Université Catholique de Louvain
Faculté de Médecine



Unité de Génétique Cellulaire
Institut de Duve
Institut Ludwig de Recherche sur le Cancer SA

Mechanisms of gene regulation in melanoma :

Interleukin-1 strongly reduces expression of MITF, and BORIS is not required for MAGE1 activation.

Olga Kholmanskikh

Thèse présentée en vue de l'obtention du grade de
Docteur en Sciences Biomédicales et Pharmaceutiques

Promoteur : Professeur Etienne De Plaen

Année académique 2010-2011

École Doctorale de Génétique et d'Immunologie
Université Catholique de Louvain

Promoteur
Professeur Etienne De Plaen
Institut Ludwig de Recherche sur le Cancer SA,
Unité de Génétique Cellulaire,
Institut de Duve, UCL, Bruxelles

Président du jury
Professeur Jean-Christophe Renauld
Institut Ludwig de Recherche sur le Cancer SA,
Unité de Médecine Expérimentale,
Institut de Duve, UCL, Bruxelles

Membres du jury

Professeur Jean-Baptiste Demoulin
Unité de Médecine Expérimentale,
Institut de Duve, UCL, Bruxelles

Professeur Patrick Jacquemin
Unité de Recherche sur les Hormones et le Métabolisme,
Institut de Duve, UCL, Bruxelles

Professeur Thomas Michiels
Unité de Virologie
Institut de Duve, UCL, Bruxelles

Professeur Lionel Larue
Institut Curie, Equipe Génétique du Développement des Mélanocytes,
Orsay, France

Docteur Jamila Louahed
GlaxoSmithKline Biologicals, Rixensart, Belgique

La réalisation de cette thèse de doctorat a bénéficié du soutien financier du Fonds National de la Recherche Scientifique - Télévie (Belgique), de la Fondation contre le Cancer (Belgique) et du Centre du Cancer (Cliniques Universitaires S^{t} Luc, UCL, Belgique).

Большое спасибо !

Évidemment, les mots de remerciements qui suivent sont dans une langue plus habituelle pour la plupart d'entre vous. Je voulais simplement dire « merci beaucoup » de tout mon coeur à de très nombreuses personnes et ces mots me viennent plus naturellement en russe.

Je souhaite faire part de toute ma gratitude au Professeur Thierry Boon pour m'avoir octroyé la possibilité de faire de la recherche pendant 9 ans dans un environnement scientifique si favorable et pour avoir diriger aux moments décisifs des projets scientifiques sur lesquels j'ai pu travailler.

Le promoteur de thèse est une personne principale dont les conseils s'avèrent essentiels pour réaliser une thèse. En ce sens, je remercie vivement le Professeur Etienne De Plaen pour son admirable capacité à guider ses étudiants et à diriger de près au quotidien un travail passionnant de recherche tout en laissant une liberté de choix des orientations. Merci pour tout ce que vous avait fait pour moi pendant mon stage d'initiation à la recherche, puis mon DES, puis ma thèse. Vous m'aviez enseigné la rigueur scientifique et des abondantes techniques de biologie cellulaire et moléculaire, dès le tout premier gel d'agarose. Merci pour votre accueil chaleureux, votre disponibilité, la confiance que vous m'aviez accordé, la lecture attentive (et en partie l'écriture) des manuscrits et pour votre gentillesse légendaire.

Mes remerciements vont également aux membres de mon comité d'encadrement, pour votre intérêt dans le travail présenté ici, la gentillesse et la patience que vous avez manifestées à mon égard durant cette thèse, et, pour certains, dès mon DES. Merci d'avoir étés toujours disponibles pour me donner des conseils pertinents, poser un regard critique extérieur sur mes résultats et pour m'avoir fait l'honneur de participer au Jury de défense de cette thèse.

Je tiens à remercier le Professeur Lionel Larue d'avoir accepté de faire partie du Jury de soutenance de ma thèse et d'avoir attendu plus d'un an avant de pouvoir juger mon travail.

*Merci à tous les co-**labo**rateurs : Marie, Axelle, Charles, Claudine, Sandra, Yurda, Lucie, Grégory, Ange, Sandrine, Laure-Alix, Thérèse,… Grâce à vous, et les cotés scientifiques et les cotés humains de cette nouvelle expérience professionnelle ont été très enrichissants et agréables à vivre. Merci aussi pour tous les bons moments passés en dehors du laboratoire. Merci beaucoup à Charles De Smet et Axelle Loriot pour m'avoir impliquée dans l'étude qui a abouti au premier article. J'ai toujours pu compter sur votre aide pour le travail que je réalisais. Je suis très reconnaissante pour votre énergie, vos idées, les discussions avec vous et votre importante contribution dans la rédaction de l'article. Merci à Ange Prisoni Jyambere d'avoir établi courageusement et laborieusement pendant son travail de maîtrise les bases de ce projet. J'espère avoir pu transmettre une partie de mon expérience à Stéphanie, Dorian, Nathalie et Julie et je leur souhaite une bonne continuation.*

Je voudrais encore remercier Nicolas Van Baren, Francis Brasseur, Jean-Christophe Renauld, Pierre Coulie et Pierre van der Bruggen pour leur expertise et les discussions enrichissantes dont j'ai pu bénéficier pour progresser au cours du deuxième projet, ainsi que pour avoir significativement amélioré la qualité de l'article. Votre expérience scientifique et votre gentillesse ont été pour moi de véritables supports. Merci à Francis, Madeleine et plusieurs autres personnes qui ont participé à l'établissement de la collection des échantillons et des lignées de mélanome. C'est un impressionnant témoignage de prise en charge de nombreux patients au cours des études cliniques. Je tiens aussi à remercier Christophe, Bernard, Marie-Claire, Laure, Annabelle, Sophie, Julie, Sabrina, Danièle, Nicolas, Gérald, Jo, Jacques et Suzanne pour leurs précieux conseils et leur aide.

Je tiens à saluer ici les personnes qui, de près ou de loin, ont contribué à la concrétisation de ce travail de thèse de doctorat. Merci à André, Paul, Encarnacion, Pascale, Manuel, Samira, Jean-Luc, Tina, Geneviève, Aline, Carine, tous et toutes toujours dévoué(e)s et toujours sympathiques.

La thèse constitue une expérience passionnante, marquante et intense. Mais elle ne serait jamais vécue ainsi s'il n'y avait pas des moments de détente avec des formidables personnes. Bolshoe spasibo à la communauté russo-internationale (Lena, Taco, Yana, Patrick, Vlad, Johanna, Hanna, Arman, Asya, Bert, Tanya, Jean-Michel, Andrei, Marina, Marc, Marion), aux Amefes boys and girls (Oli, Domi, Johan, Annick, Nicolas, Alessia, Pierre, Aline, Renzo, Dirk, Marius, Joëlle), à l'Apero team (Marina, Nicolas, Marjorie, Alexandre, Grégory, Gaëlle, Pierre, Ivan, Chrystel, Céline, Antonia), aux Télévie girls (Valérie, Amel, Violaine, Véronique), aux Martini girls (Tekla, Federica, Alexandra, Roxana), au guitare club, au groupe Dolomites, et aux ami(e)s de l'IGEAT. Je tiens surtout à remercier Yana, Lena, Lena, Vlad, Masha et Tatiana Yurievna pour leur encouragement par delà les kilomètres qui nous séparent. Spasibo à Myriam et Nehman pour leur aide et amitié.

La réalisation de cette thèse n'aurait pas été possible sans le soutien moral et affectif de mes parents et beaux-parents. De tout coeur, je souhaite beaucoup de courage à ma maman. Merci à André et Annette qui m'ont soutenu tout au long de ces années d'études et travail en Belgique. Merci de m'avoir toujours fait confiance. Ma petite sœur Irina et mon beau-frère Serge, spasibo d'être là, avec Yuri et Florence, malgré la distance. Merci infini à la famille à Perm, à Anderlecht, à Toronto, à Simferopol, à Moscou, à Dilbeek, à Ecaussinnes, à Bordeaux et bien sûr à Abel & Co en Gaume pour votre chaleur familiale et pour votre soutien.

Il y a cependant des personnes qui n'auront plus jamais l'occasion de voir cette thèse. Et pourtant c'est ici que je voudrais leur rendre un hommage. J'ai la profonde tristesse de citer mon père, dedushki Vasilii et René, Marie-Paule et Jeannine. Leur amour du travail bien fait et leurs encouragements vont toujours rester avec moi.

J'ai l'impression que toutes mes dix années de vie en Belgique se sont concentrées sur ces deux pages de remerciements initialement prévues comme les remerciements d'une thèse. Et je ne peux en aucun cas oublier encore une personne à remercier absolument. Je dédie ce travail à Mathieu, grâce à qui une histoire de thèse a commencé et a pu se terminer. Spasibo d'avoir vécu une deuxième fois [672] cette étape importante dans la vie tout en gardant patience et courage.

Spasibo également de la part d'une personne à venir…

Bruxelles, le 1er mars 2011

TABLE OF CONTENTS

LIST OF ABBREVIATIONS **9**
SUMMARY/RESUME **11**
INTRODUCTION **15**

I. Melanocyte biology and MITF transcription factor **19**
I.1 Melanocytes and melanogenesis ***19***
I.2 MITF as master regulator of melanocyte biology ***21***
I.2.1 The MITF gene and protein ***21***
I.2.2 Germline MITF mutations ***24***
I.2.3 Regulation of melanocyte biology by melanocyte-specific MITF-M isoform ***25***
I.2.3.1 Regulation of melanogenesis *25*
I.2.3.2 Regulation of melanogenesis in the course of the physiological response to ultraviolet radiation *29*
I.2.3.3 MITF transcriptional targets control proliferation and survival *33*
I.2.4 MITF expression is essential for melanocyte development ***37***
I.2.5 Role of epigenetic mechanisms in establishment and maintenance of melanocyte identity ***39***
I.2.5.1 Epigenetics and cellular identity *39*
I.2.5.2 DNA methylation *42*
I.2.5.3 Post-translational histone modifications *46*
I.2.5.4 Accessibility of nucleosomal DNA and catalyzed nucleosome remodeling *47*

II. Genetic and epigenetic events in cutaneous melanoma **49**
I.1 Melanomagenesis as a multi-step process ***49***
II.2 Genetic and epigenetic patterns in melanoma ***51***
II.3 Major signaling pathways involved in melanomagenesis ***53***
II.4 MITF expression and function in melanoma ***55***
II.5 Cancer-germline genes in melanoma: mechanisms of reactivation and known functions of their protein products ***61***

III. Immune response in cutaneous melanoma **71**
III.1 Dual role of the immune system in melanomagenesis ***71***
III.2 Activation of T cells and recognition efficiency ***74***
III.3 Antigens encoded by genes having altered expression or integrity in melanoma ***77***
III.4 Antigens encoded by melanocyte differentiation genes and autoimmunity ***78***
III.5 Major immunotherapeutic strategies in melanoma ***80***

IV. Aims of the study **81**

RESULTS **83**

PART I. Expression of BORIS in melanoma: lack of association with MAGE-A1 activation. **85**
PART II. Interleukins 1α and 1β secreted by some melanoma cell lines strongly reduce expression of MITF-M and melanocyte differentiation antigens. **105**
PART III. Deciphering mechanisms of MITF-M repression in two melanoma cell lines secreting interleukins 1α and 1β. **129**

DISCUSSION AND PERSPECTIVES **139**

I. Summary of key findings **141**
II. Quantitative approach in estimating the efficiency of antigen presentation by tumor cells **142**
III. BORIS as a potential target for immunotherapy in melanoma **144**
IV. Deciphering the role of BORIS in CG gene regulation in melanoma **145**
V. Concomitant and selective loss or downregulation of melanocyte-specific genes **150**
VI. IL-1 release in the tumor environment as a mechanism of MITF-M downregulation in melanoma **152**
VI.1 IL-1 expression in melanoma ***152***
VI.2 Why do melanoma cells make IL-1? ***153***
VI.2.1 Mechanisms underlying IL-1 release by melanoma cells *153*
VI.2.2 Mechanisms underlying constitutive IL-1 synthesis by melanoma cells *155*
VI.2.3 The failure of negative regulatory mechanisms *159*
VI.3 Mechanisms of IL-1-mediated MITF-M downregulation **161**
VI.3.1 Transcriptional regulation *161*
VI.3.2 Post-transcriptional regulation of MITF-M mRNA stability *166*
VI.3.3 Epigenetic modifications *167*
VII. Contribution of IL-1 to the tumor-immune system interactions and the tumor phenotype in the course of melanoma progression **168**
VIII. Relevance of the studied mechanisms of melanogenesis regulation in other physiological and pathological settings **174**
IX. Potential approaches in combining immunotherapy with targeted treatment in melanoma **175**

REFERENCES **181**
ANNEX **217**

LIST OF ABBREVIATIONS

α-MSH	α-Melanocyte Stimulating Hormone
AP-1	Activator Protein 1
ARF	Alternative Reading Frame
BCL2	B-cell Lymphoma 2
bHLH-LZ	basic-Helix-Loop-Helix Leucine Zipper
BORIS	Brother of the Regulator of Imprinted sites
BRM	Brahma
BRG-1	Brahma-Related protein-1
BRN2	brain-2/POU class 3 homeobox transcription factor 2
CDK	Cyclin-Dependent Kinase
CG	Cancer Germline gene
ChIP	Chromatin Immunoprecipitation
CRE	cAMP-responsive element
CREB	CRE binding factor
CTCF	CCCTC-binding factor
CTL	Cytolytic or Cytotoxic T Lymphocyte
DC	Dendritic Cell
DCT	Dopachrome Tautomerase
EDNRB	Endothelin Receptor B
EMSA	Electrophoretic Mobility Shift Assay
ERK	Extracellular signal Regulated protein Kinase
GPCR	G-Protein Coupled Receptor
gp100	glycoprotein 100
HLA	Human Leukocyte Antigen
IFN-γ	Interferon-gamma
IL	Interleukin
IL-1R	Interleukin-1 receptor
IL-1RAcP	Interleukin -1 Receptor Accessory Protein
JNK	c-Jun N-terminal kinase
KAP1	KRAB (Kruppel-associated box)-associated protein 1
kb	kilobase
kDa	kilodalton
LOH	Loss of Heterozygosity
LRO	Lysosome-Related Organelles
MAGE	Melanoma Antigen
MAPK	Mitogen-Activated Protein Kinase
MATP	Membrane-Associated Transporter Protein
MEK	MAPK/ERK kinase
MKK	MAPK kinase
MHC	Major Histocompatibility Complex
MITF	Microphthalmia-associated Transcription Factor
NEMO	NF-κB Essential Modulator
NF-κB	Nuclear Factor kappa B
OA1	Ocular Albinism 1
OCA	Oculocutaneous Albinism

PAX3	Paired box 3
PKA	Protein Kinase A
PKC	Protein Kinase C
PTEN	Phosphatase and Tensin Homolog
p90RSK	p90 ribosomal S6 kinase
Puro	puromycin resistance
qPCR	quantitative Polymerase Chain Reaction
BRAF	v-Raf murine sarcoma viral oncogene homolog B1
RAS	Rat Sarcoma oncogene
RB	Retinoblastoma protein
RGP	Radial Growth Phase
RPE	Retinal Pigment Epithelium
RT	Reverse Transcriptase
RTK	Receptor Tyrosine Kinase
SCF	Stem Cell Factor
SOX	SRY (Sex determining Region Y)-type HMG (high-mobility-group) box
SWI/SNF	Switching defective/Sucrose Non-Fermenting
STAT	Signal Transducer and Activator of Transcription
TAK1	Transforming Growth Factor β (TGF β)-Activated Kinase-1
TAP	Transporter Associated with antigen Processing
TCR	T Cell Receptor
TIL	Tumor Infiltrating Lymphocyte
TNF-α	Tumor Necrosis Factor-alpha
TSS	Transcriptional Start Site
TYR	Tyrosinase
TYRP	Tyrosinase-Related Protein
UTR	Untranslated Region
UVR	Ultraviolet Radiation
VEGF	Vascular Endothelial Growth Factor
VGP	Vertical Growth Phase

SUMMARY

Modulation of the immune system for therapeutic ends is now increasingly being employed in patients with advanced cutaneous melanoma, a deadly cancer arising from melanocytes. Cancer immunotherapy, which harnesses the protective capacity and specificity of the immune system toward eliminating cancer cells, generally capitalizes on the specific recognition of tumor antigens by cognate CTLs (cytolytic T lymphocytes). This recognition requires efficient presentation of antigenic peptides on the surface of tumor cells.

The aim of this thesis was to explore the mechanisms of transcriptional regulation of genes encoding melanoma antigens in order to identify potential strategies to increase their repertoire and the expression levels.

A group of CG (cancer-germline) genes uses primarily DNA methylation for transcriptional repression in somatic cells, whereas genome hypomethylation in tumors results in their activation and presentation of relevant antigenic peptides recognized by cognate CTLs. In the first part of this thesis, we explored the potential of protein BORIS (Brother of the Regulator of Imprinted sites) to induce and/or maintain the activation of *MAGE-A1* (Melanoma AntiGEn A1) and other CG genes in melanoma cells. BORIS expression on its own was insufficient to produce these effects and hence may not serve as a general means to increase the repertoire of melanoma antigens. The quantitative analysis of *BORIS* transcripts revealed the expression pattern characteristic to CG genes. *BORIS* activation in a substantial proportion of melanoma samples indicates the potential for immunotherapeutic targeting of BORIS-derived antigenic peptides. The identity of such peptides remains to be investigated.

The second part of this thesis focused on the mechanisms of the transcriptional regulation of MITF-M, the melanocyte-specific isoform of MITF (Microphthalmia-associated transcription factor). MITF-M is a critical transcriptional activator of melanocyte differentiation genes such as *MLANA*, *tyrosinase* and *gp100*, which encode antigenic peptides recognized on melanoma cells by cognate CTLs. The microarray gene expression studies performed at the Brussels Branch of the Ludwig Institute for Cancer Research have revealed the downregulation of melanocyte differentiation genes in melanoma cell lines exposed to inflammatory cytokines. Based on these observations, we assessed the mechanisms of *MITF*-M repression by IL-1 (interleukin 1) cytokine. This repression, either induced by exposure to recombinant IL-1 or permanent in melanoma cell lines constitutively producing IL-1, appeared to involve the NF-κB (nuclear factor kappa B) and JNK (c-Jun N-terminal kinase) signaling. The expression level of the *IL1R1* (IL-1 receptor 1) was determined as an important parameter of IL-1-mediated transcriptional changes observed in melanoma cell lines. The reduced ability of melanoma cell lines treated with IL-1 to activate differentiation antigen-specific CTL clones was mechanistically demonstrated *in vitro*. Our results indicate that the repression of melanocyte differentiation genes by IL-1 produced by stromal cells or by tumor cells themselves may represent an additional mechanism of melanoma immune escape.

In conclusion, our study got insight into mechanisms regulating the expression of melanoma antigens and pointed to the impact of genetic and epigenetic changes in melanoma cells on tumor-immune system interactions. The potential of combining immunotherapy with therapeutic strategies targeting particular genetic and epigenetic aberrations in melanoma cells should be further investigated.

RESUME

Une modulation du système immunitaire dans un but thérapeutique est de plus en plus utilisée chez les patients atteints de mélanome avancé, un cancer mortel issu des mélanocytes. L'immunothérapie du cancer tire parti de la capacité protectrice du système immunitaire et de sa spécificité pour éliminer la tumeur en se basant de façon générale sur la reconnaissance spécifique des antigènes tumoraux par les CTL (lymphocytes T cytolytiques). Cette reconnaissance requiert une présentation efficace de peptides antigéniques à la surface des cellules tumorales.

Le but de ce travail de thèse était d'étudier les mécanismes de régulation transcriptionnelle des gènes qui donnent naissance aux antigènes de mélanome pour identifier les stratégies potentielles d'augmentation de leur répertoire et de leur niveau d'expression.

Un groupe de gènes cancer-lignée germinale (CG) utilise la méthylation de l'ADN comme mécanisme de répression transcriptionnelle dans les cellules somatiques, tandis que l'hypométhylation du génome caractéristique des tumeurs aboutit à l'activation de ces gènes et à la présentation de peptides antigéniques reconnus par les CTL spécifiques. Nous avons exploré le potentiel de la protéine BORIS (en anglais, Brother of the Regulator of Imprinted sites) d'induire et/ou maintenir l'activation de *MAGE-A1* (en anglais, Melanoma AntiGEn A1) et d'autres gènes du groupe CG dans les cellules de mélanome. L'expression de BORIS n'était pas une condition suffisante pour produire ces effets et, ainsi, ne peut pas servir d'outil pour augmenter le répertoire des antigènes de mélanome. L'analyse quantitative des transcrits de *BORIS* a révélé le profil d'expression caractéristique pour des gènes du groupe CG. L'activation de *BORIS* dans une proportion substantielle des échantillons de mélanome indique le potentiel de ciblage thérapeutique des antigènes dérivés de la protéine BORIS. L'identité de ces antigènes reste à explorer.

La deuxième partie de cette thèse s'est focalisée sur des mécanismes de régulation transcriptionnelle de MITF-M, l'isoforme de MITF (en anglais, Microphthalmia-associated transcription factor) spécifique des mélanocytes. MITF-M est un activateur transcriptionnel essentiel des gènes de différenciation mélanocytaire comme *MLANA*, *tyrosinase* et *gp100.* Ces gènes codent des peptides antigéniques reconnus à la surface des cellules de mélanome par des CTL spécifiques. En utilisant la technologie des micro-damiers, les études d'expression des gènes effectués à l'Institut Ludwig pour la Recherche sur le Cancer à Bruxelles ont révélé la répression des gènes de différenciation mélanocytaire dans les lignées de mélanome traitées avec des cytokines inflammatoires. En s'appuyant sur ces observations, nous avons étudié les mécanismes de répression de *MITF*-M par la cytokine IL-1 (interleukine 1). Cette répression, soit induite par l'IL-1 recombinante, soit permanente dans des lignées de mélanomes qui produisent l'IL-1 de façon constitutive, implique les voies de signalisation de NF-κB (en anglais, nuclear factor kappa B) et JNK (en anglais, c-Jun N-terminal kinase). Le niveau d'expression d'*IL1R1* (en anglais, IL-1 receptor 1) a été déterminé comme un paramètre important des modifications transcriptionnelles induites par l'IL-1. Nous avons également démontré *in vitro* que, suite au traitement par l'IL-1, des lignées de mélanome acquièrent une capacité réduite à activer des populations clonales de CTL qui

reconnaissent spécifiquement les antigènes de différenciation. Ces résultats indiquent que la répression des gènes de différenciation mélanocytaire par l'IL-1 produit par les cellules du stroma ou par les cellules tumorales représente un mécanisme additionnel d'échappement des tumeurs à la réponse immunitaire.

En conclusion, notre étude a approfondi la connaissance des mécanismes de régulation des antigènes de mélanome et a souligné l'impact des altérations génétiques et épigénétiques sur les interactions entre les cellules de mélanome et le système immunitaire. Il est important d'explorer le potentiel qu'il y aurait à combiner l'immunothérapie avec des stratégies thérapeutiques visant des aberrations génétiques et épigénétiques propres aux cellules de mélanome.

INTRODUCTION

Cutaneous malignant melanoma is a cancer that arises from melanocytes, which are specialized cells producing pigment melanin. The first report of melanoma is found in the writings of Hippocrates, where he described "fatal black tumors with metastases". Although melanoma represents less than 5% of malignant skin tumors, it accounts for as many as 80% of all skin cancer deaths and its incidence rates are constantly rising [1, 2]. While surgical excision of a primary tumor can be an effective cure, metastatic melanoma presents a substantial clinical challenge due to its notorious resistance to radio- and chemotherapy [1]. There is an urgent need to explore novel therapeutic approaches based upon a thorough understanding of melanoma biology at the molecular and cellular levels, including melanoma interactions with the host organism and particularly with its immune system.

Enormous efforts taken to unravel the molecular mechanisms that lead to malignant transformation of melanocytes have resulted in the development of a molecular targeted therapy approach, which aims to selectively inhibit signaling pathways activated by mutations in tumor cells. The clinical efficiency of this approach has supplanted the development of relatively nonspecific cytotoxic agents, whose selectivity is based on induction of cell death in highly proliferative cell compartments, including rapidly proliferating normal cells. Although targeted therapy has demonstrated remarkable clinical benefit in melanoma, the ablation of a single target or pathway essential for tumor proliferation does not appear to produce sustained growth inhibition [3]. Thus, therapeutic interference with the different interconnected signaling pathways has been assumed to be necessary to provide the long-term efficiency of molecular targeted therapy [4].

Besides molecular selectivity in tumor killing, the protective capacity of the immune system is employed to specifically eliminate melanoma cells [5]. Demonstration of specific immune responses to tumor antigens [6] along with the possibility to follow the T lymphocyte activation during immune stimulation has opened opportunities to employ new approaches in the immunotherapy of melanoma [5]. Harnessing the immune system to target malignancy is a powerful tool, not only due to the specificity of the immune response, but also due to the potential for establishing long-lasting antitumor immunity that constitutively eliminates tumor cells and prevents the outgrowth of metastases. Although different immunotherapeutic approaches lead to limited responses in metastatic melanoma, which often do not exceed a 15% response rate threshold, long-term control of the disease and increased survival have been achieved [7, 8]. However, growing tumors manage to escape the immune response, either spontaneous or induced by immunotherapy [5], and to promote immunosuppression [9]. Therefore, maximizing the efficiency of immunotherapy in establishing active immunity against melanoma necessitates a keen understanding of the mechanisms employed by tumor cells to escape the immune response and to promote an immunosuppressive environment.

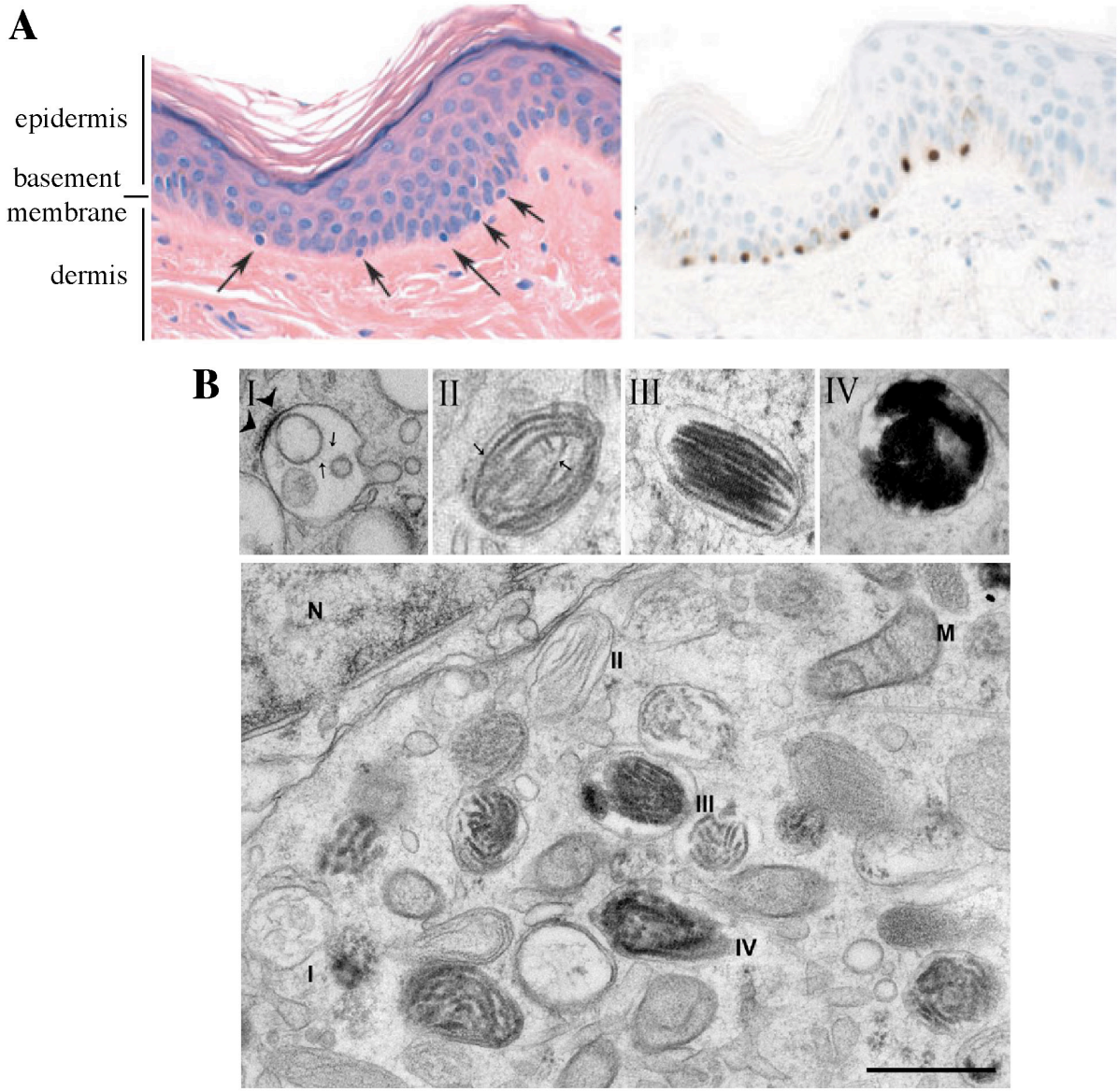


Figure 1. Melanocytes and melanogenesis. A, left. Haematoxylin and eosin stain of normal human skin. Keratinocytes of the upper layer of the epidermis contain large nuclei, which stain blue, and the dermis appears pink as a result of collagen staining. Melanocytes (arrows) located in the basal layer of the epidermis have smaller nuclei and inconspicuous cytoplasm. **A, right.** Immunohistochemical analysis of the human skin identifies melanocytes by using the monoclonal antibody D5, which stains the MITF transcription factor in their nuclei. Adapted from Lin and Fisher, 2007 (11).
B. Ultrastructural characterization of melanosomes. Electron microscopy analyses of MNT-1 human melanoma cells fixed by high pressure freezing before cryosubstitution and embedding. The four stages of melanosome development are shown in the upper panels . Stage I premelanosomes are distinguishable as round-shaped early endosomes having dense bilayered coat (arrows) and containing intralumenal vesicles (ILVs) (small arrows). Stage II premelanosomes are elongated to the shape of an American football and have fibrillar structures organized as parallel sheets (arrows) . Synthesized melanin deposits on the fibrils, resulting in their thickening and blackening in stage III melanosomes until all internal structure is masked in stage IV melanosomes. The main panel shows a typical field of MNT-1 cytoplasm near the nucleus, which contains all four stages of melanosomes. M, mitochondria; N, nucleus. Scale bar: 0,5 μm. Adapted from Raposo and Marks, 2007 (16).

I. Melanocyte biology and MITF transcription factor

I.1 Melanocytes and melanogenesis

Epidermal melanocytes represent about 4-5% of epidermal cells. They predominantly and steadily reside at epidermal-dermal junctions adjacent to the basement membrane in an average ratio 1:10 to keratinocytes [10, 11] (Fig. 1A). Whereas keratinocytes proliferate and progressively move toward the skin surface while they differentiate and keratinize, melanocytes are differentiated and rarely proliferating cells. Their homeostatic balance between differentiation and proliferation, survival and cell death is mainly controlled by keratinocytes via paracrine factors and intercellular contacts [12, 13]. Melanocyte proliferation entails detachment from the basement membrane, loss of dendrites, cell division, migration along the basement membrane and establishment of new contacts with keratinocytes and the extracellular matrix [12]. Besides keratinocytes, melanocytes communicate with other epidermal cells, such as antigen-presenting Langerhans cells, and with dermal fibroblasts [13-15].

The main function of melanocytes is melanogenesis, which includes multiple biological events. The biochemical process of tyrosine conversion into biopolymer melanin occurs within cytoplasmic organelles called melanosomes, which display unique structural features. Melanosomes belong to a distinct group of lysosome-related organelles (LROs), adapted to serve specialized functions in several cell types [16]. Unlike classical secretory granules, LROs share some proteins with lysosomes but seem to be derived from endosomes. Melanosome biogenesis entails a complex stepwise maturation process (stages I-IV), where the formation of a luminal matrix precedes the deposition on it of melanin [16] (Fig. 1B). Once matured, melanosomes themselves become melanin cargos, which are transported along microtubules from the cell center to actin-rich dendritic tips [17]. Subsequently, melanosomes are transferred into adjacent keratinocytes and form cap-like structures resembling umbrellas over the keratinocyte nuclei [17]. Each epidermal melanocyte, by virtue of its dendrites, exports melanosomes to about 30-40 keratinocytes in the upper layers of epidermis. In hair, a similar process of melanosome transfer exists, with melanosomes being passed from differentiated follicular melanocytes to hair cortical keratinocytes that cornify and make up the shaft of the hair. In contrast to skin melanocytes, melanosomes seem to be retained intracellularly in melanocytes of non-cutaneous locations (eye, inner ear, heart and leptomeninges) and in the second type of melanin-producing cells, retinal pigment epithelium (RPE) forming the cell layer behind the retina [17]. While both types of melanin-producing cells populate the uvea formed by the iris, ciliary body and choroids, it is mostly the melanin content within iris melanocytes that contributes to the impression of eye color [18]. In the stria vascularis of the inner ear, melanocytes are thought to maintain the level of the endolymphatic potassium required for the auditory nerve action potential [19].

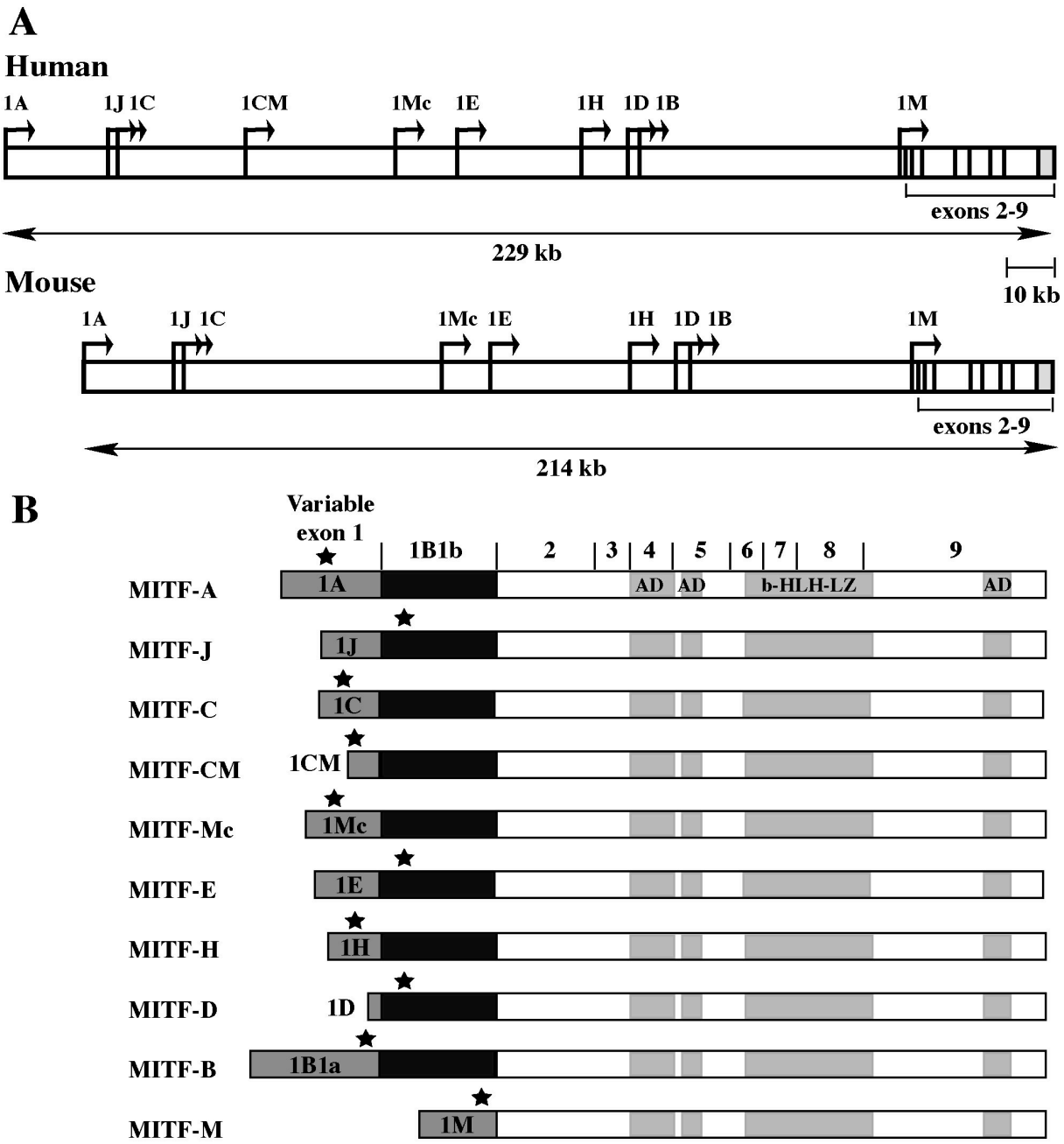


Figure 2. *MITF/Mitf* genomic organisation and MITF isoforms. A. The organisation of the human *MITF* and murine *Mitf* genes are depicted to scale with the scale bar representing 10 kb. The human locus covers 229 kb and the mouse locus about 214 kb from the beginning of the exon 1A to the end of the exon 9 (including the 3'UTR). Black bars indicate exons, the start and direction of transcription of each exon is marked with an arrow. The location of the exon 1CM in the human *MITF* is depicted as described in Shiohara *et al.*, 2009 (32). The size of exons is not shown. Modified from Hershey and Fisher, 2005 (31). **B.** Schematic representation of the structure of MITF isoforms. The isoform-specific first exons (grey) are spliced to exon 1B1b (black) and then to the common exons 2-9 (white), which encode the functional domains in light grey (AD, activation domain and b-HLH-LZ, basic/helix-loop-helix/leucine zipper). The MITF-B isoform is encoded by the entire exon 1B (1B1a/1B1b), whereas the other isoforms splice out 1B1a and contain only the later portion of exon 1B (1B1b). Exon 1CM is depicted as described in Shiohara *et al.*, 2009 (32). The predicted start ATGs are shown with asterisks. Modified from Hershey and Fisher, 2005 (31).

I.2 MITF as master regulator of melanocyte biology

Expression of numerous genes encoding enzymatic, structural and regulatory proteins that ensure melanogenesis is regulated by a remarkable protein, Microphthalmia associated transcription factor (MITF), known as transcriptional activator. MITF is the human homolog of the mouse *mi* (*Mitf*) gene product, cloned on the basis of a transgenic insertion into the *mi* (*Mitf*) locus in animals having markedly small eyes (hence the name *microphthalmia*), snow-white coat color and hearing loss [20-22]. MITF has been also proposed to be read as Melanocyte Inducible Transcription Factor, since substantial evidence points to its role as a master regulator of prenatal and postnatal melanocyte biology [23, 24]. Since its discovery, MITF has been shown to regulate a broad variety of genes, whose protein products coordinate almost every aspect of pigment cell behavior, including melanogenesis, morphology, proliferation and survival [25]. Genome-wide approaches have recently identified new potential MITF transcriptional targets [26-29]. Thus, an extensive list of 42 known and 71 potential MITF targets has been established using a two-step DNA microarray-based approach [26], while chromatin immunoprecipitation (ChIP) combined with tiling promoter microarrays (ChIP-on-chip) has predicted 117 MITF binding sites [28]. Moreover, MITF-M exerts melanocyte-specific transcriptional activity on the promoter of *DICER*, which encodes the RNase III endonuclease indispensable for the maturation of microRNAs (miRNAs) [29].

I.2.1 The MITF gene and protein

The *MITF*/*Mitf* locus of about 200 kb in length maps to chromosome 3 in human and chromosome 6 in mice and has a similar structure in the two species [30, 31](Fig. 2A). It encodes numerous MITF isoforms. Isoform MITF-M is predominantly and specifically expressed in melanocytes and shows 94.4 % of amino acid identity between human and mice. The diversity of MITF transcripts is generated by two mechanisms: alternative splicing and alternative initiation of transcription due to alternative promoter usage. *MITF*/*Mitf* locus harbors 10 alternative promoters upstream of the same number of widely spaced first exons that are individually spliced to exons 2-9 common to all *MITF* transcripts [30, 32] (Fig. 2A). The most proximal 1M promoter drives the expression of *MITF*-M transcript, in which 5' exon 1M is directly spliced to exons 2-9. Exon 1M encodes 11 amino-terminal amino acids of isoform MITF-M (Fig. 2B). The other isoforms have distinct amino termini. Six isoforms (*MITF*-A, -B, -C, -CM, -H, -MC) contain a common domain of 83 amino acid residues encoded by the 3' portion of exon 1B (1B1b) and a unique domain encoded by distinct first exons (Fig. 2B). The first exons of *MITF*-D, -E and -J transcripts are non-coding and translation is initiated directly from exon 1B1b (Fig. 2B).

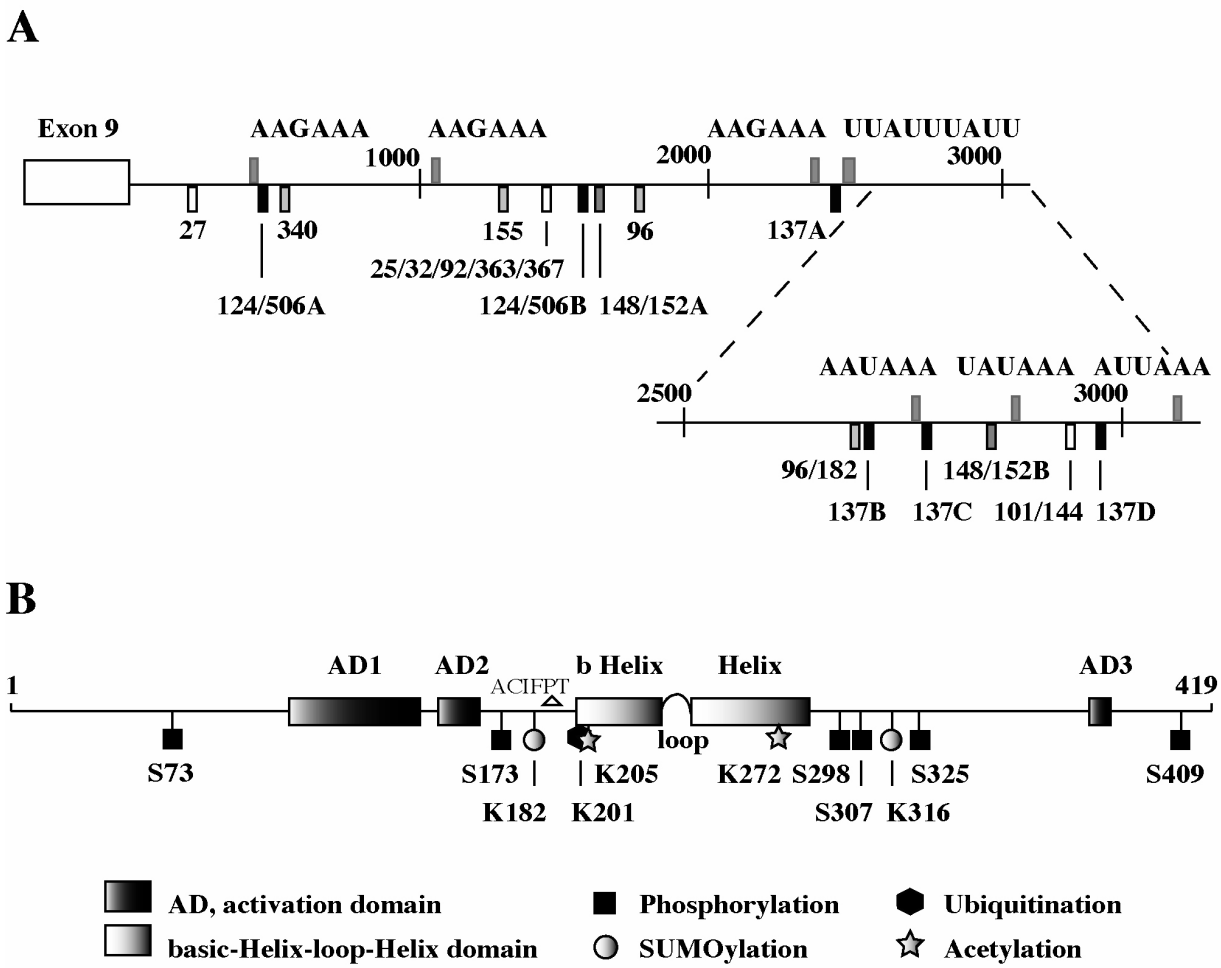


Figure 3. MITF-M 3'UTR and protein. A. Schematic presentation of the 3'UTR of the human *MITF* transcript. Potential miRNA binding sites conserved between human and mouse are indicated below the line, while potential polyadenylation sites, as well as the nonamer motif UUAUUUAUU corresponding to an AU-rich element (ARE) are indicated above the line. The coding region of exon 9 is shown, positions are numbered starting at the first nucleotide after the STOP codon. The location of seed sequences for miR-96/182 and miR-340 (light grey bars) conserved between human and mouse is depicted as described in Xu *et al.*, 2007 (42) and Goswami *et al.*, 2010 (44), respectively. The location of the predicted miR-155 seed sequence is shown. Modified from Haflidadottir *et al.*, 2010 (45).
B. A schematic drawing showing positions of protein domains in the MITF-M/Mitf-M isoform, as well as the positions of known (S73, S298, S307, S409, K182, K201, K316) and predicted (K205, K272, S325) post-translational modification sites. Activation domains are labeled AD1-AD3 and their approximate positions are shown, as only AD1 has been mapped precisely. The position of amino acids ACIFPT encoded by exon 6a is depicted. Modified from Hallsson *et al.*, 2007 (30).

If distinct amino-termini may confer functional diversity to MITF isoforms, the use of alternative promoters with distinct transcription factor-binding motifs results in cell type-specific expression differences and differential expression of *MITF* transcripts in response to external stimuli. In normal human epidermal melanocytes, *MITF*-M and *MITF*-A mRNAs represent about 80% and 18% of total MITF transcripts, respectively [33]. While expression of *MITF*-M is strictly melanocyte-specific, other *MITF* transcripts show wider pattern of expression in different cell types, while being enriched in some cell lineages: *MITF*-A and -C - in the RPE; *MITF*-D - in the RPE and osteoclasts; *MITF*-CM, -MC and -E - in mast cells [31, 32, 34-36]. In cardiomyocytes, the selective induction of *MITF*-H expression is observed following β-adrenergic stimulation [37].

In addition to the alternative usage of 5' exons, a complex pattern of splicing has been described for other exons [30, 31]. Noteworthy, alternative splicing occurs for exon 6a in all isoforms leading to production of isoforms with inclusion (+) or exclusion (-) of amino acids ACIFPT upstream of the DNA-binding basic domain [38, 39]. In cultured human and mouse melanocytes, *MITF* (+) transcripts are more abundant than *MITF* (-) transcripts (<10% of total MITF mRNA) and give rise to protein products of 419 or 413 amino acids, respectively [40].

A number of potential miRNAs target sites were found within the 3' untranslated region (3'UTR) of MITF [30], hence providing an additional level of post-transcriptional MITF regulation (Fig. 3A). Several miRNAs have been reported to negatively affect MITF mRNA expression, such as miR-96, miR-137, miR-148, miR-182 and miR-340 [41-45]. Candidate miRNAs that may be specific to the melanocytic lineage have been predicted by deep sequencing of small RNA libraries [46]. Importantly, alternative cleavage and polyadenylation generates MITF transcript variants, which have 3'UTR of different length and may be thus differentially targeted by miRNAs [44] (Fig. 3A). In normal human melanocytes, more than a half of the MITF mRNA pool is represented by transcripts containing long 3'UTR of 3 kb in length [44]. In addition, the presence of AU-rich elements (ARE), namely one nonamer motif UUAUUUAUU (Fig. 3A) and eight pentamers of AUUUA, in the 3'UTR of MITF mRNAs might modify their stability regulated via RNA-binding proteins [47].

MITF is a member of the family of basic helix-loop-helix leucine zipper (bHLH-LZ) transcription factors. All MITF isoforms share a basic DNA-binding domain (b), a helix-loop-helix (HLH) motif involved in DNA binding and dimerization as well as a leucine zipper domain (LZ) providing additional dimerization elements (Fig. 2B, 3B). The basic domain of 19 amino acids is encoded by exons 6b and 7 of MITF and allows MITF homodimers to bind target DNA regulatory regions containing consensus Enhancer box (E-box) CA*NN*TG motif, preferentially CA(C/T)GTG [25]. Binding affinity and transcriptional activity, which might depend on specific promoter context, seem to be higher for MITF (+) than for MITF (-) isoforms [48, 49]. Among four acidic transactivation domains, the activation domain 1 (AD1) encoded by exon 4 is better characterized than AD2, AD3 and AD4, which are encoded by

exons 5, 9 and *MITF*-A-specific exon 1A, respectively [30] (Fig. 2B, 3B). MITF protein is a subject to numerous post-translational modifications (Fig. 3B). Phosphorylation can decrease MITF stability and/or increase its transcriptional activity [50-52]. In addition, ubiquitination marks MITF for degradation [53] and sumoylation affects DNA binding specificity [54, 55].

I.2.2 Germline MITF mutations

Mutations of human *MITF* are associated with auditory-pigmentary syndromes such as a *MITF*-linked subset of type 2 Waardenburg Syndrome (WS2) and the highly related Tietz syndrome [56]. WS is named after the Dutch ophthalmologist Petrius Waardenburg, who first noticed that people with different colored eyes (heterochromia irides) and hypopigmented white patches in skin and hair often had a hearing impairment. With prevalence estimated to be 1/42,000-1/100,000, WS is responsible for 1-3% of total congenital deafness [57]. Within clinically and genetically heterogeneous WS, heterozygous *MITF* mutations are estimated to occur in about 15% of WS2, termed WS2A [57]. *MITF* mutations generally produce a dominantly inherited phenotype and can occur *de novo* [57]. To date, 21 *MITF* mutations have been described. Two of them lead to exon 1M splicing alterations, while most of the others are located in exons 6 to 8 encoding the bHLH-LZ motif and likely affect all MITF isoforms [56]. In Tietz syndrome, mutations in the basic domain have been documented, including the exact equivalent of the mouse *microphthalmia* mutation (ΔR217), and have been proposed to exert a dominant negative effect by sequestrating wild-type MITF in dimers deficient in DNA binding [58]. Haploinsufficiency appears to be a mechanism underlying WS2A, where the amounts of functional wild-type dimers encoded by the normal allele are insufficient, whereas the mutated allele gives rise to MITF truncation (for instance, R214TER) or affects HLH or LZ motifs leading to impaired MITF dimerization [58]. Thus, patients with Tietz syndrome have a more severe phenotype, with profound deafness being a constant feature, while patients with WS2A exhibit hearing defects of variable severity in about 70% of cases. The sensorineural hearing loss reflects the loss of melanocytes in the inner ear. Whereas patients with Tietz syndrome generally have uniform skin depigmentation (patients are born “snow white”) and vivid blue eyes, WS2 patients have patchy depigmentation areas in 10-20 % of cases and in about one third of cases exhibit heterochromia irides, white forelock or premature hair graying [56]. In mice, more than 25 either semi-dominant or recessive mutant alleles of *Mitf* have been described [59]. All mutants have reduced or absent pigmentation due to a various degree of melanocyte loss. *Mitf* homozygous mutant mice are completely white and deaf, reflecting absence of melanocytes in the skin and the inner ear, whereas other cell types are also affected, such as osteoclasts, mast cells, natural killer cells and RPE [59]. The abnormalities in the development of the RPE, which shows hyperproliferative and unpigmented phenotype, lead to defects in the expansion of the eye ball and hence to small eyes (microphthalmia) [60].

I.2.3 Regulation of melanocyte biology by melanocyte-specific MITF-M isoform

I.2.3.1 Regulation of melanogenesis

If the search of target genes for transcription factors often represents the Holy Grail, potential downstream targets of MITF-M isoform, called MITF herein later in the text, could be predicted by combining transcription factor binding information and observation of pigmentation phenotypes. Thus, mutations in genes encoding proteins with specialized function in melanogenesis are generally associated with hypomelanotic/amelanotic disorders, in which melanocyte function is reduced or absent, but viability is not affected [61]. When the viablility of melanocytes is affected, their reduced/absent number is observed in hypomelanocytic/amelanocytic disorders, such as WS [61]. The genes essential for the viability of melanocytes may be critical for the viability the whole organism, thus their regulation and function are difficult to find out in melanocytes. Much of melanogenesis comprehension came from studies in mice due to the viable and visible phenotype of mouse coat color mutants [62].

The default of melanin synthesis within viable melanocytes is observed in albinism characterized by variable loss of pigmentation in the eye, hair and skin. Impaired retina development due to defective function of neighboring RPE causes significant vision problems [61]. To date, five types of albinism have been characterized in humans and mapped to distinct pigment-related loci. Types 1 to 4 of oculocutaneous albinism (OCA) are associated with genetic mutations that affect, either directly (in OCA1) or indirectly (in OCA2-4), the activity of Tyrosinase (TYR), the rate-limiting enzyme in melanin synthesis [61, 63]. Its transcriptional regulation, post-translational activation and degradation process are the key regulation points targeted in melanogenesis defects. Mutations in *OA1* (also known as *GPR143*) are associated with type 1 ocular albinism (OA1), an X-linked disorder characterized by severe visual abnormalities, mild or absent cutaneous changes and the presence of macromelanosomes within pigment cells [64-66]. As biogenesis of melanosomes is closely related to biogenesis of LROs, mutations that affect LRO function often produce phenotypes similar to OCA, but exhibit additional systemic clinical manifestations [61].

The early studies on transcriptional regulation of genes encoding melanogenic enzymes have anticipated the existence of common transcription factors binding to elements shared between promoters of *TYR* and of two genes encoding tyrosinase-related proteins (TYRP), namely TYRP1 and TYRP2, also known as dopachrome tautomerase (DCT) [67] (Fig. 4A). Indeed, the promoter regions of these genes were found to contain a common motif AGT**CATGTG**CT named M-box [67]. The core element of M-box CATGTG corresponds to the consensus sequence of the E-box motif, which is flanked by either a 5'T and/or 3'A [25, 67]. *MITF* gene was isolated soon after and its protein product represented a suitable candidate to exert transcriptional control of melanocyte-specific genes by binding to M-boxes.

Furthermore, the expression of MITF-M isoform exclusively in melanocytes explained the cell type-specific regulation of these genes. Subsequently, MITF binding affinity and transactivating potential within promoters of melanocyte-specific genes have been extensively studied in different cellular models. Thus, electrophoretic mobility shift assays (EMSA), mutational analysis, gene reporter and ChIP assays have revealed three functional MITF binding sites within *TYR* promoter: one M-box located at positions -103 to -94 with respect to the transcription start site (TSS) and two E-box motifs at the initiator (position -12 to -7) and at the tyrosinase distal element (TDE; position -1858 to -1846) [67-72]. Similar experimental approaches demonstrated the role of MITF in the transcriptional activation of *TYRP1* and *DCT* [69, 73-75]. MITF appears to be required, but not sufficient for TYR and TYRP1 expression[76]. A *Mitf* dominant negative mutant lacking the activation domain strongly inhibited the expression of TYR and TYRP1 proteins and reduced the activity of exogenous *TYR* promoter by about 75% [76]. MITF overexpression has not resulted in induced expression of endogenous TYR and TYRP1 in human melanocytes and murine melanoma cells [76].

TYRP1 (536 aa) and DCT (552 aa) share the main structural features with TYR (529 aa) and follow similar trafficking pathways before being sorted to stage II melanosomes as mature melanogenic enzymes [77]. Newly synthesized proteins undergo processing in the endoplasmic reticulum (ER) and trans-Golgi network (TGN) by resident enzymes and chaperons to become mature glycosylated proteins having apparent molecular weight of about 70-80 kDa [78]. Mutations in *TYR* and *TYRP1*, which are associated, respectively, with OCA1 and OCA3, result in retention of misfolded proteins in ER and degradation by proteasomes [78, 79]. Moreover, post-Golgi degradation of TYR occurs via the endosomal/lysosomal system [78]. A half-life of TYR is more than 12 hours in melanocytes and may exceed 24 hours in pigmented melanoma cell lines or be less than 3 hours in amelanotic melanoma cells [80, 81]. Long amino-terminal domains that face the melanosome lumen ensure the catalytic activity of melanogenic enzymes, which control the quantity (TYR) and the quality (TYRP1, DCT, TYR) [77] of melanin types (Fig. 4A). Individual melanocytes typically synthesize both yellow-red pheomelanin and brown/black eumelanin. Their ratio is determined by the expression of melanogenic enzymes and the availability of tyrosine and cystein in the cell [77].

The TYR trafficking appears to be altered at the post-Golgi level in OCA2 and OCA4, which are associated with mutations in genes *P* (for pink-eye dilute mutant in mice) and *SLC45A2* (solute carrier family 45, member 2), respectively [82, 83]. Both P-protein (838 aa) and SLC45A2 (530 aa) have 12 predicted transmembrane domains and are thought to be melanosomal integral membrane proteins displaying similar roles in sorting of melanogenic enzymes to melanosomes and in maintaining intramelanosomal ionic equilibrium [82-85]. *SLC45A2*, also known as *AIM1* (antigen isolated from immunoselected melanoma) and *MATP* (membrane-associated transporter protein), has been found to be positively regulated by MITF at the transcriptional level [86].

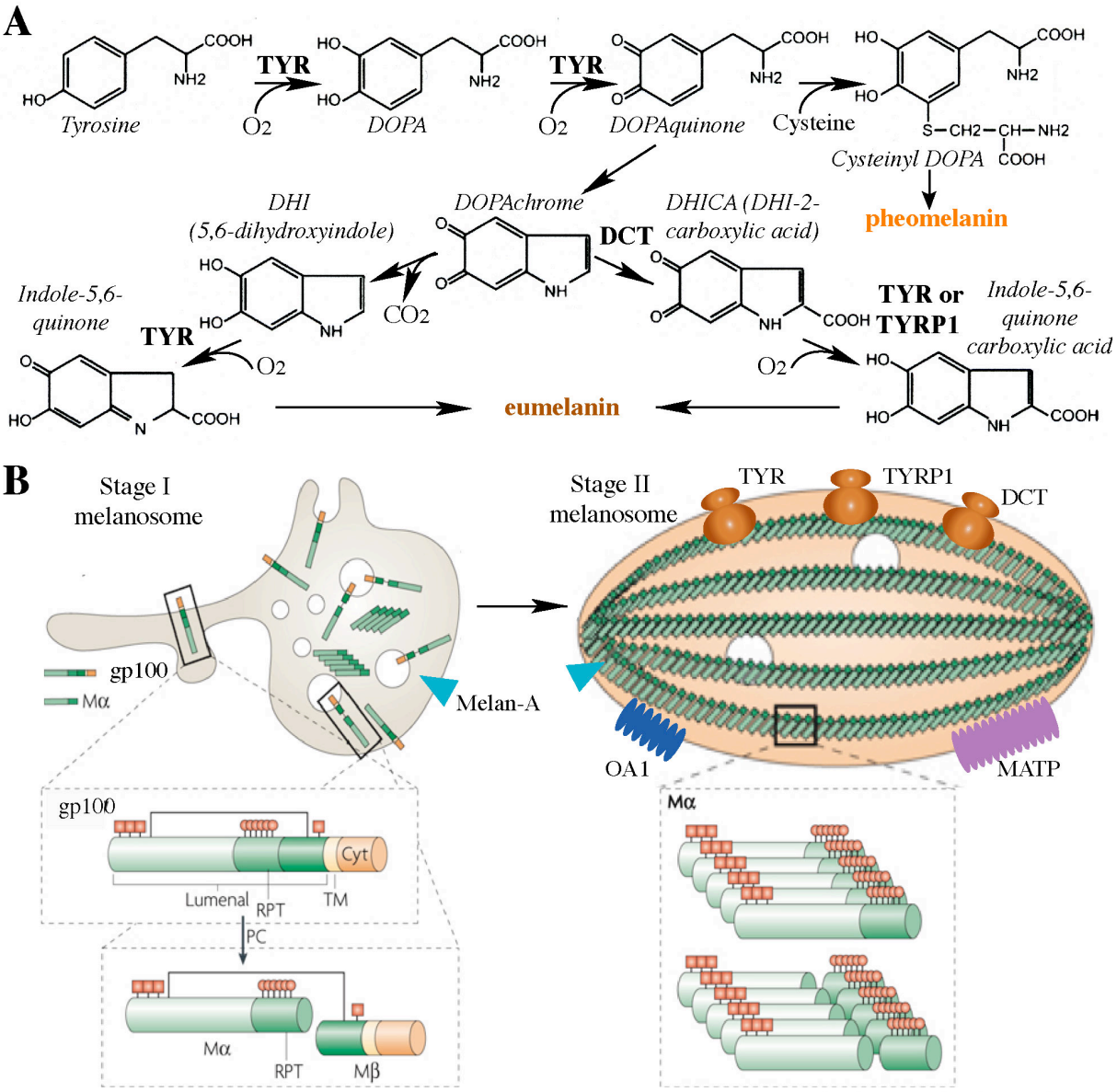


Figure 4. Melanin synthesis and melanosome biogenesis. A. Melanin synthesis pathway. Tyrosinase (TYR) performs the critical rate-limiting activity of hydroxylating tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA), which is rapidly converted to unstable DOPAquinone transformed into the structured polymers of different melanin types. Synthesis of soluble yellow-red pheomelanin requires the availability of cysteine. When cysteine is depleted, DOPAquinone spontaneously cyclizes to form an orange intermediate DOPAchrome, which gives rise to two types of eumelanin. Catalytic activities of DCT (DOPAchrome tautomerase), of TYR in humans and of tyrosinase-related protein 1 (TYRP1) in mice result in production of lighter brown moderately soluble eumelanin of low molecular weight. Alternatively, the spontaneous loss of carboxylic acid by DOPAchrome and catalytic activity of TYR lead to the production of the brown/black insoluble eumelanin of high molecular weight. Modified from Wang and Hebert, 2006 (78). **B.** Model for the formation of melanosome fibrils. The protein gp100 present on the limiting membrane and the intralumenal vesicles (ILVs) of stage I melanosomes is cleaved to Mα and Mβ fragments by a proprotein convertase (PC; bottom left). Mα fragments form small, irregular fibrils that become fully organized within stage II melanosomes (bottom right). Lumenal, transmembrane (TM) and cytoplasmic (Cyt) domains of gp100 are indicated. Repeat domain (RPT) consists of partial direct repeats required for fibril formation. Squares and circles represent *N*- and *O*-linked glycosylation, respectively. Melan-A is a structural melanosomal protein. OA1 (ocular albinism 1) is a G-protein coupled receptor binding L-DOPA. MATP (membrane-associated transporter protein) is predicted to harbor 12 transmembrane domains. Modified from Raposo and Marks, 2007 (16).

The proteins involved in melanosome biogenesis include gp100 (also known as Pmel17 or silver) and Melan-A (for melanoma antigen A; also known as MART-1, melanoma antigen recognized by T cells 1) encoded by *gp100* (*SILV*) and *MLANA*, respectively [87-89]. Based on ChIP and EMSA assays, MITF has been suggested to directly bind E-box elements within the first intron of *gp100* (CATGTG, positions +479 to +484) and in the *MLANA* promoter region (CACGTG, positions -609 to -604) [90]. Mutational analysis revealed a predominant role of these elements in mediating MITF-dependent transactivation of *gp100* and *MLANA* in gene reporter assays [90]. Furthermore, forced expression of MITF in melanoma cell lines resulted in about 2-fold higher levels of endogenous *gp100* and *MLANA* transcripts. The expression of *gp100* and *MLANA* has decreased by 90-100% from basal expression levels when dominant negative mutant of MITF (ΔR217) has been overexpressed [90]. Consistently, gp100 and Melan-A protein levels have increased in the presence of overexpressed wild-type MITF and decreased in the presence of dominant-negative MITF [90]. In EMSA assays, enhanced or disrupted MITF binding to *gp100* and *MLANA* has accompanied an increased or decreased expression of gp100 and Melan-A [90].

Consistent with their function in melanosome biogenesis, gp100 and Melan-A follow trafficking pathways distinct from melanogenic enzymes and are detected in melanosomes from the first stages of maturation [16, 91, 92] (Fig. 4B). Gp100 is a major component of a fibrillar sheet structure in melanosomes. Its expression is necessary and sufficient for fibril formation and acquirement of melanosome shape, while Melan-A appears to associate with gp100 and to modulate its function by poorly elucidated mechanisms [16, 93]. Thus, Melan-A downregulation mediated by specific siRNAs has been accompanied by reduced gp100 stability and decreased efficiency of gp100 trafficking to early melanosomes and gp100 processing [93]. The newly synthesized gp100 traffics through the secretory pathway as a 100-kDa protein termed P1, and is converted into the larger 120-kDa glycosylated form named P2 [92]. At least a fraction of the mature form is cleaved into a C-terminal Mβ fragment and an N-terminal 90-kDa Mα fragment, which serves as a basis for the fibril formation in stage I melanosomes [92] (Fig. 4B). Melan-A (118 aa) is an integral membrane protein with apparent molecular weight of 22-24 kDa. Melanosomal Melan-A accounts for 12% of its total cellular pool. In addition, Melan-A is detected in TGN, small vesicles and tubules [91]. It is modified by acylation, palmitoylation and ubiquitination [91]. Ubiquitination targets Melan-A for proteolysis in lysosomes or proteasomes [91]. The observed half-lives of Melan-A and of the newly synthesized form of gp100 (P1) are about 4-5 hours [91] and 2-3 hours [92, 93], respectively.

Polymorphisms within numerous MITF target genes have been associated with various pigmentation phenotypes [25, 94]. For instanse, *MC1R* encodes melanocortin 1 receptor (MC1R), a seven transmembrane domain G-protein coupled receptor (GPCR) expressed on the melanocyte surface, harbors highly polymorphic coding region [95]. Several of more than 70 identified allelic variants are associated with “red hair color” phenotype defined by red hair,

fair skin, inability to tan and propensity to freckle [94]. This phenotype results from the prevalence of pheomelanin over eumelanin production, since MC1R harboring particular amino acid substitutions weakly induces eumelanin synthesis [96]. Whereas, in dark skin, eumelanosomes are distributed individually in keratinocytes and remain intact throughout the epidermis, spheroid-shape pheomelanosomes are clustered above keratinocyte nuclei in fair skin and progressively degrade to ''melanin dust'' in upper layers of the epidermis [97]. Eumelanin is more stable and better protects epidermal cells from DNA damage induced by solar ultraviolet radiation (UVR) [98]. Besides acting as an UVR-absorbing and scattering sunscreen, eumelanin binds reactive oxygen species (ROS) and mutagenic intermediates of melanin biosynthesis that would otherwise favor DNA damage [98, 99]. The leakage of the toxic by-products of melanin production into the cytosol is prevented by the melanosome membrane and by wrapping of defective melanosomes by autophagosomes [100].

I.2.3.2 Regulation of melanogenesis in the course of the physiological response to ultraviolet radiation

Numerous keratinocyte-to-melanocyte signaling pathways regulating melanogenesis after UVR exposure converge on MITF (Fig. 5). Kinetic immunohistochemical studies of MITF expression in skin biopsies have demonstrated its induction within the first day of UVR exposure; increase up to 1 week, when TYR, gp100 and Melan-A expression levels are maximal; and decrease to baseline constitutive levels by week 3, when levels of melanogenic proteins, melanin content and the dendricity of melanocytes are two to three times higher than before UVR exposure [101]. Keratinocytes are primary cells that "perceive" UVR-mediated DNA damage and acivate p53, which induces genes relevant to keratinocyte-to-melanocyte signaling, such as pro-opiomelanocortin (*POMC*) [102-105]. The POMC polypeptide is cleaved to produce α-melanocyte stimulating hormone (α-MSH), adrenocorticotropic hormone (ACTH) and β-endorphin [102]. Binding of α-MSH to MC1R induces production of cyclic AMP (cAMP), which controls melanogenesis via distinct signaling pathways [106] (Fig. 5). First, activated protein kinase A (PKA) phosphorylates Ser-133 of cAMP-responsive element binding protein (CREB) transcription factor, which forms homodimers binding the cAMP-responsive element (CRE) of the 1M promoter of *MITF* [107, 108]. Second, via a PKA-independent mechanism, cAMP promotes an activation of glycogen synthase kinase 3β (GSK3β), which phosphorylates MITF on Ser-298, increasing its binding to the M-box of the *TYR* promoter [52, 109] (Fig. 5). A dominant negative mutant of MITF lacking the activation domain inhibited cAMP-induced stimulation of TYR expression [107]. Moreover, MITF-M can induce expression of protein kinase C-β (PKC-β), which can phosphorylate the cytoplasmic domain of TYR leading to a 2.5-fold higher enzyme activity [110, 111]. Finally, MC1R stimulation and increased cAMP production lead to activation of the RAS-RAF-MEK-ERK pathway [112] (Fig. 5).

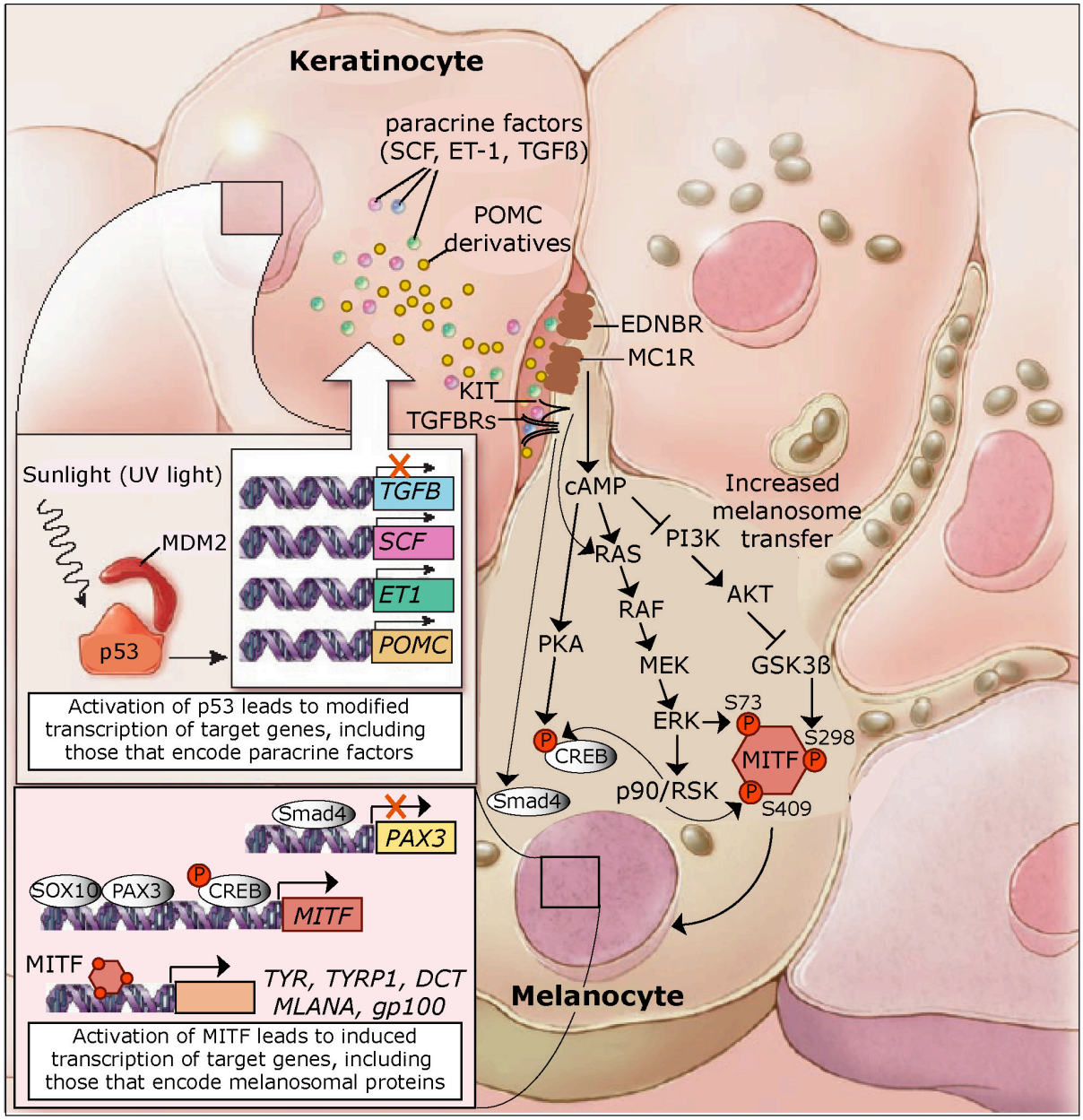


Figure 5. Keratinocyte-to-melanocyte signaling following UVR exposure. UVR induces DNA damage in keratinocytes. This leads to activation of p53 through the displacement of its negative regulators, such as murine double minute-2 (MDM2), which is known to mask p53 transactivation domain and to promote p53 nuclear export and degradation. Activated p53 induces transcription of target genes, including pro-opiomelanocortin (*POMC*), endothelin-1 (*ET1*), stem cell factor (*SCF*). In contrast, the transcription of transforming growth factor beta (*TGFB*) is reduced and results in decreased secretion of TGF-β by keratinocytes. The tanning response is mainly mediated by signaling through the MC1R on the surface of the melanocytes. This signaling is activated by POMC derivatives, such as α-melanocyte stimulating hormone (α-MSH), and leads to increased melanin synthesis and transfer of melanosomes from melanocytes to keratinocytes. Modified from Barsh and Attardi, 2007 (105).

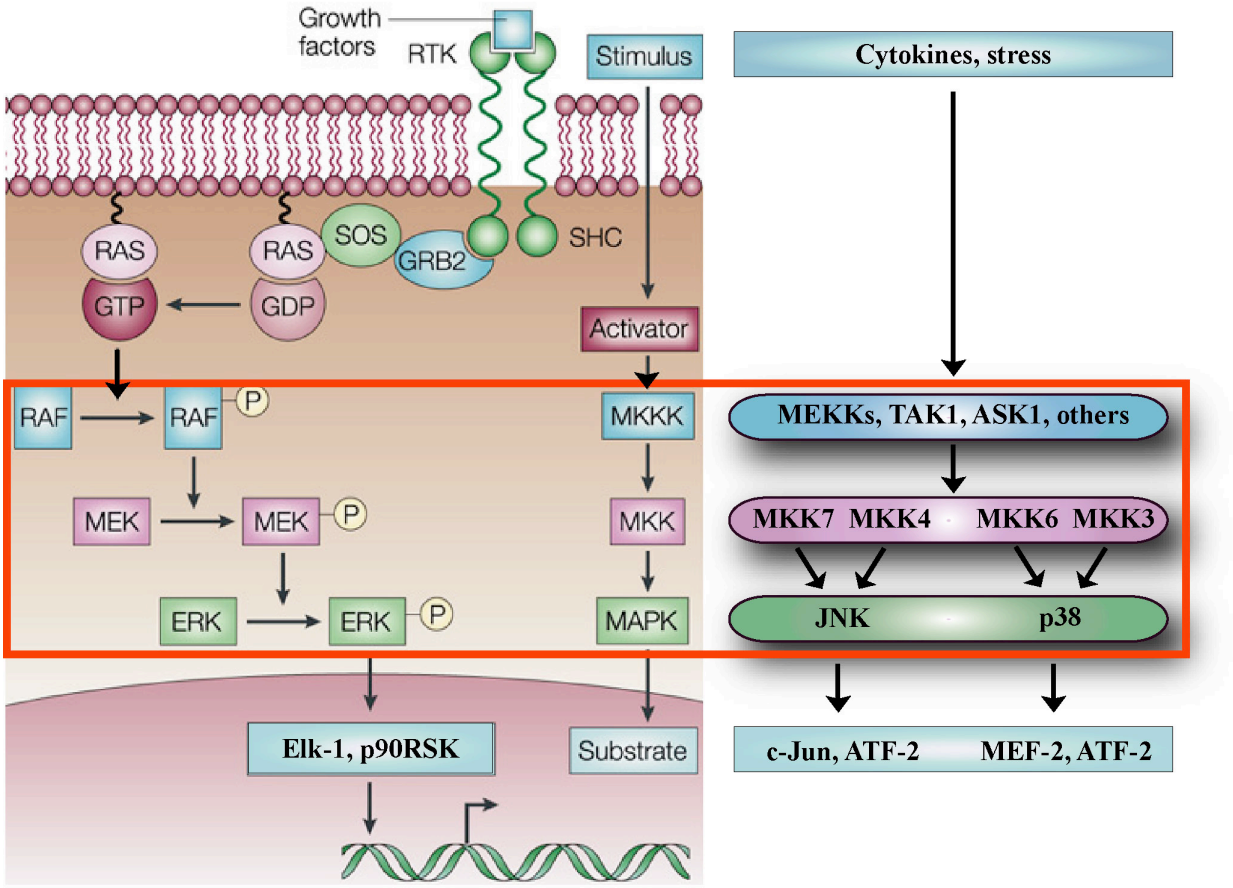


Figure 6. MAPK signal transduction pathways. The MAPK (mitogen-activated protein kinase) pathways represent membrane-to-nucleus signaling cascades. Each cascade comprises a central "core signaling module" (red box), wherein MAPKs are activated by concomitant Thr and Tyr phosphorylation catalyzed by a family of dual-specificity kinases referred to as MKKs (mitogen-activated protein kinase kinases), which, in turn, are activated by Ser/Thr phosphorylation catalyzed by several MAP3Ks (mitogen-activated protein kinase kinase kinases). Cascades leading to the activation of the ERK (extracellular signal-regulated kinase), JNK (c-Jun NH2-terminal kinases) and p38 MAPKs have been most thoroughly characterised. The ERK pathway is activated by growth factors and other mitogenic signals. After binding of ligand to its respective receptor tyrosine kinase (RTK), receptor dimerization triggers the intrinsic tyrosine kinase activity. This results in autophosphorylation of specific tyrosine residues on the intracellular portion of the receptor. These phosphorylated tyrosine residues then bind the sequence homology 2 (SH2) domains of adaptor proteins such as GRB2 (growth factor receptor-bound 2). Such a complex formation recruits SOS (son of sevenless) in close proximity to RAS on the plasma membrane. Like other G proteins, RAS cycles between the GDP-bound inactive form and the GTP-bound active form. After binding to SOS, RAS changes its conformation and associates with GTP. The GTP-bound RAS proteins (NRAS, HRAS and KRAS) act as upstream activators of RAF proteins (BRAF, ARAF and CRAF), which, in turn, phosphorylate the MEK kinases (MEK1 and MEK2), activators of the ERK kinases (ERK1 and ERK2). Activated ERKs then translocate into the nucleus where they phosphorylate specific substrates, for example transcription factor Elk-1 and 90 kDa ribosomal S6 protein kinase (p90RSK). JNK and p38 pathways target transcription factors, such as c-Jun, ATF-2 and MEF-2 (myocyte enhancer factor 2). TAK1, transforming growth factor-ß activated kinase; ASK1, apoptosis signal-regulating kinase. Modified from Chin *et al.*, 2003 (114).

Importantly for melanocyte biology and pathology, stimulation of other GPCRs than MC1R, for example the endothelin receptor B (EDNRB), as well as of numerous receptor tyrosine kinases (RTKs), such as KIT and MET, leads to the activation of the ERK pathway (Fig. 5). This pathway, together with the JNK and p38 pathways, represents one of the MAPK (mitogen-activated protein kinase) signaling cascades, which control major cell fate decisions, including proliferation and survival [113, 114] (Fig. 6). BRAF appears to be responsible for coupling RAS signals to MEK in melanocytes in response to cAMP signaling [115]. The phosphorylation and activation of ERK2 lead to phosphorylation of MITF at Ser-73 and, via phosphorylation and activation of p90RSK, to MITF phosphorylation at Ser-409, both modifications resulting in higher transcriptional activity of MITF within target promoters, notably that of *TYR* [50, 51, 112, 116, 117] (Fig. 5). Furthermore, p90RSK phosphorylates CREB at Ser-133 [118], and hence might induce *MITF* transcription. However, MITF phosphorylation at Ser-73 and Ser-409 is followed by protein ubiquitination at Lys-201 and subsequent degradation by proteasomes [51, 53]. In contrast to the signaling events mediated by GPCRs, the RTK-mediated signaling weakly stimulates melanogenesis [119, 120]. The respective ligands of EDNRB, KIT and MET receptors: endothelin-1 (EDN1), stem cell factor (SCF) and hepatocyte growth factor (HGF) are released by keratinocytes and/or fibroblasts following UVR exposure. The induced expression of these proteins has been reported to last in human skin from days to weeks [103, 121, 122]. Transcription of *EDNRB*, *KIT* and *MET* in melanocytes can be activated by MITF itself [119, 120, 123]. In conclusion, MITF is regulated in two ways after UVR exposure: via direct phosphorylation of MITF protein and via upregulation of *MITF* mRNA expression, which may compensate to some extent for MITF degradation.

Besides the cross-talk between the ERK signaling and MC1R-stimulated cAMP signaling, the specificity in melanogenesis regulation may be provided by other mechanisms. To induce *MITF*-M transcription by binding to the CRE within the 1M promoter of *MITF*, the ubiquitously expressed CREB appears to require SOX10, which is expressed in a limited number of cell lineages derived from the neural crest, including melanocytes [124] (Fig. 5, 7). SOX10 belongs to the SOX (SRY-type HMG box) family of transcription factors, which share DNA binding high-mobility-group (HMG) domain [125]. SOX10 can act synergistically with the transcription factor PAX3 (paired box 3) to activate *MITF*-M transcription [126, 127]. Within 1M *MITF* promoter, one of the SOX10 binding sites is situated immediately 5' of the more distal of the two PAX3 binding sites (PAX3-1 and PAX3-2), which are both critical for *MITF* expression [126, 127] (Fig. 7). This SOX10 binding site has been shown to overlap with the binding site for SOX9. SOX9 upregulation by cAMP pathway is accompanied by stimulation of *MITF* expression in melanocytes [128]. Whether SOX9 mediates its control of melanogenesis independently or in parallel with SOX10 remains to be elucidated. In addition, SOX10 enhances transcription from the 1M *MITF* promoter via the *MITF* distal enhancer (MDE) located about 15 kb upstream of the TSS and containing two SOX10 binding sites [129].

The transcriptional activation of MITF-M following cAMP signaling has been found to depend on the β-catenin binding via LEF-1 sites to the 1M promoter of *MITF* [130] (Fig. 7). It has been suggested that β-catenin cooperates with CREB to induce *MITF*-M transcription [130].

PAX3 is also thought to mediate the induction of MITF expression in response to UVR. Immunohistochemical analysis of human skin has demonstrated increased expression of PAX3 in the nuclei of epidermal melanocytes 3-6 hours after UVR exposure [104, 131]. In cultured melanocytes, TGF-β has been shown to negatively regulate PAX3 transcription through phosphorylated Smad4 [104]. This mechanism is potentially employed for homeostatic downregulation of *PAX3* and *MITF* in the absence of UVR exposure [104]. Since secretion of TGF-β by keratinocytes has been reduced following UVR exposure via a p53-mediated mechanism, relief of the PAX3 downregulation has been suggested to contribute to UVR-mediated *MITF* upregulation [104] (Fig. 5).

Ubiquitously expressed bHLH-LZ transcription factor USF-1 (upstream stimulatory transcription factor 1) phosphorylated on Thr-153 by UVR-activated p38 kinase has been shown to stimulate transcription of *POMC*, *MC1R*, *TYR*, *TYRP1* and *DCT* in melanocytes [132], while the subsequent acetylation of this protein on Lys-199 abrogates its transactivating capacity [132]. In addition, the activation of p38 signaling by α-MSH has been shown to promote the degradation of melanogenic enzymes [133].

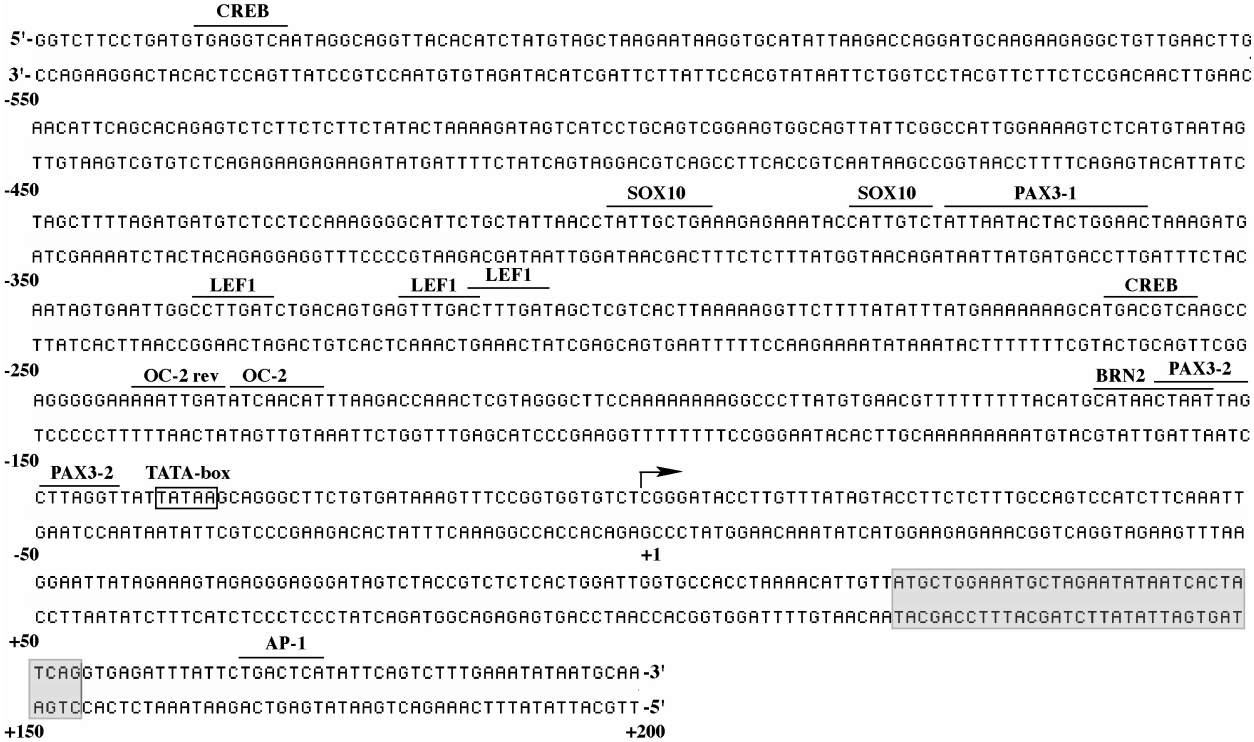


Figure 7. *MITF*-M 5' region. The sequence of the human *MITF*-M 5' region between positions -550 to +200 relative to the known TSS (marked with an arrow and +1) is shown, including the coding region of the 1M exon highlighted in a grey box and the part of the first intron that follows. Transcription factor binding sites are overlined. The location of the OC-2 (ONECUT-2) binding sites on the sense and reverse strands are indicated. Potential binding site for AP-1 (activator protein 1) is indicated. Modified from Hallsson *et al.*, 2007 (30).

I.2.3.3 MITF transcriptional targets control proliferation and survival

The differentiation of specialized cells is often linked to the cell cycle arrest. Nevertheless, differentiated melanocytes apparently maintain the capacity to proliferate. Thus, 2-3 times higher melanocyte density is observed at epidermal-dermal junctions after UVR exposure [101]. Coordinate regulation of melanocyte differentiation and proliferation may be provided by MITF, which, paradoxically, is able to exert both anti-proliferative and proliferative activities. On one hand, MITF can drive cell cycle arrest via upregulation of cyclin-dependent kinase (CDK) inhibitors, such as p21, also known as CDK inhibitor 1A (*CDKN1A*), and p16 encoded by *CDKN2A* locus [134-136] (Fig. 8A, B). MITF protein interacts with hypophosphorylated retinoblastoma protein-1 (RB1) to cooperatively stimulate p21 [134]. On the other hand, MITF can promote cell cycle progression by inducing expression of a critical cell cycle regulator *CDK2* (cyclin-dependent kinase 2), and that of *TBX2* (T-box protein 2), whose protein product represses p21 transcription [137, 138]. Through transactivation of diaphanous-related formin (*DIAPH1*), MITF can also promote the degradation of CDK inhibitor 1B (*CDKN1B*), also known as p27 [139] (Fig. 8C).

Given a low proliferative capacity of melanocytes, maintaining their survival is of particular significance for photoprotection. Whereas, after excessive UVR exposure, apoptotic keratinocytes ('sunburn cells') are quickly shed and replaced, epidermal melanocytes must continue to synthesize melanin to protect skin cells from subsequent damage. MITF appears to control the balance between survival and apoptosis of melanocytes. Thus, the enhanced level of cell death observed in *Mitf*-deficient compared to *Mitf*-expressing melanocytes points to the amount of functional Mitf as a primary determinant of resistance to UVR-induced apoptosis [140]. The propensity of *Mitf*-deficient melanocytes to undergo UVR-induced apoptosis has been accompanied by decreased ratio between anti-apoptotic protein Bcl2 (B-cell lymphoma 2) to pro-apoptotic protein Bad (Bcl2 antagonist of cell death) [140]. MITF promotes melanocyte survival through direct transcriptional activation of *BCL2* [141] and *BIRC7* (Baculoviral IAP (inhibitor of apoptosis) repeat-containing protein 7) [142]. In addition, MITF-induced expression of *MET* favors HGF-mediated signaling that protects melanocytes from apoptosis [119, 123, 141]. Furthermore, MITF promotes survival under oxidative stress via induced expression of apurinic/apyrimidinic endonuclease 1/redox factor-1 (*APEX1/Ref1*) [143]. Moreover, APEX1 protein interacts with CREB and activates hypoxia inducible factor 1 α (HIF-1α), another MITF transcriptional target [144].

To recapitulate MITF activities in relation to melanocyte differentiation, proliferation and survival, a "rheostat" model of relationship between MITF dosage and cellular fate has been proposed. Thus, high MITF expression levels would favor cell cycle arrest and differentiation, intermediate levels would promote proliferation and survival, whereas low levels would lead to cell cycle arrest and apoptosis [139, 145] (Fig. 8D).

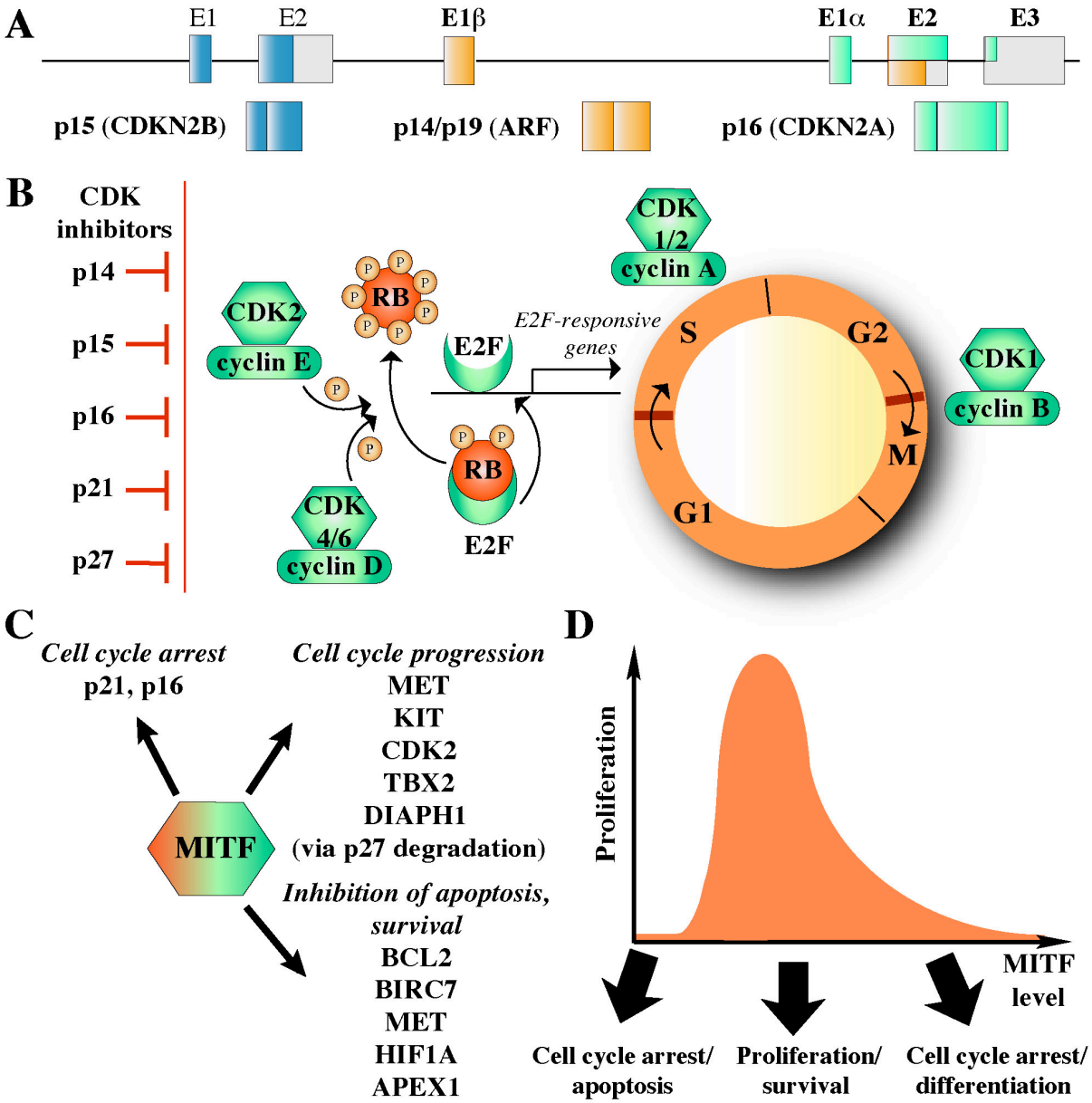


Figure 8. Regulation of cell proliferation and survival by MITF. A. The human *CDKN2* locus is depicted as a bold line, with exons indicated by boxes. *CDKN2B* encodes p15 (Ink4b in mice), whereas *CDKN2A* produces two alternative transcripts encoding p16 (Ink4a in mice) and p14 (p19 in mice). In contrast to p16 produced from exons 1α, 2 and 3, p14 or ARF (alternative reading frame) is encoded by an alternative 5' exon 1β located about 20 kb upstream of exon 1α and by the exon 2 shared with p16 and translated in alternative reading frame. The coding regions of p15, p16 and p14 are shown, respectively, in blue, orange, and green, with untranslated regions shown in light grey. The corresponding gene products are represented lower as boxes. Adapted from Gil and Peters, 2006 (134). **B.** The cell cycle progression from G1 to S phase is induced by a family of cyclins and cyclin-dependent kinases (CDK) and inhibited by a family of CDK inhibitors. In particular, G1-S checkpoint is governed by the cyclin D-CDK4/6 complex at its early phase and cyclin A or E associated with CDK2 at the later phase. Hypophosphorylated retinoblastoma protein (RB) binds the E2F transcription factor, resulting in transcriptional repression of E2F-responsive genes. In response to growth-promoting signals, the CDK2 and CDK4/6 participate in the phosphorylation of RB, causing release of RB from promoter-bound E2F, activation of E2F-responsive genes and entry into S phase. Modified from Miller and Mihm, 2006 (1). **C.** The MITF targets that control cell proliferation and survival are displayed as in Cheli *et al.*, 2010 (25). **D.** The "rheostat" model of cellular fate dependency on MITF expression levels. Modified from Gray-Schopfer *et al.*, 2007 (143).

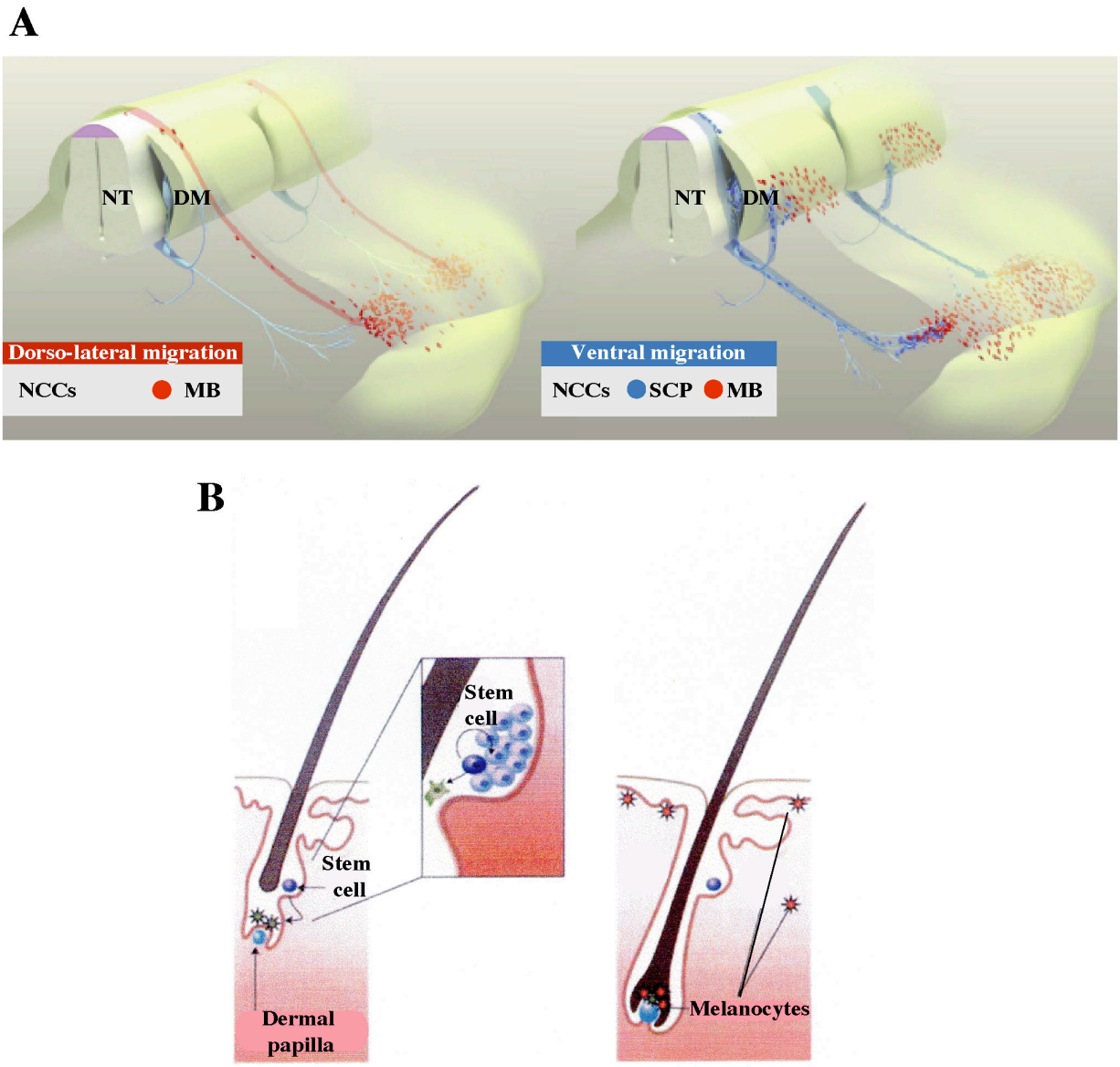


Figure 9. Melanocyte development from neural crest cells. A. Model of cellular origin and migratory pathways of melanocyte precursors, melanoblasts (MB). The population of neural crest cells (NCCs) delaminate from the dorsal edge of the neural tube (NT) (violet shading). During dorso-lateral migration (on the left, in red) NCCs adopt a melanoblast fate shortly after delamination from NT, migrate under the epidermis to a defined skin patch and expand upon reaching the final destination. NCCs migrating along the ventral pathway between the NT and the dermamyotome (DM) contribute to the neurons and glia of the peripheral nervous system. NCCs failing to adopt a neuronal fate associate with nerves and acquire a Schwann cell precursor (SCP) fate (on the right, in blue). SCPs migrate along nerves to cutaneous locations. At the end of nerves, some SCPs acquire a melanocyte fate, detach from nerves, home to the skin and expand in numbers. Adapted from Ernfors, 2010 (147). **B, left.** Melanocyte stem cells (MSCs) are found in the bulge region of the mature hair follicle. **B, middle.** During the growth phase of the hair follicle, MSCs divide generating a pluripotent stem cell and a population of melanocyte precursors. **B, right.** As the hair follicle cycle reaches resting phase the precursors become mature melanocytes and populate the hair follicle to produce pigment. Adapted from Silver *et al.*, 2006 (149).

I.2.4 MITF expression is essential for melanocyte development

Melanoblasts, unpigmented melanocyte precursors, derive from the neural crest in the second month of human embryonic life, undergo proliferation and are thought to migrate mostly via the dorso-lateral pathway throughout the developing embryo to its ventral part [146, 147] (Fig. 9A). However, recent developmental studies in model organisms, such as mouse and chicken, suggest that neural crest derivatives that migrate via the ventral pathway may also give rise to melanocytes [148, 149]. These melanocytes would arise from the Schwann cell precursors (SCPs) of the growing nerves [148, 149] (Fig. 9A). Following migration, melanoblasts reach at 15-18 weeks of human embryogenesis their final destinations, mainly the basal layer of the epidermis where they complete the differentiation process to form pigment-producing melanocytes [147]. Studies carried out mostly in mouse models suggest that melanoblasts colonizing the developing hair follicles remain undifferentiated in the hair bulge as melanocyte stem cells (MSCs) [150] (Fig. 9B). During the growth phase of the hair follicle cycle, MSCs give rise either to daughter stem cells or to cells that migrate to the bulb hair region and differentiate into mature melanocytes [150] (Fig. 9B).

Developmental studies have shown that some genes (components of the Wnt signaling pathway, *Slug, Sox9, Sox10, Pax3, Ednrb, Notch*) are essential for the development of multiple neural crest derivatives, including neurons, glial cells and melanocytes [151]. However, melanocyte lineage cells appear in particular require *Mitf* for their development. Mitf is already expressed in neural crest-derived cells before their migration and is considered, together with Kit and Dct, to be a melanoblast marker. Melanoblasts expressing *Mitf* mRNA have initially emerged in mice carrying homozygous mutation of *Mitf* and survived 2 days *in vitro* [152]. If these melanoblasts had to persist more than 2 days, the expression of a functional Mitf has been required [152]. In mice carrying heterozygous mutation of *Mitf*, melanoblasts were reduced in numbers and proliferated faster during the migratory phase *in vivo*, pointing to the dosage dependence on Mitf and suggesting its inhibitory role on proliferation [153]. These observations in mouse models as well as the loss of melanocytes documented in patients with WS2 suggest critical role of MITF in melanoblast survival, proliferation and/or inhibition of transdifferentiation towards other neural-crest lineages. Whether MITF function is required continuously or at particular stages of melanocyte development remains to be elucidated. Of known MITF targets, DICER has been proposed to be required for survival of post-migratory melanoblasts or differentiating melanocytes [29], while the postnatal survival of MSCs is thought be regulated by MITF via transcriptional activation of *BCL2* [154].

Consistently with the absolute importance of Mitf for melanocyte development, its expression and activity appear to be tightly regulated. Activation of *Mitf* expression has been proposed to require the release of transcriptional repression exerted by Foxd3 transcription factor, which has prevented *Mitf* activation by Pax3 [155]. When Foxd3 is no longer expressed,

Sox10 and Pax3 may activate Mitf expression by binding to the 1M *Mitf* promoter (Fig. 7). The 1M promoter of *Mitf* is also a direct target of Lef-1 (lymphoid enhancer factor-1) transcription factor in response to nuclear translocation of β-catenin following activation of the Wnt/β-catenin signaling pathway [156]. Moreover, MITF interaction with LEF-1 might lead to activation of its own expression mediated via three LEF-1 binding sites in the 1M promoter of *MITF* [157, 158] (Fig. 7). This might ensure efficient expression of MITF in melanoblasts at certain developmental stages [158]. Wnt1 and Wnt3a ligands likely serve as activators of the Wnt/β-catenin signaling pathway *in vivo*, since their disruption in double-knockout mice has resulted in a dramatic reduction of several neural crest derivatives, including melanoblasts [159].

Remarkably, many genes involved in melanocyte development encode proteins that constitute a common signaling network with MITF as a central unifying factor. The evidence of a "MITF network" comes from a striking overlap between the "core" hypopigmentation/deafness phenotype observed in patients with WS2A and phenotypes characteristic of other types of WS, in which "core" phenotype is accompanied by specific developmental defects due to impairment of distinct neural crest lineages [56]. "Guilty" factors identified as genetic causes of WS are either MITF transcriptional regulators (PAX3, SOX10), its transcriptional targets (SLUG, EDNRB) or belong to a common signaling pathway (EDN3, EDNRB) [56]. Some other factors have been linked to the "MITF network" via genetic studies. Thus, mutations in genes encoding KIT, its ligand SCF and transcription factor SLUG have been described in a rare autosomal dominant pigmentation disorder known as piebaldism, which is more commonly represented by ventral depigmented (melanocyte-free) areas of skin [61]. Some other transcription factors, like brain-2/POU class 3 homeobox transcription factor 2 (BRN2/POU3F2) or ONECUT-2 (OC-2), have been demonstrated to be transcriptional regulators of MITF *in vitro*, and can be potentially implicated in its regulation *in vivo* at certain stages of melanocyte development [160, 161] (Fig. 7).

The relevance of post-translational MITF modifications in melanocyte development is not completely elucidated. A substitution of Ser-298, which is targeted by GSK3β-mediated phosphorylation, to Pro-298 has been associated with WS2, suggesting the functional importance of this modification in melanocyte development [52]. Other modifications were either not studied *in vivo* or have been demonstrated to be dispensable for proper melanocyte development, such as Ser-73 and Ser-409 phosphorylation [162].

Once initiated, the maintenance of MITF expression would ensure a continued differentiation of the melanocyte lineage cells. Interestingly, SOX10, PAX3, LEF-1 and β-catenin transcription factors could act in a feed-forward circuit as they first activate MITF and then, together with MITF, regulate its transcriptional targets involved in melanogenesis [75, 157, 163-165]. The cooperative action of different sets of transcription factors within promoters of MITF target genes might be one of the factors determining temporal differences in activation of *Dct*, *Silv*, *Tyrp1*, *Mlana* and *Tyr* in melanocyte lineage cells [166-168].

I.2.5 Role of epigenetic mechanisms in establishment and maintenance of melanocyte identity

I.2.5.1 Epigenetics and cellular identity

The establishment of melanocyte identity during development requires the specific spatial distribution of melanocyte lineage cells within the developing organism accompanied by temporal regulation of gene expression patterns characteristic to each developmental stage. While some genes would be progressively activated to adjust the gene expression pattern towards a new fate upon differentiation, other genes would be stably silenced throughout cell generations to secure the acquired fate. These changes in gene expression occur through inductive mechanisms via extracellular signaling and through autonomous mechanisms, independent of signaling or mechanic interaction with other cells. As a result, melanocyte lineage cells having identical genotype would exhibit distinct gene expression profiles at different developmental stages. Once differentiated, melanocytes need to maintain their gene expression pattern and the associated phenotype in order to perform their specialized function. Thus, genes encoding proteins with housekeeping and specialized functions should be kept on, while genes encoding proteins specific to other cell types should be kept off following cell division. Such mitotic inheritance of gene expression profiles is often referred to as epigenetic [169]. It was developmental biologist Conrad Waddington who first coined this term and initially destined epigenetics to study the mechanisms that use the information of the genotype to bring the phenotype into being [170]. Following a shift in semantic definition of epigenetics, its main focus has become the mechanisms of inheritance that do not alter the genotype [169]. Nowadays, epigenetics is defined as the study of any potentially stable and heritable change in gene expression that occurs without altering the primary DNA sequence [171]. An epigenetic layer of information can be potentially transmitted through mitosis, meiosis and in some cases transgenerationally. The sum of epigenetic information that can be applied to genome by adding chemical marks either to DNA or histone proteins is termed the epigenome. While most differentiated cell types possess the same genetic background, their epigenomes established during development differ. Furthermore, epigenome is dynamically regulated through the cell cycle and in response to extrinsic stimuli [172, 173]. Importantly, some epigenetic marks, for instance certain histone modifications, reflect short-term instructions and can quickly revert in response to a signal, while others, such as DNA methylation, exert long-term instructions that might be inherited independently of the initial trigger and contribute to cellular memory [172].

The central role of MITF in the establishment and maintenance of melanocyte identity raises a fundamental question concerning the specificity of its binding within promoters of target genes in order to exert their spatial and temporal regulation. Assuming uniform

nucleotide distribution, a transcription factor like MITF recognizing a hexanucleotide E-box motif has about 1.6 millions binding sites in the diploid genome of a melanocyte lineage cell containing about 6600 Mb of DNA. Whereas the extension of the recognition motif to the M-box, differences in sequences flanking the recognition motif, signal transduction-mediated post-translational MITF modifications and its cooperative interactions with other transcription factors could partly answer the question of how binding specificity is generated, some other mechanisms should operate to restrict the ability of MITF to activate transcription to a subset of promoters.

Chromatin organization provides an essential mechanism ensuring binding specificity, since DNA wrapped around histones exhibits limited accessibility for binding of transcription factors and DNA-metabolizing enzymes. Chromatin structure allows directing the regulatory proteins to functionally relevant regions, which represent only a small proportion of the human genome. Thus, only 1.2% of the human genome encodes proteins, while a larger part of it (about 15%) is transcribed producing additionally to mRNA a large repertoire of short and long non-protein-coding RNAs [174]. Moreover the transcriptional machinery is challenged to a multifunctional usage of the same genomic space, such as alternative use of distal 5' promoters or production of overlapping transcripts from the same or opposing DNA strands [174]. The concept that genome complexity requires additional repressive mechanisms to enhance specificity of transcription initiation is supported by the evolution of epigenetic mechanisms, which, on one hand, promote transcriptional repression, and, on the other hand, ensure the fidelity of the transcriptional activation [175]. Thus, prokaryote-to-eukaryote transition was paralleled by the appearance of nucleosomes, structural and functional chromatin units connected by linker DNA (of about 38 bp in length in humans) to form 10 nm chromatin fibers [175]. Nucleosomes are considered as general transcriptional repressors, since 147 bp of nucleosomal DNA, which is wrapped 1.75 turns around an octamer of core histones, are barely accessible to binding by the transcriptional machinery (Fig. 10A). Post-translational modifications of amino-terminal tails of core histones regulate changes in chromatin condensation and, consequently, DNA accessibility [171] (Fig. 10B). The maintenance of silent and active expression states of genes during development is under the control of, respectively, Polycomb group (PcG) and Trithorax group (TrxG) proteins, which underwent marked expansion over evolutionary time [176]. And last but not least, DNA methylation (Fig. 10C) is thought to have evolved as a genome defense mechanism known as the restriction-modification system in bacteria and to have steadily acquired gene regulation function, thus becoming an essential mechanism of transcriptional repression [177]. Consistently, vertebrate genomes are dominated by methylated DNA and exhibit a global DNA methylation pattern in contrast to mosaic pattern of invertebrate genomes, wherein methylated and unmethylated domains occur in approximately equal proportions [177].

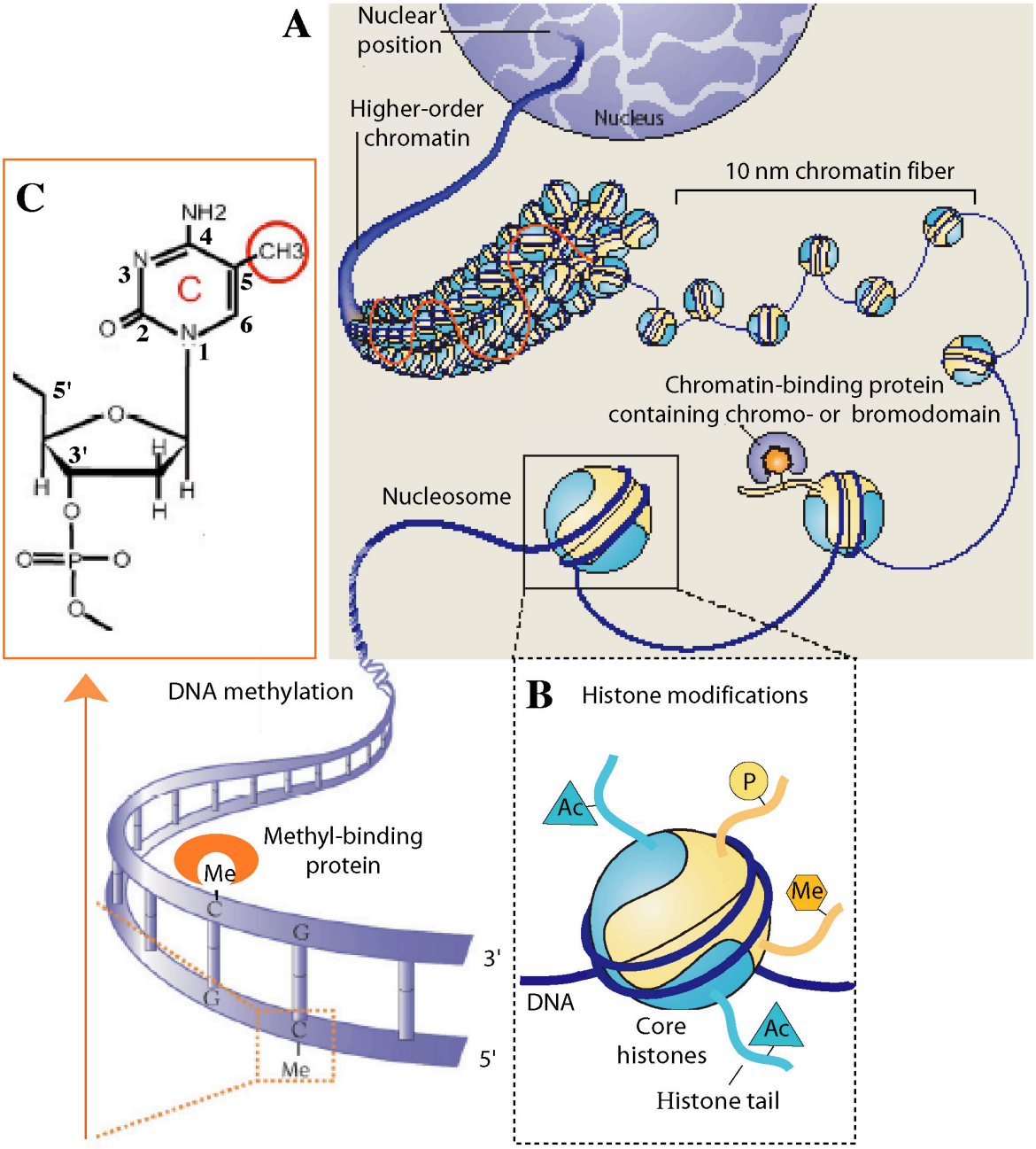


Figure 10. Epigenetic modifications. Nucleosomes are structural and functional chromatin units connected by linker DNA in a 10 nm chromatin fiber (**A**), which is fold in higher-order chromatin structures that are transcriptionally silent. The position of a particular chromosomal domain in the nucleus constitutes an additional level of instruction for gene expression. Each nucleosome (**B**) comprises two copies of each of the four core histones, H2A, H2B, H3 and H4. All four histones undergo post-translational covalent modifications at multiple sites. These modifications include acelylation (Ac), methylation (Me), phosphorylation (P), ubiquitylation, sumoylation, ADP ribosylation, deimination and proline isomerization. The presence of histone variants adds further complexity. Families of histone- or methyl-binding proteins decipher the regulatory information that is encoded by histone marks (**B**) and DNA methylation (**C**). Cytosine bases that are located 5' to guanine bases (precede them) within CpG dinucleotides often harbor methyl group at the fifth carbon position (**C**). Modified from Probst *et al.*, 2009 (170).

I.2.5.2 DNA methylation

In mammalian genomes, cytosine methylated at the fifth carbon position (5mC) constitutes ~1% of all DNA bases and is found almost exclusively within 5'-CpG-3' dinucleotides symmetrically methylated on both DNA strands [177] (Fig. 10C). CpG dinucleotides are found at about one fifth of the expected frequency in the human genome [178], supporting the notion that CpG sites have been lost during evolution due to increased mutability of 5mC to thymines [179]. The DNA methylation pattern, defined as the methylation status of CpG dinucleotide series located in *cis* on a single DNA strand, is represented by long tracts of methyl-CpG containing DNA, which are occasionally punctuated by unmethylated regions 0.5-5 kb in length called CpG islands [177]. The human genome contains about 30 000 of such "islands" [178], which represent a discrete genome fraction in several respects. While the majority of the genome is enriched in A and T nucleotides (~60%) and has methyl-CpGs approximately every 100 bp within and outside of gene bodies, CpG islands are rich in G and C content (~65%) with CpG sites occurring approximately every 10 bp [180]. Furthermore, CpG islands are unevenly distributed throughout the genome and co-localize with the 5'-UTR region and the first exon of at least 60% of protein-coding human genes [180]. In promoters containing CpG islands, also called CpG-rich promoters, the transcription can initiate in a region spanning 10-60 bp, while in CpG-poor promoters it initiates at a defined nucleotide position and mostly relies on the initiation factor TATA-box-binding protein [181]. CpG-rich promoters, which usually do not undergo DNA methylation, control the majority of ubiquitously expressed housekeeping genes and about 50% of cell type-specific genes. In order to be transcriptionally silent, CpG-rich cell type-specific genes seem to require active repression mediated by DNA methylation and PcG proteins in order to stabilize their "off" states, whereas transcriptional repression of CpG-poor promoters is not always accompanied by these epigenetic modifications [175]. CpG islands found in intra- or intergenic regions are prone to methylation and were suggested to mark the TSS of non-coding RNAs [182]. While CpG islands generally remain hypomethylated in somatic cells, a subset of them is methylated during the monoallelic silencing of parentally imprinted genes and inactivation of one of the two X chromosomes in female somatic cells [176]. Some CpG islands can be differentially methylated between somatic and germline cells or between different somatic cell types [180].

The mechanism of C5-cytosine methylation is universal in all organisms studied and involves the transfer of a methyl group from the cofactor S-adenosylmethionine to the recipient cytosine base of DNA with the release of one proton and the generation of methylated DNA and S-adenosylhomocysteine [183]. CpG dinucleotides constitute a predominant substrate for mammalian DNA methyltransferases (DNMTs), namely DNMT1, DNMT3A and DNMT3B [183]. DNMT3A and DNMT3B are able to newly modify unmethylated CpG on both DNA strands and are often referred to as *de novo* DNMTs.

DNA methylation levels change dramatically during mammalian development. After fertilization, a wave of genome-wide DNA demethylation erases almost all gametic methylation marks. Typically lowest at about the 16-cell morula stage in mice, global methylation levels start being regained in the blastocyst, preferentially in its inner cell mass [184]. The DNA methylation is acquired differentially in different cell lineages. While DNA in somatic cell lineages is heavily methylated, it is undermethylated in trophoblast-derived lineages and remains largely unmethylated in early primordial germ cells until gonadal differentiation. *De novo* methylation during gametogenesis gives rise to sex-specific DNA methylation patterns in germline cells. DNMT3A and DNMT3B, which cooperate in the establishment of DNA methylation patterns, exhibit low expression levels in adult somatic cells and are highly expressed in developing germ cells and in embryonic cells with substantial *de novo* methylation activity [185-187]. In the course of cell divisions, the active maintenance of DNA methylation pattern is mostly carried out by the ubiquitously expressed DNMT1, which exhibits *in vitro* 5- to 30-fold greater activity on one-strand methylated, than on an unmethylated template [183]. Due to its interaction with NP95 (nuclear protein 95), DNMT1 is recruited to the hemi-methylated DNA after the passage of replication fork and copies DNA methylation marks on the newly synthesized leading and lagging DNA strands [188]. DNA methylation is a stable epigenetic modification, which can be transmitted through cell divisions for at least 100 cell generations in somatic cells [189] and contributes to the control of transgenerational epigenetic inheritance [190]. DNA methylation can be removed passively, that is by preventing methylation of newly synthesized DNA. The existence and identity of enzyme(s) that might actively demethylate DNA in mammals remain controversial [191].

Investigation of DNA methylation patterns can be performed at local and genome-wide scales. Initially, it was almost entirely based on the sensitivity of the bacterial restriction enzymes to CpG methylation within their cleavage recognition site. Mapping of 5mC sites at a single-base pair resolution can be performed using bisulfite genomic sequencing [192]. The principle of this method relies on the selectivity of sodium bisulfite, which converts unmethylated cytosines to uracils, while methylated cytosines stay unaltered [192] (Fig. 11A). Converted uracils are then replaced by thymines in the course of PCR reaction using primers specific to the converted DNA region under investigation. Subsequent cloning and sequencing of PCR products can determine the original methylation state of the DNA. Genome-wide mapping of DNA methylation patterns relies on direct large-scale bisulfite DNA sequencing and indirect affinity-based approaches, which employ either a monoclonal antibody specific to methylcytosine (methylated DNA immunoprecipitation; MeDIP) or the DNA-binding domain of a methyl-CpG-binding protein (methyl-binding domain affinity purification; MAP) [177]. The samples enriched in the methylated DNA can be then tested using gene-specific (methylation-sensitive PCR, bisulfite sequencing) or genome-wide experimental readouts (DNA microarrays, direct large-scale sequencing techniques) [177].

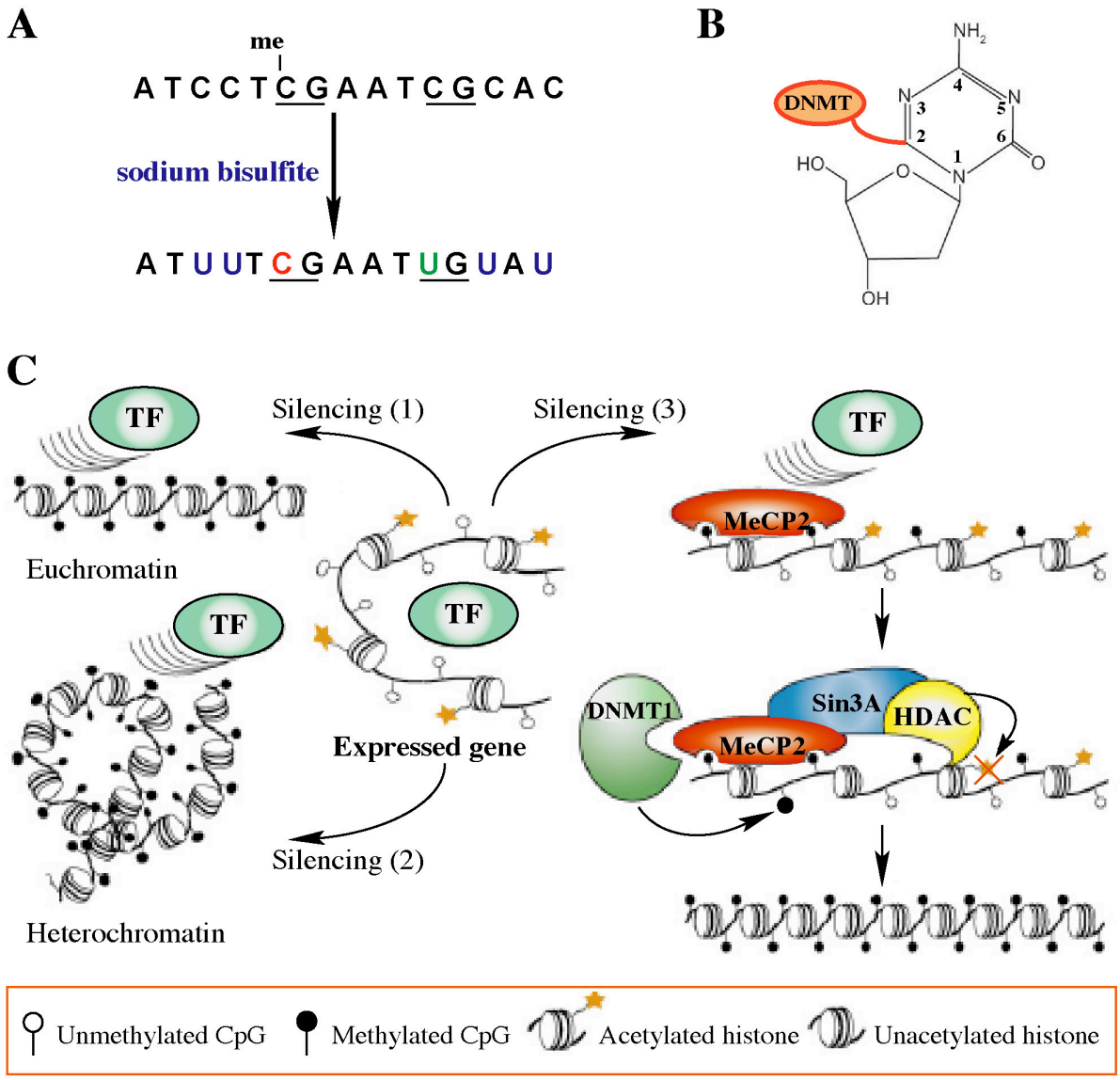


Figure 11. DNA methylation: methods of investigation and mechanisms of gene silencing. A. The principle of sodium bisulfite-mediated conversion relies on the selectivity of its action on unmethylated cytosines converted to uracils, while methylated cytosines stay unaltered. **B.** Structure and trapping mechanism of 5-aza-2'-deoxycytidine (5-azadC, also known as decitabine). The action of the DNMTs is inhibited by DNA that incorporated 5-azadC. DNMTs are covalently trapped to the modified cytosine residues, while being unable to complete the enzymatic reaction due to the nitrogen at the 5-position. Trapped DNMTs are thought to be degraded, leading to depletion of cellular DNMTs, deficient methylation of newly synthesized DNA and to global hypomethylation. Modified from Garcia *et al.*, 2010 (191).
C. Mechanisms of gene silencing. Methylated CpG sites within gene promoters can directly inhibit transcription by blocking the binding of transcription factors (TFs) (1). The formation of heterochromatin associated with methylated DNA and deacetylated histones may prevent the access for TFs to DNA (2). The silencing of a gene may result from the binding of methyl-CpG-binding proteins (e.i. MeCP2), which recruit histone deacetylases (HDAC) within repressor complexes with other proteins (e.i. Sin3A) leading to a non-permissive chromatin state that prevents a TF to bind the target promoter (3). Adapted from Vaissiere *et al.*, 2008 (192).

One of the most useful high-throughput approaches to identify genes repressed by promoter hypermethylation is a reactivation screening strategy that combines treatment of cells *in vitro* with a demethylating agent followed by global gene microarray expression analysis. Demethylating agents are also largely used for local scale DNA methylation studies. Among demethylating agents employed in fundamental and clinical studies and having similar modes of action are 5-azacitidine and 5-aza-2'-deoxycytidine (5-azadC, also known as decitabine) [193] (Fig. 11B). Once inside the cell, 5-azadC needs to be phosphorylated in order to be incorporated in DNA. DNMTs are covalently trapped to the modified cytosine residues and are thought to be degraded. This leads to deficient methylation of newly synthesized DNA and ultimately to global hypomethylation [193].

Numerous studies performed at the local and genome-wide scale have demonstrated the correlation between promoter methylation and transcriptional repression of downstream genes [177, 194]. Several mechanisms have been involved [177, 194] (Fig. 11C). Although methyl group of 5mC is hidden within the internal GC base-pairing region without interfering with the formation of hydrogen bonds, it may, however, compromise binding of transcription factors having methyl-CpG within their recognition binding sites. For instance, methylation at the CpG site located centrally within the E-box motif (CACpGTG) inhibits the binding of the USF1 transcription factor and negatively regulates gene expression [195]. Methylation sensitivity of MITF binding to CpG-containing E-box motifs has not been investigated. Methyl-CpGs also serve as tags recognized by methyl-CpG-binding proteins, which co-recruit transcriptional repressor complexes including histone deacetylase (HDACs) [196]. Besides, it has been recently demonstrated that the presence of a CpG island within the gene promoter region is associated with a destabilized nucleosome assembly, which likely enables the transcriptional activators, like Sp1, to readily gain access to the promoter binding sites in the absence of extracellular signaling and to facilitate the recruitment of general transcription factors and RNA polymerase II (Pol II) to gene promoters when signaling is present [197].

In human melanocytes, a genome-wide DNA methylation pattern has been recently studied [198]. CpG-rich promoters and promoters with intermediate CpG content have been shown to be in the majority of cases (about 90%) unmethylated or exclusively methylated at the distal part of the promoter (-1000 to -2200 bp) relative to the TSS. The CpG methylation in promoter regions proximal to the TSS (-200 to +500 bp) has been associated with transcriptional repression. The expression of genes under the control of CpG-poor promoters has not been dependent on the promoter methylation profile [198]. While the 5' regions of genes encoding *TYR* and *MLANA* apparently do not contain CpG islands, the presence of a CpG island downstream of the TSS has been reported in *gp100* [199], suggesting the potential for *gp100* promoter regulation by DNA methylation. In addition, one CpG site within the first intron of *MLANA* has been found to be unmethylated in melanocytes and *MLANA*-positive melanoma cell lines, while it was methylated in *MLANA*-negative melanoma cell lines [200].

I.2.5.3 Post-translational histone modifications

Within the nucleosome, two molecules of each core histone H2A, H2B, H3 and H4 contact DNA by their globular regions, while unstructured amino-terminal histone tails protrude outside from the core nucleosomal complex and are subject to eight types of post-translational modifications with over 60 different amino acid residues being modified [171] (Fig. 10B). Moreover, lysines of histone tails may be mono-, di- or trimethylated, while arginines may be mono- or dimethylated (symmetrically or asymmetrically) [171]. Addition of chemical marks on amino acid residues of histone tails is catalyzed by histone modifications "writers" [171]. Enzymes that remove or "erase" histone marks include K (lysine)-demethylases (KDMs) disproving the long-lasting belief concerning the irreversibility of histone methylation, a relatively stable mark. ChIP-chip and ChIP-Seq technologies using histone modification-specific antibodies have made it possible to obtain genome-wide data for several histone modifications [201, 202]. Thus, nucleosomes around the first exons carry specific epigenetic marks related to the transcriptional status of the genes. Trimethylations of lysines within the histone tail of H3, namely H3K9me3, H3K27me3 and H3K79me3 have been linked to gene repression, whereas H3K4me3 and H3K9ac - to gene activation [201, 202].

Changes of chromatin structure that accompany gene activation or repression rely on two major mechanisms. The first is a disruption of contact between nucleosomes, with histone acetylation by lysine acetyltransferases (KATs) being the most efficient to "unwrap" chromatin due to neutralization of positive charges on the amino groups of lysine residues [203]. The reversal of histone lysine acetylation by HDACs correlates with chromatin condensation and transcriptional repression. The second mechanism to modify the chromatin structure is the recruitment or prevention of docking of nonhistone proteins onto chromatin. Proteins that bind modified amino acid residues of histone tails via specific domains are often referred to as histone modification "readers" (Fig. 10A). For instance, acetylated or methylated lysines are recognized by proteins harboring bromo- or chromodomains, respectively [171]. Histones modification "readers" possess or tether enzymatic activities that further modify chromatin. For instance, the chromodomain-containing heterochromatin protein 1 (HP1) necessary for heterochromatin formation binds to H3K9me2 and H3K9me3 and recruits HDACs and lysine methyltransferases (KMTs) responsible for H3K9 methylation [204]. Within euchromatin, HP1 recruitment to gene promoters can be mediated by co-repressors, such as hypophosphorylated RB, which represses transcription from E2F-responsive promoters through its interaction with HDAC1 [171]. H3K27me3 modification linked to transcriptional repression is established by polycomb repressor complex 2 (PRC2), which contains enhancer of zeste homolog 2 protein (EZH2) carrying KMT activity. Further, H3K27me3 is recognized by chromodomain–containing components of PRC1, which stabilizes the inactive transcriptional state due to ubiquitination of histone H2A and inhibition of RNA polymerase II activity [176].

I.2.5.4 Accessibility of nucleosomal DNA and catalyzed nucleosome remodeling

Despite regularity in distribution of nucleosomes within the 10 nm chromatin fiber, genome-wide ChIP-Seq studies have revealed a depletion of nucleosomes within transcriptionally active regulatory regions, including cell-type specific ones. Nucleosome free regions (NFRs) have been identified just upstream of the TSS of protein-coding genes with well-positioned nucleosomes around their first exons [205]. NFRs are considered to be transcriptionally permissive and might allow low basal levels of leaky transcription, while the binding of transcription factors and the assembly of the transcription machinery within NFRs is required to activate genes [206]. Importantly, the distribution of nucleosomes relative to MITF-binding sites has been shown to correlate with melanocyte-specific gene expression [28]. Thus, 77% of MITF-occupied sites determined by ChIP-on-chip analysis in one melanoma cell line have been situated within NFRs surrounded by positioned nucleosomes. For instance, the TSS of *gp100* has been found to be nucleosome-free in primary melanocytes and two melanoma cell lines, but occupied by a positioned nucleosome in other cell types [28]. During differentiation, it might be advantageous to keep cell-type specific regulatory sites masked with nucleosomes unless they are in use. For instance, the initiator E-box within the *TYR* promoter might by masked by nucleosome until *TYR* is to be activated in differentiating melanocytes, while the depletion of nucleosome at this site might allow enhanced MITF binding to juxtaposed initiator E-box (position -12 to -7) and M-box (positions -103 to -94), which are separated by a DNA segment having conserved length in vertebrate species [70]. An additional level of specificity in gene regulation can be potentially provided by cooperative interactions between transcription factors in displacing nucleosomes [206]. Mechanistically, binding of one transcription factor to nucleosomal DNA, either within its rotationally exposed major groove on the nucleosome surface or within sites proximal to the linker DNA, might lead to a partially disassembled state of nucleosome and to a better accessibility of previously buried sites [206]. Such cooperative binding of two transcription factors to adjacent binding sites would require lower concentration of these factors for gene activation.

The access to DNA sites that are internal to a nucleosome requires catalyzed nucleosome remodeling exerted by ATP-dependent chromatin remodeling complexes [207]. They convert the energy of ATP hydrolysis to mechanical movement and can remodel chromatin in a number of ways to either activate or repress transcription. The mechanisms of action that lead to exposure or masking of DNA regulatory sites include creation of DNA loops on the nucleosome surface, bi-directional movement of nucleosomes along the DNA, replacement of histone subunits or eviction of nucleosomes from a particular genomic location [207]. All types of multi-subunit chromatin remodeling complexes contain a subunit with ATPase activity. The ATPase subunits of SWI/SNF (Swi**t**ching defective/**S**ucrose **n**on**f**ermenting) complexes, namely BRM (Brahma) and BRG1 (Brahma-related gene 1) have

bromodomains, which preferentially bind acetylated histones. Alternative presence of either BRG1 or BRM and specific composition of 9-12 BRG1/BRM-associated factors (BAFs) is thought to determine specificity and mode of gene regulation by SWI/SNF remodeling complexes [207]. The activity of these complexes can be enhanced by lysine acetylation of histone tails by KATs, such as CBP (cyclic AMP response element protein-binding protein) and p300 (E1A binding protein 300 kDa), classified respectively as KAT3A and KAT3B, are known co-activators recruited to chromatin by transcription factors [207].

SWI/SNF complexes and other chromatin remodeling complexes have been demonstrated to promote differentiation by coordinating gene expression events in multiple cell lineages, including melanocytes [208]. BRG1 is expressed in human melanocytes and is required for the development of neural crest derived cells [209]. It has been shown to interact with β-catenin to activate LEF/TCF target genes [210], suggesting a possible involvement of BRG1 in the transcription of *MITF* and *DCT*. Furthermore, SWI/SNF chromatin remodeling enzymes has been demonstrated to be required for transcriptional activation of a subset of MITF-regulated genes [74, 211]. Using mouse fibroblasts as cellular model for ectopic expression of MITF and dominant negative BRM or BRG1, activation of *tyr*, *tyrp1* and *dct* has been shown to be dependent on functional SWI/SNF complexes [74]. In addition, the association of MITF with co-activators CBP and p300 has been mechanistically shown to result in transcriptional activation of *TYR*, *TYRP1* and *DCT* [116, 212]. The dependence of such association on a signaling event has been demonstrated in the context of the MAP kinase signaling, where MITF phosphorylated by ERK2 on Ser-73 preferentially interacted with p300 [116]. A MITF-dependent histone acetylation, which occurs independently of SWI/SNF function, seems to be the initial event within the *tyr* and *tyrp1* promoters [74]. Thus, a model of activation of melanocyte-specific genes has been proposed, in which the E-box is 'hidden' from MITF by the chromatin structure and MITF is initially recruited to this locus through an interaction with KATs [74]. The SWI/SNF complex is recruited both by MITF and by binding via the bromodomains to acetylated histones. Remodeling of the locus then exposes the E-boxes and binding sites to other transcription factors, such as SOX10, leading to the formation of a stable multi-protein regulatory complex.

Chromatin remodeling has been proposed to be involved in the regulation of the OCA2 expression in iris melanocytes and to contribute in this way to the regulation of eye color [18]. Human T-cell leukemia virus enhancer factor (HTLF) has been suggested to act as a sequence specific DNA recognition member of a SWI/SNF chromatin remodeling complex [18]. HTLF appears to promote a relaxed chromatin state permissive for LEF-1- and MITF-mediated expression of OCA2, one of the factors determining brown eye color [18].

To parse out the exact roles of epigenetic mechanisms in the development and differentiation of melanocytes, epigenetic modifiers might be knocked out specifically in the melanocyte lineage in animal models.

II. Genetic and epigenetic events in cutaneous melanoma

II.1 Melanomagenesis as a multi-step process

Cutaneous melanomas are thought to arise from epidermal melanocytes and, potentially, from follicular melanocytes or melanocyte stem cells, whereas other types of melanoma (e.g. uveal or mucosal) derive from non-cutaneous melanocytes [213]. In addition, recent developmental studies [148] gave rise to the hypothesis that some melanomas might arise from Schwann cell precursors (SCPs) and hence follow distinct tumorigenesis process. Melanoma is among the more common causes of "metastatic cancer of unknown primary" due to indolent or unrecognized cutaneous primary lesions [213].

Epidemiological evidence implicates exposure to solar UVR spectrum, especially frequent episodes of sunburn during childhood, as a major risk factor in the etiology of melanoma [214]. The presence of multiple pigmented lesions, including banal or clinically atypical nevi or freckles, is associated with an increased melanoma risk [213]. Recent genome-wide association studies have identified genetic loci associated with pigmentation and melanoma predisposition [215]. Thus, some *MC1R* ("red hair color" alleles), *TYRP1* and *MATP* variants have been associated with melanoma risk [215]. Patients with a familial history of melanoma represent about 10% of melanoma cases and the risk of melanoma is about 2.24 times higher in individuals having at least one first-degree relative with melanoma [213]. *CDKN2A/B* is the best known familial melanoma locus affected in up to 40% of cases [213].

Transformation of skin melanocytes to cutaneous melanoma is a multi-step process also called melanomagenesis, which is generally described by the melanoma progression model based on histopathological observations [1, 122] (Fig. 12A). A benign nevus is represented by aggregated melanocytes that have lost the appropriate contact with basal keratinocytes and have aberrantly proliferated to a hyperplastic lesion. The next step toward melanoma, a dysplastic nevus, shows cytological atypia and may arise from a preexisting benign nevus or as a new lesion. Radial growth phase (RGP) melanoma, considered to be the primary malignant stage, spreads in the epidermis superficially to the basement membrane. In vertical growth phase (VGP) melanoma, nodules or nests of cells cross the basement membrane and invade the dermis. Both RGP and VGP primary melanomas can progress to metastatic melanoma. Depending on tumor thickness, mitotic index, presence of ulceration, age, gender and anatomical site, about 20-25% of all primary melanoma will spread. Melanoma cells having metastatic potential migrate, become invasive and enter the bloodstream or lymphatic vessels from which they colonize inner organs such as lung, brain and liver (Fig. 12A). Only about half of cutaneous melanomas are known to arise from nevi indicating that melanomas may emerge *de novo* in normal skin, whereas melanoma progression can occur without going through all stages [122].

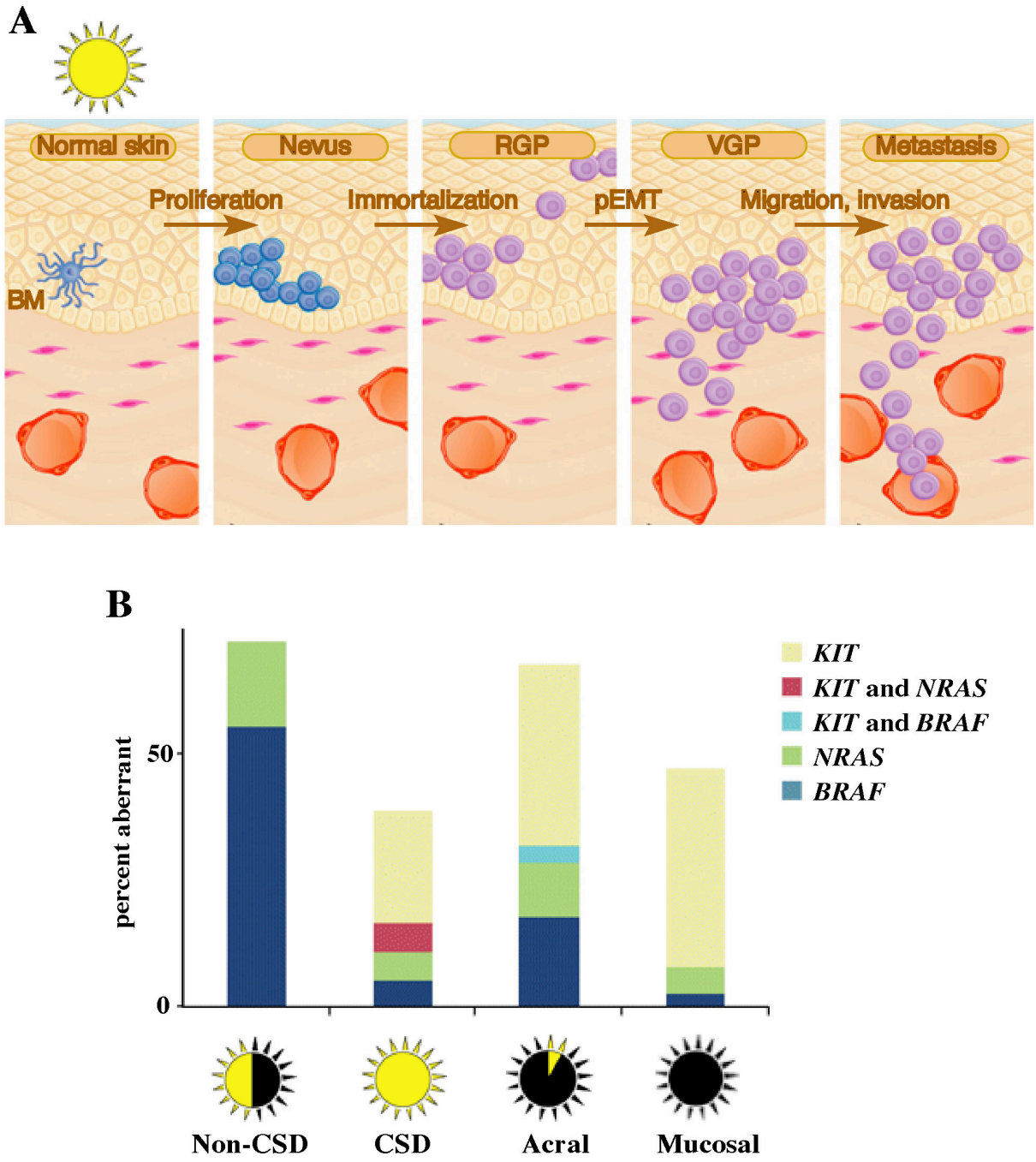


Figure 12. Melanomagenesis as a multi-step process. A. Schematic representation of melanoma progression as a multi-step process involving various cellular mechanisms. Distinct histopathological stages of melanomagenesis have been described: nevus (benign and dysplastic), radial growth phase (RGP) melanoma, vertical growth phase (VGP) melanoma and metastatic melanoma. BM, basement membrane; pEMT, pseudo-epithelial-mesenchymal transition. Modified from Zaidi *et al.*, 2008 (122). **B.** Frequency distribution of genetic alterations in BRAF, NRAS and KIT among four groups of melanoma. Non-CSD, melanomas on skin without chronic sun-induced damage; CSD, melanomas on skin with chronic sun-induced as evidenced by the presence of marked solar elastosis; acral, melanomas on the soles, palms, or sub-ungual sites; mucosal, melanomas on mucosal membranes. Adapted from Curtin *et al.*, 2006 (227).

II.2 Genetic and epigenetic patterns in melanoma

Transformation of normal melanocytes into malignant melanoma cells relies on numerous cellular mechanisms, such as proliferation, immortalization, epithelial-mesenchymal transition, migration and invasion, which are associated with numerous genetic and epigenetic alterations [216, 217] (Fig. 12). The entire genome sequencing of one melanoma cell line has revealed more than 33,000 somatic mutations present in tumor cells [218]. This catalogue of mutations carries the imprint of the past UVR-induced damage, since the vast majority (about 70%) of single base and dinucleotide substitutions are, respectively, C>T/ G>A and CC>TT/ GG>AA transitions [218]. In addition, C>T/CC>TT changes tend to affect more often CpG nucleotides [218]. Aside from the effects of UVR, many of the mutations in melanoma cells have apparently arisen as a consequence of ROS action, which preferentially causes G>T substitutions [218]. Other genome-wide studies, such as array-based comparative genomic hybridization (CGH) and single-nucleotide polymorphism arrays, determined abnormal copy numbers of multiple loci encoding mRNAs and non-coding RNAs [213, 219, 220]. Obviously, not all genetic alterations that occur in melanoma cells would be implicated in melanomagenesis. To embody this concept, terms "driver" and "passenger" mutations have been proposed [221]. "Driver" mutations are thought to confer growth, survival or any alternative selective advantage for tumor cells. They are positively selected and causally implicated in tumor development. The remainder are thought to be "passenger" mutations [221].

In melanoma, several components of the ERK pathway have been found to be frequently affected by "driver" mutations, highlighting the importance of this pathway in melanocyte lineage cells [217] (Fig. 5, 6, 12B). Those include mutations in *BRAF*, *NRAS* and *KIT*, all of which result in a constitutively active ERK pathway [213]. Somatic mutations in *BRAF* or *NRAS* have been found, respectively, in about 50-60% and 15-20% of melanoma lesions [217, 222]. Activated NRAS apparently stimulate the ERK pathway through CRAF rather than through BRAF [115]. The most common in melanoma is a T1799A mutation in exon 15 of *BRAF*, which accounts for about 90% of all *BRAF* mutations [223, 224]. It encodes the substitution of a glutamic acid for valine at codon 600 (V600E) in the BRAF kinase activation domain and results in about 500 times higher BRAF kinase activity *in vitro* [223, 224]. Other BRAF mutations (e.g. V600R, V600K) have been shown to result in increased kinase activity [225]. It has been suggested that the introduction of a negative charge and/or a switch from a small to a large hydrophobic residue at the codon 600 mimic the phosphorylation of threonine 599 and serine 602 within the BRAF activation domain thereby leading to increased BRAF activity [225]. Constitutively active $BRAF^{V600E}$ transforms immortalized melanocytes and stimulates melanoma cell proliferation and survival [226, 227]. Thus, the concept of "oncogene addiction", that emphasizes the apparent dependency of some cancers on one or a few genes for the maintenance of the malignancy, has been applied to melanomas expressing mutant BRAF [228].

Based on genome-wide genomic and gene expression profiling studies, melanoma has been proposed to constitute a heterogeneous group of tumors with different patterns of genetic alterations and distinct "driver" mutations. For instance, genome-wide CGH profiling together with analysis of individual somatic mutations by gene sequencing, allowed to classify melanomas arising in different anatomical sites with varying UV exposure histories and harbor distinctive patterns of genomic alterations into 4 groups: melanoma on skin with non-chronic sun-damage (non-CSD melanoma, for instance melanoma of the trunk, lower limbs and upper arms), which is the predominant type in Caucasians; melanoma on skin with chronic sun-damage (CSD melanoma); melanoma on palms, soles and nail bed (acral melanoma) and melanoma on mucous membrane (mucosal melanoma) [222, 229] (Fig. 12B).

Similarly to genetic alterations, mitotically heritable epigenetic changes that are advantageous to tumor progression might undergo the selection process in the course of melanomagenesis. For instance, aberrant activation of growth stimulatory pathways, which can be accomplished by gain-of-function mutations, amplifications and translocations of proto-oncogenes, may be armored by inactivation of negative regulators of these pathways by epigenetic mechanisms. Hypermethylation (gains of DNA methylation marks) within the 5' regions of genes can result in their transcriptional silencing and thereby can be an additional or alternative mechanism of gene loss-of-function [230]. Treatment of cultured melanoma cells with demethylating agents combined with global microarray gene expression analysis has allowed the identification of more than 50 genes primarily silenced by hypermethylation within their 5' regions [231]. Many of them are tumor suppressor genes [231]. In the same tumor cells, along with local hypermethylation changes, losses of methylation (hypomethylation) affect a variety of DNA sequences, including repetitive sequences dispersed throughout the genome and a limited number of genes, which are silenced by DNA methylation in normal somatic cell counterparts [232]. Hypomethylation is usually quantitatively predominant in tumors. Net loss of 20-80% of genomic 5mC has been observed in melanoma cell lines [233]. Used as a marker of global DNA hypomethylation, decreased DNA methylation of LINE-1 (long interspersed nuclear element 1) and Alu repetitive sequences has been observed in 75% and 13% of melanoma cell lines, respectively [234]. Genome-wide hypomethylation is thought to enhance genomic instability and to modify gene expression profiles via sequestration of transcriptional regulators and/or via changes in subnuclear compartmentalization or in transcription of regulatory non-coding RNAs [232]. The histone acetylation changes have been mostly assessed by treating melanoma cells with HDAC inhibitors (HDACi) [231]. HDACi have been demonstrated to activate, but also to repress gene expression in melanoma cells [231].

Although the exact order of events underlying melanomagenesis is not completely elucidated, interplay of genetic and epigenetic changes that occurs in the course of tumor progression ultimately results in the profoundly altered expression patterns of mRNAs, non-coding RNAs and, consequently, proteins in melanoma [235, 236].

II.3 Major signaling pathways involved in melanomagenesis

The constitutive activation of the ERK pathway appears to contribute to both melanoma initiation and progression. This fundamental event occurs early in melanomagenesis, since about 80 % and 10% of benign acquired nevi harbor *BRAF* and *NRAS* mutations, respectively [213]. This finding argues that such mutations are not sufficient for malignant transformation of melanocytes, as nevi remain static for decades and rarely progress to melanoma. However, it does suggest a role in the initiation of malignancy. Accordingly, experiments in transgenic mice have shown that the primary consequence of $BRAF^{V600E}$ expression specifically in melanocytes is the formation of nevi, while melanomas only arise after a long latency (about 10-12 months) in up to 70% of mice [237]. Thus, it has been suggested that mutated $BRAF^{V600E}$ has a founder role in melanomagenesis, but additional genetic and epigenetic changes are required for the development of malignant tumors [237].

A slow growth rate of nevi has been associated with cellular senescence, a mechanism considered to limit tumorigenesis [238]. Cellular senescence is characterized by the arrest of proliferation and typical morphological and molecular changes, including β-galactosidase activity at acidic pH and the formation of senescence-associated heterochromatic foci [239-241]. These senescence-associated changes have been observed in human nevi [239-241]. Cellular senescence is executed in response to telomeric shortening (replicative senescence) or following various types of stress (stress-induced senescence), including oncogene-induced senescence (OIS), DNA damage response (DDR) and oxidative stress [238]. Based on experiments in cultured cells and animal melanoma models, $BRAF^{V600E}$ expression has been suggested to lead to OIS in melanocytes, indicating that mutated $BRAF^{V600E}$ might first stimulate proliferation and then induce cell cycle arrest in nevi [237, 242, 243].

Overcoming senescence is proposed to be a necessary event for progression of melanocytic and displastic nevi, but also for initiation of primary melanomas *de novo* [242]. At the molecular level, inactivation of the p53 and p16-RB pathways critical for mediating both replicative and stress-induced senescence is associated with proliferation and immortalization of neoplastic cells [238]. In contrast to other cancers, melanomas display a low frequency of inactivating mutations in *TP53* (9%) encoding p53 and it is rather the expression of p53 downstream targets, such as the broad-spectrum cyclin/CDKs inhibitor p21 (*CDKN1A*), that is affected in melanoma [217]. Hypoacetylation of the *CDKN1A* promoter mediated by the anti-senescence factor TBX2 has been proposed as one of the mechanisms of p21 inactivation [244]. Similarly to *TP53,* inactivating mutations in the retinoblastoma gene (*RB1*) are rare in sporadic melanoma and occur in about 6% of tumors [217]. However, germline mutations in *RB1* increase up to 80-fold the lifetime risk of melanoma development in retinoblastoma survivors [213]. Importantly, the best-known familial melanoma locus, the *CDKN2A/B* locus on the chromosome 9p21 that encodes p15, p16 and p14 cell cycle inhibitors, is also frequently

altered in sporadic human melanoma by the entire spectrum of genetic and/or epigenetic alterations [217, 238] (Fig. 8A). As an example of gene silencing by both genetic and epigenetic events, p14 (ARF) has been found to be inactivated by hemizygous loss and by promoter methylation in 4/20 of sporadic melanomas showing intact p16 expression [245]. Importantly, the inactivation of p16 by the stabilized form of β-catenin has promoted an escape of melanocytes from senescence, resulting in their immortalization and promotion of melanomagenesis *in vivo* in coooperation with N-Ras activation [246]. The bypass of replicative crisis can be achieved through the activation of telomerase, namely of its subunit TERT (telomerase reverse transcriptase), that exhibits low or no expression in normal cells and is upregulated during melanoma progression [217].

The progression from RGP to VGP is considered to be the most critical point in melanoma development, because neoplastic cells appear to escape keratinocytes' control and acquire competence for growth factor- and anchorage-independent growth and metastasis [122]. For progression to the VGP, additional alterations would be required to reduce apoptosis of melanoma cells in the absence of pro-survival soluble factors produced by keratinocytes. For example, the apoptotic function of p53 can be compromised by inactivation of a key regulator of the mitochondrial apoptosis pathway, APAF1 (apoptotic protease activating factor), which is downregulated during melanoma progression due to hypermethylation of its promoter [247].

Along with the ERK signaling cascade, PI3K (phosphatidylinositol 3'-kinase)-Akt pathway has been found to play a prominent role in melanoma development [4] (Fig. 13). Increased activity of the Akt3 kinase, the predominant Akt isoform active in melanoma, occurs in up to 60% of sporadic melanomas. Akt3 can be overexpressed due to an increased *AKT3* copy number and activated due to *NRAS* mutations or inactivation of the negative regulator of PI3K signaling, PTEN, through genetic and epigenetic mechanisms [4]. Mutational profiles that simultaneously promote both ERK- and Akt3-mediated signaling appear to be favored in melanoma. Thus, *NRAS* mutations lead to stimulation of both pathways and are rarely found in concert with *BRAF* mutations, whereas combination of $BRAF^{V600E}$ with either *PTEN* loss/inactivation or activating *AKT3* mutations has been found in approximately 30% of human melanoma [219, 220, 222]. Activated Akt3 phosphorylates $BRAF^{V600E}$ at Ser-364/428 and decreases in this way its activity to levels promoting proliferation, likely facilitating the bypass of OIS induced by high $BRAF^{V600E}$ activation levels [248]. The proof-of-concept for the cooperation between $BRAF^{V600E}$ and PI3K/Akt3 signaling in melanoma development has been provided in transgenic mice, in which melanocyte-specific $BRAF^{V600E}$ expression combined with PTEN suppression has resulted in melanoma development with 100% penetrance, short latency (25-50 days) and metastases to lymph nodes and lungs [243]. Cooperative action of the ERK and PI3K/Akt3 pathways promotes proliferation and survival of melanoma cells [4]. For instance, both pathways enhance cell survival through the phosphorylation-induced inactivation of anti-apoptotic BAD and through activation of NF-κB (nuclear factor κB) [4, 249]

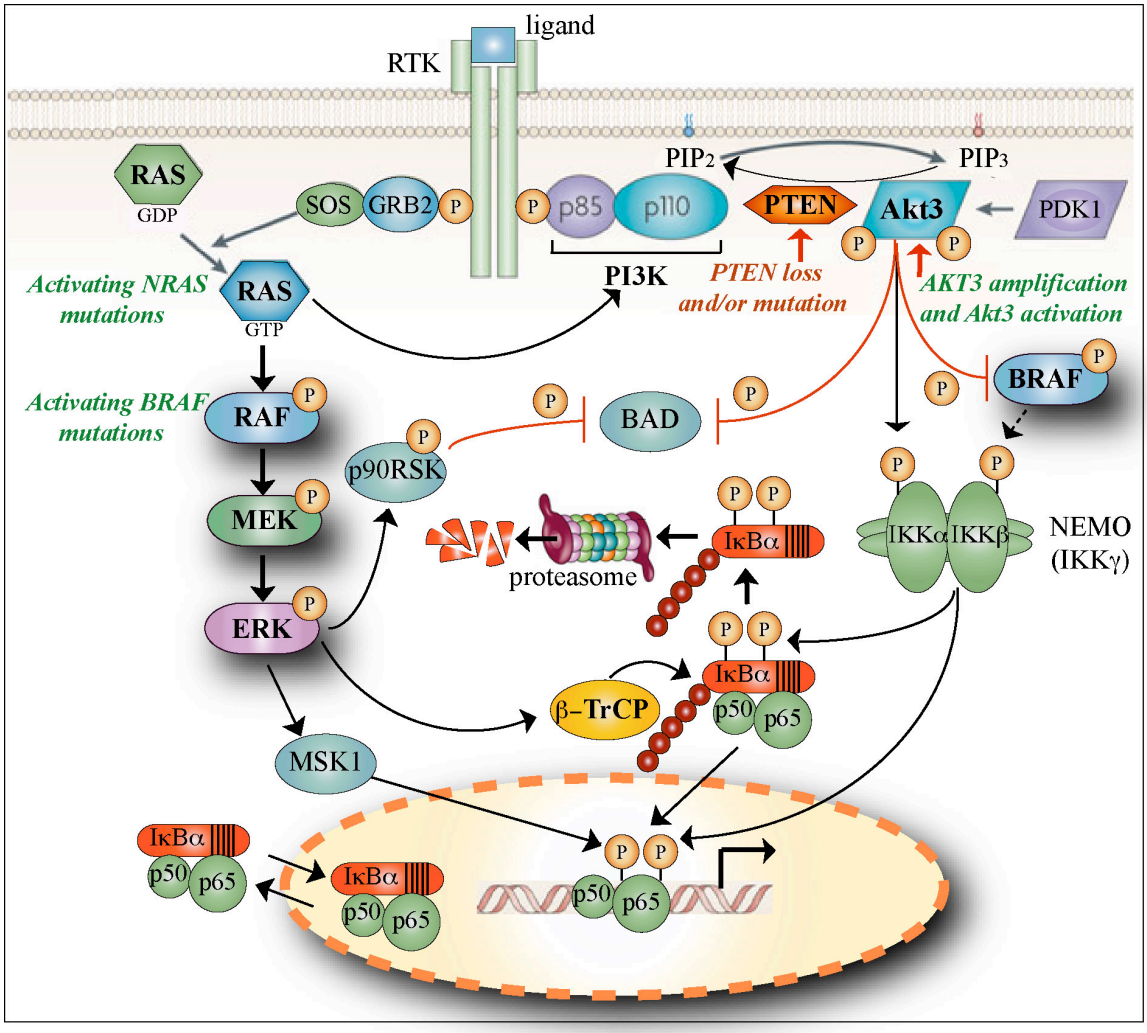


Figure 13. Selected major signaling pathways involved in melanomagenesis. Schematic representation of the ERK and the PI3K-Akt signaling pathways, which become constitutively active in melanoma. Besides activating the RAF/MEK/ERK cascade, RAS activation leads to the recruitment of the PI3K harboring regulatory (p85) and catalytic (p110) subunits to the plasma membrane and phosphorylation of PIP2 (phosphatidylinositol-4, 5-bisphosphate) to PIP3 (phosphatidyl-inositol-3, 4, 5-trisphosphate). PIP3 directly binds Akt3 and its kinase PDK1 (phosphoinositide-dependent protein kinase 1) leading to their activation. The 3'-phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome 10) dephosphorylates PIP3 and therefore terminates PI3K signaling. Activated Akt3 phosphorylates and thus reduces activity of BRAF (V600E) and pro-apoptotic BAD (BCL2-associated agonist of cell death). Similarly, BAD is inactivated by p90RSK kinase activated by ERK. Both ERK and PI3K-Akt pathways converge on the NF-κB signaling pathway. The classical NF-κB dimer (p50-p65) is present in unstimulated cells and shuttles between nuclear and cytoplasmic compartments being bound to IκBα, which inhibits its DNA binding activity. Upon stimulation, IκBα is phosphorylated by the IκB kinase (IKK) complex, comprising two catalytic kinase subunits (IKKα and IKKβ) and a regulatory protein IKKγ, known as NEMO (NF-κB essential modulator). Phosphorylation of IκBα at two specific serine residues (Ser-32 and Ser-36) acts as a docking platform for the ubiquitin ligase β–TrCP (β-transducin repeat-containing protein), which mediates ubiquitination and degradation of IκBα by proteasomes. Expression of β–TrCP is induced by ERK in melanoma cells harboring BRAF (V600E). The liberated NF-κB dimer is phosphorylated by different kinases, i.e. IKK and MSK1 (mitogen- and stress-activated kinase-1), and acetylated by p300/CBP (CREB-binding protein). Activated NF-κB induces expression of target genes encoding anti-apoptotic proteins. Inspired by Amiri and Richmond, 2005 (249).

An increasing evidence involves the NF-κB pathway in melanomagenesis [250, 251]. There are five members in the NF-κB family (p50 and p52 derived from a proteolytic processing of precursor proteins p100 and p105, RelA (p65), RelB and cRel), which can form 15 transcription factors through homo- and heterodimerization [252]. The most common is a p50-p65 dimer, which will be further referred to as NF-κB. Expression of p50 and p65 is significantly elevated in human nevi and melanomas relative to normal skin; the phosphorylated active form of p65 accumulates in the nuclei of melanomas, whereas the expression of the NF-κB inhibitor IκBα is significantly lower in metastatic melanomas than in nevi [253, 254]. Further, strong p50 nuclear staining correlates with poor prognosis for melanoma patients [253]. *In vitro* studies have shown that NF-κB binding is constitutively elevated in cultured melanoma cells having 5-10-fold higher levels of p50 and p65 in the nucleus than melanocytes [251]. BRAF mutations (for instance, V600E, K601E) may contribute to activation of NF-κB signaling via activation of IKKβ [255], enhanced expression of β-TrCP, the ubiquitin ligase targeting IκBα for ubiquitination-dependent degradation [249] and via p65 phosphorylation by MSK1, a downstream target of ERK [256] (Fig. 13).

NF-κB activates the expression of genes encoding anti-apoptotic proteins, such as inhibitor-of-apoptosis (IAP) proteins cIAP1, IAP2, survivin and BCL2-like proteins; proteins that promote migration and invasion of tumor cells, for instance metalloproteinases (MMPs) and intercellular adhesion molecule-1 (ICAM-1); cell cycle regulatory proteins (cyclin D1, CDK2) and a number of chemokines that can be constitutively expressed by melanoma cells, for instance interleukin-8 (IL-8) [251]. Overexpression of IL-8 is associated with the transition from RGP to VGP in melanoma and causes metastatic tumor growth in primary melanoma cells [250]. Transfection of highly metastatic human melanoma cells with an expression vector encoding super-repressor IκBαM, a dominant-negative form of IκBα carrying mutations at Ser-32 and Ser-36 preventing its phosphorylation and subsequent degradation, inhibited their growth after injection in nude mice and prevented lung metastasis [257]. Notably, the slow-growing subcutaneous tumors formed by the IκBαM-transfected cells exhibited a decrease in microvessel density (angiogenesis), which correlated with a decrease in the level of IL-8 and vascular endothelial growth factor (VEGF) expression [257]. Firm evidence of the requirement of the NF-κB signaling for melanomagenesis has been recently provided by significant inhibition of melanoma development in transgenic mice harboring melanocyte-specific ablation of Ikkβ in the background of Ink4a/Arf-defficiency and mutated H-Ras (G12V) [258].

The constitutively activated ERK, PI3K-Akt and NF-κB pathways may contribute to the process of pseudo-EMT (pEMT) bearing similarities to epithelial to mesenchymal transition (EMT) occuring during development [259-262]. Melanoma cells undergoing pEMT may lose expression of proteins promoting cell-cell contacts (E-cadherin (epithelial cadherin), α- and γ-catenin) and acquire mesenchymal markers (N-cadherin, fibronectin, MMP-2, MMP-9, ICAM-1) associated with increased ability for cell migration and invasion [259-262].

II.4 MITF expression and function in melanoma

Given the intimate and specific involvement of MITF in survival, growth and differentiation of melanocyte lineage cells, one might expect that MITF would discourage any deviation towards uncontrolled growth and malignancy. Apparently, the subversion of signaling pathways governing melanocyte fate decisions in melanoma inevitably leads to the implication of MITF and the products of its target genes in melanomagenesis.

Being required for the survival and proliferation of melanocyte lineage cells, MITF expression is generally maintained in melanoma and carries diagnostic and prognostic value. Immunohistochemical detection of total MITF protein levels exhibits specificity and sensitivity comparable to other melanoma diagnostic markers, such as Melan-A, tyrosinase, gp100/HMB45 (human melanoma, black 45) [263]. Intense nuclear staining for MITF protein was observed in 83% of melanocytic nevi, 56% of primary melanomas and 23% of melanoma metastases, indicating a significant reduction in MITF expression with tumor progression [264]. On one hand, low MITF levels have been linked to reduced survival rates and increased metastases in patients with intermediate thickness melanoma [265]. On the other hand, a genetic amplification of *MITF* has been found in about 10% of primary melanomas and in up to 20% of metastatic melanoma, but not in melanocytic nevi, and has been associated with a decreased 5-year survival in metastatic melanoma [266]. Finally, RT-PCR-based identification of circulating *MITF*-M expressing cells in the blood of melanoma patients after chemotherapy and melanoma resection strongly correlated with an increased risk of disease recurrence [267].

In melanoma cells, optimal MITF expression levels appear to ensure its proliferative and survival functions via activation of relevant target genes, as postulated by a "rheostat" model of relationship between MITF dosage and functional outcome [139, 145] (Fig. 8, 14).

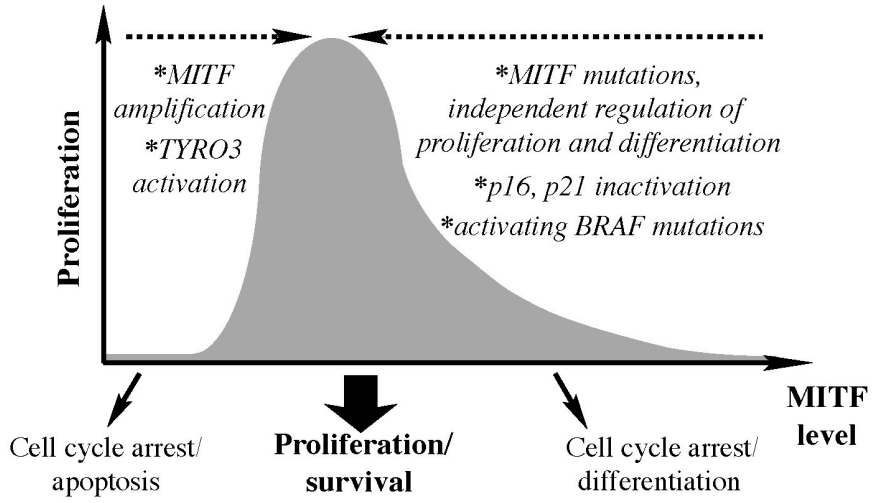


Figure 14. "Rheostat" model of MITF regulation and function in melanoma. Some mechanisms that are considered to ensure MITF expression levels compatible with proliferation and survival of melanoma cells are mentioned. Modified from Gray-Schopfer *et al.*, 2007 (143).

MITF expression has been proposed to counteract senescence response in melanoma cells and to promote their proliferation [268]. Indeed, siRNA-mediated depletion of MITF in melanoma cells harboring $BRAF^{V600E}$ has induced a phenotype displaying the hallmarks of senescence [268]. The senescence program mediated by MITF silencing has appeared to engage a DNA damage response signaling pathway known to stabilize and activate p53 [268]. Overexpression of dominant negative form of MITF in melanoma cell lines has resulted in inhibition of their growth proposed to be mediated by reduced expression of CDK2 [266].

High MITF levels, similar to those in terminally differentiated melanocytes, might lead to cell cycle arrest through induction of negative cell cycle regulators p16 and p21. Indeed, anti-proliferative effect of high MITF expression levels has been documented in melanoma cells even in the presence of the mutated $BRAF^{V600E}$ [266, 269]. This effect might be circumvented in melanoma cells via inactivation of p16 and/or p21. Among mechanisms proposed to inactivate p21 in melanoma cells are transcriptional repression by TBX2 [244] and somatic *MITF* mutations reported to occur in 16% (8/50) of melanoma metastases [270]. Five of six described mutations have conferred to MITF a reduced capacity to transactivate the *p21* promoter in gene reporter assays [270]. Interestingly, one of these mutations, termed 4TΔ2B, leads to deletion of exon 2B, which encodes the Ser-73 phosphorylation site implicated in the control of ubiquitin-dependent proteolysis of MITF. 4TΔ2B confers to MITF a higher capacity to activate *TYR* and *DCT* promoters in concert with SOX10, indicating that mutated MITF might differentially regulate proliferation and differentiation in melanoma cells [270].

Constitutive activation of ERK by $BRAF^{V600E}$ should result in MITF phosphorylation at Ser-73 and its subsequent degradation by proteasomes [51]. Accordingly, MITF expression is significantly reduced in melanocytes that ectopically express $BRAF^{V600E}$ [266, 269]. However, in melanoma cell lines and clinical melanoma samples no anti-correlation has been observed between the extent of ERK activation and the expression levels of MITF and melanosomal proteins [26, 271]. Melanoma cells likely maintain the expression of MITF at levels compatible with tumor progression via several mechanisms [145] (Fig. 14). First, amplification of *MITF* (ranging from 5 to 119 copies) has been associated with about 1.5 times higher MITF protein levels in metastatic melanoma samples [266]. Second, transcriptional *MITF* activation might occur in compensation to ERK-mediated MITF protein degradation. Thus, receptor tyrosine kinase TYRO3 able to activate *MITF* transcription through SOX10 *in vitro* has been found to be overexpressed in 50% (20/40) of melanoma samples [272], whereas melanoma cells harboring $BRAF^{V600E}$ mutation might enhance *MITF* transcription via a BRN2-dependent mechanism [273]. In human melanocytes ectopically expressing $BRAF^{V600E}$, a dominant-negative p53 and activated CDK4 and immortalized by the expression of TERT, overexpression of MITF conferred a capacity for anchorage-independent growth [266]. The propensity of MITF to amplification, its requirement for proliferation and contribution to the transformation of melanocytes suggest that it may function as a lineage-specific 'addictive oncogene' [266].

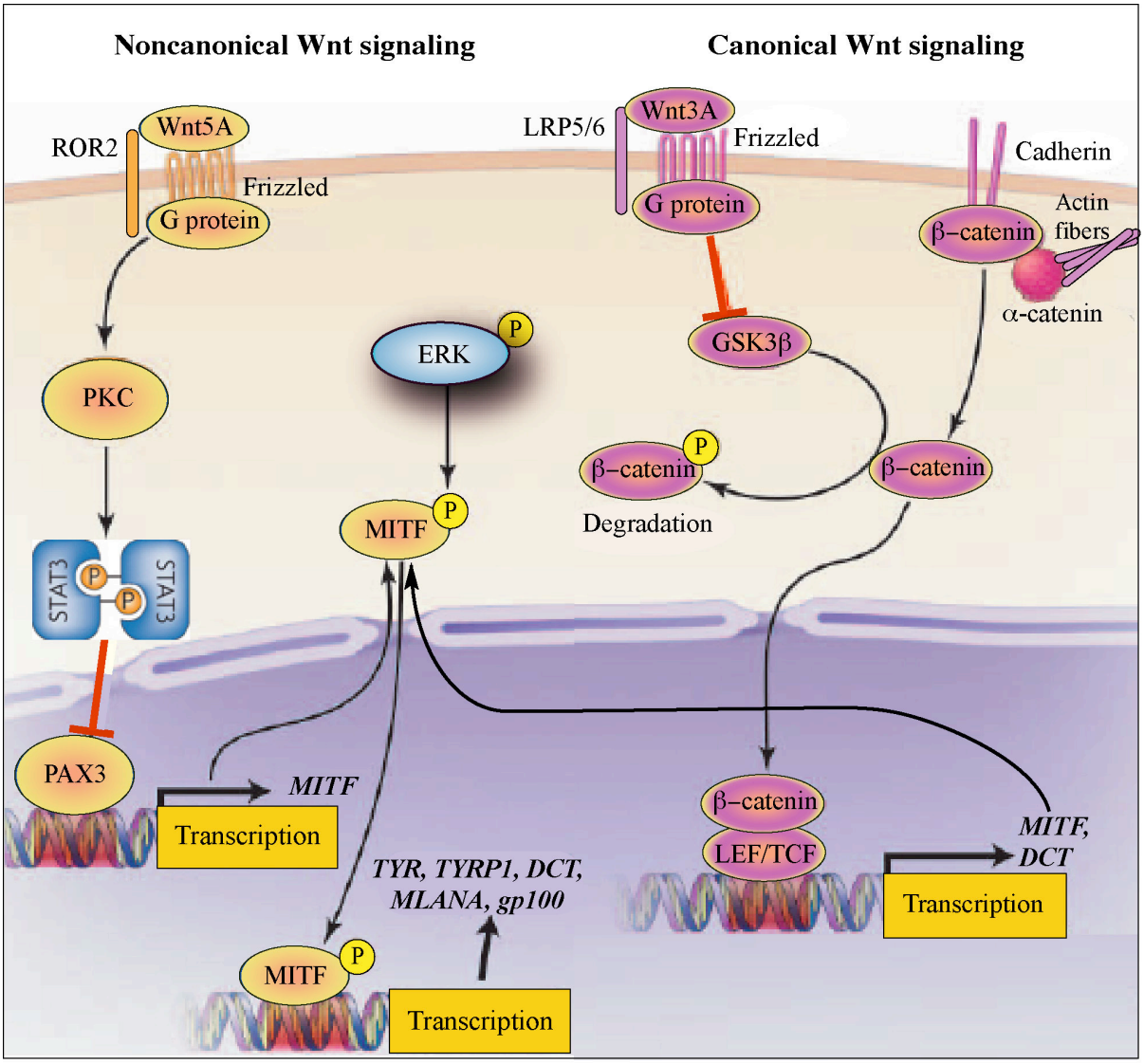


Figure 15. Schematic representation of *MITF* regulation by Wnt pathways in melanoma. Noncanonical Wnt5A ligand binds Frizzled family seven-transmembrane receptors and ROR2 co-receptor, whereas canonical Wnt ligands (Wnt1, Wnt3A) activate signaling through Frizzled receptors and either LRP5 or LRP6 (low-density-lipoprotein-receptor-related proteins). PKC (protein kinase C) is activated by Wnt5A-induced signaling and mediates the phosphorylation of STAT3, which downregulates PAX3 via an unknown mechanism. Concomitantly, downregulation of MITF and its target genes is observed. In canonical Wnt singaling, the function of β-catenin depends on it subcellular localization. When associated at the membrane with cadherins and α-catenin, it is involved in cell-cell adhesion. When dissociated from cell adhesion complex and not targeted for proteasomal degradation by the destruction complex containing GSK3β kinase, stabilized β-catenin accumulates in the cytoplasm and translocates to the nucleus. Canonical Wnt signaling leads to the inhibition of β-catenin phosphorylation by GSK3β, thus preventing the recognition of phosphorylated β-catenin by β-TrCP targeting protein for proteasomal degradation. In the nucleus, in the absence of Wnt signaling, transcription factor LEF-1 or one of the T cell factors (TCF-1, -3, -4) binds the Wnt response element within target gene promoters and generally represses transcription by recruiting co-repressors. This repression is relieved by stabilized β-catenin, which binds to either LEF-1 or one of the TCFs, interacts with co-activators and switches the activity of the complex from repression to activation of LEF/TCF target genes, including *MITF* and *DCT*. Modified from Dissanayake *et al.*, 2008 (276), Miller and Mihm, 2006 (1).

MITF-M regulation and function are intimately linked to Wnt signaling, which is predominantly mediated in melanoma cells by canonical (Wnt1, Wnt3A) and non-canonical (Wnt5A) members of the Wnt family of secreted glycoproteins [274] (Fig. 15). Although genetic and epigenetic alterations rarely affect the components of the canonical Wnt/β-catenin signaling in melanoma [275, 276], its constitutive activation assessed by nuclear β-catenin staining has been observed in about 30% of metastatic melanomas [275]. A stabilized β-catenin, which carries mutations in otherwise phosphorylated sites targeting protein for proteasomal degradation, has been shown to exert anti-proliferative effect *in vivo* in a mouse model [246]. Accordingly, nuclear staining of β-catenin has been associated with decreased proliferation of human melanoma cells *in vivo* [277]. The mechanisms leading to β-catenin localization and/or maintenance in the nucleus are poorly understood. The activation of Wnt/β-catenin signaling may be driven by the canonical Wnt ligands, either secreted by tumor cells themselves or by cells of the tumor environment. In accordance with the ability of β-catenin to directly activate *MITF* expression via a LEF/TCF binding site in the 1M promoter, the activation of Wnt/β-catenin pathway in melanoma cells treated with Wnt3A has resulted in upregulation of *MITF* and its transcriptional target genes [156, 277].

The non-canonical Wnt signaling has been proposed to mediate the inhibition of the canonical Wnt/β-catenin signaling observed during progression from benign nevi to metastasis [277]. In contrast to the canonical Wnt signaling, Wnt5A-mediated signaling leads to repression of *MITF*-M expression [278]. High levels of Wnt5A expression have been observed in late-stage melanomas and correlated with decreased patient survival [279]. The Wnt5A/PKC pathway has been shown to stimulate melanoma cell motily and invasion via induction of genes involved in pEMT and downregulation of E-cadherin [280]. Importantly, gene expression profiling of melanoma cell lines *in vitro* but also after injection into immunocompromised mice has led to the identification of "proliferative" and "invasive" signatures in late-stage tumors [281, 282]. Thus, expression of *MITF*, members of its transcriptional network (*MLANA*, *TYR*, *DCT*, *SOX10*, *EDNRB*) and lack of *Wnt5A* expression have defined a "proliferative" signature associated with high levels of proliferation, low motility and prominent sensitivity to growth inhibition by TGF-β. In contrast, in the "invasive" signature, MITF-associated genes have been downregulated, while *Wnt5A* and other genes that encode proteins promoting invasion, have been upregulated [281, 282]. This signature has been associated with low levels of proliferation, high motility and resistance to growth inhibition by TGF-β [281, 282]. Association of low MITF levels with low proliferative and high invasive potential has been also demonstrated by other studies [139, 283]. Notably, the ratio of *Wnt5A* to *MLANA* expression has been shown to be increased in visceral, but not in lymph node metastasis [278]. Thus, two types of melanoma metastasis pathways have been suggested: the mesenchymal hematogenous form of invasion, which involves pEMT and secretion of Wnt5A and MMPs, and the amoeboid, lymphatic form of invasion, which is metalloproteinase-independent [274].

II.5 Cancer-germline genes in melanoma: mechanisms of reactivation and known functions of their protein products

DNA hypomethylation in tumors, including melanoma, has been found to directly cause the activation of a particular group of germline-specific genes that rely on DNA methylation for their transcriptional repression in somatic cell lineages [233, 284-286]. Genes that belong to this group are collectively termed cancer-germline (CG) or cancer-testis (CT) genes due to their unique pattern of expression [287]. CG genes are expressed in germline cells of developing and adult testis and ovary, in trophoblast-derived cell lineages and exhibit little or no expression in adult somatic tissues [286, 288-293]. Human ES cells do not express CG genes [294]. Expression of CG genes has been found in germline-derived malignancies, such as seminoma and ovarian dysgerminoma [295, 296]. Importantly, CG genes are aberrantly activated in non-germline malignancies arising from different somatic cell lineages, with more frequent expression found in melanoma, bladder, ovarian and lung cancers [297]. In tumors that express CG genes, simultaneous expression of more than one CG gene is often observed. Thus, 3 or more CG genes have been activated in 65% of melanomas, while no expression of 9 selected CG genes has been observed in 26% of melanomas [298]. Tumors of higher histological grade and later clinical stage often show higher frequency of CG gene expression [299].

The founding member of the CG group of genes is *MAGE1* (Melanoma AntiGEn 1), later renamed *MAGE-A1*. It was discovered in 1991 by researchers of the Brussels branch of the Ludwig Institute for Cancer Research (LICR) by virtue of T-cell epitope cloning [6]. Cytolytic T lymphocytes (CTL) that recognized and lysed autologous tumor cells were found in the blood of a melanoma patient who followed a chemotherapy treatment in Mainz and had an unusually favorable clinical course [300]. Melanoma cell line MZ2-MEL (Mainz-melanoma) established from a visceral metastasis presents on its surface several antigens recognized by clonal CTL populations specific for each antigen [301]. One of such CTL epitopes has been identified as a processed protein product of *MAGE-A1*, which was found to belong to a large multigene *MAGE* family including dozens of members [302].

A total of 153 CG genes has been identified to date by T-cell epitope cloning, serum reactivity studies, *in silico* sequence homology analysis and by virtue of unique expression pattern of CG genes [287, 303]. Many of these genes have been identified and described by researchers of the Brussels branch of LICR [288, 304-311]. Remarkably, approximately half of the known CG genes belong to multigene families located on the X chromosome, representing therein about 10% of all genes [312]. The other half is represented by autosomal genes that are evenly distributed throughout the genome and do not usually form gene families [303]. CG genes localized to X chromosome have been mostly found to be expressed in premeiotic germ cells, spermatogonia and oogonia, while activation of autosomal CG genes has been mainly associated with more differentiated stages of gametogenesis [286].

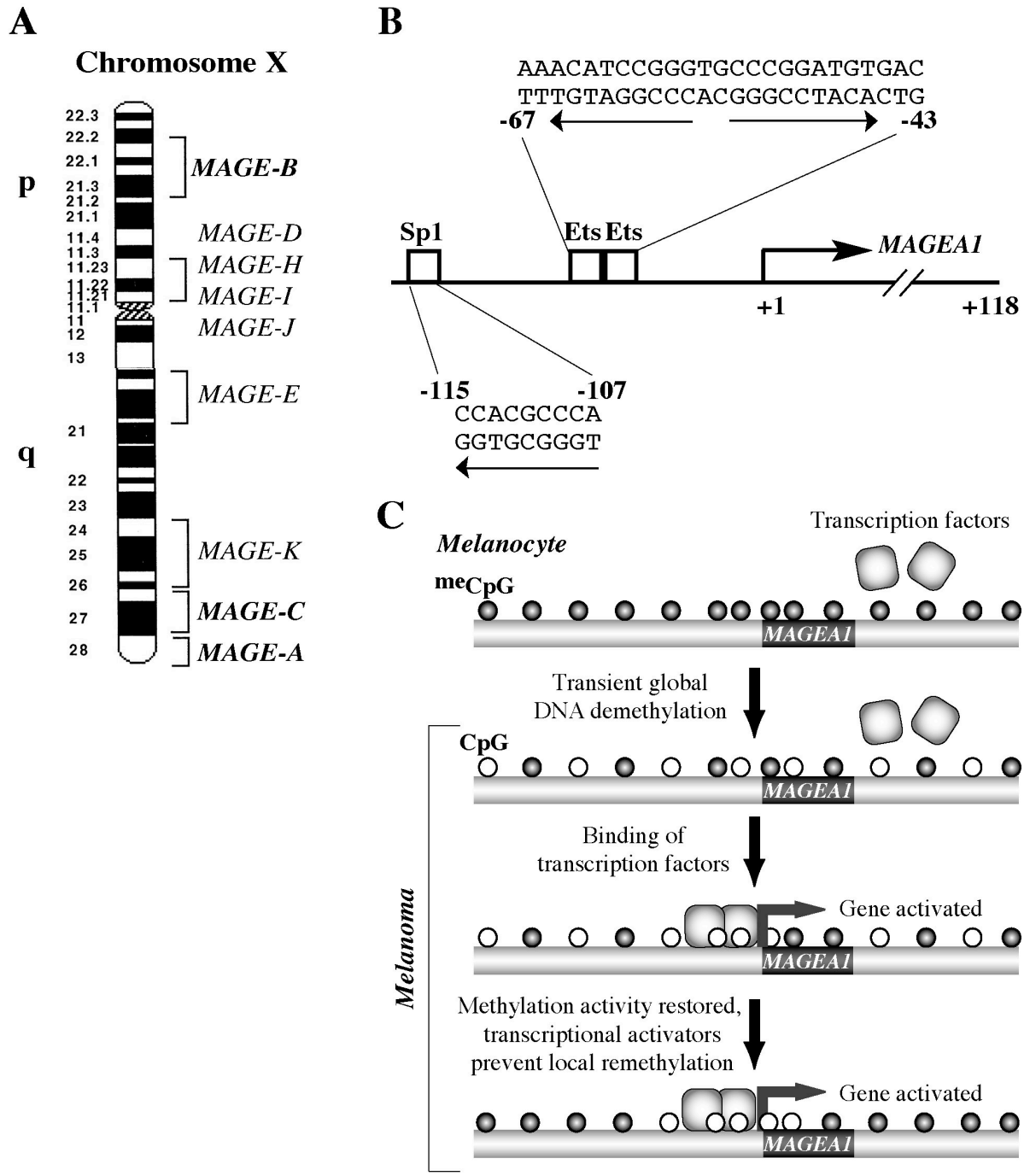


Figure 16. ***MAGE-A1*** **transcriptional regulation. A.** Locations of the human MAGE subfamilies on the X chromosome, including cancer-germline (CG) genes of *MAGE-A, MAGE-B* and *MAGE-C* subfamilies (in bold) are depicted. Locations of *MAGE-F* on the chromosome 3 and of *MAGE-G, MAGE-L2* and *Necdin* on chromosome 15 are not represented. Modified from Chomez *et al.*, 2001 (300). **B.** Schematic representation of consensus binding sites relevant for the *MAGE-A1* promoter activity. Modified from De Smet *et al.*, 1995 (317). **C.** Model for the acquisition of site-specific hypomethylation of the *MAGE-A1* promoter during melanomagenesis. Persistence of site-specific hypomethylation after the transient global DNA demethylation phase requires the continued presence of transcriptional regulators to protect the specific regions against an ongoing methylation activity. Methyl groups at CpG sites (grey dots) are shown only at the top of the bar representing DNA, even though they represent symmetrical methylation on both strands. Modified from De Smet *et al.*, 2010 (230).

The enrichment of the mammalian X chromosome for genes expressed in mitotic spermatogonia along with the paucity of genes expressed in meiotic germ cells is thought to be an evolutionary response to sex chromosome inactivation from the beginning of meiosis in spermatocytes until meiosis completion and formation of haploid spermatids [313]. The evolutionary recent expansion of CG gene families by duplication on the X chromosome, such as independent amplification of *MAGE-A* family members in human and mouse, is notable [302]. Although the expression profiles of CG genes and relevant proteins in the course of gametogenesis have been extensively studied [286, 290, 291, 293], the functions of only few of them have been elucidated [287]. Genetic studies of potential alterations of CG genes in infertility are challenging given the presumed high number of candidates and their functional redundancy. Some CG genes encode proteins with known functional domains. Thus, members of the SSX (synovial sarcoma X breakpoint) family possess a Kruppel-associated box (KRAB) domain, which mediates transcriptional repression [314, 315], whereas BRDT (bromodomain testis specific) protein containing two bromodomains has been shown to specifically bind hyperacetylated histone H4 tail and to trigger ATP-independent reorganization of the chromatin structure after induction of histone hyperacetylation by the HDAC inhibitor trichostatin A (TSA) [316].

MAGE genes are clustered at several locations mainly on the chromosome X [302] (Fig. 16A). CG genes of *MAGE-A*, *-B* and *-C* subfamilies show considerable variability of their promoter and first exon sequences, suggesting differential transcriptional control. The diversity of *MAGE* transcripts is enlarged by the production of alternative splicing variants reported for MAGE-A4a [317], MAGE-B1 [306] and MAGE-C2 [318]. The *MAGE-A1* promoter contains consensus binding sites for the transcription factors Sp1 and Ets [319] (Fig. 16B). Partial promoter deletions combined with gene reporter assays have shown that two inverted Ets motifs drive 90% of the activity of the *MAGE-A1* promoter in MZ2-MEL cells [319].

Growing evidence points to epigenetic modifications as the regulatory basis of the unique expression pattern characteristic to CG genes belonging to *MAGE-A, -B* and *-C* subfamilies and *LAGE, GAGE, SSX*, *XAGE* families [284-286, 320-322]. The primary role of DNA methylation in transcriptional regulation of CG genes has been shown by numerous studies. First, a correlation between expression of CG genes and global DNA hypomethylation has been documented [233]. Second, a significant positive correlation is frequently found between mRNA expression of CG genes and hypomethylation of their 5' regions, which generally co-localize with CpG islands [284]. Notably, hypomethylation of some CpG sites within CpG-rich promoters of cancer-germline genes is particularly relevant for transcriptional activation. For instance, hypomethylation of 6 CpG sites located between positions -30 and +30 of the *MAGE-A1* promoter is strongly associated with the activation of *MAGE-A1* [284, 323]. Third, treatment with demethylating agent 5-azadC resulted in activation of CG genes in tumor cells, and, at a less extent, in normal somatic cells that do not express CG genes [284, 324, 325].

Finally, expression of CG genes has been shown to be induced by direct targeting of DNA methyltransferases using antisense oligonucleotides and siRNAs or by recombination-based knockout in human cancer cells [286, 322, 326]. Moreover, DNA hypomethylation at CG gene promoters has been associated with an altered histone H3 modification pattern, including increased levels of acetylated lysine 9 (H3K9ac) and dimethylated lysine 4 (H3K4me2), and decreased levels of dimethylated lysine 9 (H3K9me2) [322]. Furthermore, treatment of cancer cell lines with 5-azadC and TSA synergistically induced and activated *MAGE-A1*, *-A2*, *-A3*, and *-A12* gene expression, suggesting cooperative action of DNA methylation and histone deacetylation in mediating transcriptional repression of CG genes [320].

While much is known about the mechanisms maintaining DNA methylation of CG promoters, the mechanisms underlying their hypomethylation and CG gene activation in cancer are poorly elucidated. C. De Smet and A. Loriot have performed a seminal series of experiments with the *MAGE-A1* gene to address the following question: do cancer cells expressing *MAGE-A1* possess a permanent demethylation activity targeted to the promoter of this gene? [232, 323, 326]. To address this question, a transgene containing about a 6 kb region centered to TSS of *MAGE-A1*, has been methylated *in vitro* and stably transfected in MZ2-MEL cells. Despite a continuous expression of the endogenous hypomethylated *MAGE-A1*, melanoma cells have been unable to induce the demethylation of the newly integrated *MAGE-A1* transgene even after long-term post-transfection culture [323]. Of note, the same *MAGE-A1* transgene has been efficiently demethylated following transfection into mouse embryonic stem cells, which are characterized by a high level of methylation plasticity [327]. Since permanent DNA demethylation activity has not been observed in tumor cells, a past episode of demethylation activity has been proposed to initiate *MAGE-A1* hypomethylation in the course of tumor progression [232, 326] (Fig. 16C). In line with this hypothesis, the one-week episode of global demethylation activity induced by transient antisense-mediated depletion of the DNMT1, had sufficed to convert a methylated and inactive *MAGE-A1* transgene into a permanently hypomethylated and active one [326]. Interestingly, the extremities of the transgene have been progressively remethylated in the course of cell culture, while the *MAGE-A1* 5' region has remained stably hypomethylated, suggesting its protection from *de novo* methylation due to continuous action of DNA-binding and/or chromatin modifying proteins.

The causes and underlying molecular mechanisms of anticipated transient DNA demethylation activity occurring at some point in the course of tumor progression are currently unknown [232]. DNMTs are highly expressed in most cancer cells and somatic mutations or deletions of the corresponding genes have not been reported to occur in tumor tissues [232]. The potential functional links between DNA demethylation and tumor-related cellular processes like cellular senescence, hypoxia and changes in cellular differentiation states have been proposed. Understanding of these functional links could provide insight into the mechanisms leading to DNA hypomethylation changes observed in cancer cells [232].

A central question in the tumor biology of proteins encoded by CG genes is whether their aberrant expression can contribute to tumorigenesis. A particular protein might play a tumor-advantageous or tumor-detrimental role depending on its specific function and the timing of its reactivation in the course of tumor progression. Some proteins might promote proliferation, confer resistance to apoptosis or provide tumor cells with metabolic advantages.

Recent protein-protein interaction studies based on the yeast two-hybrid (Y2H) and pull-down purification approaches have revealed that MAGE proteins participate in multi-protein complexes that influence transcriptional activity of genes and stability of proteins. MAGE proteins possess a common MAGE homology domain (MHD) of about 200 amino acids, which can be evolutionarily traced back to yeast [328] and protozoa [329] and exhibits in humans 46% amino acid identity between all MAGE family members and 70% identity between twelve members of the MAGE-A family [302, 329-331]. MHD contains two winged-helix motifs connected by a flexible linker region [302, 330-332] (Fig. 17A). Interestingly, MHD has recently emerged to represent an important site of interactions with proteins harboring a common function, rather than a common motif [331, 332]. Thus, MHD domains of distinct MAGE family members have been found to recognize disparate and apparently unrelated domains of distinct proteins that harbor a really interesting new gene (RING) domain [328, 331, 333] (Fig. 17B, C, D). Initial crystal structure studies have indicated that two winged-helix motifs of MAGE proteins might adopt particular conformational changes upon binding to unique domains of distinct RING-containing proteins [331, 332]. In addition, genome-wide Y2H studies have identified novel interactions between MAGE proteins and RING-containing proteins [332, 334]. For instance, MAGE-A1 has been found to associate with TRIM31 (tripartite motif containing 31), but the functional relevance of this interaction has not been explored [332, 334]. RING-containing proteins represent one of the classes of E3 ligases, which confer specificity to ubiquitination by recognizing target protein substrates and mediating transfer of E1 enzyme-activated ubiquitin from an E2 ubiquitin-conjugating enzyme to the substrate [335] (Fig. 17B). E2-E3 complexes can either monoubiquitinate a substrate lysine or synthesize polyubiquitin chains assembled via different lysine residues of ubiquitin (such as K48 or K63) leading to diverse effects on the substrate. These effects range from proteasome-dependent proteolysis to modulation of protein function, structure, assembly, and/or localization [335]. Based on protein purification and *in vitro* binding assays, MAGE proteins have been suggested to enhance the ubiquitin ligase activity of RING-containing proteins [331] (Fig. 17B, C). Importantly, p53 stability has been shown to be reduced by MAGE-C2, and to a lesser extent by MAGE-A2, which enhanced p53 ubiquitination mediated by E3 ubiquitin ligase KAP1 (KRAB-associated protein 1), also known as TRIM28 [331] (Fig. 17D). The regulation of p53 stability by the MAGE-C2-KAP1 complex appeared to occur independently of MDM2 (mouse double minute 2), the well-characterized E3 ubiquitin ligase for p53 [331, 336]. Moreover, MAGE-C2 has been shown to bind UNCH2, an E2 ubiquitin ligase interacting with KAP1 [331].

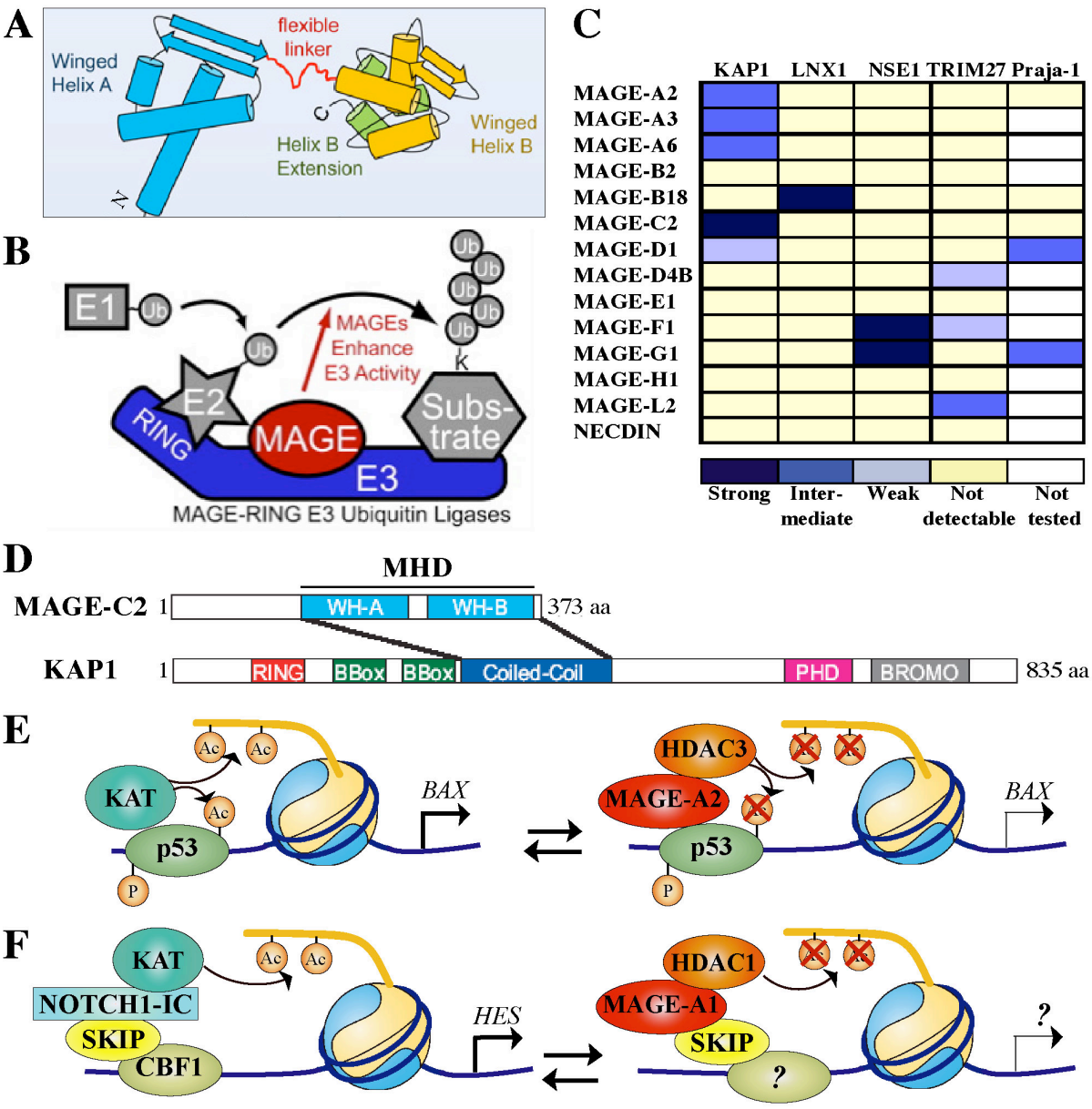


Figure 17. Some known functions of MAGE proteins. A. The conserved MAGE homology domain (MHD) comprises two winged-helix (WH) motifs connected by a flexible linker region. Adapted from Espantman and O'Shea, 2010 (330). **B.** MAGE proteins enhance the E3 ubiquitin ligase activity of RING (really interesting new gene) domain proteins. E3 ligases confer specificity to ubiquitination by recognizing target protein substrates and mediating transfer of E1 enzyme-activated ubiquitin from an E2 ubiquitin-conjugating enzyme to the substrate. E2 interactions with E3s and E1s are mutually exclusive. Adapted from Doyle et *al.*, 2010 (329). **C.** The relative binding activities of the indicated MAGEs and E3 RING domain ubiquitin ligases. Adapted from Doyle et *al.*, 2010 (329). **D.** MAGE-C2 and KAP1 (KRAB-associated protein 1) interaction. PHD, plant homeodomain; BROMO, bromodomain. Adapted from Doyle et *al.*, 2010 (329). **E.** A MAGE-A2/HDAC3 complex deacetylates p53 and histones surrounding p53-binding sites, thus weakening p53 transactivation function. KAT, lysine acetyltransferase; HDAC3, histone deacetylase 3; BAX, BCL-2 associated X protein. Inspired by Monte et *al.*, 2006 (338). **F.** MAGE-A1 counteracts transactivation mediated by intracellular NOTCH1 (NOTCH1-IC) recruited to its target promoters via interaction with SKIP (SKI-interacting protein), which binds CBF1 (C-promoter binding factor 1). MAGE-A1, via its interaction with SKIP, might recruit HDAC1 activity to promoters of target genes, whose identity is currently unknown. *HES*, Hairy and Enhancer of Split. Inspired by Laduron et *al.*, 2004 (339).

The biological relevance of observed interactions between MAGE proteins and RING-containing proteins is plausible, since different steps of gametogenesis are thought to require a high turnover of a number of proteins regulated by the ubiquitin system [337]. The appropriate turnover of p53 would be required in spermatogenesis, as transgenic male mice lacking p53 have decreased germ cell apoptosis and reduced fertility [338]. Moreover, the decreased stability of p53 mediated by MAGE-containing multi-protein complexes indicate a mechanism underlying p53 inactivation and, consequently, increased survival and resistance to chemotherapy in tumor cells harboring wild type p53 [331]. Consistently, association of KAP1 with MAGE-C2 and MAGE-A proteins has been suggested to suppress apoptosis in melanoma and colon cancer cell lines harboring wild type p53 [333, 339]. Reduced KAP1/p53 complex formation has been observed following siRNA-mediated reduction of MAGE-C2 or several MAGE-A proteins [333]. Along with its E3 ubiquitin ligase activity, KAP1 has been involved in multiple mechanisms of gene silencing, including long-range transcriptional repression in cooperation with KRAB domain-containing zinc finger proteins and action as a scaffold protein within HDAC-containing complexes [314]. Thus, KAP1 scaffold function for p53-HDAC1 interaction has been associated with deacetylation and reduced transcriptional activity of p53 [336]. Accordingly, the evidence has been provided that MAGE-A2/HDAC3 could function as a p53-repressing complex in melanoma cell lines [340]. MAGE-A2-mediated recruitment of HDAC3 resulted in hypoacetylation of p53 and of histones H3 surrounding its binding sites, thus weakening the p53-dependent transactivation function [340] (Fig. 17E). In melanoma cell lines harboring wild type p53 and treated with DNA-damaging agents, MAGE-A2 expression has provided survival advantage [340].

Other interactions of MAGE proteins shed light on their potential function [287]. Thus, S. Laduron *et al*. identified the transcriptional regulator SKIP (SKI-interacting protein) as a MAGE-A1 binding partner in a Y2H assay [341] (Fig. 17F). The association of the two proteins was shown in co-immunoprecipitation assay, while the interacting region was mapped outside the MHD in the C-terminal part of MAGE-A1 [341]. SKIP is an adaptor protein that connects DNA-binding proteins to either transcriptional repressors or activators and participates in signaling pathways mediated by vitamin D, retinoic acid, estrogens, glucocorticoids, TGF-β and NOTCH1 [341]. In gene reporter assays, MAGE-A1 was found to act as transcriptional repressor by disrupting SKIP-dependent transcriptional activation mediated by intracellular NOTCH1 [341] (Fig. 17F). MAGE-A1 is thought to repress transcription via its association with HDAC1 [341]. A Y2H assay did not reveal the association of MAGE-A1 with p53 (E. De Plaen, personal communication). Accordingly, targeting of MAGE-A1 by specific siRNA in melanoma cell line harboring wild type p53 has not resulted in apoptosis induction [333]. The physiological target genes of MAGE-A1-mediated transcriptional repression have not been found to date. The pro-apoptotic activity of MAGE-A4 has been contributed to its cleaved C-terminal part, which has interacted with Miz-1 protein and inhibited *p21* transcription [342].

In contrast to CG genes encoded by the X chromosome, far less is known about the molecular mechanisms regulating the expression of autosomal CG genes [287]. Recently, epigenetic mechanisms have been found to regulate the expression of *CTCFL* (*CTCF*-like), which was first isolated among GIPs (genes induced by p53) in a colon sarcoma cell line [343-345]. *CTCFL*, also known as *BORIS* (Brother of the Regulator of Imprinted Sites), has been described as a paralog of CTCF (CCCTC-binding factor), which plays a key role in the regulation of genomic imprinting [346, 347]. While BORIS is predominantly expressed in spermatocytes, CTCF is expressed in post-meiotic spermatids and exhibits ubiquitous expression in somatic cell lineages in a manner similar to a housekeeping gene [346, 347]. Recently, *BORIS* expression has been found in human oocytes and 4-cell embryos [348]. Data about *BORIS* expression in tumors are controversial. When assessed by RT-PCR for 40-45 amplification cycles, *BORIS* expression has been found in the vast majority of tumor cell lines and in breast, prostate and colon cancer tissues [349]. However, when measured quantitatively, the low levels of *BORIS* expression observed in prostate cancer tissues were not statistically different from those in normal prostate tissues [350]. Further, *BORIS* transcripts have not been detected in eight primary breast tumors and found in only 1 of 12 breast cancer cell lines [351]. These data were inconsistent with an earlier report describing *BORIS* expression in 7/8 breast cancer cell lines and 11/12 breast tumors [349]. Quantitatively evaluated *BORIS* expression has been extremely low or undetectable in normal ovary samples and higher in many epithelial ovary cancer tumors, although the difference has not reached statistical significance [345]. High incidence (77%) of significant BORIS expression has been demonstrated in uterine tumors [352].

There is ample evidence that *BORIS* expression is regulated by epigenetic mechanisms. First, the level of *BORIS* expression in lung and epithelial ovarian cancer tissues, as well as in testis has inversely correlated with the extent of promoter methylation [344, 345, 350, 353]. Second, *BORIS* expression has been induced in normal fibroblasts and numerous tumor cell lines by treatment with 5-azadC [344, 345, 350, 353]. Finally, *BORIS* expression in *DNMT*-deficient colon cancer cells has directly correlated with promoter DNA hypomethylation and an altered histone H3 modification pattern in a region encompassing the TSS of *BORIS* [345].

BORIS function is linked to that of its paralog CTCF. Both proteins possess, within their central part, a suite of 11 zinc fingers with similar DNA-binding capacity [354] (Fig. 18A). However, BORIS and CTCF differ significantly in their amino- and carboxy-termini, implying that they might interact with different protein complexes leading to differential function at binding sites and to distinct DNA-binding behavior [354]. If *CTCF* knockout has resulted in embryonic lethality [355], *BORIS* knockout mice are viable and fertile [356]. However, male mice have reduced testicular size accompanied by presence of multinucleated cells and significantly delayed meiosis. This phenotype could rely on reduced expression of testis-specific isoform of cerebroside sulfotransferase (CST), which is transcriptionally regulated by BORIS and harbor unmethylated promoter in testis of *BORIS* knockout mice [356].

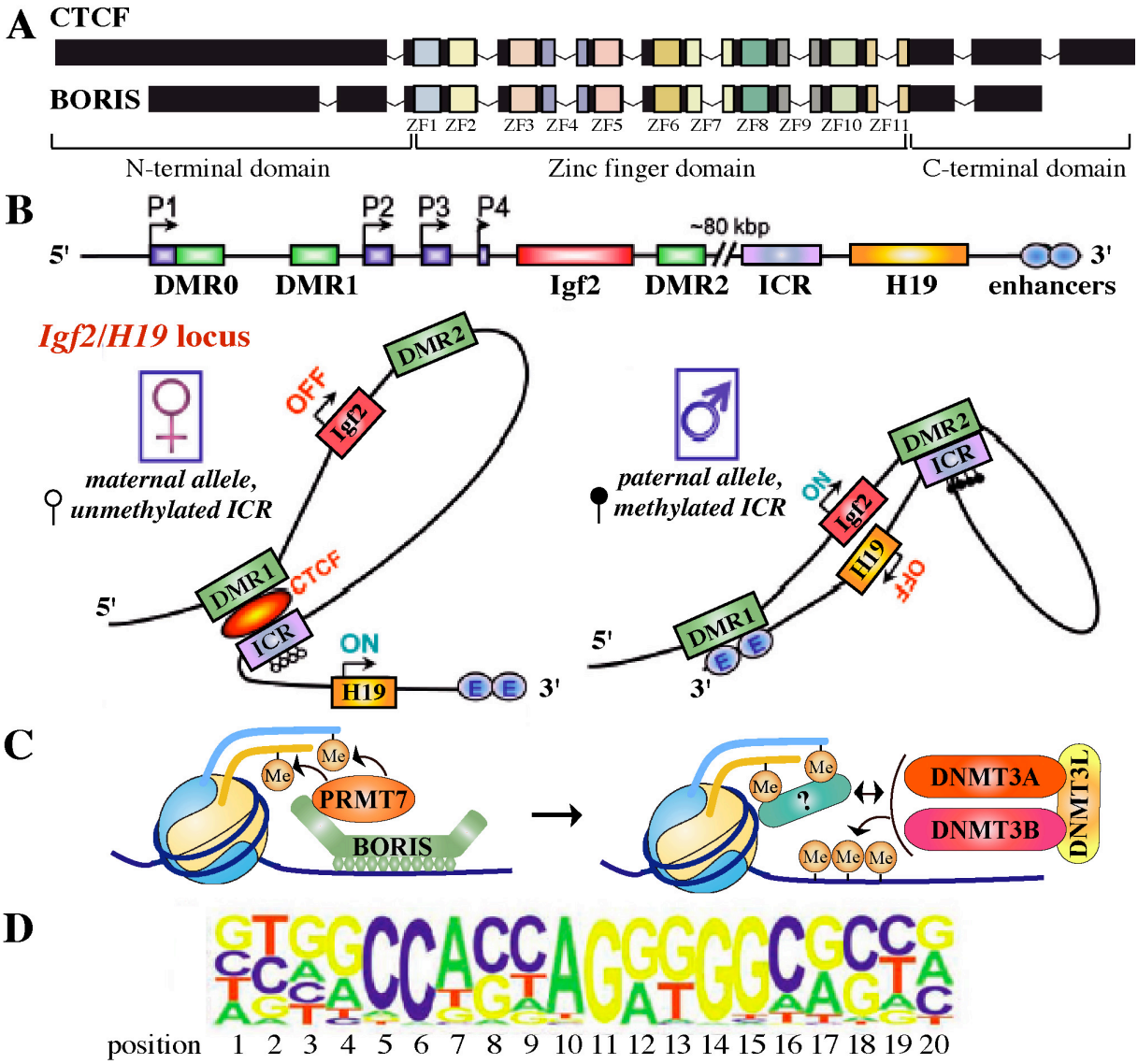


Figure 18. CTCF and BORIS function. A. Structure of CTCF and BORIS transcripts, which encode a similar zinc finger (ZF) domain, but different N- and C-terminal domains. Adapted from Hore *et al.*, 2008 (352). **B.** *Igf2/H19* imprinted locus is shown with the *Igf2* and *H19* genes and the differentially methylated DNA regions (DMR0, 1, 2, and the Imprinting Control Region, ICR). The arrows above the blue boxes show alternative promoters. Differential transcription of *Igf2* and *H19* from the maternal and paternal alleles has been described. While *Igf2* encoding a growth factor is expressed uniquely from the paternal allele, the non-coding RNA *H19* is expressed solely from the maternal allele. Both are widely expressed during embryonic development and postnatally downregulated in most tissues. On the maternal allele, the loop is mediated by CTCF binding to the unmethylated ICR and to another differentially methylated region, DMR1, upstream of the *Igf2* gene, blocking enhancer (E) access to the *Igf2* promoter and resulting in *Igf2* silencing. On the paternal allele, methylation of ICR abrogates CTCF binding and insulator activity allowing activation of the *Igf2* promoter by the enhancer. Adapted from Zlatanova and Kaiafa, 2009 (356).
C. Model of *H19* ICR methylation. The binding to ICR is assured by the zinc finger domain of BORIS, while its N-terminal part recruits PRMT7 (protein arginine methyltransferase 7), resulting in methylation of arginine residues in histone tails. Following disengagement of the BORIS-PRMT7 complex, *de novo* DNMTs might be recruited by a bridging protein to the methylated histones and methylate adjacent CpG sites in the *H19* ICR. Modified from Jelinic et al., 2006 (357).
D. DNA logo representing the CTCF binding motif. Height of each letter represents the relative frequency of occurence of the nucleotide at each position. Adapted from Kim *et al.*, 2007 (359).

One example of how BORIS and CTCF may act differently is genomic imprinting, a form of epigenetic regulation of about 100 mammalian genes, which are imprinted (marked) with their parental origin [357]. In contrast to the majority of biallelic genes, which display expression from both paternally and maternally inherited alleles, the expression of imprinted genes is non-randomly limited to one allele. For some of these genes the silent allele is always maternally inherited, while for the others allelic exclusion is always of paternal origin [357]. Imprinted genes are often found in clusters that can span more than 1 Mb and are jointly regulated through an imprinting control region (ICR), whose methylation status determines the transcriptional activity of genes within a cluster [357]. Clusters can be divided into two types. The majority of the imprinted clusters are controlled by ICRs that are methylated during oogenesis on the maternally inherited chromosome and usually overlap with promoters for antisense transcripts. In contrast, few clusters are under the control of ICRs methylated during spermatogenesis [357]. The mouse *Igf2/H19* locus is the best characterized of them [358] (Fig. 18B). If CTCF acts to "interpret" the methylation mark of this ICR in somatic tissues, BORIS appears to be essential for the establishment of differential methylation within this locus during spermatogenesis [354]. The current model contends that BORIS binds the ICR of the *Igf2/H19* locus and recruits via its amino-terminal region protein arginine methyltransferase 7 (PRMT7), which methylates nearby histones [359] (Fig. 18C). The histone methylation can then serve as a recruitment signal for the *de novo* DNMTs methylating the CpGs of the *Igf2/H19* ICR in the male germline cells, leading thereby to the activation of *Igf2*. In line with this model, BORIS has been shown to preferentially bind *in vitro* the paternal ICR of the *IGF2/H19* locus on human chromosome 11 [360]. In contrast to CTCF binding only to unmethylated DNA, BORIS binding activity is generally independent of the DNA methylation status [360]. CTCF has been proposed to have duplicated 350 million years ago to give rise to BORIS, which progressively acquired germline-specific expression and specialized function in genomic imprinting in early mammals about 210 million years ago [354].

Besides playing a central role in genomic imprinting, CTCF exerts many other functions, including context-dependent promoter activation or repression, binding to boundaries between active and repressive chromatin, insulator activity, formation of intrachromosomal loops and interchromosomal interactions and global nuclear organization via demarcation of transcriptionally silent lamina-associated domains at the periphery of the nucleus [347]. Consistent with a global role for CTCF in chromatin organization, thousands of CTCF-binding sites have been identified throughout the genome by ChIP-on-chip, ChIP-Seq and *in silico* experiments and the consensus DNA-binding sequence of about 20 bp has been established [347, 361] (Fig. 18D). A comparative analysis of ChIP-Seq data in different cell types revealed 40-60% overlap in CTCF-binding sites across cell types, suggesting that the remaining binding sites may be relevant for cell type-specific gene expression [362]. Aberrant BORIS expression in somatic malignancies might interfere with some CTCF functions.

III. Immune response in cutaneous melanoma

III.1 Dual role of the immune system in melanomagenesis

Substantial evidence indicates that the tumor-immune system interactions contribute to the fate of benign and malignant melanocytic cells in the course of melanomagenesis. This evidence is mostly based on the studies of tumor antigen-specific immune responses [5], of the infiltration of benign and malignant melanocytic lesions with immune cells [363, 364] and of the melanoma incidence in transplant recipients who received immunosuppressive treatment. An elevated melanoma risk confined to periods of transplant function in kidney transplant recipients has returned to baseline level after reduction or withdrawal of immune suppression, indicating that the fully functional immune system may prevent melanoma development [365].

Infiltration of immune cells is a well-documented observation in most cancers, including melanoma [364]. The dual role of the immune system, which is thought to eradicate tumor cells mostly at the early stages of tumorigenesis, but ultimately appears to promote tumor progression, likely reflects the complex interactions of innate (macrophages, dendritic cells, natural killer cells) and adaptive (T and B lymphocytes) immune cells with tumor cells and non-transformed stromal cells (fibroblasts, endothelial cells, keratinocytes) in the tumor environment [366]. It is the abundance of these different cell types and their interactions via direct contacts and the production of soluble factors (i.e. cytokines) that apparently dictate in which direction the immune balance is tipped at different points along the path of tumor progression. Thus, in established tumors, including melanoma, the antitumor immunity and tumor-promoting inflammation likely coexist, but the net effect of the immune system appears to be the stimulation of tumor progression [366].

The most frequently found immune cells within the tumor environment are macrophages and T cells. Macrophages can promote specific immunity by inducing T cell recruitment and activation. Despite this, the presence of macrophages within the established tumors has been accompanied by tumor-promoting functions, including secretion of growth factors, cytokines and matrix-degrading enzymes [367]. Consistently, the density of macrophages in melanoma lesions has correlated with increased angiogenesis, tumor invasion and poor prognosis [368]. Similar to macrophages, the tumor-promoting functions of T lymphocytes are mediated by cytokines. However, cytokines often play a pleiotropic role during tumor progression and mediate antitumor functions of particular subsets of T cells [366]. Among T cells harboring αβ heterodimeric T cell receptors (TCR), $CD8^+$ T cells are thought to be the major effectors of antitumor immunity due to their engagement in tumor killing as differentiated CTLs (Fig. 19A). CTLs lyse tumor cells via granule exocytosis pathway (perforin, granzyme B) and death receptor pathways, such as the Fas ligand pathway, which triggers apoptotic cell death of target cells expressing the cognate receptor Fas [369] (Fig. 19A).

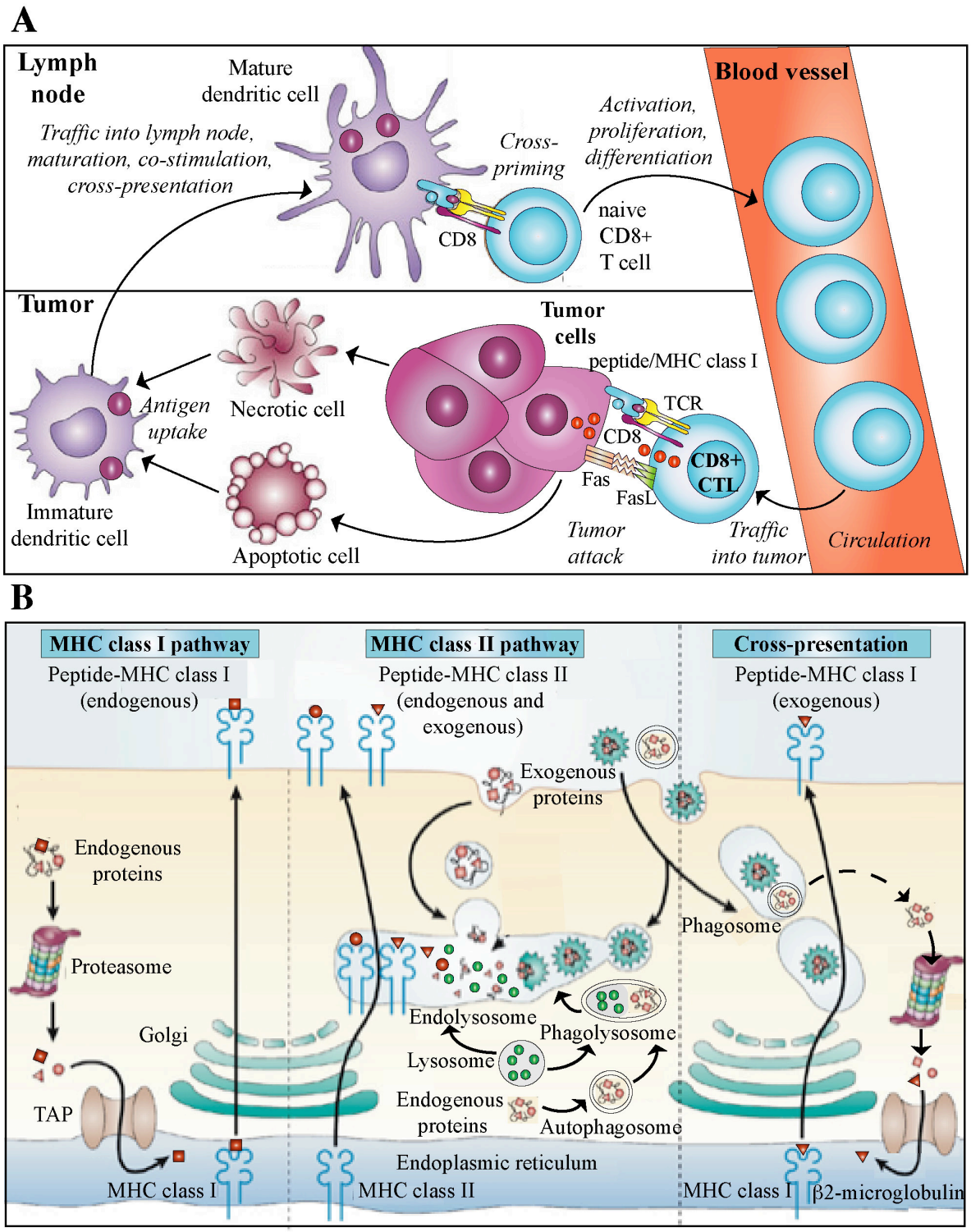


Figure 19. Antitumoral immune response. A. Schematic representaion of an effective anti-tumor immune response. The first step involves cross-presentation of antigens released from tumor cells. Dendritic cell activation and maturation is required to turn cross-presentation into a cross-priming of naive CD8+ T cells, a process co-stimulated by CD4+ T cells. Following steps are expansion, differentiation and circulation of anti-tumor CTLs, their entry into the tumor site and attack of the tumor by cytotoxic mechanisms, such as FasL (Fas ligand)-mediated and granule exocytosis-mediated apoptosis. Modified from Kurts *et al.*, 2010 (379). **B.** Crosstalks occuring between the MHC class I and class II antigen processing and presentation pathways, which generally process peptides derived, respectively, from endogenous and exogenous proteins. TAP, transporter associated with antigen processing. Modified from Villadangos and Schnorrer, 2007 (380).

Different subsets of $CD4^+$ T cells harbor either antitumor or protumor functions. Thus, Th1 (T helper 1) cells (by virtue of IFN-γ production) and in some instances Th17 cells (via production of IL-17A cytokine) provide important help that boosts cytotoxic immunity, whereas regulatory T cells (Treg) and Th2 cells generally appear to suppress antitumor immune responses via production of immunosuppressive factors [364, 366].

$CD8^+$ and subsets of $CD4^+$ T cells have been found in various proportions and isolated from tumor infiltrates, lymph nodes and the blood of melanoma patients [364, 370]. The proportions of different T cell subsets, their functional status and secretory phenotype likely influence the overall functional outcome of tumor-immune system interactions and, therefore, prognosis [364]. Generally, the higher frequency of tumor-infiltrating lymphocytes (TILs) and diffuse pattern of their distribution in VGP melanoma and regional lymph node metastases have been associated with a better prognosis [364, 371]. Immunosuppressive Treg cells are found in melanoma environment since early stages of tumor development, with higher proportions in later than in earlier melanoma stages [370]. Surprisingly, spontaneous regression usually observed in local areas in up to 20-30% of primary (especially thin) melanomas generally indicates a worse prognosis and has been associated with an almost 10-fold higher probability of regional lymph node involvement [363]. To explain this counterintuitive phenomenon, it has been hypothesized that melanoma cells spread to regional lymph nodes could increase the immune response against primary melanoma, but that the immune cells present within partially regressing lesions may not be able to destroy all malignant cells [363]. Even widely disseminated metastatic melanoma may in rare cases undergo spontaneous regression accompanied by a diffuse infiltrate of immune cells [372]. Consistently with the proposed role of the immune system in the control of the disseminated melanoma, $CD8^+$ T cells have prevented the outgrowth of metastatic melanoma cells in the bone marrow and lymph nodes of transgenic mouse melanoma model [373].

In spite of the ample evidence of antitumoral immune responses, in most established tumors containing inflammatory infiltrates the tumor-promoting effects of chronic inflammation appear to dominate [374]. Chronic cancer-associated inflammation, often described as “smoldering” to indicate a local inflammatory process that is subclinical, is mediated by immune cells, but also by tumor and stromal cells [374]. It is associated with the establishment of an immunosuppressive tumor environment, which is not only permissive but also supporting tumor progression due to production of growth and angiogenic factors by different cell types [374, 375]. The connection between inflammation and cancer is schematically represented by two pathways differentially involved in distinct types of cancer: an extrinsic pathway, driven by inflammatory conditions (viral and bacterial infections, autoimmune diseases) that increase cancer risk and/or progression, for instance in gastric cancer, and an intrinsic pathway, in which genetic events underlying neoplasia contribute to the establishment of the inflammatory tumor environment, which, in turn, promotes genetic instability [374, 375].

In melanoma, no epidemiological evidence has been provided for the involvement of the extrinsic pathway in tumorigenesis, whereas increasing evidence supports the implementation of the intrinsic pathway. Thus, the senescence-related secretory phenotype associated with the $BRAF^{V600E}$ mutation has been found to include the production of the senescence inducer Insulin-like Growth Factor Binding Protein 7 (IGFBP-7) and inflammatory cytokines, principally IL-6 and IL-8 [376]. These mediators may contribute to local immune responses once senescent tumor cells begin to die [377]. Furthermore, *in vitro* evidence has been provided for the requirement of constitutive ERK and STAT3 signaling for the secretion by melanoma cells of IL-6, IL-10 and VEGF, which exhibited immunosuppressive activity by preventing the maturation of dendritic cells [378].

In parallel to this tumor cell-intrinsic contribution to inflammation, dying tumor cells can trigger the inflammatory response [377]. Inflammatory mediators released by necrotic cells include danger-associated molecular patterns (DAMPs) such as ATP, nucleic acids, heat shock proteins (Hsp70), high mobility group box-1 (HMGB1) protein and others, which contribute to efficient antigen presentation by dendritic cells (DCs) and activation of $CD8^+$ T cells [377]. Apoptotic tumor cells are considered as less immunogenic than necrotic cells and in some cases induce tolerance when engulfed by DCs [377]. In addition, autophagy of cytoplasmic material (proteins, organelles), which can be induced by hyperactivation of $BRAF^{V600E}$, might stimulate the release of HMGB1, suggesting another link between intrinsic tumor cell events and the immune response [377, 379]. Moreover, autophagosomes are efficient antigen carriers, which can be released by dying tumor cells and engulfed by DCs, thus promoting antigen presentation [380].

III.2 Activation of T cells and recognition efficiency

Naïve $CD8^+$ T cells need to be activated initially, or "primed", by professional antigen-presenting cells (APCs), generally DCs, to become cytotoxic differentiated effectors, $CD8^+$ CTLs [381] (Fig. 19A). DCs are endowed with the capacity to acquire exogenous antigenic peptides within tumor tissues, to respond to danger signals, such as DAMPs, and subsequently to process and cross-present antigenic peptides bound to major histocompatibility (MHC) class I molecules to naïve $CD8^+$ T cells to promote their activation and clonal expansion [382] (Fig. 19B). In addition, $CD4^+$ Th1 cells recognizing antigen presented on MHC class II molecules license DCs, through co-stimulation, to promote $CD8^+$ T cell activation [383]. The binding between peptide-MHC complexes on the surface of DCs and cognate TCRs on $CD8^+$ and $CD4^+$ T cells occurs in a MHC-restricted, antigen-specific manner, whereas CD8 and CD4 co-receptors enhance this binding. Other receptor-ligand interactions mediated by co-stimulatory and adhesion molecules promote productive activation of T cells [381]. Whereas MHC class I molecules have a broad cellular distribution,

including melanocytes and their tumor counterpart, MHC class II molecules are normally expressed by professional APCs, such as DCs, macrophages and B lymphocytes, and become aberrantly expressed by melanoma cells [384].

The tumor draining lymph nodes are considered as main sites where naive $CD8^+$ T cells have the opportunity to survey DCs migrated from the tumor site [381]. After appropriate activation, antigen-primed $CD8^+$ T cells cell undergo proliferation and differentiation. Their progeny includes effector cells, which display effector CTL functions, such as release of cytokines and cytolysis, as well as memory $CD8^+$ T cells, which can generate a new wave of effector cells after re-encounter of the antigen [383]. $CD4^+$ Th1 cells appear to promote not only priming of tumor-specific $CD8^+$ T cells, but also their entry from the blood circulation to the tumor sites and secondary encounter with antigens presented on the surface of tumor cells [383]. Importantly, observations made in mouse models of antitumoral immune response to melanoma suggest that tumor itself may be a site of activation of naive tumor-specific T cells [385, 386]. Indeed, tranferred naïve $CD8^+$ T cells have appeared to infiltrate the tumor, to be primed by DCs and by tumor cells themselves, to proliferate and acquire an effector phenotype [385]. In addition, transfer of small numbers of naïve tumor-reactive $CD4^+$ T cells has been followed by their expansion, acquirement of cytotoxic activity and tumor rejection [386].

In melanoma patients, frequencies, specificities and functional status of circulating and intratumoral antigen-specific T cells have been evaluated either *in vitro*, after restimulation and amplification, generally under clonal conditions, or directly *ex vivo*, without any antigen-specific stimulation [5, 387, 388]. Following restimulation of T cells *in vitro* with autologous irradiated melanoma cells, clonal population of CTLs have been obtained that are able to specifically recognize via clonotypic TCR and subsequently lyse the same tumor cells as that used for stimulation, but also other tumor cells presenting the same antigenic peptide-HLA complex [5]. In some melanoma patients, the frequencies of tumor antigen-specific CTLs among blood $CD8^+$ T cells have been found to be higher than the background frequencies of precursor $CD8^+$ T cells recognizing a specific antigen in healthy individuals [5]. Moreover, in some patients tumor antigen-specific CTLs have been found to be highly enriched in metastases, whereas bulk populations of TILs derived from metastases have exerted cytolytic activity on fresh tumor cell preparations [5].

The failure of the immune system to completely eradicate the tumor is thought to rely on inadequate (quantitatively and/or functionally) induction of the antitumoral immune response and/or a deficient effector phase. Thus, deficient maturation of DCs, impaired effector functions of T cells and an immunosuppressive tumor environment generated by immune, stromal and tumor cells have been incriminated [9, 388, 389]. Scarce expression of proteins that give rise to tumor antigens might affect both the induction and effector phases of the antitumoral immune response. Thus, insufficient quantity of antigen engulfed by DCs and subsequently cross-presented to naïve $CD8^+$ T cells would affect their priming, whereas

inefficient presentation of antigenic peptide-MHC class I complexes on the surface of the target tumor cells would result in escape of tumor cells from the recognition and lysis by $CD8^+$ CTLs. Besides, inefficient antigen presentation by tumor cells may rely on mechanisms affecting different components of the antigen-processing and presenting machinery. Thus, the repertoire of the MHC class I-restricted antigens presented by melanoma cells can be either broadened or restrained due to differential expression of different proteasome types (standard, intermediate or immunoproteasome) and/or other proteolytic enzymes, such as insulin-degrading enzyme [390, 391]. Further, the components of MHC class I molecule, the light chain β2-microglobulin and the heavy chain represented by HLA-A, -B or -C molecule, exhibit either genetic alterations or downregulation in 16-55% of melanoma lesions, whereas TAPs (transporters associated with antigen processing) that transport antigenic peptides into the ER are estimated to be downregulated in up to 88% of melanoma lesions [384, 392-394].

The quantity of peptide-MHC complexes influences the intrinsic affinity of the TCR/peptide-MHC interaction and is one of the major parameters determining the recognition efficiency of T cells, also referred to as functional avidity [395]. The other parameters include the expression levels and spatial distribution of TCRs, CD8 co-receptors, co-stimulatory and adhesion molecules [387, 395-397]. Recognition efficiency of T cells reflects the cumulative effect of these parameters and is defined as the functional reactivity to peptide-MHC ligands [395]. It is evaluated by a several functional readouts, including the capacity of T cells to secrete effector cytokines (mainly IFN-γ and TNF-α), to lyse target cells in an antigen-specific manner or to express cell surface markers, such as CD107a, a membrane glycoprotein of cytotoxic granules [387, 395]. IFN-γ production is the most commonly used variable to detect the reactivity of CTLs against antigen-presenting targets, although cytokine-producing $CD8^+$ T cells do not necessarily harbor lytic activity and vice versa [398, 399]. The number of peptide-MHC complexes required for efficient CTL response is not definitely known and likely differs for particular tumor cell-CTL interaction. A very small proportion of the bulk of peptide-MHC complexes on the surface of the target cell is antigenic for a given TCR, and T cells appear to be sensitive to low quantities of specific antigen, with a threshold phenomenon being frequently observed [400, 401]. Thus, in a series of *in vitro* experiments, a minimum of three peptide-MHC complexes has been required to induce target cell death [401]. Moreover, it has been proposed that FasL-mediated cytotoxicity may be predominant at the lowest antigen levels (3-30 peptide-MHC complexes) and that higher antigen levels may promote the perforin-mediated transfer of granzymes [401].

In melanoma, the recognition efficiency of T cells has been extensively studied *ex vivo* and *in vitro*, mainly in the context of interactions between antigen-specific CTL clones and melanoma cells harboring cognate peptide-HLA complexes [5, 388, 401]. The origin of antigens presented on the surface of melanoma cells is highly diversified what reflects multiple genetic and epigenetic events that take place in melanoma cells in the course of tumorigenesis.

III.3 Antigens encoded by genes having altered expression or integrity in melanoma

The molecular characterization of a melanoma antigen encoded by *MAGE-A1* showed for the first time the antitumoral specificity of autologous T cells found in melanoma patients [6]. The antigen is a nonapeptide presented to CTL by HLA-A1 molecule [402]. More than three hundred peptide/HLA combinations efficiently processed and presented by human tumor cells to T cells have been described since then [403]. Many of them are induced or acquire antigenic potential due to the accumulation of genetic and epigenetic changes within tumor cells in the course of tumorigenesis. Thus, aberrant expression of *MAGE-A1*, and that of many other members of the cancer-germline (CG) group of genes, relies on the hypomethylation of their respective promoters. The protein products of CG genes give rise to numerous epitopes recognized by T cells in the context of both class I and class II HLA molecules in melanoma [5]. Quantitative analysis of *MAGE-A1* expression in a set of melanoma cell lines indicated that a threshold of about three mRNA molecules per cell was required for CTL-mediated cytolysis [404]. When quantification of antigen/HLA complexes on the cell surface was performed for a LAGE-1/LAGE-2$^{NY\text{-}ESO\text{-}1}$ -derived antigenic peptide presented by HLA-A2, the level of 10-50 complexes was found to be sufficient for efficient CTL recognition evaluated by IFN-γ production [405]. Importantly, antigens encoded by CG genes are functionally tumor specific since they derive from proteins not expressed in normal cells, except for germline and trophoblast cells, which exhibit no or weak expression of HLA molecules [5].

Some genes that are overexpressed in tumor cells relative to normal cells give rise to increased amount of antigens presented by HLA molecules on the tumor cell surface, leading to augmented T cell recognition. One example is *PRAME* (preferentially expressed antigen in melanoma) showing high prevalence of expression in melanoma lesions (about 90%) and expressed at about 100-fold higher levels in melanoma cell lines than in normal cells [406, 407].

The expression of mRNA and proteins encoded by particular families of endogenous retrovirus (HERV) sequences, which have been integrated into the genome due to ancient germline infections by exogenous retroviruses, has been demonstrated in melanoma [408]. The antigenic peptide encoded by a short ORF in the *env* (envelope) region of a *HERV-K-MEL* transcript has been found to be targeted by CTLs in a melanoma patient [409]. Recent epidemiological studies revealed reduced risk of melanoma associated with administration in early childhood of bacille Calmette-Guerin and/or vaccinia virus vaccines, which harbor antigenic determinants homologous to antigen encoded by *HERV-K-MEL*, indicating a potential anti-melanoma activity of cross-reactive T cells stimulated by vaccines [408].

Diverse aberrant protein products are detected on the surface of tumor cells thanks to the filter of the immune system discriminating “self” from “non-self” or “abnormal self”. About 20% of tumor antigens result from alterations in transcription (activation of intra-intronic promoters with sense or anti-sense transcription, transcription of pseudogenes),

translation (incompletely spliced messages, translation of small alternative open reading frames) or post-translational changes (deamidation, proteasome-dependent peptide splicing) [5].

Somatic point mutations in a number of genes have been demonstrated to give rise to new antigenic epitopes [410, 411]. In melanoma, *BRAF*, *NRAS*, *CTNNB1* (β-catenin) and *CDK4* are examples of genes whose genetic alterations are relevant for melanomagenesis and for immune recognition. BRAF and NRAS mutations that lead to constitutive activation of the ERK pathway have resulted in presentation of mutated antigens recognized by T cells [412, 413]. A serine to phenylalanine substitution at position 37 of β-catenin, which leads to protein stabilization, has generated a peptide recognized by TILs of a melanoma patient [414]. In another patient with metastatic melanoma, autologous CTLs have recognized a peptide derived from CDK4 harboring a mutation that confers resistance to p16 inhibition [415].

In a recently performed screen for mutations in coding regions of five members of MAGE family, about one third of melanoma tissue samples harbored mutations in one or more MAGE genes with the frequency of mutations in individual MAGE genes ranging from 6% in *MAGE-A1* to 16% in *MAGE-C1* [416]. Notably, 6 of 8 missense mutations identified in MAGE-A1 affect known antigenic peptides potentially altering their antigenicity [416].

III.4 Antigens encoded by melanocyte differentiation genes and autoimmunity

Surprisingly, CTL clones recognizing autologous melanoma cell lines have been found to recognize HLA-matched allogeneic melanocytes, suggesting presentation of common antigens [417]. Indeed, expression of *TYR*, *MLANA/MART-1*, *gp100/ SILV*, *TYRP-1* and *DCT/TYRP-2* genes encoding melanosomal proteins gives rise to the processing and presentation by HLA class I and II molecules of so-called differentiation antigens [5]. Differentiation antigens are self-antigens, thereby their targeting by the immune system should be avoided due to the central tolerance mechanism, which leads to deletion of T cells with high avidity to self-peptide/HLA complexes and positive selection of low avidity T cells in the thymus. In addition, self-reactive T cells can be tolerized in periphery following an encounter with an antigen in the absence of an activating stimulus and through the actions of regulatory cells. Unexpectedly, in the blood of over 70% of HLA-A2 healthy individuals, the frequencies of $CD8^+$ T cells recognizing HLA-A2-binding peptides corresponding to amino acids 27-35 and 26-35 of Melan-A have revealed to be about 100-fold higher than generally observed frequencies of naive single epitope-specific $CD8^+$ T cells [418]. Sequence homology of a Melan-A peptide sequence with a broad range of self-derived peptides is proposed to result in cross-recognition of these peptides in the context of HLA-A2 molecules in the thymus, leading to positive selection of a large pool of low avidity Melan-A-reactive T cells with diverse TCR repertoire [418]. These Melan-A-reactive $CD8^+$ T cells remain naïve and the limited locations of melanocytes might explain the immunological ignorance in the periphery [418].

In marked contrast, T cells targeting melanocyte differentiation antigens can be activated in melanoma and in rare autoimmune disorders accompanied by local melanocyte loss. The frequencies of circulating T cells directed to Melan-A, tyrosinase and gp100 epitopes are significantly higher in melanoma patients than in healthy individuals [419]. Thus, $CD8^+$ T cells recognizing HLA-A2-restricted Melan-A_{27-35} and tyrosinase$_{368-376}$ antigens were estimated to represent 0.14-0.5% and 0.19-2% of total circulating $CD8^+$ T cells, respectively [420]. In single-cell suspensions derived from melanoma metastases, the frequencies of Melan-A-reactive T cells reached up to 16% of total $CD8^+$ T cells [418]. Importantly, circulating T cells targeting differentiation antigens, but not antigen-specific TILs analyzed *ex vivo*, displayed cytotoxic and cytokine-producing activities, indicating that T cells that had a history of antigen exposure failed, however, to exert their effector function within melanoma tissues [388, 419]. Nevertheless, following appropriate *ex vivo* stimulation, TILs could recover functional competence, suggesting the reversibility of functional tolerance at least in some cases [388].

The overcome of natural immune tolerance to melanocyte differentiation antigens is thought to be the pathogenic basis of melanocyte destruction in the skin of vitiligo patients and in the eyes of patients with such potentially blinding disorders as Vogt-Koyanagi-Harada (VKH) disease and sympathetic ophthalmia [420-422]. Vitiligo, which affects approximately 0.5-1% of the population worldwide, is a skin depigmentation disorder characterized by local melanocyte loss from the dermo-epidermal junctions in association with the presence of cellular immune infiltrate in the affected skin [420]. At the edge of the vitiligo lesions, melanocytes have been found to express reduced levels of MITF and KIT proteins [423]. Some similarities have been observed between the antitumor immunity in melanoma and the autoimmunity in vitiligo, since $CD8^+$ T cells directed to differentiation antigens and displaying a memory/effector phenotype have been found in both conditions at similar frequencies [420]. However, the susceptibility of normal and tumor cells to immune responses may differ because of different tissue structure and/or inflammatory environment. Moreover, the quality of the cytotoxic responses seems to differ in the two pathologies [424]. In vitiligo patients, Melan-A-specific $CD8^+$ T cell populations have shown about six-fold higher TCR affinity to antigen, whereas particular $CD8^+$ T cell clones have exhibited higher lytic activity and capacity for IFN-γ production in response to HLA-matched melanoma cells [424]. CD8+ T lymphocytes recognizing differentiation antigens were also found in melanoma-associated vitiligo, which occurs in approximately 3% of melanoma patients [363, 425]. Melanoma-associated vitiligo has similar histological picture as classical vitiligo and represents a favorable prognostic factor in advanced melanoma [363, 425]. Thus, immune reactivity to melanocytes in vitiligo may be regarded as the effective, but exaggerated variant of an immune response often ineffective in melanoma.

III.5 Major immunotherapeutic approaches in melanoma

Immunotherapy is an important modality in the treatment of patients with malignant melanoma [5, 7, 8]. Various immunotherapeutic approaches have been developed and can be divided into active and passive. Active approaches aim to induce or enhance endogenous antitumoral immune responses. To this end, various therapeutic vaccination strategies including administration of antigenic peptides, DNA or tumor cells (from the host or another patient) simultaneously with adjuvants stimulating the specific T cell response have been used in clinical studies [5, 7]. The identification of tumor antigens recognized by autologous $CD8^+$ CTLs allowed the use of defined antigens in vaccination of melanoma patients. Clinical trials using antigenic peptides encoded by genes of the *MAGE* family, particularly *MAGE-A3* and *MAGE-A1*, and by melanocyte differentiation genes (*MLANA*, *TYR*, *gp100*) have demonstrated low toxicity, the generation of increased amounts of $CD8^+$ CTLs targeting melanoma antigens, whereas clinical responses have been observed in up to 10 % of metastatic melanoma patients [5, 7, 426].

Passive immunotherapy approach, namely adoptive T cell therapy (ACT), focuses on amplifying patients' T cells *ex vivo* followed by autologous re-infusion. Different sources of T cells have been used in ACT trials in melanoma: T cells expanded from TILs, antigen-specific T cell clones and peripheral blood T cells retrovirally transduced with T cell receptors that recognize melanoma antigens [427, 428]. Lymphodepletion using radio- and/or chemotherapy before ACT, which aims to target immunosuppressive mechanisms and to enhance antitumor responses, has yielded objective response rates of 50% to 70% in metastatic melanoma patients, some of whom experience a complete and durable response [427]. However, ACT-based strategies are complicated by *ex vivo* manipulations and difficulties to maintain a pool of effector and memory tumor-reactive T cells after transfer *in vivo*.

Besides antigen-specific immunotherapeutic strategies, antigen-non-specific approaches are employed, which take advantage of knowledge about co-stimulatory and adhesion molecules, cytokines and cytokine receptors involved in immune response [8]. Given an increasing evidence of the role of the local immunosuppressive environment in melanoma [9], several clinical strategies have been developed to block specific negative regulatory pathways that dampen antitumoral immune responses. For instance, treatment with monoclonal antibodies ipilimumab and tremelimumab, which block cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), a receptor providing an inhibitory signal for T-cell activation, has yielded objective response rates of about 15% and, importantly, significantly improved survival in metastatic melanoma patients [429, 430].

Autoimmune side-effects frequently associated with melanoma regression have been reported in up to 30% of patients that have followed various immunotherapeutic regimens, especially employing ACT and treatment with CTLA-4 blocking antibodies, [429, 431-433].

Immune-related side effects may include colitis, hepatitis and dermatitis, which are likely relevant of generally broken self-tolerance [429, 433]. Other clinical manifestations may include vitiligo, uveitis, alopecia, hair whitening and hearing loss [431-433]. These side effects generally occur secondary to antigen-targeting immunotherapy regimens and may indicate the induction of the autoimmunity to melanocyte differentiation antigens [431-433].

The exact mechanisms allowing an efficient antitumoral immune response to be reliably triggered by immunotherapy are not entirely elucidated. Their understanding would allow finding the optimal strategies to shift a tumor-promoting immunosuppressive environment to a tumor-suppressive immunoreactive one and to avoid at the same time severe autoimmune side effects.

IV. Aims of the study

The presence of T cell-mediated immune responses against specific tumor antigens in melanoma patients and recent advances in immunotherapy of melanoma support the evidence that the balance between antitumor immunity and tumor-promoting inflammation may be skewed towards tumor suppression. The end point of many immunotherapeutic strategies is the enhancement of an effective immune response to recognize and eliminate tumor cells expressing the appropriate antigen. It is obvious that generation of large numbers of antigen-specific T cells may not be sufficient to trigger tumor regression if the antigen expression is inadequate or inconsistent. The quantitative evaluation of the expression levels of genes encoding melanoma antigens allows an indirect evaluation of antigen presentation on the surface of target melanoma cells. In addition, functional *in vitro* assays using antigen-specific T cell clones can indicate whether the changes of antigen expression modify the recognition and destruction of melanoma cells by T cells. A comprehensive understanding of the factors influencing the expression of tumor antigens is crucial not only to improve antitumor immunity, but also to control undesirable responses and toxicity due to exaggerated enhancement of autoimmune responses by some immunotherapeutic strategies. Two studies were initiated at the Brussels Branch of the Ludwig Institute for Cancer Research with the goal of understanding molecular mechanisms that contribute to the transcriptional regulation of genes encoding melanoma antigens. One study aimed to assess the potential role of BORIS transcriptional regulator in activation of cancer-germline genes, such as *MAGE-A1*, in melanoma. The other study was based on an observation of the transcriptional downregulation of genes encoding melanocyte differentiation antigens by some inflammatory cytokines. It focused on the mechanisms of downregulation of MITF-M, known as the crucial transcriptional activator of genes encoding melanocyte differentiation antigens, in the presence of IL-1β and IL-1α cytokines.

RESULTS

PART I.

Expression of *BORIS* in melanoma: lack of association with MAGE-A1 activation.

Olga Kholmanskikh[1,2], Axelle Loriot[1,2], Francis Brasseur[1,2], Etienne De Plaen[1,2], and Charles De Smet[1,2*]

[1]Ludwig Institute for Cancer Research, Brussels Branch, Université Catholique de Louvain, Brussels, Belgium
[2]Cellular Genetics Unit, Université Catholique de Louvain, Brussels, Belgium

The first two authors contributed equally to this paper.

International Journal of Cancer, February 2008, 122(4): 777-784.

Running title: BORIS-independent activation of *MAGE* genes
Key words: melanoma; cancer-germline genes; cancer-testis genes; BORIS/CTCFL; tumor antigens.
Grant sponsors: Belgian Fonds National de la Recherche Scientifique (FNRS), FRSM, Belgian Fondation contre le Cancer.
DOI: 10.1002/ijc.23140
History: Received 19 March 2007; Accepted after revision 9 August 2007; Published online 23 Octobre 2007
Correspondence to: Charles De Smet, Ludwig Institute for Cancer Research, Université Catholique de Louvain, 74 Avenue Hippocrate, 1200 Bruxelles, Belgium; FAX: +32-2-762 9405; E-mail: charles.desmet@bru.licr.org

Abstract

Several genes with specific expression in germ cells show aberrant activation in different types of tumors. These genes, termed cancer-germline genes, encode tumor-specific antigens, which represent potential targets for therapeutic vaccination against cancer. The germline-specific gene *BORIS* (Brother Of the Regulator of Imprinted Sites), which encodes an 11-zinc-fingers transcriptional regulator, was recently qualified as a new cancer-germline gene, as it was found to be activated in a variety of tumor samples. Moreover, it was suggested that BORIS might be responsible for the activation of most other cancer-germline genes, including gene *MAGE-A1*, in tumors. In the present study we evaluated the frequency of *BORIS* activation in melanoma by quantitative RT-PCR. *BORIS* activation was detected in 27% (n=63) melanoma tissue samples. Surprisingly, many melanoma samples expressed *MAGE-A1* and other cancer-germline genes in the absence of *BORIS* activation, suggesting that BORIS is not an obligate factor for activation of these genes in melanoma. Consistently, forced expression of BORIS in melanoma cell lines did not induce expression of *MAGE-A1*. Our results indicate that BORIS may serve as a useful target for immunotherapy of melanoma. However, it appears that BORIS is neither necessary nor sufficient for the activation of other cancer-germline genes.

Introduction

Cancer-germline (CG) genes, also referred to as cancer-testis (CT) genes, are characterized by a unique expression pattern [287, 434]. They are expressed in male germ cells and, in some cases, in female germ cells and in trophoblast, but not in adult somatic tissues. However, CG genes are activated in a number of human cancers of different histological types. Aside from this particular expression profile, CG genes share some other characteristics, including frequent existence as multigene families, frequent mapping to chromosome X and, for many of them, immunogenicity of their protein products. Indeed, the activation of several CG genes in tumors leads to the expression of antigens that can be recognized by autologous T-cells and by antibodies [434]. As germ line cells do not express the classical HLA class I molecules and therefore cannot present antigens to T cells, antigens encoded by CG genes are strictly tumor-specific. Consequently, these antigens represent promising targets for cancer immunotherapy and are currently used in therapeutic vaccination trials of cancer patients [5].

The mechanisms underlying activation of CG genes in tumors are only partially understood. Growing evidence points to epigenetic modifications as the regulatory basis of selective expression of CG genes. DNA methylation, in particular, appears to have an essential role in the repression of these genes in normal somatic tissues [284, 286, 435]. Consistently, expression of CG genes was shown to be induced by treatment with demethylating agents, such as 5-aza-2'-deoxycytidine (5-azadC), or by direct targeting of DNA methyltransferases using antisense oligonucleotides or siRNA [322, 324, 326]. Activation of CG genes in tumor cells is associated with demethylation of their promoters, which probably occurs in the course of the genome-wide demethylation process characteristic to tumors [233, 284]. The processes leading to hypomethylation of DNA sequences in tumor cells remain obscure. We undertook to address this issue by using *MAGE-A1*, the founding member of the CG group of genes [6], as a model. Our observations suggest that hypomethylation of CG gene promoters in tumors relies on a historical event of DNA demethylation, and on the presence of appropriate transcription factors to protect their promoter against remethylation [323, 326]. The factors that are responsible for the initial DNA demethylation process and for maintaining CG gene promoters unmethylated remain to be identified.

CTCF (CCCTC-binding factor) is an 11-zing-fingers protein that binds multiple DNA sequences. Target sequences are generally unmethylated, and it has been proposed that CTCF binding might be essential to maintain this unmethylated status. As such, CTCF is involved in various aspects of gene regulation, including gene imprinting and X chromosome inactivation [436, 437]. A testis-specific paralog of CTCF, known as BORIS (Brother of the Regulator of Imprinted Sites), has been isolated [343, 346]. The amino acid sequence of the central 11-zinc-fingers region of BORIS exhibits high homology to that of CTCF, and both proteins were shown to bind overlapping DNA sequences [346, 349, 353, 359]. However, BORIS and CTCF contain

divergent amino- and carboxy-terminal domains, suggesting distinct functions. Although the exact role of BORIS remains unclear, it has been proposed that by replacing CTCF at specific genomic sites, BORIS may modify DNA methylation patterns in male germ line cells [438].

Interestingly, the gene encoding BORIS qualifies as a member of the CG group of genes, as it was shown to be aberrantly activated in different malignancies [349, 353]. The studies however, analyzed only limited amounts of tumor samples. Nevertheless, *BORIS* mRNA was detected in a remarkably high proportion (up to 90%) of cancer cell lines (including melanoma cell lines) and primary tumors [349, 353]. Like other CG genes, *BORIS* appears to be regulated by DNA methylation, as suggested by its induction following 5-azadC treatment [349, 350].

Given the presence of BORIS in tumor cells and its possible contribution to epigenetic processes, the role of BORIS in the regulation of other CG genes was recently investigated [349, 353]. One study reported derepression of the *MAGE-A1* gene and partial demethylation of its promoter upon conditional ectopic expression of BORIS in cultured normal human dermal fibroblasts [349]. Consistently, *MAGE-A1* appeared to contain a CTCF/BORIS binding site near its transcription start region. Surprisingly, binding of CTCF to this site appeared to be insensitive to the methylation status. According to the study, the activation of *MAGE-A1* in BORIS-expressing cells was associated with a switching from CTCF to BORIS occupancy at this target sequence. In addition to *MAGE-A1*, several other CG genes, including *MAGE-A2, MAGE-A4, MAGE-B1* and *LAGE-2*$^{NY\text{-}ESO\text{-}1}$, were activated in response to the ectopic expression of BORIS. Moreover, induction of *BORIS* by 5-azadC appeared to precede that of *MAGE-A1*, and it was suggested that BORIS is the essential mediator of 5-azadC for *MAGE-A1* derepression.

Considering the reported high frequency of *BORIS* expression in melanoma cell lines [349], we decided to assess the frequency of *BORIS* activation in a panel of primary and metastatic melanoma samples. The expression of *BORIS* was investigated by quantitative RT-PCR. We detected *BORIS* expression in a substantial fraction of the samples. The frequency of *BORIS* activation was however considerably lower than that of *MAGE-A1* and other CG genes. As a corollary, many melanoma samples expressed *MAGE-A1* and other CG genes in the absence of *BORIS*, suggesting that BORIS may not be involved in CG activation in melanoma. We therefore decided to assess the role of BORIS in the induction of *MAGE-A1* in melanoma cells, by testing the effect of forced expression of BORIS in two melanoma cell lines. Similar experiments were also performed in a sarcoma cell line, in immortalized human keratinocytes and in normal human fibroblasts.

Materials and methods

Tissue samples and cell lines

Tissue samples were collected at resection. They were frozen immediately in liquid nitrogen and stored at –80°C until RNA isolation. EB16-MEL, SK-MEL-23 and LB23-SAR cell lines were grown in Iscove's medium supplemented with 10% fetal bovine serum (FBS, HyClone, Perbio); 0.55 mM L-arginine HCl; 0.24 mM L-asparagine; 1.5 mM L-glutamine; 200 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 8% CO_2. Immortalized human keratinocyte cell line HaCaT [439] (received from Dr. A. Moustakas, Ludwig Institute for Cancer Research, Uppsala), normal human foreskin fibloblasts HFF2 (received from Dr. A. Decottignies, Institute of Cellular Pathology, Brussels), and 293T cells were maintained in DMEM containing 10% FBS and antibiotics.

5-Aza-2'-deoxycytidine treatment

Cells grown to 60–70% confluence were exposed to a single dose of 2μM 5-aza-2'-deoxycytidine (Sigma), and maintained in culture for a period of time corresponding to at least three population doublings (4 days for SK-MEL-23, LB23-SAR and HaCaT cell lines, 5 days for HFF2, and 6 days for EB16-MEL cell line) before RNA or DNA extraction.

Construction of pBORIS plasmid

A plasmid carrying the *BORIS* cDNA sequence was isolated from a human testis cDNA library (Clontech) by PCR screening on successive subgroups. The isolated clone however contained a truncated *BORIS* cDNA sequence, which lacked about 1 Kb of the 5' portion, including part of the open reading frame (ORF). The missing *BORIS* sequence was amplified from cDNA of an expressing non-small cell lung cancer cell line (LB61-NSCLC) using proofreading PrimeSTAR™ HS DNA polymerase (Takara Bio Inc.) with a sense primer (GAGAATTCATTATGGCAGCCACTGA) overlapping the translation start site and containing a 5' overhang with an EcoRI site, and an antisense primer (TACGAATGTGAGCGGTCATA) located in exon 9. We purified an EcoRI-Eco47III fragment (comprising exon 1 to 8) from this PCR product, and an Eco47III-XbaI fragment (exon 8 to 11) from the truncated BORIS cDNA clone. Both fragments were co-ligated within EcoRI-XbaI digested pcDNA3 vector (Invitrogen), which carries a geneticin resistance gene for selection of transfected cells. Plasmid DNA was purified with the Endofree Plasmid Maxi kit (Qiagen). The sequence of BORIS was verified using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Compared with the human *BORIS* sequence published previously [346], the sequence in pBORIS contains a G to C substitution at position 230 (relative to the transcription start site) leading to a Q50E amino acid substitution within the N-terminal part of the protein (outside of the 11-zinc-fingers

domain). This nucleotide substitution corresponds to a frequent polymorphic variant (more than 30% of the population is homozygous for this allele, see NCBI SNP reference: rs 6070128). Homozygosity for this polymorphism was also detected in a *MAGE-A1*-expressing tumor, indicating that it is not incompatible with the activation of *MAGE-A1*.

Transfections

EB16-MEL and SK-MEL-23 cells (1 x 10^6) were seeded into 75 cm^2 flasks and transfected at 50-60% confluence with a total DNA amount of 20 μg (including carrier DNA) using ExGen 500 (Fermentas, Inc., Hanover, MD) according to the instructions of the manufacturer. LB23-SAR cells were plated at 1 x 10^6 cells in 75 cm^2 flasks 1 day prior to transfection by the calcium phosphate precipitation method [402]. HaCaT cells were transfected in six-well plates with LipofectAMINE 2000 Reagent (Invitrogen, Carlsbad, CA) according to the instructions of the manufacturer. After 48 h of incubation geneticin was added in culture media and transfectants were selected for 3-4 weeks. For transient transfection experiments, cells were analyzed 72h after transfection.

Conventional and Quantitative RT-PCR

Several RNA samples of normal tissues were purchased from Clontech (BD Biosciences Clontech, Palo Alto, CA) or Ambion Inc. (Austin, TX). For the other samples, total RNA was extracted using TriPure Isolation Reagent (Roche Applied Science) or the guanidinium-isothiocyanate / cesium chloride procedure. Total RNA extracted from transiently tranfected cell lines was treated with DNase (Turbo DNA-free, Ambion Inc.), to eliminate contaminating plasmid DNA. Reverse transcription was performed on 2 µg of total RNA using oligo-dT primer as described previously [440]. The different transcripts were amplified by PCR from 1/40th of the reverse transcription reaction. Conditions of conventional RT-PCR have been described elsewhere [319]. Primers used for amplification of *BORIS* and *ACTINB* were the same for both conventional and real-time quantitative PCR. Quantitative RT-PCR amplifications were performed as previously described using the reaction mix of Eurogentec (Seraing, Belgium) [441]. The PCR primers and specific 5'-FAM/3'-TAMRA labeled probes synthesized commercially (Eurogentec) are described in Supplementary Table1. Samples for quantitative PCR were assayed in duplicate. Expression levels are represented as a ratio to 2000 *ACTINB* mRNA copies (we estimated previously that 2000 is the mean amount of *ACTINB* mRNA molecules in one cell). For the analysis of transient and stable transfectants, non-reverse transcribed RNA controls were included, and the values obtained were subtracted from their reverse transcribed counterparts.

Western blot

Nuclear extracts were prepared using the Nuclear/Cytosol fractionation kit (BioVision). Protein samples were separated on a NuPAGE® 4-12% Bis-Tris gel (Invitrogen; Carlsbad, CA) in MOPS Running Buffer, with NuPAGE® antioxidant added to the inner chamber, according to manufacturer's instructions (XCell *Sure Lock*™). Proteins from the gel were transferred to a Hybond-ECL nitrocellulose membrane (Amersham Biosciences) using XCell II™ Blot Module. Membranes were incubated in a phosphate-buffered saline blocking solution containing 2.5% dry milk and 0.1% Tween-20 for 1 hour and then probed with primary antibodies in a new blocking solution containing anti-BORIS rabbit polyclonal antibody, ab18337 (1:7500 dilution; Abcam Ltd.) or anti-p80 Ku mouse monoclonal antibody, clone GE2.9.5 (1:1000 dilution; Upstate BioScientific, Lake Placid, NY). Membranes were washed and incubated with either donkey anti-rabbit IgG-HRP sc2313 (1:10000 dilution; Santa Cruz Biotechnology) or goat anti-mouse IgG-HRP sc2005 (1:2000 dilution; Santa Cruz Biotechnology) secondary antibodies and revealed using the SuperSignal West-Femto or West-Pico chemiluminescent substrates (Pierce). Reactivity was observed using CL-X posure™ autoradiographic film (Pierce).

Sodium bisulfite genomic sequencing

Sodium bisulfite genomic sequencing of the *MAGE-A1* 5' region was performed as described previously [323].

Retrovirus production and transduction of target cells

The retroviral vector pLN-BORIS was constructed by subcloning an Ecl136II-BamHI fragment from pBORIS, comprising the human BORIS coding sequence, into BglII and StuI sites of pLNCX2 vector containing a neomycin resistance gene (Clontech, Palo Alto, CA). Recombinant retroviruses were produced by transient co-transfection of 293T cells in 100-mm tissue culture dishes with 7,5 μg of each pVPack-GP, pVPack-VSV-G (both from Stratagene) and pLNCX2 or pLN-BORIS retroviral expression vectors using the calcium phosphate precipitation method. The medium was replaced 4 h later, and virus-containing supernatants were harvested 48 h and 72 h posttransfection. Supernatants were centrifuged, filtered through a 0.45-μm filter (Millipore, Ireland) and directly used to transduce target cells. HFF2 fibroblasts (4 x 10^5 cells) were plated in 75 cm^2 flasks and 24 h later, cell medium was removed, and 10 ml of specific and control viral supernatants were added, followed 3 h later by the addition of 10 ml of cell-growing medium. The transduced cells were then incubated for an additional 48 h at 37°C before selection with geneticin. Transduced cells were selected for 25 days. Western blot analyses on nuclear extracts from pLN-BORIS-transduced HFF2 cells indicated that the BORIS protein was efficiently produced and was translocated into the nucleus (supplementary Fig. S1).

Results

Analysis of BORIS expression in melanoma

A previous study using conventional RT-PCR reported aberrant derepression of the testis-specific gene *BORIS* in a vast majority of tumor tissues and cell lines of different types [349]. The study included 10 melanoma cell lines, 9 of which scored positive for *BORIS* expression. Surprisingly, our preliminary RT-PCR experiments failed to detect a high frequency of *BORIS* expression in a series of tumor cell lines, including 5 melanoma and 2 sarcoma cell lines (Fig. 1*a*). In contrast, *CTCF* was strongly expressed in all tested samples. The discrepancy between our results and the previous study could be explained by the different number of amplification cycles performed (31 cycles instead of 40-45 cycles). High numbers of PCR cycles may however lead to the occurrence of false positives, resulting from the amplification of irrelevant background mRNA levels. To resolve this problem, we decided to use real-time quantitative RT-PCR to accurately determine the level of *BORIS* expression.

We first evaluated the level of expression of *BORIS* in normal tissues obtained from tumor-free individuals (Fig. 1*b*). As expected, only in testis did we detect a significant level of *BORIS* expression. Background levels of *BORIS* mRNA, not exceeding 0.3% of the level in testis, were detected in several somatic tissues. Based on these results, we defined a threshold level of *BORIS* activation, which corresponded to the highest level of *BORIS* mRNA in normal somatic tissues (equal to 0.1 copy of *BORIS* mRNA/2000 copies of *ACTINB* mRNA). Tumor samples exhibiting *BORIS* mRNA levels above this threshold would be scored positive for *BORIS* activation. Using these criteria, we conducted the analysis of *BORIS* expression in tissue samples of primary and metastatic melanomas. *BORIS* was found to be activated in 16% (n=25) of primary melanomas and in 34% (n=38) of metastatic melanomas (Fig. 1*c*). The actual frequency of *BORIS* activation in melanoma cells may be underestimated when assessing melanoma samples, because of the heterogeneous nature of the tumor tissues. We therefore performed the analysis of *BORIS* expression in melanoma cell lines, which represent pure populations of tumor cells. Activation of *BORIS* was observed in 59% of the cell lines (n=32) (Fig. 1*c*). Altogether, these data indicate that, although *BORIS* is activated in a substantial fraction of melanoma samples, it does not appear to be so in the vast majority of the tumors.

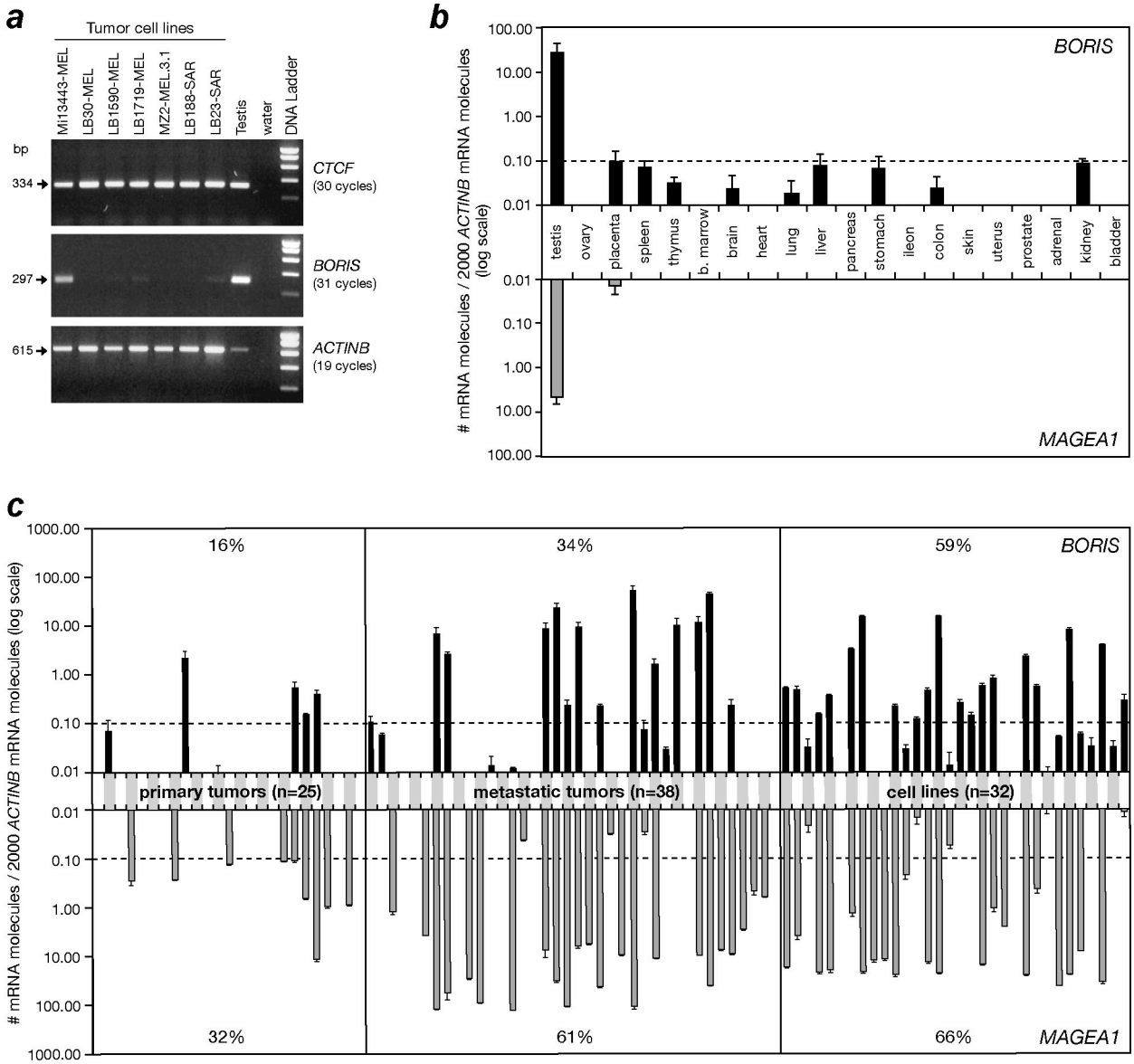


Figure 1. Expression of *BORIS* and *MAGE-A1* in normal tissues, melanoma tissues, and melanoma cell lines. *(a)* In an initial experiment, the expression of *BORIS, CTCF,* and *ACTINB* (used as an internal control) was tested by conventional RT-PCR in the indicated samples, including 5 melanoma cell lines (-MEL) and 2 sarcoma cell lines (-SAR). The numbers of PCR cycles performed are indicated. *(b)* The level of *BORIS* and *MAGE-A1* expression was evaluated in a series of normal somatic tissues, by quantitative RT-PCR. Two different RNA samples were used for each tissue. A threshold level of activation was set for *BORIS* (dashed line). It corresponds to the highest level of BORIS mRNA detected among normal somatic tissues. *(c) BORIS* and *MAGE-A1* expression levels were also evaluated by quantitative RT-PCR in a series of primary melanoma tumors, melanoma metastases, and melanoma cell lines. The percentages of samples showing activation of *BORIS* or *MAGE-A1* are indicated. Samples were scored positive for *BORIS*, when the expression level of the gene was significantly higher than the threshold level defined in panel *b* (dashed line). The same threshold level was applied to the analysis of *MAGE-A1* activation. Each value represents mean $\pm$ SE. All quantitative RT-PCR data derive from two independent experiments.

MAGE-A1 and BORIS are not always co-expressed in melanoma samples

Given the proposed role of BORIS in the activation of other CG genes [349], we decided to investigate whether CG genes were activated exclusively in melanomas expressing *BORIS*. We first assessed the expression of the *MAGE-A1* gene, because the involvement of BORIS in the regulation of this CG gene has been the most extensively studied. In normal tissues, *MAGE-A1* transcripts were found to be present in testis, and at a lower level in placenta, but were undetectable in normal somatic tissues (Fig. 1*b*). In melanoma samples, *MAGE-A1* was activated at a higher frequency than *BORIS* (*p*-value = 0.0192, double-sided *t*-test; Fig. 1*c*). As a consequence, *MAGE-A1* activation was detected in 17 melanoma tissues and 6 cell lines that scored negative for *BORIS* activation (Fig. 1*c*, Table I). We also analyzed the expression of other CG genes, namely *MAGE-A2, MAGE-A4, MAGE-B1* and *LAGE-2*$^{NY\text{-}ESO\text{-}1}$, in a fraction of the metastatic melanoma tissue samples. Activation of these CG genes, which have been proposed to be regulated by BORIS as well, was found in a substantial proportion of samples, including samples that were negative for *BORIS* activation (Table I). Taken together, these results indicate that sustained transcription of *MAGE-A1* and of other CG genes in melanoma does not rely on the presence of BORIS. There was however some level of correlation between the expression of BORIS and other CG genes in the melanoma samples (Table I). This is expected for a group of genes that share a common mechanism of activation.

TABLE I. LACK OF A STRICT ASSOCIATION BETWEEN THE EXPRESSION OF *BORIS* AND OTHER CG GENES IN MELANOMA TISSUE SAMPLES.

		BORIS expression*		Fisher's exact
Expression of other CG genes*		+	-	test (*p*-value)
MAGE-A1 (n=63)	+	14 (22%)	**17 (27%)**	0.001
	-	3 (5%)	29 (46%)	
MAGE-A2 (n=32)	+	8 (25%)	**11 (34%)**	0.035
	-	1 (3%)	12 (38%)	
MAGE-A4 (n=22)	+	6 (27%)	**6 (27%)**	0.012
	-	0	10 (46%)	
MAGE-B1 (n=32)	+	4 (13%)	**1 (3%)**	0.008
	-	4 (13%)	23 (72%)	
LAGE-2$^{NY\text{-}ESO\text{-}1}$ (n=32)	+	5 (16%)	**4 (13%)**	0.040
	-	4 (13%)	19 (59%)	

Values in parantheses are in percentages.

* The expression status was determined by quantitative RT-PCR, as described in Fig.1. Associations between the expression of a particular CG gene and *BORIS* were assessed with Fisher's exact test (*p*-values). Although significant associations were found, tumor samples expressing *MAGE* or *LAGE* genes but not *BORIS* were commonly detected (bold).

Exogenous expression of BORIS in melanoma cells does not lead to CG gene activation

One could still argue that BORIS is required only for the initial event of activation of CG genes, and that other transcription factors take over to maintain the genes active. According to this view, a transient phase of BORIS expression would be sufficient to induce permanent activation of CG genes. This may explain the lack of co-expression of *BORIS* and other CG genes in certain melanomas, in which *BORIS* expression would have been lost at some stage of the tumor development. We decided therefore to evaluate the potential of BORIS in the activation phase of *MAGE-A1* in melanoma cells, by testing the effect of forced expression of BORIS. For the experiments, we chose EB16-MEL and SK-MEL-23 melanoma cell lines, because they were initially negative for both *BORIS* and *MAGE-A1* activation. Both genes however, were induced following 5-azadC treatment, indicating that DNA methylation is involved in their repression in these cells (Fig. 2*a*). Moreover, activation of *MAGE-A1* by this treatment indicated that both cell lines possess all required factors to support expression of this gene upon demethylation. EB16-MEL and SK-MEL-23 cells were transfected with increasing amounts of a BORIS-encoding plasmid (pBORIS) or with the empty vector, and were analyzed 72 hours after transfection. Quantitative RT-PCR analyses revealed high levels of BORIS expression upon transfection with pBORIS (Fig. 2*a*). Furthermore, Western blot analyses on nuclear protein extracts of BORIS-transfected cells indicated that the protein was efficiently produced and was translocated into the nucleus (Fig. 2*b*). Exogenous expression of BORIS, however, did not induce significant increases in the expression of *MAGE-A1*, as measured by quantitative RT-PCR (Fig. 2*a*).

One possible explanation for the lack of activation of *MAGE-A1* in transfected cells is that the time of exposure of the gene to BORIS was insufficient to support its activation. Derepression of epigenetically silenced genes may indeed require longer time and more cell generations. We therefore decided to derive stable transfectants in order to test the effect of long-term expression of BORIS on the activation of *MAGE-A1*. EB16-MEL and SK-MEL-23 cells that had been transfected with pBORIS were selected according to their resistance to geneticin, which was conferred by the transgene vector. Pools of transfectants, representing several hundreds of clones, were used for subsequent analyses without further subcloning, in order to avoid the possibility of selecting clones not representative of the entire transfected population. Quantitative RT-PCR analyses on EB16-MEL and SK-MEL-23 transfected populations indicated that the levels of *BORIS* expression in these cell cultures were comparable to those observed in cell lines in which the gene is constitutively expressed (compare Fig. 2*c* and Fig. 1*c*). As shown in figure 2*c*, even one month after transfection, forced expression of *BORIS* in both melanoma cell lines did not result in the activation of *MAGE-A1*. We also tested the expression of other CG genes, including *MAGE-A2, MAGE-A4, MAGE-B1* and *LAGE-2*$^{NY\text{-}ESO\text{-}1}$, in the transfected populations, but none of them were induced in the *BORIS*-expressing cells (supplementary Fig. S2). Taken together, these data indicate that over-expression of BORIS is not sufficient to induce activation of CG genes in melanoma cells.

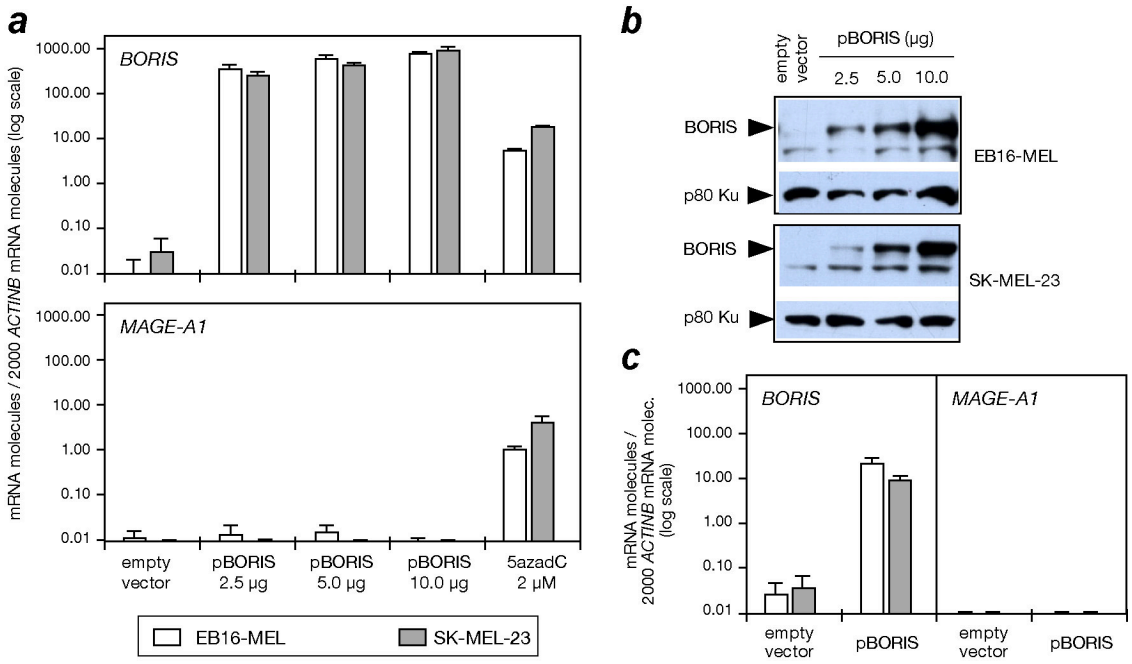


Figure 2. Forced expression of BORIS does not lead to *MAGE-A1* activation in melanoma cell lines. *(a)* EB16-MEL and SK-MEL-23 melanoma cell lines were transfected with increasing amounts of the vector pBORIS, which contains the entire open reading frame of *BORIS*, or with the empty vector. *BORIS* and *MAGE-A1* expression levels were assessed by quantitative RT-PCR 72 hours after transfection. The expression level of these genes was also measured after treatment of the cells with the methylation inhibitor 5-azadC. Each value represents mean ± SE. *(b)* Western blot analyses with antibodies directed against BORIS were performed on nuclear extracts from EB16-MEL and SK-MEL-23 transfectants. Detection of p80Ku was performed to verify equal loading in the different lanes. *(c)* EB16-MEL and SK-MEL-23 cells were transfected with pBORIS or the empty vector, and selected according to their resistance to geneticin, which was conferred by the vector. Four weeks after transfection, the stable transfectants were assessed for *BORIS* and *MAGE-A1* expression levels by quantitative RT-PCR. Each value represents mean ± SE. All quantitative RT-PCR data derive from two independent experiments.

***MAGE-A1* methylation patterns following ectopic expression of *BORIS* in melanoma cells**

We examined whether the ectopic expression of *BORIS* in both melanoma cell lines induced methylation changes in the 5' region of *MAGE-A1*. To this end, DNA was extracted from EB16-MEL and SK-MEL-23 cells that had been stably transfected with either pBORIS or the empty vector. DNA was also obtained from both cell lines exposed to 5-azadC. We applied the sodium bisulfite genomic sequencing method to analyze the methylation status of 17 CpG sites located between positions -81 and +169 relative to the transcription start site of *MAGE-A1*. Whereas this region of *MAGE-A1* showed marked demethylation following 5-azadC treatment, its overall methylation level was not significantly affected by the ectopic expression of *BORIS* (Fig. 3). In SK-MEL-23 cells however, a cluster of five CpGs located within the first intron of *MAGE-A1,* between positions +129 and +169, showed a reduced level of methylation in the pBORIS-transfected group (6.7% methylated CpGs), as compared with the empty vector control group (21.1%, *p*-value = 0.005, Chi-square test). This observation is in accordance with the study by Vatolin *et al.*, which showed partial demethylation of the same five CpGs in human fibroblasts that were induced to express BORIS [349]. However, demethylation of these intronic CpG sites does not appear to influence

the transcriptional status of *MAGE-A1*. We showed previously that the activation of *MAGE-A1* is strongly associated with the demethylation of six CpGs located closer to the transcription start site, between positions -30 and +30 [284, 323]. This cluster of CpGs was mostly methylated (>80%) in both *MAGE-A1*-negative melanoma cell lines, and remained so even after long-term expression of *BORIS* (Fig. 3). In contrast, these CpGs were significantly demethylated in the 5-azadC-treated cells (<45% methylated CpGs), where *MAGE-A1* is activated. Together these data indicate that the CpGs that impact on the transcription of *MAGE-A1* remain methylated in melanoma cells that were induced to express *BORIS*.

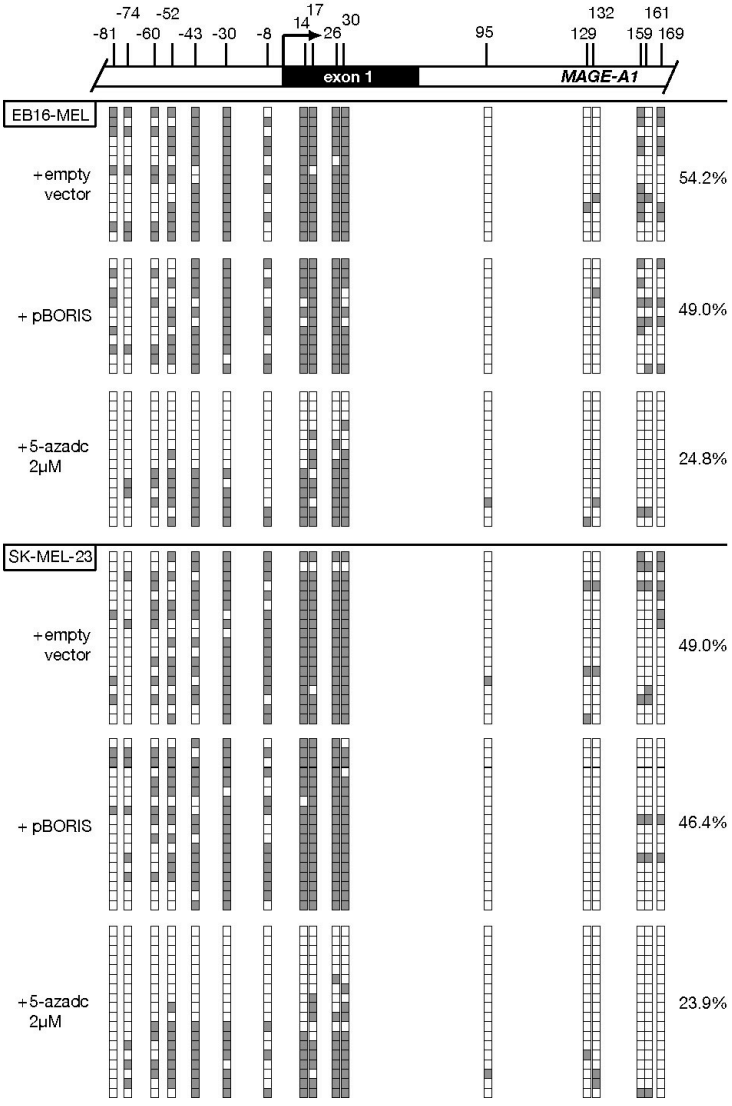


Figure 3. Methylation status of the 5' region of *MAGE-A1* in melanoma cells transfected with pBORIS. DNA was extracted from EB16-MEL and SK-MEL-23 cells either four weeks after transfection with pBORIS or the empty vector, or 72 hours after exposure to 5-azadC. DNA samples were treated with sodium bisulfite, and the 5' region of *MAGE-A1* was amplified by PCR. The amplified region of *MAGE-A1* is represented at the top with vertical bars at the location of CpG dinucleotides, a broken arrow at the transcription start site, and a black box corresponding to the first exon. PCR products were cloned, and several clones were sequenced. The deduced CpG methylation patterns are represented by the squares: filled squares, methylated cytosines; empty squares, unmethylated cytosines. Each line derives from the sequence of one clone, and gives the methylation patterns of a single DNA molecule. The overall level of methylation within the region is given (percent methylated CpGs).

Lack of BORIS-induced activation of MAGE-A1 in other cell types

In order to extend the transfection experiments to other cell types, we decided to assess the effect of BORIS over-expression in a human sarcoma cell line (LB23-SAR), in immortalized human keratinocytes (HaCaT), and in normal human fibroblasts (HFF2). None of these cells expressed *BORIS* nor *MAGE-A1* above background levels before treatment (Fig. 4). *MAGE-A1* was induced by 5-azadC in all three cell cultures. In contrast, induction of *BORIS* was very weak in 5-azadC-treated HaCaT and HFF2 cells (Fig. 4). This observation is inconsistent with the model proposed by Vatolin *et al*., in which CG gene induction by 5-azadC relies on prior activation of *BORIS* [349]. LB23-SAR and HaCaT cells were transfected with pBORIS, while HFF2 fibroblasts were transduced with a BORIS-expressing retroviral vector (pLN-BORIS). Cells were thereafter selected for their resistance to geneticin, which was conferred by the vectors. Quantitative RT-PCRs, which were performed 3-4 weeks after transfection, indicated that all three selected populations expressed *BORIS*. However, forced *BORIS* expression in these cells did not result in the activation of *MAGE-A1* (Fig. 4). These data indicate that the lack of induction of *MAGE-A1* following forced expression of BORIS is not restricted to melanoma cells.

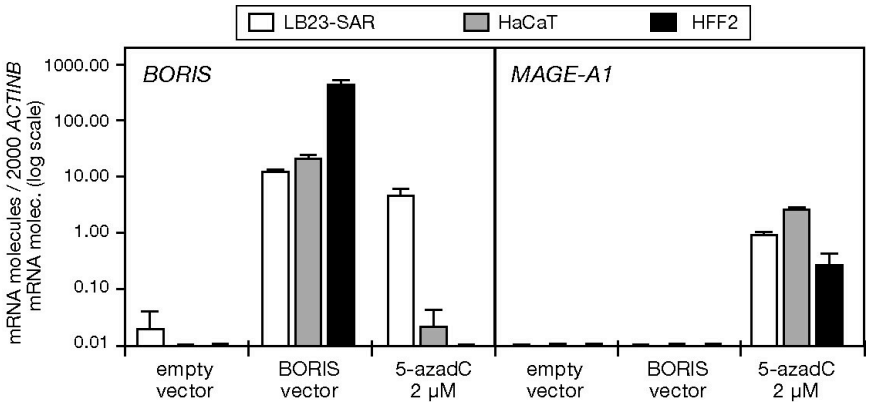


Figure 4. Lack of induction of *MAGE-A1* by BORIS in nonmelanoma cell types. LB23-SAR sarcoma cells, HaCaT immortalized human keratinocytes, and HFF2 normal human fibroblasts were transfected or transduced with a BORIS-expressing vector or with the corresponding empty vector, and selected according to their resistance to geneticin, which was conferred by the vectors. Three to four weeks after transfection or transduction, the selected cell populations were assessed for *BORIS* and *MAGE-A1* expression levels by quantitative RT-PCR. The level of expression of these genes was also assessed in LB23-SAR, HaCaT or HFF2 cells that had been exposed to 5-azadC. Each value represents mean $\pm$ SE. All quantitative RT-PCR data derive from two independent experiments.

Discussion

Tumor-specific antigens encoded by CG genes represent attractive targets for cancer immunotherapy. The germline-specific gene *BORIS* was recently defined as a new CG gene, as it was found to be activated in different malignancies. Because our group is currently focusing on antitumor vaccinations in melanoma patients, we decided to assess the frequency of activation of *BORIS* in melanoma. We found activation of *BORIS* in a substantial fraction of melanoma tissue samples (27%). The level of *BORIS* mRNA in several melanoma samples was similar to that in testis, where the gene is naturally expressed, and where the protein could be readily detected [346, 350]. This level of expression should be sufficient to allow efficient recognition of BORIS-derived antigenic peptides by T lymphocytes. BORIS peptide sequences that can be processed properly to be presented at the cell surface by HLA class I molecules, remain to be identified.

The frequency of *BORIS* activation appeared to be higher in metastatic melanoma samples, as compared with primary lesions. This tendency has already been observed for other CG genes [434]. This may reflect a positive role of *BORIS* and other CG genes in the metastatic process, and therefore selection for expression of these genes during progression of the disease. However, to date there is no clear evidence that CG genes play such a role. Another explanation for the higher frequency of CG gene expression in metastases may relate to the process of activation of these genes. Thus, the DNA demethylation process, which underlies the activation of CG genes and probably of *BORIS* as well, may coincide with one of the numerous steps associated with tumor progression. In this case, the chance that this demethylation process occurred would be higher in tumors that are at later stages of progression. Of note, the increased frequency of CG gene expression in advanced tumors supports the notion that CG gene expression in tumors is due to an activation process associated with malignant development, rather than the expansion of precursor cancer stem cells, in which these genes were constitutively unmethylated and active.

It was previously reported that the frequency of *BORIS* activation in tumors is generally higher than that of other CG genes [349]. While this appears to be true in certain types of tumors, such as uterine cancers [352], our study indicates that the frequency of activation of *BORIS* in melanoma is significantly lower than that of *MAGE-A1*. Another recent study also reported limited activation of *BORIS* in prostate and bladder carcinoma [350]. Contradictory results about the frequency of *BORIS* activation in tumors may be due to leaky transcription of the gene in several tissues, which may lead to the occurrence of false positives when analyzing tumor samples with a sensitive but not quantitative RT-PCR assay.

While numerous studies confirmed that DNA demethylation is an essential step towards CG gene activation in tumors, the mechanisms underlying this demethylation process remain unknown. The recent proposal that BORIS could be involved in this epigenetic

process, relies on the observation that CG genes became demethylated and activated in normal human fibroblasts that were forced to express BORIS [349, 353]. This effect was attributed to the ability of BORIS to bind nearby the promoter of CG genes, and to antagonize the transcriptional silencing imposed by CTCF, when bound to the same DNA sites. Demethylation and activation of CG genes in tumors was therefore proposed to result from the aberrant activation of BORIS, and the consequent CTCF to BORIS switch in occupancy of regulatory regions. As such, induction of *BORIS* expression in tumors appeared as a novel interesting strategy to increase antitumor immunity via activation of numerous antigen-encoding CG genes [442, 443].

Several results in our study indicate that BORIS is not the primary regulator of CG gene expression. Firstly, our detailed expression analysis in a large panel of melanoma tissues revealed that a substantial proportion of tumors express *MAGE-A1* and other CG genes in the absence of *BORIS* activation. Therefore, BORIS does not appear to be necessary to maintain expression of CG genes in melanoma. Secondly, forced expression of BORIS in two melanoma cell lines by either transient or stable transfection of a BORIS-encoding vector, did not result in the activation of *MAGE-A1* or other CG genes. Both cell lines evidently possessed all required factors to support expression of CG genes upon demethylation, since these genes were efficiently induced by 5-azadC treatment. Therefore, the lack of CG gene induction by BORIS appears to be due to its inability to direct DNA demethylation within CG gene promoters. We conclude that BORIS is not sufficient to induce CG gene expression in melanoma cells and that other, yet unidentified, factors are involved in this process. These conclusions do not apply only to melanoma cells, since forced expression of BORIS failed to induce *MAGE-A1* and other CG genes (data not shown) in other cell types as well. Our data, which show lack of induction of CG genes in *BORIS*-expressing human fibroblasts, differ from those previously reported [349]. The reason for this discrepancy is unclear. Altogether, our results indicate that over-expression of BORIS may not serve as a general means to induce CG genes in tumors.

Besides its putative role in the activation of CG genes in tumors, BORIS was also proposed to induce expression of CG genes in normal male germ cells. However, the timing of expression of BORIS and of the other CG genes during germ cell development does not fit with this suggestion. Thus, whereas BORIS was detected exclusively in the nucleus of spermatocytes in adult human testis [346, 350], most CG genes, including *MAGE-A1* and *LAGE-2*$^{NY\text{-}ESO\text{-}1}$, are already expressed in fetal gonocytes and become silenced at the spermatogonia to spermatocyte transition stage [291, 293, 444]. Therefore, the appearance of BORIS in human testicular germ cells coincides with repression rather than activation of CG genes. This implies that factors other than BORIS are involved in the activation of CG genes during early male germ cell development. These factors, as well as those underlying CG gene derepression in tumors, still need to be identified.

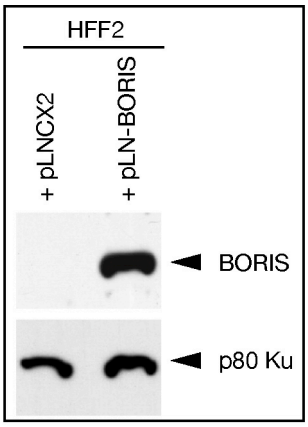
Acknowledgments

The authors would like to acknowledge Dr. Thierry Boon for critical reading of the manuscript. We would also like to thank A. Prisoni for his abundant contribution to this work, Dr. B. Lethé for providing the reagents for *LAGE-2* quantitative PCR, and Dr. Anabelle Decottignies for her help in designing retroviral transduction experiments. We appreciate the excellent work provided by M. Swinarska in preparing and analyzing melanoma specimens. This work was funded by the Belgian 'Fonds National de la Recherche Scientifique' (FNRS), FRSM and by the Belgian 'Fondation contre le Cancer'. A.L. is a postdoctoral researcher of FNRS. O.K. was supported by a 'Télévie' grant from the FNRS.

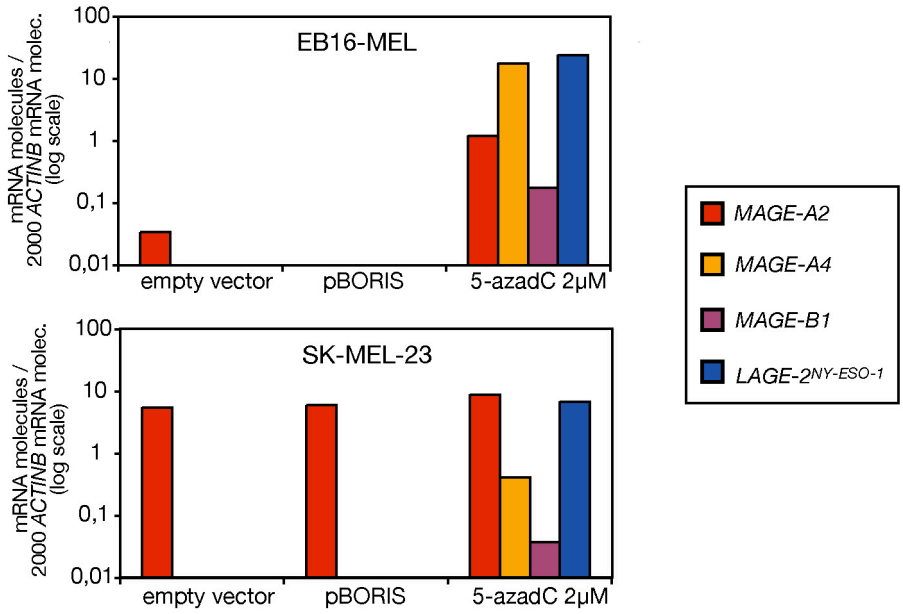
Supporting information.

Supplementary Table 1. Primers and probes used in conventional and quantitative RT-PCRs

Gene	Sense primer	Antisense primer	Probe
CTCF	5'-CAT ggC AAG ACA TgC TgA T-3'	5'- TgC ACC TgT ATT CTg gTC TTC-3'	
BORIS	5'- CCC TCA TTC AgC ACC AgA A -3'	5'- CTg ATC CAC ACT TCT CCg AA -3'	5' - 6FAM - CCg ACA gAA gCA ACT TCT AAA CgC T - TAMRA -3'
MAGE-A1	5'- gCC gAA ggA ACC TgA CC -3'	5'- ACT ggg TTg CCT CTg TCg -3'	5' - 6FAM - TTC ATT gCC CAg CTC CTg CCC ACA CT -TAMRA -3'
MAGE-A2	5'- AAg TAg gAC CCg Agg CAC Tg -3'	5'- gAA gAg gAA gAA gCg gTC Tg -3'	5'- 6FAM - CAT TgA Agg AgA AgA TCT gCC TgT ggg TCT TC - TAMRA -3'
MAGE-A4	5'- CCA CTA CCA TCA gCT TCA CTT gC -3'	5'- CTT CTC ggA ACA Agg ACT CTg C -3'	5' - 6FAM - Agg CAA CCC AAT gAg ggT TCC AgC - TAMRA -3'
MAGE-B1	5'- CCC gAg CgA gCT TAA ggA gT -3'	5'- gTC AgA TTC ggT ACA TgA CAC Ag -3'	5'- 6FAM - Tgg TCg ggA gTC AAg gTg CCA CAT CTC CTg - TAMRA -3'
LAGE-2/NY-ESO-1	5'- CgC CTg CTT gAg TTC TAC C -3'	5'- CAC TgC gTg ATC CAC ATC AAC A -3'	5'- 6FAM - TCA gTA TgT TgC Cgg ACA CAg TgA ACT C - TAMRA -3'
ACTINB	5'- ggC ATC gTg ATg gAC TCC g -3'	5'- gCT ggA Agg Tgg ACA gCg A -3'	5'- 6FAM - TCA AgA TCA TTg CTC CTC CTg AgC gC - TAMRA -3'



Supplementary figure 1. Production of BORIS protein in HFF2 human fibroblasts transduced with the pLN-BORIS retroviral vector. HFF2 cells were transduced with the BORIS-encoding retroviral vector pLN-BORIS or with the empty vector pLNCX2. Transduced cells were selected in geneticin. A Western blot analysis with antibodies directed against BORIS was performed on nuclear extracts from both selected cell populations. Detection of p80Ku was performed to verify equal loading in the different lanes.



Supplementary figure 2. Forced expression of BORIS does not lead to activation of other CG genes in melanoma cell lines. EB16-MEL and SK-MEL-23 cells were transfected with pBORIS or the empty vector, and selected according to their resistance to geneticin, which was conferred by the vector. Four weeks after transfection, the stable transfectants were assessed for CG gene expression levels by quantitative RT-PCR. Expression of CG genes was also evaluated after treatment with 5-azadC. Each value represents mean of duplicate.

PART II.

Interleukins 1α and 1β secreted by some melanoma cell lines strongly reduce expression of MITF-M and melanocyte-differentiation antigens.

Olga Kholmanskikh[1,2], Nicolas van Baren[1,2], Francis Brasseur[1,2], Sabrina Ottaviani[1,2], Julie Vanacker[2], Nathalie Arts[1,2], Pierre van der Bruggen[1,2], Pierre Coulie[2] and Etienne De Plaen[1,2 *]

[1]Ludwig Institute for Cancer Research, Brussels Branch, Université Catholique de Louvain, 74 avenue Hippocrate, UCL7459, B1200 Brussels, Belgium

[2]Cellular Genetics Unit, de Duve Institute, Université Catholique de Louvain, 74 avenue Hippocrate, UCL7459, B1200 Brussels, Belgium

International Journal of Cancer, October 2010, 127(7): 1625-1636.

Running title: Repression of melanocyte differentiation antigens by IL-1

Key words: interleukin 1, MITF, melanoma, antigen, CTL

Abbreviations: MITF : Microphtalmia-associated transcription factor, CTL : Cytolytic T lymphocyte, RT-PCR : Reverse transcriptase-polymerase chain reaction, IL-1: interleukin 1

Grant sponsors: Fondation contre le Cancer (Belgium), Fonds de la Recherche Scientifique Médicale (Belgium), The Fonds National de la Recherche Scientifique (Belgium) and the Fondation Salus Sanguinis (Belgium)

DOI: 10.1002/ijc.25182

History: Received 2 October 2009; Accepted after revision 7 January 2010; Published online 22 January 2010

Correspondence to: Etienne De Plaen, Ludwig Institute for Cancer Research Ltd, Brussels branch, 74 Avenue Hippocrate-UCL7459, B1200 Brussels, Belgium, Fax: +32-2-762 9405, E-mail: Etienne.Deplaen@bru.licr.org

Abstract

We report that melanoma cell lines expressing the interleukin-1 receptor exhibit 4- to 10-fold lower levels of mRNA of microphthalmia-associated transcription factor (*MITF*-M) when treated with interleukin-1β. This effect is NF-κB and JNK-dependent. MITF-M regulates the expression of melanocyte differentiation genes such as *MLANA*, *tyrosinase* and *gp100*, which encode antigens recognized on melanoma cells by autologous cytolytic T lymphocytes (CTL). Accordingly, treating some melanoma cells with IL-1β reduced by 40-100% their ability to activate such antimelanoma CTL. Finally, we observed large amounts of biologically active IL-1α or IL-1β secreted by two melanoma cell lines that did not express *MITF*-M, suggesting an autocrine *MITF*-M downregulation. We estimate that about 13% of melanoma cell lines are MITF-M-negative and secrete IL-1 cytokines. These results indicate that the repression of melanocyte-differentiation genes by IL-1 produced by stromal cells or by tumor cells themselves may represent an additional mechanism of melanoma immune escape.

Introduction

Melanoma is a prime target of immunotherapy notably because spontaneous antitumoral T cell responses are frequent in melanoma patients [5, 445]. Surprisingly, it was found that many of these T lymphocytes recognized differentiation antigens shared by normal melanocytes and melanoma cells [417]. These antigens are encoded by melanocyte-specific genes such as *TYR* (*tyrosinase), MLANA* (*Melan-A/Mart-1*), *SILV* (*pMel17/gp100*), *TYRP1* (*tyrosinase-related protein*), or *TYRP2*. $CD8^+$ and $CD4^+$ T cells recognizing differentiation antigens were found in the blood and among tumor-infiltrating lymphocytes of melanoma patients [5]. The presence of these T cells is usually not associated with autoimmunity, even though approximately 3% of melanoma patients develop vitiligo-like skin hypopigmentation [425]. The lymphocytes infiltrating these lesions were reported to be mostly $CD8^+$ T cells directed against differentiation antigens [446].

The antitumoral immune responses, either spontaneous or stimulated by immunotherapy, do usually not lead to complete eradication of melanoma, even when the recruitment of T cells to the tumor site is successful. This may be explained by multiple mechanisms of inhibition of T cell function or of tumor antigen expression. Antitumoral T cells can be inhibited by immunosuppressive cells such as regulatory T cells, immunosuppressive dendritic cells or myeloid-derived suppressor cells [447, 448]. Expression of the tryptophan-catabolizing enzyme indoleamine 2,3-dioxygenase (IDO) in metastatic melanoma tumor sites deprives surrounding T lymphocytes of tryptophan thereby suppressing their function [449]. Melanoma cells themselves can alter the efficacy of antitumor responses. Engagement of the inhibitory programmed death receptor-1 (PD-1) on T cells by its ligand PD-L1 on tumor cells inhibits the proliferation of T cells and their production of IFN-γ and IL-10 [450, 451].

Melanoma cells can express both PD-L1 and IDO in response to IFN-γ, a cytokine produced by activated T cells and natural killer cells. Another cytokine produced by activated T lymphocytes is TNF-α [452]. TNF-α was reported to be present in primary and metastatic melanoma samples [453], where macrophages are considered to be its main source. The number of melanoma macrophages was shown to increase with tumor stage and angiogenesis [368]. IFN-γ and TNF-α modulate the expression of many genes that contribute to the tumor-immune system interaction [454, 455]. Interestingly, many genes that are induced by IFN-γ are also induced by TNF-α, and in most cases induction is synergistic and attributed to the activation of transcription factors IRF-1 and NF-κB (reviewed in Ref. [454]).

There is therefore growing evidence that the production of cytokines in the tumor environment may help melanoma cells to resist elimination by T lymphocytes. To find additional mechanisms of tumor escape, we used the Affymetrix microarray technology and explored the transcriptional profiles of melanoma cell lines treated with IFN-α, IFN-γ and TNF-α.

Materials and methods

Tissue samples, cell lines, media and reagents

Tissue samples were collected at resection. They were frozen immediately in liquid nitrogen and stored at –80°C until RNA isolation. Melanoma cell lines were from our laboratory, except for SK-MEL-23 obtained from Dr E. Stockert (Memorial Sloan-Kettering Cancer Center, New York). They were grown in Iscove's modified Dulbecco's medium (IMDM) (Invitrogen Life technologies; Carlsbad, CA) supplemented with 10% fetal bovine serum (HyClone, Perbio, Erembodegem-Aalst, Belgium); 0.55 mM L-arginine, 0.24 mM L-asparagine, 1.5 mM L-glutamine (AAG); 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 8% CO_2. T cells were cultured in IMDM supplemented with 10% human serum, AAG, antibiotics, human recombinant IL-2 50 U/ml (Chiron Healthcare SAS, Suresnes, France) and IL-7 10 ng/ml (R&D Systems, Minneapolis, MN). Melanoma cells were treated with TNF-α 10 ng/ml (R&D Systems), IFNα-2a 1,000 U/ml (Roferon-A, Roche, Nutley, NJ), IFN-γ 100 U/ml (PeproTech, Rocky Hill, NJ), IL-1β 10 ng/ml (PeproTech) or IL-1α 10 ng/ml (PeproTech). Anakinra (Kineret, rh-met-IL-1Ra, Amgen, Thousand Oaks, CA) is a recombinant, nonglycosylated form of human IL-1Ra. JNK inhibitor SP600125 and MEK inhibitor PD98059 were purchased from Sigma-Aldrich (St. Louis, MO). MEK inhibitor U0126 was purchased from BIOMOL International (Plymouth Meeting, PA).

Retroviral transduction

Recombinant retroviruses were produced in 293T cells, as described [456]. Target LB2259-MEL cells were plated in 75 cm^2 flasks; 24 and 48 h later, culture medium was removed, and viral supernatant was added, followed 3 h later by the addition of fresh cell-growing medium. Seventy-two hours after transduction with recombinant retroviruses harboring a Flag-IκBαM (S32A/S36A)-IRES-puromycin expression cassette, a stably infected puromycin-resistant population was obtained after selection in 0.8 μg/ml puromycin (Sigma-Aldrich). A puromycin-resistant population harboring the empty retroviral vector was also generated to serve as a negative control. Retroviral expression vectors were kindly provided by Dr. K. Marcu (Bologna, Italy and State University of New York, Stony Brook, New York) [457].

Quantitative RT-PCR

RNA samples were prepared from cell lines and surgical specimens using the guanidinium isothiocyanate / cesium chloride procedure or the InnuPREP RNA Mini Kit (Analytik Jena AG bio solutions, Jena, Germany). Total RNA was treated with TURBO DNAse (Turbo DNA-free™ Kit, Ambion, Austin, TX). The first-strand cDNA was produced from 2 μg of total RNA with the Superscript II reverse transcriptase and an oligo-dT primer, according to the manufacturer's protocol. The cDNA was then treated with 2 U of RNase H (Fermentas,

St. Leon-Rot, Germany), and 1/40 of the reaction volume was used for PCR. Quantitative RT-PCR amplifications were performed with the PCR core kit (Eurogentec, Seraing, Belgium). PCR amplifications were performed in a final volume of 25 μL with 0.625 U of HotGoldStar DNA polymerase (Eurogentec), 200 nM of each primer, 100 nM of probe, 200 μM of each dNTP, and 5 mM of $MgCl_2$ in an ABI Prism 7700 Sequence Detector (Applied Biosystems, Halle, Belgium) with the following conditions: 94°C for 10 min, 45 cycles of 94°C for 15 s, and 62°C for 1 min or for 1 min 30 (*ACTB*). The PCR primers and specific 5'-FAM/3'-TAMRA labeled probes (Eurogentec) are described in Supporting Information Table 1. Samples for quantitative PCR were assayed at least in duplicate. Expression levels were normalized to 2,000 *β-actin* mRNA copies (we estimated previously that 2,000 is the mean amount of *β-actin* transcripts per cell). The statistical analyses were performed using the Student's t-test. P-values of less than 0.05 (double sided) were considered as statistically significant.

Western blot

Cells were homogenized in lysis buffer containing 0.1% sodium dodecyl sulfate, 1% sodium deoxycholate, 0.5% NP-40 and 1/50 per milliliter of a protease inhibitor cocktail complete mini, EDTA-free tablet (Roche Diagnostics, Mannheim, Germany) in PBS. Protein samples were separated on a NuPAGE® 4-12% Bis-Tris gel (Invitrogen Life technologies) in MOPS Running Buffer, with NuPAGE® antioxidant added to the inner chamber, according to the manufacturer's instructions (XCell *Sure Lock*™). Proteins were transferred from the gel to a Hybond-ECL nitrocellulose membrane (GE healthcare, Chalfont St Giles, UK) using the XCell II™ Blot Module. Membranes were incubated in a blocking solution containing 2.5% dry milk and 0.1% Tween-20 in PBS for 1 hour and then incubated with the following primary antibodies: anti-IκBα (C-21) rabbit polyclonal antibody sc-371 (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), anti-β-actin monoclonal antibody, clone AC-15 A5441 (1:10,000 dilution; Sigma-Aldrich), anti-Melan-A mouse monoclonal antibody, clone A103 (1:1,000 dilution; Novocastra lab., Newcastle upon Tyne, UK), anti-tyrosinase mouse monoclonal antibody (T311) sc-20035 (1:1,000 dilution; Santa Cruz Biotechnology) or monoclonal anti-Flag M2 antibody F 1804 (1:1,000 dilution; Sigma-Aldrich). αPep13h antibody against the C-terminus of human gp100 was kindly provided by Dr. M. Marks (University of Pennsylvania, Philadelphia, PA). Membranes were then washed with PBS, 0.1% Tween-20 and incubated with the following secondary antibodies: either goat anti-mouse IgG-HRP sc-2005 (1:1,000 dilution; Santa Cruz Biotechnology) or anti-rabbit IgG-HRP #7074 (1:1,000 dilution; Cell Signaling, Danvers, MA). If needed, membranes were stripped with the Restore Western blot Stripping buffer (Thermo Fisher Scientific, Waltham, MA) before reprobing. Immunoreactivity was revealed using the SuperSignal West Femto or West Pico chemiluminescent substrates (Thermo Fisher Scientific) followed by exposure to Hyperfilm ECL (GE healthcare).

CTL clones: stimulation conditions and recognition assay based on IFN-γ production

CTLs (3 x10^5) were cultured in 24-well plates with 1 x10^6 irradiated allogeneic EBV-B feeder cells (LG2-EBV) and stimulated with EBV-B cells (3x10^5) previously incubated for 1 hr at 37°C with 1 μg/ml of peptide and washed. All peptides were >90% pure, as indicated by analytical high-pressure liquid chromatography.

Tumor cells were tested for their ability to stimulate the production of IFN-g by CTL clones. 10,000 cells of CTL clones Melan-A_{26-35}/HLA-A2 or $gp100_{209-217}$/HLA-A2, or 30,000 cells of CTL clone $tyrosinase_{369-377}$/HLA-A2 were added in the microwells containing 20,000 tumor cells, in a total volume of 200 μl of complete IMDM. After overnight culture, IFN-γ released in the supernatant was measured by ELISA using reagents from Medgenix Diagnostics-Biosource (Fleurus, Belgium).

Cytokine production assay

Melanoma cells (1 x 10^6) were seeded into 75 cm^2 flasks and supernatants collected after 72 h were analyzed for IL-1α and IL-1β production using Bio-Plex Human Cytokine assays (Bio-Rad).

Gene expression microarrays

We examined the gene expression profile of 5 melanoma cell lines (DDHK62-MEL, LB2259-MEL, LB2586-MEL, LB2117-MEL, EB81-MEL) treated with IFN-α 1,000 U/ml, IFN-γ 100 U/ml or TNF-α 10 ng/ml for 72 hours. Cells were collected at rest or after stimulation with cytokines. RNA was extracted using the guanidinium isothiocyanate / cesium chloride procedure. Microarray assays were conducted according to the Affymetrix Genechip Expression Analysis Technical manual. Briefly, biotin-labeled antisense cRNA was prepared from 5 μg of total RNA using the one-cycle target labeling procedure, and hybridized to HG-U133A2.0 gene chips (Affymetrix, Santa Clara, CA), which were then washed and stained with the Fluidics Station 450 (Affymetrix), and scanned on the Gene Chip Scanner 3000 (Affymetrix). Data acquisition and processing were conducted with Affymetrix Genechips Operating Software, Excel and Access softwares. We compared the profiles of the 5 cell lines observed before and after cytokine treatment, focusing on genes that are relevant to antitumoral immune responses (Supporting Information Fig. 1). The Affymetrix data were deposited in GEO under accession number GSE19428.

Results

Exploring the effect of cytokines on melanoma cells, we compared the gene expression profiles of 5 human melanoma lines treated over 72 hours with IFN-α, IFN-γ or TNF-α. Both IFN-γ and TNF-α downregulated genes *MLANA*, which encodes melanoma differentiation antigens recognized by CTL [88], and *MATP/AIM1,* which codes for a membrane-associated transporter protein, another melanoma antigen recognized by autologous T cells [458]. These results are in line with published data showing reduced expression of *MLANA, TRP-1, tyrosinase* and *gp100* in melanoma cells incubated with IFN-γ or TNF-α [459, 460] and the absence of effect with IFN-α [461]. We sought to extend our study to another inflammatory cytokine, namely IL-1. There are three founding members of the IL-1 gene family, two of which are agonists, IL-1α and IL-1β, and the third is IL-1 receptor antagonist (IL-1Ra) (reviewed in Ref. [462]). IL-1α and IL-1β bind the same membrane-bound type 1 IL-1 receptor (IL-1R1), which then associates with the IL-1-receptor accessory protein (IL-1RAcP) to form a complex that allows intracellular signaling. There is also a type-2 IL-1 receptor (IL-1R2) that lacks an intracellular signaling domain and, therefore, functions as a decoy receptor. Along with stimulating differentiation and function of immune surveillance cells, IL-1 contributes to increased tumor invasiveness, metastasis and angiogenesis in chronic inflammatory conditions (reviewed in Ref. [463]).

Downregulation of MITF-M by interleukin-1β

The expression of genes *MLANA* and *MATP/AIM1* is positively regulated by the melanocyte-specific isoform of microphthalmia-associated transcription factor (MITF-M) [86, 90]. We, therefore, quantified *MITF*-M expression in two melanoma cell lines, LB2259-MEL and CP50-MEL, treated with IL-1β (Fig. 1). An important and sustained reduction of *MITF*-M expression was observed, with a maximal effect after 4 hours of treatment. This kinetics was similar to that observed for the downregulation of *MITF*-M by TNF-α (Fig. 1), while the effect of IFN-γ took longer and was maximal only after 3 days (Fig. 1). IFN-α had no effect on *MITF*-M expression in these cells.

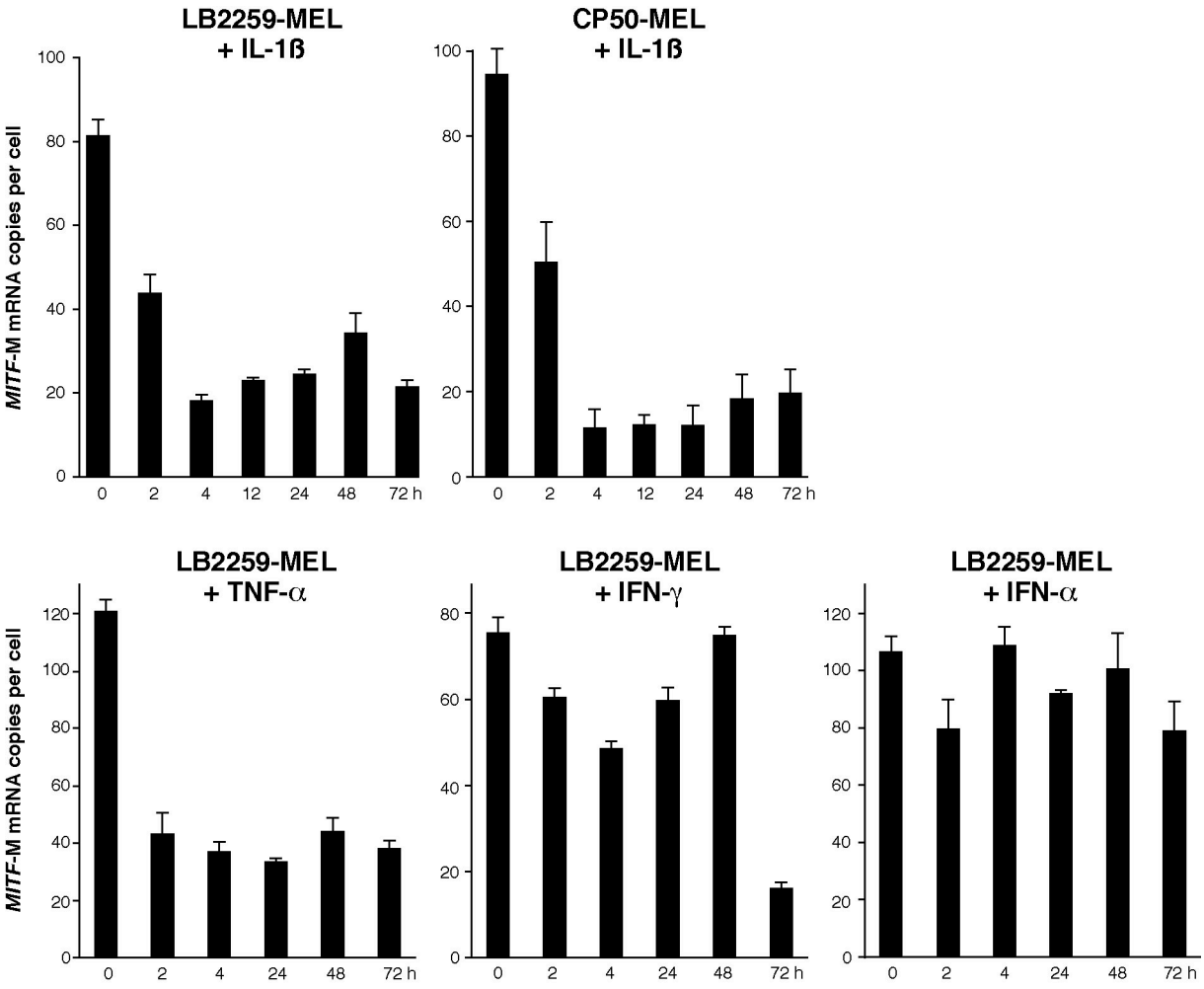


Figure 1. Time course of *MITF*-M expression in melanoma cell lines treated with cytokines. Melanoma cell lines LB2259-MEL or CP50-MEL were incubated for the indicated times with IL-1β (10 ng/ml), TNF-α (10 ng/ml), IFN-γ (100 U/ml), or IFN-α (1000 U/ml). Transcript levels of *MITF*-M and *ACTB* (used as an internal control) were evaluated by quantitative RT-PCR. Data (means ± SE) for IL-1β, IFN-α and TNF-α were obtained from three independent experiments, whereas data for IFN-γ were obtained from one experiment, with triplicate incubations.

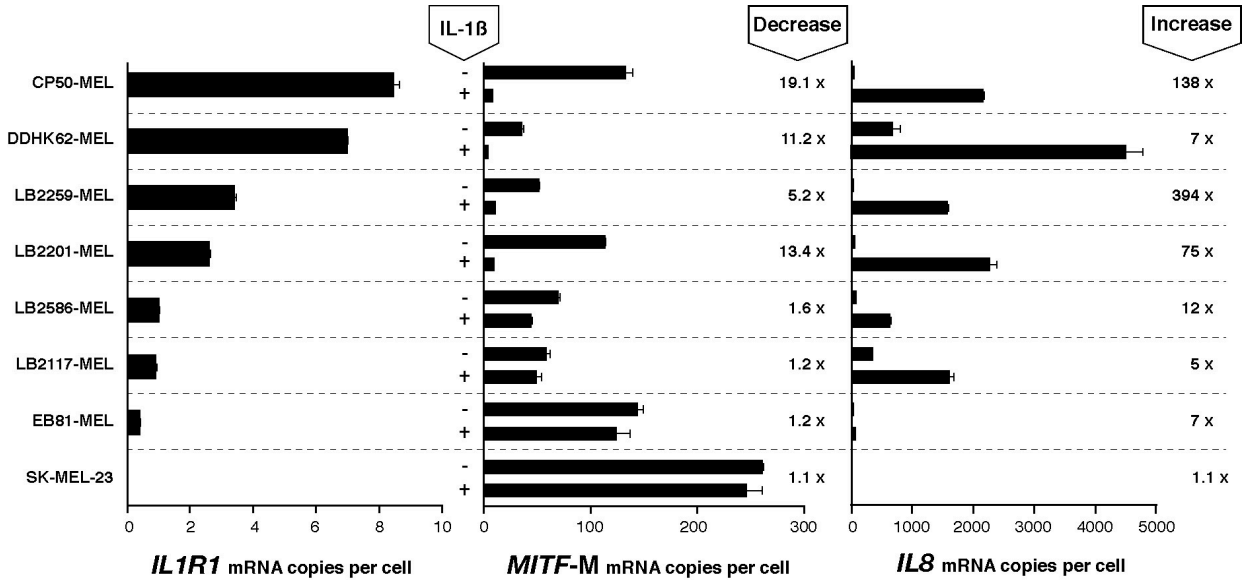


Figure 2. IL-1β downregulates the expression of *MITF*-M in some melanoma cell lines. *MITF*-M repression after 4 h of IL-1β treatment is observed in melanoma cell lines expressing high levels of IL-1 receptor transcripts. Transcript levels of *IL1R1*, *MITF*-M, *IL8* and *ACTB* (used as an internal control) were evaluated by quantitative RT-PCR in triplicate. Each value represents mean ± SE.

To extend our observations, we treated a panel of melanoma cell lines with IL-1β. We observed a wide range of *MITF*-M inhibition, with about one third of the lines being not responsive to IL-1β. As shown in Fig. 2, the extent of *MITF*-M inhibition appeared to be correlated to the expression of gene *IL1R1* encoding the IL-1 receptor. We also observed a correlation between *MITF*-M inhibition and the expression of the *IL8* gene, known to be induced after activation of the IL-1 receptor and encoding chemokine CXCL8 produced by some melanoma cell lines (Fig. 2) [464, 465].

Signaling through IL-1R1 involves the NF-κB and JNK pathways. We inhibited NF-κB activation in melanoma cells by expressing the super-repressor of IκBα, IκBαM. This mutant IκBα exerts a permanent inhibition of NF-κB because it cannot be phosphorylated and is, therefore, not degraded by proteasomes. LB2259-MEL cells transduced with retroviruses coding for IκBαM were treated with IL-1β for 4h. The levels of MITF-M transcripts were only 1.6-fold lower, as compared to 4.6-fold lower in control cells (Fig. 3*a*). This result confirmed the NF-κB involvement in *MITF*-M regulation by IL-1β.

We investigated the JNK pathway by treating LB2259-MEL cells with the ATP-competitive inhibitor of JNK, SP600125. We observed that the levels of *MITF*-M transcripts were 2.2-fold lower, while they were 6.7-fold lower in the absence of inhibitor, confirming the implication of the JNK pathway (Fig. 3*b*).

Finally, we explored whether the NF-kB and the JNK pathways had additive effects. When IκBαM-expressing LB2259-MEL cells, which exhibited a 2.1-fold lower expression of *MITF*-M following IL-1β treatment, were treated with the JNK inhibitor, no effect of IL-1ß on *MITF*-M expression could be detected (p-value = 0.35, unpaired *t*-test; Fig. 3*c*). We concluded that the IL-1ß-mediated *MITF*-M downregulation in *IL1R1*-expressing melanoma cell lines is NF-κB and JNK-dependent, and that inactivation of both pathways abrogates the repressive effect of IL-1β.

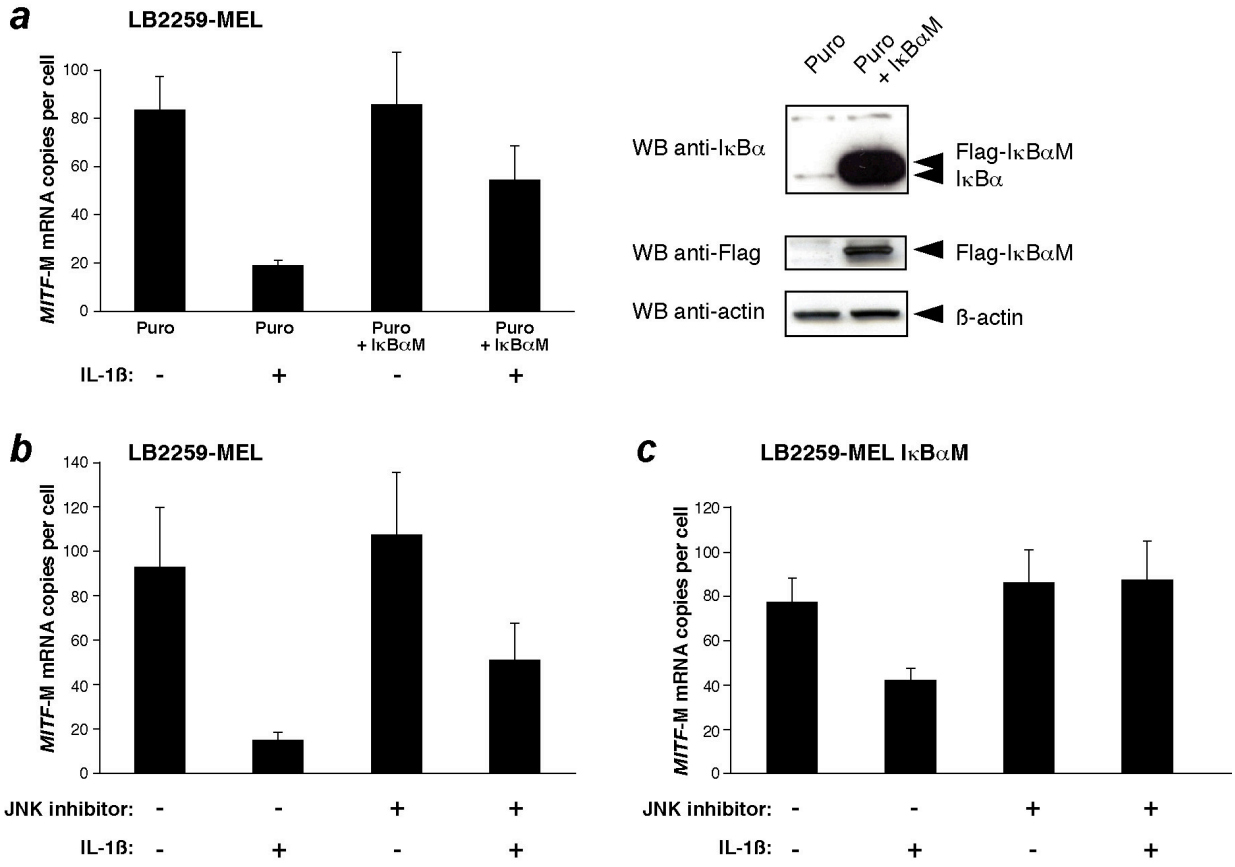


Figure 3. JNK and NF-κB pathways involved in the downregulation of *MITF*-M by IL-1β. *(a)* Stable expression of IκBαM, a repressor of NF-κB activation, decreases IL-1β-induced downregulation of *MITF*-M. LB2259-MEL cells were transduced with retroviruses encoding puromycin resistance and a flagged IκBαM, or puromycin resistance alone. Stable puromycin-resistant cell populations were obtained and treated with IL-1β (10 ng/ml) for 4 h. *MITF*-M expression was measured by quantitative RT-PCR and normalized to *ACTB* expression (means ± SD of 5 samples from 2 experiments). Western blot analyses were carried out on whole cell protein extracts with antibodies against IκBα, the Flag epitope, and β-actin. *(b)* JNK inhibition decreases IL-1β-induced downregulation of *MITF*-M. LB2259-MEL cells were pretreated with JNK inhibitor SP600125 (25 μM) for 1h before the addition of IL-1β. *MITF*-M expression was measured 4h later and normalized to *ACTB* expression (means ± SD of 6 samples from 4 experiments). *(c)* Effect of inactivating both the NF-κB and JNK pathways on *MITF*-M repression by IL-1β. LB2259-MEL cells expressing IκBαM were treated with SP600125 for 1h before the addition of IL-1ß. *MITF*-M expression was measured 4h later and normalized to *ACTB* expression (means ± SD of 5 samples from 3 experiments).

IL-1β reduces the recognition of melanoma cells by CTL against melanocyte differentiation antigens

Since IL-1β decreases the expression of *MITF*-M and MITF-M activates the expression of melanocyte differentiation genes such as *MLANA, tyrosinase* and *gp100*, we tested the effect of IL-1β on the expression of these genes. For two melanoma lines displaying *MITF*-M down-regulation by IL-1β, namely LB2259-MEL and CP50-MEL, culture over 4 days in the presence of IL-1β lead to a reduction of MLANA, tyrosinase and gp100 transcripts and proteins (Fig. 4*a*). This resulted in a decreased recognition by CTL clones recognizing MLANA or tyrosinase antigenic peptides (Fig. 4*b*). Surprisingly, no effect was observed with a CTL clone recognizing a gp100 peptide (Fig. 4*b*), even though the amount of protein gp100 was clearly decreased after IL-1β treatment (Fig. 4*a*).

For melanoma cells SK-MEL-23, which do not express *IL1R1* (Fig. 2), IL-1β had no effect on the expression of melanocyte differentiation genes and proteins (Fig. 4*a*), and no effect on the recognition of melanocyte differentiation antigens by CTL (Fig. 4*b*). We conclude that IL-1β can contribute to decreasing the sensitivity of melanoma cells to recognition by CTL targeting melanocyte differentiation antigens.

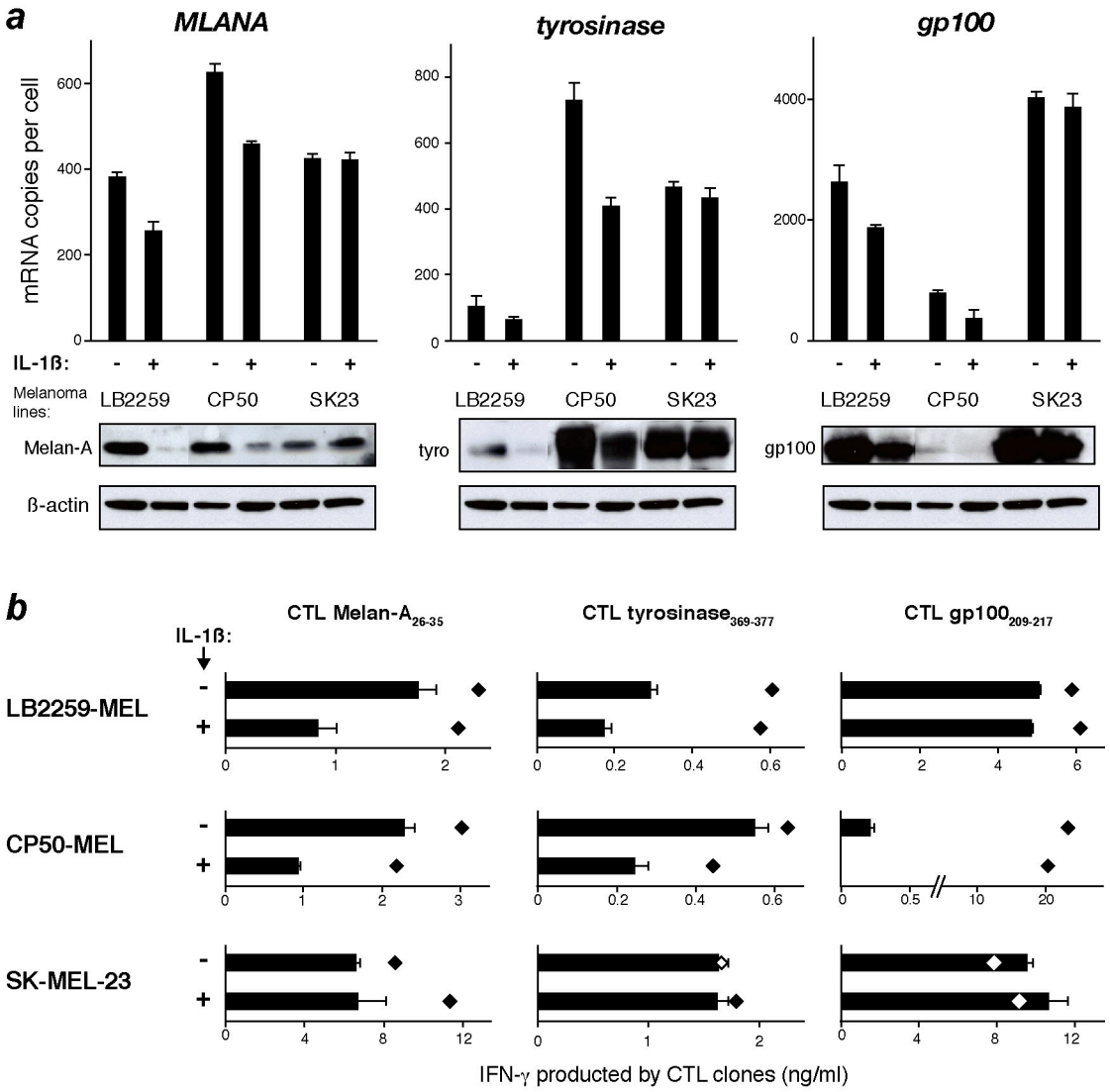


Figure 4. IL-1β downregulates expression of melanocyte differentiation genes. *(a)* Effect of IL-1β on mRNA and protein levels of melanocyte differentiation genes. The indicated melanoma cell lines were treated with IL-1β (10 ng/ml added every day) for 4 days. *MLANA*, *tyrosinase*, *gp100* and *ACTB* (used as internal control) mRNA levels were analyzed by quantitative RT-PCR in triplicate. Each value represents mean ± SE. Protein extracts (25 μg) were analyzed by Western blotting using the indicated antibodies. *(b)* HLA-A2-positive melanoma cell lines were treated with IL-1β for 4 days (10 ng/ml added every day) and cocultured overnight with 3 HLA-A2-restricted CTL clones. IFN-γ produced by activated CTL was measured by ELISA. Results are means ± SD of triplicate measurements. Diamonds indicate IFN-γ production obtained when 5 μg/ml of the relevant antigenic peptide was added to the melanoma cells 1h before CTL addition, as a control for a maximum CTL activation.

Bioactive IL-1α and IL-1β are produced by MITF-M-negative melanoma cells

We explored the possibility that some melanoma tumors might not express *MITF*-M, and therefore resist attack by CTL recognizing melanocyte differentiation antigens, by producing IL-1β. Indeed, production of IL-1 cytokines by melanoma lines has been previously reported [466, 467]. In our collection of 205 melanoma cell lines, we observed that 19% did not express gene *MLANA*. Expression of *MLANA* was correlated with that of *MITF*-M, as 19 out of 19 *MLANA*-positive cell lines expressed *MITF*-M whereas 20 out of 22 *MLANA*-negative cell lines did not express *MITF*-M (<1 *MITF*-M mRNA copy per cell). Thus, we considered that 17% of the cell lines in our collection were *MITF*-M-negative. Screening the supernatants of 11 MITF-M-negative lines for the production of IL-1α or IL-1β by Bio-Plex immunoassay, we found 9 positive supernatants with total IL-1 levels ranging from 20 to 7,240 pg/ml. These supernatants reduced by > 20% *MITF-M* expression in LB2259-MEL cells. For 8 supernatants, the effect was blocked by the addition of the IL-1 receptor antagonist IL-1Ra. In parallel, we tested 19 supernatants of MITF-M-positive lines and found that total IL-1 was either undetectable or present at levels below 12 pg/ml. We conclude that about 13% of human melanoma lines are MITF-M-negative and produce bioactive IL-1.

In this small screening, melanoma lines EB16-MEL and KUL84-MEL were the highest IL-1 producers. In Table 1, these two *MITF*-M-negative lines are compared to two *MITF*-M-positive lines. EB16-MEL cells transcribe the *IL1B* gene at a high level, and secrete immunoreactive IL-1β in the culture medium. It is therefore likely that EB16-MEL cells have an activated NLRP3 inflammasome pathway, which is required to activate caspase-1 and process the pro-IL-1β gene product into mature and secreted IL-1β. For KUL84-MEL cells, *IL1B* transcription is low and no IL-1β could be detected in the culture medium (Table 1). However, these cells, as well as EB16-MEL, transcribe gene *IL1A* and secrete IL-1α (Table 1). Two genes activated by IL-1 cytokines, *IL8* and *CCL20*, were 10-1,000-fold more expressed in EB16-MEL and KUL84-MEL than in the control melanoma lines, suggesting an autocrine activity of the produced IL-1 (Table 1). Finally, the supernatants of the two IL-1 producing lines inhibited *MITF*-M expression and activated *IL8* expression in melanoma cells LB2259-MEL (Fig. 5). These effects were blocked in the presence of IL-1Ra (Fig. 5), and dose-response curves of recombinant IL-1α or IL-1β indicated specific activities matching those observed in the melanoma supernatants (Fig. 5), suggesting that IL-1 were the main soluble factors responsible for *MITF*-M downregulation.

To verify that melanoma cells could also produce IL-1 *in vivo*, we analyzed frozen tumor tissues corresponding to the 4 melanoma lines shown in the first part of Table 1. Expression levels of *MITF*-M, *IL1A*, *IL1B*, and of the IL-1-induced genes *IL8* and *CCL20* correlated with those observed in the melanoma lines (Table 1). Even though other cells than the melanoma cells could contribute to IL-1 production in these tumors, the results strongly suggest that the melanoma cells were producing IL-1 *in vivo*.

	Gene expression (mRNA copies/cell)					Cytokine production (pg/mL)	
	MITF-M	*IL1β*	*IL1α*	*IL8*	*CCL20*	IL-1β	IL-1α
Melanoma cell lines							
EB16-MEL	0.0 ± 0.0	1380.7 ± 240.9	112.0 ± 47.7	1683.6 ± 657.8	14.8 ± 8.5	2050 ± 310	5187 ± 113
KUL84-MEL	0.0 ± 0.0	18.4 ± 6.9	142.7 ± 11.4	631.7 ± 37.2	22.7 ± 2.8	<3	1327 ± 37
LB2259-MEL	59.1 ± 14.4	0.1 ± 0.0	0.0 ± 0.0	2.1 ± 1.4	0.0 ± 0.0	<3	<2
LB2439-MEL	152.3 ± 28.2	0.0 ± 0.0	0.0 ± 0.0	6.8 ± 1.7	0.2 ± 0.1	<3	<2
Tumor tissues							
EB16	0.0 ± 0.0	347.4 ± 97.1	0.3 ± 0.1	261.8 ± 66.3	4.9 ± 1.9		
KUL84	0.1 ± 0.1	15.2 ± 11.7	4.8 ± 1.6	57.9 ± 6.8	38.3 ± 11.7		
LB2259	45.7 ± 1.3	0.3 ± 0.1	0.0 ± 0.0	0.4 ± 0.1	0.1 ± 0.0		
LB2439	54.2 ± 6.6	0.5 ± 0.1	0.0 ± 0.0	0.4 ± 0.1	0.2 ± 0.0		

Table 1. Transcription and production of IL-1 by melanoma cell lines and tumors.
mRNA levels of *MITF*-M, *IL1B*, *IL1A*, *IL8*, *CCL20* and *ACTB* (used as an internal control) were evaluated by quantitative RT-PCR in melanoma cell lines and corresponding melanoma tissues. For each cell line or tissue, results were obtained from 3 independent RNA preparations, except for tissues LB2259 and LB2439 where a single cDNA was analyzed in 3 independent PCR measurements. Each value represents mean ± SD. The levels of secreted IL-1α and IL-1β were measured in three independently collected supernatants with the bead-based Bio-Plex immunoassay. Each value represents mean ± SD.

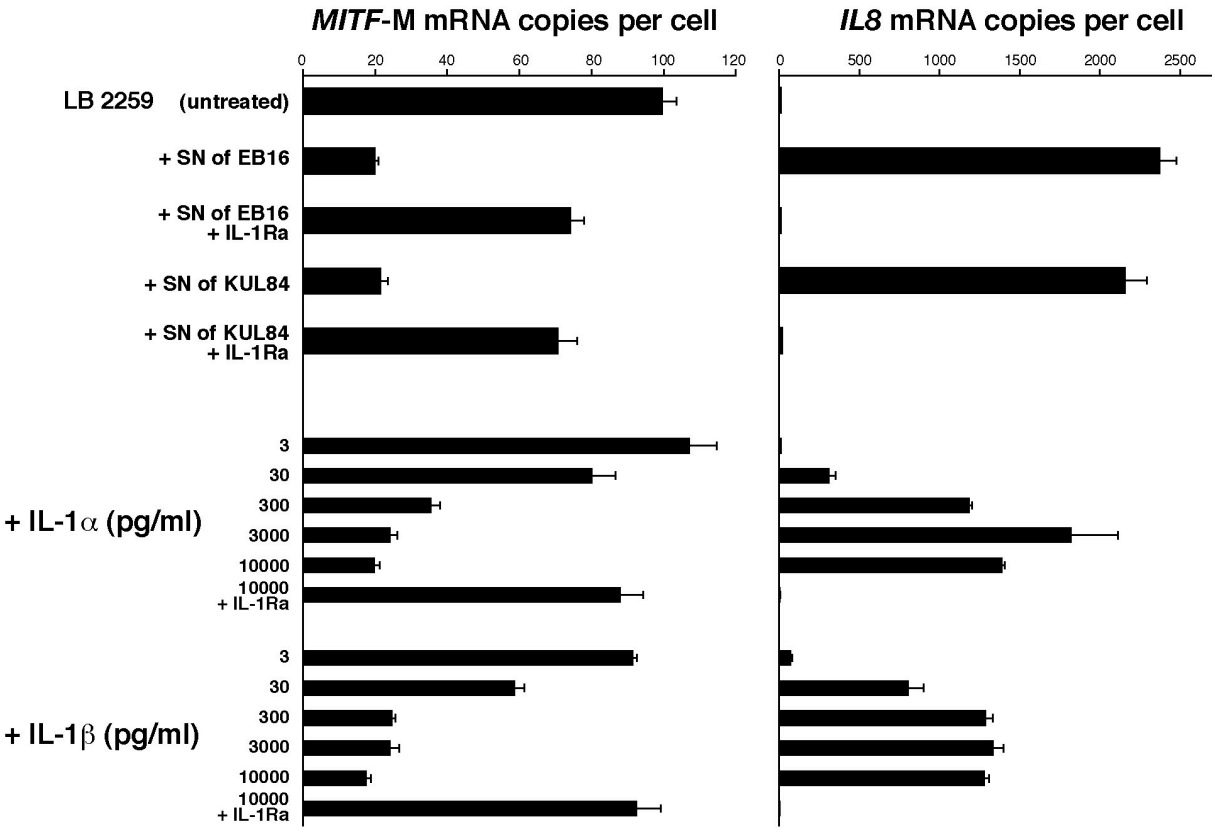


Figure 5. Bioactive IL-1 secreted by melanoma cell lines. LB2259-MEL cells were incubated 4 h with culture supernatants (SN) of the indicated melanoma cell lines, in the presence or absence of 10 μg/ml of IL-1Ra, or with increasing concentrations of recombinant human IL-1α or IL-1β. The expression levels of *MITF*-M and *IL8*, as a control of the biological activity of the IL-1 cytokines, was assessed by quantitative RT-PCR and normalized to *ACTB* expression. Each value represents mean ± SE from one representative experiment with triplicate incubations with supernatants and duplicate incubations with recombinant IL-1.

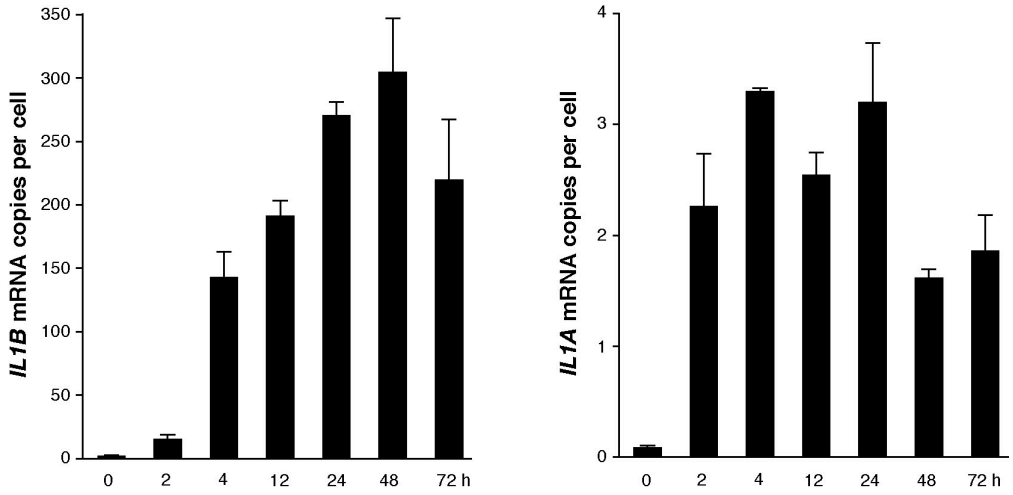


Figure 6. IL-1β upregulates its own expression and expression of *IL1A* in CP50-MEL cells. Time course of *IL1B* and *IL1A* upregulation by IL-1β. CP50-MEL melanoma cells were treated with IL-1β (10 ng/ml) for the indicated time periods. Quantification of *IL1B* and *IL1A* transcripts normalized to those of *ACTB* shows means ± SE of three and two independent experiments, respectively.

Considering that IL-1α and IL-1β can activate their own transcription through functional NF-κB sites present in the promoters of both genes [468, 469], we explored the possibility of a permanent IL-1 autocrine loop in melanoma cells. Melanoma cells CP50-MEL express gene *MITF*-M and do not express genes *IL1A* and *IL1B*. In the presence of IL-1β, genes *IL1A* and *IL1B* were induced (Fig. 6). When the cells were deprived of IL-1β after one week of exposure, the transcriptional activation of *IL1B* lasted 3 days but then declined to background level (data not shown). Therefore, other factors than the IL-1R1 transduction pathway are necessary for the permanent *IL1B* gene activation that is present in some melanoma cells.

Nevertheless, in KUL84-MEL cell line with established IL-1 cytokine production, treatment with the IL-1Ra receptor antagonist for one week lead to 2-20 fold lower levels of *IL1A*, *IL1B* and *IL8* transcripts, but to a weak increase in *MITF-M* expression (Supporting Information Fig 2).

Discussion

It is known that murine and human melanoma cells incubated with cytokines such as TNF-α, IFN-γ and TGF-β, have reduced mRNA levels of *TYR*, *MLANA*, *TRP1*, and *SILV* [459, 460, 470, 471]. According to our results, this reduction most likely originates from the down-regulation of transcription factor MITF-M by these cytokines. We provide the first evidence that IL-1α and IL-1β are additional cytokines that reduce the expression of *MITF*-M. It is noteworthy that IFN-α, which is currently used to treat melanoma patients, did not affect *MITF*-M expression. This supports further development of this cytokine as an immunological adjuvant combined with melanoma vaccines.

The activation of NF-κB that is observed in many cancers including melanoma, regulates the expression of genes that prevent apoptosis and promote malignant proliferation, metastasis, or angiogenesis [472]. NF-κB is usually described as a transcriptional activator. We show here that genes encoding melanocyte differentiation antigens are repressed by NF-κB and JNK activation. The mechanism of this repression remains to be elucidated. The signal induced by IL-1β could act on the *MITF*-M promoter. We tested this promoter in front of a reporter *luciferase* gene but failed to detect any repression after IL-1β treatment (data not shown)**.** Alternatively, the signal could increase the MITF-M transcript degradation rate. One possibility is that IL-1β and TNF-α induce miRNAs that target the MITF-M mRNAs. In line with this hypothesis, a recent study describes the NF-κB-dependent induction of miRNA-146 targeting the 3'UTRs of the mRNAs encoding TNF receptor-associated factor 6 (TRAF6) and IL-1 receptor-associated kinase 1 (IRAK1), two proteins involved in IL-1R1 signaling [473]. Another miRNA, miRNA-137, was reported to reduce *MITF*-M expression in melanoma cell lines [41]. We could not document an induction of miRNA-137 by IL-1β (data not shown).

We report here that the level of expression of the IL-1 receptor (*IL1R1)* is an important parameter to determine the ability of melanoma cells to respond to IL-1. However, when we expressed *IL1R1* in SK-MEL-23 cells, we observed the induction of *IL8* after IL-1β treatment, but not the repression of *MITF*-M (data not shown). This observation suggests that there are distinct mechanisms downstream of the NF-κB and JNK pathways that lead either to *IL8* activation or to *MITF*-M down-regulation.

The critical role of an activated MAPK pathway in melanoma growth and progression has prompted attempts to develop inhibitors of this pathway [474]. *In vitro*, these inhibitors consistently enhance *MLANA* or *SILV* transcript levels, as reported for 70% of the melanoma cell lines treated with the ERK inhibitors PD98059 and U0126 [271]. We found that in the LB2259-MEL human melanoma cells the repressive effect of IL-1β on *MITF*-M expression was dependent on both NF-κB and JNK activation. *MITF*-M down-regulation induced by IL-1β was not mediated by ERK activation since we found that inhibitors PD98059 and U0126 did not counteract this effect (data not shown). In agreement with our findings, a study on murine melanoma cells B16 showed that the inhibition of *TYR* expression by TNF-α was

mainly mediated by NF-κB activation [459]. Given the overlap in signaling pathways employed by TNF-α and IL-1 cytokines (reviewed in Ref. [475]), we suggest that NF-κB and JNK are important mediators in the observed down-regulation of differentiation genes in melanoma cells incubated with these inflammatory cytokines. Detailed signaling mechanisms should therefore be explored further.

The decreased expression of melanocyte differentiation genes after IL-1β treatment should decrease the recognition of melanoma cells by T lymphocytes specific for differentiation antigens. This is what we found with two antimelanoma CTL clones, including a CTL recognizing the immunodominant peptide Melan-A/MART1$_{26-35}$, presented by HLA-A2. In a previous study, IFN-γ was shown to reduce by up to 78% the cytotoxicity of anti-Melan-A/MART-1$_{26-35}$ CTL, despite the stimulatory effect of IFN-γ on MHC class I expression and antigen presentation [460]. This reduction is most likely due to the induction of the immunoproteasome, which does not process the Melan-A/MART-1$_{26-35}$ peptide [476]. Data of a recent study suggest that IL-1β is not able to induce the immunoproteasome [477]. Therefore, we conclude that the main effect of IL-1β on CTL activation was to reduce tyrosinase, Melan-A/MART-1 and gp100 protein levels. Surprisingly, the activation of CTL directed against the gp100$_{209-217}$ antigen on LB2259-MEL cells was not affected by IL-1β, although there was a clear decrease in the amount of gp100 protein. One explanation would be that saturating levels of the gp100 antigenic peptide may be available to CTL, even after IL-1β treatment. Alternatively, IL-1β may promote post-proteasomal processing and intracellular peptide transport that may be required for recognition of the gp100 antigen, as there is some evidence that peptide gp100$_{209-217}$ is not directly produced by the proteasome [478].

Cytokines are produced by immune, stromal and inflammatory cells, but also by tumor cells. We found that IL-1α and IL-1β are clearly secreted in large amounts by some melanoma cells that do not express *MITF*-M. This is in line with data indicating that soluble proteins secreted by antigen-negative melanoma cells, such as cytokine oncostatin M, are capable of down-regulating the expression of melanocyte differentiation genes [479, 480]. It is also in line with the observation that *IL-1* is up-regulated in melanoma samples and that patients with IL-1 producing tumors have generally bad prognoses [481]. At first sight, interference with the IL-1 signaling in melanoma might, therefore, appear as a meaningful strategy to overcome the immunotherapeutic problem of low antigen expression. In an attempt to increase the expression of melanocyte differentiation antigens, we incubated melanoma cells that express very low amounts of MITF-M and produce IL-1 with the IL-1Ra receptor antagonist. We observed that this treatment was not sufficient to reactivate significantly *MITF*-M expression. We have, therefore, preliminary evidence that the silencing of differentiation genes in IL-1-producing melanoma cells is difficult to reverse. The difficulties in restoring the expression of differentiation genes once it is lost have been previously reported and the establishment of a nuclear context leading to their transcriptional repression has been proposed [482, 483] .

In our experience, an activated IL-1 pathway is an event that is detected in only about 13% of melanoma cell lines. By screening public microarray databases, we observed a similar frequency of 13% among large series of melanoma cell lines, and an even lower frequency of 4% among melanoma metastases (data not shown). Nevertheless, these data do not exclude the possibility of local and hardly detectable IL-1 production either by melanoma cells themselves or by stromal macrophages and fibroblasts [368, 484], resulting in downregulation of differentiation antigens in neighboring tumor cells. Therefore, additional attempts should be performed in order to determine spatial and temporal changes in expression of IL-1 cytokines and differentiation antigens preferably at the cellular level and at different stages of melanoma progression. This could lead to a better understanding of the relevance of IL-1 production within melanoma tissues.

Acknowledgements

This work was supported by grants from the Fondation contre le Cancer (Belgium), the Fonds de la Recherche Scientifique Médicale (Belgium), the Fonds National de la Recherche Scientifique (Belgium) and the Fondation Salus Sanguinis (Belgium). J.V. is a Research Fellow with the Fonds National de la Recherche Scientifique (Belgium). O.K. was supported by a Télévie Grant from the Fonds National de la Recherche Scientifique (Belgium) and the Centre du Cancer (Cliniques Universitaires Saint-Luc, UCL, Belgium).

We thank Dr. Thierry Boon and Dr. Benoît Van den Eynde for critical reading of the manuscript. We also thank Dr. S. Shibahara (Tohoku University School of Medicine, Japan) for providing reporter constructs for the M promoter of the human *MITF* gene, Dr. M. Martin (Justus-Liebig-University Giessen, Germany) and Dr. W. Falk (University of Regensburg, Germany) for the *IL1R1* expression vector and Dr. B. Lethé for providing the reagents for *tyrosinase* and *gp100* quantitative PCR. We thank Suzanne Depelchin for excellent editorial assistance, Jerôme Degueldre for samples and data managing, Magdalena Swinarska, Thérèse Aerts and Vinh Ha Thi for expert technical assistance.

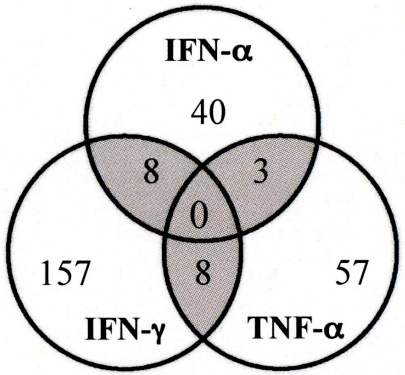
Supporting information.

Supplementary Table 1. Primers and probes used in quantitative RT-PCRs.

Gene	Sense primer	Antisense primer	Probe
CTCF	5'-CAT ggC AAG ACA TgC TgA T-3'	5'- TgC ACC TgT ATT CTg gTC TTC-3'	
BORIS	5'- CCC TCA TTC AgC ACC AgA A -3'	5'- CTg ATC CAC ACT TCT CCg AA -3'	5'- 6FAM - CCg ACA gAA gCA ACT TCT AAA CgC T - TAMRA -3'
MAGE-A1	5'- gCC gAA ggA ACC TgA CC -3'	5'- ACT ggg TTg CCT CTg TCg -3'	5'- 6FAM - TTC ATT gCC CAg CTC CTg CCC ACA CT -TAMRA -3'
MAGE-A2	5'- AAg TAg gAC CCg Agg CAC Tg -3'	5'- gAA gAg gAA gAA gCg gTC Tg -3'	5'- 6FAM - CAT TgA Agg AgA AgA TCT gCC TgT ggg TCT TC - TAMRA -3'
MAGE-A4	5'- CCA CTA CCA TCA gCT TCA CTT gC -3'	5'- CTT CTC ggA ACA Agg ACT CTg C -3'	5'- 6FAM - Agg CAA CCC AAT gAg ggT TCC AgC - TAMRA -3'
MAGE-B1	5'- CCC gAg CgA gCT TAA ggA gT -3'	5'- gTC AgA TTC ggT ACA TgA CAC Ag -3'	5'- 6FAM - Tgg TCg ggA gTC AAg gTg CCA CAT CTC CTg - TAMRA -3'
LAGE-2/NY-ESO-1	5'- CgC CTg CTT gAg TTC TAC C -3'	5'- CAC TgC gTg ATC CAC ATC AAC A -3'	5'- 6FAM - TCA gTA TgT TgC Cgg ACA CAg TgA ACT C - TAMRA -3'
ACTINB	5'- ggC ATC gTg ATg gAC TCC g -3'	5'- gCT ggA Agg Tgg ACA gCg A -3'	5'- 6FAM - TCA AgA TCA TTg CTC CTC CTg AgC gC - TAMRA -3'

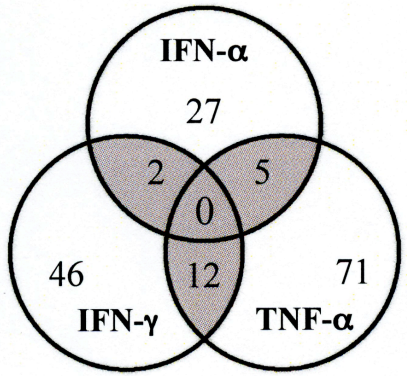
Supplementary Figure 1. Genes up-regulated or down-regulated by inflammatory cytokines in melanoma cell lines.

Up-regulated in 5 cell lines



		Genbank	fold IFN-α	fold IFN-γ	fold TNF-α
IFN-α IFN-γ					
IFI44L	interferon-induced protein 44-like	NM_006820	7-240	2-16	
HLA-C	major histocompatibility complex, class I, C	U62824	2-8	9-17	
HLA-F	major histocompatibility complex, class I, F	AW514210	2-4	6-8	
IFI27	interferon, alpha-inducible protein 27	NM_005532	2-1696	2-123	
IRF7	interferon regulatory factor 7	NM_004030	3-12	2-5	
TAP1	transporter 1, ATP-binding cassette	NM_000593	3-6	4-17	
TBC1D3	TBC1 domain family, member 3	AL136860	3-16	3-16	
TRIM22	tripartite motif-containing 22	AA083478	4-21	4-21	
IFN-α TNF-α					
BIRC3	baculoviral IAP-repeat-containing 3	U37546	2-21		30-1078
GIP2	interferon alpha-inducible protein	NM_005101	4-83		2-7
ISG20	interferon stimulated gene 20kDa	NM_002201	2-14		2-26
TNF-α IFN-γ					
C1S	complement component 1, s subcomponent	M18767		4-51	3-22
CTSS	cathepsin S	BC002642		9-29	9-80
CXCL11	chemokine (C-X-C motif) ligand 11	AF030514		3-13	4-125
CXCL11	chemokine (C-X-C motif) ligand 11	AF002985		3-89	34-547
CXCL2	chemokine (C-X-C motif) ligand 2	M57731		2-25	4-375
HLA-B	major histocompatibility complex, class I, B	D83043		6-15	2-11
HLA-C	major histocompatibility complex, class I, C	L42024		3-13	2-6
	Homo sapiens cDNA clone, expressed in placenta	AU157078		4-26	2-43

Down-regulated in 4 or 5 cell lines



		Genbank	fold IFN-α	fold IFN-γ	fold TNF-α
IFN-α IFN-γ					
	arrestin, beta 1	NM_004041	1-13	3-21	
	cDNA clone IMAGE:22392	T87225	0,4-25	2-40	
IFN-α TNF-α					
ITGB1BP3	integrin beta 1 binding protein 3	NM_014446	1-6		0,3-6
	hypothetical protein MGC5566	NM_024049	2-7		1-10
SOS2	son of sevenless homolog 2	BF692958	1-15		1-15
	cDNA clone DKFZp566B193	AL050047	1-6		2-9
	cDNA clone EUROIMAGE 295344	AL109707	1-14		1-24
TNF-α IFN-γ					
CSPG2	chondroitin sulfate proteoglycan 2 versican[1],	NM_004385		3-12	1-4
	DKFZP586A0522 protein	NM_014033		3-213	2-7
GALNT3	GalNAc-T3	BF063271		13-97	1-275
GYG2	glycogenin 2	U94364		3-47	1-70
MATP	membrane associated transporter	NM_016180		2-11	1-12
MATP	membrane associated transporter	BC003597		2-12	1-5
MLANA	melan-A	NM_005511		2-15	1-136
PCDH7	BH (brain-heart)-protocadherin	NM_002589		3-39	1-72

Five melanoma cell lines were treated for 72h with IFN-α, IFN-γ or TNF-α or left untreated, and their gene expression profiles were analyzed using Affymetrix HG-U133A2.0 gene chips. Six lists were generated, with genes whose expression was at least 2-fold higher or lower relative to untreated cells in at least 4 of the 5 cell lines. The number of genes in these lists is indicated in the Venn diagrams.

[1] Reduced expression of chondroitin sulfate proteoglycan 2 (CSPG2, versican) in the presence of IFN-γ (2- to 11-fold lower levels) and TNF-α (2- to 53-fold lower levels) was observed for 4 other transcripts (BF590263, D32039, R94644, BF218922).

Supplementary Figure 2 is represented in Results – Part III (Figure 2).

PART III.

Deciphering mechanisms of *MITF*-M repression in two melanoma cell lines secreting interleukins 1α and 1ß.

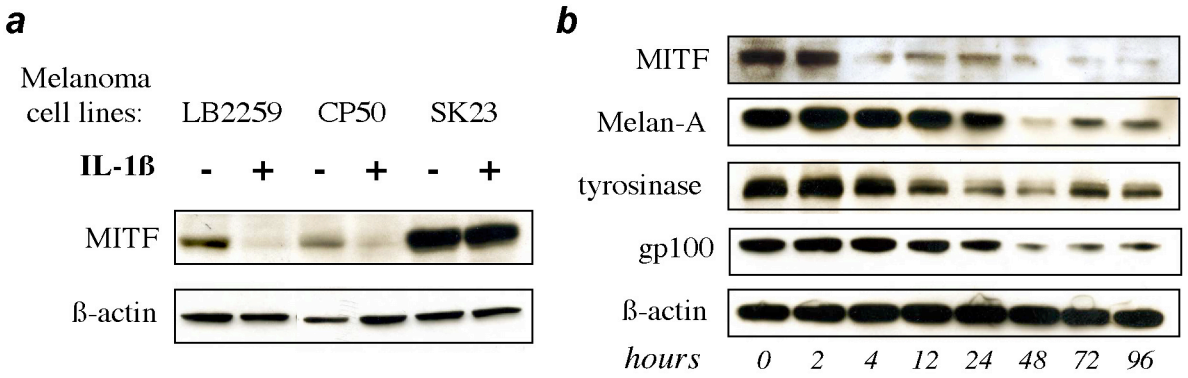


Figure 1. IL-1ß downregulates the expression of MITF and other melanocyte differentiation proteins. (*a*) Effect of IL-1ß on MITF protein levels. Indicated melanoma cell lines were treated with IL-1ß for 4 days (10 ng/mL added every day). Protein extracts (25μg) were analyzed by Western blot using anti-MITF antibody (Gentaur Ltd.) and anti-ß-actin antibody (used as internal control). (*b*) Time course of MITF, Melan-A, tyrosinase and gp100 expression in LB2259-MEL cells incubated for the indicated times with IL-1ß (10 ng/ml). Protein extracts (25μg) were analyzed by Western blot using indicated antibodies.

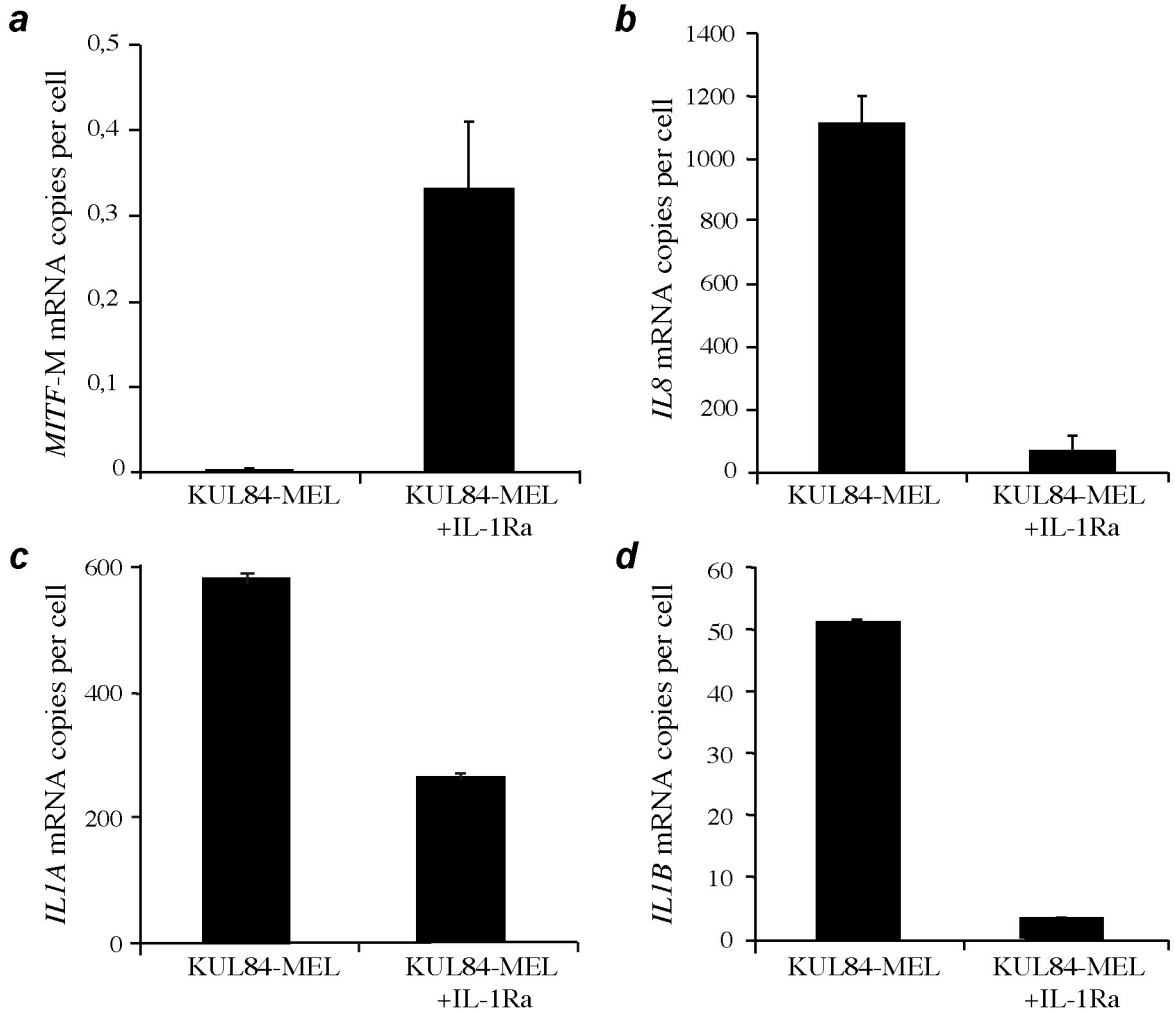


Figure 2. Incubation with IL-1Ra leads to a modest increase of *MITF*-M in KUL84-MEL cells. KUL84-MEL cells were incubated 7 days in the presence or absence of IL-1 receptor antagonist (IL-1Ra, 10 μg/ml). The expression levels of *MITF*-M, *IL1A*, *IL1B* and *IL8*, as a control of the biological activity of the IL-1 cytokines, were assessed by quantitative RT-PCR and normalized to *ACTB* expression. Each value represents mean ± SD from one representative experiment with triplicate incubations.

Our previous study and studies of other groups have demonstrated an autonomous production of IL-1α and IL-1β cytokines by some melanoma cell lines (Results - Part II). Moreover, we have estimated that about 13% of melanoma cell lines established at the Brussels Branch of the Ludwig Institute for Cancer Research exhibit simultaneous release of IL-1 cytokines and repression of *MITF*-M expression (Results - Part II). Furthermore, *MITF*-M-negative cell lines do not express MITF-M transcriptional target genes *MLANA*, *TYR* and *gp100* or express them at a low levels (0-1.4; 0-13.2; 0-2.5 mRNA copies per cell, respectively), as assessed by quantitative RT-PCR analysis performed on 10 melanoma cell lines secreting IL-1 cytokines and on two cell lines that do not produce them (data not shown). Furthermore, *MITF*-M-positive melanoma cell lines expressing *IL-1R1* showed reduced mRNA and protein expression levels of Melan-A, tyrosinase and gp100 concomitantly with *MITF*-M downregulation following treatment with recombinant IL-1β (Results - Part III, Fig. 1 and Results - Part II, Fig. 1 and 4). All together, these findings support a hypothesis that autonomous production of IL-1 cytokines might be responsible for initiation and/or maintenance of permanent MITF-M repression and downregulation of differentiation genes in a subset of melanoma cell lines. We decided, therefore, to explore the mechanisms of *MITF*-M repression in two melanoma cell lines producing high levels of IL-1.

Inhibition of IL1-R1 signaling by antagonist IL-1Ra weakly reactivates MITF-M expression

IL-1α and IL-1β stimulate their own and each other's transcription, and hence might, by triggering the IL-1R1 receptor, initiate and maintain a positive feed-forward loop of their own production. Such constitutive production of IL-1α and IL-1β by melanoma cell lines might lead to a permanent repression of *MITF*-M. *MITF*-M-negative KUL84-MEL and EB16-MEL cell lines produce IL-1 and express *IL1R1* at levels (3 and 2 mRNA copies per cell, respectively) associated with efficient IL-1 signaling (Results - Part II, Fig. 2). To assess the functional role of IL-1R1 activation in MITF-M repression, these cell lines were cultured for one week in the presence of the IL-1 receptor antagonist (IL-1Ra). IL-1Ra was used at a concentration of 10 μg/ml, which efficiently blocks IL-1R1-mediated activation of *IL8*, a transcriptional target of IL-1 (Results - Part II, Fig. 5). In KUL84-MEL cells, this treatment resulted in a weak reactivation of *MITF*-M expression (Results - Part III, Fig. 2*a*), whereas 2- to 20-fold lower levels of *IL1A*, *IL1B* and *IL8* transcripts were observed (Results - Part III, Fig. 2*b*, *c*, *d*). In EB16-MEL cells, the same treatment led to 2-3 fold lower levels of *IL1A*, *IL1B* and *IL8* transcripts, while *MITF-M* was not reactivated (data not shown). In conclusion, blocking of IL-1R1 signaling did not lead to considerable reactivation of *MITF*-M expression, whereas it resulted in reduced constitutive expression of *IL1A*, *IL1B* and *IL8*.

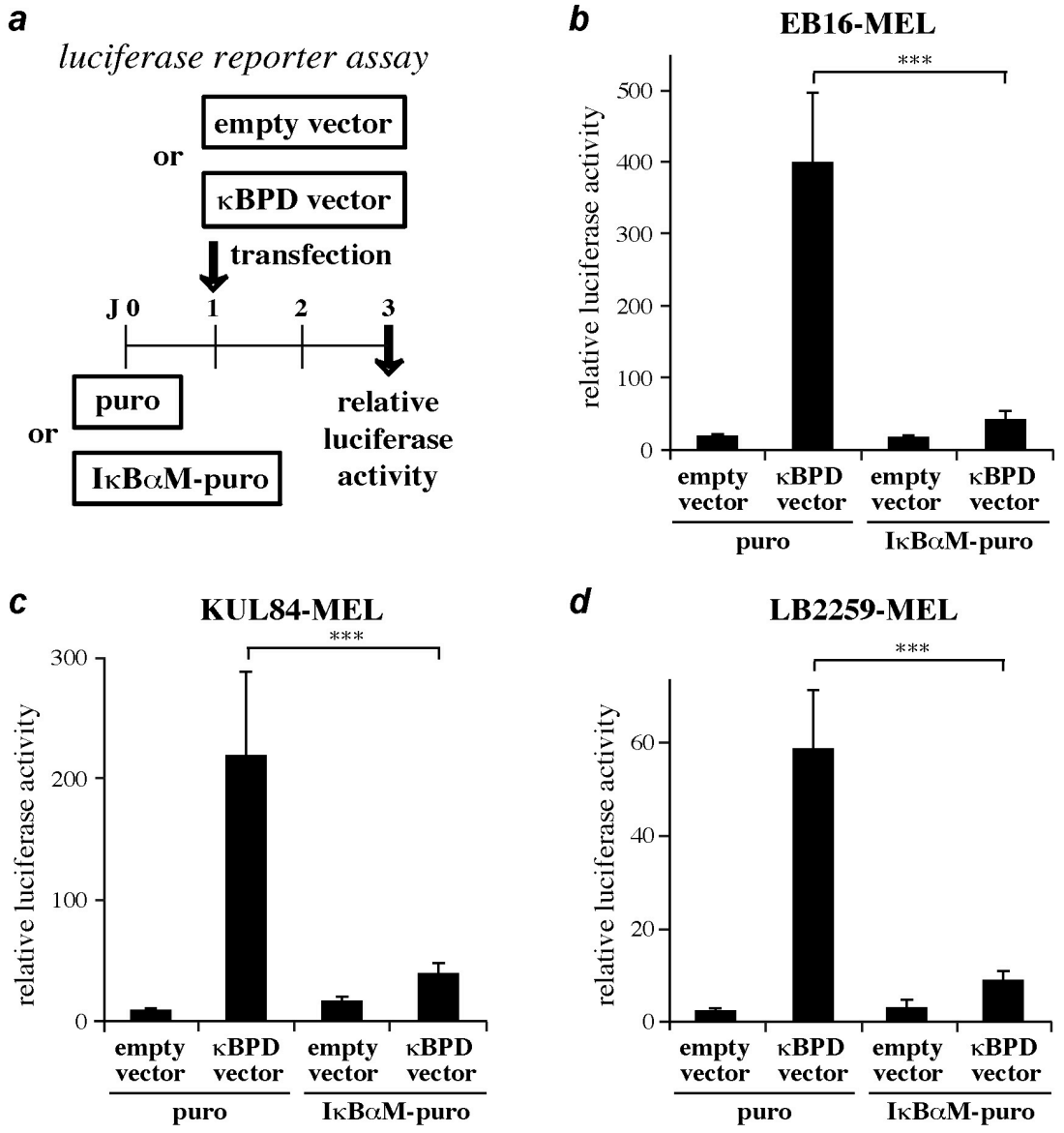


Figure 3. Constitutive NF-κB activity is inhibited by the stable expression of IκBαM in melanoma cell lines. (*a*) Melanoma cell lines were transduced with retroviruses encoding puromycin resistance and super-repressor IκBαM, or puromycin resistance alone. Stable puromycin-resistant cell populations were seeded in 12-well plates (100,000 cells per well) 24 hours before transfection using ExGen500 (Fermentas). Cells were transfected with 2,000 ng of either pGL3-Basic plasmid or reporter plasmid containing two palindromic NF-κB-binding sites (κBPD) upstream of the firefly luciferase coding sequence (both vectors were kindly provided by L. Dumoutier, LICR) as well as with 1 ng of pGL4-75 vector (containing the Renilla luciferase gene) used as internal control. After 48 hours, cells were lyzed and both luciferase activities were assayed with the Dual Luciferase Reporter Assay System kit (Promega). NF-κB transcritional activity was measured by NF-κB-driven firefly luciferase normalized to Renilla luciferase and multiplied by 1,000 to obtain relative luciferase activity units. (*b, c*) Three independently obtained stable populations of the indicated cells were assayed in triplicate transfections. Mean values ± SD are shown. (*d*) LB2259-MEL cells that express either puromycin resistance gene alone or together with IκBαM were assayed in triplicate transfections in three independent experiments. Mean values ± SD are shown. Statistical significance by Student's t-test: ***p<0.001.

Inhibition of constitutive NF-κB activity by IκBαM does not restore MITF-M expression

Blocking of the NF-κB activity in *MITF*-M-positive melanoma cell lines led to a significantly lower extent of the IL-1β-induced *MITF*-M downregulation (Results - Part II, Fig. 3*a*). Hence, we sought to investigate whether permanent *MITF*-M repression could be relieved in IL-1-producing melanoma cell lines if we inhibit the NF-κB signaling. Two IL-1-producing melanoma cell lines, KUL84-MEL and EB16-MEL, showed constitutive NF-κB activity in gene reporter assays with luciferase-encoding vector containing two NF-κB-binding motifs (Results - Part III, Fig. 3*a*, *b*, *c*). This activity was largely inhibited by the stable expression of the super-repressor of IκBα, IκBαM (Results - Part III, Fig. 3*b*, *c*). Compared with *MITF*-M-positive LB2259-MEL cells, which do not constitutively release IL-1 cytokines, KUL84-MEL and EB16-MEL cells exhibited a 3.7-fold and 6.8-fold higher NF-κB activity, respectively (Results - Part III, Fig. 3*b*, *c*, *d*).

Expression of the super-repressor IκBαM in KUL84-MEL cells led to reactivation of *MITF*-M expression to levels of 0.05±0.01 (n=6) transcript per cell (Results - Part III, Fig. 4*b*, lane 3), but did not result in reactivation of *MITF*-M in EB16-MEL cells (data not shown). As to expression levels of *IL1A*, *IL1B* and *IL8* transcripts, they were, respectively, 4-, 16- and 34-fold lower in IκBαM-expressing KUL84-MEL cells than in control cells (Fig. 4*b*, compare lanes 1 and 3), whereas 4-fold lower levels of secreted IL-1 cytokines were detected after 3 days of culture (Fig. 4*c*, compare lanes 1 and 3). In EB16-MEL cells, the levels of IL-1α and IL-1β measured in the supernatants after one day of culture were respectively 6- and 14-fold lower in IκBαM-expressing cells than in control cells (data not shown). These observations demonstrate that inhibition of constitutive NF-κB activity in *MITF*-M-negative melanoma cell lines secreting IL-1 is not sufficient to relieve permanent *MITF*-M repression.

JNK inhibition results in a higher level of MITF-M reactivation in KUL84-MEL cells

We demonstrated the IL-1-mediated activation of the JNK pathway in LB2259-MEL cells and the engagement of this pathway in IL-1β-induced *MITF*-M downregulation (Results - Part II, Fig. 3*b* and Results - Part III, Fig. 5*a*). Consistent with the failure of a MEK inhibitor PD98059 to counteract the repression of *MITF*-M by IL-1β (data not shown), we did not observe a significant IL-1β-induced activation of the ERK pathway in LB2259-MEL cells as assessed by Western blot using an antibody specific for phosphorylated residues of ERK1/2 (Results - Part III, Fig. 5*b*). Accordingly to observed constitutive ERK1/2 activation (Fig. 5*b*), LB2259-MEL cells appeared to harbor the heterozygous T1799A mutation at the codon 600 of *BRAF* ($BRAF^{V600E}$) (Results - Part III, Fig. 5*c*). The same mutation was observed in KUL84-MEL and EB16-MEL, and the wild-type *BRAF* allele at the codon 600 was not detected, indicating that loss of heterozygosity (LOH) occurs at this locus (Fig. 5*c*).

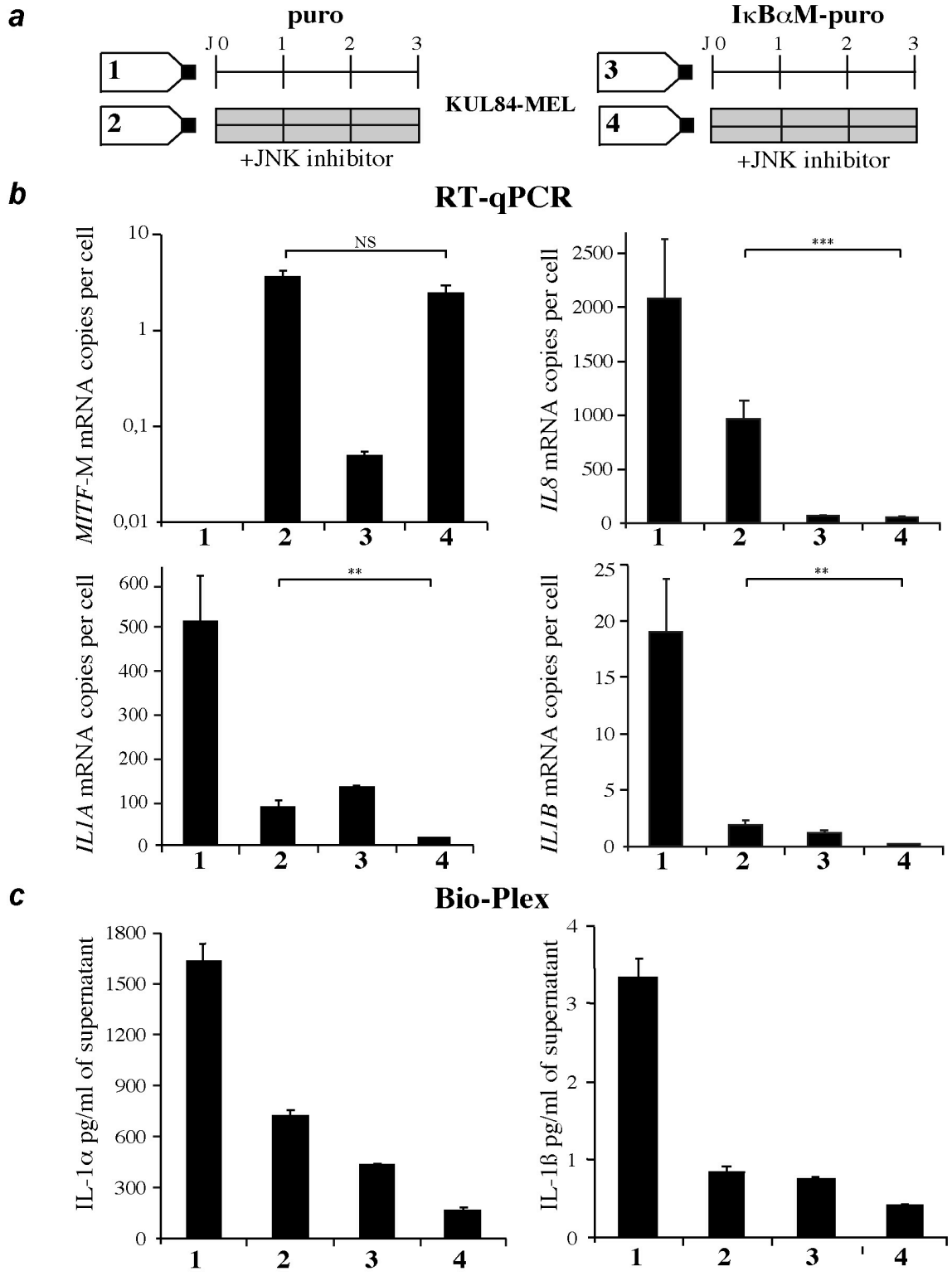


Figure 4. Stable expression of IκBαM and treatment with JNK inhibitor increase *MITF*-M expression in KUL84-MEL cells. (*a*) KUL84-MEL cells were transduced with retroviruses encoding puromycin resistance and super-repressor IκBαM, or puromycin resistance alone. Stable puromycin-resistant cell populations were treated with JNK inhibitor SP600125 (25 μM) or left untreated for 3 days. (*b*) *MITF*-M, *IL1A*, *IL1B* and *IL8* expression was measured by quantitative RT-PCR and normalized to *ACTB* expression. Each value represents mean ± SD from three independently obtained populations. Statistical significance by Student's t-test: NS, not significant; **p<0.01; ***p<0.001. (*c*) The levels of IL-1α and IL-1β were measured in duplicate in the supernatants of puromycin-resistant cell populations of KUL84-MEL cells with the Bio-Plex immunoassay. Each value represents mean ± SD from one representative cell population.

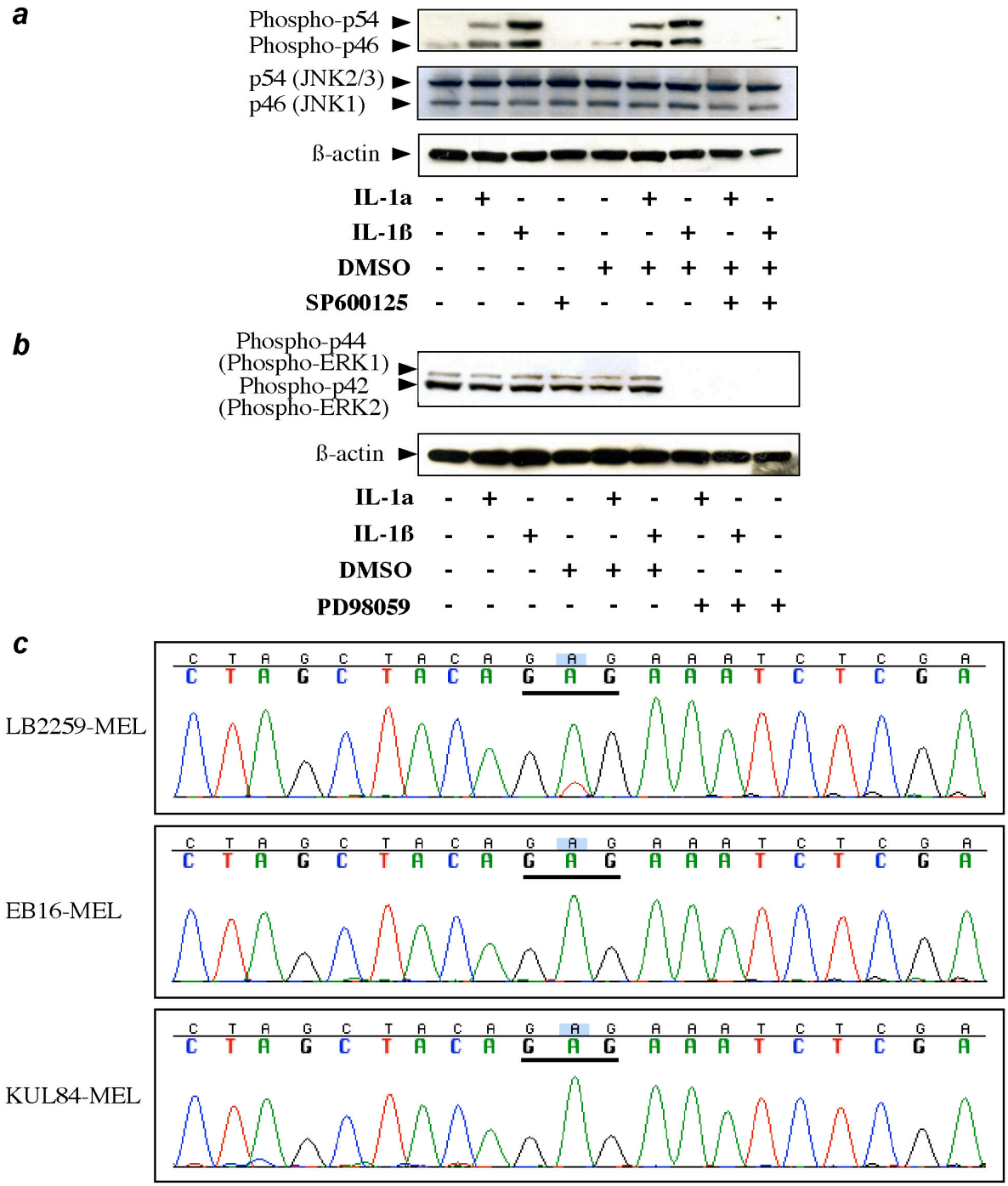


Figure 5. IL-1 induces phosphorylation of JNK, but not ERK in LB2259-MEL melanoma cell line. (*a*, *b*) LB2259-MEL cells (100,000) were were seeded in 6-well plates 2 day before stimulation with recombinant IL-1 cytokines (10 ng/ml) for 10 min. Where mentioned, cells were preincubated for 1 h in the presence of dimethylsulfoxide (DMSO), 25 μM SP600125 JNK inhibitor or 50 μM PD98059 MEK inhibitor. Total lysates were analyzed by Western blot with anti-phospho-JNK1/2/3 or anti-phospho-ERK1/2 antibody (Cell signaling). The membranes were then reprobed with anti-JNK (Cell signaling) and/or anti-ß-actin (Sigma-Aldrich) antibodies as a control. (*c*) Sequence analysis of the BRAF codon 600 (underlined) in melanoma cell lines. Exon 15 fragment was amplified from purified DNA of indicated cell lines using LUR411-LUR412 primers kindly provided by C. Lurquin (LICR) and sequenced using DYEnamic ET Dye Terminator kit and Big-Dye® X terminator purification method (Amersham Biosciences). The sequence electropherogram shows a predominance of the peak corresponding to the mutated nucleotide at the codon 600 (GAG=Glu) over the one of the wild-type allele (GTG=Val) in LB2259-MEL cells and loss of normal allele in two other melanoma cell lines.

To investigate the involvement of the JNK pathway in permanent repression of *MITF*-M, we treated KUL84-MEL cells with JNK inhibitor SP600125 for 3 days. In addition, IκBαM-expressing KUL84-MEL cells were subjected to the same treatment to assess the effect of simultaneous JNK and NF-κB inhibition (Results – Part III, Fig. 4). JNK inhibition alone or in combination with NF-κB inhibition led to reactivation of *MITF*-M. Its expression levels reached 2.4-3.5 mRNA copies per cell and were not significantly different between the two conditions (Fig 4*b*, compare lanes 2 and 4). In contrast, expression of *IL1A*, *IL1B* and *IL8* transcripts was significantly higher in KUL84-MEL cells treated with SP600125 compared to IκBαM-expressing cells subjected to the same treatment (Fig 4*b*, compare lanes 2 and 4). Accordingly, inhibition of the JNK pathway alone or together with the NF-κB pathway led to decreased levels of IL-1 cytokines released in the supernatants of KUL84-MEL cells (Fig. 4*c*). In EB16-MEL cells, *MITF*-M expression was not reactivated by inhibition of the NF-κB and JNK pathways (data not shown). Therefore, neither separate nor simultaneous inhibition of the NF-κB and JNK pathways is sufficient to reactivate *MITF*-M expression in *MITF*-M-negative melanoma cell lines up to levels of 30-250 mRNA copies per cell observed in *MITF*-M-positive melanoma cell lines (Results - Part II, Fig. 2). Hence other mechanisms might be involved in permanent *MITF*-M repression in IL-1 producing melanoma cells.

Prolonged treatment of MITF-M positive cells with IL-1β does not lead to activation of autonomous IL-1 production and permanent MITF-M inhibition

To explore the potential primary role of prolonged IL-1 exposure in permanent repression of *MITF*-M, we tried to establish an autonomous IL-1 production in *MITF*-M-positive melanoma cells. We used CP50-MEL cells, which exhibit induction of *IL1A* and *IL1B* after IL-1β exposure (Results - Part II, Fig. 6). After one week of everyday treatment with IL-1β, CP50-MEL cells were either left in the presence of IL-1β for another 3 days or washed and left untreated for 3 or 6 days (Results - Part III, Fig. 6). When, after the episode of IL-1β exposure, CP50-MEL cells were deprived of this cytokine for 3 or 6 days, the expression of *MITF*-M transcripts was not significantly different from that in non-treated cells and remained significantly decreased in cells continuously exposed to IL-1β (Fig. 6*b*). Significant induction of *IL1B* was conserved after a 3-day deprivation of CP50-MEL cells of exogenous IL-1β (Fig. 6*b*, lane 2). It was accompanied by the release of endogenous IL-1β in supernatants at levels of 9.3±1.5 pg/ml (n=3) (Fig. 6*c*, lane 2). Deprivation of IL-1β for another 3 days led to decreased *IL1B* activation and IL-1β secretion (Fig. 6*b*, *c*, lane 3). Significant induction of *IL1A* and release of endogenous IL-1α in supernatants of CP50-MEL cells were observed after a 10-day exposure to IL-1β (Fig. 6*b*, *c*, lane 4). Thus, CP50-MEL cells were not able to maintain *IL-1* production and *MITF*-M downregulation after a one-week episode of IL-1β exposure, suggesting the involvement of some additional mechanism(s) in the establishment of constitutive IL-1 production and permanent *MITF*-M repression.

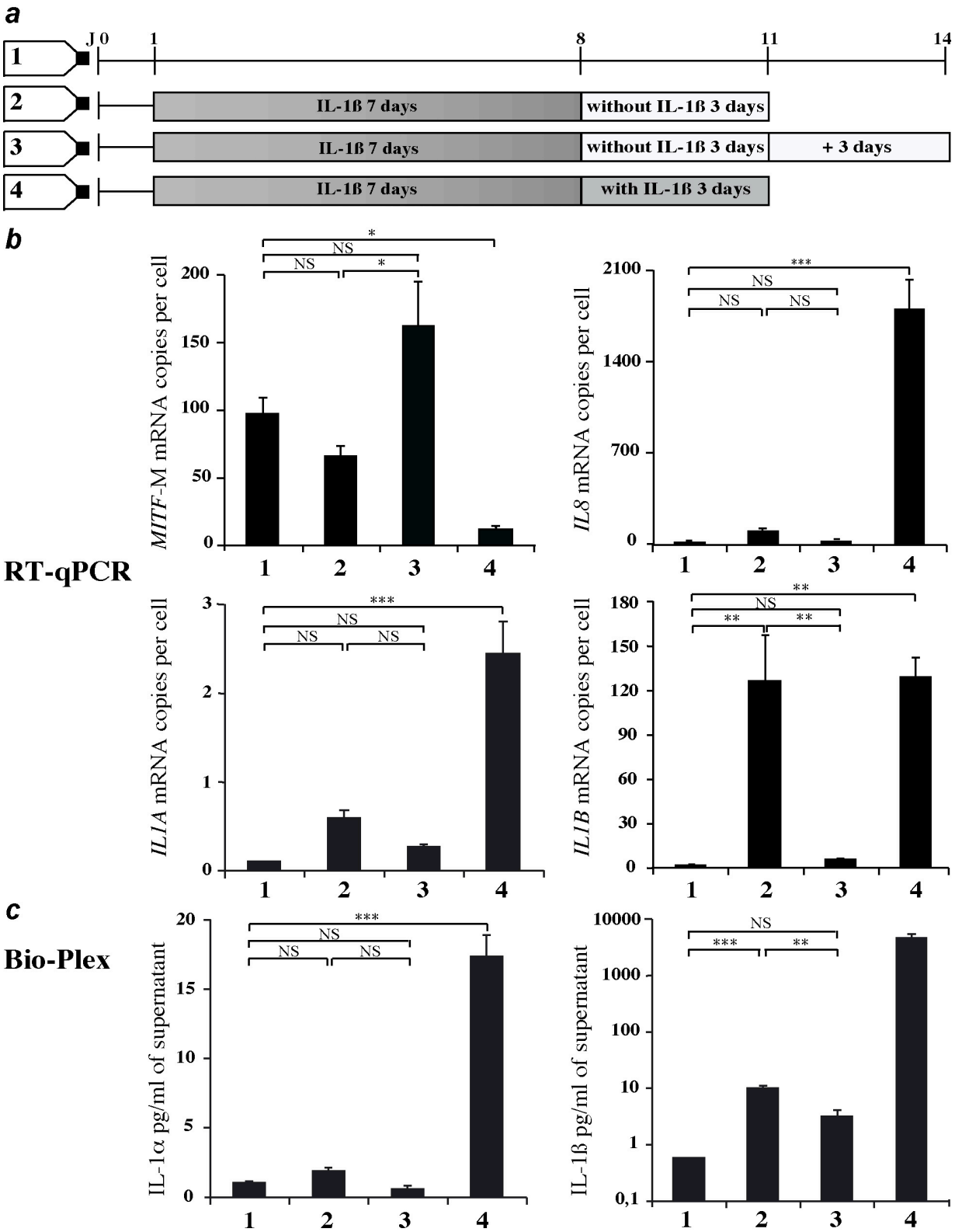


Figure 6. Prolonged treatment with IL-1ß does not lead to the establishment of constitutive IL-1 production and permanent *MITF*-M repression. (*a*) CP50-MEL cells were treated with IL-1ß (10ng/ml) for 7 days and then either left in the presence of cytokine for another 3 days (condition 4) or washed, switched to medium without IL-1ß and left for 3 or 6 days (conditions 2 and 3). (*b*) The expression levels of *MITF*-M, *IL8, IL1A* and *IL1B* were assessed by quantitative RT-PCR and normalized to *ACTB* expression. Each value represents mean ± SD from one experiment with triplicate incubations. (*c*) Concentrations of IL-1α and IL-1β were measured in 3 independently collected supernatants of CP50-MEL cells using the Bio-Plex immunoassay (mean ± SD). Statistical significance by Bonferroni Multiple Comparisons Test (test post-hoc ANOVA): NS, not significant; *p<0.05; **p<0.01; ***p<0.001.

DISCUSSION AND PERSPECTIVES

The evolution of the tumorigenic process is thought to depend on the balance of determinants that either promote or restrain tumor progression. Interactions of tumor cells with immune cells contribute to this dynamic process and the dominance of factors favoring immune tolerance over immune activation may underlie tumor progression in the presence of tumor-reactive T cells. Efficient interaction of a $CD8^+$ CTL with a target tumor cell is a key event in the anti-tumor immune response, which leads to the destruction of the tumor cell. This response is primarily driven by T-cell receptor recognition of a specific peptide-HLA complex, with peptide recognition being the most important factor to confer target specificity. The versatile character of tumor-immune system interactions, in which each interaction partner shapes its own phenotype and that of the other partner, is illustrated by the action of cytokines, which are membrane-bound or secreted proteins produced by the immune cells but also by the tumor cells and other cells of the tumor environment. Understanding tumor-immune system interactions can lead to the development of new therapeutic approaches.

I. Summary of key findings

To explore particular aspects of cell contact-dependent and -independent interactions between tumor cells and immune cells in melanoma, we evaluated the expression levels of antigen-encoding genes in melanoma samples and assessed the mechanisms governing transcriptional regulation of these genes using *in vitro* models of cultured cell lines.

In the first study (Results - Part I), we provided a quantitative analysis of mRNA expression levels of two cancer-germline genes, *MAGE-A1* and *BORIS*, in a panel of primary and metastatic melanoma tissues and in established melanoma cell lines. A potential role of BORIS in the activation of *MAGE-A1* and other CG genes in melanoma was assessed *in vitro* in transient and stable transfection experiments. It appeared that BORIS expression is neither necessary nor sufficient for the activation of other CG genes.

In the second study (Results - Part II and III), we demonstrated the inhibitory effect of recombinant IL-1α and IL-1β cytokines on the levels of MITF mRNA and protein in melanoma cell lines bearing IL-1R1. We showed that this effect is NF-κB and JNK-dependent. Concomitantly with the reduction of MITF protein levels, we observed a decreased expression of its transcriptional target genes *MLANA*, *TYR* and *gp100*. The reduced ability of IL-1β-treated melanoma cell lines to activate CTL clones, which specifically recognize differentiation antigens derived from Melan-A, TYR and gp100 proteins, was mechanistically demonstrated *in vitro*. Finally, we observed concomitant MITF repression and secretion of biologically active IL-1α and IL-1β in a subset (about 13%) of melanoma cell lines and demonstrated the involvement of NF-κB and JNK pathways in this phenotype.

II. Quantitative approach in estimating the efficiency of antigen presentation by tumor cells

The mechanisms underlying an uneventful co-existence of effector $CD8^+$ CTLs and target tumor cells *in vivo* remain among the central subjects of tumor immunology. The lack of effectiveness of antigen-specific $CD8^+$ CTLs may be explained by the inadequacy of tumor cells as targets. It is possible that functional $CD8^+$ CTLs attracted to a tumor site may, however, be incapable to recognize and lyse tumor cells presenting insufficient quantities of peptide/HLA complexes on their surface. One reason of inefficient antigen presentation may be absent or reduced expression of genes encoding the relevant antigenic peptides. If silencing of either CG genes or genes giving rise to differentiation antigens should undoubtedly prevent recognition and lysis of melanoma cells by cognate $CD8^+$ CTLs, the relevance of either sparse reactivation or downregulated expression of these genes is difficult to anticipate *in vivo*. In the tumor environment, productive engagements between TCRs and peptide/HLA complexes might occur when a certain balance between TCR binding affinity and availability of ligand is achieved. In borderline conditions of reduced availability of peptide/HLA complexes and/or suboptimal TCR affinity, efficient CTL recognition may considerably depend on factors extrinsic to TCR-ligand interaction, for instance soluble immunomodulators present in the tumor environment [485].

The availability of a large repertoire of melanoma antigens presented at quantities that are optimal for recognition by T cells would favor spontaneous but also vaccination-induced antitumoral immune responses. In fact, T cells present in regressing melanoma metastasis or blood of vaccinated patients have been shown to recognize tumor antigens absent from therapeutic vaccines suggesting that vaccination has reawakened antitumoral T cell responses to other antigens than those targeted by vaccination [486-489]. Therefore, the quantitative approach in evaluating the expression of antigens presented by melanoma cells appears indispensable not only for diagnostic, but also for immunotherapeutic purposes, since it determines the eligibility of a patient for therapeutic vaccination and assists monitoring of the clinical response.

Quantitative determination of peptide-HLA complex density on the surface of tumor cells within melanoma lesions could allow a direct evaluation of the susceptibility of tumor cells to CTL killing. For this purpose, antibodies generated against specific peptide-HLA complexes might be employed, but their limited availability and strict specificity to particular peptide-HLA combinations preclude their wide use [490-493]. RT-PCR-based quantitative measurement of mRNA expression offers an indirect estimation of the amount of the relevant antigenic peptide presented at the tumor cell surface. This technical approach can be more beneficial than semi-quantitative immunohistochemical analysis of relevant protein expression when small tumor samples have to be analyzed and specific monoclonal antibodies are not available.

Tumor cell lines isolated from melanoma lesions are capable of restimulating *in vitro* autologous T lymphocytes and are sensitive to lysis by isolated antigen-specific CTL clones in more than 90% of instances [5]. Although the phenotypes of melanoma cell lines may differ from the phenotypes of melanoma cells *in vivo* due to the distinct adaptation to culture conditions, the co-culture of melanoma cells with antigen-specific CTLs may bear some relevance to the *in vivo* conditions. Moreover, quantitative analysis of parameters determining the functional outcome of tumor cell-CTL interactions can be readily performed *in vitro*.

The variable expression of genes encoding melanoma antigens appears to be functionally significant since it has been associated with variable efficiency of recognition of target tumor cells by CTLs [404, 494, 495]. A correlation has been found between the levels of mRNA and protein expression, on one hand, and the extent of recognition efficiency of the relevant antigens by specific CTLs, on the other hand [404, 495]. The threshold of the lowest mRNA expression level that results in recognition of a relevant antigen by specific CTL clones has been established [404, 495]. The threshold of 3 *MAGE-A1* mRNA copies per cell has been associated with the susceptibility of melanoma cells to lysis by CTL clones recognizing MAGE-A1-derived antigens [404]. The majority (76%) of *MAGE-A1*-expressing melanoma cell lines exhibited the levels of *MAGE-A1* transcripts that exceeded this threshold (Results - Part I, Fig. 1*c*). In our second study, CP50-MEL cells had the lowest levels of gp100 mRNA and protein expression. These expression levels further decreased after treatment with IL-1β, and recognition of treated cells by the cognate CTL clone was no more observed. These data indicated that the expression level of gp100 decreased under the threshold compatible with an efficient CTL recognition (Results - Part II, Fig. 4). In accordance with our study, lower mRNA and protein expression levels of gp100 and Melan-A in a panel of melanoma cell lines have been associated with lower efficiency of recognition by cognate CTL clones [495]. Similarly to IL-1β that reduced the capacity of melanoma cell lines to activate differentiation antigen-specific CTL clones in our study (Results - Part II, Fig. 4), Wnt5A has been shown to repress the expression of MITF, Melan-A and gp100 proteins and to render melanoma cells less competent for CTL activation [278]. These overall observations indicate that the lower expression levels of mRNA and proteins encoded by melanocyte differentiation genes would lead to decreased supply and presentation of relevant antigenic peptides by melanoma cells. Besides expression levels of relevant mRNA and protein, other parameters might account for the efficiency of antigen recognition by specific CTLs. These parameters would include post-translational modifications of the relevant protein [496, 497], its misfolding and degradation rates [133, 498], the efficiency of processing of antigenic peptides, the expression of HLA molecules, the affinity and competition of intracellular peptides for HLA molecules [499] and the expression and distribution of co-receptors, co-stimulatory and adhesion molecules [500]. The respective weight of these parameters in the overall functional outcome could be investigated *in vitro* in order to get insight into the mechanisms governing tumor cell-CTL interactions *in vivo*.

III. BORIS as a potential target for immunotherapy in melanoma

We showed by quantitative RT-PCR that BORIS is aberrantly activated in a substantial proportion of primary and metastatic melanoma tissues (16% and 34%, respectively) and melanoma cell lines (59%). Although this frequency is lower than the 77% of *BORIS*-expressing uterine cancer tissue samples [352], it is similar to that reported for other CG genes in primary (11-36%) and metastatic (22-76%) melanoma [299]. Frequency and patterns of BORIS expression in melanoma tissues could be investigated in a larger number of melanoma samples both at the mRNA and protein levels. The expression of *BORIS* and the methylation status of its promoter assessed in melanoma lesions or in serum of melanoma patients could serve as a diagnostic biomarker as well as a potential tool to predict the disease outcome, the likelihood of metastatic spreading and the probability of response to a treatment. The investigation of the immunogenic potential of BORIS might help to identify new target antigens, which may be preferential targets in individual anti-tumor immune responses determined by the natural HLA polymorphism and the individual T cell repertoire.

The critical step in exploring the immunogenicity of BORIS in melanoma should be to find out if the level of BORIS expression in tumor cells is sufficient to produce antigenic peptides and whether these peptides are efficiently processed and presented in the context of HLA molecules to antigen-specific T cells. Since BORIS-derived peptides have not been found among epitopes eliciting T cell responses in melanoma or in other types of cancer, the "reverse immunology" approach of tumor antigen identification could be applied. On the basis of the well-established algorithms in the publicly available databases for predicting HLA-allele-restricted epitopes [501], numerous BORIS-derived peptides match consensus sequences for presentation by different HLA class I and class II alleles. These candidate peptides might be synthesized, assessed for their binding affinity to the relevant HLA molecules and their capacity to activate *in vitro* T cells isolated from melanoma patients. Antigen-specific T cell clones might be then established and assessed for their capacity to lyse BORIS-expressing tumor cells. Recently, the indirect evidence of BORIS immunogenicity has been provided in mice vaccinated with a plasmid encoding a mouse BORIS lacking the DNA-binding domain [442, 502]. The splenocytes of the vaccinated mice exhibited cytolytic activity against several *BORIS*-expressing mouse tumor cell lines. The epitope(s) eliciting this immune response remain(s) to be identified [442, 502].

Finally, the evidence should be provided that BORIS-derived antigens are strictly tumor-specific in order to avoid an immunotherapy-induced autoimmune response against normal cells. In contrast to *MAGE-A1*, whose expression was found to be restricted to testis and placenta, several normal somatic tissues exhibited low expression levels of *BORIS* mRNA, which did not exceed the threshold of 0.1 copy of *BORIS* mRNA/2,000 copies of *ACTINB* mRNA, that is about 0.3% of the level in testis (Results - Part I, Fig. 1*b*).

Accordingly, other studies in normal somatic tissues have reported either absence or low expression of *BORIS* transcripts in semi-quantitative RT-PCR experiments, and absence of BORIS protein detection by immunohistochemistry [289, 346, 442]. Some other CG genes, especially those mapped to autosomes, have been found to be expressed in a limited number of somatic tissues, albeit at levels not exceeding 1.5% of their expression levels in testis [297, 311]. It has been often debated whether this so-called "leaky" mRNA expression corresponds to a biologically significant level of protein [289, 297]. Insightful studies of mRNA and protein stability are likely needed for each CG gene. Until now, low expression levels of CG gene transcripts have not been associated with immunohistochemically detectable levels of the corresponding proteins and are unlikely to result in the presence of immunologically relevant levels of peptide/HLA complexes on the surface of normal cells [297]. Nevertheless, one cannot exclude the possibility of high and immunologically relevant BORIS expression by particular cell types within normal tissues. Therefore, if BORIS-derived peptides efficiently presented in the context of HLA molecules are identified, it should be essential to quantitatively evaluate BORIS mRNA and protein levels associated with the immunologically relevant levels of antigen presentation. In melanoma samples, the expression levels of *BORIS* transcripts varied greatly and reached up to 50 copies of *BORIS* mRNA/2,000 copies of *ACTINB* mRNA (Results - Part I, Fig. 1*c*). In order to assess the immunotherapeutic potential of BORIS activation in melanoma, it would be of relevance to directly measure by specific antibodies the quantity of peptide/HLA complexes that might elicit specific T cell responses.

IV. Deciphering the role of BORIS in CG gene regulation in melanoma

We explored the hypothesis that BORIS overexpression in melanoma cells might induce and/or maintain the activation of CG genes and, therefore, represent a new strategy to increase the repertoire of antigens presented on the surface of tumor cells. However, our data and recent data of other researchers argue against this hypothesis. First, in many melanoma specimens the activation of *MAGE-A1* and other CG genes was found in the absence of *BORIS* activation (Results - Part I, Fig. 1*c* and Table 1), indicating that BORIS is not required for the maintenance of CG gene expression. Similarly, glioma cells have expressed several CG genes in the absence of *BORIS* expression [503]. Second, neither transient nor stable forced expression of BORIS in melanoma cell lines that do not express *BORIS* was sufficient to derepress the expression of MAGE-A1 and other CG genes (Results - Part I, Fig. 2). This finding held true for ovarian cancer cell lines forced to express BORIS using an adenoviral transduction approach [504] and was extended to non-malignant cell types, such as human fibroblasts, immortalized human keratinocytes (Results - Part I, Fig. 4) and immortalized ovarian epithelial cells [504]. Thus, no effect of forced BORIS expression has been documented by several studies in contradiction to data reported by another group of researchers [349, 505].

Explicitly, they have documented the activation of *MAGE-A1*, *-A2*, *-A4*, *-B1*, *LAGE-1* and *LAGE-2*$^{NY\text{-}ESO\text{-}1}$ in normal human fibroblasts and that of *MAGE-A2*, *-A3/6*, *-A4* and *-A11* in immortalized oral keratinocyte cells within 3 days of conditional transient BORIS overexpression [349, 505]. The origin of the observed discrepancy is unlikely to be cell type-dependent as neither human fibroblasts nor human keratinocytes used in our study showed activation of *MAGE-A1, -A2*, *-A4*, *-B1* and *LAGE-2*$^{NY\text{-}ESO\text{-}1}$ following forced BORIS expression. Moreover, all cell types used in above-mentioned studies appear to possess necessary factors to drive the expression of CG genes when their epigenetic repression is relieved by treatment with demethylating agent 5-azadC. Finally, conflicting findings call into question the intriguing proposal that BORIS reactivation may functionally contribute to 5-azadC-mediated induction of MAGE-A1 and other CG genes. If a previous study in human fibroblasts treated with 5-azadC has reported the induction of *BORIS* at an earlier time point (8 hours) than *MAGE-A1* (48 hours) [349], our time-course study in the same type of cells showed *MAGE-A1* activation after 3 days of 5-azadC treatment, obviously in the absence of *BORIS* activation (data not shown). In ovarian cancer cells, the kinetics of 5-azadC-induced gene reactivation and promoter hypomethylation of CG genes (*MAGE-A1*, *LAGE-2*$^{NY\text{-}ESO\text{-}1}$, *XAGE-1*) has been reported to be similar to that of *BORIS* [504] . This kinetics has not been affected by siRNA-mediated silencing of *BORIS* activation [504] in contradiction with data obtained in human fibroblasts [349]. Moreover, neither the methylation of CG gene promoters nor global DNA methylation parameters, such as total genomic 5mdC levels and the methylation levels of the common repetitive element LINE-1, have been affected by ectopic expression of BORIS in ovarian epithelial cells and ovarian cancer cell lines [504]. In accordance with the study in human fibroblasts [349], we observed local and partial demethylation of 5 CpG sites located between positions +129 and +169 within the first intron of MAGE-A1 in one of two melanoma cell lines ectopically expressing BORIS (Results - Part I, Fig. 3). However, 6 CpG sites between positions -30 and +30, whose methylation status has been previously shown to have a major impact on *MAGE-A1* transcriptional activity [284, 323], remained methylated consistently with *MAGE-A1* silencing (Results - Part I, Fig. 2*c* and Fig. 3). Based on these observations, we concluded that BORIS expression on its own is insufficient to induce the expression of *MAGE-A1* and other CG genes in melanoma cells and, consequently, may not serve as a general means to increase the repertoire of melanoma antigens.

Although BORIS does not seem to be the primary regulator of CG gene expression, some recent findings do not exclude the possibility that BORIS may contribute to the regulation of CG genes and/or to the global epigenetic changes in tumors, including melanoma. The co-expression of BORIS and its paralog CTCF within the same cells is an abnormal phenomenon specific to tumor cells and its functional consequences are poorly understood. Understanding the mechanisms that would underlie BORIS recruitment to its target sites is likely to provide insight into its potential role in gene regulation in tumor cells.

The presence of shared 11 zinc finger DNA-binding domains likely provides BORIS and CTCF with similar DNA binding specificities and affinities. However, interactions with different proteins via distinct amino- and carboxy-termini might influence their DNA-binding behavior. It has been recently demonstrated that competition between BORIS and CTCF for the same DNA binding sites may occur when both proteins are present in equal amounts [506]. The occurrence and frequency of such situation in tumors are poorly explored, whereas mRNA and protein stability of BORIS and CTCF in tumor cells has not been investigated. Although we did not quantitatively assess the levels of CTCF expression in melanoma specimens, our preliminary RT-PCR data indicate that CTCF is expressed at high levels in all tested melanoma samples. Quantitative expression analysis of breast cancer cell lines has revealed the constant abundance of *CTCF* mRNA, with levels being at least 9,000-fold higher than those of *BORIS* mRNA, thus raising the question whether the corresponding BORIS protein levels would be sufficient to compete with CTCF at DNA binding sites [351]. Another study reported the quantitative analysis of *BORIS* and *CTCF* mRNA levels in ten lung cancer cell lines: only one out of five *BORIS*-expressing cell lines harbored higher *BORIS* than *CTCF* expression, whereas in four cell lines the levels of *CTCF* transcripts exceeded those of *BORIS* at least 100 times [353]. Thus, currently there is no evidence that BORIS expression levels are superior or comparable to those of CTCF in the majority of tumor samples, suggesting that the competition model is rarely applicable. In addition to quantitative RT-PCR, immunohistochemistry and/or Western blot approaches could be used to assess the mRNA and protein levels of BORIS and CTCF in melanoma cell lines and tissues. Whether BORIS is able to displace CTCF from its binding sites in melanoma cells *in vivo* could be investigated in genome-wide and local scale ChIP-based studies of regions bound by BORIS and CTCF in melanoma cells compared to those bound by CTCF in melanocytes. CTCF depletion using siRNA could help to determine the CTCF/BORIS ratio leading to the competitive occupancy of their target sites.

Recent reports strongly suggest that the specificity of DNA-binding by either BORIS or CTCF is conferred by the methylation status of their target sequences [360, 361]. If CTCF binding is generally limited to unmethylated DNA, BORIS DNA-binding activity appears to be methylation-insensitive and to depend on physical occupancy of unmethylated sites by CTCF [360]. BORIS has been proposed to be able to bind methylated sites, but also unmethylated sites when CTCF is displaced [360]. One report has provided evidence that DNMT1 and DNMT3B expression levels influence the respective amounts of BORIS and CTCF binding to the promoter of *BAG-1* (BCL2-associated athanogene) [506]. In human colon carcinoma cell line HCT116 harboring similar BORIS and CTCF protein levels, BORIS binding to BORIS/CTCF binding sites within the *BAG-1* promoter has increased as a function of DNMT1 and/or DNMT3B overexpression. In contrast, CTCF binding has increased when DNMT1 and/or DNMT3B have been deleted [506].

In addition, BORIS binding to *BAG-1* has been accompanied by increased expression of this gene and chromatin permissive changes in the histone H3 methylation status of its promoter [506]. In contrast, non-permissive chromatin status (decreased H3K4me2/H3K9me2 ratio) and reduced *BAG-1* expression have been observed concomitantly with the occupancy of the *BAG-1* promoter by CTCF [506]. Based on a recent observation in the same HCT116 cell line, BORIS has been proposed to act as a scaffold protein that assembles complexes containing chromatin-modifying enzymes within gene promoters [507]. Within the promoters of *Myc* and *BRCA1*, BORIS has been proposed to bind via its N-terminal domain a chaperone protein BAT3 (HLA-B associated transcript 3) and to recruit SET1A/ASH2 complex that possesses H3K4 methyltransferase activity [507]. Concomitantly with the recruitment of this complex, increased transcriptional activity of *Myc* and *BRCA1* has been observed [507].

In the light of these recent findings, it would be of interest to explore whether BORIS might regulate the histone H3 methylation status within the *MAGE-A1* promoter or within promoters of other CG genes in melanoma cells. To exert this function, BORIS needs to be recruited to the *MAGE-A1* promoter. BORIS binding to the *MAGE-A1* promoter has been demonstrated by ChIP-qPCR assay in NCCIT cell line derived from testicular tumor and in lung cancer cell line H1299, which exhibits 4-fold higher mRNA levels of *BORIS* than those of *CTCF* [349, 353]. The specificity of either BORIS or CTCF binding to the *MAGE-A1* promoter has been proposed to be determined by the CTCF/BORIS ratio [349, 353]. The second factor that might determine binding specificity is the methylation status of the common CTCF/BORIS binding region in the *MAGE-A1* promoter mapped by EMSA studies to the S9 fragment corresponding to the positions +47 to +183 downstream of the *MAGE-A1* TSS [349] (Fig. 1).

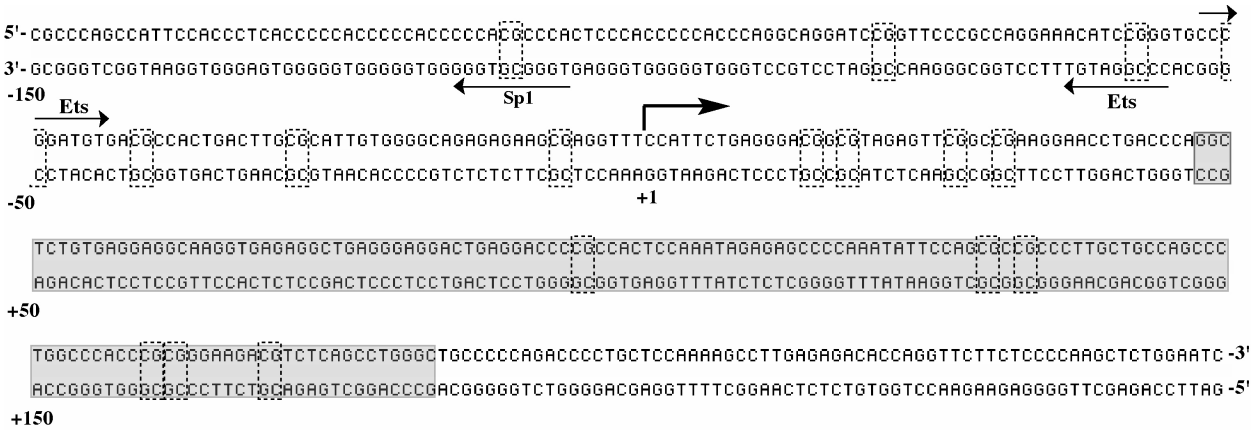


Figure 1. *MAGE-A1* 5' region. The sequence of the 5' region of the human *MAGE-A1* between positions -150 to +250 relative to the known TSS (marked with an arrow and +1) is shown. Transcription factor binding sites are indicated with arrows. The distribution of CpG dinucleotides is indicated as depicted in Kholmanskikh *et al.*, 2008 (454). In addition, CpG within the Sp1 binding site is shown. The S9 fragment determined by EMSA as a region recognized by the 11ZF domain of CTCF and BORIS is highlighted in a grey box as depicted in Vatolin *et al.*, 2005 (347).

In ChIP-qPCR assay, the occupancy of the S9 region by BORIS has increased after treatment of human fibroblasts with 5-azadC, suggesting preferential binding of BORIS to hypomethylated sequences [349]. However, EMSA assay has demonstrated methylation-insensitive binding to the S9 fragment of the 11ZF DNA-binding domain of CTCF and BORIS [349]. Whether the methylation status of 6 CpG nucleotides situated within the S9 region might determine BORIS binding specificity in melanoma cells, could be explored by ChIP assay using anti-BORIS antibody. The pulled-down DNA could be subjected to bisulfite conversion and subsequent methylation-specific PCR spanning the S9 region. In parallel, the pulled-down chromatin could be examined for histone modifications that might occur within the *MAGE-A1* promoter region following BORIS binding.

The third factor that might underlie the specificity of DNA binding by BORIS could be its interaction with transcription factors operating within the *MAGE-A1* promoter. For instance, BORIS recruitment might be favored by its interaction with the Sp1 transcription factor, which has been shown to physically associate with BORIS, but not with CTCF, in co-immunoprecipitation experiments [508]. Consistently, the co-recruitment of Sp1 together with BORIS to the *LAGE-2*$^{NY\text{-}ESO\text{-}1}$ promoter has been demonstrated in lung cancer cell line H1299 [508]. Since the consensus binding site for Sp1 within the *MAGE-A1* promoter (Fig. 1) has been shown to be functionally relevant for *MAGE-A1* transcriptional activity [319], reciprocal dependence of BORIS and Sp1 recruitment could be investigated in ChIP and EMSA assays.

Finally, the potential association between global DNA methylation changes and either CTCF or BORIS binding to DNA target sites could be explored in melanoma. In the course of tumor progression, DNA hypomethylation changes might lead to exposure of new CTCF binding sites. The consequent spatial redistribution of CTCF might ultimately lead to the sequestration of the major part of the CTCF pool, for example within hypomethylated repetitive regions of the genome, providing in this way unoccupied binding sites for BORIS. Given the global role of CTCF in chromatin organization [347], the resulting functional consequences, such as disruption of insulator boundaries and abnormal gene expression, are expected to have a genome-wide character. T cell-tumor cell interactions could also be modified since CTCF mediates multiple long-range intra- and inter-chromosomal interactions, including those that govern the expression of HLA molecules [509]. CTCF/BORIS chromatin binding distribution and expression of genes relevant for tumor-immune system interactions (CG genes, HLAs) could be investigated in BORIS-negative and BORIS-positive melanoma cell lines after induction of DNA hypomethylation by transient depletion of DNMTs.

In conclusion, recently reported data support a role of BORIS as an epigenetic regulator of gene expression due to its specific interactions with other proteins. Additional experimental investigation is required to determine BORIS potential to synergize or to compete with other epigenetic modifiers and to regulate gene expression in melanoma.

V. Concomitant and selective loss or downregulation of melanocyte-specific genes

The concomitant loss or downregulation of several targets for effector T cells would confer an advantage for tumor cells and avert the benefits of immunotherapy. The absence of immunoreactivity to three melanoma markers (Melan-A, tyrosinase and gp100) has been found in 4/25 (16%) of advanced primary and 7/41 (17%) of metastatic melanoma samples, and none of *MLANA*, *TYR* and *TYRP1* transcripts has been detected in 4/35 (11%) of metastatic melanoma specimens [510, 511]. Patients bearing metastatic melanomas with no or low expression of at least two genes encoding differentiation antigens have had a significantly worse prognosis [511]. The frequency of *MITF*-M repression occuring concomitantly with loss or downregulation of the other melanocyte differentiation genes has not been reported and is worth to be evaluated in a large number of melanoma tissue samples. The lack of immunoreactivity to MITF and simultaneous loss of other melanocytic markers have been observed to occur in about 80% of a desmoplastic variant of cutaneous melanoma [512, 513]. Our preliminary data support the hypothesis that coordinated loss or downregulation of melanocyte differentiation genes can be associated with downregulation of *MITF*-M. Indeed, we estimated that about 17% of melanoma cell lines harbor a *MITF*-M-negative phenotype (Results - Part II). This phenotype was associated with absent or low levels of *MLANA*, *TYR* and *gp100* expression in 12 melanoma cell lines tested (data not shown). Similarly, in the other study repression of *MITF*-M has been accompanied by repression of three MITF-M target genes (*TYR*, *TYRP1*, *DCT*) in 3 of 17 (17%) melanoma cell lines [482].

The selectively lost expression of particular melanocyte differentiation genes and relevant proteins has been observed in melanoma tissues and established cell lines [510, 511, 513, 514]. Of note, the visibly non-pigmented phenotype of melanoma cell lines does not necessarily imply that they have lost the expression of tyrosinase. Instead, tyrosinase may be retained in the ER and rapidly degraded because of the elevated proton export properties of melanoma cells [515]. Selective loss of *MLANA* or *TYRP1* expression has been observed by us (Results - Part II) and others [483] in *MITF*-M-expressing melanoma cell lines, suggesting that MITF-M expression is a requirement, but not the sole prerequisite for the expression of melanocyte differentiation genes in melanoma. Moreover, forced expression of MITF-M in *MITF*-M-negative melanoma cell lines has not restored the expression of the endogenous melanocyte differentiation genes and visible pigmentation, while it has led to activation of an exogenous *TYR* promoter in gene reporter assays [482, 516]. These observations might be explained by an insufficient concentration and/or activity of other transcriptional factors cooperatively acting with MITF-M (for instance, SOX10), and/or by a predominance of repressor(s) on promoters of melanocyte differentiation genes. Accordingly, the requirement of *de novo* protein synthesis for selective *TYRP1* repression together with the EMSA data have suggested the inhibition of MITF-M binding to the *TYRP1* promoter by an unknown repressor [483].

Several common regulatory mechanisms might underlie coordinate downregulation of *MITF*-M and other melanocyte differentiation genes. First, their transcriptional activation critically requires the recruitment of SWI/SNF chromatin remodeling complexes, which contain one of the two catalytic subunits, BRM or BRG1 [211, 517]. In melanoma cell lines, siRNA-mediated depletion of BRG1 has resulted in 80-90% reduction of *MITF*-M and *TYR* expression, whereas about 60% decrease has been observed after the depletion of BRM [517]. Several essential components of the SWI/SNF chromatin remodeling complexes have been found to be downregulated in cancer [518, 519]. It would be of interest to evaluate whether their expression is altered in *MITF*-M-negative melanoma cell lines. For instance, the expression of the SNF5 component of the SWI/SNF chromatin remodeling complexes has been shown to be downregulated in melanoma compared to dysplastic nevi as well as in advanced melanoma compared to less metastatic melanoma [519].

The implication of other epigenetic mechanisms, such as DNA methylation and post-transcriptional histone modifications, in the repression of melanocyte differentiation genes could be investigated in ChIP assays and by using inhibitors and/or siRNAs targeting different epigenetic modifiers. The prolonged absent or reduced expression of MITF-M and, consequently, of other melanocyte differentiation genes, might lead to repressive epigenetic modifications within promoters of these genes and ultimately to make them unresponsive to MITF-M transcriptional activity. The dynamic epigenetic changes occurring within promoters of melanocyte differentiation genes in the absence of MITF-M expression as well as following its re-expression might be investigated using the ectopic expression of MITF-M-specific shRNAs under the control of an inducible promoter.

Alternative regulatory mechanism that might underlie coordinate downregulation of *MITF*-M and other melanocyte differentiation genes may involve overexpression of miRNAs, either known to target MITF (Introduction, Fig. 3) or miRNAs recently found to be specifically expressed in melanocyte lineage cells [46]. Interestingly, some miRNAs have been predicted to target several melanocyte-specific transcripts, for instance miR-137 (*MITF*, *TYR*) and miR-221/222 (*SOX10*, *TYR*) [520]. Investigation of miRNA expression profiles in melanoma cells in relation with coordinate or selective silencing of melanocyte differentiation genes could be carried out. *MITF*-M-negative and *MITF*-M-positive melanoma cell lines could be used for this purpose.

In conclusion, MITF-M expression appears to be necessary but not sufficient for the expression of genes that give rise to differentiation antigens in melanoma. Understanding the mechanisms underlying coordinate and selective regulation of melanocyte differentiation genes might serve for diagnostic and immunotherapeutic purposes in melanoma patients.

VI. IL-1 release in the tumor environment as a mechanism of MITF-M downregulation in melanoma

We suggested that one of the mechanisms leading to *MITF*-M downregulation in melanoma could be the production of IL-1α and IL-1β cytokines in the tumor environment. Several lines of evidence support this hypothesis (Results - Part II and III). First, IL-1α and IL-1β cytokines, either recombinant or released by melanoma cells, inhibit *MITF*-M expression in melanoma cell lines in an IL-1R1-dependent manner *in vitro*. Second, *MITF*-M repression in IL-1-producing melanoma cell line KUL84-MEL was relieved, albeit modestly, by the treatment with IL-1Ra, a natural antagonist of IL-1R1. Third, inhibition of the major IL-1R1 transduction pathways counteracted *MITF*-M downregulation. Finally, IL-1 production and *MITF*-M downregulation were estimated to occur simultaneously in about 13% of melanoma cell lines and about 4% of metastatic melanoma tissues. The biological relevance of these observations *in vivo* could be uncovered by investigating the mechanisms responsible for *MITF*-M downregulation in melanoma cells and the production of IL-1 in the melanoma environment.

VI.1 IL-1 expression in melanoma

Immunohistochemical analysis of melanoma tissues with anti-IL-1β antibodies has revealed immunoreactivity of melanoma cells and of the stromal compartment containing macrophages and fibroblasts [484, 521, 522]. Moreover, the expression of IL-1α and IL-1β has appeared to increase with melanoma progression [522]. *IL1B* transcripts have been found in metastatic melanoma tissues at levels ranging from 1.5 to 450 mRNA copies/2,000 *ACTB* mRNA copies [467]. Our RT-qPCR analysis revealed similar levels of *IL1B* transcripts in two metastatic melanoma tissues (Results - Part II, Table 1). As to the expression levels of *IL1A* transcripts, they were generally low and did not exceed 5 mRNA copies/2,000 *ACTB* mRNA copies in metastatic melanoma tissues [467] (Results - Part II, Table 1). In the serum of melanoma patients, IL-1α and IL-1β circulate, respectively, at average levels of 174±92 pg/ml and 406±97 pg/ml (n=179), which are 13-fold and 10-fold higher than in healthy individuals [523]. Local concentrations of IL-1 in melanoma tissues have not been investigated and might be higher than the circulating ones. Of note, local IL-1β levels observed in muscles during experimental sepsis in rats peaked at 1 ng/ml, whereas the levels of circulating IL-1β were about 200 pg/ml [524]. In accordance with other studies [466, 467, 521, 525], we found that some melanoma cell lines release bioactive IL-1 cytokines in the supernatant, with levels ranging from 12 to 5,187 pg/ml for IL-1α and 5 to 2,050 pg/ml for IL-1β (Results - Part II).

VI.2 Why do melanoma cells make IL-1?

Accumulating evidence indicates that a deregulation of numerous mechanisms controlling IL-1 production at the level of transcription, mRNA stability, translation and proteolytic processing occurs in some melanoma cell lines and melanoma lesions (Fig. 2).

VI.2.1 Mechanisms underlying IL-1 release by melanoma cells

Some melanoma cell lines are able to constitutively release biologically active IL-1β in the extracellular space [521], whereas macrophages need two distinct stimuli: the first stimulus (Signal 1) induces transcription and translation of the biologically inactive precursor pro-IL-1β (31 kDa) devoid of secretory signal peptide and the second stimulus (Signal 2) is needed for caspase-1 activation with subsequent processing of pro-IL-1β and ATP-stimulated release of biologically active mature IL-1β (17 kDa) into the extracellular space [526]. Activation of caspase-1 requires autocatalysis of procaspase-1 by the NLRP3 inflammasome, a multi-protein complex containing nucleotide-binding domain and leucine-rich repeat containing protein 3 (NLRP3) [462, 527]. One recent report has documented the presence of constitutively active NLRP3 inflammasome (autoactive Signal 2) in metastatic melanoma cells *in vitro* and *in vivo* [521]. Consistently, the constitutive release of IL-1β by melanoma cells has been reduced by inhibition of caspase-1 or the use of siRNA directed against the inflammasome component ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain (CARD)) [521]. While primary melanoma cells have harbored assembled NLRP3 inflammasome, the release of IL-1β by these cells has necessitated either activation of the IL-1R1 by IL-1α or co-stimulation with muramyl dipeptide together with IL-1α [521]. The mechanisms underlying constitutive activation of the NLRP3 inflammasome in melanoma cells and the activation status of the NLRP3 inflammasome in melanocytes remain to be investigated.

In the light of these findings, melanoma cell lines that were found to constitutively release biologically active IL-1β in our study might be assessed for caspase-1 activation by investigating the presence of its cleaved form (p20) in cell lysates. Furthermore, our preliminary data suggest that CP50-MEL melanoma cells, which do not spontaneously release IL-1β, might contain active NLRP3 inflammasome. Indeed, CP50-MEL cells treated with IL-1α for two days were found to release IL-1β at levels of about 25 pg/ml (data not shown), which are similar to those observed in IL-1α-treated primary (4-31 pg/ml) and metastatic melanoma cell lines (33-120 pg/ml) harboring active NLRP3 inflammasome [521]. Interestingly, the levels of intracellular IL-1β, most probably pro-IL-1β, have been shown to be 6.5-80-fold higher than the levels of secreted IL-1β, indicating that only a minor fraction of pro-IL-1β is processed and released by melanoma cells [521].

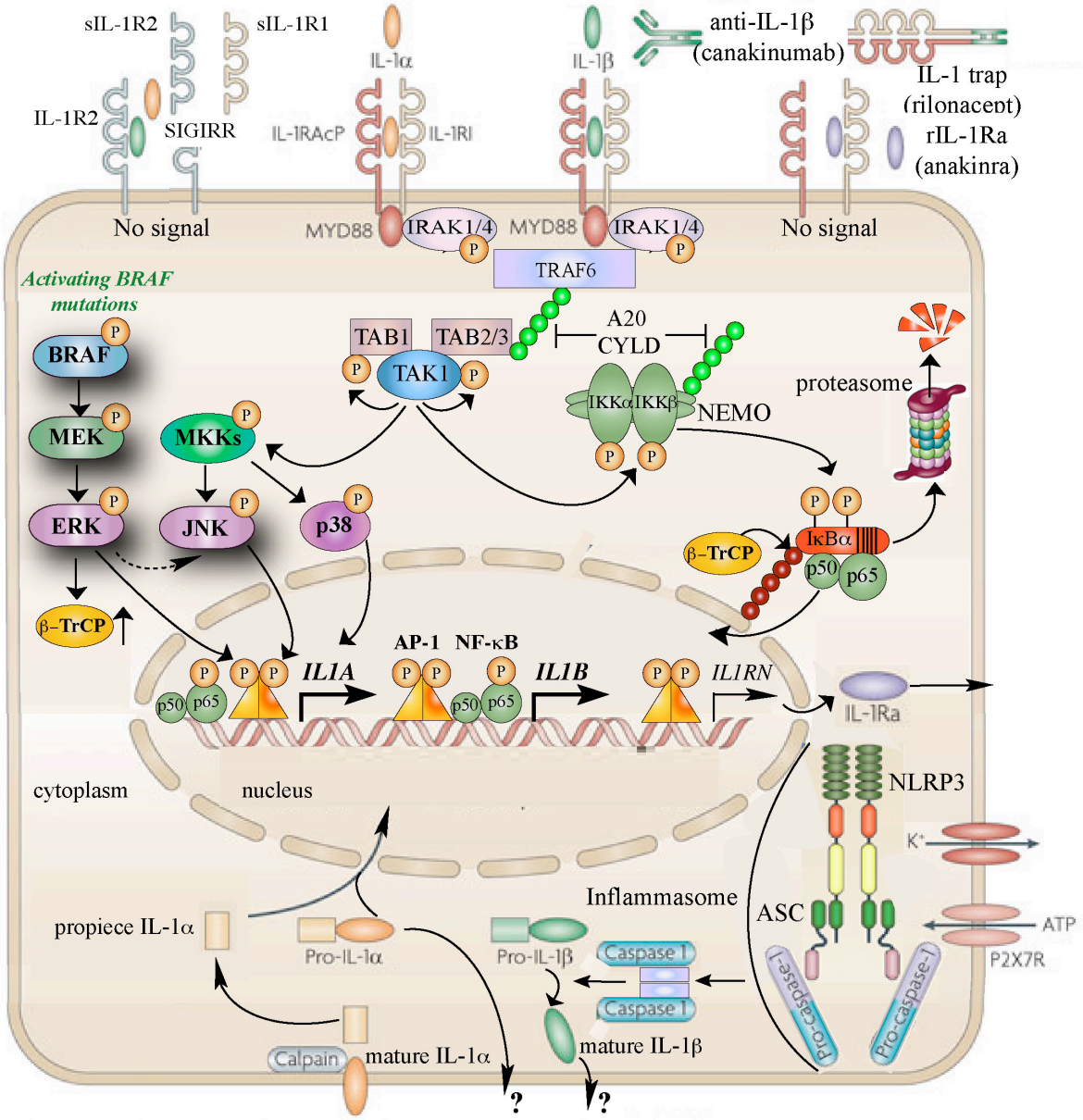


Figure 2. A model of constitutive IL-1 synthesis and release by some melanoma cells. Some melanoma cells synthesize IL-1 due to constitutive transcriptional activation of *IL1A* and *IL1B* by IL-1R1-mediated signaling (**autoactive signal 1**). Binding of IL-1 to the IL-1R1 recruits IL-1R accessory protein (IL-1RAcP). This results in the recruitment of myeloid differentiation factor 88 (MYD88), phosphorylation of IL-1R-associated kinase 1 and 4 (IRAK1/4), K63 polyubiquitination (green circles) of TNF receptor-associated factor 6 (TRAF6) and recruitment of TAK1 (TGFß-activated kinase) byTAK1-binding 2/3 proteins (TAB2/3). TAK1 phosphorylates the IKK complex, which in turn phosphorylates MKKs and IκBα, mediating its degradation. Activated MAPKs, including JNK, p38 and constitutively activated ERKs, translocate to the nucleus, where they can phosphorylate several transcription factors of the bZIP family, like c-Jun and c-Fos, which might cooperate with NF-κB in transcriptional activation of *IL1A* and *IL1B*. Release of biologically active IL-1α and IL-1β requires proteolytical cleavage of their precursors by calpain and caspase 1, respectively. Activated caspase 1 is produced by cleavage of pro-caspase 1 by the NLRP3 inflammasome, which is consitutively activated in melanoma cells (**autoactive signal 2**). ATP activates the P2X7 purinergic ion receptor allowing potassium efflux and the lowering of intracellular potassium concentrations, thus triggerring caspase 1 and IL-1β processing and secretion. A natural IL-1R1 antagonist (IL-1Ra), IL-1R2, SIGIRR (single immunoglobulin IL-1-receptor related molecule) and secreted sIL1-R1/sIL-1R2 negatively regulate IL-1R1 signaling. Drugs used to neutralize IL-1 activity are shown. Modified from Kopf *et al.*, 2010 (525).

IL-1α and IL-1β in their recombinant mature forms (17 kDa) bind to the same receptors and mediate similar biological functions [462, 463]. However, they may have different functional roles based on their cellular compartmentalization [462, 463]. Whereas IL-1β is solely active as a secreted product, IL-1α is rarely released by normal cells other than macrophages and is mainly active as an intracellular precursor form (pro-IL-1α) or as a surface-bound form, which acts in a juxtacrine manner through the IL-1R1 [462, 463, 528]. Intracellular pro-IL-1α is constitutively present in keratinocytes, fibroblasts and epithelial cells. Pro-IL-1α can be processed by the Ca^{2+}-dependent protease calpain into the C-terminal mature form (17 kDa) and the N-terminal cleavage product – the propiece of IL-1α (16kDa) [529] (Fig. 2). Pro-IL-1α and the propiece of IL-1α are suggested to act in an intracrine manner and to translocate into the nucleus due to a N-terminal nuclear localization sequence [530-532]. Although pro-IL-1α is not a substrate of caspase-1, recent studies have indicated that secretion of bioactive IL-1α by macrophages and keratinocytes requires functional NLRP3 inflammasome and activated caspase-1 [533]. Both pro-IL-1α and mature IL-1α can be released in the extracellular space and activate IL-1R1. The extracellular export of IL-1α, which lacks secretory signal peptide, appears to rely on stress signal-induced transmembrane translocation of IL-1α in a complex with S100A13, a protein harboring chaperone-like function [534]. The identity of IL-1α form(s) released into the supernatants of melanoma cell lines could be determined by Western blot. Determining the identity, quantity and localization of IL-1α forms in melanoma cells, as well as understanding the mechanisms of IL-1α cleavage and release (i.e. imbalance between the expression of calpain and calpain inhibitors), would help to uncover the role of this cytokine in melanoma pathogenesis.

VI.2.2 Mechanisms underlying constitutive IL-1 synthesis by melanoma cells

Given that the NLRP3 inflammasome appears to be activated in melanoma cells that spontaneously release IL-1β, but also in those that need an additional signal for IL-1β release, the constitutive transcriptional activation of *IL1B* (autoactive Signal 1) might be particularly important for constitutive IL-1β production [521] (Fig. 3). In agreement with previous studies [468, 469, 535, 536], our data indicate the involvement of the NF-κB pathway in constitutive transcriptional activation of *IL1A* and *IL1B* (Results - Part III, Fig. 4). In IL-1 producing melanoma cell lines, NF-κB constitutively activated by IL-1R1-mediated signaling would, in turn, induce expression of *IL1A* and *IL1B* by binding to their promoters and ensure the maintenance of the autoregulatory positive feedback loop of IL-1 production [521, 536]. In accordance with this model, IL-1-producing melanoma cell lines harbored constitutive NF-κB binding activity and exhibited reduced transcription of *IL1A* and *IL1B* after IL-1Ra-mediated blockade of IL-1R1 signaling (Results - Part III, Fig. 2 and 3). The relevance of the proposed model could be further validated in *IL1A*/*IL1B* promoter-driven reporter assays.

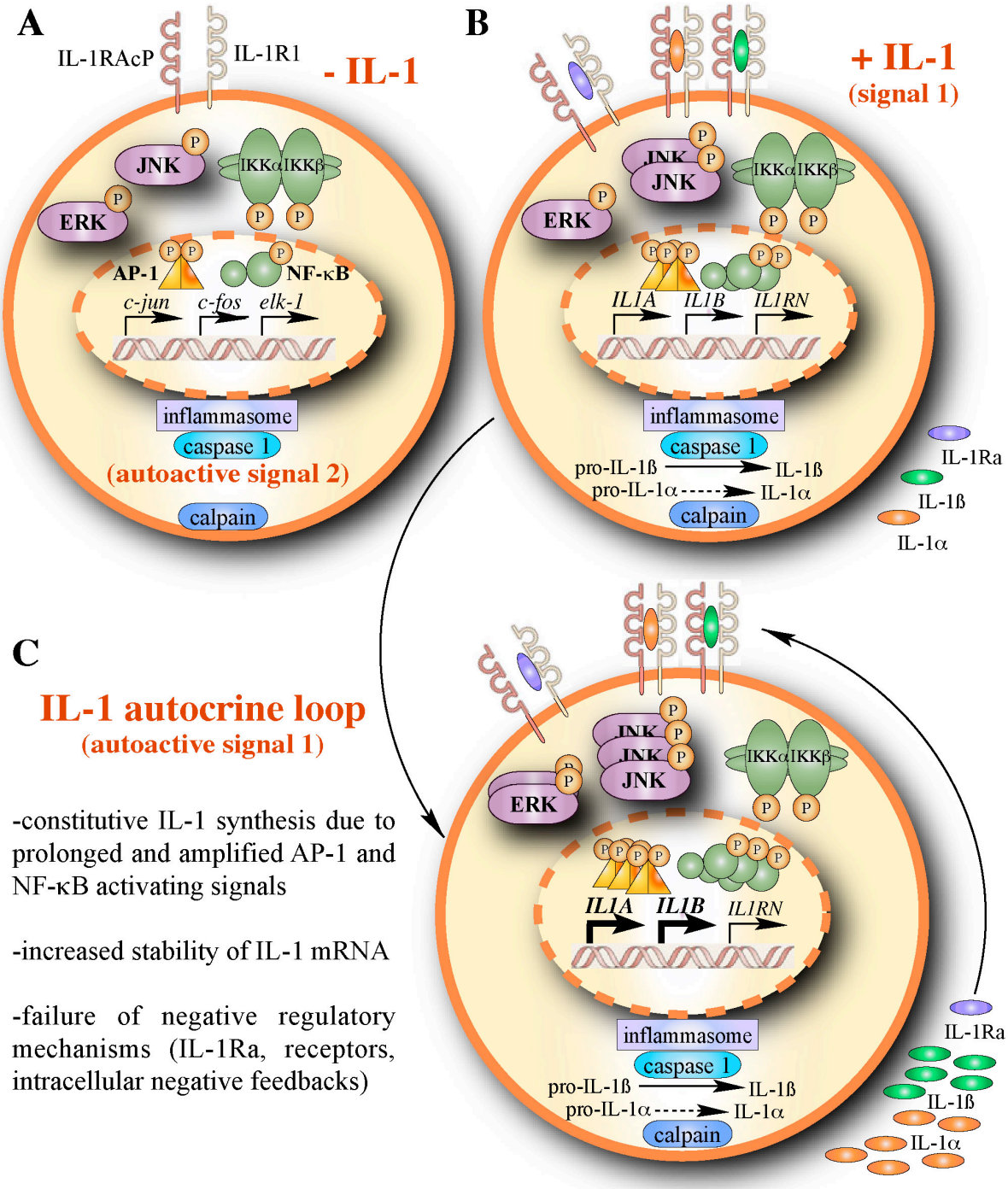


Figure 3. Potential mechanisms leading to constitutive IL-1 production by some melanoma cells. A. Melanoma cells do not produce IL-1 cytokines, but inflammasome is constitutively activated (**autoactive signal 2**). **B.** To synthesize and release IL-1, melanoma cells need stimulation with IL-1 (**signal 1**) and/or with other cytokines (for instance, TNF-α) and, in early stage melanoma cells, co-stimulation. Both IL-1 synthesis and release are reversible due to the involvement of negative regulatory mechanisms. **C.** Some melanoma cells acquire the capacity to constitutively synthesize IL-1 (**autoactive signal 1**) via activation of an autocrine IL-1R1-mediated loop. The prevalence of the amplified prolonged activating signals over the negative regulatory mechanisms might result in a switch to constitutive IL-1 production. Inspired by Okamoto *et al.*, 2010 (519).

Further, our data suggest the engagement of the JNK pathway in the constitutive activation of *IL1A* and *IL1B* (Results - Part III, Fig. 4). The JNK, ERK and p38 MAPK pathways frequently share their upstream regulators and downstream effectors (Introduction, Fig. 6) [113, 537]. Selective and combined inhibition of these pathways using various approaches (specific inhibitors, siRNA silencing, dominant negative mutants) would help to dissect the extent of their involvement in the induced and constitutive *IL1A* and *IL1B* transcription. Cross-talks between MAPK pathways are usually highly dependent on the cell context [113, 537]. In melanoma cells harboring the $BRAF^{V600E}$ mutation, a rewiring mechanism linking ERK signaling to JNK signaling has been reported [538]. Constitutively active ERK has been shown to increase c-Jun levels through its stabilization and activation of c-*jun* transcription [538]. c-Jun, in turn, has increased the transcription of RACK-1, an adaptor protein required for the activation of JNK, which phosphorylates c-Jun and increases its stability and transactivation potential [538]. EB16-MEL and KUL84-MEL cells harbor activating $BRAF^{V600E}$ mutation and apparently exhibit loss of heterozygosity of the wild-type allele at the codon 600 of *BRAF* (Results - Part III, Fig. 5*c*). This genotype has been reported to occur in 11% (3/28) of melanoma cell lines harboring $BRAF^{V600E}$ mutations [539]. As $BRAF^{V600E}$ is a gain-of-function mutation, loss of the wild-type BRAF competitor could promote the effectiveness of the mutant $BRAF^{V600E}$ variant. Therefore, the ERK-JNK feed-forward loop might be activated in EB16-MEL and KUL84-MEL cells. It would be of interest to confirm the suggested *BRAF* genotype by cloning and sequencing of exon 15 PCR products obtained from these cell lines and from corresponding melanoma lesions. Moreover, the extent of the ERK and JNK activity could be investigated in these samples.

A common downstream effector of MAPK pathways is activator protein 1 (AP-1) transcription factor, which possesses functionally relevant binding sites within *IL1A* and *IL1B* promoters [469, 540, 541]. The binding of homo- or heterodimeric AP-1 complexes to *IL1A* and *IL1B* promoters and the identity of AP-1 subunits could be investigated in IL-1-producing melanoma cell lines using EMSA, ChIP and gene reporter assays. AP-1 dimers binding *IL1A* and *IL1B* promoters might contain of the members of Jun (c-Jun, JunB, JunD) and Fos (c-Fos, FosB, Fra1, Fra2) families, which contain basic region (b) and leucine zipper (Zip) domains [542]. In pancreatic cancer cells constitutively producing IL-1α, AP-1 complexes containing c-Fos have been shown to bind two AP-1 sites within the *IL1A* promoter [469]. In melanoma cells harboring a $BRAF^{V600E}$ mutation, the constitutively activated ERK signaling would result in increased expression of c-Jun and *c-fos*, which encodes a heteromeric partner of c-Jun [538, 543]. The relative importance of AP-1 activity in the induced and constitutive transcription of *IL1A* and *IL1B* could be evaluated by selectively targeting AP-1 subunits using siRNAs or by expressing TAM67, a dominant-negative mutant of c-Jun devoid of N-terminal transactivation domain [544]. TAM67 generally inhibits AP-1 activity regardless of the specific binding partner owing to its promiscuous interaction with most AP-1 proteins [542].

Finally, our data obtained in KUL84-MEL cells suggest the cooperative action of the JNK and NF-κB pathways in the constitutive activation of *IL1A* and *IL1B* (Results – Part III, Fig. 4). The cross-talks between the NF-κB pathway and JNK or other MAPK pathways might occur at several signal transduction levels and ultimately result in simultaneous activation and cooperative action of AP-1 and NF-κB within *IL1A* and *IL1B* promoters (Discussion, Fig. 2 and 3; Introduction, Fig. 13). First, several upstream kinases of the JNK pathway, such as TAK1, MEKK1, MEKK3, have been demonstrated to concomitantly activate the IκB kinase complexes [545-547]. Second, a discovered scaffold protein, named POSH, has been shown to participate in the activation of the JNK pathway and the nuclear translocation of NF-κB [548]. Third, mutated $BRAF^{V600E}$ might contribute to constitutive NF-κB activity via activation of IKKβ [255], enhanced expression of β-TrCP targeting IκBα for ubiquitination-dependent degradation [249] and via p65 phosphorylation by MSK1, a downstream target of ERK [256]. Moreover, c-Raf-induced transformation of NIH 3T3 mice fibroblasts required NF-κB activation, and the initiating role of the ERK pathway in the establishment of an NF-κB activation-dependent IL-1 autocrine loop has been proposed [549]. In addition, the association between the presence of a *NRAS* activating mutation and the expression of *IL1A*, *IL6* and *TNFA* by clonal melanoma populations derived from the single melanoma lesion has been established, but the state of NF-κB activity has not been explored [550]. Fourth, NF-κB has been found to exert a transcriptional control of the components of the AP-1 complexes by inducing the expression of Elk-1, which is phosphorylated by ERK and induces the expression of *c-fos* and *fosB* [551].

Finally, cooperative action of NF-κB and AP-1 transcription factors might be mediated via their enhanced DNA binding and/or transactivation potential within *IL1A* and *IL1B* promoters. Physical interactions between AP-1 (c-Jun/c-Fos) and NF-κB (p65/p50) components have been demonstrated in living cells using the bimolecular fluorescence complementation (BIFC) assay [552]. Furthermore, the overexpression of NF-κB subunits has been shown to potentiate transcription from an AP-1 dependent promoter in gene reporter assays and vice versa [553]. Moreover, NF-κB binding to its target sequences has been shown to be suppressed in c-Jun-deficient cells [554]. The cooperative action of AP-1 and NF-κB transcription factors within *IL1A* and *IL1B* promoters could be assessed by EMSA, ChIP and gene reporter assays.

Other transcription factors than AP-1 and NF-κB as well as some epigenetic mechanisms may contribute to constitutive transcription of *IL1A* and *IL1B* in melanoma cells [555, 556]. Differential expression of *IL1A* and *IL1B* in the same melanoma cell line supports this notion (Results - Part II, Table 1). The activation of *IL1A* in a clonal population of A375 melanoma cells constitutively producing IL-1α has been reported to require, on one hand, the binding of Sp1 or Sp3 to the *IL1A* promoter and, on the other hand, reduced activity of HDAC1 [557].

VI.2.3 The failure of negative regulatory mechanisms

To prevent an inappropriate and aberrantly sustained response following activation of IL-1R1, a variety of extracellular and intracellular feedback pathways negatively regulating IL-1R1-mediated signaling have evolved (Fig. 2). These include the competitive inhibition of ligand binding to IL-1R1 by the secreted isoform of IL-1Ra encoded by *IL1RN* locus; expression of the membrane decoy receptor IL-1R2 and inhibitory receptor SIGIRR [462]; intramembrane proteolysis and release of soluble forms of IL-1R1, IL-1R2 and IL-1RAcP (IL-1R accessory protein), which compete with membrane receptors for ligand binding [462, 558]; post-translational modifications and production of dominant negative splice variants of the components of the NF-κB pathway [559], *de novo* synthesis of IκBα and action of deubiquitinating enzymes A20 and CYLD [560, 561]. The failure of any of these negative feedback mechanisms might favor IL-1 production by melanoma cells.

Our data obtained in IL-1-negative CP50-MEL cells demonstrated the reversibility of transcriptional changes induced by IL-1 exposure (Results – Part III, Fig. 6), suggesting the activation of negative feedback mechanisms preventing the establishment of the autocrine IL-1 loop in these cells. Consistently, the release of IL-1Ra in the culture medium was observed following treatment with IL-1β. The levels of IL-1Ra (371±36 pg/ml, n=3) exceeded about 22-fold the levels of *de novo* produced IL-1α (17±1 pg/ml, n=3), as measured by Bio-Plex immunoassay in the supernatants of CP50-MEL cells left in the presence of IL-1β for 10 days (data not shown). To investigate the role of potentially deficient negative regulators in the establishment of the autocrine IL-1 loop and to determine their identity, long-term IL-1 treatment of CP50-MEL melanoma cells could be combined with siRNA screening using spontaneous IL-1 production as a read-out. Precisely, *IL1RN*-specific siRNAs could be used.

Remarkably, IL-1-producing melanoma cell lines exhibit a deficient negative regulation by IL-1Ra. 10- to 200-fold greater amounts of recombinant IL-1Ra (17 kDa) are required to efficiently inhibit the binding of IL-1α and IL-1β to IL-1R1 *in vitro* [562]. Notably, the supernatants of EB16-MEL cells producing about 7 ng/ml of total IL-1 contained only 121±25 pg/ml (n=3) of IL-1Ra, and the supernatants of KUL84-MEL cells were devoid of it (data not shown). Within the melanoma environment, IL-1Ra produced by non-melanoma cells might compete with IL-1 for binding to IL-1R1. This could explain the higher levels of auto-activated *IL1A* and *IL1B* expression in melanoma cell lines than in the corresponding melanoma tissues (Results - Part II, Table 1). Interestingly, several antigens derived from non-mutated IL-1Ra protein have been found to be recognized by CTLs on the surface of melanoma cell lines, indicating IL-1Ra production by some melanoma cells [563]. The levels of circulating IL-1Ra are worth to be explored in melanoma patients. In patients with other types of cancer, the increased levels of circulating IL-1Ra (over 150-400 pg/ml observed in healthy individuals) have been associated with either increased or decreased disease severity [564].

The mechanisms underlying the imbalance in IL-1Ra/IL-1 ratio in some melanoma cell lines are unknown and several possibilities could be explored. First, polymorphisms within *IL1A*, *IL1B* and *IL1RN* may result in altered synthesis of IL-1α, IL-1β and IL-1Ra, respectively [565]. Second, differences in transcriptional regulation of *IL1RN*, such as independency on the NF-κB activity, might account for its differential synthesis in melanoma cells [566]. Third, the presence of AU-rich elements (ARE) within the 3'UTR of *IL1B*, but not *IL1RN* transcripts, might account for their differential post-transcriptional regulation.

The IL-1R1/IL-1R2 imbalance might also contribute to a deregulation of IL-1 control in melanoma. Of interest is the finding of differential *IL1R1* expression by melanoma cell lines (Results - Part II, Fig. 2). In order to assess whether this observation reflects the *in vivo* situation, immunohistochemical analysis of melanoma tissues, *in situ* binding assay [567] or RT-qPCR analysis of *IL1R1* expression in laser capture microdissected melanoma cells could be performed. The functional significance of differential *IL1R1* expression is not clear. On one hand, prevalent binding of IL-1 to IL-1R1 on the surface of melanoma cells, which generally outnumber other cell types in tumor environment, might dampen immunostimulatory actions of IL-1 *in situ*. In addition, the expression of IL-1R1 would be required for the establishment of the autocrine IL-1 loop in some melanoma cells. On the other hand, melanoma cells with no or low expression of IL-1R1 would not compete for IL-1 in the tumor environment, exhibit reduced sensitivity to IL-1 triggering and maintain in this way the expression of differentiation antigens to elicit antitumoral immune responses.

The transcriptional silencing of *IL1R1* might rely on epigenetic mechanisms. For instance, local extrinsic factors might induce *IL1R1* silencing, which might be consolidated due to intrinsic melanoma-related epigenetic changes. In line with this hypothesis, the repression of *OSMRβ* , which encodes receptor for oncostatin M (OSM), appears to rely on increased histone deacetylation within its promoter [568]. In addition, differential regulation of alternative *IL1R1* promoters might occur in tumor cells [569].

To understand the relationships between IL-1 producing and IL-1 responding cell types within the tumor environment, an extensive analysis of mRNA and protein expression of the components of the IL-1 system could be performed in a large number of samples from different melanoma stages. It would be appropriate to compare mRNA and protein expression profiles from melanoma tissues with those exhibited by melanoma cell lines established from these tissues. In order to overcome the problem of cell heterogeneity and sub-regional differences in mRNA profiles in tumor samples, such comparisons would require laser capture microdissection of the tumor and stromal cells in combination with an immunohistochemical analysis of tumor tissues.

In conclusion, our study revealed disequilibrium in IL-1 control in melanoma cells. Some underlying mechanisms were suggested and might be explored in melanoma and other types of cancer.

VI.3 Mechanisms of IL-1-mediated MITF-M downregulation

Whereas IL-1-induced *MITF*-M repression appears to be reversible in *MITF*-M-positive melanoma cell lines, it might become constitutive and hardly reversible in melanoma cells constitutively producing IL-1 cytokines. Since solely IL-1 exposure was insufficient to initiate constitutive IL-1 production in melanoma cells, the primary causative role of IL-1 in permanent *MITF*-M downregulation could not be experimentally established. However, we suggested that a subset of melanoma cells chronically exposed to IL-1 cytokines in the tumor environment might acquire a capacity to autonomously produce IL-1 and hence to maintain *MITF*-M downregulation. The role of IL-1 in *MITF*-M downregulation *in vivo* could be indirectly assessed by the analysis of *MITF*-M transcript expression in microdissected melanoma cells adjacent to IL-1-producing stromal cells. Mechanistically, IL-1-mediated repression of *MITF*-M might be exerted at transcriptional, post-transcriptional and epigenetic levels. If some proposed mechanisms might promote both inducible and constitutive *MITF*-M downregulation, the others might solely operate to permanently silence *MITF*-M expression.

VI.3.1 Transcriptional regulation

Our study linked for the first time the downregulation of *MITF*-M expression with the activation of NF-κB and JNK pathways. Based on our data, it is tempting to propose that the previously reported NF-κB-mediated inhibition of *TYR* transcription could primarily imply the downregulation of *MITF*-M expression [459]. In our study, LB2259-MEL cells exhibited constitutive NF-κB activity (Results - Part III, Fig. 3), which was likely further induced by IL-1 signaling and contributed in this way to *MITF*-M repression (Results - Part II, Fig. 3). Consistently with the constitutive triggering of IL-1R1 in IL-1-producing KUL84-MEL and EB16-MEL cells, they showed, respectively, about 4- and 7-fold higher constitutive NF-κB activity than LB2259-MEL cells (Results - Part III, Fig. 3). However, inhibition of the NF-κB signaling did not result in considerable derepression of *MITF*-M expression in IL-1-producing melanoma cell lines (Results – Part III, Fig. 4), suggesting that constitutive NF-κB activity is not the major mechanism responsible for the maintenance of permanent *MITF*-M repression.

In contrast, inhibition of the JNK signaling appeared to counteract both IL-1-induced and constitutive *MITF*-M repression (Results - Part II, Fig. 3 and Results - Part III, Fig. 4). Both constitutive and IL-1-induced JNK activation might underly the observed effects. An ERK-JNK feed-forward loop [538] might be constitutively active in both *MITF*-M-positive and *MITF*-M negative cell lines that harbor a heterozygous T1799A mutation in *BRAF* (LB2259-MEL and CP50-MEL cells) or, in addition, seemingly carry out the loss of heterozygosity at the codon 600 of *BRAF* (KUL84-MEL and EB16-MEL cells).

Consistently, phosphorylation of JNK1 and ERK1/2 was observed in LB2259-MEL cells in the absence of stimulation with IL-1 (Results - Part III, Fig. 5*c*). Moreover, the activated ERK pathway seemed to consitutively downregulate *MITF*-M expression in LB2259-MEL cells since their treatment with MEK inhibitors PD98059 and U0126 resulted in about 1.3-1.5-fold higher *MITF*-M expression (data not shown). The functional outcome of the selectively activated JNK pathway on *MITF*-M expression could be explored in these cells by the ectopic expression of the gain-of-function fusion protein of JNK and its upstream kinase MKK7 [570]. The extent of the constitutive ERK activity and its impact on permanent *MITF*-M repression are worth to be investigated in IL-1-producing melanoma cell lines.

The constitutive activation of the ERK, JNK and NF-κB pathways might provide higher levels of expression and activity of transcription factors that are available for IL-1-mediated *MITF*-M repression. Therefore, lower IL-1 concentration might be needed to incite *MITF*-M downregulation in *MITF*-M positive cell lines. Candidate transcription factors that might mediate *MITF*-M repression either directly or via intermediate action of other transcription factor(s) or miRNA(s) include NF-κB and AP-1. *In silico* analysis of the 5' region of *MITF* (positions -2000 to +2000 relative to TSS) did not reveal the presence of consensus NF-κB-binding sites, whereas one AP-1 binding site TGACTCA is located in the first intron of *MITF*-M (Introduction, Fig. 7). Expression levels of AP-1 subunits, their post-translational modifications and interactions with other proteins [571] might determine a function of AP-1 complexes within the 5' region of *MITF*-M. NF-κB might indirectly contribute to the function of AP-1 complexes by increasing *c-fos* transcription via binding to its first intron [572] or by inducing the expression of Elk-1 [551]. The AP-1 complexes might contain bZip transcription factors of Jun, Fos and activating transcription factor (ATF) families [542, 571]. Besides binding to 7-bp AP-1 sites as homodimers or heterodimers with Fos proteins, Jun proteins can bind 8-bp CRE sites as heterodimers with ATF proteins. The activity of c-Jun-ATF2 heterodimer is enhanced via phosphorylation of the transactivation domains of c-Jun and ATF2 by JNK [542]. JunB-ATF2 complexes have been shown to suppress SOX10 transcription and hence to repress MITF expression in about 50% of melanoma cell lines [573].

The presence of the CRE and AP-1 sites within the 5' region of *MITF*-M (Introduction, Fig. 7) confers the potential for the context-dependent modulation of *MITF*-M transcription due to differential binding of CREB and AP-1 transcription factors to these *cis*-elements. Of the two CRE located upstream of the *MITF*-M TSS, the proximal one has been shown to bind phosphorylated CREB and to mediate basal and cAMP-stimulated expression of *MITF*-M [108] (Introduction, Fig. 5). In contrast, an inducer of AP-1 activity, TPA (12-*O*-tetradecanoylphorbol-13-acetate), has been proposed to inhibit *TYR* transcription via decreased MITF-M binding to M-box in the *TYR* promoter due to reduced MITF-M expression [574]. Based on previously reported data, three possible scenarios could explain the negative regulation of *MITF*-M transcription following activation of IL-1 signaling (Fig. 4A).

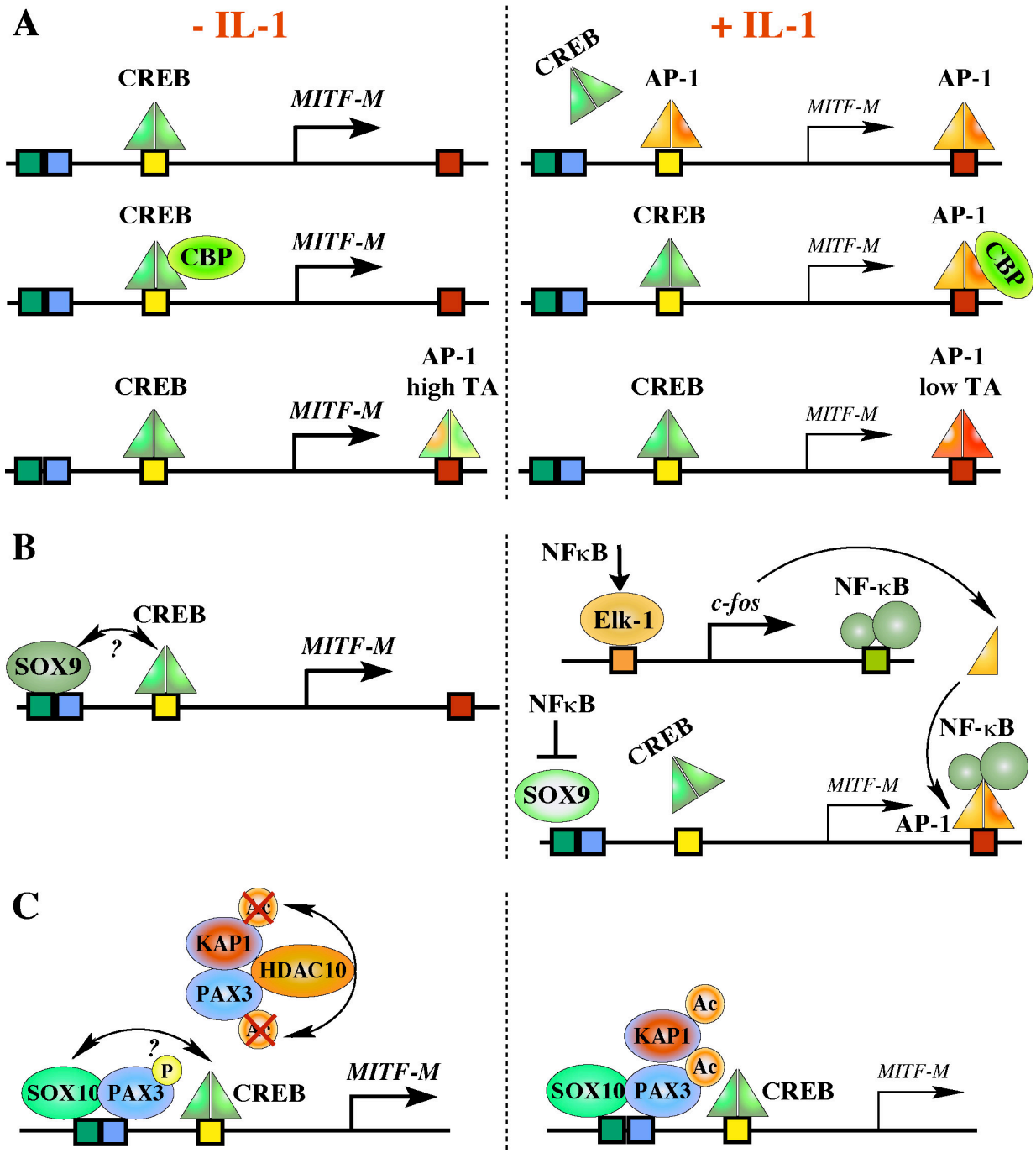


Figure 4. Potential mechanisms of transcriptional MITF-M repression by IL-1.
A. Hypothetical mechanisms of *MITF*-M repression by AP-1 (activator protein 1) complexes via CREB (CRE binding protein) displacement, sequestration of an intracellular pool of CBP (CREB-binding protein) and binding of AP-1 complexes with low transcriptional activity (TA). CRE (cAMP response element) and AP-1 binding sites within the *MITF*-M promoter are represented by yellow and red boxes, respectively. **B.** NF-κB might be involved in transcriptional *MITF*-M repression via induction of AP-1 subunit expression and inhibition of *SOX9* expression. Adjacent SOX10/SOX9 and PAX3 binding sites are depicted as green and blue boxes, respectively. **C.** PAX3 might be subjected to post-translational modifications (phosphorylation, acetylation) that might influence its DNA and protein binding and/or transcriptional activity (activation or repression) within the *MITF*-M promoter.

First, AP-1 complexes might competitively bind CRE and prevent transcriptional activation mediated by phospho-CREB. Second, AP-1 and CREB transcription factors might compete for binding with the limiting amounts of intracellular CBP, a common co-activator [575, 576]. Finally, AP-1 dimers with higher transcriptional potential might be concurrently replaced following IL-1 signaling by dimers *containing* subunits with weak transactivation capacity, such as JunB, JunD, Fra1 and Fra2 [542, 577]. In addition, NF-κB might interfere with the action of SOX9, an activator of *MITF*-M transcription, which, similarly to SOX10, might be required for the transactivating function of phospho-CREB (Fig. 4B). Indeed, it has been reported that NF-κB indirectly affects the stability of the *SOX9* transcript by a poorly understood mechanism [578].

In LB2259-MEL cells, *de novo* protein synthesis appears to be required for IL-1-induced *MITF*-M downregulation, since the treatment of these cells with the translational inhibitor cycloheximide (CHX) counteracted the inhibitory effect of IL-1 (data not shown). Moreover, the CHX treatment alone resulted in an increased abundance of *MITF*-M transcripts, suggesting that *de novo* protein synthesis is required for the homeostatic downregulation of *MITF*-M mRNA levels. Previously, CHX-dependent homeostatic downregulation of *MITF*-M has been proposed to involve proximal CRE site within the 1M promoter of *MITF* [108]. *De novo* protein synthesis has been suggested to be required for the downregulation of phospho-CREB levels by protein phosphatase 1α (PP1α), while CHX treatment has resulted in the decline in PP1α and the maintenance of phospho-CREB levels [108]. To fit a model of competitive CREB/AP-1 binding to CRE site, CHX treatment might in addition reduce *de novo* protein synthesis of the AP-1 subunits executing negative transcriptional activity within the 1M promoter of *MITF*. The CHX sensitivity of DNA binding by CREB or AP-1 within 5' *MITF* region may be investigated in ChIP-qPCR assays.

To further investigate the potential involvement of AP-1 in *MITF*-M regulation, dominant-negative mutants of AP-1 subunits could be used, for instance TAM67, which is able to suppress both AP-1 and NF-κB transactivation activities [544], and A-Fos, a dominant negative variant of c-Fos, which inhibits endogenous AP-1 DNA binding activity [575]. A-Fos forms non-functional dimers with wild type c-Fos via its acidic domain complementary in charge distribution to the basic region of c-Fos [575]. The functional relevance of the AP-1 site found within the *MITF*-M promoter could be investigated in gene reporter assays. Since we did not observe IL-1-induced repression of luciferase reporter activity driven by the 1M promoter of *MITF* comprising 15 kb upstream and 95 bp downstread of its TSS [129] (data not shown), the vector that contains a part of the first intron of *MITF*-M could be used. The time-course study of AP-1 and NF-κB nuclear co-localization following IL-1R1 signaling could be investigated in living cells using BIFC assay [552] and temporally related to *MITF*-M inhibition.

Other transcription factors than AP-1 and NF-κB might contribute to *MITF*-M repression in response to IL-1 signaling. BRN2 has been shown to be transcriptional regulator

of *MITF*-M (Introduction, Fig. 7). It has been found to repress *MITF*-M transcription in melanoma cells [283, 579]. ChIP-on-chip and ChIP-qPCR assays have revealed four BRN2 binding sites within the 5' region of *MITF*-M, two upstream and two downstream of its TSS [579]. The occupancy of these sites by BRN2 in the conditions of induced and constitutive IL-1R1 signaling could be assessed by ChIP-qPCR assay. The portion of *MITF*-M that contains all BRN2 binding sites, including the one situated at +1800 bp relative to TSS [579], could be used to drive the expression of luciferase in gene reporter assays. Since phosphorylation of BRN2 has been documented to reduce its DNA binding activity [580], its dephosphorylation by a currently unknown phosphatase following IL-1 signaling, could result in increased binding and repression of the *MITF*-M promoter.

PAX3, which acts as either transcriptional activator or repressor, has been found to be modified by phosphorylation [581] and acetylation [582]. One possibility is that IL-1-induced dephosphorylation of PAX3 decreases its transactivating potential within the *MITF*-M promoter and/or impairs PAX3 interaction with other proteins, such as SOX10 [127] (Fig. 4C). Interaction of PAX3 and KAP1 with HDAC10 in a ternary complex has been shown to relieve the repression of *MITF*-M by keeping PAX3 and KAP1 co-repressor deacetylated [582]. Triggering of the IL-1 signaling might result in acetylation of PAX3 and KAP1, leading to repression of *MITF*-M (Fig. 4C).

In addition, PAX3 and MITF have been show to be downregulated downstream of the PKC-mediated STAT3 phosphorylation in Wnt5A-treated melanoma cells [278]. This signaling pathway might be further explored taking in account our findings of the JNK and NF-κB involvement in *MITF*-M repression. Indeed, JNK activation downstream of the Wnt5A signaling as well as phosphorylation of JNK by PKC have been demonstrated [583, 584], but the downstream events that could lead to *MITF*-M repression have not been studied in melanoma cells. In addition, persistently activated STAT3 has been shown to maintain constitutive NF-κB activity in melanoma cells by promoting the nuclear retention of the acetylated p65 subunit of NF-κB [585]. Moreover, IL-1-producing melanoma cell lines secrete IL-6 [521], a powerful inducer of STAT3 signaling, which has been shown to mediate PAX3, MITF-M and TYR downregulation by poorly understood mechanisms [586]. Therefore, the cooperative action of the JNK, NF-κB and STAT3 pathways in Wnt5A- and IL-1-mediated *MITF*-M repression could be investigated in melanoma cells using loss-of-function approaches.

Finally, long-range influence of distal *cis*-acting regulatory elements could interfere with *MITF*-M transcriptional regulation. Regulatory elements located within the other nine alternative *MITF* promoters (Introduction, Fig. 2) might act as enhancers or silencers for the *MITF*-M promoter activity. IL-1 signaling might either abolish enhancer activity or favor silencer function. Given that *MITF* spans over 200 kb (Introduction, Fig. 2), the relevance of distal *cis*-acting regulatory elements is technically difficult to assess in gene reporter assays, which, besides, do not always reproduce natural promoter context.

Over the past years, various models for the long-range transcriptional control have been put forward. These include intrachromosomal looping, interchromosomal *trans*-interactions, heterochromatic suburbs and transcription factories [587]. For instance, NF-κB has been shown to bind not only transcribed regions, but also repeated Alu elements throughout the genome and to induce formation of intra- and interchromosomal loops following environmental stimuli [588, 589]. Global analysis of long-range *MITF* regulation may be done using newly developed high-throughput technologies based on chromosome conformation capture (3C) assays, which assess the physical proximity between DNA sequences as they occur in the nucleus [590]. Another technology, the 4C assay (3C-on-Chip or Circular 3C) employs microarrays following inverse PCR that amplify all DNA fragments ligated to a genomic element of interest [591]. 4C assay might be used to identify all genomic regions that interact with *MITF*-M promoter region in the absence and presence of IL-1 exposure.

VI.3.2 Post-transcriptional regulation of MITF-M mRNA stability

Contributing to the negative feedback response following IL-1R1 signaling, miRNA-146a, miRNA-146b and miRNA-155 have been reported to target components of the IL-1R1-mediated signaling pathway [473, 592]. The transcription of these miRNAs is induced by NF-κB and AP-1 transcription factors [473, 593, 594], and their increased levels following IL-1 exposure might lead to *MITF*-M downregulation. Interestingly, we found one miR-155 target site (AGCAUUAA [595]) in the 3'UTR of *MITF* (Introduction, Fig. 3). Moreover, the repression of *MITF* expression by miRNA-155 has been recently reported in an *in vitro* model of macrophage differentiation [596]. Therefore, the expression of miR-155 could be assessed in LB2259-MEL cells treated with IL-1 and in IL-1-producing melanoma cell lines. Alternatively, a microarray-based screen to identify miRNAs induced by IL-1R1 signaling could be performed. Depletion of MITF-M mRNA and protein by miRNAs is expected to be reversible as it would lead to the downregulation of DICER known as transcriptional MITF target [29] and, consequently, to decreased maturation of *de novo* produced miRNAs.

The presence of ARE sites (Introduction, Fig. 3) in *MITF*-M mRNA could be a determinant factor of its stability in melanoma cells. An imbalance between RNA-binding proteins that favor mRNA decay, such as tristetraprolin (TTP), and those that stabilize mRNA, such as Human antigen R (HuR), is frequently observed in cancer [47]. Following inflammatory stimuli, such as IL-1 and TNF-α, numerous transcripts exhibit a reduction of their decay rate due to increased phosphorylation and inactivation of TTP and increased recruitment of HuR to their AREs [47]. The competition for the limiting intracellular amount of ARE-regulating proteins might lead to their sequestration predominantly by AREs of cytokine-induced transcripts, leading to reduced mRNA stability of other ARE-regulated transcripts, including *MITF*-M mRNA.

The differential binding of TTP and HuR to the 3'UTR of MITF before and after IL-1 treatment could be assessed by RNA immunoprecipitation using anti-HuR and anti-TTP antibodies [597] combined with quantitative RT-PCR. The p38 MAPK pathway is essential for the regulation of ARE-mediated post-transcriptional events [47]. The observation of p38 phosphorylation in LB2259-MEL cells treated with IL-1 (data not shown), prompts further analysis of p38 contribution to *MITF*-M regulation.

Shortening of 3'UTRs by alternative cleavage and polyadenylation is frequently observed in cancer cells [598]. In melanoma cell lines, *MITF*-M transcripts with short 3'UTRs, which should escape miRNA-mediated degradation and ARE-mediated regulation, predominate over *MITF*-M transcripts with intermediate and long 3'UTRs [44]. Therefore, it would be of interest to assess the correlation between the content of shorter *MITF*-M transcripts in melanoma cell lines and the level of *MITF*-M expression after IL-1 treatment.

VI.3.3 Epigenetic modifications

There is compelling evidence that activation of signaling pathways is accompanied by epigenetic changes within gene promoters [173], and the relevance of such changes in carcinogenesis has been recently demonstrated in the context of JAK2 signaling [599]. The activation of the MAPK signaling cascades has been associated with acetylation, phosphorylation and methylation changes of histone H3 and H4 amino acid residues [173, 600-602]. In IL-1-stimulated cells, phosphorylation of c-Jun has promoted its interaction with the p65 NF-κB subunit, dissociation of c-Jun/p65 from HDACs and concomitant histone acetylation within target promoter regions [554, 601]. In addition, NF-κB signaling has been involved in transcriptional activation of histone demethylase expression [603], suggesting that constitutive NF-κB activity might result in permanent changes of histone methylation patterns.

We suggested the involvement of some epigenetic mechanisms in permanent *MITF*-M downregulation. It is possible that the normally transient binding of negative regulatory factors to the *MITF*-M promoter after IL-1 exposure then leads to epigenetic changes that maintain the repressive *MITF* state following cell divisions. Whether induced and constitutive IL-1R1 signaling in melanoma cells is associated with chromatin changes within the 5' region of *MITF*-M could be determined by ChIP analysis using monoclonal antibodies to specific histone modifications. DNA methylation might mediate a sustained repression of *MITF*-M. However, its 5' region contains few CpG nucleotides and hence appears less susceptible to this form of epigenetic silencing. Nevertheless, methylation of a single CpG site, for instance located within the CRE of the 1M promoter of *MITF*, might affect DNA binding affinity of the relevant transcription factors. To test this hypothesis, the treatment of KUL84-MEL cells with 5-azadC alone or in combination with a JNK inhibitor could be performed and followed by ChIP-qPCR assay to assess the binding of CREB and AP-1 to the CRE-containing region.

The character of epigenetic changes might differ in IL-1-producing melanoma cells. Since treatment of EB16-MEL cells with 5-azadC did not result in *MITF*-M reactivation (data not shown), the *MITF*-M promoter might harbor a completely locked heterochromatin state insensitive to hypomethylating treatment in these cells. Of note, RT-PCR product was obtained with primers amplifying a region corresponding to exon 2 common to all MITF isoforms, indicating that at least one of them was expressed in these cells. Accordingly, *MITF*-M negative melanoma cell lines analyzed in another study have not been found to harbor any abnormality or a chromosomal translocation involving the *MITF* gene [482].

VII. Contribution of IL-1 to the tumor-immune system interactions and the tumor phenotype in the course of melanoma progression

The role of IL-1α and IL-1β as key mediators of both antitumor immunity and tumor-promoting inflammation is emerging and starts to be elucidated in cutaneous melanoma [375, 463]. The currently available data indicate that in the early stages of tumor development, IL-1 might support the induction of the antitumoral immune response, while in advanced tumors it appears to promote tumor progression (Fig. 5) [375, 463]. In view of these opposing functions, the cellular source, compartmentalization and quantity of IL-1 production might follow dynamic changes in the course of tumor development. In early stages of melanoma development, IL-1α and IL-1β might be produced by resident skin keratinocytes after UV exposure [604, 605]. Circulating monocytes, which are among the first immune cells to be attracted by tumor-derived chemokines, migrate from the blood into hypoxic tumor tissue and differentiate *in situ* into macrophages and DCs (Fig. 5). These cells are able to release IL-1β when stimulated by danger-associated molecular patterns, which include IL-1α released from stressed or dying tumor and stromal cells in the hypoxic environment [521, 526, 606, 607]. IL-1β, in turn, promotes priming of IFN-γ-producing tumor antigen-specific $CD8^+$ T cells and stimulates activation and proliferation of $CD4^+$ T cells [606, 608].

Thus, IL-1 cytokines might serve as a 'danger signal' for mounting antitumoral immunity in early stages of tumor development by recruiting immune cells and enhancing antigen presentation [463]. However, IL-1β production in the tumor environment increases the rate of accumulation of myeloid-derived suppressor cells (MDSC) exhibiting T cell suppressive activity [609-611] (Fig. 5). In melanoma patients, increased frequency of circulating MDSC has been documented [612]. These melanoma-associated MDSC inhibited proliferation and IFN-γ production by $CD8^+$ T cells *in vitro* and produced high levels of intracellular arginase leading to increased catabolism of L-arginine thus depriving T cells of this amino acid [612]. In addition, tryptophan starvation of T cells might be promoted by IL-1 due to induction of indoleamine 2,3-dioxygenase expression by DCs [613].

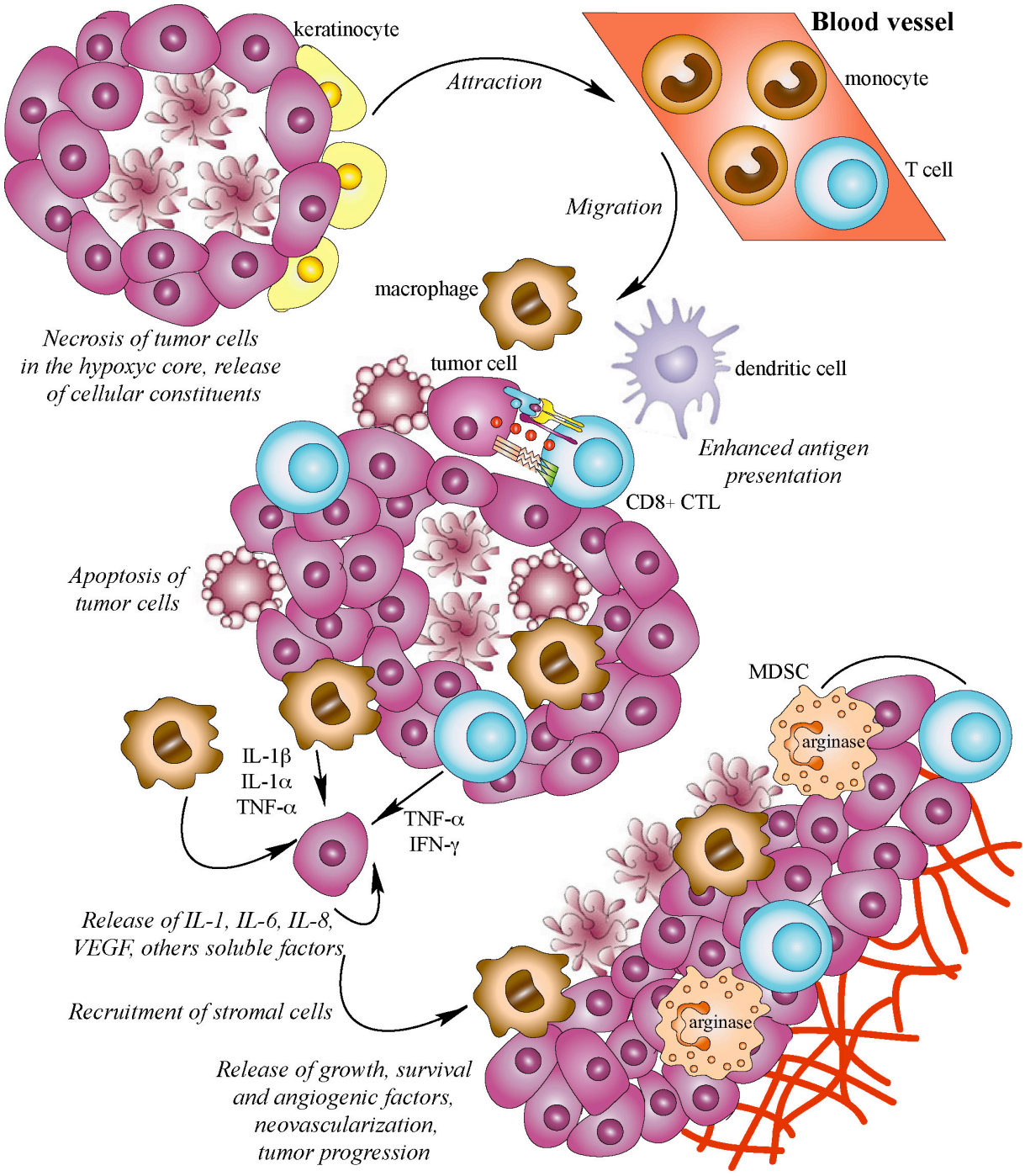


Figure 5. A model of IL-1 function in the melanoma environment. T cells and monocyte-derived cells infiltrate the tumor after being attracted by tumor-secreted chemokines, cytokines or cell-death associated molecular patterns (DAMPs), including IL-1α, which can also be released together with IL-1β from UV-exposed keratinocytes. Macrophages and dendritic cells can secrete IL-1β, which enhances antigen presentation and amplifies the immune response. Activated CD8+ CTLs lyse tumor cells and secrete IFN-γ and TNF-α, which can induce *IL1A* and *IL1B* expression by melanoma cells. The interactions between melanoma cells and macrophages may be reciprocal and self-perpetuating, leading to further recruitment of macrophages and an amplification of IL-1 production in the tumor environment. IL-1β increases the rate of accumulation of myeloid-derived supressor cells (MDSC) and their T cell suppressive activity. Some melanoma cells might acquire the capacity to autonomously produce IL-1, thereby enhancing angiogenesis and leading to tumor progression. Inspired by Okamoto *et al.*, 2010 (519).

The tumor-mediated production of IL-1 cytokines, which are able to stimulate antitumoral immune response, appears counterintuitive. Consistently, in the early stages of melanoma development, tumor cells apparently lack the capacity to actively release IL-1 [521]. In contrast, overactivation of the IL-1/IL-1R1 system in advanced tumors seems meaningful since it might contribute to the establishment of the immunosuppressive tumor environment. In a human melanoma/nude mouse model, the change in IL-1α compartmentalization has appeared to determine its function, since the antitumoral activities attributed to the cytosolic and membrane-bound forms [463] have not been observed when IL-1α has been actively secreted by melanoma cells [614]. Immune cells might contribute to the initiation and maintenance of IL-1 production by tumor cells via cytokine-mediated interactions. Thus, macrophage-derived IL-1 might promote the initiation of an autoregulatory IL-1 production by melanoma cells. The interactions between melanoma cells and macrophages may be reciprocal and self-perpetuating leading to an amplification of the IL-1 production in the tumor environment. Melanoma-derived IL-1β and its downstream mediators (IL-8, IL-6, CCL2) may enhance macrophage chemotaxis and promote angiogenesis mediated by pro-angiogenic factors, such as VEGF produced by tumor cells, macrophages and endothelial cells [521, 615]. Recent studies clearly emphasize the requirement of NF-κB activation in both macrophages and tumor cells for maintaining the inflammatory circuits that promote tumor progression [251, 616]. Besides IL-1 itself, other effector cytokines could stimulate IL-1 synthesis by melanoma cells, such as TNF-α, a T-cell and macrophage-produced pleiotropic cytokine, which potently induces NF-κB activity [617]. We observed that TNF-α was able to stimulate *IL1B* transcription in LB2259-MEL cells, whereas our preliminary data suggest constitutive production of TNF-α by KUL84-MEL cells (data not shown). In addition, IFN-γ induced *IL1B* expression, but not IL-1β release by LB2259-MEL and CP50-MEL cells (data not shown), suggesting that this cytokine might enhance IL-1 synthesis in melanoma cells. In macrophages, IFN-γ treatment have resulted in the activation of caspase-1 and the release of endogenous ATP, thus enhancing IL-1β release [526].

It is possible, that a combination of prolonged amplified activating signals with a failure of negative feedback mechanisms allows to reach a certain threshold and to switch to the constitutive IL-1 production by melanoma cells (Fig. 3, 5). This model could explain how multiple signaling pathways, such as the MAPK and NF-κB pathways, may cooperate to initiate and maintain an IL-1 autoregulatory loop. If the threshold model is operational, than the amplitude and the duration of the positive and negative regulatory signals should be considered. For instance, the presence of the $BRAF^{V600E}$ mutation in melanoma cells might enforce activating signals via the constitutive activation of ERK, JNK and NF-κB pathways. The role of BRAF mutations (such as V600E or V600R (1798-99GT→AG) as observed in DDHK62-MEL cells) in activating intracellular signaling pathways might be assessed by using loss-of-function approach, such as the depletion of the mutated BRAF by siRNAs [378, 618].

The apparent involvement of the mutated $BRAF^{V600E}$ in orchestrating downstream events that underlie the production of inflammatory cytokines [376, 378] indicates that this genetic event occurring early in melanomagenesis might participate in the build-up of an inflammatory environment in the course of tumor progression.

There is ample experimental evidence indicating that constitutive IL-1-production by melanoma cells might promote tumor progression to metastatic disease [467, 564, 614, 619]. Invasion of tumor cells in the extracellular tissues, their adhesion to endothelial cells prior to gaining access to the lymphatic or blood vessels, survival during circulation and establishment of angiogenesis at a secondary site requires the coordinated expression of a large number of genes. Several IL-1-regulated genes that encode adhesion molecules, chemokines and matrix metalloproteases (MMPs) have been shown to be essential for tumor invasion and metastasis [467, 564]. Transcriptional activation of many such genes by AP-1 cooperatively with NF-κB hints at mechanisms by which coordinate expression of functionally related sets of genes relevant to tumorigenesis is achieved [620]. For instance, protein products of *IL8* and *VEGF* are relevant to angiogenesis, whereas both AP-1 and NF-κB can cooperatively induce their expression via AP-1 and NF-κB binding sites for *IL8* and solely via AP-1 binding site for *VEGF* [621-623]. The NF-κB and AP-1 activation might be enhanced in circulating metastatic melanoma cells due to an increased likelihood of exposure to viral infection. In some types of cancer, viral infection has been linked to IL-1β production by tumor cells, constitutive NF-κB and AP-1 activity and increased expression of particular miRNAs, including miR-155 [594, 624, 625]. It would be of interest to identify which genes are coordinately regulated by AP-1 and NF-κB transcription factors in response to transient and constitutive IL-1 exposure to better define and specifically inhibit the mechanisms that underlie this cooperation. The microarray studies of gene expression combined with loss-of-function approaches targeting AP-1 and/or NF-κB activity might be used for this purpose.

Hypo-pigmentation appears to be one of the biological characteristics of metastasis-competent melanoma cells. In a model of melanoma metastasis using intravital imaging, only a sub-population of less pigmented B16F2 mouse melanoma cells has been able to leave the primary tumor and to enter vessels draining from the tumor [626]. Interestingly, the less pigmented phenotype of motile and intravasated cells has not been maintained in secondarily formed highly pigmented metastatic tumors, implying switching between pigmentation states as melanoma cells metastasize [626]. A switch between high and low pigment production has been associated with changes in mRNA expression patterns [626]. Thus, upregulation of *TGFβ2* and activated TGF-β signaling have characterized poorly pigmented cells collected *in vivo*, consistent with the TGF-β-induced hypo-pigmentation and cell motility observed *in vitro* [626]. Interestingly, dose-dependent cross-talk has been described between the TGF-β and IL-1β signaling pathways, suggesting that a hypo-pigmenting phenotype might result from a functional overlap between these pathways [627].

Phenotypic switching affecting pigmentation has been similarly shown *in vivo* in immunocompromised mice subcutaneously injected either with *MITF*-negative human melanoma cell lines harboring an 'invasive' expression signature or *MITF*-positive cell lines exhibiting a 'proliferative' expression signature [282]. After injection of melanoma cells, tumors have developed at different time periods, which have been shorter for melanoma cells harboring the 'proliferative' signature [282]. Remarkably, all developed tumors contained both *MITF*-positive and *MITF*-negative cells, suggesting that 'proliferative' and 'invasive' transcriptional signatures reflect environment-dependent interchangeable programs [282].

The diversity of transcriptional programs in melanoma cell lines obtained from late-stage tumors is frequently described. It is tempting to suggest that constitutive IL-1 production by melanoma cells might dictate a particular expression pattern. This pattern might be characterized using microarray technology. The absence of MITF expression and the regulation of numerous genes involved in invasion by IL-1 indicate that IL-1-producing melanoma cell lines might constitute a subgroup harboring an 'invasive', rather than a 'proliferative' signature. Accordingly, 8 of 9 MITF-M negative melanoma cell lines producing IL-1 exhibited doubling time of at least 80 hours, a feature observed in only 3 out of 14 *MITF*-M-positive melanoma cell lines (data not shown). Immunohistochemical staining of melanoma tissues corresponding to established melanoma cell lines with anti-Ki67 and anti-MITF antibodies could indicate whether there are differential rates of proliferation between MITF-negative and MITF-positive melanoma cells *in vivo*. However, MITF-negative melanoma cells might achieve their proliferative and survival equilibrium by capitalizing an activation of the NF-κB and MAPKs pathways [113, 251, 537]. In line with the substitutive role of active NF-κB in ensuring survival of melanoma cells, we repeatedly observed lower numbers of surviving cells among IκBαM-expressing than in control cell populations (data not shown). While our *in vitro* data suggest that once it is switched off, the differentiation program is hard to reactivate (Results - Part III), the downregulation of *MITF* and other differentiation genes might be reversible *in vivo*. The capacity of melanoma cells to switch between phenotypes would probably depend on the environmental conditions and on the degree of survival dependency of melanoma cells on IL-1 signaling. Thus, some melanoma cells might acquire a "cytokine addiction" state incompatible with the capacity to switch between different phenotypes.

Melanoma cells that express low amounts of differentiation antigens might have a higher capacity to escape immune response after exposure to systemic T cells in the circulation and, consequently, to harbor a higher propensity to metastasize and to form macrometastases at the secondary sites. Recent studies in a transgenic mouse model of melanoma suggest that dissemination of tumor cells occurs early in the tumor development, but that tumor cells may remain dormant or form micrometastases because their outgrowth to macrometastases is prevented by the antitumor action of $CD8^+$ T cells [373, 628, 629]. The depletion

of $CD8^+$ T cells in this model has significantly accelerated outgrowth of mostly visceral metastases, which have exhibited downregulation of MHC molecules and of differentiation antigens [628, 629]. Therefore, $CD8^+$ T cells might control tumor cells that migrate to visceral sites and delay the development of metastases until antigenic loss variants are eventually selected. These observations in animal models are reminiscent of that made in melanoma patients. Indeed, some melanoma patients have shown initially favorable clinical response after antigen-specific immunotherapy, but have ultimately experienced the delayed outgrowth of visceral metastases with selective or coordinate loss of differentiation antigens targeted by the immunotherapy [630-632]. These observations suggest that a selection process of antigen loss variants may occur in the face of an efficient antitumoral immune response.

What could be the contribution of IL-1 production in tumor environment to the diversity of antigen expression levels observed in melanoma cells? A subset of melanoma cells might be refractory to the inhibitory action of IL-1 on *MITF*-M, for instance due to the downregulation of *IL1R1* or genomic amplification of *MITF*. These cells may keep expression of MITF-M at levels compatible with proliferation and that of differentiation antigens at levels compatible with efficient recognition by T cells. Another group of MITF-M-expressing melanoma cells might respond to IL-1 signaling by transiently repressing *MITF*-M and by producing small amounts of IL-1, which are not sufficient to induce potent immune responses capable to counteract immunosuppressive mechanisms. Finally, melanoma cells might switch to constitutive production of high levels of IL-1, which are sufficient to promote potent antitumoral immune responses. Nevertheless, these cells might escape immune control due to the immunoselection of variants harboring loss of several differentiation antigens. Moreover, some of *MITF*-M-negative melanoma cells, similarly to EB16-MEL cells, may exhibit low or no expression of CG genes (Results - Part I), and, therefore, harbor an increased capacity to escape the immune response. In contrast to EB16-MEL cells, KUL84-MEL cells express high levels of *MAGE-A1*, *-A2*, *-A3*, *-A4*, *-A10* (F. Brasseur, personal communication), which might give rise to antigenic peptides efficiently recognized by cognate CTLs. However, constitutive IL-1 production accompanied by high constitutive NF-κB activity and survival capacity might confer these cells a resistance to the apoptotic cell death induced by CTLs. In accordance with this hypothesis, restoration of the apoptotic death program by treatment with proteasome inhibitor bortezomib has resulted in sensitization of melanoma cells toward T-cell mediated lysis *in vitro* [633]. The potential of the NF-κB pathway to confer melanoma cells resistance to immune destruction could be investigated by evaluating the lysis of IκBαM-expressing KUL84-MEL cells by CTLs targeting MAGE-A-derived antigenic peptides.

In conclusion, the immune response that is appearantly generated early in the melanomagenesis with the aim to eradicate the tumor and inhibit its outgrowth, could induce adaptive alterations in tumor and immune cells. These changes might ultimately promote tumor progression and favor escape of tumor cells from the antitumoral immune response.

VIII. Relevance of the studied mechanisms of melanogenesis regulation in other physiological and pathological settings

Expression of IL-1 cytokines in epidermis might represent one of the negative feedback mechanisms regulating melanogenesis. On one hand, keratinocytes release IL-1α and IL-1β cytokines *in vitro* following exposure to UVR, which induces activation of inflammasomes [604, 605]. On the other hand, IL-1α treatment of cultured human melanocytes has been shown to elicit an inhibitory effect on melanogenesis, with markedly reduced tyrosinase activity [634]. Therefore, IL-1-mediated repression of *MITF*-M might prevent the exaggerated induction of melanogenesis and the presentation of differentiation antigens at levels that could elicit autoimmune response to melanocytes. Similarly, a homeostasic role in the regulation of melanogenesis via NF-κB signaling has been proposed for TNF-α secreted by UV-exposed keratinocytes [459]. In addition, the finding that α-MSH, which is released by keratinocytes and melanocytes following UV exposure, is able to negatively regulate TNF-α-induced NF-κB activity in melanocytes *in vitro* suggests a potential antagonism of α-MSH-induced and TNF-α-induced signaling mechanisms in order to ascertain homeostatic regulation of melanogenesis [635].

The pigmentation phenotype associated with germline mutations affecting components of different signaling pathways could indirectly indicate their functional involvement in the regulation of melanogenesis. For instance, mutations in genes that encode the RAS/BRAF/MEK components of the ERK signaling pathway are associated with so-called "RASopathies", a group of rare developmental syndromes [636]. Pigmentation abnormalities occur in 2-9% of cases and are represented by hyperpigmentation, "café-au-lait" spots, pigmented nevi and lentigines. Lentigines are black-brown spots whose number progressively increases up to thousands in patients with LEOPARD (Lentigines, ECG conduction abnormalities, Ocular hypertelorism, Pulmonic stenosis, Abnormal genitalia, Retardation of growth, sensorineural Deafness) syndrome, known as multiple lentigines syndrome [637, 638].

Germline mutations in *NEMO* encoding an essential component of the NF-κB pathway lead to a X-linked disorder Incontinentia Pigmenti (IP), which is lethal *in utero* in males and specifically affects females [639]. A shorter version of NEMO produced in 70-80% of IP patients retains the ability to interact with IKKα and IKKβ, but is no longer able to activate the NF-κB pathway in response to stimulation [639]. IP is characterized by skin inflammation that usually starts within 2 weeks of birth and is followed by the accumulation of highly pigmented melanocytes along the lines of Blaschko, which are considered to overlap with the migratory melanoblast pathway. The cutaneous lesions then regress leaving behind hypopigmented areas [639].

It would be of interest to explore whether the pigmentation changes observed in the above mentioned pathologies rely on molecular mechanisms that involve *MITF*-M regulation.

IX. Potential approaches in combining immunotherapy with targeted treatment in melanoma

The elucidation of the molecular and cellular mechanisms underlying tumor-immune system interactions would allow rational design of strategies that tilt the balance in the favor of the antitumoral immune response over the tumor-promoting inflammation.

The therapeutic strategies aimed to increase the repertoire and expression levels of melanoma antigens might improve an endogenous immune response and enhance the efficacy of the immunotherapy. Studies focused on the mechanisms of regulation of CG and differentiation genes in melanoma have underscored an important issue. The absent or poor expression of these genes does not appear to rely on irreversible processes altering gene integrity, but rather on mechanisms that are reversible and can be potentially targeted by specific treatment.

Convincing data indicate that epigenetic modulators, including hypomethylating agents and histone deacetylase inhibitors (HDACi), promote the coordinate expression of multiple CG genes, as well as genes encoding MHC class I molecules [640]. Therefore, a combination of immunotherapy with treatment by epigenetic modulators might enhance CTL-directed killing of melanoma cells with increased number of peptide/MHC cell surface complexes. As the opportunity to reverse DNA methylation pattern occurs primarily in the course of replication, hypomethylating agents would preferentially target proliferating malignant cells. Used at low doses to avoid toxicity, Food and Drug Administration (FDA)-approved hypomethylating agents azacitidine and decitabine have shown good clinical responses in patients with hematological malignancies, such as myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML), with recent data confirming an overall survival benefit [641]. One of the HDACi, vorinostat, has been approved by FDA for treating cutaneous T-cell lymphoma and more than 8 other HDACi undergo active clinical investigation in different types of cancer [642]. Importantly, in a small scale study in patients with MDS and AML, combined treatment with azacitidine and HDACi sodium valproate has resulted in induction of MAGE-specific CTL responses in a half of patients, the majority of which have achieved a clinical response [643]. Thus, the proof-of-concept for the use of epigenetic modulators to induce antitumoral immune responses has been provided *in vivo*. However, the specificity of these agents remains an issue, since they may reactivate, along with CG genes and tumor suppressor genes, the expression of pro-metastatic genes [644], whereas the treatment-induced hypomethylation of the genome might lead to genetic instability of tumor cells. Based on *in vitro* experiments using breast cancer cell lines, the design of new hypomethylating agents that could selectively target DNMT1 has been proposed [644]. The use of HDACi in combination with immunotherapy also needs an insightful investigation in different types of cancer since HDACs can modify histones but also non-histone proteins [645].

Moreover, HDACs have differential effects on the expression of pro- and anti-inflammatory cytokines and their inhibition can enhance the immune response in one case, but repress it in other [645]. Moreover, several HDACi have repressed MITF-M expression in melanoma cells *in vitro* and in a humanized skin model *in vivo* [646], thus calling into question their use in combination with an immunotherapy targeting differentiation antigens. Nevertheless, HDACi treatment exhibited significant growth suppression in a human melanoma xenograft model in accordance with previously observed *in vitro* induction of growth arrest in melanoma cell lines [646, 647]. The use of epigenetic modifiers in combination with an immunotherapy targeting antigens encoded by CG genes might be beneficial for patients with metastatic cutaneous melanomas harboring no expression of both CG and differentiation genes (as observed in EB16-MEL cells) and in patients with a desmoplastic variant of melanoma, which generally exhibits a similar antigen-loss phenotype [512]. In conclusion, additional investigations are required to determine the potential for synergy of epigenetic modifiers and immunotherapy.

Restoring the expression of *MITF*-M and genes encoding differentiation antigens appears to be challenging from a therapeutic perspective, since multiple mechanisms appear to be involved and need to be simultaneously targeted. Further deciphering the mechanisms of *MITF*-M regulation in melanoma cells might help to tightly control its expression in order to obtain levels fitting the chosen treatment modality. If we consider the proposed "rheostat" model of relationship between MITF-M dosage and functional outcome (Introduction, Fig. 8 and 14), reactivation of *MITF*-M expression might lead to increased proliferation of melanoma cells, which could then become more sensitive to cytotoxic drugs targeting proliferating cells. Further, pushing MITF-M-positive melanoma cells to express even higher MITF-M levels might result in slower proliferation rate and higher expression of differentiation antigens, features suitable for immunotherapeutic approaches.

Within the set of cytotoxic drugs, inhibitors of topoisomerases have been recently proposed to be used at low doses in combination with immunotherapy due to their capacity to enhance the expression of differentiation antigens, to increase the recognition of melanoma cells by cognate CTLs and to dampen regulatory T cells [648]. Another recent study has assessed the effects of the ERK pathway inhibitors on the recognition efficiency of melanoma cells by CTLs[649]. Importantly, this study has emphasized that the effects of systemically used drugs on tumor and immune cells should not be considered in isolation from each other [649]. Indeed, treatment of melanoma cells with the selective inhibitor of $BRAF^{V600E}$, PLX4032, has led to increased expression of differentiation genes (*MITF*, *MLANA*, *gp100*, *TYRP1*, *DCT*) and enhanced recognition by antigen-specific CTLs, whose proliferation and function were not compromised by treatment with this inhibitor. In contrast, MEK inhibitors (such as U0126) had a similar effect on the expression of differentiation genes, but deleterious effects on proliferation and function of T cells, indicating that these drugs are not appropriate to combine targeted melanoma therapy and immunotherapy [649].

Whereas monotherapy with MEK inhibitors yielded modest response rates in clinical trials, clinical responses to PLX4032 have been achieved in up to 70% of patients harboring melanoma with $BRAF^{V600E}$ [3]. The remarkable clinical activity of PLX4032 is encouraging, but a median duration of response was only about 8 months, suggesting the emergence of resistance through compensatory activity of alternative signaling pathways. Moreover, melanomas with wild-type BRAF, including those with mutant NRAS, are not appropriate for treatment with specific BRAF inhibitors, since they provoke increased CRAF activity as adverse effect [650]. Therefore, a combination of BRAF/MAPK-targeted therapy with other signal transduction inhibitors, for instance NF-κB pathway inhibitors, has been proposed [4, 651].

Much effort has been devoted to the development of highly specific pharmacological NF-κB inhibitors, which are increasingly being used in preclinical and clinical studies for the treatment of human malignancies, including melanoma [652, 653]. Most of them exert their action on the IKK complex (mainly IKKβ), on IκBα phosphorylation or on proteasome activity [251, 654, 655]. Recent preclinical data suggest that IKKβ inhibition would be particularly beneficial in chronic inflammatory conditions [656]. However, prolonged NF-κB inhibition can result in broad suppression of immunity and increased propensity for liver damage [366]. Therefore, NF-κB inhibition targeted to tumor cells would be desired to avoid immunosuppression and to enhance the sensitivity of melanoma cells to CTL-mediated lysis. The proof-of-concept for tumor-targeted NF-κB inhibition has been recently provided *in vitro* and in a xenograft melanoma model [657]. For this purpose, a recombinant fusion protein of the natural inhibitor of intracellular NF-κB activity (IκBα) with a single-chain antibody targeting the high-molecular weight melanoma-associated glycoprotein gp240 [657] has been used. Other approaches might include siRNA delivery to tumor cells using either systemic administration of siRNA-containing nanoparticles [658] or shRNA-producing recombinant viruses with substituted ligands that would selectively target tumor-expressed receptors or other tumor surface molecules [659].

Based on our *in vitro* data, we suggest that simultaneous inactivation of the NF-κB and JNK pathways would be deleterious to melanoma cells particularly in the conditions of the chronic exposure to inflammatory cytokines, namely IL-1. Many JNK inhibitors and several cell-permeable peptide inhibitors, such as JNKI1, which blocks interaction of JNK with its substrate, or BI-78D3, which interferes with JNK binding to the JNK-interacting protein 1 (JIP1) scaffold, have been developed and might be used in the clinic in the near future [537, 660]. Local delivery or selective targeting of JNK inhibitors to melanoma cells would probably be more advantageous to avoid potential undesirable effects on immune cells [661]. Thus, JNK inhibition has been shown to rescue $CD8^+$ CTLs reactive to Melan-A-derived antigenic peptide from an activation-induced cell death, but has also interfered with the functional ability of CTLs to secrete IFN-γ [662]. Strategies to modulate the tumor environment offer a complementary perspective, since signaling pathways aberrantly activated in stromal cells might be targeted by specific therapeutic approaches.

Our study of IL-1 production by melanoma cells pointed to the individuality in tumor-immune system interactions and, consequently, indicated the possibility of personalized treatment. Thus, it might be beneficial to neutralize IL-1 activity in a group of advanced melanoma patients with markedly increased activation of the IL-1/IL-1R1 system [521, 564]. Nevertheless, the possibility of therapeutically regulating IL-1 production must be thoroughly investigated in individual melanoma patients because of opposing activities of IL-1 in immunity and differential responses of melanoma cells to IL-1 [463]. If immunosuppressive effects of IL-1 are predominant, blocking the IL-1 activity might help to derepress antitumor immunity, which might then be reinforced by the immunostimulatory effects of IL-1. Therefore, cyclic rather than continuous treatment regimen with IL-1 blockers would likely be more efficient. If there is no evidence that tumor cells release IL-1α, whereas the IL-1β production is significant and is associated with the recruitment of immunosuppressive cell populations, anti-IL-1β monoclonal antibodies, such as canakinumab, might be used [663]. Among other IL-1 blocking agents approved for treating chronic inflammatory diseases are rilonacept, an "IL-1 trap" fusion protein composed of the extracellular chains of IL-1R1 and IL-1RAcP with the Fc segment of IgG, and anakinra, recombinant IL-1Ra, which is used in ongoing National Institutes of Health trial as an anti-angiogenic therapy in patients with cutaneous melanoma [564]. Although treatment with IL-1 blocking agents has been associated with the increase of bacterial non-opportunistic infections, no toxicity has been documented [564]. If there is no evidence of immunosuppressive activity of IL-1 in particular melanoma lesions, but counteracting its hypopigmenting activity is desirable in the course of immunotherapy, then the use of α-MSH might represent an alternative option. Indeed, the relevance of α-MSH/MC1R signaling has been shown not only for the induction of differentiation genes, but also for counteracting NF-κB signaling in melanoma cells and for inducing cytotoxic activity of $CD8^+$ T cells, which harbor functional MC1R [664, 665]. In addition, a derivative of the C-terminal part of α-MSH, a tripeptide KdPT (Lys-D-Pro-Thr), which is collinear with amino acids 193-95 of the human IL-1β, has been shown to suppress IL-1β-mediated NF-κB activation and induction of *IL6* and *IL8* expression, possibly via interaction with the third Ig-like C2 domain of IL-1R1 [666].

Numerous genomic, transcriptomic and proteomic studies show a high heterogeneity of melanoma tumors. This heterogeneity relies on genetic and epigenetic changes acquired in the course of melanomagenesis and on host background factors, such as gene polymorphisms. The resulting functional heterogeneity of melanoma cells, due to the interdependence and cross-talks between signaling pathways, might translate into distinct capacities to respond to spontaneous and immunotherapy-stimulated immunity. Therefore, defining the unique characteristics of a tumor together with the genetic background of a patient may lead to a personalized prediction of therapeutic outcomes and help segregate suitable candidates for immunotherapy, which may be combined with other targeted melanoma treatment regimens.

For instance, polymorphisms in genes encoding cytokine receptors [667] or MC1R as well as their expression levels are worth to be explored as potential predictive biomarkers since these parameters might ultimately affect the efficacy of immune intervention in specific subsets of patients. Further, transcriptomic high-throughput profiling of melanoma tissues and cell lines has uncountable value in determining predictive biomarkers for clinical benefit of treatment approaches as well as for therapeutic monitoring. Finally, antibody-based proteomic approaches, such as reverse-phase protein microarrays (RPPA), are a powerful tool to analyze signaling maps through the quantitative study of phosphorylation states of signaling pathway components in a small amount of formalin-fixed and paraffin-embedded (FFPE) tissues [668, 669]. In addition, RPPA combined with laser capture microdissection [670] might allow to quantitatively analyze the protein phosphorylation patterns in tumor cells in different tissue contexts and to evaluate the expression of proteins giving rise to melanoma antigens. Besides its predictive therapeutic value, such functional profiling might indicate whether the combination of immunotherapy with selective inhibition of particular signaling pathways would be required to achieve a better therapeutic outcome.

To uncover changes in the regulation of signaling and, consequently, their relevance for the choice of therapeutic strategies, it is important to determine not only whether a particular signaling pathway is constitutively active, but also to determine how the quality (magnitude and duration) of signaling responses differ in cancer cells. Thus, a failure of signal pathway components cannot be assessed without providing a signaling input, whereas potentially important differences in the activity of signaling networks cannot be measured by observing basal signaling alone. A clinically relevant signaling profile, for instance in response to cytokines, might be obtained by measuring the responsiveness of signaling networks in *ex vivo* melanoma cell suspensions. Determining the phenotypes of melanoma cells at a single cell level by flow cytometry [671] might be performed before and after induction of a particular signaling pathway. For instance, antibodies to differentiation antigens [649], to the IL-1R1 receptor and phospho-antibodies to components of the IL-1 signaling pathway might be used to predict the responsiveness of melanoma cells to IL-1 in the tumor environment.

Given the apparently high and, at present, unpredictable degree of individuality in tumor-immune system interactions, optimization of the treatment regimens and their combinations according to the individual genetic background, genetic and epigenetic changes in melanoma cells, stage of melanoma progression and the state of melanoma-related inflammation might improve the therapeutic efficiency in a way to trigger antitumoral immune responses and to neutralize the pro-tumor actions of the chronic inflammation.

REFERENCES

1. Miller AJ, Mihm MC, Jr. Melanoma. N Engl J Med 2006;355:51-65.

2. Garbe C, Leiter U. Melanoma epidemiology and trends. Clin Dermatol 2009;27:3-9.

3. Davies MA, Samuels Y. Analysis of the genome to personalize therapy for melanoma. Oncogene 2010;29:5545-55.

4. Smalley KS. Understanding melanoma signaling networks as the basis for molecular targeted therapy. J Invest Dermatol 2010;130:28-37.

5. Boon T, Coulie PG, Van den Eynde BJ, van der Bruggen P. Human T cell responses against melanoma. Annu Rev Immunol 2006;24:175-208.

6. van der Bruggen P, Traversari C, Chomez P, Lurquin C, De Plaen E, Van den Eynde B, Knuth A, Boon T. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. Science 1991;254:1643-7.

7. Schadendorf D, Algarra SM, Bastholt L, Cinat G, Dreno B, Eggermont AM, Espinosa E, Guo J, Hauschild A, Petrella T, Schachter J, Hersey P. Immunotherapy of distant metastatic disease. Ann Oncol 2009;20 Suppl 6:vi41-50.

8. Alexandrescu DT, Ichim TE, Riordan NH, Marincola FM, Di Nardo A, Kabigting FD, Dasanu CA. Immunotherapy for melanoma: current status and perspectives. J Immunother 2010;33:570-90.

9. Gajewski TF. Failure at the effector phase: immune barriers at the level of the melanoma tumor microenvironment. Clin Cancer Res 2007;13:5256-61.

10. Lu S, Slominski A, Yang SE, Sheehan C, Ross J, Carlson JA. The correlation of TRPM1 (Melastatin) mRNA expression with microphthalmia-associated transcription factor (MITF) and other melanogenesis-related proteins in normal and pathological skin, hair follicles and melanocytic nevi. J Cutan Pathol 2010;37 Suppl 1:26-40.

11. Lin JY, Fisher DE. Melanocyte biology and skin pigmentation. Nature 2007;445:843-50.

12. Haass NK, Smalley KS, Li L, Herlyn M. Adhesion, migration and communication in melanocytes and melanoma. Pigment Cell Res 2005;18:150-9.

13. Hirobe T. Role of keratinocyte-derived factors involved in regulating the proliferation and differentiation of mammalian epidermal melanocytes. Pigment Cell Res 2005;18:2-12.

14. Yamaguchi Y, Itami S, Watabe H, Yasumoto K, Abdel-Malek ZA, Kubo T, Rouzaud F, Tanemura A, Yoshikawa K, Hearing VJ. Mesenchymal-epithelial interactions in the skin: increased expression of dickkopf1 by palmoplantar fibroblasts inhibits melanocyte growth and differentiation. J Cell Biol 2004;165:275-85.

15. Nestle FO, Di Meglio P, Qin JZ, Nickoloff BJ. Skin immune sentinels in health and disease. Nat Rev Immunol 2009;9:679-91.

16. Raposo G, Marks MS. Melanosomes--dark organelles enlighten endosomal membrane transport. Nat Rev Mol Cell Biol 2007;8:786-97.

17. Wasmeier C, Hume AN, Bolasco G, Seabra MC. Melanosomes at a glance. J Cell Sci 2008;121:3995-9.

18. Sturm RA, Larsson M. Genetics of human iris colour and patterns. Pigment Cell Melanoma Res 2009;22:544-62.

19. Tachibana M. Sound needs sound melanocytes to be heard. Pigment Cell Res 1999;12:344-54.

20. Hodgkinson CA, Moore KJ, Nakayama A, Steingrimsson E, Copeland NG, Jenkins NA, Arnheiter H. Mutations at the mouse microphthalmia locus are associated with defects in a gene encoding a novel basic-helix-loop-helix-zipper protein. Cell 1993;74:395-404.

21. Hughes MJ, Lingrel JB, Krakowsky JM, Anderson KP. A helix-loop-helix transcription factor-like gene is located at the mi locus. J Biol Chem 1993;268:20687-90.

22. Tachibana M, Perez-Jurado LA, Nakayama A, Hodgkinson CA, Li X, Schneider M, Miki T, Fex J, Francke U, Arnheiter H. Cloning of MITF, the human homolog of the mouse microphthalmia gene and assignment to chromosome 3p14.1-p12.3. Hum Mol Genet 1994;3:553-7.

23. Tachibana M. MITF: a stream flowing for pigment cells. Pigment Cell Res 2000;13:230-40.

24. Levy C, Khaled M, Fisher DE. MITF: master regulator of melanocyte development and melanoma oncogene. Trends Mol Med 2006;12:406-14.

25. Cheli Y, Ohanna M, Ballotti R, Bertolotto C. Fifteen-year quest for microphthalmia-associated transcription factor target genes. Pigment Cell Melanoma Res 2010;23:27-40.

26. Hoek KS, Schlegel NC, Eichhoff OM, Widmer DS, Praetorius C, Einarsson SO, Valgeirsdottir S, Bergsteinsdottir K, Schepsky A, Dummer R, Steingrimsson E. Novel MITF targets identified using a two-step DNA microarray strategy. Pigment Cell Melanoma Res 2008;21:665-76.

27. Ozsolak F, Poling LL, Wang Z, Liu H, Liu XS, Roeder RG, Zhang X, Song JS, Fisher DE. Chromatin structure analyses identify miRNA promoters. Genes Dev 2008;22:3172-83.

28. Ozsolak F, Song JS, Liu XS, Fisher DE. High-throughput mapping of the chromatin structure of human promoters. Nat Biotechnol 2007;25:244-8.

29. Levy C, Khaled M, Robinson KC, Veguilla RA, Chen PH, Yokoyama S, Makino E, Lu J, Larue L, Beermann F, Chin L, Bosenberg M, et al. Lineage-specific transcriptional regulation of DICER by MITF in melanocytes. Cell 2010;141:994-1005.

30. Hallsson JH, Haflidadottir BS, Schepsky A, Arnheiter H, Steingrimsson E. Evolutionary sequence comparison of the Mitf gene reveals novel conserved domains. Pigment Cell Res 2007;20:185-200.

31. Hershey CL, Fisher DE. Genomic analysis of the Microphthalmia locus and identification of the MITF-J/Mitf-J isoform. Gene 2005;347:73-82.

32. Shiohara M, Shigemura T, Suzuki T, Tanaka M, Morii E, Ohtsu H, Shibahara S, Koike K. MITF-CM, a newly identified isoform of microphthalmia-associated transcription factor, is expressed in cultured mast cells. Int J Lab Hematol 2009;31:215-26.

33. Yasumoto K, Amae S, Udono T, Fuse N, Takeda K, Shibahara S. A big gene linked to small eyes encodes multiple Mitf isoforms: many promoters make light work. Pigment Cell Res 1998;11:329-36.

34. Amae S, Fuse N, Yasumoto K, Sato S, Yajima I, Yamamoto H, Udono T, Durlu YK, Tamai M, Takahashi K, Shibahara S. Identification of a novel isoform of microphthalmia-associated transcription factor that is enriched in retinal pigment epithelium. Biochem Biophys Res Commun 1998;247:710-5.

35. Oboki K, Morii E, Kataoka TR, Jippo T, Kitamura Y. Isoforms of mi transcription factor preferentially expressed in cultured mast cells of mice. Biochem Biophys Res Commun 2002;290:1250-4.

36. Takemoto CM, Yoon YJ, Fisher DE. The identification and functional characterization of a novel mast cell isoform of the microphthalmia-associated transcription factor. J Biol Chem 2002;277:30244-52.

37. Tshori S, Gilon D, Beeri R, Nechushtan H, Kaluzhny D, Pikarsky E, Razin E. Transcription factor MITF regulates cardiac growth and hypertrophy. J Clin Invest 2006;116:2673-81.

38. Bismuth K, Maric D, Arnheiter H. MITF and cell proliferation: the role of alternative splice forms. Pigment Cell Res 2005;18:349-59.

39. Murakami M, Iwata Y, Funaba M. Expression and transcriptional activity of alternative splice variants of Mitf exon 6. Mol Cell Biochem 2007;303:251-7.

40. Primot A, Mogha A, Corre S, Roberts K, Debbache J, Adamski H, Dreno B, Khammari A, Lesimple T, Mereau A, Goding CR, Galibert MD. ERK-regulated differential expression of the Mitf 6a/b splicing isoforms in melanoma. Pigment Cell Melanoma Res 2010;23:93-102.

41. Bemis LT, Chen R, Amato CM, Classen EH, Robinson SE, Coffey DG, Erickson PF, Shellman YG, Robinson WA. MicroRNA-137 targets microphthalmia-associated transcription factor in melanoma cell lines. Cancer Res 2008;68:1362-8.

42. Xu S, Witmer PD, Lumayag S, Kovacs B, Valle D. MicroRNA (miRNA) transcriptome of mouse retina and identification of a sensory organ-specific miRNA cluster. J Biol Chem 2007;282:25053-66.

43. Segura MF, Hanniford D, Menendez S, Reavie L, Zou X, Alvarez-Diaz S, Zakrzewski J, Blochin E, Rose A, Bogunovic D, Polsky D, Wei J, et al. Aberrant miR-182 expression promotes melanoma metastasis by repressing FOXO3 and microphthalmia-associated transcription factor. Proc Natl Acad Sci U S A 2009;106:1814-9.

44. Goswami S, Tarapore RS, Teslaa JJ, Grinblat Y, Setaluri V, Spiegelman VS. MicroRNA-340-mediated degradation of microphthalmia-associated transcription factor mRNA is inhibited by the coding region determinant-binding protein. J Biol Chem 2010;285:20532-40.

45. Haflidadottir BS, Bergsteinsdottir K, Praetorius C, Steingrimsson E. miR-148 regulates Mitf in melanoma cells. PLoS One 2010;5:e11574.

46. Stark MS, Tyagi S, Nancarrow DJ, Boyle GM, Cook AL, Whiteman DC, Parsons PG, Schmidt C, Sturm RA, Hayward NK. Characterization of the Melanoma miRNAome by Deep Sequencing. PLoS One 2010;5:e9685.

47. Khabar KS. Post-transcriptional control during chronic inflammation and cancer: a focus on AU-rich elements. Cell Mol Life Sci 2010;67:2937-55.

48. Hemesath TJ, Steingrimsson E, McGill G, Hansen MJ, Vaught J, Hodgkinson CA, Arnheiter H, Copeland NG, Jenkins NA, Fisher DE. microphthalmia, a critical factor in melanocyte development, defines a discrete transcription factor family. Genes Dev 1994;8:2770-80.

49. Steingrimsson E, Tessarollo L, Pathak B, Hou L, Arnheiter H, Copeland NG, Jenkins NA. Mitf and Tfe3, two members of the Mitf-Tfe family of bHLH-Zip transcription factors, have important but functionally redundant roles in osteoclast development. Proc Natl Acad Sci U S A 2002;99:4477-82.

50. Hemesath TJ, Price ER, Takemoto C, Badalian T, Fisher DE. MAP kinase links the transcription factor Microphthalmia to c-Kit signalling in melanocytes. Nature 1998;391:298-301.

51. Wu M, Hemesath TJ, Takemoto CM, Horstmann MA, Wells AG, Price ER, Fisher DZ, Fisher DE. c-Kit triggers dual phosphorylations, which couple activation and degradation of the essential melanocyte factor Mi. Genes Dev 2000;14:301-12.

52. Takeda K, Takemoto C, Kobayashi I, Watanabe A, Nobukuni Y, Fisher DE, Tachibana M. Ser298 of MITF, a mutation site in Waardenburg syndrome type 2, is a phosphorylation site with functional significance. Hum Mol Genet 2000;9:125-32.

53. Xu W, Gong L, Haddad MM, Bischof O, Campisi J, Yeh ET, Medrano EE. Regulation of microphthalmia-associated transcription factor MITF protein levels by association with the ubiquitin-conjugating enzyme hUBC9. Exp Cell Res 2000;255:135-43.

54. Miller AJ, Levy C, Davis IJ, Razin E, Fisher DE. Sumoylation of MITF and its related family members TFE3 and TFEB. J Biol Chem 2005;280:146-55.

55. Murakami H, Arnheiter H. Sumoylation modulates transcriptional activity of MITF in a promoter-specific manner. Pigment Cell Res 2005;18:265-77.

56. Pingault V, Ente D, Dastot-Le Moal F, Goossens M, Marlin S, Bondurand N. Review and update of mutations causing Waardenburg syndrome. Hum Mutat 2010;31:391-406.

57. Read AP, Newton VE. Waardenburg syndrome. J Med Genet 1997;34:656-65.

58. Smith SD, Kelley PM, Kenyon JB, Hoover D. Tietz syndrome (hypopigmentation/deafness) caused by mutation of MITF. J Med Genet 2000;37:446-8.

59. Steingrimsson E, Copeland NG, Jenkins NA. Melanocytes and the microphthalmia transcription factor network. Annu Rev Genet 2004;38:365-411.

60. Bharti K, Nguyen MT, Skuntz S, Bertuzzi S, Arnheiter H. The other pigment cell: specification and development of the pigmented epithelium of the vertebrate eye. Pigment Cell Res 2006;19:380-94.

61. Dessinioti C, Stratigos AJ, Rigopoulos D, Katsambas AD. A review of genetic disorders of hypopigmentation: lessons learned from the biology of melanocytes. Exp Dermatol 2009;18:741-9.

62. Bennett DC, Lamoreux ML. The color loci of mice--a genetic century. Pigment Cell Res 2003;16:333-44.

63. Oetting WS, Fryer JP, Shriram S, King RA. Oculocutaneous albinism type 1: the last 100 years. Pigment Cell Res 2003;16:307-11.

64. Schiaffino MV, Tacchetti C. The ocular albinism type 1 (OA1) protein and the evidence for an intracellular signal transduction system involved in melanosome biogenesis. Pigment Cell Res 2005;18:227-33.

65. Palmisano I, Bagnato P, Palmigiano A, Innamorati G, Rotondo G, Altimare D, Venturi C, Sviderskaya EV, Piccirillo R, Coppola M, Marigo V, Incerti B, et al. The ocular albinism type 1 protein, an intracellular G protein-coupled receptor, regulates melanosome transport in pigment cells. Hum Mol Genet 2008;17:3487-501.

66. Lopez VM, Decatur CL, Stamer WD, Lynch RM, McKay BS. L-DOPA is an endogenous ligand for OA1. PLoS Biol 2008;6:e236.

67. Lowings P, Yavuzer U, Goding CR. Positive and negative elements regulate a melanocyte-specific promoter. Mol Cell Biol 1992;12:3653-62.

68. Bentley NJ, Eisen T, Goding CR. Melanocyte-specific expression of the human tyrosinase promoter: activation by the microphthalmia gene product and role of the initiator. Mol Cell Biol 1994;14:7996-8006.

69. Yasumoto K, Yokoyama K, Takahashi K, Tomita Y, Shibahara S. Functional analysis of microphthalmia-associated transcription factor in pigment cell-specific transcription of the human tyrosinase family genes. J Biol Chem 1997;272:503-9.

70. Aksan I, Goding CR. Targeting the microphthalmia basic helix-loop-helix-leucine zipper transcription factor to a subset of E-box elements in vitro and in vivo. Mol Cell Biol 1998;18:6930-8.

71. Yasumoto K, Mahalingam H, Suzuki H, Yoshizawa M, Yokoyama K. Transcriptional activation of the melanocyte-specific genes by the human homolog of the mouse Microphthalmia protein. J Biochem 1995;118:874-81.

72. Galibert MD, Carreira S, Goding CR. The Usf-1 transcription factor is a novel target for the stress-responsive p38 kinase and mediates UV-induced Tyrosinase expression. Embo J 2001;20:5022-31.

73. Bertolotto C, Busca R, Abbe P, Bille K, Aberdam E, Ortonne JP, Ballotti R. Different cis-acting elements are involved in the regulation of TRP1 and TRP2 promoter activities by cyclic AMP: pivotal role of M boxes (GTCATGTGCT) and of microphthalmia. Mol Cell Biol 1998;18:694-702.

74. de la Serna IL, Ohkawa Y, Higashi C, Dutta C, Osias J, Kommajosyula N, Tachibana T, Imbalzano AN. The microphthalmia-associated transcription factor requires SWI/SNF enzymes to activate melanocyte-specific genes. J Biol Chem 2006;281:20233-41.

75. Lang D, Lu MM, Huang L, Engleka KA, Zhang M, Chu EY, Lipner S, Skoultchi A, Millar SE, Epstein JA. Pax3 functions at a nodal point in melanocyte stem cell differentiation. Nature 2005;433:884-7.

76. Gaggioli C, Busca R, Abbe P, Ortonne JP, Ballotti R. Microphthalmia-associated transcription factor (MITF) is required but is not sufficient to induce the expression of melanogenic genes. Pigment Cell Res 2003;16:374-82.

77. Olivares C, Solano F. New insights into the active site structure and catalytic mechanism of tyrosinase and its related proteins. Pigment Cell Melanoma Res 2009;22:750-60.

78. Wang N, Hebert DN. Tyrosinase maturation through the mammalian secretory pathway: bringing color to life. Pigment Cell Res 2006;19:3-18.

79. Toyofuku K, Wada I, Valencia JC, Kushimoto T, Ferrans VJ, Hearing VJ. Oculocutaneous albinism types 1 and 3 are ER retention diseases: mutation of tyrosinase or Tyrp1 can affect the processing of both mutant and wild-type proteins. Faseb J 2001;15:2149-61.

80. Halaban R, Cheng E, Zhang Y, Moellmann G, Hanlon D, Michalak M, Setaluri V, Hebert DN. Aberrant retention of tyrosinase in the endoplasmic reticulum mediates accelerated degradation of the enzyme and contributes to the dedifferentiated phenotype of amelanotic melanoma cells. Proc Natl Acad Sci U S A 1997;94:6210-5.

81. Watabe H, Valencia JC, Yasumoto K, Kushimoto T, Ando H, Muller J, Vieira WD, Mizoguchi M, Appella E, Hearing VJ. Regulation of tyrosinase processing and trafficking by organellar pH and by proteasome activity. J Biol Chem 2004;279:7971-81.

82. Brilliant MH. The mouse p (pink-eyed dilution) and human P genes, oculocutaneous albinism type 2 (OCA2), and melanosomal pH. Pigment Cell Res 2001;14:86-93.

83. Chen K, Manga P, Orlow SJ. Pink-eyed dilution protein controls the processing of tyrosinase. Mol Biol Cell 2002;13:1953-64.

84. Cheli Y, Luciani F, Khaled M, Beuret L, Bille K, Gounon P, Ortonne JP, Bertolotto C, Ballotti R. α-MSH and Cyclic AMP elevating agents control melanosome pH through a protein kinase A-independent mechanism. J Biol Chem 2009;284:18699-706.

85. Sturm RA. A golden age of human pigmentation genetics. Trends Genet 2006;22:464-8.

86. Du J, Fisher DE. Identification of Aim-1 as the underwhite mouse mutant and its transcriptional regulation by MITF. J Biol Chem 2002;277:402-6.

87. Theos AC, Truschel ST, Raposo G, Marks MS. The Silver locus product Pmel17/gp100/Silv/ME20: controversial in name and in function. Pigment Cell Res 2005;18:322-36.

88. Coulie PG, Brichard V, Van Pel A, Wolfel T, Schneider J, Traversari C, Mattei S, De Plaen E, Lurquin C, Szikora JP, Renauld JC, Boon T. A new gene coding for a differentiation antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. J Exp Med 1994;180:35-42.

89. Kawakami Y, Eliyahu S, Delgado CH, Robbins PF, Rivoltini L, Topalian SL, Miki T, Rosenberg SA. Cloning of the gene coding for a shared human melanoma antigen recognized by autologous T cells infiltrating into tumor. Proc Natl Acad Sci U S A 1994;91:3515-9.

90. Du J, Miller AJ, Widlund HR, Horstmann MA, Ramaswamy S, Fisher DE. MLANA/MART1 and SILV/PMEL17/GP100 are transcriptionally regulated by MITF in melanocytes and melanoma. Am J Pathol 2003;163:333-43.

91. De Maziere AM, Muehlethaler K, van Donselaar E, Salvi S, Davoust J, Cerottini JC, Levy F, Slot JW, Rimoldi D. The melanocytic protein Melan-A/MART-1 has a subcellular localization distinct from typical melanosomal proteins. Traffic 2002;3:678-93.

92. Berson JF, Harper DC, Tenza D, Raposo G, Marks MS. Pmel17 initiates premelanosome morphogenesis within multivesicular bodies. Mol Biol Cell 2001;12:3451-64.

93. Hoashi T, Watabe H, Muller J, Yamaguchi Y, Vieira WD, Hearing VJ. MART-1 is required for the function of the melanosomal matrix protein PMEL17/GP100 and the maturation of melanosomes. J Biol Chem 2005;280:14006-16.

94. Sturm RA. Molecular genetics of human pigmentation diversity. Hum Mol Genet 2009;18:R9-17.

95. Aoki H, Moro O. Involvement of microphthalmia-associated transcription factor (MITF) in expression of human melanocortin-1 receptor (MC1R). Life Sci 2002;71:2171-9.

96. D'Orazio JA, Nobuhisa T, Cui R, Arya M, Spry M, Wakamatsu K, Igras V, Kunisada T, Granter SR, Nishimura EK, Ito S, Fisher DE. Topical drug rescue strategy and skin protection based on the role of Mc1r in UV-induced tanning. Nature 2006;443:340-4.

97. Yamaguchi Y, Brenner M, Hearing VJ. The regulation of skin pigmentation. J Biol Chem 2007;282:27557-61.

98. Hennessy A, Oh C, Diffey B, Wakamatsu K, Ito S, Rees J. Eumelanin and pheomelanin concentrations in human epidermis before and after UVB irradiation. Pigment Cell Res 2005;18:220-3.

99. Chen KG, Valencia JC, Gillet JP, Hearing VJ, Gottesman MM. Involvement of ABC transporters in melanogenesis and the development of multidrug resistance of melanoma. Pigment Cell Melanoma Res 2009;22:740-9.

100. Lazova R, Klump V, Pawelek J. Autophagy in cutaneous malignant melanoma. J Cutan Pathol 2010;37:256-68.

101. Coelho SG, Choi W, Brenner M, Miyamura Y, Yamaguchi Y, Wolber R, Smuda C, Batzer J, Kolbe L, Ito S, Wakamatsu K, Zmudzka BZ, et al. Short- and long-term effects of UV radiation on the pigmentation of human skin. J Investig Dermatol Symp Proc 2009;14:32-5.

102. Cui R, Widlund HR, Feige E, Lin JY, Wilensky DL, Igras VE, D'Orazio J, Fung CY, Schanbacher CF, Granter SR, Fisher DE. Central role of p53 in the suntan response and pathologic hyperpigmentation. Cell 2007;128:853-64.

103. Murase D, Hachiya A, Amano Y, Ohuchi A, Kitahara T, Takema Y. The essential role of p53 in hyperpigmentation of the skin via regulation of paracrine melanogenic cytokine receptor signaling. J Biol Chem 2009;284:4343-53.

104. Yang G, Li Y, Nishimura EK, Xin H, Zhou A, Guo Y, Dong L, Denning MF, Nickoloff BJ, Cui R. Inhibition of PAX3 by TGF-beta modulates melanocyte viability. Mol Cell 2008;32:554-63.

105. Barsh G, Attardi LD. A healthy tan? N Engl J Med 2007;356:2208-10.

106. Busca R, Ballotti R. Cyclic AMP a key messenger in the regulation of skin pigmentation. Pigment Cell Res 2000;13:60-9.

107. Bertolotto C, Abbe P, Hemesath TJ, Bille K, Fisher DE, Ortonne JP, Ballotti R. Microphthalmia gene product as a signal transducer in cAMP-induced differentiation of melanocytes. J Cell Biol 1998;142:827-35.

108. Price ER, Horstmann MA, Wells AG, Weilbaecher KN, Takemoto CM, Landis MW, Fisher DE. alpha-Melanocyte-stimulating hormone signaling regulates expression of microphthalmia, a gene deficient in Waardenburg syndrome. J Biol Chem 1998;273:33042-7.

109. Khaled M, Larribere L, Bille K, Aberdam E, Ortonne JP, Ballotti R, Bertolotto C. Glycogen synthase kinase 3beta is activated by cAMP and plays an active role in the regulation of melanogenesis. J Biol Chem 2002;277:33690-7.

110. Park HY, Perez JM, Laursen R, Hara M, Gilchrest BA. Protein kinase C-beta activates tyrosinase by phosphorylating serine residues in its cytoplasmic domain. J Biol Chem 1999;274:16470-8.

111. Park HY, Wu C, Yonemoto L, Murphy-Smith M, Wu H, Stachur CM, Gilchrest BA. MITF mediates cAMP-induced protein kinase C-beta expression in human melanocytes. Biochem J 2006;395:571-8.

112. Busca R, Abbe P, Mantoux F, Aberdam E, Peyssonnaux C, Eychene A, Ortonne JP, Ballotti R. Ras mediates the cAMP-dependent activation of extracellular signal-regulated kinases (ERKs) in melanocytes. Embo J 2000;19:2900-10.

113. Wellbrock C, Karasarides M, Marais R. The RAF proteins take centre stage. Nat Rev Mol Cell Biol 2004;5:875-85.

114. Chin L. The genetics of malignant melanoma: lessons from mouse and man. Nat Rev Cancer 2003;3:559-70.

115. Dumaz N, Hayward R, Martin J, Ogilvie L, Hedley D, Curtin JA, Bastian BC, Springer C, Marais R. In melanoma, RAS mutations are accompanied by switching signaling from BRAF to CRAF and disrupted cyclic AMP signaling. Cancer Res 2006;66:9483-91.

116. Price ER, Ding HF, Badalian T, Bhattacharya S, Takemoto C, Yao TP, Hemesath TJ, Fisher DE. Lineage-specific signaling in melanocytes. C-kit stimulation recruits p300/CBP to microphthalmia. J Biol Chem 1998;273:17983-6.

117. Molina DM, Grewal S, Bardwell L. Characterization of an ERK-binding domain in microphthalmia-associated transcription factor and differential inhibition of ERK2-mediated substrate phosphorylation. J Biol Chem 2005;280:42051-60.

118. Xing J, Ginty DD, Greenberg ME. Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase. Science 1996;273:959-63.

119. McGill GG, Haq R, Nishimura EK, Fisher DE. c-Met expression is regulated by Mitf in the melanocyte lineage. J Biol Chem 2006;281:10365-73.

120. Sato-Jin K, Nishimura EK, Akasaka E, Huber W, Nakano H, Miller A, Du J, Wu M, Hanada K, Sawamura D, Fisher DE, Imokawa G. Epistatic connections between microphthalmia-associated transcription factor and endothelin signaling in Waardenburg syndrome and other pigmentary disorders. Faseb J 2008;22:1155-68.

121. Mildner M, Mlitz V, Gruber F, Wojta J, Tschachler E. Hepatocyte growth factor establishes autocrine and paracrine feedback loops for the protection of skin cells after UV irradiation. J Invest Dermatol 2007;127:2637-44.

122. Zaidi MR, Day CP, Merlino G. From UVs to metastases: modeling melanoma initiation and progression in the mouse. J Invest Dermatol 2008;128:2381-91.

123. Beuret L, Flori E, Denoyelle C, Bille K, Busca R, Picardo M, Bertolotto C, Ballotti R. Up-regulation of MET expression by alpha-melanocyte-stimulating hormone and MITF allows hepatocyte growth factor to protect melanocytes and melanoma cells from apoptosis. J Biol Chem 2007;282:14140-7.

124. Huber WE, Price ER, Widlund HR, Du J, Davis IJ, Wegner M, Fisher DE. A tissue-restricted cAMP transcriptional response: SOX10 modulates alpha-melanocyte-stimulating hormone-triggered expression of microphthalmia-associated transcription factor in melanocytes. J Biol Chem 2003;278:45224-30.

125. Harris ML, Baxter LL, Loftus SK, Pavan WJ. Sox proteins in melanocyte development and melanoma. Pigment Cell Melanoma Res 2010;23:496-513.

126. Potterf SB, Furumura M, Dunn KJ, Arnheiter H, Pavan WJ. Transcription factor hierarchy in Waardenburg syndrome: regulation of MITF expression by SOX10 and PAX3. Hum Genet 2000;107:1-6.

127. Bondurand N, Pingault V, Goerich DE, Lemort N, Sock E, Le Caignec C, Wegner M, Goossens M. Interaction among SOX10, PAX3 and MITF, three genes altered in Waardenburg syndrome. Hum Mol Genet 2000;9:1907-17.

128. Passeron T, Valencia JC, Bertolotto C, Hoashi T, Le Pape E, Takahashi K, Ballotti R, Hearing VJ. SOX9 is a key player in ultraviolet B-induced melanocyte differentiation and pigmentation. Proc Natl Acad Sci U S A 2007;104:13984-9.

129. Watanabe K, Takeda K, Yasumoto K, Udono T, Saito H, Ikeda K, Takasaka T, Takahashi K, Kobayashi T, Tachibana M, Shibahara S. Identification of a distal enhancer for the melanocyte-specific promoter of the MITF gene. Pigment Cell Res 2002;15:201-11.

130. Bellei B, Pitisci A, Catricala C, Larue L, Picardo M. Wnt/beta-catenin signaling is stimulated by alpha-melanocyte-stimulating hormone in melanoma and melanocyte cells: implication in cell differentiation. Pigment Cell Melanoma Res 2010.

131. Medic S, Ziman M. PAX3 expression in normal skin melanocytes and melanocytic lesions (naevi and melanomas). PLoS One 2010;5:e9977.

132. Corre S, Primot A, Baron Y, Le Seyec J, Goding C, Galibert MD. Target gene specificity of USF-1 is directed via p38-mediated phosphorylation-dependent acetylation. J Biol Chem 2009;284:18851-62.

133. Bellei B, Maresca V, Flori E, Pitisci A, Larue L, Picardo M. p38 regulates pigmentation via proteasomal degradation of tyrosinase. J Biol Chem 2010;285:7288-99.

134. Carreira S, Goodall J, Aksan I, La Rocca SA, Galibert MD, Denat L, Larue L, Goding CR. Mitf cooperates with Rb1 and activates p21Cip1 expression to regulate cell cycle progression. Nature 2005;433:764-9.

135. Loercher AE, Tank EM, Delston RB, Harbour JW. MITF links differentiation with cell cycle arrest in melanocytes by transcriptional activation of INK4A. J Cell Biol 2005;168:35-40.

136. Gil J, Peters G. Regulation of the INK4b-ARF-INK4a tumour suppressor locus: all for one or one for all. Nat Rev Mol Cell Biol 2006;7:667-77.

137. Carreira S, Liu B, Goding CR. The gene encoding the T-box factor Tbx2 is a target for the microphthalmia-associated transcription factor in melanocytes. J Biol Chem 2000;275:21920-7.

138. Prince S, Carreira S, Vance KW, Abrahams A, Goding CR. Tbx2 directly represses the expression of the p21(WAF1) cyclin-dependent kinase inhibitor. Cancer Res 2004;64:1669-74.

139. Carreira S, Goodall J, Denat L, Rodriguez M, Nuciforo P, Hoek KS, Testori A, Larue L, Goding CR. Mitf regulation of Dia1 controls melanoma proliferation and invasiveness. Genes Dev 2006;20:3426-39.

140. Hornyak TJ, Jiang S, Guzman EA, Scissors BN, Tuchinda C, He H, Neville JD, Strickland FM. Mitf dosage as a primary determinant of melanocyte survival after ultraviolet irradiation. Pigment Cell Melanoma Res 2009;22:307-18.

141. McGill GG, Horstmann M, Widlund HR, Du J, Motyckova G, Nishimura EK, Lin YL, Ramaswamy S, Avery W, Ding HF, Jordan SA, Jackson IJ, et al. Bcl2 regulation by the melanocyte master regulator Mitf modulates lineage survival and melanoma cell viability. Cell 2002;109:707-18.

142. Dynek JN, Chan SM, Liu J, Zha J, Fairbrother WJ, Vucic D. Microphthalmia-associated transcription factor is a critical transcriptional regulator of melanoma inhibitor of apoptosis in melanomas. Cancer Res 2008;68:3124-32.

143. Liu F, Fu Y, Meyskens FL, Jr. MiTF regulates cellular response to reactive oxygen species through transcriptional regulation of APE-1/Ref-1. J Invest Dermatol 2009;129:422-31.

144. Busca R, Berra E, Gaggioli C, Khaled M, Bille K, Marchetti B, Thyss R, Fitsialos G, Larribere L, Bertolotto C, Virolle T, Barbry P, et al. Hypoxia-inducible factor 1{alpha} is a new target of microphthalmia-associated transcription factor (MITF) in melanoma cells. J Cell Biol 2005;170:49-59.

145. Gray-Schopfer V, Wellbrock C, Marais R. Melanoma biology and new targeted therapy. Nature 2007;445:851-7.

146. Thomas AJ, Erickson CA. The making of a melanocyte: the specification of melanoblasts from the neural crest. Pigment Cell Melanoma Res 2008;21:598-610.

147. Gleason BC, Crum CP, Murphy GF. Expression patterns of MITF during human cutaneous embryogenesis: evidence for bulge epithelial expression and persistence of dermal melanoblasts. J Cutan Pathol 2008;35:615-22.

148. Adameyko I, Lallemend F, Aquino JB, Pereira JA, Topilko P, Muller T, Fritz N, Beljajeva A, Mochii M, Liste I, Usoskin D, Suter U, et al. Schwann cell precursors from nerve innervation are a cellular origin of melanocytes in skin. Cell 2009;139:366-79.

149. Ernfors P. Cellular origin and developmental mechanisms during the formation of skin melanocytes. Exp Cell Res 2010;316:1397-407.

150. Nishimura EK, Granter SR, Fisher DE. Mechanisms of hair graying: incomplete melanocyte stem cell maintenance in the niche. Science 2005;307:720-4.

151. Silver DL, Hou L, Pavan WJ. The genetic regulation of pigment cell development. Adv Exp Med Biol 2006;589:155-69.

152. Opdecamp K, Nakayama A, Nguyen MT, Hodgkinson CA, Pavan WJ, Arnheiter H. Melanocyte development in vivo and in neural crest cell cultures: crucial dependence on the Mitf basic-helix-loop-helix-zipper transcription factor. Development 1997;124:2377-86.

153. Hornyak TJ, Hayes DJ, Chiu LY, Ziff EB. Transcription factors in melanocyte development: distinct roles for Pax-3 and Mitf. Mech Dev 2001;101:47-59.

154. Mak SS, Moriyama M, Nishioka E, Osawa M, Nishikawa S. Indispensable role of Bcl2 in the development of the melanocyte stem cell. Dev Biol 2006;291:144-53.

155. Thomas AJ, Erickson CA. FOXD3 regulates the lineage switch between neural crest-derived glial cells and pigment cells by repressing MITF through a non-canonical mechanism. Development 2009;136:1849-58.

156. Takeda K, Yasumoto K, Takada R, Takada S, Watanabe K, Udono T, Saito H, Takahashi K, Shibahara S. Induction of melanocyte-specific microphthalmia-associated transcription factor by Wnt-3a. J Biol Chem 2000;275:14013-6.

157. Yasumoto K, Takeda K, Saito H, Watanabe K, Takahashi K, Shibahara S. Microphthalmia-associated transcription factor interacts with LEF-1, a mediator of Wnt signaling. Embo J 2002;21:2703-14.

158. Saito H, Yasumoto K, Takeda K, Takahashi K, Fukuzaki A, Orikasa S, Shibahara S. Melanocyte-specific microphthalmia-associated transcription factor isoform activates its own gene promoter through physical interaction with lymphoid-enhancing factor 1. J Biol Chem 2002;277:28787-94.

159. Ikeya M, Lee SM, Johnson JE, McMahon AP, Takada S. Wnt signalling required for expansion of neural crest and CNS progenitors. Nature 1997;389:966-70.

160. Jacquemin P, Lannoy VJ, O'Sullivan J, Read A, Lemaigre FP, Rousseau GG. The transcription factor onecut-2 controls the microphthalmia-associated transcription factor gene. Biochem Biophys Res Commun 2001;285:1200-5.

161. Cook AL, Sturm RA. POU domain transcription factors: BRN2 as a regulator of melanocytic growth and tumourigenesis. Pigment Cell Melanoma Res 2008;21:611-26.

162. Bauer GL, Praetorius C, Bergsteinsdottir K, Hallsson JH, Gisladottir BK, Schepsky A, Swing DA, O'Sullivan TN, Arnheiter H, Bismuth K, Debbache J, Fletcher C, et al. The role of MITF phosphorylation sites during coat color and eye development in mice analyzed by bacterial artificial chromosome transgene rescue. Genetics 2009;183:581-94.

163. Hou L, Arnheiter H, Pavan WJ. Interspecies difference in the regulation of melanocyte development by SOX10 and MITF. Proc Natl Acad Sci U S A 2006;103:9081-5.

164. Ludwig A, Rehberg S, Wegner M. Melanocyte-specific expression of dopachrome tautomerase is dependent on synergistic gene activation by the Sox10 and Mitf transcription factors. FEBS Lett 2004;556:236-44.

165. Galibert MD, Yavuzer U, Dexter TJ, Goding CR. Pax3 and regulation of the melanocyte-specific tyrosinase-related protein-1 promoter. J Biol Chem 1999;274:26894-900.

166. Baxter LL, Pavan WJ. Pmel17 expression is Mitf-dependent and reveals cranial melanoblast migration during murine development. Gene Expr Patterns 2003;3:703-7.

167. Aydin IT, Beermann F. Melanocyte and RPE-specific expression in transgenic mice by mouse MART-1/Melan-A/mlana regulatory sequences. Pigment Cell Melanoma Res 2009;22:854-6.

168. Mackenzie MA, Jordan SA, Budd PS, Jackson IJ. Activation of the receptor tyrosine kinase Kit is required for the proliferation of melanoblasts in the mouse embryo. Dev Biol 1997;192:99-107.

169. Holliday R. Epigenetics: a historical overview. Epigenetics 2006;1:76-80.

170. Van Speybroeck L. From epigenesis to epigenetics: the case of C. H. Waddington. Ann N Y Acad Sci 2002;981:61-81.

171. Kouzarides T. Chromatin modifications and their function. Cell 2007;128:693-705.

172. Probst AV, Dunleavy E, Almouzni G. Epigenetic inheritance during the cell cycle. Nat Rev Mol Cell Biol 2009;10:192-206.

173. Delcuve GP, Rastegar M, Davie JR. Epigenetic control. J Cell Physiol 2009;219:243-50.

174. Kapranov P, Willingham AT, Gingeras TR. Genome-wide transcription and the implications for genomic organization. Nat Rev Genet 2007;8:413-23.

175. Mohn F, Schubeler D. Genetics and epigenetics: stability and plasticity during cellular differentiation. Trends Genet 2009;25:129-36.

176. Mills AA. Throwing the cancer switch: reciprocal roles of polycomb and trithorax proteins. Nat Rev Cancer 2010;10:669-82.

177. Suzuki MM, Bird A. DNA methylation landscapes: provocative insights from epigenomics. Nat Rev Genet 2008;9:465-76.

178. Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J, Devon K, Dewar K, Doyle M, FitzHugh W, Funke R, Gage D, et al. Initial sequencing and analysis of the human genome. Nature 2001;409:860-921.

179. Sawan C, Vaissiere T, Murr R, Herceg Z. Epigenetic drivers and genetic passengers on the road to cancer. Mutat Res 2008;642:1-13.

180. Illingworth RS, Bird AP. CpG islands--'a rough guide'. FEBS Lett 2009;583:1713-20.

181. Carninci P, Sandelin A, Lenhard B, Katayama S, Shimokawa K, Ponjavic J, Semple CA, Taylor MS, Engstrom PG, Frith MC, Forrest AR, Alkema WB, et al. Genome-wide analysis of mammalian promoter architecture and evolution. Nat Genet 2006;38:626-35.

182. Illingworth R, Kerr A, Desousa D, Jorgensen H, Ellis P, Stalker J, Jackson D, Clee C, Plumb R, Rogers J, Humphray S, Cox T, et al. A novel CpG island set identifies tissue-specific methylation at developmental gene loci. PLoS Biol 2008;6:e22.

183. Goll MG, Bestor TH. Eukaryotic cytosine methyltransferases. Annu Rev Biochem 2005;74:481-514.

184. Santos F, Hendrich B, Reik W, Dean W. Dynamic reprogramming of DNA methylation in the early mouse embryo. Dev Biol 2002;241:172-82.

185. Okano M, Bell DW, Haber DA, Li E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell 1999;99:247-57.

186. Robertson KD, Uzvolgyi E, Liang G, Talmadge C, Sumegi J, Gonzales FA, Jones PA. The human DNA methyltransferases (DNMTs) 1, 3a and 3b: coordinate mRNA expression in normal tissues and overexpression in tumors. Nucleic Acids Res 1999;27:2291-8.

187. Kaneda M, Okano M, Hata K, Sado T, Tsujimoto N, Li E, Sasaki H. Essential role for de novo DNA methyltransferase Dnmt3a in paternal and maternal imprinting. Nature 2004;429:900-3.

188. Bostick M, Kim JK, Esteve PO, Clark A, Pradhan S, Jacobsen SE. UHRF1 plays a role in maintaining DNA methylation in mammalian cells. Science 2007;317:1760-4.

189. Schubeler D, Lorincz MC, Cimbora DM, Telling A, Feng YQ, Bouhassira EE, Groudine M. Genomic targeting of methylated DNA: influence of methylation on transcription, replication, chromatin structure, and histone acetylation. Mol Cell Biol 2000;20:9103-12.

190. Skinner MK. What is an epigenetic transgenerational phenotype? F3 or F2. Reprod Toxicol 2008;25:2-6.

191. Ooi SK, Bestor TH. The colorful history of active DNA demethylation. Cell 2008;133:1145-8.

192. Frommer M, McDonald LE, Millar DS, Collis CM, Watt F, Grigg GW, Molloy PL, Paul CL. A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. Proc Natl Acad Sci U S A 1992;89:1827-31.

193. Garcia JS, Jain N, Godley LA. An update on the safety and efficacy of decitabine in the treatment of myelodysplastic syndromes. Onco Targets Ther 2010;3:1-13.

194. Vaissiere T, Sawan C, Herceg Z. Epigenetic interplay between histone modifications and DNA methylation in gene silencing. Mutat Res 2008;659:40-8.

195. Kumari D, Usdin K. Interaction of the transcription factors USF1, USF2, and alpha - Pal/Nrf-1 with the FMR1 promoter. Implications for Fragile X mental retardation syndrome. J Biol Chem 2001;276:4357-64.

196. Nan X, Ng HH, Johnson CA, Laherty CD, Turner BM, Eisenman RN, Bird A. Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. Nature 1998;393:386-9.

197. Ramirez-Carrozzi VR, Braas D, Bhatt DM, Cheng CS, Hong C, Doty KR, Black JC, Hoffmann A, Carey M, Smale ST. A unifying model for the selective regulation of inducible transcription by CpG islands and nucleosome remodeling. Cell 2009;138:114-28.

198. Koga Y, Pelizzola M, Cheng E, Krauthammer M, Sznol M, Ariyan S, Narayan D, Molinaro AM, Halaban R, Weissman SM. Genome-wide screen of promoter methylation identifies novel markers in melanoma. Genome Res 2009;19:1462-70.

199. Stennett LS, Riker AI, Kroll TM, ChaMberlin J, Miki T, Nickoloff BJ, Le Poole IC. Expression of gp100 and CDK2 in melanoma cells is not co-regulated by a shared promoter region. Pigment Cell Res 2004;17:525-32.

200. van Dinten LC, Pul N, van Nieuwpoort AF, Out CJ, Jager MJ, van den Elsen PJ. Uveal and cutaneous melanoma: shared expression characteristics of melanoma-associated antigens. Invest Ophthalmol Vis Sci 2005;46:24-30.

201. Barski A, Cuddapah S, Cui K, Roh TY, Schones DE, Wang Z, Wei G, Chepelev I, Zhao K. High-resolution profiling of histone methylations in the human genome. Cell 2007;129:823-37.

202. Wang Z, Zang C, Rosenfeld JA, Schones DE, Barski A, Cuddapah S, Cui K, Roh TY, Peng W, Zhang MQ, Zhao K. Combinatorial patterns of histone acetylations and methylations in the human genome. Nat Genet 2008;40:897-903.

203. Shogren-Knaak M, Ishii H, Sun JM, Pazin MJ, Davie JR, Peterson CL. Histone H4-K16 acetylation controls chromatin structure and protein interactions. Science 2006;311:844-7.

204. Fanti L, Pimpinelli S. HP1: a functionally multifaceted protein. Curr Opin Genet Dev 2008;18:169-74.

205. Schones DE, Cui K, Cuddapah S, Roh TY, Barski A, Wang Z, Wei G, Zhao K. Dynamic regulation of nucleosome positioning in the human genome. Cell 2008;132:887-98.

206. Jiang C, Pugh BF. Nucleosome positioning and gene regulation: advances through genomics. Nat Rev Genet 2009;10:161-72.

207. Clapier CR, Cairns BR. The biology of chromatin remodeling complexes. Annu Rev Biochem 2009;78:273-304.

208. de la Serna IL, Ohkawa Y, Imbalzano AN. Chromatin remodelling in mammalian differentiation: lessons from ATP-dependent remodellers. Nat Rev Genet 2006;7:461-73.

209. Eroglu B, Wang G, Tu N, Sun X, Mivechi NF. Critical role of Brg1 member of the SWI/SNF chromatin remodeling complex during neurogenesis and neural crest induction in zebrafish. Dev Dyn 2006;235:2722-35.

210. Barker N, Hurlstone A, Musisi H, Miles A, Bienz M, Clevers H. The chromatin remodelling factor Brg-1 interacts with beta-catenin to promote target gene activation. Embo J 2001;20:4935-43.

211. Keenen B, Qi H, Saladi SV, Yeung M, de la Serna IL. Heterogeneous SWI/SNF chromatin remodeling complexes promote expression of microphthalmia-associated transcription factor target genes in melanoma. Oncogene 2009.

212. Sato S, Roberts K, Gambino G, Cook A, Kouzarides T, Goding CR. CBP/p300 as a co-factor for the Microphthalmia transcription factor. Oncogene 1997;14:3083-92.

213. Chin L, Garraway LA, Fisher DE. Malignant melanoma: genetics and therapeutics in the genomic era. Genes Dev 2006;20:2149-82.

214. Bennett DC. Ultraviolet wavebands and melanoma initiation. Pigment Cell Melanoma Res 2008;21:520-4.

215. Gerstenblith MR, Shi J, Landi MT. Genome-wide association studies of pigmentation and skin cancer: a review and meta-analysis. Pigment Cell Melanoma Res 2010;23:587-606.

216. Larue L, Beermann F. Cutaneous melanoma in genetically modified animals. Pigment Cell Res 2007;20:485-97.

217. Bennett DC. How to make a melanoma: what do we know of the primary clonal events? Pigment Cell Melanoma Res 2008;21:27-38.

218. Pleasance ED, Cheetham RK, Stephens PJ, McBride DJ, Humphray SJ, Greenman CD, Varela I, Lin ML, Ordonez GR, Bignell GR, Ye K, Alipaz J, et al. A comprehensive catalogue of somatic mutations from a human cancer genome. Nature 2010;463:191-6.

219. Stark M, Hayward N. Genome-wide loss of heterozygosity and copy number analysis in melanoma using high-density single-nucleotide polymorphism arrays. Cancer Res 2007;67:2632-42.

220. Jonsson G, Dahl C, Staaf J, Sandberg T, Bendahl PO, Ringner M, Guldberg P, Borg A. Genomic profiling of malignant melanoma using tiling-resolution arrayCGH. Oncogene 2007;26:4738-48.

221. Stratton MR, Campbell PJ, Futreal PA. The cancer genome. Nature 2009;458:719-24.

222. Curtin JA, Fridlyand J, Kageshita T, Patel HN, Busam KJ, Kutzner H, Cho KH, Aiba S, Brocker EB, LeBoit PE, Pinkel D, Bastian BC. Distinct sets of genetic alterations in melanoma. N Engl J Med 2005;353:2135-47.

223. Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, Teague J, Woffendin H, Garnett MJ, Bottomley W, Davis N, Dicks E, et al. Mutations of the BRAF gene in human cancer. Nature 2002;417:949-54.

224. Wan PT, Garnett MJ, Roe SM, Lee S, Niculescu-Duvaz D, Good VM, Jones CM, Marshall CJ, Springer CJ, Barford D, Marais R. Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. Cell 2004;116:855-67.

225. Houben R, Becker JC, Kappel A, Terheyden P, Brocker EB, Goetz R, Rapp UR. Constitutive activation of the Ras-Raf signaling pathway in metastatic melanoma is associated with poor prognosis. J Carcinog 2004;3:6.

226. Wellbrock C, Ogilvie L, Hedley D, Karasarides M, Martin J, Niculescu-Duvaz D, Springer CJ, Marais R. V599EB-RAF is an oncogene in melanocytes. Cancer Res 2004;64:2338-42.

227. Hingorani SR, Jacobetz MA, Robertson GP, Herlyn M, Tuveson DA. Suppression of BRAF(V599E) in human melanoma abrogates transformation. Cancer Res 2003;63:5198-202.

228. Weinstein IB, Joe A. Oncogene addiction. Cancer Res 2008;68:3077-80; discussion 80.

229. Curtin JA, Busam K, Pinkel D, Bastian BC. Somatic activation of KIT in distinct subtypes of melanoma. J Clin Oncol 2006;24:4340-6.

230. Jones PA, Baylin SB. The epigenomics of cancer. Cell 2007;128:683-92.

231. Howell PM, Jr., Liu S, Ren S, Behlen C, Fodstad O, Riker AI. Epigenetics in human melanoma. Cancer Control 2009;16:200-18.

232. De Smet C, Loriot A. DNA hypomethylation in cancer: Epigenetic scars of a neoplastic journey. Epigenetics 2010;5.

233. De Smet C, De Backer O, Faraoni I, Lurquin C, Brasseur F, Boon T. The activation of human gene MAGE-1 in tumor cells is correlated with genome-wide demethylation. Proc Natl Acad Sci U S A 1996;93:7149-53.

234. Tellez CS, Shen L, Estecio MR, Jelinek J, Gershenwald JE, Issa JP. CpG island methylation profiling in human melanoma cell lines. Melanoma Res 2009;19:146-55.

235. Haqq C, Nosrati M, Sudilovsky D, Crothers J, Khodabakhsh D, Pulliam BL, Federman S, Miller JR, 3rd, Allen RE, Singer MI, Leong SP, Ljung BM, et al. The gene expression signatures of melanoma progression. Proc Natl Acad Sci U S A 2005;102:6092-7.

236. Mueller DW, Bosserhoff AK. Role of miRNAs in the progression of malignant melanoma. Br J Cancer 2009;101:551-6.

237. Dhomen N, Reis-Filho JS, da Rocha Dias S, Hayward R, Savage K, Delmas V, Larue L, Pritchard C, Marais R. Oncogenic Braf induces melanocyte senescence and melanoma in mice. Cancer Cell 2009;15:294-303.

238. Ha L, Merlino G, Sviderskaya EV. Melanomagenesis: overcoming the barrier of melanocyte senescence. Cell Cycle 2008;7:1944-8.

239. Michaloglou C, Vredeveld LC, Soengas MS, Denoyelle C, Kuilman T, van der Horst CM, Majoor DM, Shay JW, Mooi WJ, Peeper DS. BRAFE600-associated senescence-like cell cycle arrest of human naevi. Nature 2005;436:720-4.

240. Bandyopadhyay D, Curry JL, Lin Q, Richards HW, Chen D, Hornsby PJ, Timchenko NA, Medrano EE. Dynamic assembly of chromatin complexes during cellular senescence: implications for the growth arrest of human melanocytic nevi. Aging Cell 2007;6:577-91.

241. Gray-Schopfer VC, Cheong SC, Chong H, Chow J, Moss T, Abdel-Malek ZA, Marais R, Wynford-Thomas D, Bennett DC. Cellular senescence in naevi and immortalisation in melanoma: a role for p16? Br J Cancer 2006;95:496-505.

242. Michaloglou C, Vredeveld LC, Mooi WJ, Peeper DS. BRAF(E600) in benign and malignant human tumours. Oncogene 2008;27:877-95.

243. Dankort D, Curley DP, Cartlidge RA, Nelson B, Karnezis AN, Damsky WE, Jr., You MJ, DePinho RA, McMahon M, Bosenberg M. Braf(V600E) cooperates with Pten loss to induce metastatic melanoma. Nat Genet 2009;41:544-52.

244. Vance KW, Carreira S, Brosch G, Goding CR. Tbx2 is overexpressed and plays an important role in maintaining proliferation and suppression of senescence in melanomas. Cancer Res 2005;65:2260-8.

245. Freedberg DE, Rigas SH, Russak J, Gai W, Kaplow M, Osman I, Turner F, Randerson-Moor JA, Houghton A, Busam K, Timothy Bishop D, Bastian BC, et al. Frequent p16-independent inactivation of p14ARF in human melanoma. J Natl Cancer Inst 2008;100:784-95.

246. Delmas V, Beermann F, Martinozzi S, Carreira S, Ackermann J, Kumasaka M, Denat L, Goodall J, Luciani F, Viros A, Demirkan N, Bastian BC, et al. Beta-catenin induces immortalization of melanocytes by suppressing p16INK4a expression and cooperates with N-Ras in melanoma development. Genes Dev 2007;21:2923-35.

247. Soengas MS, Capodieci P, Polsky D, Mora J, Esteller M, Opitz-Araya X, McCombie R, Herman JG, Gerald WL, Lazebnik YA, Cordon-Cardo C, Lowe SW. Inactivation of the apoptosis effector Apaf-1 in malignant melanoma. Nature 2001;409:207-11.

248. Cheung M, Sharma A, Madhunapantula SV, Robertson GP. Akt3 and mutant V600E B-Raf cooperate to promote early melanoma development. Cancer Res 2008;68:3429-39.

249. Liu J, Suresh Kumar KG, Yu D, Molton SA, McMahon M, Herlyn M, Thomas-Tikhonenko A, Fuchs SY. Oncogenic BRAF regulates beta-Trcp expression and NF-kappaB activity in human melanoma cells. Oncogene 2007;26:1954-8.

250. Ueda Y, Richmond A. NF-kappaB activation in melanoma. Pigment Cell Res 2006;19:112-24.

251. Amiri KI, Richmond A. Role of nuclear factor-kappa B in melanoma. Cancer Metastasis Rev 2005;24:301-13.

252. Hoffmann A, Baltimore D. Circuitry of nuclear factor kappaB signaling. Immunol Rev 2006;210:171-86.

253. Gao K, Dai DL, Martinka M, Li G. Prognostic significance of nuclear factor-kappaB p105/p50 in human melanoma and its role in cell migration. Cancer Res 2006;66:8382-8.

254. McNulty SE, del Rosario R, Cen D, Meyskens FL, Jr., Yang S. Comparative expression of NFkappaB proteins in melanocytes of normal skin vs. benign intradermal naevus and human metastatic melanoma biopsies. Pigment Cell Res 2004;17:173-80.

255. Ikenoue T, Hikiba Y, Kanai F, Tanaka Y, Imamura J, Imamura T, Ohta M, Ijichi H, Tateishi K, Kawakami T, Aragaki J, Matsumura M, et al. Functional analysis of mutations within the kinase activation segment of B-Raf in human colorectal tumors. Cancer Res 2003;63:8132-7.

256. Vermeulen L, De Wilde G, Van Damme P, Vanden Berghe W, Haegeman G. Transcriptional activation of the NF-kappaB p65 subunit by mitogen- and stress-activated protein kinase-1 (MSK1). Embo J 2003;22:1313-24.

257. Huang S, DeGuzman A, Bucana CD, Fidler IJ. Nuclear factor-kappaB activity correlates with growth, angiogenesis, and metastasis of human melanoma cells in nude mice. Clin Cancer Res 2000;6:2573-81.

258. Yang J, Splittgerber R, Yull FE, Kantrow S, Ayers GD, Karin M, Richmond A. Conditional ablation of Ikkb inhibits melanoma tumor development in mice. J Clin Invest 2010;120:2563-74.

259. Lin K, Baritaki S, Militello L, Malaponte G, Bevelacqua Y, Bonavida B. The Role of B-RAF Mutations in Melanoma and the Induction of EMT via Dysregulation of the NF-kappaB/Snail/RKIP/PTEN Circuit. Genes Cancer 2010;1:409-20.

260. Julien S, Puig I, Caretti E, Bonaventure J, Nelles L, van Roy F, Dargemont C, de Herreros AG, Bellacosa A, Larue L. Activation of NF-kappaB by Akt upregulates Snail expression and induces epithelium mesenchyme transition. Oncogene 2007;26:7445-56.

261. Massoumi R, Kuphal S, Hellerbrand C, Haas B, Wild P, Spruss T, Pfeifer A, Fassler R, Bosserhoff AK. Down-regulation of CYLD expression by Snail promotes tumor progression in malignant melanoma. J Exp Med 2009;206:221-32.

262. Gupta PB, Kuperwasser C, Brunet JP, Ramaswamy S, Kuo WL, Gray JW, Naber SP, Weinberg RA. The melanocyte differentiation program predisposes to metastasis after neoplastic transformation. Nat Genet 2005;37:1047-54.

263. Dorvault CC, Weilbaecher KN, Yee H, Fisher DE, Chiriboga LA, Xu Y, Chhieng DC. Microphthalmia transcription factor: a sensitive and specific marker for malignant melanoma in cytologic specimens. Cancer 2001;93:337-43.

264. Nazarian RM, Prieto VG, Elder DE, Duncan LM. Melanoma biomarker expression in melanocytic tumor progression: a tissue microarray study. J Cutan Pathol 2010;37 Suppl 1:41-7.

265. Salti GI, Manougian T, Farolan M, Shilkaitis A, Majumdar D, Das Gupta TK. Micropthalmia transcription factor: a new prognostic marker in intermediate-thickness cutaneous malignant melanoma. Cancer Res 2000;60:5012-6.

266. Garraway LA, Widlund HR, Rubin MA, Getz G, Berger AJ, Ramaswamy S, Beroukhim R, Milner DA, Granter SR, Du J, Lee C, Wagner SN, et al. Integrative genomic analyses identify MITF as a lineage survival oncogene amplified in malignant melanoma. Nature 2005;436:117-22.

267. Koyanagi K, O'Day SJ, Gonzalez R, Lewis K, Robinson WA, Amatruda TT, Kuo C, Wang HJ, Milford R, Morton DL, Hoon DS. Microphthalmia transcription factor as a molecular marker for circulating tumor cell detection in blood of melanoma patients. Clin Cancer Res 2006;12:1137-43.

268. Giuliano S, Cheli Y, Ohanna M, Bonet C, Beuret L, Bille K, Loubat A, Hofman V, Hofman P, Ponzio G, Bahadoran P, Ballotti R, et al. Microphthalmia-associated transcription factor controls the DNA damage response and a lineage-specific senescence program in melanomas. Cancer Res 2010;70:3813-22.

269. Wellbrock C, Marais R. Elevated expression of MITF counteracts B-RAF-stimulated melanocyte and melanoma cell proliferation. J Cell Biol 2005;170:703-8.

270. Cronin JC, Wunderlich J, Loftus SK, Prickett TD, Wei X, Ridd K, Vemula S, Burrell AS, Agrawal NS, Lin JC, Banister CE, Buckhaults P, et al. Frequent mutations in the MITF pathway in melanoma. Pigment Cell Melanoma Res 2009;22:435-44.

271. Kono M, Dunn IS, Durda PJ, Butera D, Rose LB, Haggerty TJ, Benson EM, Kurnick JT. Role of the mitogen-activated protein kinase signaling pathway in the regulation of human melanocytic antigen expression. Mol Cancer Res 2006;4:779-92.

272. Zhu S, Wurdak H, Wang Y, Galkin A, Tao H, Li J, Lyssiotis CA, Yan F, Tu BP, Miraglia L, Walker J, Sun F, et al. A genomic screen identifies TYRO3 as a MITF regulator in melanoma. Proc Natl Acad Sci U S A 2009;106:17025-30.

273. Wellbrock C, Rana S, Paterson H, Pickersgill H, Brummelkamp T, Marais R. Oncogenic BRAF regulates melanoma proliferation through the lineage specific factor MITF. PLoS One 2008;3:e2734.

274. O'Connell MP, Weeraratna AT. Hear the Wnt Ror: how melanoma cells adjust to changes in Wnt. Pigment Cell Melanoma Res 2009;22:724-39.

275. Rimm DL, Caca K, Hu G, Harrison FB, Fearon ER. Frequent nuclear/cytoplasmic localization of beta-catenin without exon 3 mutations in malignant melanoma. Am J Pathol 1999;154:325-9.

276. Worm J, Christensen C, Gronbaek K, Tulchinsky E, Guldberg P. Genetic and epigenetic alterations of the APC gene in malignant melanoma. Oncogene 2004;23:5215-26.

277. Chien AJ, Moore EC, Lonsdorf AS, Kulikauskas RM, Rothberg BG, Berger AJ, Major MB, Hwang ST, Rimm DL, Moon RT. Activated Wnt/beta-catenin signaling in melanoma is associated with decreased proliferation in patient tumors and a murine melanoma model. Proc Natl Acad Sci U S A 2009;106:1193-8.

278. Dissanayake SK, Olkhanud PB, O'Connell MP, Carter A, French AD, Camilli TC, Emeche CD, Hewitt KJ, Rosenthal DT, Leotlela PD, Wade MS, Yang SW, et al. Wnt5A regulates

expression of tumor-associated antigens in melanoma via changes in signal transducers and activators of transcription 3 phosphorylation. Cancer Res 2008;68:10205-14.

279. Da Forno PD, Pringle JH, Hutchinson P, Osborn J, Huang Q, Potter L, Hancox RA, Fletcher A, Saldanha GS. WNT5A expression increases during melanoma progression and correlates with outcome. Clin Cancer Res 2008;14:5825-32.

280. Dissanayake SK, Wade M, Johnson CE, O'Connell MP, Leotlela PD, French AD, Shah KV, Hewitt KJ, Rosenthal DT, Indig FE, Jiang Y, Nickoloff BJ, et al. The Wnt5A/protein kinase C pathway mediates motility in melanoma cells via the inhibition of metastasis suppressors and initiation of an epithelial to mesenchymal transition. J Biol Chem 2007;282:17259-71.

281. Hoek KS, Schlegel NC, Brafford P, Sucker A, Ugurel S, Kumar R, Weber BL, Nathanson KL, Phillips DJ, Herlyn M, Schadendorf D, Dummer R. Metastatic potential of melanomas defined by specific gene expression profiles with no BRAF signature. Pigment Cell Res 2006;19:290-302.

282. Hoek KS, Eichhoff OM, Schlegel NC, Dobbeling U, Kobert N, Schaerer L, Hemmi S, Dummer R. In vivo switching of human melanoma cells between proliferative and invasive states. Cancer Res 2008;68:650-6.

283. Goodall J, Carreira S, Denat L, Kobi D, Davidson I, Nuciforo P, Sturm RA, Larue L, Goding CR. Brn-2 represses microphthalmia-associated transcription factor expression and marks a distinct subpopulation of microphthalmia-associated transcription factor-negative melanoma cells. Cancer Res 2008;68:7788-94.

284. De Smet C, Lurquin C, Lethe B, Martelange V, Boon T. DNA methylation is the primary silencing mechanism for a set of germ line- and tumor-specific genes with a CpG-rich promoter. Mol Cell Biol 1999;19:7327-35.

285. Sigalotti L, Coral S, Nardi G, Spessotto A, Cortini E, Cattarossi I, Colizzi F, Altomonte M, Maio M. Promoter methylation controls the expression of MAGE2, 3 and 4 genes in human cutaneous melanoma. J Immunother 2002;25:16-26.

286. Koslowski M, Bell C, Seitz G, Lehr HA, Roemer K, Muntefering H, Huber C, Sahin U, Tureci O. Frequent nonrandom activation of germ-line genes in human cancer. Cancer Res 2004;64:5988-93.

287. Simpson AJ, Caballero OL, Jungbluth A, Chen YT, Old LJ. Cancer/testis antigens, gametogenesis and cancer. Nat Rev Cancer 2005;5:615-25.

288. De Plaen E, Arden K, Traversari C, Gaforio JJ, Szikora JP, De Smet C, Brasseur F, van der Bruggen P, Lethe B, Lurquin C, et al. Structure, chromosomal localization, and expression of 12 genes of the MAGE family. Immunogenetics 1994;40:360-9.

289. Scanlan MJ, Simpson AJ, Old LJ. The cancer/testis genes: review, standardization, and commentary. Cancer Immun 2004;4:1.

290. Nelson PT, Zhang PJ, Spagnoli GC, Tomaszewski JE, Pasha TL, Frosina D, Caballero OL, Simpson AJ, Old LJ, Jungbluth AA. Cancer/testis (CT) antigens are expressed in fetal ovary. Cancer Immun 2007;7:1.

291. Gjerstorff MF, Kock K, Nielsen O, Ditzel HJ. MAGE-A1, GAGE and NY-ESO-1 cancer/testis antigen expression during human gonadal development. Hum Reprod 2007;22:953-60.

292. Jungbluth AA, Silva WA, Jr., Iversen K, Frosina D, Zaidi B, Coplan K, Eastlake-Wade SK, Castelli SB, Spagnoli GC, Old LJ, Vogel M. Expression of cancer-testis (CT) antigens in placenta. Cancer Immun 2007;7:15.

293. Gaskell TL, Esnal A, Robinson LL, Anderson RA, Saunders PT. Immunohistochemical profiling of germ cells within the human fetal testis: identification of three subpopulations. Biol Reprod 2004;71:2012-21.

294. Loriot A, Reister S, Parvizi GK, Lysy PA, De Smet C. DNA methylation-associated repression of cancer-germline genes in human embryonic and adult stem cells. Stem Cells 2009;27:822-4.

295. Hara I, Hara S, Miyake H, Yamanaka K, Nagai H, Gohji K, Arakawa S, Kamidono S. Expression of MAGE genes in testicular germ cell tumors. Urology 1999;53:843-7.

296. Hoei-Hansen CE, Kraggerud SM, Abeler VM, Kaern J, Rajpert-De Meyts E, Lothe RA. Ovarian dysgerminomas are characterised by frequent KIT mutations and abundant expression of pluripotency markers. Mol Cancer 2007;6:12.

297. Caballero OL, Chen YT. Cancer/testis (CT) antigens: potential targets for immunotherapy. Cancer Sci 2009;100:2014-21.

298. Sahin U, Tureci O, Chen YT, Seitz G, Villena-Heinsen C, Old LJ, Pfreundschuh M. Expression of multiple cancer/testis (CT) antigens in breast cancer and melanoma: basis for polyvalent CT vaccine strategies. Int J Cancer 1998;78:387-9.

299. Brasseur F, Rimoldi D, Lienard D, Lethe B, Carrel S, Arienti F, Suter L, Vanwijck R, Bourlond A, Humblet Y, et al. Expression of MAGE genes in primary and metastatic cutaneous melanoma. Int J Cancer 1995;63:375-80.

300. Knuth A, Wolfel T, Klehmann E, Boon T, Meyer zum Buschenfelde KH. Cytolytic T-cell clones against an autologous human melanoma: specificity study and definition of three antigens by immunoselection. Proc Natl Acad Sci U S A 1989;86:2804-8.

301. Van den Eynde B, Hainaut P, Herin M, Knuth A, Lemoine C, Weynants P, van der Bruggen P, Fauchet R, Boon T. Presence on a human melanoma of multiple antigens recognized by autologous CTL. Int J Cancer 1989;44:634-40.

302. Chomez P, De Backer O, Bertrand M, De Plaen E, Boon T, Lucas S. An overview of the MAGE gene family with the identification of all human members of the family. Cancer Res 2001;61:5544-51.

303. Almeida LG, Sakabe NJ, deOliveira AR, Silva MC, Mundstein AS, Cohen T, Chen YT, Chua R, Gurung S, Gnjatic S, Jungbluth AA, Caballero OL, et al. CTdatabase: a knowledge-base of high-throughput and curated data on cancer-testis antigens. Nucleic Acids Res 2009;37:D816-9.

304. De Backer O, Arden KC, Boretti M, Vantomme V, De Smet C, Czekay S, Viars CS, De Plaen E, Brasseur F, Chomez P, Van den Eynde B, Boon T, et al. Characterization of the GAGE genes that are expressed in various human cancers and in normal testis. Cancer Res 1999;59:3157-65.

305. Boel P, Wildmann C, Sensi ML, Brasseur R, Renauld JC, Coulie P, Boon T, van der Bruggen P. BAGE: a new gene encoding an antigen recognized on human melanomas by cytolytic T lymphocytes. Immunity 1995;2:167-75.

306. Lurquin C, De Smet C, Brasseur F, Muscatelli F, Martelange V, De Plaen E, Brasseur R, Monaco AP, Boon T. Two members of the human MAGEB gene family located in Xp21.3 are expressed in tumors of various histological origins. Genomics 1997;46:397-408.

307. Lethe B, Lucas S, Michaux L, De Smet C, Godelaine D, Serrano A, De Plaen E, Boon T. LAGE-1, a new gene with tumor specificity. Int J Cancer 1998;76:903-8.

308. Lucas S, De Plaen E, Boon T. MAGE-B5, MAGE-B6, MAGE-C2, and MAGE-C3: four new members of the MAGE family with tumor-specific expression. Int J Cancer 2000;87:55-60.

309. Lucas S, De Smet C, Arden KC, Viars CS, Lethe B, Lurquin C, Boon T. Identification of a new MAGE gene with tumor-specific expression by representational difference analysis. Cancer Res 1998;58:743-52.

310. Martelange V, De Smet C, De Plaen E, Lurquin C, Boon T. Identification on a human sarcoma of two new genes with tumor-specific expression. Cancer Res 2000;60:3848-55.

311. Loriot A, Boon T, De Smet C. Five new human cancer-germline genes identified among 12 genes expressed in spermatogonia. Int J Cancer 2003;105:371-6.

312. Ross MT, Grafham DV, Coffey AJ, Scherer S, McLay K, Muzny D, Platzer M, Howell GR, Burrows C, Bird CP, Frankish A, Lovell FL, et al. The DNA sequence of the human X chromosome. Nature 2005;434:325-37.

313. Khil PP, Smirnova NA, Romanienko PJ, Camerini-Otero RD. The mouse X chromosome is enriched for sex-biased genes not subject to selection by meiotic sex chromosome inactivation. Nat Genet 2004;36:642-6.

314. Groner AC, Meylan S, Ciuffi A, Zangger N, Ambrosini G, Denervaud N, Bucher P, Trono D. KRAB-zinc finger proteins and KAP1 can mediate long-range transcriptional repression through heterochromatin spreading. PLoS Genet 2010;6:e1000869.

315. Smith HA, McNeel DG. The SSX family of cancer-testis antigens as target proteins for tumor therapy. Clin Dev Immunol 2010;2010:150591.

316. Pivot-Pajot C, Caron C, Govin J, Vion A, Rousseaux S, Khochbin S. Acetylation-dependent chromatin reorganization by BRDT, a testis-specific bromodomain-containing protein. Mol Cell Biol 2003;23:5354-65.

317. De Plaen E, Naerhuyzen B, De Smet C, Szikora JP, Boon T. Alternative promoters of gene MAGE4a. Genomics 1997;40:305-13.

318. Ma W, Germeau C, Vigneron N, Maernoudt AS, Morel S, Boon T, Coulie PG, Van den Eynde BJ. Two new tumor-specific antigenic peptides encoded by gene MAGE-C2 and presented to cytolytic T lymphocytes by HLA-A2. Int J Cancer 2004;109:698-702.

319. De Smet C, Courtois SJ, Faraoni I, Lurquin C, Szikora JP, De Backer O, Boon T. Involvement of two Ets binding sites in the transcriptional activation of the MAGE1 gene. Immunogenetics 1995;42:282-90.

320. Wischnewski F, Pantel K, Schwarzenbach H. Promoter demethylation and histone acetylation mediate gene expression of MAGE-A1, -A2, -A3, and -A12 in human cancer cells. Mol Cancer Res 2006;4:339-49.

321. Gure AO, Wei IJ, Old LJ, Chen YT. The SSX gene family: characterization of 9 complete genes. Int J Cancer 2002;101:448-53.

322. James SR, Link PA, Karpf AR. Epigenetic regulation of X-linked cancer/germline antigen genes by DNMT1 and DNMT3b. Oncogene 2006;25:6975-85.

323. De Smet C, Loriot A, Boon T. Promoter-dependent mechanism leading to selective hypomethylation within the 5' region of gene MAGE-A1 in tumor cells. Mol Cell Biol 2004;24:4781-90.

324. Weber J, Salgaller M, Samid D, Johnson B, Herlyn M, Lassam N, Treisman J, Rosenberg SA. Expression of the MAGE-1 tumor antigen is up-regulated by the demethylating agent 5-aza-2'-deoxycytidine. Cancer Res 1994;54:1766-71.

325. Karpf AR, Lasek AW, Ririe TO, Hanks AN, Grossman D, Jones DA. Limited gene activation in tumor and normal epithelial cells treated with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine. Mol Pharmacol 2004;65:18-27.

326. Loriot A, De Plaen E, Boon T, De Smet C. Transient down-regulation of DNMT1 methyltransferase leads to activation and stable hypomethylation of MAGE-A1 in melanoma cells. J Biol Chem 2006;281:10118-26.

327. Loriot A, Sterpin C, De Backer O, De Smet C. Mouse embryonic stem cells induce targeted DNA demethylation within human MAGE-A1 transgenes. Epigenetics 2008;3:38-42.

328. Sergeant J, Taylor E, Palecek J, Fousteri M, Andrews EA, Sweeney S, Shinagawa H, Watts FZ, Lehmann AR. Composition and architecture of the Schizosaccharomyces pombe Rad18 (Smc5-6) complex. Mol Cell Biol 2005;25:172-84.

329. Lopez-Sanchez N, Gonzalez-Fernandez Z, Niinobe M, Yoshikawa K, Frade JM. Single mage gene in the chicken genome encodes CMage, a protein with functional similarities to mammalian type II Mage proteins. Physiol Genomics 2007;30:156-71.

330. Barker PA, Salehi A. The MAGE proteins: emerging roles in cell cycle progression, apoptosis, and neurogenetic disease. J Neurosci Res 2002;67:705-12.

331. Doyle JM, Gao J, Wang J, Yang M, Potts PR. MAGE-RING protein complexes comprise a family of E3 ubiquitin ligases. Mol Cell 2010;39:963-74.

332. Espantman KC, O'Shea CC. aMAGEing new players enter the RING to promote ubiquitylation. Mol Cell 2010;39:835-7.

333. Yang B, O'Herrin SM, Wu J, Reagan-Shaw S, Ma Y, Bhat KM, Gravekamp C, Setaluri V, Peters N, Hoffmann FM, Peng H, Ivanov AV, et al. MAGE-A, mMage-b, and MAGE-C proteins form complexes with KAP1 and suppress p53-dependent apoptosis in MAGE-positive cell lines. Cancer Res 2007;67:9954-62.

334. Rual JF, Venkatesan K, Hao T, Hirozane-Kishikawa T, Dricot A, Li N, Berriz GF, Gibbons FD, Dreze M, Ayivi-Guedehoussou N, Klitgord N, Simon C, et al. Towards a proteome-scale map of the human protein-protein interaction network. Nature 2005;437:1173-8.

335. Deshaies RJ, Joazeiro CA. RING domain E3 ubiquitin ligases. Annu Rev Biochem 2009;78:399-434.

336. Wang C, Ivanov A, Chen L, Fredericks WJ, Seto E, Rauscher FJ, 3rd, Chen J. MDM2 interaction with nuclear corepressor KAP1 contributes to p53 inactivation. Embo J 2005;24:3279-90.

337. Baarends WM, Roest HP, Grootegoed JA. The ubiquitin system in gametogenesis. Mol Cell Endocrinol 1999;151:5-16.

338. Yin Y, Stahl BC, DeWolf WC, Morgentaler A. p53-mediated germ cell quality control in spermatogenesis. Dev Biol 1998;204:165-71.

339. Yang B, O'Herrin S, Wu J, Reagan-Shaw S, Ma Y, Nihal M, Longley BJ. Select cancer testes antigens of the MAGE-A, -B, and -C families are expressed in mast cell lines and promote cell viability in vitro and in vivo. J Invest Dermatol 2007;127:267-75.

340. Monte M, Simonatto M, Peche LY, Bublik DR, Gobessi S, Pierotti MA, Rodolfo M, Schneider C. MAGE-A tumor antigens target p53 transactivation function through histone deacetylase recruitment and confer resistance to chemotherapeutic agents. Proc Natl Acad Sci U S A 2006;103:11160-5.

341. Laduron S, Deplus R, Zhou S, Kholmanskikh O, Godelaine D, De Smet C, Hayward SD, Fuks F, Boon T, De Plaen E. MAGE-A1 interacts with adaptor SKIP and the deacetylase HDAC1 to repress transcription. Nucleic Acids Res 2004;32:4340-50.

342. Sakurai T, Itoh K, Higashitsuji H, Nagao T, Nonoguchi K, Chiba T, Fujita J. A cleaved form of MAGE-A4 binds to Miz-1 and induces apoptosis in human cells. J Biol Chem 2004;279:15505-14.

343. Kostic C, Shaw PH. Isolation and characterization of sixteen novel p53 response genes. Oncogene 2000;19:3978-87.

344. Renaud S, Pugacheva EM, Delgado MD, Braunschweig R, Abdullaev Z, Loukinov D, Benhattar J, Lobanenkov V. Expression of the CTCF-paralogous cancer-testis gene, brother of the regulator of imprinted sites (BORIS), is regulated by three alternative promoters modulated by CpG methylation and by CTCF and p53 transcription factors. Nucleic Acids Res 2007;35:7372-88.

345. Woloszynska-Read A, James SR, Link PA, Yu J, Odunsi K, Karpf AR. DNA methylation-dependent regulation of BORIS/CTCFL expression in ovarian cancer. Cancer Immun 2007;7:21.

346. Loukinov DI, Pugacheva E, Vatolin S, Pack SD, Moon H, Chernukhin I, Mannan P, Larsson E, Kanduri C, Vostrov AA, Cui H, Niemitz EL, et al. BORIS, a novel male germ-line-specific protein associated with epigenetic reprogramming events, shares the same 11-zinc-finger domain with CTCF, the insulator protein involved in reading imprinting marks in the soma. Proc Natl Acad Sci U S A 2002;99:6806-11.

347. Phillips JE, Corces VG. CTCF: master weaver of the genome. Cell 2009;137:1194-211.

348. Monk M, Hitchins M, Hawes S. Differential expression of the embryo/cancer gene ECSA(DPPA2), the cancer/testis gene BORIS and the pluripotency structural gene OCT4, in human preimplantation development. Mol Hum Reprod 2008;14:347-55.

349. Vatolin S, Abdullaev Z, Pack SD, Flanagan PT, Custer M, Loukinov DI, Pugacheva E, Hong JA, Morse H, 3rd, Schrump DS, Risinger JI, Barrett JC, et al. Conditional expression of the CTCF-paralogous transcriptional factor BORIS in normal cells results in demethylation and derepression of MAGE-A1 and reactivation of other cancer-testis genes. Cancer Res 2005;65:7751-62.

350. Hoffmann MJ, Muller M, Engers R, Schulz WA. Epigenetic control of CTCFL/BORIS and OCT4 expression in urogenital malignancies. Biochem Pharmacol 2006;72:1577-88.

351. Hines WC, Bazarov AV, Mukhopadhyay R, Yaswen P. BORIS (CTCFL) is not expressed in most human breast cell lines and high grade breast carcinomas. PLoS One 2010;5:e9738.

352. Risinger JI, Chandramouli GV, Maxwell GL, Custer M, Pack S, Loukinov D, Aprelikova O, Litzi T, Schrump DS, Murphy SK, Berchuck A, Lobanenkov V, et al. Global expression analysis of cancer/testis genes in uterine cancers reveals a high incidence of BORIS expression. Clin Cancer Res 2007;13:1713-9.

353. Hong JA, Kang Y, Abdullaev Z, Flanagan PT, Pack SD, Fischette MR, Adnani MT, Loukinov DI, Vatolin S, Risinger JI, Custer M, Chen GA, et al. Reciprocal binding of CTCF and BORIS to the NY-ESO-1 promoter coincides with derepression of this cancer-testis gene in lung cancer cells. Cancer Res 2005;65:7763-74.

354. Hore TA, Deakin JE, Marshall Graves JA. The evolution of epigenetic regulators CTCF and BORIS/CTCFL in amniotes. PLoS Genet 2008;4:e1000169.

355. Heath H, Ribeiro de Almeida C, Sleutels F, Dingjan G, van de Nobelen S, Jonkers I, Ling KW, Gribnau J, Renkawitz R, Grosveld F, Hendriks RW, Galjart N. CTCF regulates cell cycle progression of alphabeta T cells in the thymus. Embo J 2008;27:2839-50.

356. Suzuki T, Kosaka-Suzuki N, Pack S, Shin DM, Yoon J, Abdullaev Z, Pugacheva E, Morse HC, 3rd, Loukinov D, Lobanenkov V. Expression of a testis-specific form of Gal3st1 (CST), a gene essential for spermatogenesis, is regulated by the CTCF paralogous gene BORIS. Mol Cell Biol 2010;30:2473-84.

357. Ideraabdullah FY, Vigneau S, Bartolomei MS. Genomic imprinting mechanisms in mammals. Mutat Res 2008;647:77-85.

358. Zlatanova J, Caiafa P. CCCTC-binding factor: to loop or to bridge. Cell Mol Life Sci 2009;66:1647-60.

359. Jelinic P, Stehle JC, Shaw P. The testis-specific factor CTCFL cooperates with the protein methyltransferase PRMT7 in H19 imprinting control region methylation. PLoS Biol 2006;4:e355.

360. Nguyen P, Cui H, Bisht KS, Sun L, Patel K, Lee RS, Kugoh H, Oshimura M, Feinberg AP, Gius D. CTCFL/BORIS is a methylation-independent DNA-binding protein that preferentially binds to the paternal H19 differentially methylated region. Cancer Res 2008;68:5546-51.

361. Kim TH, Abdullaev ZK, Smith AD, Ching KA, Loukinov DI, Green RD, Zhang MQ, Lobanenkov VV, Ren B. Analysis of the vertebrate insulator protein CTCF-binding sites in the human genome. Cell 2007;128:1231-45.

362. Cuddapah S, Jothi R, Schones DE, Roh TY, Cui K, Zhao K. Global analysis of the insulator binding protein CTCF in chromatin barrier regions reveals demarcation of active and repressive domains. Genome Res 2009;19:24-32.

363. Speeckaert R, Van Geel N, Vermaelen KV, Lambert J, Van Gele M, Speeckaert MM, Brochez L. Immune reactions in benign and malignant melanocytic lesions: lessons for immunotherapy. Pigment Cell Melanoma Res 2010.

364. Oble DA, Loewe R, Yu P, Mihm MC, Jr. Focus on TILs: prognostic significance of tumor infiltrating lymphocytes in human melanoma. Cancer Immun 2009;9:3.

365. Vajdic CM, van Leeuwen MT, Webster AC, McCredie MR, Stewart JH, Chapman JR, Amin J, McDonald SP, Grulich AE. Cutaneous melanoma is related to immune suppression in kidney transplant recipients. Cancer Epidemiol Biomarkers Prev 2009;18:2297-303.

366. Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. Cell 2010;140:883-99.

367. Sica A, Allavena P, Mantovani A. Cancer related inflammation: the macrophage connection. Cancer Lett 2008;267:204-15.

368. Torisu H, Ono M, Kiryu H, Furue M, Ohmoto Y, Nakayama J, Nishioka Y, Sone S, Kuwano M. Macrophage infiltration correlates with tumor stage and angiogenesis in human malignant melanoma: possible involvement of TNFalpha and IL-1alpha. Int J Cancer 2000;85:182-8.

369. de Saint Basile G, Menasche G, Fischer A. Molecular mechanisms of biogenesis and exocytosis of cytotoxic granules. Nat Rev Immunol 2010;10:568-79.

370. De Panfilis G, Campanini N, Santini M, Mori G, Tognetti E, Maestri R, Lombardi M, Froio E, Ferrari D, Ricci R. Phase- and stage-related proportions of T cells bearing the transcription factor FOXP3 infiltrate primary melanoma. J Invest Dermatol 2008;128:676-84.

371. Clemente CG, Mihm MC, Jr., Bufalino R, Zurrida S, Collini P, Cascinelli N. Prognostic value of tumor infiltrating lymphocytes in the vertical growth phase of primary cutaneous melanoma. Cancer 1996;77:1303-10.

372. Kalialis LV, Drzewiecki KT, Klyver H. Spontaneous regression of metastases from melanoma: review of the literature. Melanoma Res 2009;19:275-82.

373. Umansky V, Abschuetz O, Osen W, Ramacher M, Zhao F, Kato M, Schadendorf D. Melanoma-specific memory T cells are functionally active in Ret transgenic mice without macroscopic tumors. Cancer Res 2008;68:9451-8.

374. Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. Nature 2008;454:436-44.

375. Colotta F, Allavena P, Sica A, Garlanda C, Mantovani A. Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. Carcinogenesis 2009;30:1073-81.

376. Kuilman T, Michaloglou C, Vredeveld LC, Douma S, van Doorn R, Desmet CJ, Aarden LA, Mooi WJ, Peeper DS. Oncogene-induced senescence relayed by an interleukin-dependent inflammatory network. Cell 2008;133:1019-31.

377. Green DR, Ferguson T, Zitvogel L, Kroemer G. Immunogenic and tolerogenic cell death. Nat Rev Immunol 2009;9:353-63.

378. Sumimoto H, Imabayashi F, Iwata T, Kawakami Y. The BRAF-MAPK signaling pathway is essential for cancer-immune evasion in human melanoma cells. J Exp Med 2006;203:1651-6.

379. Maddodi N, Huang W, Havighurst T, Kim K, Longley BJ, Setaluri V. Induction of autophagy and inhibition of melanoma growth in vitro and in vivo by hyperactivation of oncogenic BRAF. J Invest Dermatol 2010;130:1657-67.

380. Li Y, Wang LX, Yang G, Hao F, Urba WJ, Hu HM. Efficient cross-presentation depends on autophagy in tumor cells. Cancer Res 2008;68:6889-95.

381. Kurts C, Robinson BW, Knolle PA. Cross-priming in health and disease. Nat Rev Immunol 2010;10:403-14.

382. Villadangos JA, Schnorrer P. Intrinsic and cooperative antigen-presenting functions of dendritic-cell subsets in vivo. Nat Rev Immunol 2007;7:543-55.

383. Steer HJ, Lake RA, Nowak AK, Robinson BW. Harnessing the immune response to treat cancer. Oncogene 2010;29:6301-13.

384. Mendez R, Aptsiauri N, Del Campo A, Maleno I, Cabrera T, Ruiz-Cabello F, Garrido F, Garcia-Lora A. HLA and melanoma: multiple alterations in HLA class I and II expression in human melanoma cell lines from ESTDAB cell bank. Cancer Immunol Immunother 2009;58:1507-15.

385. Thompson ED, Enriquez HL, Fu YX, Engelhard VH. Tumor masses support naive T cell infiltration, activation, and differentiation into effectors. J Exp Med 2010;207:1791-804.

386. Quezada SA, Simpson TR, Peggs KS, Merghoub T, Vider J, Fan X, Blasberg R, Yagita H, Muranski P, Antony PA, Restifo NP, Allison JP. Tumor-reactive CD4(+) T cells develop cytotoxic activity and eradicate large established melanoma after transfer into lymphopenic hosts. J Exp Med 2010;207:637-50.

387. Rubio V, Stuge TB, Singh N, Betts MR, Weber JS, Roederer M, Lee PP. Ex vivo identification, isolation and analysis of tumor-cytolytic T cells. Nat Med 2003;9:1377-82.

388. Zippelius A, Batard P, Rubio-Godoy V, Bioley G, Lienard D, Lejeune F, Rimoldi D, Guillaume P, Meidenbauer N, Mackensen A, Rufer N, Lubenow N, et al. Effector function of human tumor-specific CD8 T cells in melanoma lesions: a state of local functional tolerance. Cancer Res 2004;64:2865-73.

389. Ladanyi A, Kiss J, Somlai B, Gilde K, Fejos Z, Mohos A, Gaudi I, Timar J. Density of DC-LAMP(+) mature dendritic cells in combination with activated T lymphocytes infiltrating primary cutaneous melanoma is a strong independent prognostic factor. Cancer Immunol Immunother 2007;56:1459-69.

390. Guillaume B, Chapiro J, Stroobant V, Colau D, Van Holle B, Parvizi G, Bousquet-Dubouch MP, Theate I, Parmentier N, Van den Eynde BJ. Two abundant proteasome subtypes that uniquely process some antigens presented by HLA class I molecules. Proc Natl Acad Sci U S A 2010;107:18599-604.

391. Parmentier N, Stroobant V, Colau D, de Diesbach P, Morel S, Chapiro J, van Endert P, Van den Eynde BJ. Production of an antigenic peptide by insulin-degrading enzyme. Nat Immunol 2010;11:449-54.

392. Maleno I, Aptsiauri N, Cabrera T, Gallego A, Paschen A, Lopez-Nevot MA, Garrido F. Frequent loss of heterozygosity in the beta2-microglobulin region of chromosome 15 in primary human tumors. Immunogenetics 2010.

393. Tao J, Li Y, Liu YQ, Li L, Liu J, Shen X, Shen GX, Tu YT. Expression of transporters associated with antigen processing and human leucocyte antigen class I in malignant melanoma and its association with prognostic factors. Br J Dermatol 2008;158:88-94.

394. Kageshita T, Ishihara T, Campoli M, Ferrone S. Selective monomorphic and polymorphic HLA class I antigenic determinant loss in surgically removed melanoma lesions. Tissue Antigens 2005;65:419-28.

395. McMahan RH, Slansky JE. Mobilizing the low-avidity T cell repertoire to kill tumors. Semin Cancer Biol 2007;17:317-29.

396. Moore TV, Lyons GE, Brasic N, Roszkowski JJ, Voelkl S, Mackensen A, Kast WM, Le Poole IC, Nishimura MI. Relationship between CD8-dependent antigen recognition, T cell functional avidity, and tumor cell recognition. Cancer Immunol Immunother 2009;58:719-28.

397. Demotte N, Stroobant V, Courtoy PJ, Van Der Smissen P, Colau D, Luescher IF, Hivroz C, Nicaise J, Squifflet JL, Mourad M, Godelaine D, Boon T, et al. Restoring the association of the T cell receptor with CD8 reverses anergy in human tumor-infiltrating lymphocytes. Immunity 2008;28:414-24.

398. Snyder JE, Bowers WJ, Livingstone AM, Lee FE, Federoff HJ, Mosmann TR. Measuring the frequency of mouse and human cytotoxic T cells by the Lysispot assay: independent regulation of cytokine secretion and short-term killing. Nat Med 2003;9:231-5.

399. Nguyen LT, Yen PH, Nie J, Liadis N, Ghazarian D, Al-Habeeb A, Easson A, Leong W, Lipa J, McCready D, Reedijk M, Hogg D, et al. Expansion and characterization of human melanoma tumor-infiltrating lymphocytes (TILs). PLoS One 2010;5:e13940.

400. Weidanz JA, Nguyen T, Woodburn T, Neethling FA, Chiriva-Internati M, Hildebrand WH, Lustgarten J. Levels of specific peptide-HLA class I complex predicts tumor cell susceptibility to CTL killing. J Immunol 2006;177:5088-97.

401. Purbhoo MA, Irvine DJ, Huppa JB, Davis MM. T cell killing does not require the formation of a stable mature immunological synapse. Nat Immunol 2004;5:524-30.

402. Traversari C, van der Bruggen P, Luescher IF, Lurquin C, Chomez P, Van Pel A, De Plaen E, Amar-Costesec A, Boon T. A nonapeptide encoded by human gene MAGE-1 is recognized on HLA-A1 by cytolytic T lymphocytes directed against tumor antigen MZ2-E. J Exp Med 1992;176:1453-7.

403. Lucas S, Coulie PG. About human tumor antigens to be used in immunotherapy. Semin Immunol 2008;20:301-7.

404. Lethe B, van der Bruggen P, Brasseur F, Boon T. MAGE-1 expression threshold for the lysis of melanoma cell lines by a specific cytotoxic T lymphocyte. Melanoma Res 1997;7 Suppl 2:S83-8.

405. Purbhoo MA, Sutton DH, Brewer JE, Mullings RE, Hill ME, Mahon TM, Karbach J, Jager E, Cameron BJ, Lissin N, Vyas P, Chen JL, et al. Quantifying and imaging NY-ESO-1/LAGE-1-derived epitopes on tumor cells using high affinity T cell receptors. J Immunol 2006;176:7308-16.

406. Ikeda H, Lethe B, Lehmann F, van Baren N, Baurain JF, de Smet C, Chambost H, Vitale M, Moretta A, Boon T, Coulie PG. Characterization of an antigen that is recognized on a melanoma showing partial HLA loss by CTL expressing an NK inhibitory receptor. Immunity 1997;6:199-208.

407. Griffioen M, Kessler JH, Borghi M, van Soest RA, van der Minne CE, Nouta J, van der Burg SH, Medema JP, Schrier PI, Falkenburg JH, Osanto S, Melief CJ. Detection and functional analysis of CD8+ T cells specific for PRAME: a target for T-cell therapy. Clin Cancer Res 2006;12:3130-6.

408. Singh S, Kaye S, Gore ME, McClure MO, Bunker CB. The role of human endogenous retroviruses in melanoma. Br J Dermatol 2009;161:1225-31.

409. Schiavetti F, Thonnard J, Colau D, Boon T, Coulie PG. A human endogenous retroviral sequence encoding an antigen recognized on melanoma by cytolytic T lymphocytes. Cancer Res 2002;62:5510-6.

410. Parmiani G, De Filippo A, Novellino L, Castelli C. Unique human tumor antigens: immunobiology and use in clinical trials. J Immunol 2007;178:1975-9.

411. Sensi M, Anichini A. Unique tumor antigens: evidence for immune control of genome integrity and immunogenic targets for T cell-mediated patient-specific immunotherapy. Clin Cancer Res 2006;12:5023-32.

412. Sharkey MS, Lizee G, Gonzales MI, Patel S, Topalian SL. CD4(+) T-cell recognition of mutated B-RAF in melanoma patients harboring the V599E mutation. Cancer Res 2004;64:1595-9.

413. Linard B, Bezieau S, Benlalam H, Labarriere N, Guilloux Y, Diez E, Jotereau F. A ras-mutated peptide targeted by CTL infiltrating a human melanoma lesion. J Immunol 2002;168:4802-8.

414. Robbins PF, El-Gamil M, Li YF, Kawakami Y, Loftus D, Appella E, Rosenberg SA. A mutated beta-catenin gene encodes a melanoma-specific antigen recognized by tumor infiltrating lymphocytes. J Exp Med 1996;183:1185-92.

415. Wolfel T, Hauer M, Schneider J, Serrano M, Wolfel C, Klehmann-Hieb E, De Plaen E, Hankeln T, Meyer zum Buschenfelde KH, Beach D. A p16INK4a-insensitive CDK4 mutant targeted by cytolytic T lymphocytes in a human melanoma. Science 1995;269:1281-4.

416. Caballero OL, Zhao Q, Rimoldi D, Stevenson BJ, Svobodova S, Devalle S, Rohrig UF, Pagotto A, Michielin O, Speiser D, Wolchok JD, Liu C, et al. Frequent MAGE mutations in human melanoma. PLoS One 2010;5.

417. Anichini A, Maccalli C, Mortarini R, Salvi S, Mazzocchi A, Squarcina P, Herlyn M, Parmiani G. Melanoma cells and normal melanocytes share antigens recognized by HLA-A2-restricted cytotoxic T cell clones from melanoma patients. J Exp Med 1993;177:989-98.

418. Romero P, Valmori D, Pittet MJ, Zippelius A, Rimoldi D, Levy F, Dutoit V, Ayyoub M, Rubio-Godoy V, Michielin O, Guillaume P, Batard P, et al. Antigenicity and immunogenicity of Melan-A/MART-1 derived peptides as targets for tumor reactive CTL in human melanoma. Immunol Rev 2002;188:81-96.

419. Mortarini R, Piris A, Maurichi A, Molla A, Bersani I, Bono A, Bartoli C, Santinami M, Lombardo C, Ravagnani F, Cascinelli N, Parmiani G, et al. Lack of terminally differentiated tumor-specific CD8+ T cells at tumor site in spite of antitumor immunity to self-antigens in human metastatic melanoma. Cancer Res 2003;63:2535-45.

420. Garbelli S, Mantovani S, Palermo B, Giachino C. Melanocyte-specific, cytotoxic T cell responses in vitiligo: the effective variant of melanoma immunity? Pigment Cell Res 2005;18:234-42.

421. Damico FM, Bezerra FT, Silva GC, Gasparin F, Yamamoto JH. New insights into Vogt-Koyanagi-Harada disease. Arq Bras Oftalmol 2009;72:413-20.

422. Sen HN, Nussenblatt RB. Sympathetic ophthalmia: what have we learned? Am J Ophthalmol 2009;148:632-3.

423. Kitamura R, Tsukamoto K, Harada K, Shimizu A, Shimada S, Kobayashi T, Imokawa G. Mechanisms underlying the dysfunction of melanocytes in vitiligo epidermis: role of SCF/KIT protein interactions and the downstream effector, MITF-M. J Pathol 2004;202:463-75.

424. Palermo B, Garbelli S, Mantovani S, Scoccia E, Da Prada GA, Bernabei P, Avanzini MA, Brazzelli V, Borroni G, Giachino C. Qualitative difference between the cytotoxic T lymphocyte responses to melanocyte antigens in melanoma and vitiligo. Eur J Immunol 2005;35:3153-62.

425. Quaglino P, Marenco F, Osella-Abate S, Cappello N, Ortoncelli M, Salomone B, Fierro MT, Savoia P, Bernengo MG. Vitiligo is an independent favourable prognostic factor in stage III and IV metastatic melanoma patients: results from a single-institution hospital-based observational cohort study. Ann Oncol 2010;21:409-14.

426. Coulie PG, Karanikas V, Lurquin C, Colau D, Connerotte T, Hanagiri T, Van Pel A, Lucas S, Godelaine D, Lonchay C, Marchand M, Van Baren N, et al. Cytolytic T-cell responses of cancer patients vaccinated with a MAGE antigen. Immunol Rev 2002;188:33-42.

427. Dudley ME, Yang JC, Sherry R, Hughes MS, Royal R, Kammula U, Robbins PF, Huang J, Citrin DE, Leitman SF, Wunderlich J, Restifo NP, et al. Adoptive cell therapy for patients with metastatic melanoma: evaluation of intensive myeloablative chemoradiation preparative regimens. J Clin Oncol 2008;26:5233-9.

428. Burns WR, Zheng Z, Rosenberg SA, Morgan RA. Lack of specific gamma-retroviral vector long terminal repeat promoter silencing in patients receiving genetically engineered lymphocytes and activation upon lymphocyte restimulation. Blood 2009;114:2888-99.

429. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, Gonzalez R, Robert C, Schadendorf D, Hassel JC, Akerley W, van den Eertwegh AJ, et al. Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med 2010;363:711-23.

430. Tarhini AA, Iqbal F. CTLA-4 blockade: therapeutic potential in cancer treatments. Onco Targets Ther 2010;3:15-25.

431. Yeh S, Karne NK, Kerkar SP, Heller CK, Palmer DC, Johnson LA, Li Z, Bishop RJ, Wong WT, Sherry RM, Yang JC, Dudley ME, et al. Ocular and systemic autoimmunity after successful tumor-infiltrating lymphocyte immunotherapy for recurrent, metastatic melanoma. Ophthalmology 2009;116:981-9 e1.

432. Caspi RR. Immunotherapy of autoimmunity and cancer: the penalty for success. Nat Rev Immunol 2008;8:970-6.

433. Attia P, Phan GQ, Maker AV, Robinson MR, Quezado MM, Yang JC, Sherry RM, Topalian SL, Kammula US, Royal RE, Restifo NP, Haworth LR, et al. Autoimmunity correlates with tumor regression in patients with metastatic melanoma treated with anti-cytotoxic T-lymphocyte antigen-4. J Clin Oncol 2005;23:6043-53.

434. Van Der Bruggen P, Zhang Y, Chaux P, Stroobant V, Panichelli C, Schultz ES, Chapiro J, Van Den Eynde BJ, Brasseur F, Boon T. Tumor-specific shared antigenic peptides recognized by human T cells. Immunol Rev 2002;188:51-64.

435. Kaneda A, Tsukamoto T, Takamura-Enya T, Watanabe N, Kaminishi M, Sugimura T, Tatematsu M, Ushijima T. Frequent hypomethylation in multiple promoter CpG islands is associated with global hypomethylation, but not with frequent promoter hypermethylation. Cancer Sci 2004;95:58-64.

436. Chao W, Huynh KD, Spencer RJ, Davidow LS, Lee JT. CTCF, a candidate trans-acting factor for X-inactivation choice. Science 2002;295:345-7.

437. Ohlsson R, Renkawitz R, Lobanenkov V. CTCF is a uniquely versatile transcription regulator linked to epigenetics and disease. Trends Genet 2001;17:520-7.

438. Klenova EM, Morse HC, 3rd, Ohlsson R, Lobanenkov VV. The novel BORIS + CTCF gene family is uniquely involved in the epigenetics of normal biology and cancer. Semin Cancer Biol 2002;12:399-414.

439. Boukamp P, Petrussevska RT, Breitkreutz D, Hornung J, Markham A, Fusenig NE. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. J Cell Biol 1988;106:761-71.

440. Weynants P, Lethe B, Brasseur F, Marchand M, Boon T. Expression of mage genes by non-small-cell lung carcinomas. Int J Cancer 1994;56:826-9.

441. Jacobs JF, Brasseur F, Hulsbergen-van de Kaa CA, van de Rakt MW, Figdor CG, Adema GJ, Hoogerbrugge PM, Coulie PG, de Vries IJ. Cancer-germline gene expression in pediatric solid tumors using quantitative real-time PCR. Int J Cancer 2007;120:67-74.

442. Ghochikyan A, Mkrtichyan M, Loukinov D, Mamikonyan G, Pack SD, Movsesyan N, Ichim TE, Cribbs DH, Lobanenkov VV, Agadjanyan MG. Elicitation of T cell responses to histologically unrelated tumors by immunization with the novel cancer-testis antigen, brother of the regulator of imprinted sites. J Immunol 2007;178:566-73.

443. Loukinov D, Ghochikyan A, Mkrtichyan M, Ichim TE, Lobanenkov VV, Cribbs DH, Agadjanyan MG. Antitumor efficacy of DNA vaccination to the epigenetically acting tumor promoting transcription factor BORIS and CD80 molecular adjuvant. J Cell Biochem 2006;98:1037-43.

444. Pauls K, Schorle H, Jeske W, Brehm R, Steger K, Wernert N, Buttner R, Zhou H. Spatial expression of germ cell markers during maturation of human fetal male gonads: an immunohistochemical study. Hum Reprod 2006;21:397-404.

445. Rosenberg SA, Packard BS, Aebersold PM, Solomon D, Topalian SL, Toy ST, Simon P, Lotze MT, Yang JC, Seipp CA, et al. Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma. A preliminary report. N Engl J Med 1988;319:1676-80.

446. Le Gal FA, Avril MF, Bosq J, Lefebvre P, Deschemin JC, Andrieu M, Dore MX, Guillet JG. Direct evidence to support the role of antigen-specific CD8(+) T cells in melanoma-associated vitiligo. J Invest Dermatol 2001;117:1464-70.

447. Zou W. Regulatory T cells, tumour immunity and immunotherapy. Nat Rev Immunol 2006;6:295-307.

448. Filipazzi P, Valenti R, Huber V, Pilla L, Canese P, Iero M, Castelli C, Mariani L, Parmiani G, Rivoltini L. Identification of a new subset of myeloid suppressor cells in peripheral blood of melanoma patients with modulation by a granulocyte-macrophage colony-stimulation factor-based antitumor vaccine. J Clin Oncol 2007;25:2546-53.

449. Uyttenhove C, Pilotte L, Theate I, Stroobant V, Colau D, Parmentier N, Boon T, Van den Eynde BJ. Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase. Nat Med 2003;9:1269-74.

450. Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, Fitz LJ, Malenkovich N, Okazaki T, Byrne MC, Horton HF, Fouser L, et al. Engagement of the PD-1

immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. J Exp Med 2000;192:1027-34.

451. Blank C, Mackensen A. Contribution of the PD-L1/PD-1 pathway to T-cell exhaustion: an update on implications for chronic infections and tumor evasion. Cancer Immunol Immunother 2007;56:739-45.

452. Schwartzentruber DJ, Topalian SL, Mancini M, Rosenberg SA. Specific release of granulocyte-macrophage colony-stimulating factor, tumor necrosis factor-alpha, and IFN-gamma by human tumor-infiltrating lymphocytes after autologous tumor stimulation. J Immunol 1991;146:3674-81.

453. Sander B, Boeryd B. Tumor necrosis factor-alpha expression in human primary malignant malanoma and it relationship to tumor infiltration by CD3+ cells. Int J Cancer 1996;66:42-7.

454. Boehm U, Klamp T, Groot M, Howard JC. Cellular responses to interferon-gamma. Annu Rev Immunol 1997;15:749-95.

455. Strehl B, Seifert U, Kruger E, Heink S, Kuckelkorn U, Kloetzel PM. Interferon-gamma, the functional plasticity of the ubiquitin-proteasome system, and MHC class I antigen processing. Immunol Rev 2005;207:19-30.

456. Kholmanskikh O, Loriot A, Brasseur F, De Plaen E, De Smet C. Expression of BORIS in melanoma: lack of association with MAGE-A1 activation. Int J Cancer 2008;122:777-84.

457. Penzo M, Massa PE, Olivotto E, Bianchi F, Borzi RM, Hanidu A, Li X, Li J, Marcu KB. Sustained NF-kappaB activation produces a short-term cell proliferation block in conjunction with repressing effectors of cell cycle progression controlled by E2F or FoxM1. J Cell Physiol 2009;218:215-27.

458. Harada M, Li YF, El-Gamil M, Rosenberg SA, Robbins PF. Use of an in vitro immunoselected tumor line to identify shared melanoma antigens recognized by HLA-A*0201-restricted T cells. Cancer Res 2001;61:1089-94.

459. Englaro W, Bahadoran P, Bertolotto C, Busca R, Derijard B, Livolsi A, Peyron JF, Ortonne JP, Ballotti R. Tumor necrosis factor alpha-mediated inhibition of melanogenesis is dependent on nuclear factor kappa B activation. Oncogene 1999;18:1553-9.

460. Le Poole IC, Riker AI, Quevedo ME, Stennett LS, Wang E, Marincola FM, Kast WM, Robinson JK, Nickoloff BJ. Interferon-gamma reduces melanosomal antigen expression and recognition of melanoma cells by cytotoxic T cells. Am J Pathol 2002;160:521-8.

461. Certa U, Seiler M, Padovan E, Spagnoli GC. Interferon-a sensitivity in melanoma cells: detection of potential response marker genes. Recent Results Cancer Res 2002;160:85-91.

462. Dinarello CA. Immunological and inflammatory functions of the interleukin-1 family. Annu Rev Immunol 2009;27:519-50.

463. Apte RN, Voronov E. Is interleukin-1 a good or bad 'guy' in tumor immunobiology and immunotherapy? Immunol Rev 2008;222:222-41.

464. Schadendorf D, Moller A, Algermissen B, Worm M, Sticherling M, Czarnetzki BM. IL-8 produced by human malignant melanoma cells in vitro is an essential autocrine growth factor. J Immunol 1993;151:2667-75.

465. Singh RK, Varney ML. Regulation of interleukin 8 expression in human malignant melanoma cells. Cancer Res 1998;58:1532-7.

466. Bennicelli JL, Elias J, Kern J, Guerry Dt. Production of interleukin 1 activity by cultured human melanoma cells. Cancer Res 1989;49:930-5.

467. Elaraj DM, Weinreich DM, Varghese S, Puhlmann M, Hewitt SM, Carroll NM, Feldman ED, Turner EM, Alexander HR. The role of interleukin 1 in growth and metastasis of human cancer xenografts. Clin Cancer Res 2006;12:1088-96.

468. Hiscott J, Marois J, Garoufalis J, D'Addario M, Roulston A, Kwan I, Pepin N, Lacoste J, Nguyen H, Bensi G, et al. Characterization of a functional NF-kappa B site in the human interleukin 1 beta promoter: evidence for a positive autoregulatory loop. Mol Cell Biol 1993;13:6231-40.

469. Niu J, Li Z, Peng B, Chiao PJ. Identification of an autoregulatory feedback pathway involving interleukin-1alpha in induction of constitutive NF-kappaB activation in pancreatic cancer cells. J Biol Chem 2004;279:16452-62.

470. Kim DS, Park SH, Park KC. Transforming growth factor-beta1 decreases melanin synthesis via delayed extracellular signal-regulated kinase activation. Int J Biochem Cell Biol 2004;36:1482-91.

471. Gollob JA, Sciambi CJ, Huang Z, Dressman HK. Gene expression changes and signaling events associated with the direct antimelanoma effect of IFN-gamma. Cancer Res 2005;65:8869-77.

472. Naugler WE, Karin M. NF-kappaB and cancer-identifying targets and mechanisms. Curr Opin Genet Dev 2008;18:19-26.

473. Taganov KD, Boldin MP, Chang KJ, Baltimore D. NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. Proc Natl Acad Sci U S A 2006;103:12481-6.

474. Cohen C, Zavala-Pompa A, Sequeira JH, Shoji M, Sexton DG, Cotsonis G, Cerimele F, Govindarajan B, Macaron N, Arbiser JL. Mitogen-actived protein kinase activation is an early event in melanoma progression. Clin Cancer Res 2002;8:3728-33.

475. Verstrepen L, Bekaert T, Chau TL, Tavernier J, Chariot A, Beyaert R. TLR-4, IL-1R and TNF-R signaling to NF-kappaB: variations on a common theme. Cell Mol Life Sci 2008;65:2964-78.

476. Morel S, Levy F, Burlet-Schiltz O, Brasseur F, Probst-Kepper M, Peitrequin AL, Monsarrat B, Van Velthoven R, Cerottini JC, Boon T, Gairin JE, Van den Eynde BJ. Processing of some antigens by the standard proteasome but not by the immunoproteasome results in poor presentation by dendritic cells. Immunity 2000;12:107-17.

477. Leblond J, Hubert-Buron A, Bole-Feysot C, Ducrotte P, Dechelotte P, Coeffier M. Regulation of proteolysis by cytokines in the human intestinal epithelial cell line HCT-8: role of IFNgamma. Biochimie 2006;88:759-65.

478. Nagorsen D, Servis C, Levy N, Provenzano M, Dudley ME, Marincola FM, Levy F. Proteasomal cleavage does not determine immunogenicity of gp100-derived peptides gp100 209-217 and gp100 209-217T210M. Cancer Immunol Immunother 2004;53:817-24.

479. Kurnick JT, Ramirez-Montagut T, Boyle LA, Andrews DM, Pandolfi F, Durda PJ, Butera D, Dunn IS, Benson EM, Gobin SJ, van den Elsen PJ. A novel autocrine pathway of tumor escape from immune recognition: melanoma cell lines produce a soluble protein that diminishes expression of the gene encoding the melanocyte lineage melan-A/MART-1 antigen through down-modulation of its promoter. J Immunol 2001;167:1204-11.

480. Durda PJ, Dunn IS, Rose LB, Butera D, Benson EM, Pandolfi F, Kurnick JT. Induction of "antigen silencing" in melanomas by oncostatin M: down-modulation of melanocyte antigen expression. Mol Cancer Res 2003;1:411-9.

481. Lewis AM, Varghese S, Xu H, Alexander HR. Interleukin-1 and cancer progression: the emerging role of interleukin-1 receptor antagonist as a novel therapeutic agent in cancer treatment. J Transl Med 2006;4:48.

482. Vachtenheim J, Novotna H, Ghanem G. Transcriptional repression of the microphthalmia gene in melanoma cells correlates with the unresponsiveness of target genes to ectopic microphthalmia-associated transcription factor. J Invest Dermatol 2001;117:1505-11.

483. Fang D, Tsuji Y, Setaluri V. Selective down-regulation of tyrosinase family gene TYRP1 by inhibition of the activity of melanocyte transcription factor, MITF. Nucleic Acids Res 2002;30:3096-106.

484. Li L, Dragulev B, Zigrino P, Mauch C, Fox JW. The invasive potential of human melanoma cell lines correlates with their ability to alter fibroblast gene expression in vitro and the stromal microenvironment in vivo. Int J Cancer 2009;125:1796-804.

485. Salazar-Onfray F, Lopez MN, Mendoza-Naranjo A. Paradoxical effects of cytokines in tumor immune surveillance and tumor immune escape. Cytokine Growth Factor Rev 2007;18:171-82.

486. Lurquin C, Lethe B, De Plaen E, Corbiere V, Theate I, van Baren N, Coulie PG, Boon T. Contrasting frequencies of antitumor and anti-vaccine T cells in metastases of a melanoma patient vaccinated with a MAGE tumor antigen. J Exp Med 2005;201:249-57.

487. Germeau C, Ma W, Schiavetti F, Lurquin C, Henry E, Vigneron N, Brasseur F, Lethe B, De Plaen E, Velu T, Boon T, Coulie PG. High frequency of antitumor T cells in the blood of melanoma patients before and after vaccination with tumor antigens. J Exp Med 2005;201:241-8.

488. Butterfield LH, Ribas A, Dissette VB, Amarnani SN, Vu HT, Oseguera D, Wang HJ, Elashoff RM, McBride WH, Mukherji B, Cochran AJ, Glaspy JA, et al. Determinant spreading

associated with clinical response in dendritic cell-based immunotherapy for malignant melanoma. Clin Cancer Res 2003;9:998-1008.

489. Lally KM, Mocellin S, Ohnmacht GA, Nielsen MB, Bettinotti M, Panelli MC, Monsurro V, Marincola FM. Unmasking cryptic epitopes after loss of immunodominant tumor antigen expression through epitope spreading. Int J Cancer 2001;93:841-7.

490. Chames P, Hufton SE, Coulie PG, Uchanska-Ziegler B, Hoogenboom HR. Direct selection of a human antibody fragment directed against the tumor T-cell epitope HLA-A1-MAGE-A1 from a nonimmunized phage-Fab library. Proc Natl Acad Sci U S A 2000;97:7969-74.

491. Denkberg G, Reiter Y. Recombinant antibodies with T-cell receptor-like specificity: novel tools to study MHC class I presentation. Autoimmun Rev 2006;5:252-7.

492. Stewart-Jones G, Wadle A, Hombach A, Shenderov E, Held G, Fischer E, Kleber S, Nuber N, Stenner-Liewen F, Bauer S, McMichael A, Knuth A, et al. Rational development of high-affinity T-cell receptor-like antibodies. Proc Natl Acad Sci U S A 2009;106:5784-8.

493. Verma B, Neethling FA, Caseltine S, Fabrizio G, Largo S, Duty JA, Tabaczewski P, Weidanz JA. TCR mimic monoclonal antibody targets a specific peptide/HLA class I complex and significantly impedes tumor growth in vivo using breast cancer models. J Immunol 2010;184:2156-65.

494. Cormier JN, Panelli MC, Hackett JA, Bettinotti MP, Mixon A, Wunderlich J, Parker LL, Restifo NP, Ferrone S, Marincola FM. Natural variation of the expression of HLA and endogenous antigen modulates CTL recognition in an in vitro melanoma model. Int J Cancer 1999;80:781-90.

495. Riker AI, Kammula US, Panelli MC, Wang E, Ohnmacht GA, Steinberg SM, Rosenberg SA, Marincola FM. Threshold levels of gene expression of the melanoma antigen gp100 correlate with tumor cell recognition by cytotoxic T lymphocytes. Int J Cancer 2000;86:818-26.

496. Ostankovitch M, Altrich-Vanlith M, Robila V, Engelhard VH. N-glycosylation enhances presentation of a MHC class I-restricted epitope from tyrosinase. J Immunol 2009;182:4830-5.

497. Engelhard VH, Altrich-Vanlith M, Ostankovitch M, Zarling AL. Post-translational modifications of naturally processed MHC-binding epitopes. Curr Opin Immunol 2006;18:92-7.

498. Ostankovitch M, Robila V, Engelhard VH. Regulated folding of tyrosinase in the endoplasmic reticulum demonstrates that misfolded full-length proteins are efficient substrates for class I processing and presentation. J Immunol 2005;174:2544-51.

499. Thompson LW, Garbee CF, Hibbitts S, Brinckerhoff LH, Pierce RA, Chianese-Bullock KA, Deacon DH, Engelhard VH, Slingluff CL, Jr. Competition among peptides in melanoma vaccines for binding to MHC molecules. J Immunother 2004;27:425-31.

500. Voss CY, Deola S, Fleisher TA, Marincola FM. Increased effector-target cell conjugate formation due to HLA restricted specific antigen recognition. Immunol Res 2009;45:13-24.

501. Lundegaard C, Lund O, Buus S, Nielsen M. Major histocompatibility complex class I binding predictions as a tool in epitope discovery. Immunology 2010;130:309-18.

502. Mkrtichyan M, Ghochikyan A, Loukinov D, Davtyan H, Ichim TE, Cribbs DH, Lobanenkov VV, Agadjanyan MG. DNA, but not protein vaccine based on mutated BORIS antigen significantly inhibits tumor growth and prolongs the survival of mice. Gene Ther 2008;15:61-4.

503. Yawata T, Nakai E, Park KC, Chihara T, Kumazawa A, Toyonaga S, Masahira T, Nakabayashi H, Kaji T, Shimizu K. Enhanced expression of cancer testis antigen genes in glioma stem cells. Mol Carcinog 2010;49:532-44.

504. Woloszynska-Read A, James SR, Song C, Jin B, Odunsi K, Karpf AR. BORIS/CTCFL expression is insufficient for cancer-germline antigen gene expression and DNA hypomethylation in ovarian cell lines. Cancer Immun 2010;10:6.

505. Smith IM, Glazer CA, Mithani SK, Ochs MF, Sun W, Bhan S, Vostrov A, Abdullaev Z, Lobanenkov V, Gray A, Liu C, Chang SS, et al. Coordinated activation of candidate proto-oncogenes and cancer testes antigens via promoter demethylation in head and neck cancer and lung cancer. PLoS One 2009;4:e4961.

506. Sun L, Huang L, Nguyen P, Bisht KS, Bar-Sela G, Ho AS, Bradbury CM, Yu W, Cui H, Lee S, Trepel JB, Feinberg AP, et al. DNA methyltransferase 1 and 3B activate BAG-1 expression via recruitment of CTCFL/BORIS and modulation of promoter histone methylation. Cancer Res 2008;68:2726-35.

507. Nguyen P, Bar-Sela G, Sun L, Bisht KS, Cui H, Kohn E, Feinberg AP, Gius D. BAT3 and SET1A form a complex with CTCFL/BORIS to modulate H3K4 histone dimethylation and gene expression. Mol Cell Biol 2008;28:6720-9.

508. Kang Y, Hong JA, Chen GA, Nguyen DM, Schrump DS. Dynamic transcriptional regulatory complexes including BORIS, CTCF and Sp1 modulate NY-ESO-1 expression in lung cancer cells. Oncogene 2007;26:4394-403.

509. Majumder P, Gomez JA, Chadwick BP, Boss JM. The insulator factor CTCF controls MHC class II gene expression and is required for the formation of long-distance chromatin interactions. J Exp Med 2008;205:785-98.

510. de Vries TJ, Fourkour A, Wobbes T, Verkroost G, Ruiter DJ, van Muijen GN. Heterogeneous expression of immunotherapy candidate proteins gp100, MART-1, and tyrosinase in human melanoma cell lines and in human melanocytic lesions. Cancer Res 1997;57:3223-9.

511. Takeuchi H, Kuo C, Morton DL, Wang HJ, Hoon DS. Expression of differentiation melanoma-associated antigen genes is associated with favorable disease outcome in advanced-stage melanomas. Cancer Res 2003;63:441-8.

512. Lim E, Browning J, MacGregor D, Davis ID, Cebon JS. Desmoplastic melanoma: comparison of expression of differentiation antigens and cancer testis antigens. Melanoma Res 2006;16:347-55.

513. Ohsie SJ, Sarantopoulos GP, Cochran AJ, Binder SW. Immunohistochemical characteristics of melanoma. J Cutan Pathol 2008;35:433-44.

514. Eberle J, Garbe C, Wang N, Orfanos CE. Incomplete expression of the tyrosinase gene family (tyrosinase, TRP-1, and TRP-2) in human malignant melanoma cells in vitro. Pigment Cell Res 1995;8:307-13.

515. Halaban R, Patton RS, Cheng E, Svedine S, Trombetta ES, Wahl ML, Ariyan S, Hebert DN. Abnormal acidification of melanoma cells induces tyrosinase retention in the early secretory pathway. J Biol Chem 2002;277:14821-8.

516. Selzer E, Wacheck V, Lucas T, Heere-Ress E, Wu M, Weilbaecher KN, Schlegel W, Valent P, Wrba F, Pehamberger H, Fisher D, Jansen B. The melanocyte-specific isoform of the microphthalmia transcription factor affects the phenotype of human melanoma. Cancer Res 2002;62:2098-103.

517. Vachtenheim J, Ondrusova L, Borovansky J. SWI/SNF chromatin remodeling complex is critical for the expression of microphthalmia-associated transcription factor in melanoma cells. Biochem Biophys Res Commun 2010;392:454-9.

518. Reisman D, Glaros S, Thompson EA. The SWI/SNF complex and cancer. Oncogene 2009;28:1653-68.

519. Becker TM, Haferkamp S, Dijkstra MK, Scurr LL, Frausto M, Diefenbach E, Scolyer RA, Reisman DN, Mann GJ, Kefford RF, Rizos H. The chromatin remodelling factor BRG1 is a novel binding partner of the tumor suppressor p16INK4a. Mol Cancer 2009;8:4.

520. Philippidou D, Schmitt M, Moser D, Margue C, Nazarov PV, Muller A, Vallar L, Nashan D, Behrmann I, Kreis S. Signatures of microRNAs and selected microRNA target genes in human melanoma. Cancer Res 2010;70:4163-73.

521. Okamoto M, Liu W, Luo Y, Tanaka A, Cai X, Norris DA, Dinarello CA, Fujita M. Constitutively active inflammasome in human melanoma cells mediating autoinflammation via caspase-1 processing and secretion of interleukin-1beta. J Biol Chem 2010;285:6477-88.

522. Moretti S, Pinzi C, Spallanzani A, Berti E, Chiarugi A, Mazzoli S, Fabiani M, Vallecchi C, Herlyn M. Immunohistochemical evidence of cytokine networks during progression of human melanocytic lesions. Int J Cancer 1999;84:160-8.

523. Yurkovetsky ZR, Kirkwood JM, Edington HD, Marrangoni AM, Velikokhatnaya L, Winans MT, Gorelik E, Lokshin AE. Multiplex analysis of serum cytokines in melanoma patients treated with interferon-alpha2b. Clin Cancer Res 2007;13:2422-8.

524. Borge BA, Kalland KH, Olsen S, Bletsa A, Berggreen E, Wiig H. Cytokines are produced locally by myocytes in rat skeletal muscle during endotoxemia. Am J Physiol Heart Circ Physiol 2009;296:H735-44.

525. Burrows FJ, Haskard DO, Hart IR, Marshall JF, Selkirk S, Poole S, Thorpe PE. Influence of tumor-derived interleukin 1 on melanoma-endothelial cell interactions in vitro. Cancer Res 1991;51:4768-75.

526. Netea MG, Nold-Petry CA, Nold MF, Joosten LA, Opitz B, van der Meer JH, van de Veerdonk FL, Ferwerda G, Heinhuis B, Devesa I, Funk CJ, Mason RJ, et al. Differential requirement for the activation of the inflammasome for processing and release of IL-1beta in monocytes and macrophages. Blood 2009;113:2324-35.

527. Kopf M, Bachmann MF, Marsland BJ. Averting inflammation by targeting the cytokine environment. Nat Rev Drug Discov 2010;9:703-18.

528. Kaplanski G, Farnarier C, Kaplanski S, Porat R, Shapiro L, Bongrand P, Dinarello CA. Interleukin-1 induces interleukin-8 secretion from endothelial cells by a juxtacrine mechanism. Blood 1994;84:4242-8.

529. Kavita U, Mizel SB. Differential sensitivity of interleukin-1 alpha and -beta precursor proteins to cleavage by calpain, a calcium-dependent protease. J Biol Chem 1995;270:27758-65.

530. Stevenson FT, Turck J, Locksley RM, Lovett DH. The N-terminal propiece of interleukin 1 alpha is a transforming nuclear oncoprotein. Proc Natl Acad Sci U S A 1997;94:508-13.

531. Wessendorf JH, Garfinkel S, Zhan X, Brown S, Maciag T. Identification of a nuclear localization sequence within the structure of the human interleukin-1 alpha precursor. J Biol Chem 1993;268:22100-4.

532. Werman A, Werman-Venkert R, White R, Lee JK, Werman B, Krelin Y, Voronov E, Dinarello CA, Apte RN. The precursor form of IL-1alpha is an intracrine proinflammatory activator of transcription. Proc Natl Acad Sci U S A 2004;101:2434-9.

533. Keller M, Ruegg A, Werner S, Beer HD. Active caspase-1 is a regulator of unconventional protein secretion. Cell 2008;132:818-31.

534. Prudovsky I, Mandinova A, Soldi R, Bagala C, Graziani I, Landriscina M, Tarantini F, Duarte M, Bellum S, Doherty H, Maciag T. The non-classical export routes: FGF1 and IL-1alpha point the way. J Cell Sci 2003;116:4871-81.

535. Cogswell JP, Godlevski MM, Wisely GB, Clay WC, Leesnitzer LM, Ways JP, Gray JG. NF-kappa B regulates IL-1 beta transcription through a consensus NF-kappa B binding site and a nonconsensus CRE-like site. J Immunol 1994;153:712-23.

536. Kimura H, Inukai Y, Takii T, Furutani Y, Shibata Y, Hayashi H, Sakurada S, Okamoto T, Inoue J, Oomoto Y, Onozaki K. Molecular analysis of constitutive IL-1alpha gene expression in human melanoma cells: autocrine stimulation through NF-kappaB activation by endogenous IL-1alpha. Cytokine 1998;10:872-9.

537. Wagner EF, Nebreda AR. Signal integration by JNK and p38 MAPK pathways in cancer development. Nat Rev Cancer 2009;9:537-49.

538. Lopez-Bergami P, Huang C, Goydos JS, Yip D, Bar-Eli M, Herlyn M, Smalley KS, Mahale A, Eroshkin A, Aaronson S, Ronai Z. Rewired ERK-JNK signaling pathways in melanoma. Cancer Cell 2007;11:447-60.

539. Tsao H, Goel V, Wu H, Yang G, Haluska FG. Genetic interaction between NRAS and BRAF mutations and PTEN/MMAC1 inactivation in melanoma. J Invest Dermatol 2004;122:337-41.

540. Bensi G, Mora M, Raugei G, Buonamassa DT, Rossini M, Melli M. An inducible enhancer controls the expression of the human interleukin 1 beta gene. Cell Growth Differ 1990;1:491-7.

541. Stylianou E, Saklatvala J. Interleukin-1. Int J Biochem Cell Biol 1998;30:1075-9.

542. Lopez-Bergami P, Lau E, Ronai Z. Emerging roles of ATF2 and the dynamic AP1 network in cancer. Nat Rev Cancer 2010;10:65-76.

543. Pratilas CA, Taylor BS, Ye Q, Viale A, Sander C, Solit DB, Rosen N. (V600E)BRAF is associated with disabled feedback inhibition of RAF-MEK signaling and elevated transcriptional output of the pathway. Proc Natl Acad Sci U S A 2009;106:4519-24.

544. Li JJ, Rhim JS, Schlegel R, Vousden KH, Colburn NH. Expression of dominant negative Jun inhibits elevated AP-1 and NF-kappaB transactivation and suppresses anchorage independent growth of HPV immortalized human keratinocytes. Oncogene 1998;16:2711-21.

545. Shim JH, Xiao C, Paschal AE, Bailey ST, Rao P, Hayden MS, Lee KY, Bussey C, Steckel M, Tanaka N, Yamada G, Akira S, et al. TAK1, but not TAB1 or TAB2, plays an essential role in multiple signaling pathways in vivo. Genes Dev 2005;19:2668-81.

546. Lee FS, Hagler J, Chen ZJ, Maniatis T. Activation of the IkappaB alpha kinase complex by MEKK1, a kinase of the JNK pathway. Cell 1997;88:213-22.

547. Yao J, Kim TW, Qin J, Jiang Z, Qian Y, Xiao H, Lu Y, Qian W, Gulen MF, Sizemore N, DiDonato J, Sato S, et al. Interleukin-1 (IL-1)-induced TAK1-dependent Versus MEKK3-dependent NFkappaB activation pathways bifurcate at IL-1 receptor-associated kinase modification. J Biol Chem 2007;282:6075-89.

548. Tapon N, Nagata K, Lamarche N, Hall A. A new rac target POSH is an SH3-containing scaffold protein involved in the JNK and NF-kappaB signalling pathways. Embo J 1998;17:1395-404.

549. Vale T, Ngo TT, White MA, Lipsky PE. Raf-induced transformation requires an interleukin 1 autocrine loop. Cancer Res 2001;61:602-7.

550. Castelli C, Sensi M, Lupetti R, Mortarini R, Panceri P, Anichini A, Parmiani G. Expression of interleukin 1 alpha, interleukin 6, and tumor necrosis factor alpha genes in human melanoma clones is associated with that of mutated N-RAS oncogene. Cancer Res 1994;54:4785-90.

551. Fujioka S, Niu J, Schmidt C, Sclabas GM, Peng B, Uwagawa T, Li Z, Evans DB, Abbruzzese JL, Chiao PJ. NF-kappaB and AP-1 connection: mechanism of NF-kappaB-dependent regulation of AP-1 activity. Mol Cell Biol 2004;24:7806-19.

552. Hu CD, Chinenov Y, Kerppola TK. Visualization of interactions among bZIP and Rel family proteins in living cells using bimolecular fluorescence complementation. Mol Cell 2002;9:789-98.

553. Stein B, Baldwin AS, Jr., Ballard DW, Greene WC, Angel P, Herrlich P. Cross-coupling of the NF-kappa B p65 and Fos/Jun transcription factors produces potentiated biological function. Embo J 1993;12:3879-91.

554. Wolter S, Doerrie A, Weber A, Schneider H, Hoffmann E, von der Ohe J, Bakiri L, Wagner EF, Resch K, Kracht M. c-Jun controls histone modifications, NF-kappaB recruitment, and RNA polymerase II function to activate the ccl2 gene. Mol Cell Biol 2008;28:4407-23.

555. van Rietschoten JG, Verzijlbergen KF, Gringhuis SI, van der Pouw Kraan TC, Bayley JP, Wierenga EA, Jones PA, Kooter JM, Verweij CL. Differentially methylated alleles in a distinct region of the human interleukin-1alpha promoter are associated with allele-specific expression of IL-1alpha in CD4+ T cells. Blood 2006;108:2143-9.

556. Hashimoto K, Oreffo RO, Gibson MB, Goldring MB, Roach HI. DNA demethylation at specific CpG sites in the IL1B promoter in response to inflammatory cytokines in human articular chondrocytes. Arthritis Rheum 2009;60:3303-13.

557. Enya K, Hayashi H, Takii T, Ohoka N, Kanata S, Okamoto T, Onozaki K. The interaction with Sp1 and reduction in the activity of histone deacetylase 1 are critical for the constitutive gene expression of IL-1 alpha in human melanoma cells. J Leukoc Biol 2008;83:190-9.

558. Elzinga BM, Twomey C, Powell JC, Harte F, McCarthy JV. Interleukin-1 receptor type 1 is a substrate for gamma-secretase-dependent regulated intramembrane proteolysis. J Biol Chem 2009;284:1394-409.

559. Leeman JR, Gilmore TD. Alternative splicing in the NF-kappaB signaling pathway. Gene 2008;423:97-107.

560. Sun SC. CYLD: a tumor suppressor deubiquitinase regulating NF-kappaB activation and diverse biological processes. Cell Death Differ 2010;17:25-34.

561. Hymowitz SG, Wertz IE. A20: from ubiquitin editing to tumour suppression. Nat Rev Cancer 2010;10:332-41.

562. Arend WP. Interleukin-1 receptor antagonist: discovery, structure and properties. Prog Growth Factor Res 1990;2:193-205.

563. Mitchell MS, Kan-Mitchell J, Minev B, Edman C, Deans RJ. A novel melanoma gene (MG50) encoding the interleukin 1 receptor antagonist and six epitopes recognized by human cytolytic T lymphocytes. Cancer Res 2000;60:6448-56.

564. Dinarello CA. Why not treat human cancer with interleukin-1 blockade? Cancer Metastasis Rev 2010;29:317-29.

565. Vamvakopoulos J, Green C, Metcalfe S. Genetic control of IL-1beta bioactivity through differential regulation of the IL-1 receptor antagonist. Eur J Immunol 2002;32:2988-96.

566. Bondeson J, Browne KA, Brennan FM, Foxwell BM, Feldmann M. Selective regulation of cytokine induction by adenoviral gene transfer of IkappaBalpha into human macrophages: lipopolysaccharide-induced, but not zymosan-induced, proinflammatory cytokines are inhibited, but IL-10 is nuclear factor-kappaB independent. J Immunol 1999;162:2939-45.

567. Rot A. In situ binding assay for studying chemokine interactions with endothelial cells. J Immunol Methods 2003;273:63-71.

568. Lacreusette A, Nguyen JM, Pandolfino MC, Khammari A, Dreno B, Jacques Y, Godard A, Blanchard F. Loss of oncostatin M receptor beta in metastatic melanoma cells. Oncogene 2007;26:881-92.

569. Li Q, Zhang H, Chen Q, Quan N. Existence of seven human IL-1R1 promoters. J Inflamm Res 2010;2010:17-24.

570. Hoffmann E, Ashouri J, Wolter S, Doerrie A, Dittrich-Breiholz O, Schneider H, Wagner EF, Troppmair J, Mackman N, Kracht M. Transcriptional regulation of EGR-1 by the interleukin-1-JNK-MKK7-c-Jun pathway. J Biol Chem 2008;283:12120-8.

571. Hazzalin CA, Mahadevan LC. MAPK-regulated transcription: a continuously variable gene switch? Nat Rev Mol Cell Biol 2002;3:30-40.

572. Charital YM, van Haasteren G, Massiha A, Schlegel W, Fujita T. A functional NF-kappaB enhancer element in the first intron contributes to the control of c-fos transcription. Gene 2009;430:116-22.

573. Shah M, Bhoumik A, Goel V, Dewing A, Breitwieser W, Kluger H, Krajewski S, Krajewska M, Dehart J, Lau E, Kallenberg DM, Jeong H, et al. A role for ATF2 in regulating MITF and melanoma development. PLoS Genet 2010;6:e1001258.

574. Bertolotto C, Bille K, Ortonne JP, Ballotti R. In B16 melanoma cells, the inhibition of melanogenesis by TPA results from PKC activation and diminution of microphthalmia binding to the M-box of the tyrosinase promoter. Oncogene 1998;16:1665-70.

575. Guberman AS, Scassa ME, Giono LE, Varone CL, Canepa ET. Inhibitory effect of AP-1 complex on 5-aminolevulinate synthase gene expression through sequestration of cAMP-response element protein (CRE)-binding protein (CBP) coactivator. J Biol Chem 2003;278:2317-26.

576. Manna PR, Stocco DM. Crosstalk of CREB and Fos/Jun on a single cis-element: transcriptional repression of the steroidogenic acute regulatory protein gene. J Mol Endocrinol 2007;39:261-77.

577. Yang S, Meyskens FL, Jr. Alterations in activating protein 1 composition correlate with phenotypic differentiation changes induced by resveratrol in human melanoma. Mol Pharmacol 2005;67:298-308.

578. Sitcheran R, Cogswell PC, Baldwin AS, Jr. NF-kappaB mediates inhibition of mesenchymal cell differentiation through a posttranscriptional gene silencing mechanism. Genes Dev 2003;17:2368-73.

579. Kobi D, Steunou AL, Dembele D, Legras S, Larue L, Nieto L, Davidson I. Genome-wide analysis of POU3F2/BRN2 promoter occupancy in human melanoma cells reveals Kitl as a novel regulated target gene. Pigment Cell Melanoma Res 2010;23:404-18.

580. Sunabori T, Tokunaga A, Nagai T, Sawamoto K, Okabe M, Miyawaki A, Matsuzaki Y, Miyata T, Okano H. Cell-cycle-specific nestin expression coordinates with morphological changes in embryonic cortical neural progenitors. J Cell Sci 2008;121:1204-12.

581. Miller PJ, Dietz KN, Hollenbach AD. Identification of serine 205 as a site of phosphorylation on Pax3 in proliferating but not differentiating primary myoblasts. Protein Sci 2008;17:1979-86.

582. Lai IL, Lin TP, Yao YL, Lin CY, Hsieh MJ, Yang WM. Histone deacetylase 10 relieves repression on the melanogenic program by maintaining the deacetylation status of repressors. J Biol Chem 2010;285:7187-96.

583. Lopez-Bergami P, Habelhah H, Bhoumik A, Zhang W, Wang LH, Ronai Z. RACK1 mediates activation of JNK by protein kinase C [corrected]. Mol Cell 2005;19:309-20.

584. McDonald SL, Silver A. The opposing roles of Wnt-5a in cancer. Br J Cancer 2009;101:209-14.

585. Lee H, Herrmann A, Deng JH, Kujawski M, Niu G, Li Z, Forman S, Jove R, Pardoll DM, Yu H. Persistently activated Stat3 maintains constitutive NF-kappaB activity in tumors. Cancer Cell 2009;15:283-93.

586. Kamaraju AK, Bertolotto C, Chebath J, Revel M. Pax3 down-regulation and shut-off of melanogenesis in melanoma B16/F10.9 by interleukin-6 receptor signaling. J Biol Chem 2002;277:15132-41.

587. Papantonis A, Cook PR. Genome architecture and the role of transcription. Curr Opin Cell Biol 2010;22:271-6.

588. Apostolou E, Thanos D. Virus Infection Induces NF-kappaB-dependent interchromosomal associations mediating monoallelic IFN-beta gene expression. Cell 2008;134:85-96.

589. Nolis IK, McKay DJ, Mantouvalou E, Lomvardas S, Merika M, Thanos D. Transcription factors mediate long-range enhancer-promoter interactions. Proc Natl Acad Sci U S A 2009;106:20222-7.

590. Dekker J, Rippe K, Dekker M, Kleckner N. Capturing chromosome conformation. Science 2002;295:1306-11.

591. Simonis M, Klous P, Splinter E, Moshkin Y, Willemsen R, de Wit E, van Steensel B, de Laat W. Nuclear organization of active and inactive chromatin domains uncovered by chromosome conformation capture-on-chip (4C). Nat Genet 2006;38:1348-54.

592. Ceppi M, Pereira PM, Dunand-Sauthier I, Barras E, Reith W, Santos MA, Pierre P. MicroRNA-155 modulates the interleukin-1 signaling pathway in activated human monocyte-derived dendritic cells. Proc Natl Acad Sci U S A 2009;106:2735-40.

593. Yin Q, Wang X, McBride J, Fewell C, Flemington E. B-cell receptor activation induces BIC/miR-155 expression through a conserved AP-1 element. J Biol Chem 2008;283:2654-62.

594. Gatto G, Rossi A, Rossi D, Kroening S, Bonatti S, Mallardo M. Epstein-Barr virus latent membrane protein 1 trans-activates miR-155 transcription through the NF-kappaB pathway. Nucleic Acids Res 2008;36:6608-19.

595. O'Connell RM, Chaudhuri AA, Rao DS, Baltimore D. Inositol phosphatase SHIP1 is a primary target of miR-155. Proc Natl Acad Sci U S A 2009;106:7113-8.

596. Mann M, Barad O, Agami R, Geiger B, Hornstein E. miRNA-based mechanism for the commitment of multipotent progenitors to a single cellular fate. Proc Natl Acad Sci U S A 2010;107:15804-9.

597. Yeh CH, Hung LY, Hsu C, Le SY, Lee PT, Liao WL, Lin YT, Chang WC, Tseng JT. RNA-binding protein HuR interacts with thrombomodulin 5'untranslated region and represses internal ribosome entry site-mediated translation under IL-1 beta treatment. Mol Biol Cell 2008;19:3812-22.

598. Mayr C, Bartel DP. Widespread shortening of 3'UTRs by alternative cleavage and polyadenylation activates oncogenes in cancer cells. Cell 2009;138:673-84.

599. Dawson MA, Bannister AJ, Gottgens B, Foster SD, Bartke T, Green AR, Kouzarides T. JAK2 phosphorylates histone H3Y41 and excludes HP1alpha from chromatin. Nature 2009;461:819-22.

600. Chang Q, Zhang Y, Beezhold KJ, Bhatia D, Zhao H, Chen J, Castranova V, Shi X, Chen F. Sustained JNK1 activation is associated with altered histone H3 methylations in human liver cancer. J Hepatol 2009;50:323-33.

601. Lee KY, Ito K, Hayashi R, Jazrawi EP, Barnes PJ, Adcock IM. NF-kappaB and activator protein 1 response elements and the role of histone modifications in IL-1beta-induced TGF-beta1 gene transcription. J Immunol 2006;176:603-15.

602. Ndlovu N, Van Lint C, Van Wesemael K, Callebert P, Chalbos D, Haegeman G, Vanden Berghe W. Hyperactivated NF-{kappa}B and AP-1 transcription factors promote highly accessible chromatin and constitutive transcription across the interleukin-6 gene promoter in metastatic breast cancer cells. Mol Cell Biol 2009;29:5488-504.

603. De Santa F, Totaro MG, Prosperini E, Notarbartolo S, Testa G, Natoli G. The histone H3 lysine-27 demethylase Jmjd3 links inflammation to inhibition of polycomb-mediated gene silencing. Cell 2007;130:1083-94.

604. Kondo S, Sauder DN, Kono T, Galley KA, McKenzie RC. Differential modulation of interleukin-1 alpha (IL-1 alpha) and interleukin-1 beta (IL-1 beta) in human epidermal keratinocytes by UVB. Exp Dermatol 1994;3:29-39.

605. Feldmeyer L, Keller M, Niklaus G, Hohl D, Werner S, Beer HD. The inflammasome mediates UVB-induced activation and secretion of interleukin-1beta by keratinocytes. Curr Biol 2007;17:1140-5.

606. Ghiringhelli F, Apetoh L, Tesniere A, Aymeric L, Ma Y, Ortiz C, Vermaelen K, Panaretakis T, Mignot G, Ullrich E, Perfettini JL, Schlemmer F, et al. Activation of the NLRP3 inflammasome in dendritic cells induces IL-1beta-dependent adaptive immunity against tumors. Nat Med 2009;15:1170-8.

607. Cohen I, Rider P, Carmi Y, Braiman A, Dotan S, White MR, Voronov E, Martin MU, Dinarello CA, Apte RN. Differential release of chromatin-bound IL-1alpha discriminates between necrotic and apoptotic cell death by the ability to induce sterile inflammation. Proc Natl Acad Sci U S A 2010;107:2574-9.

608. Ben-Sasson SZ, Hu-Li J, Quiel J, Cauchetaux S, Ratner M, Shapira I, Dinarello CA, Paul WE. IL-1 acts directly on CD4 T cells to enhance their antigen-driven expansion and differentiation. Proc Natl Acad Sci U S A 2009;106:7119-24.

609. Bunt SK, Sinha P, Clements VK, Leips J, Ostrand-Rosenberg S. Inflammation induces myeloid-derived suppressor cells that facilitate tumor progression. J Immunol 2006;176:284-90.

610. Song X, Krelin Y, Dvorkin T, Bjorkdahl O, Segal S, Dinarello CA, Voronov E, Apte RN. CD11b+/Gr-1+ immature myeloid cells mediate suppression of T cells in mice bearing tumors of IL-1beta-secreting cells. J Immunol 2005;175:8200-8.

611. Ostrand-Rosenberg S. Myeloid-derived suppressor cells: more mechanisms for inhibiting antitumor immunity. Cancer Immunol Immunother 2010;59:1593-600.

612. Poschke I, Mougiakakos D, Hansson J, Masucci GV, Kiessling R. Immature immunosuppressive CD14+HLA-DR-/low cells in melanoma patients are Stat3hi and overexpress CD80, CD83, and DC-sign. Cancer Res 2010;70:4335-45.

613. Muller AJ, Sharma MD, Chandler PR, Duhadaway JB, Everhart ME, Johnson BA, 3rd, Kahler DJ, Pihkala J, Soler AP, Munn DH, Prendergast GC, Mellor AL. Chronic inflammation that facilitates tumor progression creates local immune suppression by inducing indoleamine 2,3 dioxygenase. Proc Natl Acad Sci U S A 2008;105:17073-8.

614. Chirivi RG, Chiodoni C, Musiani P, Garofalo A, Bernasconi S, Colombo MP, Giavazzi R. IL-1alpha gene-transfected human melanoma cells increase tumor-cell adhesion to endothelial cells and their retention in the lung of nude mice. Int J Cancer 1996;67:856-63.

615. Carmi Y, Voronov E, Dotan S, Lahat N, Rahat MA, Fogel M, Huszar M, White MR, Dinarello CA, Apte RN. The role of macrophage-derived IL-1 in induction and maintenance of angiogenesis. J Immunol 2009;183:4705-14.

616. Mancino A, Lawrence T. Nuclear factor-kappaB and tumor-associated macrophages. Clin Cancer Res 2010;16:784-9.

617. Balkwill F. Tumour necrosis factor and cancer. Nat Rev Cancer 2009;9:361-71.

618. Thomas NE, Alexander A, Edmiston SN, Parrish E, Millikan RC, Berwick M, Groben P, Ollila DW, Mattingly D, Conway K. Tandem BRAF mutations in primary invasive melanomas. J Invest Dermatol 2004;122:1245-50.

619. Giavazzi R, Garofalo A, Bani MR, Abbate M, Ghezzi P, Boraschi D, Mantovani A, Dejana E. Interleukin 1-induced augmentation of experimental metastases from a human melanoma in nude mice. Cancer Res 1990;50:4771-5.

620. Ozanne BW, Spence HJ, McGarry LC, Hennigan RF. Transcription factors control invasion: AP-1 the first among equals. Oncogene 2007;26:1-10.

621. Bancroft CC, Chen Z, Dong G, Sunwoo JB, Yeh N, Park C, Van Waes C. Coexpression of proangiogenic factors IL-8 and VEGF by human head and neck squamous cell carcinoma involves coactivation by MEK-MAPK and IKK-NF-kappaB signal pathways. Clin Cancer Res 2001;7:435-42.

622. Lev DC, Ruiz M, Mills L, McGary EC, Price JE, Bar-Eli M. Dacarbazine causes transcriptional up-regulation of interleukin 8 and vascular endothelial growth factor in melanoma cells: a possible escape mechanism from chemotherapy. Mol Cancer Ther 2003;2:753-63.

623. Patel PS, Varney ML, Dave BJ, Singh RK. Regulation of constitutive and induced NF-kappaB activation in malignant melanoma cells by capsaicin modulates interleukin-8 production and cell proliferation. J Interferon Cytokine Res 2002;22:427-35.

624. Chong JM, Sakuma K, Sudo M, Osawa T, Ohara E, Uozaki H, Shibahara J, Kuroiwa K, Tominaga S, Hippo Y, Aburatani H, Funata N, et al. Interleukin-1beta expression in human gastric carcinoma with Epstein-Barr virus infection. J Virol 2002;76:6825-31.

625. Motsch N, Pfuhl T, Mrazek J, Barth S, Grasser FA. Epstein-Barr virus-encoded latent membrane protein 1 (LMP1) induces the expression of the cellular microRNA miR-146a. RNA Biol 2007;4:131-7.

626. Pinner S, Jordan P, Sharrock K, Bazley L, Collinson L, Marais R, Bonvin E, Goding C, Sahai E. Intravital imaging reveals transient changes in pigment production and Brn2 expression during metastatic melanoma dissemination. Cancer Res 2009;69:7969-77.

627. Lu T, Tian L, Han Y, Vogelbaum M, Stark GR. Dose-dependent cross-talk between the transforming growth factor-beta and interleukin-1 signaling pathways. Proc Natl Acad Sci U S A 2007;104:4365-70.

628. Lengagne R, Graff-Dubois S, Garcette M, Renia L, Kato M, Guillet JG, Engelhard VH, Avril MF, Abastado JP, Prevost-Blondel A. Distinct role for CD8 T cells toward cutaneous tumors and visceral metastases. J Immunol 2008;180:130-7.

629. Eyles J, Puaux AL, Wang X, Toh B, Prakash C, Hong M, Tan TG, Zheng L, Ong LC, Jin Y, Kato M, Prevost-Blondel A, et al. Tumor cells disseminate early, but immunosurveillance limits metastatic outgrowth, in a mouse model of melanoma. J Clin Invest 2010;120:2030-9.

630. Yee C, Thompson JA, Byrd D, Riddell SR, Roche P, Celis E, Greenberg PD. Adoptive T cell therapy using antigen-specific CD8+ T cell clones for the treatment of patients with metastatic melanoma: in vivo persistence, migration, and antitumor effect of transferred T cells. Proc Natl Acad Sci U S A 2002;99:16168-73.

631. Voelter V, Pica A, Laurent J, Rimoldi D, Bouzourene H, Sajadi A, Matter M, Romero P, Rufer N, Speiser DE. An unusual case of metastatic melanoma sensitive to chemotherapy and immunotherapy, with late immune escape in the brain. Cancer Immun 2008;8:6.

632. Khong HT, Wang QJ, Rosenberg SA. Identification of multiple antigens recognized by tumor-infiltrating lymphocytes from a single patient: tumor escape by antigen loss and loss of MHC expression. J Immunother 2004;27:184-90.

633. Seeger JM, Schmidt P, Brinkmann K, Hombach AA, Coutelle O, Zigrino P, Wagner-Stippich D, Mauch C, Abken H, Kronke M, Kashkar H. The proteasome inhibitor bortezomib sensitizes melanoma cells toward adoptive CTL attack. Cancer Res 2010;70:1825-34.

634. Swope VB, Abdel-Malek Z, Kassem LM, Nordlund JJ. Interleukins 1 alpha and 6 and tumor necrosis factor-alpha are paracrine inhibitors of human melanocyte proliferation and melanogenesis. J Invest Dermatol 1991;96:180-5.

635. Haycock JW, Wagner M, Morandini R, Ghanem G, Rennie IG, Mac Neil S. Alpha-melanocyte-stimulating hormone inhibits NF-kappaB activation in human melanocytes and melanoma cells. J Invest Dermatol 1999;113:560-6.

636. Tidyman WE, Rauen KA. The RASopathies: developmental syndromes of Ras/MAPK pathway dysregulation. Curr Opin Genet Dev 2009;19:230-6.

637. Roberts A, Allanson J, Jadico SK, Kavamura MI, Noonan J, Opitz JM, Young T, Neri G. The cardiofaciocutaneous syndrome. J Med Genet 2006;43:833-42.

638. Sarkozy A, Digilio MC, Dallapiccola B. Leopard syndrome. Orphanet J Rare Dis 2008;3:13.

639. Courtois G, Gilmore TD. Mutations in the NF-kappaB signaling pathway: implications for human disease. Oncogene 2006;25:6831-43.

640. Karpf AR. A potential role for epigenetic modulatory drugs in the enhancement of cancer/germ-line antigen vaccine efficacy. Epigenetics 2006;1:116-20.

641. McCormack SE, Warlick ED. Epigenetic approaches in the treatment of myelodysplastic syndromes: clinical utility of azacitidine. Onco Targets Ther 2010;3:157-65.

642. Tan J, Cang S, Ma Y, Petrillo RL, Liu D. Novel histone deacetylase inhibitors in clinical trials as anti-cancer agents. J Hematol Oncol 2010;3:5.

643. Goodyear O, Agathanggelou A, Novitzky-Basso I, Siddique S, McSkeane T, Ryan G, Vyas P, Cavenagh J, Stankovic T, Moss P, Craddock C. Induction of a CD8+ T-cell response to the MAGE cancer testis antigen by combined treatment with azacitidine and sodium valproate in patients with acute myeloid leukemia and myelodysplasia. Blood 2010;116:1908-18.

644. Chik F, Szyf M. Effects of specific DNMT-gene depletion on cancer cell transformation and breast cancer cell invasion; towards selective DNMT inhibitors. Carcinogenesis.

645. Villagra A, Sotomayor EM, Seto E. Histone deacetylases and the immunological network: implications in cancer and inflammation. Oncogene 2010;29:157-73.

646. Yokoyama S, Feige E, Poling LL, Levy C, Widlund HR, Khaled M, Kung AL, Fisher DE. Pharmacologic suppression of MITF expression via HDAC inhibitors in the melanocyte lineage. Pigment Cell Melanoma Res 2008;21:457-63.

647. Boyle GM, Martyn AC, Parsons PG. Histone deacetylase inhibitors and malignant melanoma. Pigment Cell Res 2005;18:160-6.

648. Haggerty TJ, Dunn IS, Rose LB, Newton EE, Martin S, Riley JL, Kurnick JT. Topoisomerase inhibitors modulate expression of melanocytic antigens and enhance T cell recognition of tumor cells. Cancer Immunol Immunother 2010.

649. Boni A, Cogdill AP, Dang P, Udayakumar D, Njauw CN, Sloss CM, Ferrone CR, Flaherty KT, Lawrence DP, Fisher DE, Tsao H, Wargo JA. Selective BRAFV600E inhibition enhances T-cell recognition of melanoma without affecting lymphocyte function. Cancer Res 2010;70:5213-9.

650. Heidorn SJ, Milagre C, Whittaker S, Nourry A, Niculescu-Duvas I, Dhomen N, Hussain J, Reis-Filho JS, Springer CJ, Pritchard C, Marais R. Kinase-dead BRAF and oncogenic RAS cooperate to drive tumor progression through CRAF. Cell 2010;140:209-21.

651. Gray-Schopfer VC, Karasarides M, Hayward R, Marais R. Tumor necrosis factor-alpha blocks apoptosis in melanoma cells when BRAF signaling is inhibited. Cancer Res 2007;67:122-9.

652. Nakanishi C, Toi M. Nuclear factor-kappaB inhibitors as sensitizers to anticancer drugs. Nat Rev Cancer 2005;5:297-309.

653. Baud V, Karin M. Is NF-kappaB a good target for cancer therapy? Hopes and pitfalls. Nat Rev Drug Discov 2009;8:33-40.

654. Schon M, Wienrich BG, Kneitz S, Sennefelder H, Amschler K, Vohringer V, Weber O, Stiewe T, Ziegelbauer K, Schon MP. KINK-1, a novel small-molecule inhibitor of IKKbeta, and the susceptibility of melanoma cells to antitumoral treatment. J Natl Cancer Inst 2008;100:862-75.

655. Yan Liu X, Robinson D, Veach RA, Liu D, Timmons S, Collins RD, Hawiger J. Peptide-directed suppression of a pro-inflammatory cytokine response. J Biol Chem 2000;275:16774-8.

656. Eckmann L, Nebelsiek T, Fingerle AA, Dann SM, Mages J, Lang R, Robine S, Kagnoff MF, Schmid RM, Karin M, Arkan MC, Greten FR. Opposing functions of IKKbeta during acute and chronic intestinal inflammation. Proc Natl Acad Sci U S A 2008;105:15058-63.

657. Zhou H, Liu Y, Cheung LH, Kim S, Zhang W, Mohamedali KA, Anand P, Hittelman WN, Aggarwal BB, Rosenblum MG. Characterization and mechanistic studies of a novel melanoma-targeting construct containing IkappaBa for specific inhibition of nuclear factor-kappaB activity. Neoplasia 2010;12:766-77.

658. Davis ME, Zuckerman JE, Choi CH, Seligson D, Tolcher A, Alabi CA, Yen Y, Heidel JD, Ribas A. Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles. Nature 2010;464:1067-70.

659. Tas SW, Vervoordeldonk MJ, Tak PP. Gene therapy targeting nuclear factor-kappaB: towards clinical application in inflammatory diseases and cancer. Curr Gene Ther 2009;9:160-70.

660. Gao YJ, Cheng JK, Zeng Q, Xu ZZ, Decosterd I, Xu X, Ji RR. Selective inhibition of JNK with a peptide inhibitor attenuates pain hypersensitivity and tumor growth in a mouse skin cancer pain model. Exp Neurol 2009;219:146-55.

661. Conze D, Krahl T, Kennedy N, Weiss L, Lumsden J, Hess P, Flavell RA, Le Gros G, Davis RJ, Rincon M. c-Jun NH(2)-terminal kinase (JNK)1 and JNK2 have distinct roles in CD8(+) T cell activation. J Exp Med 2002;195:811-23.

662. Norell H, Martins da Palma T, Lesher A, Kaur N, Mehrotra M, Naga OS, Spivey N, Olafimihan S, Chakraborty NG, Voelkel-Johnson C, Nishimura MI, Mukherji B, et al. Inhibition of superoxide generation upon T-cell receptor engagement rescues Mart-1(27-35)-reactive T cells from activation-induced cell death. Cancer Res 2009;69:6282-9.

663. Lachmann HJ, Kone-Paut I, Kuemmerle-Deschner JB, Leslie KS, Hachulla E, Quartier P, Gitton X, Widmer A, Patel N, Hawkins PN. Use of canakinumab in the cryopyrin-associated periodic syndrome. N Engl J Med 2009;360:2416-25.

664. Loser K, Brzoska T, Oji V, Auriemma M, Voskort M, Kupas V, Klenner L, Mensing C, Hauschild A, Beissert S, Luger TA. The neuropeptide alpha-melanocyte-stimulating hormone is critically involved in the development of cytotoxic CD8+ T cells in mice and humans. PLoS One 2010;5:e8958.

665. Eves PC, MacNeil S, Haycock JW. alpha-Melanocyte stimulating hormone, inflammation and human melanoma. Peptides 2006;27:444-52.

666. Mastrofrancesco A, Kokot A, Eberle A, Gibbons NC, Schallreuter KU, Strozyk E, Picardo M, Zouboulis CC, Luger TA, Bohm M. KdPT, a tripeptide derivative of alpha-melanocyte-stimulating hormone, suppresses IL-1 beta-mediated cytokine expression and signaling in human sebocytes. J Immunol 2010;185:1903-11.

667. Gu F, Qureshi AA, Niu T, Kraft P, Guo Q, Hunter DJ, Han J. Interleukin and interleukin receptor gene polymorphisms and susceptibility to melanoma. Melanoma Res 2008;18:330-5.

668. Brennan DJ, O'Connor DP, Rexhepaj E, Ponten F, Gallagher WM. Antibody-based proteomics: fast-tracking molecular diagnostics in oncology. Nat Rev Cancer 2010;10:605-17.

669. Brase JC, Mannsperger H, Frohlich H, Gade S, Schmidt C, Wiemann S, Beissbarth T, Schlomm T, Sultmann H, Korf U. Increasing the sensitivity of reverse phase protein arrays by antibody-mediated signal amplification. Proteome Sci 2010;8:36.

670. Charboneau L, Tory H, Chen T, Winters M, Petricoin EF, 3rd, Liotta LA, Paweletz CP. Utility of reverse phase protein arrays: applications to signalling pathways and human body arrays. Brief Funct Genomic Proteomic 2002;1:305-15.

671. Irish JM, Kotecha N, Nolan GP. Mapping normal and cancer cell signalling networks: towards single-cell proteomics. Nat Rev Cancer 2006;6:146-55.

672. Van Criekingen M. La rénovation résidentielle à Montréal et à Bruxelles. Dynamiques, impacts sociaux et rôle des pouvoirs publics. Thèse de doctorat en Sciences Géographiques, Université Libre de Bruxelles, 2001.

ANNEX

Published online August 17, 2004

4340–4350 *Nucleic Acids Research, 2004, Vol. 32, No. 14*
doi:10.1093/nar/gkh735

MAGE-A1 interacts with adaptor SKIP and the deacetylase HDAC1 to repress transcription

Sandra Laduron, Rachel Deplus[1], Sifang Zhou[2], Olga Kholmanskikh, Danièle Godelaine, Charles De Smet, S. Diane Hayward[2], François Fuks[1], Thierry Boon and Etienne De Plaen*

Ludwig Institute for Cancer Research, Brussels branch, and Cellular Genetics Unit, Université Catholique de Louvain, Brussels B1200, Belgium, [1]Laboratory of Molecular Virology, C.P. 614, Faculty of Medicine, Free University of Brussels, B1070, Belgium and [2]Department of Pharmacology and Molecular Science, Johns Hopkins University School of Medicine, Baltimore, MD 21231, USA

Received as resubmission June 27, 2004; Accepted July 12, 2004

ABSTRACT

MAGE-A1 **belongs to a family of 12 genes that are active in various types of tumors and silent in normal tissues except in male germ-line cells. The *MAGE*-encoded antigens recognized by T cells are highly tumor-specific targets for T cell-oriented cancer immunotherapy. The function of MAGE-A1 is currently unknown. To analyze it, we attempted to identify protein partners of MAGE-A1. Using yeast two-hybrid screening, we detected an interaction between MAGE-A1 and Ski Interacting Protein (SKIP). SKIP is a transcriptional regulator that connects DNA-binding proteins to proteins that either activate or repress transcription. We show that MAGE-A1 inhibits the activity of a SKIP-interacting transactivator, namely the intracellular part of Notch1. Deletion analysis indicated that this inhibition requires the binding of MAGE-A1 to SKIP. Moreover, MAGE-A1 was found to actively repress transcription by binding and recruiting histone deacetylase 1 (HDAC1). Our results indicate that by binding to SKIP and by recruiting HDACs, MAGE-A1 can act as a potent transcriptional repressor. MAGE-A1 could therefore participate in the setting of specific gene expression patterns for tumor cell growth or spermatogenesis.**

INTRODUCTION

The gene *MAGE-A1* was identified because it encodes a human melanoma antigen recognized by cytolytic T lymphocytes (1). It belongs to a family of 12 genes, the *MAGE-A* cluster located on the X chromosome in region q28 (2). The *MAGE-A* genes are expressed in tumors of various histological types (1,3,4), but they are silent in normal adult tissues except in male germ-line cells (5–7).

A search for the gene responsible for the sex-reversal phenotype led to the identification of a second cluster of *MAGE* genes, *MAGE-B*, located in Xp21.3 (8–11). A third cluster, *MAGE-C*, was identified and located in Xq26–27 (11,12). The expression pattern of the *MAGE-B* and *C* genes is similar to that of the *MAGE-A* genes.

In contrast to the *MAGE-A*, *B* and *C* genes, two genes constituting a distantly related fourth cluster, *MAGE-D*, were found to be expressed in many normal tissues (13,14). Additional *MAGE* genes families with such a ubiquitous expression pattern, *MAGE-E* to *L*, were identified through database screening (15).

By comparing the sequences of all the MAGE proteins, we identified a stretch of ~200 amino acids which was named the 'MAGE conserved domain' (15). The rest of the protein sequences vary widely from one family to another.

The function of protein MAGE-D1 has been partially elucidated. The rat protein NRAGE (neurotrophin receptor-interacting MAGE homolog), the ortholog of human MAGE-D1 and mouse Dlxin-1, was found to bind to the cytosolic domain of the p75 neurotrophin receptor (p75NTR) (16). Upon binding, NRAGE prevents the formation of a complex between p75NTR and tyrosine kinase receptor TrkA. This retards cell cycle progression and initiates an apoptotic cascade leading to neuronal cell death (16,17). In agreement with these data, NRAGE and p75NTR are co-expressed in a region of the developing nervous system where p75NTR mediates developmental apoptosis. In addition, NRAGE was found recently to induce apoptosis through interaction with the axon guidance receptor, UNC5H1 (18). On the other hand, Dlxin-1, the murine ortholog of NRAGE, exerts a function in the nucleus: it acts as a transcriptional activator of Dlx5, a homeodomain protein of the Dlx family that plays a critical role in skeletal development (19). This transcriptional function may be regulated by the tyrosine kinase receptor, Ror2 since this receptor appeared capable of sequestering Dlxin-1 at the plasma membrane and endoplasmic reticulum (20).

Computer search of the Protein Sequence Database revealed that MAGE proteins display a weak similarity with necdin because it also contains a 'MAGE conserved domain'. The murine necdin gene (*Ndn*) is highly expressed in post-mitotic neurons in the brain stem and hypothalamus (21,22). Mice deficient in necdin show early postnatal lethality due to respiratory distress and hypotonia, as observed in Prader–Willi syndrome (23–25). Necdin seems to act as a neuron-specific

*To whom correspondence should be addressed at Ludwig Institute for Cancer Research, 74 Avenue Hippocrate-UCL 74.59, B1200 Brussels, Belgium. Tel: +322 764 7479; Fax: +322 762 9405; Email: Etienne.Deplaen@bru.licr.org

 © Oxford University Press 2004; all rights reserved

post-mitotic growth suppressor, functionally similar to the retinoblastoma suppressor protein, Rb. Ectopic expression of *Ndn* suppresses proliferation of NIH/3T3 fibroblasts and colony formation of SAOS-2 osteosarcoma cells (26,27). Necdin interacts with the E2F1 and p53 transcription factors, repressing their transcriptional activity (27,28). Furthermore, necdin associates with the p75 neurotrophin receptor (29). These observations suggest that necdin induces cell cycle arrest and controls neuronal apoptosis through interactions with E2F1 and p75NTR (30).

The functions of the MAGE-A group of proteins, which are present only in tumor and germ-line cells, are largely unknown. MAGE-A4 has been identified as binding to gankyrin, an abundant protein in hepatocellular carcinomas (31). This protein has been reported to associate with Rb and to compete with p16 for binding to cyclin-dependent kinase CDK4, increasing both the phosphorylation and degradation of Rb. MAGE-A4 was shown to suppress both anchorage-independent growth *in vitro* and tumor formation of gankyrin-expressing cells in nude mice. Interaction with gankyrin was not observed for proteins MAGE-A1, MAGE-A2 and MAGE-A12 (31).

The subcellular localization of MAGE-A proteins seems to vary from one member of the family to another. MAGE-A1 and MAGE-A3 were reported to be located in the cytosol of melanoma cells (32–34). MAGE-A1 and MAGE-A4 have been detected in both the cytoplasm and the nucleus of spermatogonia (7). MAGE-A10 and MAGE-A11 have been shown to be located predominantly in the nucleus of tumor cells (35,36). MAGE-A proteins may exert different functions according to their subcellular localizations. To gain insight into MAGE-A functions, we used the yeast two-hybrid system to identify protein partners of MAGE-A1.

MATERIALS AND METHODS

Plasmids

Yeast expression plasmids. Plasmids pGBT9 and pAS2-1 encoding the Gal4(1–147) DNA-binding domain, pACT2 and pGAD424 encoding the Gal4(768–881) activation domain, pTD1 encoding SV40 large T antigen (84–708) and pVA3 encoding mouse p53 (72–390) were purchased from Clontech. The MAGE-A1 open reading frame (ORF) and truncated versions of the MAGE-A1 ORF were obtained by PCR amplification with native *Pfu* DNA polymerase (Stratagene). The PCR products were cloned in frame with the Gal4 DNA-binding domain sequence of pGBT9 or pAS2-1. The MAGE-A1 ORF was excised from pGBT9 and subcloned in frame with the Gal4 activation domain of pGAD424. A BamHI/BglII fragment carrying the Ski Interacting Protein (SKIP)-coding region was isolated from the pACT2 plasmid and ligated into the BamHI site of pGBT9 in frame with the Gal4 DNA-binding domain. The human testis cDNA library in pACT2 was purchased from Clontech.

Eukaryotic expression plasmids. Expression vectors encoding the Gal4(1–147) DNA-binding domain (GH250), Gal4 (1–147)–SKIP (JH274) and the full-length cytoplasmic domain (amino acids 1747–2531) of rat Notch1-IC (pBOS-FCDN1) were provided by Diane Hayward (Johns Hopkins University School of Medicine, Baltimore, MA) (37). The reporter plasmid 5xGal4TK-CAT was also obtained from Diane Hayward. Expression vectors for Luciferase (pTKluc) and Gal4(1–147) fused to the activation domain of HNF-6 (Gal4BD Nterm) were provided by F.Lemaigre (Hormone and Metabolic Research Unit, Université Catholique de Louvain and Institute of Cellular Pathology, Brussels). The pcDNAI/Amp expression vector carrying a complete MAGE-A1 cDNA (pcDNAI/Amp-M1) was provided by C.Lurquin (Ludwig Institute, Brussels).

The sequence encoding a 9 amino acid hemagglutinin (HA) epitope tag (YPYDVPDYA) was obtained by PCR on vector pACT2 with primers LAD 31: 5′-CGGGATCCGACTATGGCTTACCCATAC-3′ and LAD32: 5′-CGGAATTCGAGCGTAATCTGGAAC-3′. The PCR product was digested with restriction enzymes BamHI and EcoRI. An EcoRI/XhoI fragment carrying the SKIP coding region was excised from the pACT2 plasmid and ligated with HA into the BamHI/XhoI sites of pcDNAI/Amp, placing the SKIP-coding region in frame with (and downstream of) the HA epitope. MAGE-A1 ORF was excised from plasmid pGBT9 and cloned in frame with the Gal4 DNA-binding domain of GH250.

Vectors expressing deleted versions of MAGE-A1 were obtained by cloning PCR products obtained with native *Pfu* DNA polymerase in vector pcDNAI/Amp digested with HindIII and XhoI. The deleted MAGE-1 ORFs were inserted between their native 5′- and 3′-untranslated regions obtained by PCR. The deleted ORF encoding MAGE-A1$_{1–279}$ and the 5′-untranslated region were obtained by PCR on vector pcDNAI/Amp-M1 with primers LAD 108 (forward): 5′-CCCAAGCTTCCATTCTGAGGGACG-3′ and LAD14 (reverse): 5′-GCGGATCCTCAGACTTTCACATAGCTGGTTTC-3′. The 1040 bp product was digested with HindIII and BamHI and ligated to a 540 bp fragment corresponding to the 3′-untranslated region. This fragment was obtained by PCR with primers LAD 110 (forward): 5′-CGGGATCCGCATGAGTTGCAGCCA-3′ and LAD 111 (reverse): 5′-CCGCTCGAGACAGGAAGAATTCTTTA-3′ and was digested with BamHI and XhoI. The ORF encoding MAGE-A1$_{99–309}$ and the 3′-untranslated region were obtained with primers LAD 112 (forward): 5′-CGGGATCCGTCATCATGCGAGCAGTAATCA-3′ and LAD 111 (reverse). The 1170 bp product was digested with BamHI and XhoI and ligated to a 200 bp fragment corresponding to the 5′-untranslated region. This fragment was obtained by PCR with LAD 108 (forward): 5′-CCCAAGCTTCCATTCTGAGGGACG-3′ and LAD 109 (reverse): 5′-CGGGATCCTCTCGTCAGGGCAGCA-3′ and digested with HindIII and BamHI. The ORF encoding MAGE-A1$_{99–279}$ was obtained by PCR with primers LAD 112 (forward) and LAD14 (reverse). The 540 bp product was digested with BamHI and ligated to both the 200 bp fragment corresponding to the 5′-untranslated region and the 540 bp fragment corresponding to the 3′-untranslated region.

pcDNA3-HDAC1-F, pING14A-HDAC1 and the reporter construct (Gal4)$_4$–tkLuc have been described previously (38,39).

Yeast two-hybrid assays

Plasmid DNA was transformed into yeast cells using the lithium acetate method and the Clontech's protocol.

DNA of plasmid pAS2-1/MAGE-A1 was cotransformed with DNA of the testis cDNA library into yeast strain CG-1945 (Clontech). Transformed His^+ CG-1945 cells were selected onto medium lacking histidine (−His). In our conditions, 5 mM 3-aminotriazole (3-AT), a chemical inhibitor of imidazoleglycerolphosphate dehydratase (*HIS3*) which restores histidine auxotrophy, was always required in the −His medium. It completely abrogated the growth of yeast cells containing only plasmid pAS2-1/MAGE-A1. His^+ colonies were grown in 5 ml −His liquid medium to eliminate some false positives. Plasmids isolated from His^+ colonies were then transferred in Y190 yeast strain (Clontech). This strain has a higher *LacZ* reporter expression. Transformed His^+ Y190 cells were assayed for β-galactosidase with *O*-nitrophenyl β-D-galactopyranoside (ONPG) as substrate according to a Clontech's protocol. β-Galactosidase units were calculated as in the Clontech's protocol. To obtain more reproducible values in the β-galactosidase assays, plasmid DNA of His^+LacZ^+ yeast colonies was isolated and transformed with the pGBT9/MAGE-1 construct into yeast strain PJ69-4A, a kind gift of Philip James (40). This strain contains three reporter genes (*HIS3*, *ADE2* and *LacZ*), each driven by a different promoter (*GAL1*, *GAL2* or *GAL7* promoter) inducible by the Gal4 transcription factor. According to a protocol sent out by Philip James with the strain, transformed PJ69-4A cells were first selected on medium lacking histidine in the presence of 1 mM 3-AT. His^+ colonies were then plated on a medium lacking adenine to screen for the ADE2 marker. Finally, liquid cultures of His^+Ade^+ colonies were assayed for β-galactosidase.

Sequence analysis

Sequencing was performed using the dideoxy-mediated chain termination method with the BigDye Terminator Cycle sequencing kit and an ABI Prism 310 genetic analyser (Applied Biosystems).

Co-immunoprecipitations and western blot analysis

COS-7 cells seeded at 10^6 cells per 10 cm diameter culture dish were transiently transfected with 8 μg of expression plasmid and 48 μl of LIPOFECTAMIN reagent (Invitrogen). Approximately 48 h after transfection, cells were washed with phosphate-buffered saline (PBS) and lysed for 30 min on ice in 1 ml of ice-cold NP-40 lysis buffer [150 mM NaCl, 1% NP-40, 50 mM Tris–HCl, pH 8.0, and 1/50 per milliliter of a protease inhibitor cocktail tablet (Complete, Roche) in PBS]. For the co-immunoprecipitation of HA–SKIP with the truncated forms of MAGE-A1, cells were lysed in DH buffer (0.1% sodium dodecyl sulfate, 1% sodium deoxycholate, 0.5% NP-40 and 1/50 per milliliter of a protease inhibitor cocktail tablet in PBS). The cell extract was clarified by centrifugation for 10 min at 12 000 *g* at 4°C in a Sigma 1K15 microcentrifuge. The supernatant was preincubated for 3 h at 4°C with 50 μl of a protein G-agarose suspension (Roche) on a rocking platform. Beads were pelleted by centrifugation for 20 s at 12 000 *g*. Precleared lysate was incubated at 4°C for 1–2 h on a rocking platform with 5 μl of polyclonal anti-MAGE-A1 serum LBK7-2 [a goat K7 serum directed against the recombinant MAGE-A1 (1–309) protein made by Chiron]. A mixture of 5 μl of polyclonal anti-MAGE-A1 serum LBK7-2 and 100 μl of monoclonal anti-MAGE-A1 antibody supernatant 6C1 (a gift of Donata Rimoldi, LICR Lausanne) (41), was used in the co-immunoprecipitation of HA–SKIP with the truncated forms of MAGE-A1. Fifty microliters of protein G-agarose were added to the cell extract and the mixture was incubated for 3–12 h at 4°C on a rocking platform. The beads were then washed 4 to 5 times with 1 ml of DH buffer and mixed with 50 μl of gel-loading buffer. Samples (10 μl) were subjected to electrophoresis using a 10% denaturing polyacrylamide gel. For the co-immunoprecipitation of HA–SKIP with MAGE-A1, western blot analysis was performed using anti-HA high-affinity rat monoclonal antibody (1/1000) (Roche), sheep anti-rat IgG-biotin $F(ab')_2$ fragments (1/400) (Roche), streptavidin–horseradish peroxidase (HRP) conjugate (1/2500) (Amersham Pharmacia Biotech) and the ECL Western Blotting Detection Reagents (Amersham Pharmacia Biotech). Alternatively, western blot analysis was performed using the anti-MAGE-A1 6C1 hybridoma supernatant (one-half to one-fiftieth) and the goat anti-mouse IgG–HRP (1/2000) (BD Transduction Laboratories).

For the co-immunoprecipitation of histone deacetylase 1 (HDAC1) with MAGE-A1, COS cell lysates in 100 mM Tris–HCl, pH 7.4, 10% glycerol, 0.5% NP-40 and 1/50 per milliliter of a protease inhibitor cocktail tablet were incubated with 5 μl of pre-immune or 5 μl of polyclonal anti-MAGE-A1 goat serum, as described above. Immunoprecipitates were washed 4 times with 500 μl of IPH buffer (50 mM Tris–HCl, pH 8, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40 and 1/50 per milliliter of a protease inhibitor cocktail tablet). Western blots of the immunoprecipitates were analyzed either with the anti-MAGE-A1 6C1 antibody as above or with the anti-HDAC1 mouse monoclonal antibody (clone 2E10, Upstate) (1/2000), the goat anti-mouse IgG–HRP (1/2000) (Santa Cruz Biotechnology) and the Supersignal West Pico chemiluminescent substrate (Pierce).

Cell culture, transfection, CAT and luciferase assays

HeLa cells were maintained in DMEM plus 10% fetal calf serum and plated at 1.2×10^5 cells per well in 12-well plates (Nunc) 1 day prior to transfection. Cells were transfected using LIPOFECTIN and PLUS Reagent (Invitrogen). Cells received 0.25 μg of the expression plasmid encoding Gal4(1–147)-SKIP, Notch1-IC, Gal4(1–147), MAGE-A1 (intact or deleted), 0.4 μg of the 5xGal4TK-CAT reporter plasmid and 0.5 μg of pTKluc plasmid as an internal control for transfection efficiency. Plasmid DNA was purified with the Endofree Plasmid Maxi kit (Qiagen). At 48 h after transfection, cells were washed twice with PBS, lysed in 100 μl of Reporter Gene Assay Lysis Buffer (Roche) for 15 min at 4°C. Cell debris were removed by centrifugation for 2 min at maximum speed at 4°C. For luciferase assays, 40 μl of cell lysate were loaded into a luminometer TD20/20 (Promega) and reaction was started by addition of 100 μl of Luciferase Assay Reagent (Roche) and chemiluminescence was counted for 12 s. For chloramphenicol acetyltransferase (CAT) assays, 40 μl of cell lysate were incubated for 5 min at 65°C to destroy deacetylases. The test was then performed in 1.5 ml translucent, colorless, microcentrifuge tubes with the *Quan*-T-CAT assay system, as recommended by Amersham Pharmacia Biotech. Each experiment was repeated at least twice.

Transfected HeLa cells were separated into nuclear and cytosolic fractions using the Nuclear/Cytosol fractionation kit (BioVision). They were analyzed by western blotting with the following antibodies: anti-MAGE-A1 clone 6C1, anti-HDAC1 clone 2E10 (Upstate) and anti-α-Tubulin clone B-7 (Santa Cruz Biotechnology, 1/2000).

Monolayer cultures of U2OS human osteosarcoma cells were maintained in DMEM supplemented with 10% fetal calf serum at 37°C in 5% CO_2 atmosphere. Twenty-four hours after plating, the exponentially growing U2OS cells were transfected with polyethylene imine (PEI) (Euromedex). All transfections were carried out with the same total amount of DNA (1 μg). Four hours after transfection, the cells were washed once in PBS and recovered with fresh medium. After 24 h of incubation, luciferase assays were performed with the Promega luciferase Assay System according to the manufacturer's instructions. Transfection efficiencies were normalized using a cotransfected plasmid encoding β-galactosidase. For trichostatin A (TSA) treatment, U2OS cells were transfected with the $(Gal4)_4$–tkLuc reporter and a vector encoding Gal4bd/MAGE-A1, and incubated for an additional 24 h, either in the presence or absence of TSA (100 nM; Waco BioProducts) before harvesting. Luciferase assays were then performed as described above.

MBP fusions proteins, *in vitro* translations and pull-down assays

The vector expressing a fusion protein of maltose-binding protein (MBP) with MAGE-A1 was constructed by ligating a BamHI/EcoRI fragment carrying the MAGE-A1 coding region isolated from the pGBT9 plasmid into vector pMAL-C2 (New England Biolabs). For *in vitro* transcription and translation, we used the following constructs: pcDNA3.1-Myc HDAC1-F and pcDNA3.1MycHis HDAC4. We expressed MBP and MBP fusion proteins in *Escherichia coli* TOP 10F′ (Invitrogen) and proteins from crude bacterial lysates were purified using Amylose resin (New England BioLabs) according to the manufacturer's instructions. *In vitro* transcription/translation was performed using the TNT system (Promega). MBP pull-down experiments were performed essentially as described previously (42).

RESULTS

MAGE-A1 interacts with SKIP in yeast cells

To identify proteins interacting with MAGE-A1, we used a yeast two-hybrid system with reporter genes controlled by the Gal4 transcription factor. The bait was protein MAGE-A1 fused to the Gal4 DNA-binding domain (Gal4bd). It was obtained by cloning the full-length ORF of *MAGE-A1* into vector pAS2-1, which produces a high level of fusion protein. The preys, fused with the Gal4 transcriptional activation domain (Gal4ad), were produced from a human testis cDNA library prepared in vector pACT2. The first round of screening was conducted in yeast strain CG-1945 which contains reporter gene *HIS3* controlled by Gal4, using medium deficient in histidine. Since Gal4bd/MAGE-A1 exerted a certain degree of transactivation on its own, selection occurred in the presence of 5 mM 3-aminotriazole to increase histidine dependence. Under these conditions, no colonies were observed with pAS2/MAGE-A1 alone. From approximately 7×10^5 yeast transformants, nine His^+ colonies were recovered.

Table 1. *In vivo* interaction of MAGE proteins with SKIP

Ga14bd fusions	Ga14ad fusions	β-Gal activity
MAGE-A1	—	8.8
—	SKIP	0.8
MAGE-A1	SKIP	52.1
p53	SV40 T	123.6

Yeast PJ69-4A cells were transformed with vectors expressing Gal4 DNA-binding domain (Gal4bd) and Gal4 activation domain (Gal4ad) fusion proteins. β-Galactosidase activity was quantified using liquid cultures. Interaction between murine p53 and SV40 large T antigen was taken as a positive control.

For the second round of screening, pACT2 plasmids were isolated from the His^+ colonies and tested for their ability to encode proteins interacting with Gal4bd/MAGE-A1 produced by vector pGBT9 at a much lower level than by vector pAS2-1. This screening used a yeast strain in which gene *LacZ* is controlled by Gal4. One plasmid was repeatedly positive. Interaction was conserved when the testis/Gal4ad and Gal4bd/MAGE-A1 associations were switched. The positive clone contained a 2.1 kb cDNA including the complete ORF of gene *SKIP*, also designated *NcoA-62* (Nuclear receptor coactivator; 62 kDa) (43,44). This gene codes for an adaptor protein involved in transcription regulation.

The β-galactosidase activity was reproducibly 2 to 6 times higher in cells co-expressing Gal4bd/MAGE-A1 and Gal4ad/SKIP than in cells expressing Gal4bd/MAGE-A1 or Gal4ad/SKIP alone (Table 1 and Figure 1). To identify in the 309 amino acids MAGE-A1 protein the region that interacted with SKIP, constructs were prepared that coded for truncated versions of MAGE-A1 fused to Gal4bd (Figure 1). A comparison of $MAGE\text{-}A1_{1–295}$ (amino acids 1–295) and the complete $MAGE\text{-}A1_{1–309}$ indicated that the negatively charged region composed of the 14 C-terminal residues of MAGE-A1 is required for SKIP interaction. The ability of MAGE-A1 to autonomously stimulate the reporter gene was lost when amino acids 279–295 were deleted.

MAGE-A1 interacts with SKIP in mammalian cells

To verify the interaction observed in yeast, we used a co-immunoprecipitation approach in mammalian cells. To this end, the simian COS-7 cells were cotransfected with vectors expressing MAGE-A1 and SKIP. The latter was tagged with an influenza HA epitope. Immunoprecipitations were carried out on cell extracts with an anti-MAGE-A1 antiserum, followed by western blotting using an anti-HA antibody. As shown in Figure 2, MAGE-A1 interacted specifically with SKIP.

Using constructs producing truncated forms of MAGE-A1, we found that $MAGE\text{-}A1_{99–309}$ also coprecipitated with HA–SKIP (Figure 3A, *lane 7*), whereas $MAGE\text{-}A1_{1–279}$ and $MAGE\text{-}A1_{99–279}$ did not (Figure 3A, *lanes 5* and *9*). These results indicated that the C-terminal region of MAGE-A1 was required for the interaction with SKIP in mammalian cells as well as in yeast.

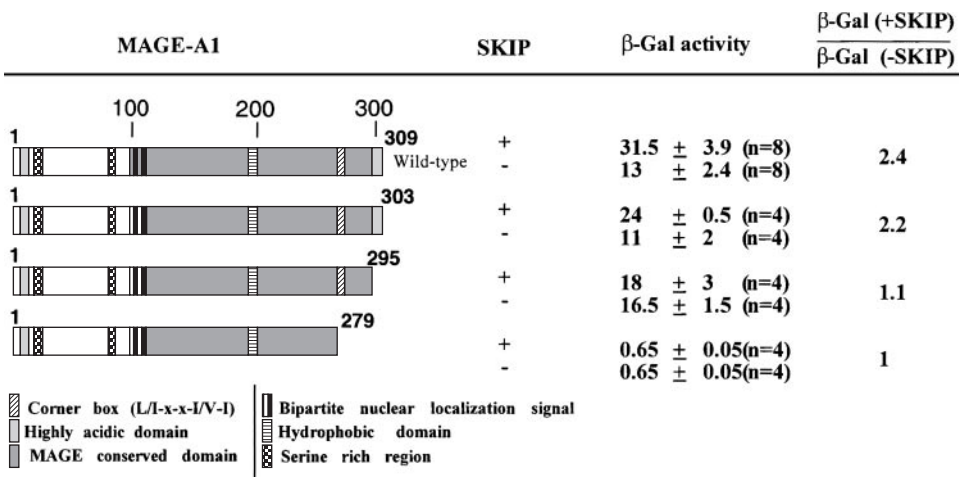


Figure 1. Interaction between deleted forms of MAGE-A1 and SKIP in the yeast two-hybrid system. Truncated *MAGE-A1* sequences were inserted in vector pGBT9 and *SKIP* in vector pACT2. These constructs were transformed in yeast PJ69-4A cells. β-Galactosidase activity was quantified using liquid cultures. Results are presented as mean ± S.D. of *n* tests. CoRNR box (L/I-x-x-I/V-I) involved in interactions with nuclear hormone receptors is found in nuclear corepressors like SMRT and N-CoR (61).

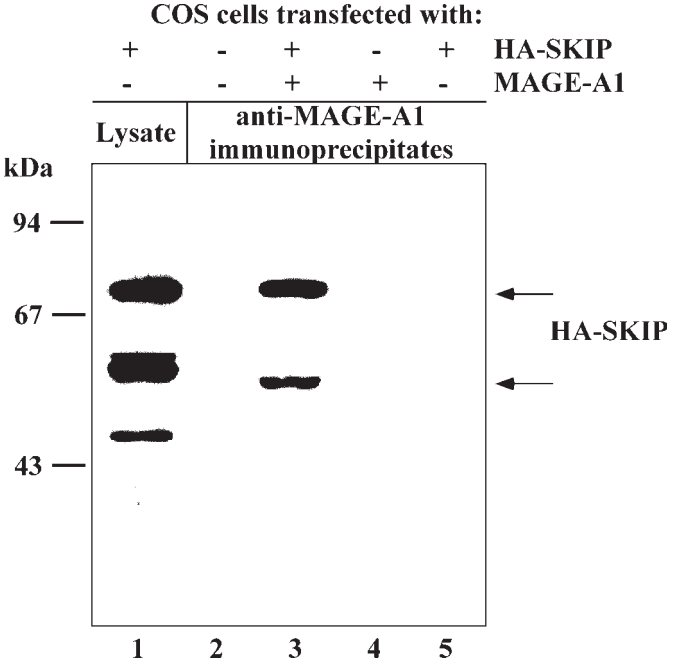


Figure 2. Immunoprecipitation of MAGE-A1 and HA–SKIP. Approximately 2×10^6 COS-7 cells were transiently transfected with HA–SKIP (lanes 1, 3 and 5) and MAGE-A1 expression plasmids (lanes 3 and 4). Cell extracts were incubated with an anti-MAGE-A1 polyclonal antibody. One-hundredth of whole cell lysate of SKIP-positive cells (lane 1) and immune complexes were separated by SDS–polyacrylamide gel electrophoresis, blotted and probed with an anti-HA antibody. HA–SKIP has already been observed as two bands of ~66 and 55 kDa in COS cells (44).

MAGE-A1 counteracts Notch1-IC-mediated transactivation

Having shown the association of MAGE-A1 with SKIP, we wished to assess its functional consequences. SKIP has been described to be involved in the Notch1 signaling pathway. Notch1 is a transmembrane receptor that mediates intercellular communications and directs cell fate decisions during development. Ligand binding to Notch1 induces an intramembrane proteolytic cleavage that releases the intracellular domain of the receptor, Notch1-IC, which then enters the nucleus and regulates the expression of a number of genes. One of the DNA-binding proteins that interact with Notch1-IC is the C-promoter binding factor 1 (CBF1) (45). In the absence of Notch1-IC, CBF1 inhibits transcription by binding SKIP and the SMRT–corepressor complex which contains HDACs (Figure 4) (37). Repression is relieved by the presence of Notch1-IC, which binds to SKIP and CBF1, displaces the repression complex and activates transcription by recruiting histone acetyltransferases (HATs) and also coactivators such as Mastermind (Mam), which stabilizes the complex of Notch1-IC and CBF1 (37,46,47).

We examined whether MAGE-A1 could interfere with the SKIP-dependent transcriptional activity of Notch1-IC. Transient transfection into HeLa cells of a plasmid encoding a Gal4bd/SKIP fusion protein together with a *CAT* reporter gene containing Gal4 DNA-binding sites near its promoter, led to transcriptional repression of the low level of expression observed with the reporter gene alone (Figure 5A). As shown previously (37), when Gal4bd/SKIP was cotransfected with Notch1-IC, a considerable increase in transcription was observed, whereas in the absence of Gal4bd/SKIP, the effect of Notch1-IC was negligible. Finally, when we cotransfected MAGE-A1 together with Gal4bd/SKIP and Notch1-IC, this led to significant transcriptional repression. Increasing the amount of Notch1-IC with a constant amount of MAGE-A1 gradually increased the expression of the *CAT* reporter, but even at the highest dose of transfected Notch1-IC plasmid, repression by MAGE-A1 remained efficient (Figure 5A).

Conversely, MAGE-A1 repressed transcription of the reporter activated by Gal4bd/SKIP and Notch1-IC in a dose-dependent manner (Figure 5B). This was not due to an autonomous non-specific repressor activity of MAGE-A1, as we did not observe a dose-dependent repression when the same promoter was activated by Gal4bd fused to the activation domain of HNF-6 in the presence of increasing amounts of MAGE-A1 (Figure 5C).

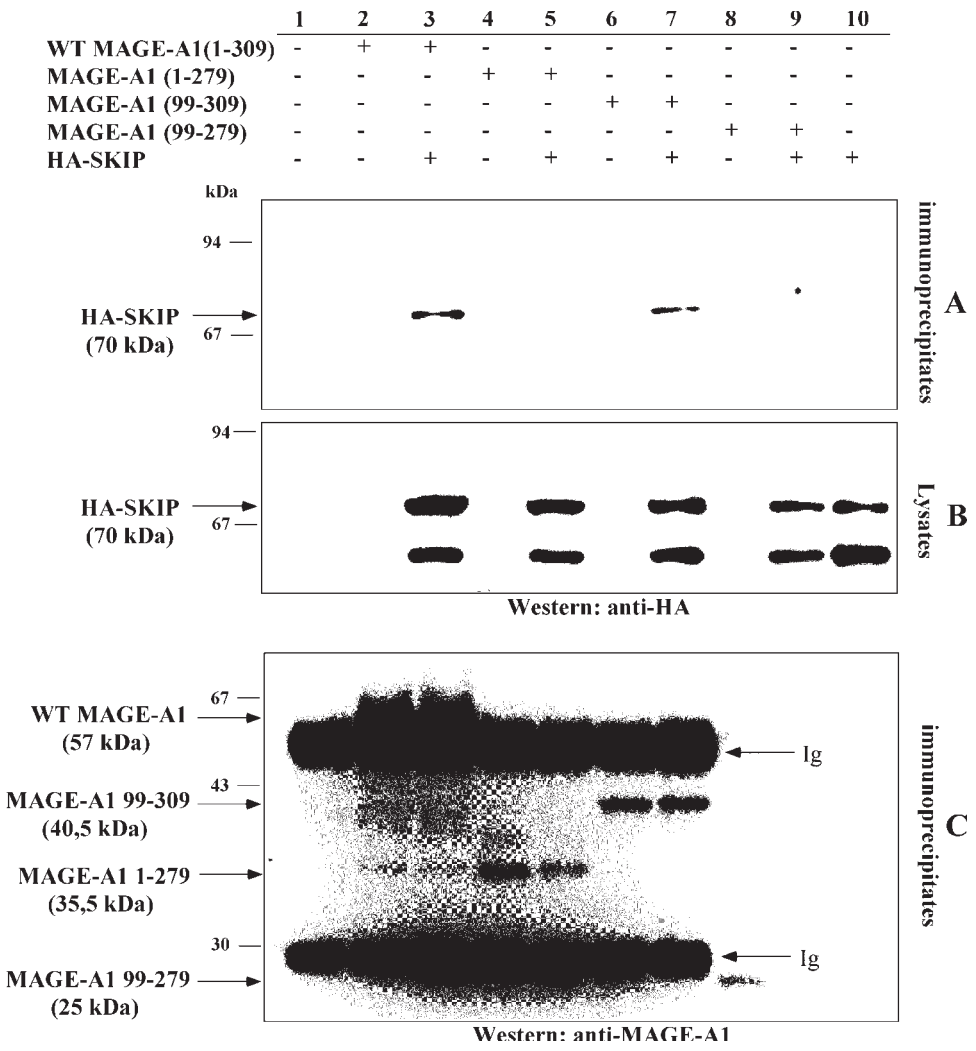


Figure 3. Binding of HA–SKIP to truncated forms of MAGE-A1 in mammalian cells. Approximately 2×10^6 COS-7 cells were transiently transfected with a HA–SKIP expression plasmid (lanes 3, 5, 7, 9 and 10) and with plasmids expressing intact or deleted forms of MAGE-A1: wild-type MAGE-A1 (WT MAGE-A1) (lanes 2 and 3), MAGE-A1$_{1–279}$ (lanes 4 and 5), MAGE-A1$_{99–309}$ (lanes 6 and 7), MAGE-A1$_{99–279}$ (lanes 8 and 9). Half of each cell lysate was immunoprecipitated with a mixture of a polyclonal anti-MAGE-A1 antibody and a monoclonal anti-MAGE-A1 antibody [(**A**,**C**) lanes 1–7] or only the polyclonal anti-MAGE-A1 antibody [(A,C) lanes 8–10]. Immune complexes (*A*,C) or one-hundredth of whole cell lysates (**B**) used for immunoprecipitation were separated by SDS–PAGE, blotted and probed with a monoclonal anti-HA antibody (A,B) or with a monoclonal anti-MAGE-A1 antibody (C). Bands marked as Ig display the light and the heavy chains of the immunoglobulins used for the immunoprecipitation.

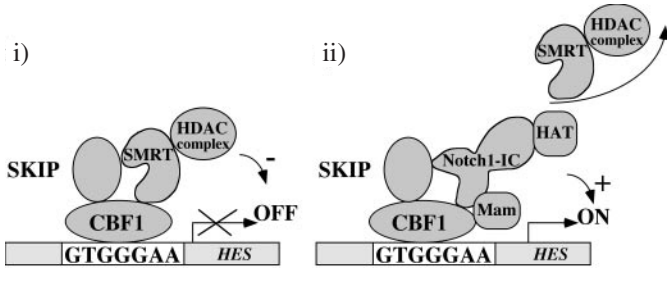


Figure 4. Transactivation by the intracellular part of Notch. Model for Notch1-IC activation on the *HES* (Hairy/Enhancer of Split) promoter regulated by CBF1 according to Zhou *et al*. (37). (**i**) The SMRT–corepressor complex containing HDAC binds to SKIP; (**ii**) Notch1-IC interacts with SKIP and CBF1, displaces the SMRT–corepressor complex and activates the transcription of the *HES* genes through the recruitment of Mastermind (Mam) and HATs.

To determine the cellular localization of the exogenous MAGE-A1, cell fractionation experiments were performed on transfected HeLa cells. These experiments revealed that cytosolic expression of MAGE-A1 was predominant, but that a significant fraction of MAGE-A1 was present in the nucleus (Figure 5D). We could not detect Notch1-IC on these immunoblots. This was not unexpected since Notch1-IC is known to be instable and to act at very low nuclear concentrations (48). However, in COS cells transfected in exactly the same conditions as the HeLa cells of Figure 5B, we found that transfected *Notch1-IC* was expressed and that the levels of Notch1-IC did not decrease when the amounts of MAGE-A1 in the cell increased (data not shown).

To determine the region of MAGE-A1 that is required for the inhibition of Notch1-IC-mediated transactivation, we tested deleted forms of MAGE-A1 in the reporter assay.

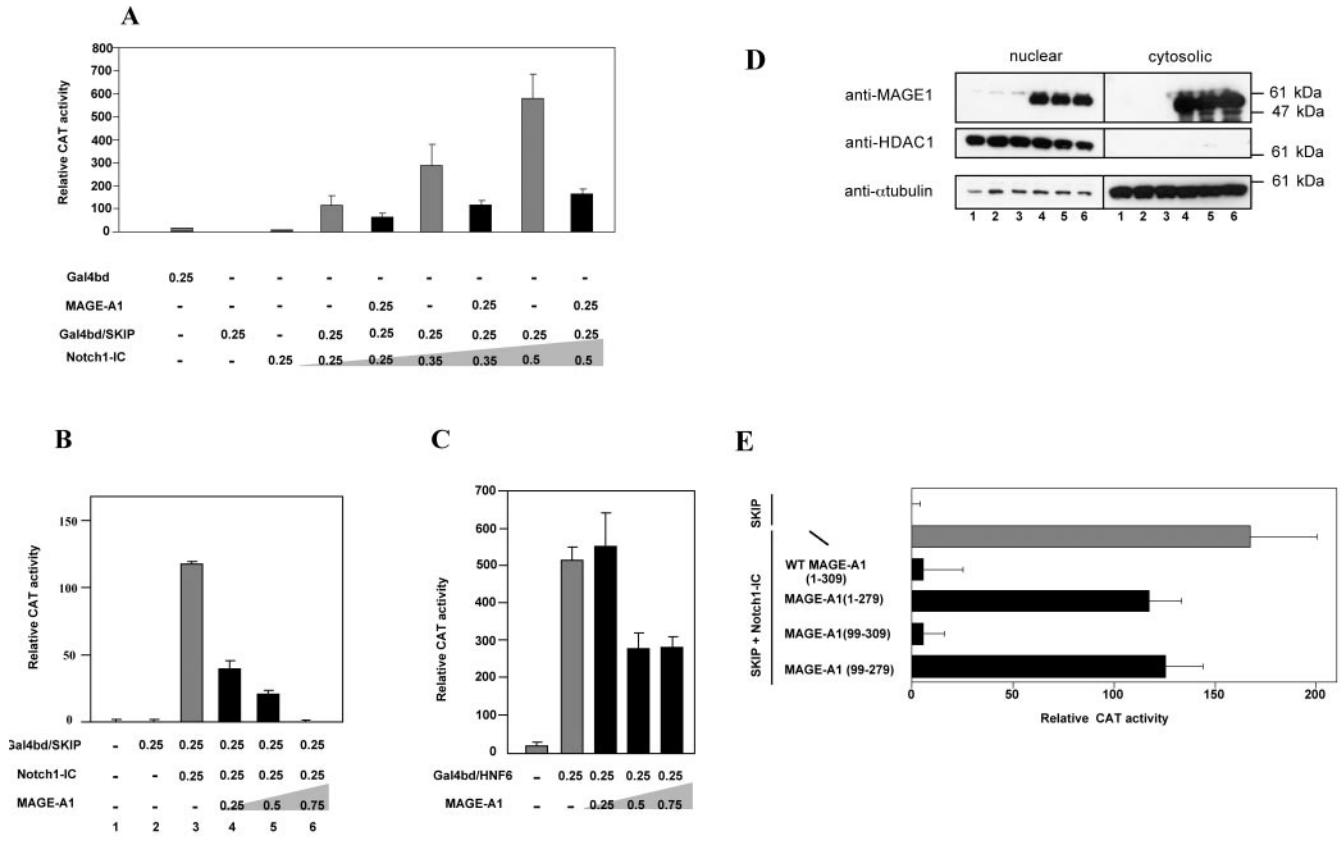


Figure 5. MAGE-A1 prevents Notch1-IC transactivation. (**A**) An aliquot of 0.25 μg of an expression vector encoding Gal4bd, Gal4bd/SKIP, Notch1-IC or MAGE-A1 was transfected into HeLa cells together with 0.4 μg of the 5xGal4TK-CAT reporter plasmid. CAT activities measured on cell lysates were normalized to the luciferase activity obtained by transfecting 0.5 μg of pTKluc. The amount of DNA was kept constant at 1.65 μg by adding an irrelevant pcDNAI/Amp recombinant plasmid. Data are means of duplicates. (**B**) Relative CAT activity measured in HeLa cells transfected with the 5xGal4TK-CAT reporter (0.4 μg), increasing amounts (0.25–0.75 μg) of the expression vector coding for MAGE-A1 and the vectors producing Gal4bd/SKIP (0.25 μg) and Notch1-IC (0.25 μg). Data are means of triplicates. Comparable total amounts of DNA were transfected. (**C**) Relative CAT activity measured in HeLa cells transfected with the 5xGal4TK-CAT reporter (0.4 μg), increasing amounts (0.25–0.75 μg) of the expression vector coding for MAGE-A1 and a vector encoding Gal4bd fused to the activation domain of HNF-6 (0.25 μg). Data are means of quadruplicates. Comparable total amounts of DNA were transfected. (**D**) Cellular localization of MAGE-A1 in transfected HeLa cells. Transfections were performed as described in Figure 5B. Nuclear and cytosolic extracts were analyzed by western blotting for the presence of MAGE-A1 with the 6C1 monoclonal antibody. Purity of the fractions was evaluated by incubating the western blot with antibodies against HDAC1 (a nuclear localized protein) and α-tubulin (a cytoplasmic localized protein). Molecular weights in kiloDaltons are indicated on the right. (**E**) Repression of Notch1-IC transactivation requires that MAGE-A1 binds to SKIP. An aliquot of 0.25 μg of a vector producing Gal4bd/SKIP, Notch1-IC, wild-type MAGE-A1 (WT MAGE-A1) or deleted versions of MAGE-A1 (two clones per construct) together with 0.4 μg of the 5xGal4TK-CAT reporter plasmid were transfected into HeLa cells. Data are means of quadruplicates for each MAGE-A1 construct.

MAGE-A1$_{99–309}$ reduced Notch1-IC transactivation by a factor of about 30, whereas MAGE-A1$_{1–279}$ or MAGE-A1$_{99–279}$ had no significant effect (Figure 5E). As the MAGE-A1 C-terminus mediates the binding to SKIP (Figures 1 and 3), this suggests that the repression of Notch1-IC transactivation requires an interaction between SKIP and MAGE-A1.

As reported previously, Gal4bd/SKIP repression is likely to be due to recruitment of the SMRT–HDAC repressor complex whereas Notch1-IC upregulation is caused by Notch1-IC competition with the SMRT–corepressor complex and recruitment of coactivators (Figure 6) (37,49). Our experiments indicate that, by binding to SKIP MAGE-A1 inhibits Notch1-IC transactivation. MAGE-A1 could achieve this inhibitory effect either by displacing Notch1-IC from SKIP or by masking Notch1 sites which bind transcriptional coactivators while leaving Notch1-IC attached to SKIP. A third possibility would be that, after binding to SKIP MAGE-A1 functions as an active repressor.

MAGE-A1 recruits deacetylase HDAC1

We investigated whether MAGE-A1 had the ability to repress transcription upon binding autonomously to the promoter. To this end, we transiently transfected cells with a Gal4bd/MAGE-A1 construct together with a luciferase reporter gene driven by a tk promoter preceded by four Gal4 DNA-binding sites. MAGE-A1 inhibited transcription in a dose-dependent manner when linked to the Gal4 DNA-binding domain (Figure 7A). This suggests that MAGE-A1 can autonomously repress transcription.

Active repression of transcription can be mediated through recruitment of HDAC, resulting in nucleosome

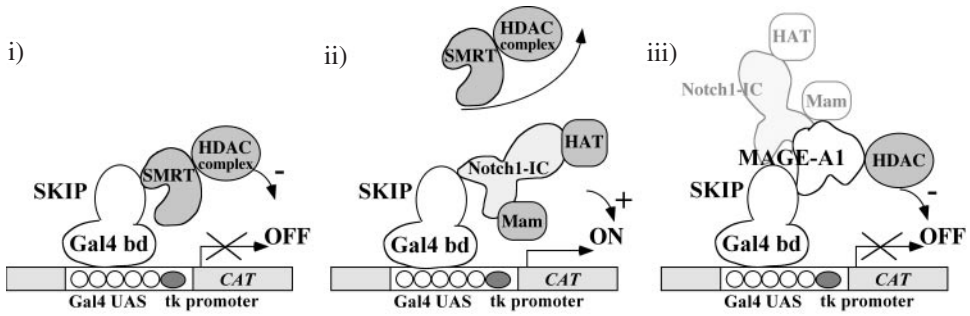


Figure 6. MAGE-A1 counteracts Notch1-IC transactivation and recruits HDAC. (**i** and **ii**) Summary of experimental data using Gal4bd/SKIP, Notch1-IC and the *CAT* reporter according to the model of Zhou *et al*. (37). (**iii**) By binding to SKIP and recruiting HDAC, MAGE-A1 counteracts Notch1-IC transactivation and thereby represses transcription. Endogenous proteins are indicated by shaded forms; proteins expressed upon transfection are indicated by unshaded forms.

remodeling (50). To test whether MAGE-A1 may repress transcription through HDAC, we incubated cells expressing Gal4bd/MAGE-A1 with the HDAC inhibitor, TSA. This resulted in a significant relieve of MAGE-A1-mediated transcriptional repression (Figure 7B).

We, therefore, examined whether MAGE-A1 could associate with HDAC1 and/or HDAC4 that belong to Class I and Class II HDAC, respectively. To this end, we performed *in vitro* pull-down experiments using a MBP–MAGE-A1 fusion protein produced in *E.coli* and *in vitro* translated ^{35}S-labelled HDAC1 and HDAC4. As shown in Figure 7C, MBP–MAGE-A1 bound specifically to full-length HDAC1 (lane 3) whereas MBP alone did not (lane 2). No binding was observed with HDAC4 (Figure 7C).

We then examined whether the association between MAGE-A1 and HDAC1 could occur *in vivo*, in COS cells that were transiently transfected with HDAC1 and MAGE-A1 expression plasmids. MAGE-A1 was immunoprecipitated with the anti-MAGE-A1 goat serum. Co-immunoprecipitated HDAC1 was detected by western blotting using an anti-HDAC1 antibody (Figure 7D, lane 3). No HDAC1 was detected when a goat preimmune serum was used to immunoprecipitate the lysate (Figure 7D, lane 2).

Finally, we tested the functional relevance of the interaction between MAGE-A1 and HDAC1 by using transient transfection of a reporter gene containing Gal4 DNA-binding sites and a construct expressing a Gal4bd/MAGE-A1 fusion protein. In this assay, we used a limiting amount of Gal4bd-MAGE-A1 that moderately repressed luciferase activity. Cotransfection of Gal4bd/MAGE-A1 with HDAC1 synergistically enhanced transcriptional repression (Figure 7E), indicating a co-operative effect of MAGE-A1 and HDAC1. Taken together, these experiments strongly suggest that MAGE-A1 can actively repress transcription through the recruitment of HDAC1.

DISCUSSION

Our results indicate that MAGE-A1 interacts with transcriptional regulator SKIP, through its C-terminal part. In line with these results, we observed that MAGE-A4, the only other MAGE in which this region is conserved, also binds to SKIP, whereas MAGE-A10 and MAGE-D2, which display different C-terminal parts, do not bind to SKIP in the yeast two-hybrid system (data not shown).

SKIP intervenes in signaling pathways involving vitamin D, retinoic acid, estrogens, glucocorticoids, Notch1-IC and TGF-β (37,43,51,52). It can have either an activator or repressor role in transcription because it is an adaptor protein that connects DNA-binding proteins such as Smad3, the vitamin D receptor, CBF1 or MyoD, to proteins that activate or repress transcription. For example, the steroid receptor coactivator (SRC), which has a HAT activity, forms a ternary complex with SKIP and the liganded vitamin D receptor (51,53). Ski, the protein that was used as bait to fish SKIP, similarly binds to the DNA-binding protein Smad and recruits a repression complex including HDAC (54).

Another repression complex was shown to bind to SKIP and CBF1 (37). In the presence of Notch1-IC, the repression complex is detached from SKIP and recruitment of an activation complex including HATs is facilitated by SKIP (37,47). In our experimental system, MAGE-A1 counteracted the transcriptional activation mediated by Notch1-IC (Figure 6). A possible explanation of this effect is that MAGE-A1 functions as a passive repressor dissociating Notch1-IC from SKIP, thereby preventing the recruitment of the transcriptional activation complex. However, the observation that the repressive effect of MAGE-A1 was not relieved by increasing the amount of transfected Notch1-IC plasmid (Figure 5A) suggests that MAGE-A1 and Notch1-IC did not compete to bind to SKIP. Another possible mechanism of passive repression could be that, upon binding to SKIP, MAGE-A1 masked the activation domain of Notch1-IC. Such a mechanism of passive repression was reported for the MAGE-related protein, necdin, which binds to the transactivation domain of E2F1 and p53 (27,28). MAGE-A1 is not involved in this pathway, as we found that it does not interact with E2F1 or p53 in the yeast two-hybrid system (data not shown).

We investigated whether MAGE-A1 could actively repress transcription. Active repression could be exerted by interfering with the assembly of the basal transcription complex near the transcription-initiation site, by disrupting the interaction between DNA-bound transcription activators and the transcription machinery or by recruiting corepressors, such as HDAC. We observed that MAGE-A1 binds and recruits HDAC1, which modifies chromatin, but may also target non-histone proteins involved in transcriptional regulation (38). Recruitment of HDAC by MAGE-A1 could be sufficient to inhibit Notch1-IC transactivation, but we cannot exclude that, in our experimental setting, MAGE-A1 also inhibited transcription by destabilizing the binding of transcriptional coactivators to Notch1-IC.

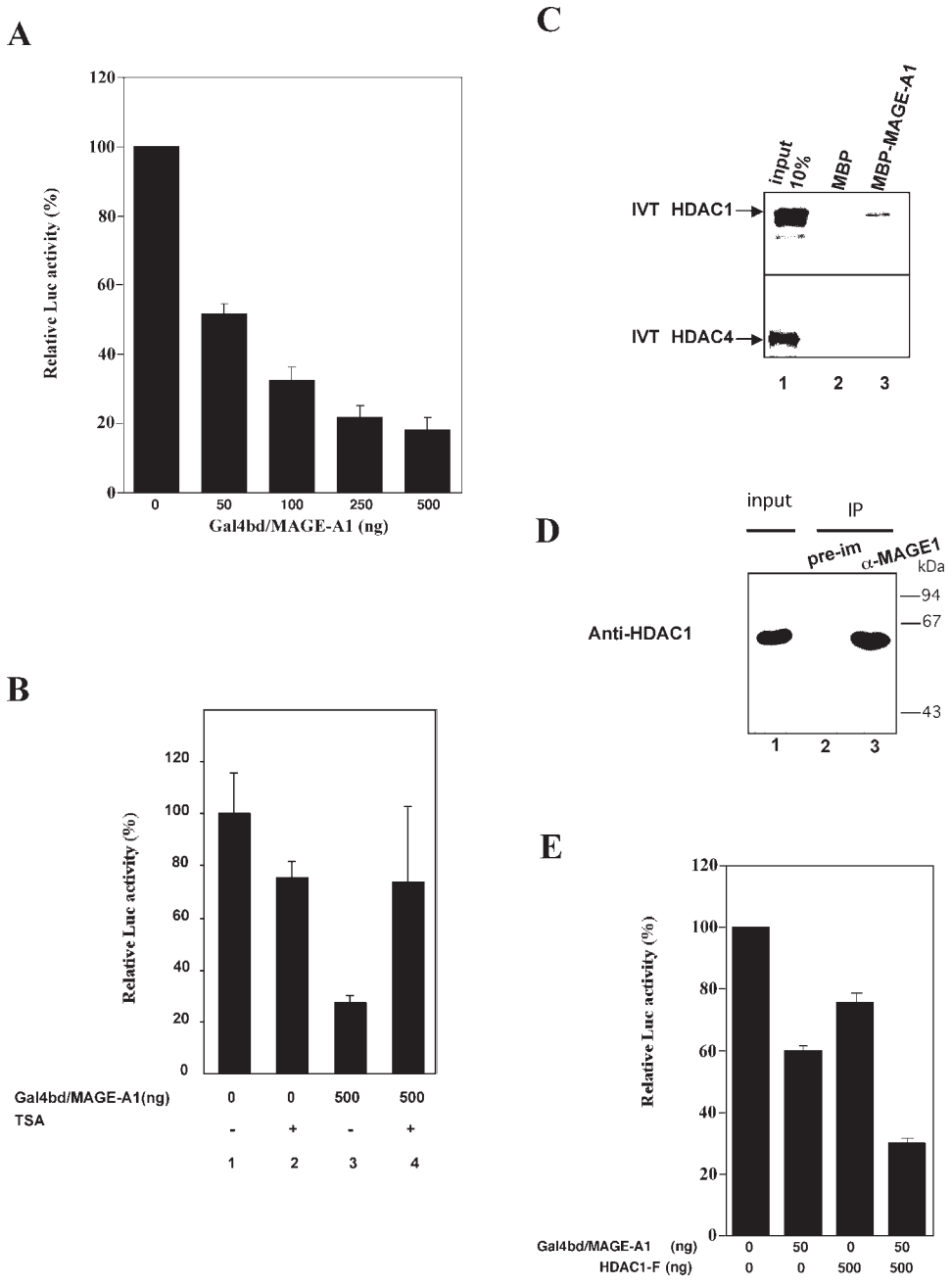


Figure 7. MAGE-A1 actively represses transcription through the recruitment of the histone deacetylase, HDAC1. (**A**) MAGE-A1 actively inhibits transcription when fused to Gal4bd. U2OS cells were transiently transfected with 250 ng of $(Gal4)_4$–tkLuc reporter with increasing amounts (50–500 ng) of a vector coding for Gal4bd/MAGE-A1. The amount of transfected DNA was kept constant to 500 ng by adding DNA of a vector producing Gal4bd. Whole cell extracts were used in luciferase assays. The basal activity of the reporter in the presence of Gal4bd is normalized to a value of 100%. Transfection efficiencies were normalized using β-galactosidase activity. The results are the average of seven independent transfections with error bars displaying standard deviations. (**B**) MAGE-A1-mediated repression is sensitive to the HDAC inhibitor TSA. U2OS cells were transiently transfected with 250 ng of the $(Gal4)_4$–tkLuc reporter and 500 ng of the vector encoding Gal4bd/MAGE-A1. Four hours after transfection, cells were treated (lanes 2 and 4) or not (lanes 1 and 3) with 200 nM TSA. The relative luciferase activities were determined as described in Figure 7A. The results are the average of at least two independent transfections with error bars displaying standard deviations. (**C**) MAGE-A1 interacts with HDAC1. Full-length ^{35}S-radiolabelled HDAC1 and HDAC4 were obtained by *in vitro* translation (IVT) and incubated with equivalent amounts of maltose binding protein (MBP) (lane 2) or MBP–MAGE-A1 fusion protein (lane 3). IVT HDAC1 and HDAC4 are indicated by an arrow on the left. Lane 1, ^{35}S-radiolabelled HDAC1 (top) or HDAC4 (bottom), input (10%). (**D**) HDAC1 co-immunoprecipitates with MAGE-A1. COS cells were cotransfected with expression vectors encoding MAGE-A1 and HDAC1. A lysate was analyzed by immunoblotting with an anti-HDAC1 monoclonal antibody, directly (lane 1, input 4%) or after immunoprecipitation (IP) with preimmune goat serum (lane 2) or anti-MAGE-A1 goat serum (lane 3). (**E**) MAGE-A1 requires the deacetylase HDAC1 for transcriptional repression. U2OS cells were transiently transfected with 250 ng of the $(Gal4)_4$–tkLuc reporter and, as indicated, with 50 ng of a vector encoding Gal4bd/MAGE-A1 and/or 500 ng of a vector producing HDAC1-F (HDAC1 tagged with a Flag epitope). Cells were then harvested and assayed for luciferase activity. The basal activity of the reporter is normalized to a value of 100%. Transfection efficiencies were normalized using β-galactosidase activity. The results of a representative experiment done in duplicate are shown. In the absence of synergy, the expected value for the relative luciferase activity is 46%, which corresponds to the product of the activity measured in the presence of MAGE-A1 alone (60%) × the activity measured in the presence of HDAC1 alone (76%).

Such a combination of passive and active repression mechanisms was previously demonstrated for retinoblastoma protein, Rb. It binds to the activation domain of transcription factor E2F1, preventing it to interact with the CBP coactivator (55,56). In addition, Rb recruits HDAC and actively represses the E2F-regulated promoters of genes required for the S phase of the cell cycle (38,57).

Since MAGE-A1 acts as a transcriptional regulator, a fraction of the MAGE-A1 protein produced in mammalian cells must reach the nucleus. In transfected HeLa cells, we observed that MAGE-A1 was not exclusively localized in the cytosol, but that a significant fraction of it was present in the nucleus (Figure 5D). Some reports described that MAGE-A1 was located only in the cytosol of melanoma cells and spermatogonia (32,33,58), but another report described that MAGE-A1 and MAGE-A4 proteins are located in both the cytoplasm and the nucleus of spermatogonia (7). MAGE-A1 may be located predominantly in the cytoplasm and may translocate to the nucleus upon appropriate stimulation of the cell.

Experiments performed in our experimental system clearly indicated that, upon binding to SKIP, MAGE-A1 exerted a potent transcriptional repression. Since SKIP is involved in several signaling pathways, repression by MAGE-A1 bound to SKIP may influence gene expression from many different promoters. As the testis is the only healthy adult organ where human *MAGE-A* and murine *Mage-a* genes are expressed, proteins encoded by these genes may play a role in spermatogenesis (7,59). It is therefore possible that, by binding to SKIP and by inhibiting Notch1-IC transactivation, MAGE-A1 participates in cell fate choices during spermatogenesis. However, we were not able to demonstrate that MAGE-A1 could repress the expression of an endogenous Notch1-IC target gene (data not shown). This may be because MAGE-A1, in contrast to Notch1-IC or SMRT, does not bind to the activator CBF1 but binds only to SKIP, a protein that could not be absolutely required for Notch1-IC signal transduction. The possibility that MAGE-A1 regulates the expression of genes involved in spermatogenesis through other signaling pathways remains to be investigated. On the other hand, MAGE-A1 is expressed in tumor cells of different histological types. Altered HAT and/or HDAC activities have been observed in many cancer cells (60). By recruiting HDAC1 to genes that remain to be identified, MAGE-A1 may contribute to these alterations and favor tumor cell growth.

ACKNOWLEDGEMENTS

We thank Prof. Pierre Coulie for critical comments on the manuscript and C.Blondiaux for excellent assistance. R.D. and F.F. were supported by the Belgian FNRS ('FRIA' and 'Chercheur qualifié du FNRS', respectively). This work was supported by a 'Télévie' grant from the Belgian Fonds National de la Recherche Scientifique (FNRS).

REFERENCES

1. van der Bruggen,P., Traversari,C., Chomez,P., Lurquin,C., De Plaen,E., Van den Eynde,B., Knuth,A. and Boon,T. (1991) A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science*, **254**, 1643–1647.
2. Rogner,U.C., Wilke,K., Steck,E., Korn,B. and Poustka,A. (1995) The melanoma antigen gene (MAGE) family is clustered in the chromosomal band Xq28. *Genomics*, **29**, 725–731.
3. Brasseur,F., Marchand,M., Vanwijck,R., Hérin,M., Lethé,B., Chomez,P. and Boon,T. (1992) Human gene MAGE-1, which codes for a tumor rejection antigen, is expressed by some breast tumors. *Int. J. Cancer*, **52**, 839–841.
4. Weynants,P., Lethé,B., Brasseur,F., Marchand,M. and Boon,T. (1994) Expression of MAGE genes by non-small-cell lung carcinomas. *Int. J. Cancer*, **56**, 826–829.
5. De Plaen,E., Arden,K., Traversari,C., Gaforio,J.J., Szikora,J.-P., De Smet,C., Brasseur,F., van der Bruggen,P., Lethé,B., Lurquin,C. *et al.* (1994) Structure, chromosomal localization and expression of twelve genes of the MAGE family. *Immunogenetics*, **40**, 360–369.
6. De Smet,C., Lurquin,C., van der Bruggen,P., De Plaen,E., Brasseur,F. and Boon,T. (1994) Sequence and expression pattern of the human MAGE2 gene. *Immunogenetics*, **39**, 121–129.
7. Takahashi,K., Shichijo,S., Noguchi,M., Hirohata,M. and Itoh,K. (1995) Identification of MAGE-1 and MAGE-4 proteins in spermatogonia and primary spermatocytes of testis. *Cancer Res.*, **55**, 3478–3482.
8. Muscatelli,F., Walker,A.P., De Plaen,E., Stafford,A.N. and Monaco,A.P. (1995) Isolation and characterization of a new MAGE gene family in the Xp21.3 region. *Proc. Natl Acad. Sci. USA*, **92**, 4987–4991.
9. Dabovic,B., Zanaria,E., Bardoni,B., Lisa,A., Bordignon,C., Russo,V., Matessi,C., Traversari,C. and Camerino,G. (1995) A family of rapidly evolving genes from the sex reversal critical region in Xp21. *Mamm. Genome*, **6**, 571–580.
10. Lurquin,C., De Smet,C., Brasseur,F., Muscatelli,F., Martelange,V., De Plaen,E., Brasseur,R., Monaco,A.P. and Boon,T. (1997) Two members of the human *MAGEB* gene family located in Xp.21.3 are expressed in tumors of various histological origins. *Genomics*, **46**, 397–408.
11. Lucas,S., De Plaen,E. and Boon,T. (2000) *MAGE-B5*, *MAGE-B6*, *MAGE-C2* and *MAGE-C3*: four new members of the *MAGE* family with tumor-specific expression. *Int. J. Cancer*, **87**, 55–60.
12. Lucas,S., De Smet,C., Arden,K.C., Viars,C.S., Lethé,B., Lurquin,C. and Boon,T. (1998) Identification of a new MAGE gene with tumor-specific expression by representational difference analysis. *Cancer Res.*, **58**, 743–752.
13. Lucas,S., Brasseur,F. and Boon,T. (1999) A new *MAGE* gene with ubiquitous expression does not code for known MAGE antigens recognized by T cells. *Cancer Res.*, **59**, 4100–4103.
14. Pold,M., Zhou,J., Chen,G.L., Hall,J.M., Vescio,R.A. and Berenson,J.R. (1999) Identification of a new, unorthodox member of the MAGE gene family. *Genomics*, **59**, 161–167.
15. Chomez,P., De Backer,O., Bertrand,M., De Plaen,E., Boon,T. and Lucas,S. (2001) An overview of the MAGE gene family with the identification of all human members of the family. *Cancer Res.*, **61**, 5544–5551.
16. Salehi,A.H., Roux,P.P., Kubu,C.J., Zeindler,C., Bhakar,A., Tannis,L.L., Verdi,J.M. and Barker,P.A. (2000) NRAGE, a novel MAGE protein, interacts with the p75 neurotrophin receptor and facilitates nerve growth factor-dependent apoptosis. *Neuron*, **27**, 279–288.
17. Salehi,A.H., Xanthoudakis,S. and Barker,P.A. (2002) NRAGE, a p75 neurotrophin receptor-interacting protein, induces caspase activation and cell death through a JNK-dependent mitochondrial pathway. *J. Biol. Chem.*, **277**, 48043–48050.
18. Williams,M.E., Strickland,P., Watanabe,K. and Hinck,L. (2003) UNC5H1 induces apoptosis via its juxtamembrane region through an interaction with NRAGE. *J. Biol. Chem.*, **278**, 17483–17490.
19. Masuda,Y., Sasaki,A., Shibuya,H., Ueno,N., Ikeda,K. and Watanabe,K. (2001) Dlxin-1, a novel protein that binds Dlx5 and regulates its transcriptional function. *J. Biol. Chem.*, **276**, 5331–5338.
20. Matsuda,T., Suzuki,H., Oishi,I., Kani,S., Kuroda,Y., Komori,T., Sasaki,A., Watanabe,K. and Minami,Y. (2003) The receptor tyrosine kinase Ror2 associates with the melanoma-associated antigen (MAGE) family protein Dlxin-1 and regulates its intracellular distribution. *J. Biol. Chem.*, **278**, 29057–29064.
21. Uetsuki,T., Takagi,K., Sugiura,H. and Yoshikawa,K. (1996) Structure and expression of the mouse necdin gene. Identification of a postmitotic neuron-restrictive core promoter. *J. Biol. Chem.*, **271**, 918–924.
22. Niinobe,M., Koyama,K. and Yoshikawa,K. (2000) Cellular and subcellular localization of necdin in fetal and adult mouse brain. *Dev. Neurosci.*, **22**, 310–319.

23. Gerard,M., Hernandez,L., Wevrick,R. and Stewart,C.L. (1999) Disruption of the mouse necdin gene results in early post-natal lethality. *Nature Genet*., **23**, 199–202.
24. Muscatelli,F., Abrous,D.N., Massacrier,A., Boccaccio,I., Le Moal,M., Cau,P. and Cremer,H. (2000) Disruption of the mouse Necdin gene results in hypothalamic and behavioral alterations reminiscent of the human Prader–Willi syndrome. *Hum. Mol. Genet*., **9**, 3101–3110.
25. Tsai,T.F., Jiang,Y.H., Bressler,J., Armstrong,D. and Beaudet,A.L. (1999) Paternal deletion from Snrpn to Ube3a in the mouse causes hypotonia, growth retardation and partial lethality and provides evidence for a gene contributing to Prader–Willi syndrome. *Hum. Mol. Genet*., **8**, 1357–1364.
26. Hayashi,Y., Matsuyama,K., Takagi,K., Sugiura,H. and Yoshikawa,K. (1995) Arrest of cell growth by necdin, a nuclear protein expressed in postmitotic neurons. *Biochem. Biophys. Res. Commun*., **213**, 317–324.
27. Taniura,H., Taniguchi,N., Hara,M. and Yoshikawa,K. (1998) Necdin, a postmitotic neuron-specific growth suppressor, interacts with viral transforming proteins and cellular transcription factor E2F1. *J. Biol. Chem*., **273**, 720–728.
28. Taniura,H., Matsumoto,K. and Yoshikawa,K. (1999) Physical and functional interactions of neuronal growth suppressor necdin with p53. *J. Biol. Chem*., **274**, 16242–16248.
29. Tcherpakov,M., Bronfman,F.C., Conticello,S.G., Vaskovsky,A., Levy,Z., Niinobe,M., Yoshikawa,K., Arenas,E. and Fainzilber,M. (2002) The p75 neurotrophin receptor interacts with multiple MAGE proteins. *J. Biol. Chem*., **277**, 49101–49104.
30. Kuwako,K., Taniura,H. and Yoshikawa,K. (2004) Necdin-related MAGE proteins differentially interact with the E2F1 transcription factor and the p75 neurotrophin receptor. *J. Biol. Chem*., **279**, 1703–1712.
31. Nagao,T., Higashitsuji,H., Nonoguchi,K., Sakurai,T., Dawson,S., Mayer,R.J., Itoh,K. and Fujita,J. (2003) MAGE-A4 interacts with the liver oncoprotein gankyrin and suppresses its tumorigenic activity. *J. Biol. Chem*., **278**, 10668–10674.
32. Schultz-Thater,E., Juretic,A., Dellabona,P., Luscher,U., Siegrist,W., Harder,F., Heberer,M., Zuber,M. and Spagnoli,G.C. (1994) MAGE-1 gene product is a cytoplasmic protein. *Int. J. Cancer*, **59**, 435–439.
33. Amar-Costesec,A., Godelaine,D., Stockert,E., van der Bruggen,P., Beaufay,H. and Chen,Y.T. (1994) The tumor protein MAGE-1 is located in the cytosol of human melanoma cells. *Biochem. Biophys. Res. Commun*., **204**, 710–715.
34. Kocher,T., Schultz-Thater,E., Gudat,F., Schaefer,C., Casorati,G., Juretic,A., Willimann,T., Harder,F., Heberer,M. and Spagnoli,G.C. (1995) Identification and intracellular location of MAGE-3 gene product. *Cancer Res*., **55**, 2236–2239.
35. Jurk,M., Kremmer,E., Schwarz,U., Forster,R. and Winnacker,E.L. (1998) MAGE-11 protein is highly conserved in higher organisms and located predominantly in the nucleus. *Int. J. Cancer*, **75**, 762–766.
36. Rimoldi,D., Salvi,S., Reed,D., Coulie,P., Jongeneel,V.C., De Plaen,E., Brasseur,F., Rodriguez,A.M., Boon,T. and Cerottini,J.-C. (1999) cDNA and protein characterization of human MAGE-10. *Int. J. Cancer*, **82**, 901–907.
37. Zhou,S., Fujimuro,M., Hsieh,J.J., Chen,L., Miyamoto,A., Weinmaster,G. and Hayward,S.D. (2000) SKIP, a CBF1-associated protein, interacts with the ankyrin repeat domain of NotchIC to facilitate NotchIC function. *Mol. Cell. Biol*., **20**, 2400–2410.
38. Brehm,A., Miska,E.A., McCance,D.J., Reid,J.L., Bannister,A.J. and Kouzarides,T. (1998) Retinoblastoma protein recruits histone deacetylase to repress transcription. *Nature*, **391**, 597–601.
39. Yu,J., Angelin-Duclos,C., Greenwood,J., Liao,J. and Calame,K. (2000) Transcriptional repression by blimp-1 (PRDI-BF1) involves recruitment of histone deacetylase. *Mol. Cell. Biol*., **20**, 2592–2603.
40. James,P., Halladay,J. and Craig,E.A. (1996) Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. *Genetics*, **144**, 1425–1436.
41. Rimoldi,D., Salvi,S., Schultz-Thater,E., Spagnoli,G.C. and Cerottini,J.C. (2000) Anti-MAGE-3 antibody 57B and anti-MAGE-1 antibody 6C1 can be used to study different proteins of the MAGE-A family. *Int. J. Cancer*, **86**, 749–751.
42. Fuks,F., Burgers,W.A., Godin,N., Kasai,M. and Kouzarides,T. (2001) Dnmt3a binds deacetylases and is recruited by a sequence-specific repressor to silence transcription. *EMBO J*., **20**, 2536–2544.
43. Baudino,T.A., Kraichely,D.M., Jefcoat,S.C.,Jr, Winchester,S.K., Partridge,N.C. and MacDonald,P.N. (1998) Isolation and characterization of a novel coactivator protein, NCoA-62, involved in vitamin D-mediated transcription. *J. Biol. Chem*., **273**, 16434–16441.
44. Dahl,R., Wani,B. and Hayman,M.J. (1998) The Ski oncoprotein interacts with Skip, the human homolog of Drosophila Bx42. *Oncogene*, **16**, 1579–1586.
45. Fortini,M.E. and Artavanis-Tsakonas,S. (1994) The suppressor of hairless protein participates in notch receptor signaling. *Cell*, **79**, 273–282.
46. Kitagawa,M., Oyama,T., Kawashima,T., Yedvobnick,B., Kumar,A., Matsuno,K. and Harigaya,K. (2001) A human protein with sequence similarity to Drosophila mastermind coordinates the nuclear form of notch and a CSL protein to build a transcriptional activator complex on target promoters. *Mol. Cell. Biol*., **21**, 4337–4346.
47. Kurooka,H. and Honjo,T. (2000) Functional interaction between the mouse notch1 intracellular region and histone acetyltransferases PCAF and GCN5. *J. Biol. Chem*., **275**, 17211–17220.
48. Schroeter,E.H., Kisslinger,J.A. and Kopan,R. (1998) Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain. *Nature*, **393**, 382–386.
49. Hsieh,J.J., Henkel,T., Salmon,P., Robey,E., Peterson,M.G. and Hayward,S.D. (1996) Truncated mammalian Notch1 activates CBF1/RBPJk-repressed genes by a mechanism resembling that of Epstein–Barr virus EBNA2. *Mol. Cell. Biol*., **16**, 952–959.
50. Kouzarides,T. (1999) Histone acetylases and deacetylases in cell proliferation. *Curr. Opin. Genet. Dev*., **9**, 40–48.
51. Zhang,C., Baudino,T.A., Dowd,D.R., Tokumaru,H., Wang,W. and MacDonald,P.N. (2001) Ternary complexes and cooperative interplay between NCoA-62/Ski-interacting protein and steroid receptor coactivators in vitamin D receptor-mediated transcription. *J. Biol. Chem*., **276**, 40614–40620.
52. Leong,G.M., Subramaniam,N., Figueroa,J., Flanagan,J.L., Hayman,M.J., Eisman,J.A. and Kouzmenko,A.P. (2001) Ski-interacting protein interacts with Smad proteins to augment transforming growth factor-beta-dependent transcription. *J. Biol. Chem*., **276**, 18243–18248.
53. MacDonald,P.N., Baudino,T.A., Tokumaru,H., Dowd,D.R. and Zhang,C. (2001) Vitamin D receptor and nuclear receptor coactivators: crucial interactions in vitamin D-mediated transcription. *Steroids*, **66**, 171–176.
54. Liu,X., Sun,Y., Weinberg,R.A. and Lodish,H.F. (2001) Ski/Sno and TGF-beta signaling. *Cytokine Growth Factor Rev*., **12**, 1–8.
55. Trouche,D., Cook,A. and Kouzarides,T. (1996) The CBP co-activator stimulates E2F1/DP1 activity. *Nucleic Acids Res*., **24**, 4139–4145.
56. Trouche,D. and Kouzarides,T. (1996) E2F1 and E1A(12S) have a homologous activation domain regulated by RB and CBP. *Proc. Natl Acad. Sci. USA*, **93**, 1439–1442.
57. Brehm,A. and Kouzarides,T. (1999) Retinoblastoma protein meets chromatin. *Trends Biochem. Sci*., **24**, 142–145.
58. Jungbluth,A.A., Stockert,E., Chen,Y.T., Kolb,D., Iversen,K., Coplan,K., Williamson,B., Altorki,N., Busam,K.J. and Old,L.J. (2000) Monoclonal antibody MA454 reveals a heterogeneous expression pattern of MAGE-1 antigen in formalin-fixed paraffin embedded lung tumours. *Br. J. Cancer*, **83**, 493–497.
59. Clotman,F., De Backer,O., De Plaen,E., Boon,T. and Picard,J. (2000) Cell- and stage-specific expression of MAGE genes during mouse spermatogenesis. *Mamm. Genome*, **11**, 696–699.
60. Jones,P.A. and Baylin,S.B. (2002) The fundamental role of epigenetic events in cancer. *Nature Rev. Genet*., **3**, 415–428.
61. Hu,X. and Lazar,M.A. (1999) The CoRNR motif controls the recruitment of corepressors by nuclear hormone receptors. *Nature*, **402**, 93–96.