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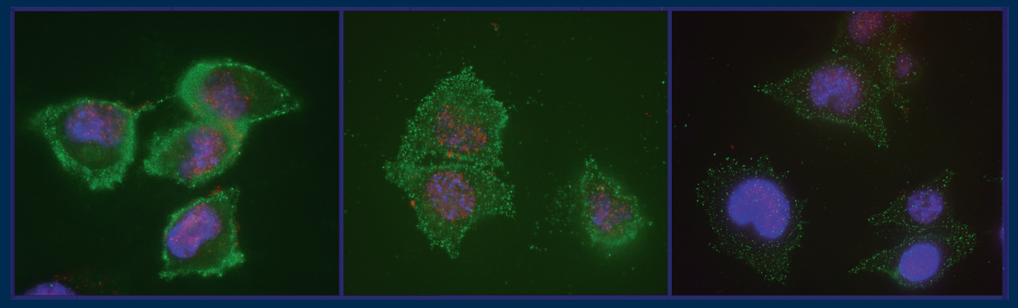

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To
My parents Jalal and Parvin
&
Shahryar and Armin

List of publications

The thesis is based on the following publications, which are referred to in the text by Roman numerals as follows:

I. **Eskandarpour M**, Hashemi J, Kanter L, Ringborg U, Platz A, Hansson J. Frequency of UV-Inducible *NRAS* Mutations in Melanomas of Patients With Germline *CDKN2A* Mutations. *Journal of the National Cancer Institute 2003; 95(11):790-798.*

II. **Eskandarpour M,** Kiaii S, Zhu C, Castro J, Sakko AJ, Hansson J. Suppression of Oncogenic *NRAS* by RNA Interference Induces Apoptosis of Human Melanoma Cells. *International Journal of Cancer 2005; 115, 65-73.*

III. **Eskandarpour M**, Huang F, Reeves KA, Clark E, Hansson J. Oncogenic *NRAS* has a Pivotal Role in the Malignant Phenotype of Human Melanoma Cells. *Submitted for publication.*

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Abbreviations

ARF	alternative reading frame
bFGF	basic fibroblast growth factor
CDK	cyclin dependent kinase
CDKN2A	cyclin dependent kinase inhibitor 2A
CGH	comparative genomic hybridization
CIP1	CDK interacting protein 1 (CDKN1A)
CMM	cutaneous malignant melanoma
CPD	cyclobutane pyrimidine dimmer
ERK	extracellular signal regulated kinase
FAK	focal adhesion kinase
FTI	farnesyltransferase inhibitors
GEF	guanine nucleotide exchange factor
GPCR	G-protein-coupled receptor
HDM2	human double minute 2
MAPK	mitogen activated protein kinase
MC1R	melanocortin 1 receptor
MEK	MAP-ERK kinase
MLCK	myosin light chain kinase
MMP	matrix metalloprotease
NF-κB	nuclear factor-kappa B
NMA	neuromedin A
Nore 1	novel ras effector 1
PI3K	phosphoinositide 3 kinase
PIP3	phosphatidylinositol tri-phosphate
PKC	protein kinase C
PTEN	phosphatase and tensin homolog on chromosome ten
RASSF	RAS association domain family protein
RB	retinoblastoma
RGP	radial growth phase
RNAi	RNA interference
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
SCF	stem cell factor
siRNA	small interfering RNA
uPA	urokinase plasminogen activator
uPAR	urokinase plasminogen activator receptor
UV	ultraviolet
VEGF	vascular epithelial growth factor
VGP	vertical growth phase
WAF1	wildtype p53 activated fragment 1 (CDKN1A)

Abstract

Cutaneous malignant melanoma is an aggressive tumor of melanocytes in the skin with rapidly increasing incidence. Patients with advanced disease have a poor prognosis since the tumor is usually resistant to current therapies. Therefore, the development of novel strategies for preventing and treating melanoma is important. To explore novel therapies we need to find appropriate targets and for that knowledge about the biology of melanoma is important. There is growing evidence suggesting that NRAS has an important role in tumorigenesis and tumor maintenance in malignant melanoma and that the RAS-RAF-ERK signaling pathway is constitutively activated through multiple mechanisms, one of which is activating mutations in *NRAS* gene.

In an initial study, we investigated the occurrence of activating mutation in the *NRAS* gene in a subset of patients with hereditary melanoma carrying germ line *CDKN2A* alterations. From this study we found differences in the frequency of *NRAS* mutations between hereditary and sporadic melanomas. Activating mutations in *NRAS* codon 61 were found in 95% (20/21) of primary hereditary melanomas but in only 10% (1/10) of sporadic melanomas. We also detected multiple activating *NRAS* mutations in tumor cells from different regions of individual primary hereditary melanomas. Activating mutations that were detected in the primary melanomas of these patients were also retained in their metastases. We also found that *NRAS* mutations are present in potential precursor lesions (dysplastic nevi). We concluded that the high frequency of *NRAS* codon 61 mutations detected in these hereditary melanomas may be the result of a hypermutability phenotype associated with the hereditary predisposition for melanoma development in patients with germline *CDKN2A* mutations.

The presence of a mutant *NRAS* oncogene in sporadic and familial melanomas implies that the *NRAS* oncogene may be an important target for prevention and treatment of melanomas. Therefore, to better define the role of this oncogene in melanoma development, we specifically targeted this mutant oncogene using RNAi techniques and studied the effect of suppression of mutant *NRAS* on melanoma cell lines. Suppression of oncogenic *NRAS* in these cell lines resulted in decreased proliferation, increased apoptosis as well as decreased phosphorylation of ERK and Akt, and also reduced expression of NF-κB and cyclin D1 downstream in the NRAS signaling pathway. To follow up this investigation, we studied the effect of siRNA against mutant *NRAS* on

gene expression profiles in melanoma cell lines which carry oncogenic *NRAS* mutations. We could show the impact of knockdown of the *NRAS* oncogene on different cellular processes. For instance, we observed a disability of cells with respect to migration and invasion, which is accompanied by down-regulation of EphA2, uPAR and cytoskeleton proteins such as leupaxin, α-actinin, paxillin, and vinculin. These cells also showed inhibition of cell proliferation accompanied by downregulation of two cyclins, cyclin D1, cyclin E2, and up-regulation of HBP1 repressor. In summary, we conclude that the use of siRNAs against $NRAS^{Q61R}$ is an important tool in suppressing oncogenic NRAS signaling, which might contribute to the development of more specific melanoma therapy in the subset of patients with tumor with *NRAS* mutations.

Cutaneous malignant melanoma

Cutaneous malignant melanoma (CMM) is a tumor derived from transformed genetically altered epidermal melanocytes in the skin, as a result of complex interactions between genetics, and environmental factors. Melanocytes, which are localized in the basal layer of the epidermis, synthesize and transfer melanin pigment to surrounding keratinocytes, thereby protecting these cells from harmful exposure to UV radiation (1). Melanoma is a most rapidly increasing malignancy. In some parts of the word, especially in Western countries with Caucasian populations, the number of new cases of melanoma each year is increasing faster than any other cancer (2, 3). In Sweden, the incidence of CMM has been rising rapidly during the last decades. At present, the overall incidence of cutaneous melanoma in Sweden is approximately 2100 cases per year corresponding to yearly incidence of approximately 25.5/100,000 for males and 21.3/100,000 for females and it is the eight most common cancer in Sweden (4). CMM is the most dangerous type of human skin cancer since the tumors may metastasize and often are resistant to current systemic therapy. Unfortunately, the rising incidence of cutaneous melanomas has been accompanied by an increased mortality.

Melanoma tumor progression

Clinical and histological studies have resulted in defining relatively distinct steps of melanoma development and progression. Step 0, normal melanocytes; step 1, common acquired and congenital nevi with structurally normal melanocytes; step 2, dysplastic nevi with structural and architectural atypia; step 3, melanoma in situ (MIS) and radial growth phase (RGP), nontumorigenic primary melanomas without metastatic competence; step 4, vertical growth phase (VGP), tumorigenic primary melanomas with competence for metastasis; and step 5, metastatic melanoma. As in any neoplastic system, individual melanomas can skip steps in their development, appearing without identifiable intermediate lesions (5).

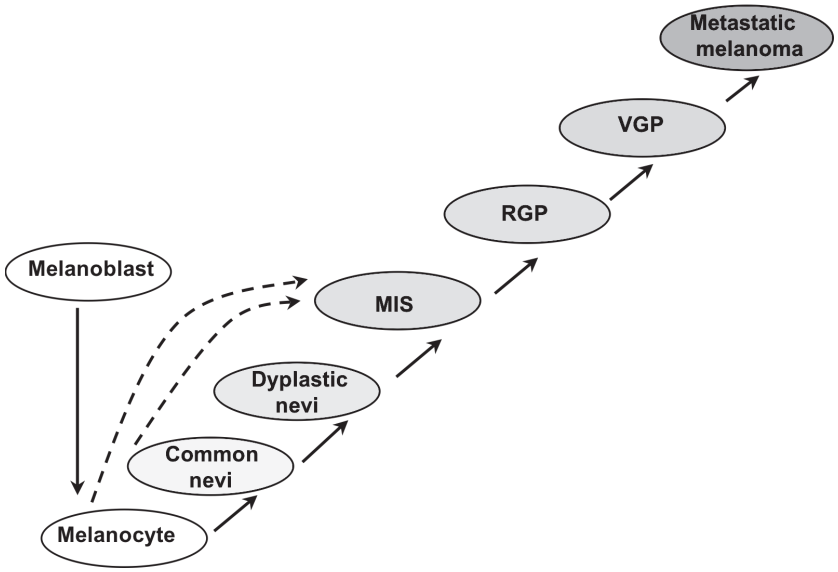

Figure 1. Melanoma tumor progression. The model, developed by Drs. Clark, Elder, and Guerry, implies that melanoma commonly develops and progresses in a sequence of steps. However, melanoma may develop directly from normal (and precursor) cells. The roles of melanocyte stem cells, melanoblasts (immature melanocytes) in melanogenesis remain poorly defined. (modified from DeVita et al. (5) and www.wistar.org/herlyn/.

The progression from each stage to the next is associated with specific biologic changes, which are based on experimental models and clinical and histopathologic observations. The transition from the mature melanocyte to the formation of a nevus is characterized by a disruption of cell-cell cross-talk between melanocytes and keratinocytes, which leads to an escape of the melanocyte from the regulatory control of keratinocytes. Thus, nevus cells show limited proliferation and cells in common acquired nevi have no apparent chromosomal aberrations. Nevi can develop not just through a stimulatory event, but also through loss of control of keratinocytes over melanocytes. Progression from the melanocyte or common acquired nevus cell to a dysplastic nevus or RGP melanoma most likely involves the onset of genetic aberrations. The cells show cytologic atypia, they can separate from the basement membrane without undergoing apoptosis, and the entire lesion shows architectural atypia. Cells from RGP lesions have biologic properties in vitro that are intermediate between benign and malignant. VGP primary melanomas are characterized as expanding nodules that invade deep into the dermis. VGP primary melanomas are

highly aneuploid. Biologically, the cells are relatively plastic, some also acquire metastatic competence. Metastatic cells show a high level of genetic instability, and phenotypic plasticity, depending on the environment and any selective pressure placed on the cells (5, 6). Metastatic cells are highly motile and independent of growth factors, and have acquired the capacity to invade other tissues and organs

Melanoma risk factors

As in most types of cancers, there are two sets of factors that present significant risk for melanoma in humans: host characteristics and environmental factors.

Epidemiologic studies have identified host factors important for risk of melanoma. These include family history of melanoma, alterations in melanoma susceptibility genes, number and type of nevi, skin type and pigmentation (7). Melanoma is more common in individuals with fair skin, blue or green eyes, red or blond hair, many freckles and in individuals who react to sunlight by burning rather than tanning.

UV radiation is the most important environmental factor in the development of melanoma. Intermittent repeated exposures to sunlight from childhood are epidemiologically shown to be a major cause of melanoma. UVB, a minor component of sunlight reaching the earth, is experimentally demonstrated to be the most effective radiation to induce skin cancer in animals. UVB can cause DNA damage, particularly cyclobutane pyrimidine dimmers (CPDs) and (6-4) photoproducts, which may induce mutations in the epidermal cells, leading to the development of cancer. UVB is also known to upregulate gene expression through intracellular signal transduction pathways, which may contribute to the development of skin cancer at the tumor promotion stage. In addition, UVB is proven to suppress immune reactions, and to induce tolerance to antigens, which have been applied topically or systemically in experimental animals. These three effects of UVB on the skin are believed to cooperatively contribute to producing skin cancer in humans (8). UVA is the major portion (approximately 95%) of UV light reaching the earth surface and it is reported to lead to benign and malignant tumor formation. UVA penetrates through the epidermis deep into the dermis. UVA-mediated cellular damage occurs primarily through the formation of reactive oxygen species (ROS). After UVA exposure, singlet oxygen, H_2O_2 (hydrogen peroxide), superoxide and hydroxyl free radicals are generated. These interact and can cause damage to cellular proteins, lipids and saccharides. UVA can also indirectly produce structural damage to the DNA and 8-

oxo-guanine is the most common lesion inhibits DNA repair as well as affecting numerous signal transduction pathways and impairing the immune system (9).

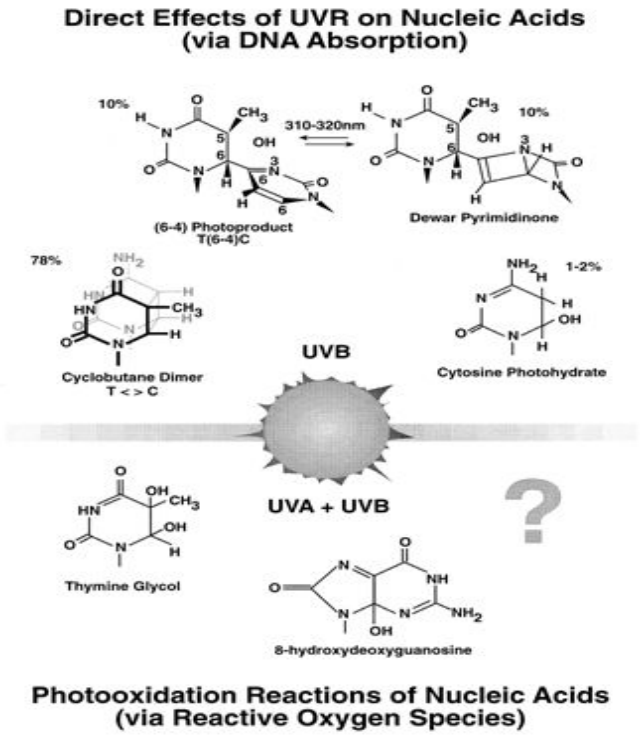

Figure 2. UV radiation is subdivided into three wavelength bands; UVA (320-400 nm), UVB (290-320 nm) and UVC (220-290 nm). UVA and UVB radiation are proved to produce DNA damage directly and indirectly through oxidative stress. UVB induces formation of cyclobutane pyrimidine dimmers and 6-4 photoproducts. The wavelength of UVA is too long to be absorbed by DNA, therefore causing DNA damage via reactive oxygen species (ROS). Modified from http://images.google.com/

Pathways involved in melanoma biology

Several of the key alterations in melanoma tumorigenesis affect the regulation of cellular proliferation and viability, including the RAS-RAF-ERK, PI3K-AKT and $p16^{INK4}$/CDK4/RB pathways (10, 11). There is growing evidence suggesting a key role for the RAS-RAF-ERK (MAPK) pathway in the development of malignant

melanoma (11-13). To review the roles of oncogenes and tumor suppressor genes involved in melanoma biology, I will first give a general view of these pathways.

RAS signaling pathway

The RAS gene family is among the most frequently activated oncogenes in human cancer. RAS proteins are small monomeric GTPases that play a key role in transducing growth signals from cell surface receptors to the nucleus. Activating point mutations in RAS promote cellular transformation by growth factor independent stimulation of cell proliferation and cell survival. In humans, three *RAS* genes have been identified: *HRAS*, *NRAS* and *KRAS*. The RAS proteins display high sequence conservation (14). Like other GTPases, RAS proteins function as regulated GDP-GTP binary switches. Extracellular signals are received by membrane-bound receptors such as G-protein-coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs). These receptors activate guanine nucleotide exchange factors (GEFs), which then cause transient activation of RAS. Activated RAS-GTP adopts a conformation that facilitates binding to, and activation of, downstream effectors. RAS signaling is terminated by RAS GAP-mediated stimulation of hydrolysis of bound GTP to GDP, and release of the bound effector (15). The most common *RAS* mutations in tumors occur at sites critical for RAS regulation. Single point mutations in codons 12, 13, 59 and 61 completely abrogate the GAP-induced GTP hydrolysis of RAS. Unlike normal RAS, oncogenic *RAS* remains constitutively in the active GTP-bound form. Thus, the transforming properties of oncogenic *RAS* are based on continuous activation of its down-stream effectors (16).

The three main RAS effectors, RAF kinase, RAL-GEFs, and PI3K, bind the same region of RAS-GTP, the 32-40 domain. All three effectors increase their in vivo activity after RAS binding (17) (Figure 2).

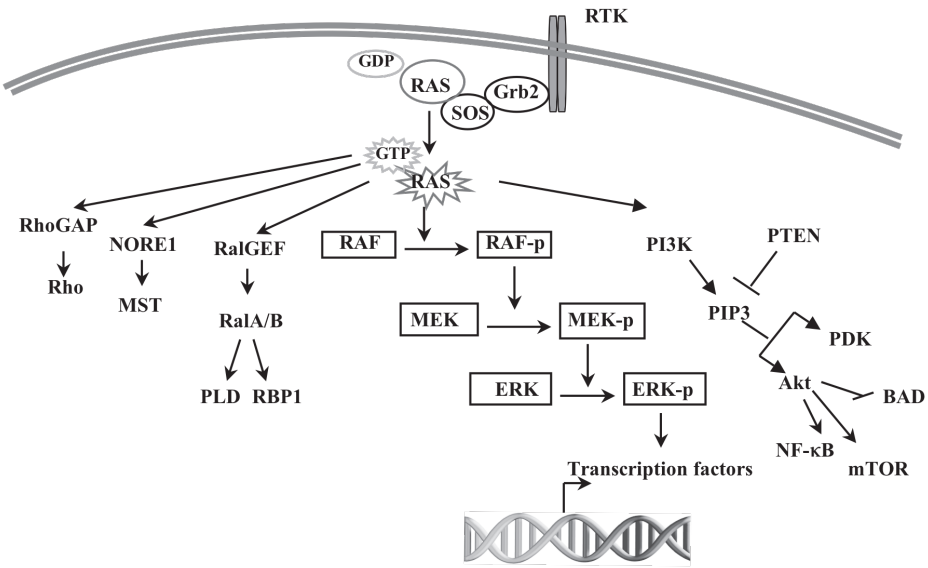

Figure 3. RAS is a GTPase, which is activated by GDP to GTP exchange. Active RAS is able to stimulate many effector proteins. These include RhoGAP, GEFs such as RalGDS, and a number of protein kinases such as PI3K, PKC, RAF, MEK, and ERK.

RAS-RAF-ERK signaling

The best known RAS stimulated pathway, which has been most directly linked to growth promotion activities, starts with activation of the RAF family of serine-threonine kinase. There are three members of the RAF class: ARAF, BRAF and CRAF. Recent studies suggest that RAS interacts with the amino terminal portion of RAF located in the cytoplasm in a complex with 14-3-3 protein, which is an essential cofactor of RAF kinase activity(18). This interaction causes a RAF conformational change unmasking one or more residues of phosphorylation and stabilizing a new catalytically active RAF conformation. After these conformational changes, RAF is anchored in the plasma membrane. The RAF-RAS binding is transitory and once attached to plasma membrane, RAF activity becomes independent of RAS and is no longer influenced by dominant negative *RAS* mutations. The phosphorylated RAF activates a series of kinases in a cascade that by amplifying low level cell membrane signals, modulate the activity of several cytoplasmic and nuclear factors (19). Finally, the signals transmitted to the nucleus determine the activation of transcriptional factors, such as the members of Ets family (20). These transcriptional factors

influence the expression of specific genes encoding proteins involved in the control of cellular proliferation and/or differentiation (17) (Figure 2).

RAS-PI3K-AKT signaling

Another well-characterized RAS effector is PI3K, which has a role in both cell proliferation and survival. PI3-kinases are lipid kinases that phosphorylate the 3'-OH position of inositol phospholipids. RAS-GTP can bind and activate the catalytic subunit of this enzyme that generates PI(3,4,5)P3 (phosphatidylinositol tri-phosphate) by phosphorylating PI(4,5)P2 in the 3-position. PI(3,4,5)P3 acts directly as a second messenger, binding several cytoskeleton kinase proteins and modulating their activity by conformational changes and/or membrane translocation. PI3K class I is consisted of a 110 kDa catalytic subunit and an 85 kDa regulatory subunit and are activated by RAS or by RTKs. The p110 catalytic subunit contains the RBD (RAS binding domain), to which RAS-GTP binds. The catalytic subunit also contains a p85 binding domain and the kinase domain. A major downstream target of PI3K is the serine/threonine kinase AKT (also called PKB). In mammals, three different isoforms of AKT (AKT1, 2, 3) have been described. This protein regulates extracellular growth signals using the lipid phosphatidylinositol phosphate (PIP3) as an intracellular second messenger. In the presence of growth factor signaling, the intracellular level of PIP3 rises, leading to phosphorylation of AKT, which is known to promote cell cycle progression and inhibit apoptosis. PTEN, is a negative regulator of the PI3K-AKT pathway (21). PTEN regulates PIP3 levels, and its inactivation results in accumulation of PIP3, AKT hyperphosphorylation, and enhanced cell survival and proliferation (22). The PI3K-AKT pathway is often hyperactive in melanoma. In addition, elevated phospho-AKT levels appear to correlate adversely with patient survival (21) (Figure 2).

RAS-RAL signaling

Another class of RAS effectors is the GEF family (RalGDS) that serve as activators of the RAL small monomeric GTPases. RAL seems to interact with Cdc42 and RAC-GAP. Rho, RAC, and Cdc42 constitute another family of monomeric G proteins that play an important role in cytoskeleton remodeling and activate kinases regulating the activity of various transcriptional factors. The signaling activity of RAS GTPases occurs not only through engagement of direct effectors, but also by the recruitment of

other GTPases, especially other members of the RAS subfamily (e.g. Rap) and members of the Rho subfamily (e.g. RhoA, Rac1 and cdc42). This hierarchical networking between different RAS isoforms is controlled, in part, by interactions with GEFs, GAPs and downstream effectors. For instance, RalGEFs are important in RAS-mediated transformation. RalGEFs, such as RalGDS, link RAS signaling to the activation of the small GTPases RalA and RalB. In human cells, the RAS effector loop mutant that preferentially activates RalGDS was able to transform cells (23) (Figure 2).

Other effectors of RAS signaling pathway

Other potential effectors are AF-6, protein kinase C-zeta (PKC-zeta), and Nore1. RAS may use the AF-6 effector to modulate intercellular binding and communication. PKC-zeta displays homology with RAF. A recent study suggests that PKC zeta can activate the RAS pathway independently of RAS (24). Recently, members of the RASSF (RAS association domain family protein) gene family that potentially act as tumor suppressors have been identified as candidate RAS effectors. Loss of expression of Nore1 (novel RAS effector 1) and RASSF1, members of the RASSF gene family, has been observed in a variety of cancers (25). The interaction of RAS with Nore1 has been shown to regulate apoptosis (26) (Figure 2).

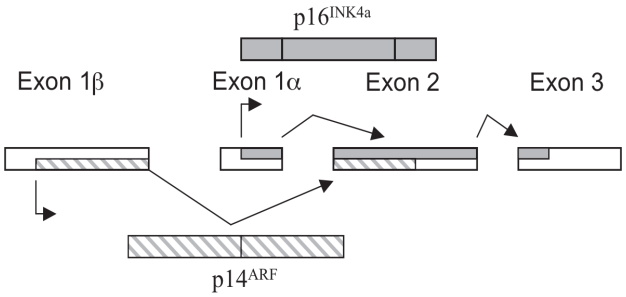

Figure 4. The INK4A/ARF locus is located on human chromosome 9p21. The two products of the INK4A/ARF locus encode $p16^{INK4A}$ and $p14^{ARF}$ ($p19^{ARF}$ in mice). $p16I^{NK4A}$ indirectly regulates RB function and $p14^{ARF}$ indirectly stabilizes p53.

RB and p53 pathways

The mammalian INK4A-ARF locus uniquely encodes two distinct proteins, $p14^{ARF}$ and $p16^{INK4A}$, which both function in cell cycle control and tumor suppression, and are involved in two separate pathways: $p16^{INK4}$-RB and $p14^{ARF}$-p53, respectively (Figure 4). These two gene products are transcriptionally initiated from separate promoters and read in two different reading frames: $p16^{INK4A}$, referred to as INK4A, and $p14^{ARF}$, referred to as ARF. INK4A positively regulates the RB tumor suppressor by inhibiting CDK4, while wildtype ARF protein forms a complex with HDM2 and p53 and blocks nuclear export of both p53 and HDM2, leading to p53 stabilization and activation in nucleus (27) Figure 5.

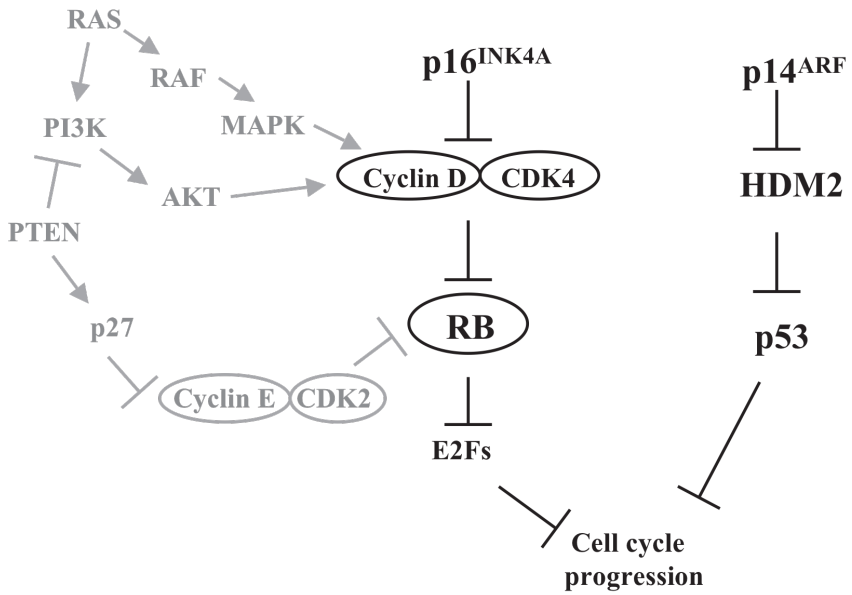

Figure 5. The ARF-p53 and $p16^{INK4A}$-RB pathways. RAS cooperates with RB pathway and affect cell cycle progression by upregulating Cyclin D, CDK4, and/or Cyclin E/CDK2, which ultimately inactivate RB.

Mutations in oncogenes and suppressor genes in melanoma:

The molecular genetic alterations during melanoma development are partially characterized. Mutations in many different genes, such as *NRAS*, *BRAF* and *PTEN* and mutations and deletions of *CDKN2A* have all been implicated in the pathogenesis

of malignant melanoma. Selected genetic alterations in malignant melanomas are summarized in table 1(25).

Table 1: Genetic alterations in malignant melanoma. Modified from Dohmem et al. 2007.

Gene type	Gene	Alteration frequency in melanoma	Alteration type(s)
Oncogenes	*BRAF*	50-70%	mutation
	NRAS	15-30%	mutation
	AKT3	43-60%	overexpression
	CCND1	6-44%	amplification
	MITF	10-16%	amplification
Tumor suppressors	*CDKN2A*	30-70%	deletion, mutation or silencing
	PTEN	20-40%	deletion or mutation
	APAF-1	40%	silencing
	P53	10%	loss or mutation

RAS: RAS genes are among the most frequently mutated genes in human cancers, but display distinct spectra of mutations of *NRAS*, *HRAS*, and *KRAS* in different malignancies. Human melanomas carry mutations almost exclusively in *NRAS* (5-36%), with 90% of mutations localized to codon 61 (28-30). *HRAS* and *KRAS* are much less frequently mutated. *NRAS* mutations are found in approximately 10% of common acquired nevi (31, 32) and it was recently determined that 81% (26/32) of congenital nevi harbor mutations in *NRAS,* while *BRAF* mutations were absent in such lesions (33). Albino et al have reported that 24% of cultured metastatic and 12% of noncultured primary and metastatic melanomas carry *NRAS* alterations, although others reported higher frequencies in primary tumors and have suggested that mutations correlate with metastasis or disease progression (34). In one study, however 33% of primary and 26% of metastatic melanoma samples were found to harbor mutations in the *NRAS* gene (35, 36). Activating *NRAS* mutations have been correlated with the nodular melanoma subtype and with sun exposure (37-40). The presence of *NRAS* mutations in tumor associated nevi and RGP lesions suggests that *NRAS* activation occurs at an early stage during melanoma development (35, 36).

RAF: The RAF family of serine-threonine kinases function downstream of RAS in signal transduction. The most commonly mutated component of RAS-RAF-ERK pathway in melanoma is BRAF. *BRAF* is mutated in 50 % - 70% of melanomas and the most common mutation is a glutamic acid for valine substitution at position 600

(V600E) (36, 41). About 80% of benign nevi carry this mutation as well, but so far no mutations have been detected in uveal melanomas (42). The *BRAF* gene resides at chromosome 7q34, a chromosomal region that is frequently amplified in melanoma tumors (43).

PI3K: Mutations of *PIK3CA* (encoding the p110 alpha catalytic subunit of PI3K) are rarely detected in melanoma, and are found in less than 1% of primary melanomas and 3% of melanoma metastases, with no evidence of amplification of any PI3K subunit in primary melanomas by array CGH (44, 45).

AKT: Constitutive activation of *AKT* has been shown to be a potent oncogenic lesion for melanocyte transformation (46). AKT3 is the major isoform deregulated in melanoma. DNA copy gains involving the *AKT3* locus have been described in melanoma, and selective *AKT3* activation may characterize 40%–60% of sporadic tumors (47). Recent data have suggested that activation of different AKT isotypes may result in distinct effects on cell proliferation and survival. For example, among the three AKT isotypes, AKT3 was correlated most strongly with melanoma tumor progression, and targeted AKT3 depletion triggered apoptotic signaling (47).

PTEN: PTEN is another important element in signal transduction altered in human melanomas. PTEN was identified as a tumor suppressor candidate from the chromosome region 10q23-24 which is frequently deleted in gliomas and melanomas (48, 49). Cytogenetic evidence showed that 10q loss is an early and frequent event in melanomas. *PTEN* encodes a protein with extensive homology to dual specificity protein phosphatases and, like RAS, it is implicated in the pathways that control apoptosis through AKT. PTEN, is a negative regulator of the PI3K-AKT pathway. When AKT is phosphorylated, it has several activities but functions mainly to antagonize apoptosis. Several groups have shown that PTEN loss impairs apoptosis. In melanoma, allelic loss or altered expression of PTEN occurs in 20% and 40% of tumors, respectively (21, 50), although somatic point mutations and homozygous deletions are rarely observed. Functionally, ectopic expression of PTEN in *PTEN*-deficient melanoma cells can abolish phospho-AKT activity, induce apoptosis, and suppress growth, tumorigenicity, and metastasis (51, 52). Interestingly, *NRAS* and *PTEN* appear to be mutually exclusive in melanoma suggesting, that *NRAS* mutations

and *PTEN* alterations may have overlapping functions in melanoma (53). *BRAF* mutations and *PTEN* alterations on the other hand, have been described to coexist in the same melanoma cell lines suggesting that *BRAF* activation and *PTEN* loss can cooperate to activate ERK and AKT in melanoma (10).

c-KIT: The *c-KIT* gene encodes a RTK that serves as the receptor for SCF (stem cell factor). Immunohistochemical studies have linked progressive loss of *c-KIT* expression with the transition from benign nevi to primary and metastatic melanomas (54). A recurrent L576P mutation in *c-KIT* has recently been reported in melanoma. Among 153 cases examined, Willmore and colleagues identified four metastatic melanomas with robust expression of c-Kit on immunohistochemistry and three of them harbored a L576P mutation with selective loss of the normal allele. L576P is a known GIST-associated mutation that maps to the 5' juxtamembrane domain where most activating *KIT* mutations cluster (55, 56). In a study of 102 primary melanomas mutations and/or copy number increases of *C-KIT* were found in 39% of mucosal, 36% of acral, and 28% of melanomas on chronically sun-damaged skin, but not in any melanomas on skin without chronic sun damage. Seventy-nine percent of tumors with mutations and 53% of tumors with multiple copies of *KIT* demonstrated increased KIT protein levels (57).

$p16^{INK4A}$: Sporadic and familial melanomas have been associated with mutations, loss of heterozygosity, and deletions in the CDKN2A locus, which is important for the normal progression of the cell cycle. Somatic inactivation of CDKN2A ($p16^{INK4A}$ and $p14^{ARF}$) is frequently detected in melanoma cell lines but less commonly in primary tumors (58). Biallelic *CDKN2A* deletions however, have been reported in about 45% of CMM metastases and associated with adverse prognosis, emphasizing the importance of this locus in disease progression (59).
Expression of $p16^{INK4A}$ has been reported to correlate inversely with aggressive melanoma behavior; nevertheless, mutations at this locus also have been detected in normal melanocytes and in benign compound nevi lacking signs of clinical or histologic atypia (27).

p53 and $p14^{ARF}$: Mutations in TP53 (the p53 gene) are the most common contributors to the etiology of neoplastic disorders, but their role in the pathogenesis

of melanoma has not been established. TP53 mutations are in infrequent in primary human melanoma, thus, there is no apparent correlation between TP53 gene rearrangements or altered expression of the p53 protein and progression of melanocytic lesions. Nevertheless, some authors suggest that p53 could have a more complex role in the pathogenesis of melanoma by acting on downstream effector genes, such as HDM2, GADD45, and CIP1/WAF1 (27). ARF has been thought to function predominantly as a positive regulator of the p53 tumor suppressor through inhibition of HDM2. Loss of ARF could thus explain the lack of TP53 mutations in melanomas. However, experimental evidence has recently showed, that ARF functions as a tumor suppressor by inducing p53-independent senescence. Accordingly, ARF– and p53- deficient mice do not exhibit identical tumor phenotypes, and ARF interacts with a variety of other proteins, including E2F1, Myc, NF-κB, and can function independently of p53 in ribosome biosynthesis, DNA damage response, apoptosis, and autophagy (60).

Chromosomal abnormalities in melanoma

All studies on chromosomal aberrations, from cytogenetics to CGH analysis, show a noticeable diversity in genomic aberrations, reflecting the heterogeneous nature of CMM. Some of the aberrations are listed below.

Sporadic dysplastic nevi show a high rate of loss of heterozygosity of chromosomes 1p and 9q31; some variants show a predominant allelic deletion at chromosome 9p21 (INK4 locus). In sporadic melanomas, chromosomes 1, 6, 7, 9, and 10 are most commonly affected. In chromosome 1, structural rearrangements are frequent and include translocations or deletions of 1p12–22.3, loss of heterozygosity in 1p3, and deletion of 1p36.3 (61). In addition, one study found a linkage to chromosome 1p36 (61). The abnormalities in chromosome 7 consist of chromosomal losses or gains, with the latter associated with increased expression of the receptor for epidermal growth factor located on 7p12-13 (62). Loss of chromosome 10 is frequently associated with melanoma progression, possibility related to loss of the NMA (neuromedin A) gene, located at 10p11.2-12.3, which is a potential inhibitor of metastatic capability (63).

The results of array-based comparative genomic hybridization (array-CGH) analyses of melanocytic neoplasms have shown different patterns of chromosomal aberrations

in benign melanocytic nevi and melanoma. In melanomas the genetic alterations depended on anatomical site, Clark's histogenetic type, and sun-exposure pattern. Melanomas on acral sites have significantly more aberrations involving chromosomes 5p, 11q, 12q, and 15, as well as focused gene amplifications. Melanomas classified as lentigo malignant melanomas or occurring on severely sun-damaged skin showed markedly more frequent losses of chromosomes 17p and 13q (48). A recent genome-wide study in melanocytic lesions, using array-CGH, showed the most common overlapping regions with losses were mapped to 9p24.3-q13, 10 and 11q14.1-qter, whereas copy number gains were most frequent on chromosomes 1q, 7, 17q and 20q. Amplifications were defined for oncogenes such as MITF (3p14), CCND1 (11q13), MDM2 (12q15), CCNE1 (19q12) and NOTCH2 (1p12) (64).

Gain of chromosome 7q is common in CMM suggesting that BRAF, located on 7q34, is a target for gene amplification (65). Moreover, cyclin D1, a down-stream target of the MAPK pathway and a $p16^{INK4A}$ antagonist, is amplified in acral lentigiuous CMM in which *BRAF* and *NRAS* mutations are infrequent. Frequent findings of homozygous deletions of the 9p21 locus confirmed the importance of the INK4 gene locus(66). Homozygous deletions on 10q23 where *PTEN* gene is located are also frequent in melanoma (67).

Susceptibility genes in melanoma

Two genes conferring susceptibility to melanoma have been identified within high-risk families, *CDKN2A* and *CDK4*. Both of these genes are important in controlling cell division. As stated above, *CDKN2A* codes for two proteins, $p16^{IKN4A}$, a tumor suppressor, which has a key role in the CDK4–cyclin D–retinoblastoma protein (RB) pathway and in the regulation of the G1 checkpoint of the cell cycle and $p14^{ARF}$, important in the p53 pathway (Figure 5).

Germline *CDKN2A* mutations occur in many patients with a hereditary predisposition to melanoma (68-71). Overall, approximately half of all melanoma-prone families, the disease shows genetic linkage to 9p21, the chromosome arm where the *CDKN2A* gene is located, and approximately 40% of these families carry germline mutations in *CDKN2A*. In Sweden, a *CDKN2A* mutation consisting of a 3-base-pair (bp) insertion leading to an extra arginine residue in codon 113 in exon 2 (113insR) has been identified in several Swedish families (68, 69). Aitken et al. in 1999 found mutations

of *CDKN2A* in 10.3% of a population of high-risk families from Australia. They estimated that 0.2% of melanoma in Australia was due to mutations in *CDKN2A*(72).

Many of the recurrent mutations in $p16^{INK4A}$ that have been described are founder mutations dating back up to 100 generations, including the 113insR a Swedish founder mutation (73). Such founder mutations have been described in a number of different populations. Families with mutations in *CDKN2A* that affect only $p14^{ARF}$ are much less common than mutations that affect $p16^{INK4A}$ with or without affecting $p14^{ARF}$ (7, 74).

A recent study characterized a germ line deletion, including the entire INK4/ARF locus in a French melanoma-neural system tumor family and identified a new large antisense RNA (named ANRIL), whose expression co-clusters with ARF. The identification of this large antisense noncoding RNA could be important in cancer molecular genetics both in hereditary predisposition to melanoma and in somatic alteration of the p15/CDKN2B-p16/CDKN2A-p14/ARF cluster observed in a large proportion of cancers (75).

Germline mutations of CDK4, an RB-kinase that is inhibited by INK4A, have also been identified in a small number of melanoma-prone kindreds in different populations (US, UK, Norway, France, Latvian, Australia). *CDK4* is located on chromosome 12q14. So far, only seven kindreds carrying *CDK4* germline mutations have been documented. Two US families carry an R24C mutation and the remaining carry R24H germ line CDK4 mutations (76). These mutations thus target a conserved arginine residue (R24) and render the mutant protein insensitive to inhibition by the INK4 class of cell cycle inhibitors. Thus, the *CDK4* germline mutation identified, abolishes the binding of CDK4 to $p16^{INK4A}$, providing further evidence that impaired p16-mediated cell cycle regulation may predispose carriers to melanoma development. Melanomas from patients harboring these germline *CDK4* mutations do not demonstrate somatic INK4A inactivation, and inactivation of INK4A and CDK4 activation are thus mutually exclusive (77). Germ line mutations in *CDKN2A* and *CDK4* genes, however, only account for minority of families with melanoma. Mutations in these genes are more frequent in families with many affected cases with CMM, and they are thus regarded as high risk genes.

Variations in other genes have also been associated with increased risk of melanoma, particularly MC1R (the melanocortin 1 receptor gene). The MC1R gene, located on 16p24, which is involved in the regulation of melanin production by melanocytes, is a

low-risk susceptibility gene. Some polymorphic variants of MC1R cause a switch from eumelanin (brown-black) to pheomelanin (red-yellow) synthesis and are associated with sensitive skin type, poor tanning ability and red hair color. It has been shown that such MC1R variants are also associated with the risk of developing both sporadic and hereditary melanoma and acts as a risk modifier in some melanoma-prone families with *CDKN2A* mutations (78). Some MC1R genetypes thus increased the melanoma penetrance in *CDKN2A* gene carriers from 18 to 55% in Dutch melanoma families (79).

Cooperation of RAS and CDKN2A

Indications of cooperation of activation of oncogenic *RAS* accompanied by inactivation of the *CDKN2A* locus (INK4A, ARF) mostly comes from melanoma animal models. Transgenic mice that express a mutant form of *HRAS* specifically in melanocytes using the tyrosinase (tyr) promoter showed melanocytic hyperplasia with intense skin pigmentation, which after treatment with carcinogens progressed into skin melanoma with metastasis formation in lymph nodes and lung (80). Breeding of Tyr::*H-RAS*^{V12G} transgenic mice on an INK4A/ARF-deficient background resulted in the development of highly vascularized but amelanotic melanomas resembling nodular melanoma (81). No metastasis was observed in these mice. Melanoma tumors regressed when *HRAS*^{V12G} expression was removed in an inducible melanoma model. This suggests that RAS signaling is essential for both initiation and maintenance of melanoma (82, 83).

In contrast, a transgenic mouse line which expresses the oncogenic form of human *NRAS* (*NRAS*^{Q61K}) in melanocytes on an *INK4A*-deficient background developed melanotic melanomas with high penetrance, which acquire a metastatic phenotype, spreading to lymph nodes and other distal sites (e.g., lung and liver), thus mimicking the human condition (84).

Moreover, it has also been shown in an animal model that oncogenic *NRAS* collaborates with deficiency in ARF, to fully transform melanocytes. The role of ARF in melanocyte transformation was further characterized in this model. The results showed that in concert with mutant *NRAS*, the loss of ARF, but not p53 alone, allowed melanocytes to form tumors readily in vivo (85).

RAS and cell cycle regulation

Oncogenic *RAS* often deregulates the S-phase entry, and therefore cellular RAS is likely to function as part of the cell cycle control. The role of RAS in cell proliferation and cell cycle control is highlighted by several findings. RAS is activated by serum and various growth factors, and its expression in quiescent immortal mouse fibroblasts leads to cell cycle entry and transformation (86). Treatment of quiescent cells with neutralizing RAS-antibody, in contrast, prevents S-phase entry by serum (87). Besides the growth factor-induced RAS activation, RAS becomes activated independently of extracellular signals in mid-G1-phase, demonstrating the involvement of RAS activity at multiple steps during the G1-phase and S-transition. These results suggest an important role for RAS in cell cycle entry (24). The exact mechanisms of RAS-action in cell cycle regulation are largely unknown. However, cyclin D1, a regulatory subunit of several CDKs, was shown to be upregulated in RAS-transformed cells, leading to an increased proliferation rate or shortened G1-phase of the cells (88). These effects are reversed by cyclin D1 antisense oligonucleotides. RAS activates *CCND1* (encoding cyclin D1) transcription, and dominant negative *RAS* prevents serum induction of cyclin D1 protein (89). RAS-effectors including RAF and MEK have also been shown to increase cyclin D1 expression. Probable mediators of cyclin D1 induction by RAS are AP-1-complexes composed of Jun and Fos transcription factors. Their levels and activities are regulated by RAS and deletion of AP-1 sequences in the *CCND1* promoter abolishes induction by RAS (90). It has been shown that activated ERK causes enhanced expression of immediate early genes, including the AP-1 protein family. Subsequently, delayed early genes, including *CCND*, are induced. The Cyclin D-CDK4/6 complex then initiates RB phosphorylation, which activates the E2F family of transcription factors and induces expression of target genes, including *CCNE* (encoding cyclin E). The Cyclin E-CDK2 complex further phosphorylates RB and activates the E2F family. This positive feed back loop drives the cells to S phase entry (91).

Another possible downstream effector of RAS-induced cell cycle changes is $p27^{Kip1}$ (p27). Although RAS leads to an increased level and CDK-binding of cyclin D1 in quiescent cells, the CDK-complexes formed, are inactive in many cases and unable to promote cell cycle progression due to bound p27. Only after growth factor

stimulation, p27 is downregulated and S-phase entry occurs. Similarly, activation of MEK in serum-starved cells increases the amount of inactive CDK/cyclin D complexes, since p27 is not degraded as it is in response to serum though contrasting results have also been obtained. Although RAS is unable to repress p27 levels in the quiescent state, the RAS pathway seems to be required for p27 downregulation by serum (91). The RAF-MEK-ERK pathway is perhaps, the best characterized effector pathway causing the downregulation of p27 by RAS. Moreover, inhibition of PI3K blocks growth factor-induced downregulation of p27, suggesting a role also for this effector in RAS-mediated p27 downregulation (89).

RAS and cell invasion

Oncogenic RAS proteins stimulate a number of effector pathways that end in the transcriptional activation of genes, which control migration, invasion and angiogenesis. Several ERK substrates have been implicated in cell migration, such as myosin light chain kinase (MLCK), calpain proteases, focal adhesion kinase (FAK) and paxillin (92). There are also some reports that show a synergistic interaction between integrin-mediated cell adhesion and ERK activation in cell migration (93). Since integrins are thought to regulate RAS activation via focal adhesion kinase (FAK), it shows a feedback loop in which mutant *RAS* leads to its own activation. RAS deregulation of Rho GTPase function, which are important regulators of cell-cell and cell-substrate contacts, may also cause significant changes in cellular adhesion (23).

Oncogenic *RAS* stimulates the expression of several proteases that break down the extracellular matrix including urokinase plasminogen activator (uPA), its receptor (uPAR) and matrix metalloproteases (MMP-2 and MMP-9) (94). Optimal stimulation of uPA and MMP-9 gene expression by RAS requires signaling through MAPK and JNK responsive promoter elements(95). Local degradation of the extracellular matrix by these enzymes promotes tumor angiogenesis. Therefore, theses enzymes have critical role in the control of both tumor cell migration and tumor angiogenesis.

RAS oncogenes stimulate the production of growth factors involved in angiogenesis including VEGF and bFGF. Since oncogenic RAS promotes VEGF expression, tumor harboring mutant *RAS* often express high levels of VEGF, one of the most potent angiogenesis stimulating growth factors. Inhibition of mutant *KRAS* gene expression

in human colon cancer cells, either through expression of a ribozyme or through antisense oligonucleotides, inhibited the expression of VEGF. In addition, tumor-derived cell lines transfected with an activated *HRAS* gene showed increased expression of VEGF-A (95). Furthermore, inhibition of RAS signaling (either by dominant negative RAS or by farnesyltransferase inhibitors (FTIs) prevents efficient hypoxia-induced VEGF mRNA synthesis and protein secretion in malignant human astrocytoma cells that do not harbor *RAS* mutations. Thus, the induction of VEGF synthesis by hypoxia requires activation of the RAS pathway (95).

Oncogenic RAS reduces the production of anti-angiogenic factors including thrombospondin-1 and thrombospondin-2. It has been shown that whereas tumor cells expressing activated RAS show elevated levels of VEGF, the levels of thrombospondin-1 and thrombospondin-2 are dramatically reduced. These two extracellular matrix glycoproteins are negative regulators of angiogenesis. Repression of thrombospondin expression was found in cells expressing either one of the three RAS isoforms H-, K-, or NRAS. It seems that c-Jun activation by RAS mediates, or at least contributes to, thrombospondin gene repression in RAS- transformed cells (96).

RAS in differentiation, growth arrest and senescence

Although RAS was originally characterized as a protein with mitogenic and transforming potential, it has been later shown to have remarkably diverse effects on cell growth. Besides proliferation and transformation, RAS has been implicated in cellular events including differentiation, growth arrest, and senescence.

Microinjection of *HRAS* oncoprotein into mouse pheochromocytoma PC12 cells leads to reversible neuronal differentiation and growth arrest (97). Expression of oncogenic *HRAS* results in growth arrest of rat Schwann cells, whereas these cells are transformed when RAS co-operates with SV40 large T antigen, adenoviral E1A, or Myc, and growth arrest is absent. Similar results have been obtained in these cells with RAF-1 (CRAF), which causes growth arrest via p53-dependent induction of p21 without affecting p27 levels (98). In murine fibroblasts moderate RAF expression is accompanied by cell cycle progression whereas a robust RAF signal leads to p53-independent accumulation of p21 and p21-dependent cell cycle arrest, a phenomenon not seen in p21-/- cells (98).

Although RAS-induced growth arrest seems to partly rely on p21, also other

regulators are required. In primary human and rodent fibroblasts oncogenic *HRAS* induces accumulation of the negative growth regulators p53, p21 and $p16^{INK4A}$ and cellular senescence with irreversible growth arrest. Activation of the MAPK pathway may lead to opposing growth effects; cell senescence as well as proliferation or transformation, but the factors determining the response have so far not been identified. Determinants of the final outcome are suggested to include downstream cell cycle regulators but also the strength and duration of the RAS-RAF-MAPK signaling, as seen in the case of RAF (99, 100). Thus it seems that the ability of RAS to trigger either growth or apoptosis depends on the balance of interactions between pro-growth, pro-survival and pro-death effectors (24).

Oncogenic RAS and human cancer

As mentioned above, *RAS* genes are among the most frequently mutated genes in human cancers, but different malignancies display different frequencies and spectra of mutations in *NRAS*, *HRAS*, and *KRAS*. The important role of RAS in the regulation of cell growth and differentiation is verified by the fact that approximately 35% of neoplasias display mutations in this gene family, especially in the codons 12, 13, 59 and 61. In vivo mutations in *RAS* genes are not equally distributed between the RAS isoforms in different malignancies. The *KRAS* gene is mutated in nonsmall cell lung cancer (33%), colorectal cancer (44%), and pancreas cancer (90%), liver cancer (30%), and acute myelogenous leukemia (30%); *HRAS* gene is mutated in bladder cancer (10%), while kidney cancer (10%) and thyroid carcinomas have mutations in all three *RAS* genes (24). *NRAS* is the most frequently mutated gene in a wide variety of human leukaemias with an incidence of up to 60% in chronic myelomonocytic leukaemia and up to 40% in acute myelogenous leukaemia (101). Mutations of codon 61 of *NRAS* in thyroid follicular tumors occurred in 23% and 18% of atypical adenomas and follicular carcinomas, respectively. *NRAS* mutations was also found in liver 30% of cancer (OMIM:http://www.ncbi.nlm.nih.gov/).

Targeting the RAS oncogenes

RAS proteins are intracellular key transducers of growth signals regulated by cell surface receptors. Since a high percentage of human tumors harbor oncogenic *RAS* mutants this oncoprotein could be an appropriate target for drug design. The major anti-RAS therapy approaches, at present, include the prevention of RAS membrane localization by farnesyltransferase inhibitors (102), and inhibition of RAS protein expression by antisense oligonucleotides, and RNA interference (RNAi) (16, 103, 104). Farnesylation of RAS is essential for its membrane localization and thereby for its signaling function and cell transforming activity. This lipid modification is catalyzed by farnesyltransferase. The first strategy to inhibit RAS activity in tumor cells was the development of farnesyltransferase inhibitors (FTIs), which prevent RAS localization.

FTIs appeared to have anti-tumor effects in different studies. As single agents, FTIs have significant activity in myeloid leukemia's, but in solid tumors the effects appear to be modest (16). The nature of the FTI effects is highly complex. It appears that KRAS and NRAS proteins can also be modified by geranylgeranyl protein transferase, particularly when cells are treated with FTIs. This geranylgeranyl modification enables KRAS and NRAS to remain associated with the cell membrane. In addition, it has become clear that cell types with no *RAS* mutation are also sensitive to FTIs. Therefore, it is likely that the anti-tumor effects of FTIs are mediated by the inhibition of farnesylation of other proteins in addition to RAS (105).

Antisense inhibition of RAS protein expression appeared to have partial anti-tumor effects in human lung cancer cells and transformed fibroblast. The therapeutic use of these antisense oligonucleotides was limited, since they were rapidly degraded by intracellular nucleases. Moreover, they appeared to have sequence independent cytotoxic and antiproliferative effects (16).

The different approaches to inhibit Ras signaling in cancer cells are not specific for oncogenic *RAS*. A technique that can be used to differentiate between mutated and wild-type transcripts is RNA interference (RNAi). RNAi is a sequence specific post-transcriptional gene silencing mechanism induced by double-stranded RNA (dsRNA) molecules. A major advantage of RNAi-mediated anti-RAS cancer therapy, in contrast to other RAS based therapies, is the specific targeting of oncogenic *RAS*. This might be crucial, since inhibition of wild type RAS function in cells could interfere

with normal cell viability. The targeting of oncogenic *RAS* by RNAi can serve as a highly specific therapy. It has been investigated that suppression of the $KRAS^{G12V}$ mutation by retroviral vector-mediated RNAi inhibited the anchorage-independent growth in vitro, and growth in nude mice, of human pancreatic cells, although no significant effect was found on proliferation under adherent conditions (106). Furthermore, suppression of the $HRAS^{G12V}$ mutation in a human ovarian cancer cell line by retroviral vector-mediated RNAi has been demonstrated to decrease proliferation, increase G0/G1 arrest and apoptosis, and decrease tumor growth in nude mice (107).

Aims

The overall aim of this thesis was:

To estimate the frequency of *NRAS* alterations in malignant melanomas in patients. with germline *CDKN2A* mutations.

To establish the time of appearance of *NRAS* alterations during tumor development.

To define the role of these alterations in melanoma development in these high-risk individuals.

To specifically suppress the expression of mutant *NRAS* by siRNA in melanoma cells.

To determine whether suppression of mutant *NRAS* is sufficient to reverse the effects of oncogenic *NRAS* in human melanoma cells.

To study the role of the *NRAS* oncogene in the malignant phenotype of melanoma.

To define which genes and pathways involved in the malignant phenotype are regulated by mutant *NRAS*.

Results and Conclusions

The thesis is based on three publications with the overall aim to investigate the importance of activating mutations in the *NRAS* proto-oncogene in human cutaneous melanoma. In the first study *NRAS* mutations were analyzed in primary and metastatic melanoma tumors in patients with hereditary melanomas and germline *CDKN2A* mutations. In the two later studies the importance of mutant *NRAS* in human melanoma cells was explored by knocking down the mutant gene using RNAi.

Paper I

Frequency of UV-Inducible *NRAS* Mutations in Melanomas of Patients With Germline *CDKN2A* Mutations, *Journal of the National Cancer Institute 2003; 95(11):790-798.*

Germline alterations of the cyclin-dependent kinase inhibitor 2A (*CDKN2A*) are important genetic factors in familial predisposition to melanoma. Activating mutations of the *NRAS* proto-oncogene are among the most common somatic genetic alterations in cutaneous malignant melanomas. We performed a study in which *NRAS* mutations in melanomas from 25 patients in six Swedish melanoma-prone families carrying germline *CDKN2A* mutations were compared with *NRAS* mutations in melanomas from patients with sporadic melanomas. Five families carried the Swedish founder 113insR germline mutation in exon 2 of *CDKN2A*, and one family carried a P48L germline mutation in exon 1 of *CDKN2A*.

Genomic DNA was extracted from biopsy samples including primary melanomas, metastatic melanomas, and dysplastic nevi, using laser capture microscopy techniques. DNA was also extracted from 10 biopsy samples from patients with sporadic melanomas. *NRAS* was analyzed using polymerase chain reaction, single-strand conformation polymorphism analysis, and nucleotide sequence analysis.

Activating mutations in *NRAS* codon 61, all of which were either Q61K or Q61R mutations, were found in 95% (20/21) of primary hereditary melanomas but in only 10% (1/10) of sporadic melanomas ($P<.001$) from patients with no family history of melanoma. Multiple activating *NRAS* mutations were detected in tumor cells from different regions of individual primary melanomas in nine patients. The same *NRAS* mutations that were present in the primary tumors were also detected in all metastases

from these patients, indicating a clonal relationship between melanoma cells in primary and metastatic melanoma tumors. However, additional *NRAS* mutations at sites other than at codon 61 were also present in these metastases. The presence of *NRAS* mutations in dysplastic nevi and in an in situ melanoma tumor suggest that *NRAS* mutations may be early events in the development of melanoma in individuals with germline *CDKN2A* mutations. In contrast, no *NRAS* mutations were detected in common nevi and normal skin tissue biopsies.

We conclude that the high frequency of *NRAS* codon 61 mutations in these hereditary melanomas may be the result of a hypermutability phenotype associated with a hereditary predisposition for melanoma development in patients with germline *CDKN2A* mutations. The results also suggest that mutant *NRAS* may be an important target for specific anticancer prevention and therapy in melanoma patients who carry codon 61 mutations.

Paper II

Suppression of Oncogenic *NRAS* by RNA Interference Induces Apoptosis of Human Melanoma Cells. *International Journal of Cancer 2005; 115, 65-73.*

Studies on melanoma tumors have confirmed that oncogenic activation of *NRAS* constitutes the predominant *RAS* alteration in cutaneous melanoma, with *NRAS* codon 61 mutations being the most common alterations. Oncogenic *NRAS* activates a series of signal transduction pathways. A complex relationship between *RAS* activation, apoptosis, and cellular proliferation has been demonstrated. Most likely, activated RAS is able to protect cells from apoptosis either through activation of AKT via PI3K, or through activation of NF-κB. RAS may also play a role in maintaining the proliferative capacity of cells in a process involving the RAS-RAF-MAPK pathway.

To date, the role of oncogenic *NRAS* in melanoma remains only partially defined and no current therapies are directed at specifically suppressing oncogenic *NRAS* in human melanoma tumors. The aim of this study was to investigate the effects of suppressing oncogenic *NRAS* in human melanoma cell lines *in vitro*. We first investigated whether it is possible to specifically suppress the expression of the codon 61 $NRAS^{Q61R}$ mutation in melanoma cells using siRNA. We then determined whether the suppression was sufficient to abolish the effects of oncogenic *NRAS* in human melanoma cells. Using both small interfering RNA- and plasmid based-RNA

interference techniques, oncogenic *NRAS* was specifically suppressed in two human melanoma cell lines, 224 and BL, that harbor a $NRAS^{Q61R}$ mutation. Suppression of oncogenic *NRAS* in these cell lines resulted in decreased proliferation, increased apoptosis as well as decreased phosphorylation of ERK and AKT, in the NRAS signaling pathway and reduced expression of NF-κB and cyclin D1. In contrast, RNA interference directed at wild type *NRAS* had no significant effect on the proliferation and apoptosis of 224 cells or two human melanoma cell lines (A375 and 397) containing wild type *NRAS* but a codon 600 GTG (V) to GAG (E) mutation in *BRAF*. These data suggest that oncogenic *NRAS* is crucial for proliferation in melanomas that harbor the codon 61 *NRAS* mutation and that suppression of oncogenic *NRAS* in such melanomas by RNA interference may prove a useful future therapeutic option.

Paper III

Oncogenic *NRAS* has a Pivotal Role in the Malignant Phenotype of Human Melanoma Cells. Submitted for publication

One of the major goals of melanoma research is to identify molecular targets for the development of novel treatment strategies. Activating mutations in the *NRAS* gene, which occur predominantly in codon 61 (Q61R, Q61K) can be good therapeutic targets since these alterations are among the most common genetic events in malignant melanoma. These mutations affect regulation of cellular growth and viability, which contribute to the malignant phenotype.

We demonstrated in paper II that suppression of oncogenic *NRAS* by siRNA inhibited proliferation and induced apoptosis in human melanoma cells, suggesting that oncogenic *NRAS* is crucial for proliferation and resistance to apoptosis in melanomas.

With the aim of improving our understanding of the role of this oncogene in melanoma biology and to identify possible specific molecular therapeutic targets in this malignant disease, we used gene expression profiling as a method for global characterization of gene expression alterations that resulted from treatment of melanoma cells with siRNA specifically targeting $NRAS^{Q61R}$. Thirty-one probe sets were identified whose expression was significantly altered by siRNA against $NRAS^{Q61R}$ in two melanoma cell lines. The genes with altered expression are involved in several functions, including modulation of cell growth, invasion and migration. The results suggest that down

regulation of cyclin E2 and cyclin D1 and also up-regulation of a negative cell cycle regulator (HBP1) in *NRAS*^{Q61R} knockdown cells contribute to the inhibition of cell proliferation. Moreover, staining these cells with β-galactosidase showed 10% positive cells indicating premature senescence in some of these transfected cells. Furthermore, suppression of oncogenic *NRAS* results in a disability of cells in migration and invasion, which is accompanied by down-regulation of EphA2 (a receptor tyrosine kinase), uPAR (urokinase receptor) and cytoskeleton proteins such as leupaxin, α-actinin, paxillin, and vinculin.

In summary, these studies provide strong support for the conclusion that suppression of oncogenic *NRAS* by siRNA can induce growth arrest and inhibit invasion of human melanoma cells, which may be the basis of the development of more specific melanoma therapy in the subset of patients with *NRAS* mutations.

Future perspectives

As in any form of research, the answer to each scientific question leads to additional novel questions and possibilities. The main directions of future work could be as follows:

Paper I:

To extend the project with analysis of *NRAS* alterations in more melanocytic lesions from hereditary melanomas carrying germline *CDKN2A* alterations.

To determine the frequency of *NRAS* mutations in hereditary melanomas without germline *CDKN2A* alterations.

To study the gene expression profiling in melanoma tumors with activating *NRAS* mutations

To analyze other genes involved in melanoma development in hereditary melanomas like *BRAF, c-kit, PIK3CA, PTEN* genes, to define the involvement of such genes in hereditary melanomas.

Paper II and III:

To analyze the expression of main gene products identified in gene expression profiling after knockdown of the *NRAS* oncogene in clinical materials including primary and metastatic melanomas.

To study the function of the genes identified by gene expression profiling by ectopic expression and knocking down of the genes in melanoma cell lines.

To further evaluate the potential use of siRNA against $NRAS^{Q61R}$ *in vivo*.

To investigate the function of HBP1 and Txnip inhibitors in melanoma cells and study a possible connection to the generation of ROS in *NRAS* suppressed melanoma cells.

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