrix Inc., CA, USA) at the Bioinformatics and Expression Analysis Core Facility (Karolinska Institute, Sweden). The raw .CEL files generated from this analysis were processed using the Agilent GeneSpring GX 13 software package (Agilent Technologies, Santa Clara, CA, USA). The exon-level data were summarized using the RMA16

algorithm (Bolstad et al., 2003), and the baseline was transformed to the median of all samples. Entities are filtered based on their signal intensity values between the 20th and 100th percentiles in at least 1 sample. The cut-off fold change was ± 2 ($p < 0.01$). The data were quality assessed before and after normalization through the inspection of hierarchical clustering trees and principal component analysis (PCA) plots. For the gene-level experiment, the data were normalized using a quantile algorithm (Bolstad et al., 2003), and the baseline was transformed to the median of all samples. The data has been deposited in the Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) accession number GSE83922. The gene id conversion was performed by converting the EntrezGene IDs to official gene symbols.

Gene ontology and network analysis

Gene ontology term analyses using gene symbols were performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID; <https://david.ncifcrf.gov>) (Huang et al., 2009a; Huang et al., 2009b). Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, <http://www.ingenuity.com/>) was used to generate the networks of direct relationships between molecules and for upstream regulator analysis.

Gene set enrichment analysis

GSE3189 identifier was used to import microarray data from a public dataset (Talantov et al., 2005) previously analyzed on an Affymetrix Hu133A microarray containing 22,000 probe sets into the Agilent GeneSpring GX 13 software package. In this study, the majority of the primary melanomas had a thickness of < 4 mm and the majority of nevi represented compound and intradermal nevi. GSEA was used to identify common hallmarks of the experimental system (young vs senescent melanocytes) and the public dataset (melanoma vs nevi) (Talantov et al., 2005) and to compare derived senescence associated gene sets to the entire ranked gene list from the dataset (Subramanian et al., 2005). The results with p -values ≤ 0.05 and false discovery rates ≤ 0.25 were considered significant.

Western blot and dot blot analysis

Melanocyte cell cultures were prepared for immunoblot as previously described (Appelqvist et al., 2011). Immunodetection was performed with monoclonal mouse antibodies against p16 and p53 (both from Santa Cruz), a monoclonal rabbit antibody against tubulin β -3 (Novus Biologicals) and polyclonal rabbit antibodies against Bid (BD Pharmingen) and Ki-67 (Abcam) followed by the corresponding horseradish peroxidase (DAKO, Sweden)-conjugated secondary antibody. The membranes were reprobated with glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Biogenesis, Poole, UK) as an internal control.

Densitometric quantification of the bands was performed using Image Lab Software (Version 4.1, BioRAD).

Tubulin β -3 silencing

siRNA transfection against tubulin β -3 was performed using 5 nM TUBB3 siRNA (SI02636655, SI02780883, Qiagen, Germantown, MD, USA) and 6 μ l HiPerFect Transfection Reagent for 24 h. Optimal transfection conditions for the cells were determined by titration using Alexa Fluor 555 labeled non-silencing siRNA with a scrambled sequence without homology to mammalian genes (AATTCTCCGAACGTGTCACGT, Qiagen). This siRNA was used as negative control, and siRNA targeting Lamin A/C (SI03650332, Qiagen) served as positive control, as recommended by the manufacturer.

Cell migration and proliferation

To determine migration, the Cultrex[®] 96 well Migration Assay was used (Trevigen Inc., Helgerman Ct. Gaithersburg, MD, USA). The top invasion chambers (8- μ m pore size) were uncoated. Cells (5×10^4 cells/well) were grown in the top chamber, and the migrated cells were dissociated and incubated with Calcein-AM; the assay chamber was analyzed using a VICTOR X3 multiple counter (λ_{ex} 485/ λ_{em} 520 nm, Perkin Elmer, MA, USA). Reproducibility was confirmed by internal reference (4 individual cell lines, each value is mean of three replicates). Proliferation was assessed in cultures 24 h after seeding using the WST-1 cell proliferation reagent (1:10, Roche Diagnostics GmbH, Mannheim, Germany). Absorbance was measured at 450 nm and 650 nm to determine proliferation and background absorbance from the medium using VICTOR X3. Nuclear morphology was evaluated in fixed cells stained with 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes). Nuclear morphology was assessed in minimums of 200 nuclei in each sample.

Melanocytic lesions

The study included 5 benign melanocytic nevi, 5 dysplastic melanocytic nevi, 5 melanomas in situ, 5 primary malignant melanomas stadium pT1a and 5 primary malignant melanomas stadium pT2a-pT4a, as presented in Table 2 (Ethical permission; Dnr 2013-31431). Clinical data were not available. The slides were stained with hematoxylin and eosin and 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.

Immunohistochemistry and image analysis

Serial sections (5 μm) of formalin-fixed paraffin-embedded tissue were processed for routine immunohistochemistry. Antibody staining was performed with anti-tubulin β -3 (1:100), Melan-A, (Dako; 1:50) and Ki-67 (1:500). Samples were incubated with a polymer linked to alkaline phosphatase (MACH2), and were visualized using vulcan fast red (both from Histolab, Göteborg, Sweden). The slides were blinded and the staining intensity and staining pattern of tubulin β -3 was determined by K Lundmark and K Orfanidis independently. The slides were also scanned using an Aperio Scanner, viewed on the Imagescope software (Leica) and image analysis of staining intensities was performed on blinded images. The same number of melanocytic areas (300x200 μm) from the superficial and deep intradermal part of each lesion (of similar melanocytic content in paired selections) was randomly selected, and 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/ij/>). From luminosity histograms, the red chromophore density was calculated using

the slightly modified formula, $\frac{\sum(255-Value) \times Count}{Area (pixels^2)}$, as previously described (Amber, 2015; Billings et al., 2015). Lesions with no or only superficial intradermal melanocytic component were excluded from this analysis.

Cell cycle analysis

Cell cycle distribution was assayed as described by Vindelöv et al. (Vindelöv et al., 1983). After washing in PBS the cells were trypsinated pelleted and resuspended in citrate buffer (250 mM sucrose, 40 mM trisodium citrate-dihydrate and 5% DMSO, pH 7.6). Nuclei were isolated after digestion in 0.03% trypsin. Finally, cells were stained with 0.37 mM propidium iodide and 2 mM spermine tetrahydrochloride and subjected to flow cytometry (Gallios flow cytometer, Beckman Coulter Inc., USA). In each experiment, data from 5 000 cells was obtained and the histograms of DNA content were analyzed using the Kaluza software (Beckman Coulter Inc.).

Statistical analysis

Microarray data were evaluated with unpaired t-test and corrected for multiple testing using the Benjamini–Hochberg method (Huang et al., 2009a). Differentially expressed genes with a fold change $\geq \pm 2$ and p-value ≤ 0.01 were included in further analyses. GSEA used a permutation test procedure to evaluate the significance of enrichments; $p \leq 0.05$ and false discovery rate ≤ 0.25 were used as the thresholds. Fisher's exact test, as implemented in IPA and other pathway analysis applications, was used

to assess the significance of the association between a pathway and the differentially expressed genes. The image analyses of the staining intensities of histological specimens were compared using student's t-test for independent samples. One-way ANOVA and Dunnett post-hoc analyses were used for multiple comparisons between groups in the tubulin β -3 inhibition studies.

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Figure legends

Figure 1. Melanocyte senescence model system

Human melanocytes from 4 different donors were isolated and cultured for 3 days (young) and until they stopped dividing and displayed senescent morphology (minimum of 30 days). **A.** Representative images of light microscopy and β -galactosidase staining. Scale bar=5 μ m. **B.** Analysis of β -galactosidase activity. Similar symbols indicate samples from the same donor, and the horizontal line indicates mean value, * $p < 0.05$. **C.** Immunoblotting of Bid, p53, and p16 using GAPDH as loading control and a dot blot of Ki-67. The corresponding young and senescent melanocytes are marked Y1-4 and S1-4, respectively. **D.** Hierarchical clustering analysis of the young and senescent cell cultures according to the respective gene expression profiles showing the Euclidean similarity measure with Wards linkage rule. **E.** Principal component analysis using the entire set of mRNAs as variables of young (blue) and senescent (red) cultures.

Figure 2. Construction of the signaling network involving TUBB3 expression

Human melanocytes from 4 donors were cultured for 3 days (young) and until they stopped dividing and displayed senescent morphology (minimum of 30 days). Gene expression was analyzed using microarrays. The list of differentially expressed genes was transformed into a set of relevant networks using the Ingenuity pathways knowledge base. **A.** The second highest inter-connected network annotated “Cancer, Endocrine System Disorders, Hematological Disease” identified TUBB3 as the most connected gene. The node color indicates up-regulation (white) or down-regulation (grey) of significant genes. **B.** Predicted upstream regulators identified by Ingenuity pathway analysis of senescent compared to young melanocyte cultures. **C.** Immunoblotting for tubulin β -3 in 4 young (Y1-4) and the corresponding senescent (S1-4) melanocytes. GAPDH was used as a loading control.

Figure 3. Senescence-associated gene set enrichment analysis in the combined datasets of the cell culture model and a public dataset of nevi and melanomas

Human melanocytes from 4 donors were cultured for 3 days (young) and until they stopped dividing and displayed senescent morphology (minimum of 30 days). Gene expression was analyzed by microarrays and compared with a public gene set of 45 primary melanomas and 18 benign skin nevi (Talantov et al., 2005) utilizing the GSEA of up- and down-regulated gene sets in senescence with respect to the ranked expression of the public data set. **A.** Enrichment plot of up-regulated genes in senescent versus young melanocytes. Normalized enrichment score = 1.62, nominal p -value < 0.002, FDR q -value < 0.002. **B.** Heat map displaying the GSEA leading edge up-regulated genes that exhibited increased expression in nevi

versus melanomas. **C.** Enrichment plot of down-regulated genes in senescent versus young melanocytes. Normalized enrichment score =2.03, nominal p-value<0.001, FDR q-value<0.001. **D.** Heat map displaying the GSEA leading edge down-regulated genes that exhibited increased expression in melanoma versus nevi. The rows in the heat maps show the genes, and the columns show each individual sample. The scale bar shows the color-coded differential expression, whereas the range of colors shows the range of expression values (high-red, low-blue).

Figure 4. Analysis of tubulin β -3 in melanocytic nevi and primary melanomas

Immunostaining of tubulin β -3 and image analysis of staining intensities in **A.** benign melanocytic nevus, **B.** dysplastic nevus and **C.** primary malignant melanoma pT2a-pT4a. Melanocytic areas (300x200 μ m) from the superficial and deep intradermal part of each lesion (of similar melanocytic content in paired selections) were randomly selected, and the staining intensities were determined. Each symbol in the graph represents lesions from different individuals. **D.** Benign nevus (n=5), **E.** dysplastic nevus (n=4) and **F.** primary malignant melanoma pT2a-pT4a (n=5). Low magnification images, bar=1 mm; high magnification images, bar=20 μ m.

Figure 5. Effect on proliferation and migration upon silencing of tubulin β -3 and the addition of microtubule-destabilizing drugs

Human melanocyte (obtained from 4 different individuals; MC1-4) and melanoma cell cultures (WM115, WM793, WM278 and FM55P) were depleted of tubulin β -3 by treatment with two different siRNA sequences (siRNA1; S1 and siRNA2; S2) or were exposed to the microtubule-destabilizing drugs vinblastine (100 nM, 24 h) or colchicine (10 μ M, 30 min). Immunoblotting of tubulin β -3 expression in siRNA-depleted **A.** melanocytes and **B.** melanoma cell cultures. Proliferation estimated by the WST-1 assay in **C.** melanocytes and **D.** melanoma cell cultures (n=4). Flow cytometric cell cycle analysis by quantification of DNA content using propidium iodide. Percentage of cells in G0/G1 (black bars), S (grey bars) and G2/M (white bars) phase, in **E.** melanocytes and **F.** melanoma cell cultures (n=4). Migration as estimated using the Cultrex[®] 96 well Migration Assay of **G.** melanocytes and **H.** melanoma cell cultures (n=4). Similar symbols indicate samples from the same donor/melanoma cell line, and the horizontal lines indicate mean values, *p<0.05.

Figure 6. Senescence and apoptosis analysis after tubulin β -3 silencing

Human melanocyte (4 individuals; MC1-4) and melanoma cell cultures (WM115, WM793, WM278 and FM55P) were depleted of tubulin β -3 by treatment with two different siRNA sequences (siRNA1 and siRNA2). Analysis of β -galactosidase activity in **A.** melanocytes and **B.** melanoma cell cultures (n=4).

Similar symbols indicate samples from the same donor, and the horizontal line indicates mean value, * $p < 0.05$. Immunoblotting of p16 and Bid in siRNA-depleted **C.** melanocytes and **D.** melanoma cell cultures (n=4).

Supplementary figure S1. Common hallmarks of the cellular model system and a public dataset of nevi and melanoma

Gene expression was analyzed using microarrays in young and senescent human melanocytes from 4 donors and compared with a public gene set of 45 primary melanomas and 18 benign skin nevi (Talanov et al., 2005). Results of GSEA reveal enrichment plots for the common hallmarks. $p < 0.05$, FDR < 0.25 .

Supplementary figure S2. Tubulin β -3 immunostaining in melanocytic lesions

Two consecutive sections of dysplastic nevus stained for **A.** tubulin β -3 and **B.** Melan-A, bar=1 mm. Tubulin β -3 immunostaining of **C.** melanoma in situ and **D.** primary malignant melanoma, pT1a, bar=1 mm. **E.** Tubulin β -3 immunostaining of primary malignant melanoma, pT3b, depicting the disordered staining pattern (upper inset) and the strong staining at the invasive front (lower inset), bar=100 μ m.

Supplementary figure S3. Tubulin β -3 expression in melanocyte and melanoma cell lines.

Human melanocyte (n=4 individuals, MC1-4) and melanoma cell cultures (WM115, WM793, WM278 and FM55P) were depleted of tubulin β -3 by treatment with two different siRNA sequences (siRNA1 and siRNA2). Immunoblotting of tubulin β -3 expression in siRNA-depleted (Control; C, siRNA1; S1, siRNA2; S2) **A.** melanocytes and **B.** melanoma cell cultures.

Supplementary figure S4. Cell cycle analysis by flow cytometry.

Primary melanoma cells (FM55P) were stained with propidium iodide and DNA content analyzed in **A.** representative image of a control sample and **B.** sample depleted of tubulin β -3 by siRNA.

Supplementary Tables

Supplementary Table S1. Genes upregulated in senescent melanocytes compared to young.

Supplementary Table S2. Genes downregulated in senescent melanocytes compared to young.

Supplementary Table S3. IPA network analysis of differentially expressed genes in senescent versus young melanocytes.

Table 1. Gene ontology term analyses using DAVID presenting down- and up regulated genes in senescence melanocyte cultures as compared to young cultures ($p < 0.05$).

Term	Count	P-value	Genes
M phase¹	28	1.72E-15	KIF23, ADCY3, KIFC1, KIF22, KNTC1, PKMYT1, AURKB, CDCA8, NCAPH, DDX11, MC1R, H2AFX, TUBG1, FANCA, CDCA5, TUBB3 , CDCA3, EXO1, MKI67, CCNF, ESPL1, UBE2C, TACC3, RAD54L, CDC25A, NCAPD2, RAD51, FANCD2, ZWINT
Cell cycle phase¹	30	8.74E-15	KIF23, ADCY3, KIFC1, KIF22, KNTC1, PKMYT1, AURKB, GTSE1, CDCA8, NCAPH, DDX11, MC1R, H2AFX, TUBG1, FANCA, CDCA5, TUBB3 , CDCA3, EXO1, MKI67, CCNF, POLE, ESPL1, UBE2C, TACC3, RAD54L, CDC25A, NCAPD2, RAD51, FANCD2, ZWINT
Cell cycle¹	38	2.08E-13	KIF23, ADCY3, CLSPN, KIF22, KIFC1, KNTC1, PKMYT1, AURKB, GTSE1, CDCA8, CDC45, NCAPH, MCM7, MC1R, DDX11, HJURP, H2AFX, TUBG1, THBS1, FANCA, CDCA5, TUBB3 , CDCA3, EXO1, MKI67, POLE, CCNF, ESPL1, UBE2C, TACC3, RAD54L, GSG2, CDC25A, NCAPD2, RAD51, UHRF1, FANCD2, ZWINT, CHAF1A
DNA packaging¹	17	5.45E-13	HIST1H2AB, HIST1H2BB, HIST1H3J, HIST1H4L, HIST1H2BF, HIST1H1A, HIST1H2AD, HIST1H2BH, NCAPD2, HIST2H2AB, NCAPH, HIST1H2BM, HIST1H4A, HJURP, NAA10, HIST1H2AI, HIST1H3B, HIST1H3D, H2AFX, CHAF1A, CDCA5, HIST1H3G, HIST1H3H
Cell cycle process¹	31	3.96E-12	KIF23, ADCY3, KIFC1, KIF22, KNTC1, PKMYT1, AURKB, GTSE1, CDCA8, NCAPH, MC1R, DDX11, H2AFX, TUBG1, THBS1, FANCA, CDCA5, TUBB3 , CDCA3, EXO1, MKI67, CCNF, POLE, ESPL1, UBE2C, TACC3, RAD54L, CDC25A, NCAPD2, RAD51, FANCD2, ZWINT
Cell cycle arrest²	5	1.78E-03	ING4, HBP1, FOXO4, SESN1, TP53INP1
Intracellular signaling cascade²	12	4.77E-02	ING4, GPR155, ADCY1, DOK5, DUSP16, TLR1, PLCD4, RAB6B, RHOD, FOXO4, PPARGC1A, RAB27A

¹Selected GO categories for downregulated genes in senescent vs young melanocytes.²Selected GO categories for upregulated genes in senescent vs young melanocytes

Table 2. Histopathologic characterization of the melanocytic skin lesions¹.

	Characteristics	Thickness ² (mm)	Anatomic level ³
Benign nevus	compound acquired	0.4	III
	compound acquired	2.1	IV
	intra-dermal congenital	3.6	IV
	intra-dermal congenital	5.2	IV
	intra-dermal congenital	6	IV
Dysplastic nevus	intraepidermal	0	I
	compound	0.4	III
	compound	1.1	IV
	compound	1.3	IV
	compound	1.4	IV
Melanoma in situ	without intra-dermal component	0	I
	without intra-dermal component	0	I
	benign intra-dermal component	0.2	II
	benign intra-dermal component	0.4	II
	benign intra-dermal component	0.4	II
Primary malignant melanoma	pT1a, SSM ⁴	0.3	II
	pT1a, SSM	0.4	II
	pT1a, SSM	0.6	II
	pT1a, SSM	0.6	II
	pT1a, SSM	0.7	III
	pT2a, SMM, with intra-dermal satellite	1.2	III
	pT3b, nodular	2.1	III
	pT3a, other	2.2	III
	pT3a, nodular	3.3	IV
	pT4a, nodular	4.1	III

¹All cases were found without chronic sun-induced damage; no lesions were from facial or acral skin.

²Thickness represents the measurement of the nevus/ malignant melanoma cells from the granular cell layer to the deepest extension of the lesion.

³The anatomic level refers to the deepest extension of the nevus/ malignant melanoma into the papillary dermis, reticular dermis and subcutaneous tissue.

⁴Superficial spreading melanoma.

Figure 1. Orfanidis et al., 2016

Figure 2. Orfanidis et al., 2016

Figure 4. Orfanidis et al.,2016

Figure 5. Orfanidis et al., 2016

Figure 6. Orfanidis et al., 2016.