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Germline *CDKN2A/ARF* Alterations in

Human Melanoma

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Cover figure: Subcellular localization of wild—type p16 in MCF7 cell line (Figure 4 in paper IV).

To my Parents

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Approximately 10% of cases of human cutaneous malignant melanoma (CMM) have been estimated to occur in individuals with a familial predisposition, frequently in association with dysplastic nevus syndrome (DNS). The genetics of familial melanoma is complex and heterogeneous. To date only two melanoma predisposing genes have been identified. The *CDKN2A/ARF* locus on human chromosome 9p21 encodes two distinct cell cycle regulatory proteins, p16 and p14ARF. Germline alterations in the *CDKN2A* gene have been detected in approximately 20% of CMM families. Germline mutations have also been reported in the *CDK4* gene on chromosome 12q15, in a few families.

The primary aim of this thesis was to investigate alterations in the *CDKN2A/ARF* locus among individuals belonging to Swedish families with melanoma heredity and to study their biological consequences.

The relationships between germline *CDKN2A* mutations, melanoma and DNS phenotype were studied in five Swedish melanoma families with the mutations: a proline to leucine substitution (P48L), and an arginine insertion (113insR). We found significant correlations between *CDKN2A* mutations and CMM/DNS. Our results are consistent with the hypothesis that germline *CDKN2A* mutations and DNS both contribute to melanoma predisposition and may lead to early onset melanoma when present in the same individual.

The 113insR germline mutation was detected in 17 Swedish melanoma families. Haplotype analysis, using microsatellite markers in the 9p21 region, showed that these families share a common allele at markers close to *CDKN2A*, suggesting that 113insR is a founder mutation. Statistical analysis of meiotic recombination events in the 9p21 region indicated that the mutation arose approximately 2000 years ago.

We screened 80 individuals with multiple primary melanomas (MPM) for germline *CDKN2A* mutations. Mutations were detected in 11% of these patients and the majority of them had a family history of melanoma. A novel 24 base pair deletion, which included codons 62-69 (Δ62-69) was detected in one individual belonging to a family with melanoma heredity. *CDKN2A* mutation screening of individuals with MPM may thus identify high-risk families.

We confirmed the role of the P48L and Δ62-69 *CDKN2A* germline mutations in the development of melanoma tumors by demonstrating that the mutant p16 proteins are functionally abnormal and do not bind to CDK4 or CDK6.

Due to overlapping open reading frames in exon 2 of *CDKN2A*, the p16Δ62-69 also causes an in frame deletion of residues 77-84 in p14ARF. Our studies in cultured tumor cells showed that partial functional loss in p14ARFΔ77-84 may complement the defective p16Δ62-69 mutant and may contribute to melanoma development in patients carrying the 24 bp deletion in *CDKN2A*.

The high rate of *CDKN2A* inactivation in human cancer encouraged us to address the mechanisms and frequency of *Cdkn2a/b* gene loss during immortalization of *Ras*-transformed rat embryo fibroblast clones. We found homozygous deletions of *Cdkn2a/b* in all established cell lines studied. The mechanisms for loss of heterozygosity (LOH) were mitotic non-disjunction, deletion/rearrangements and, rarely, mitotic recombination. The frequency of *Cdkn2a/b* gene deletions was estimated to be approximately 2×10^{-8}/cell/generation.

LIST OF PAPERS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals (I-V).

I.   **Hashemi J.**, Linder S., Platz A. and Hansson J. Melanoma development in relation to non-functional p16/INK4A protein and dysplastic nevus syndrome in Swedish melanoma kindreds.
Melanoma Research 9: 21-30, 1999.

II.   **Hashemi J.**, Bendahl P-O., Sandberg T., Platz A., Linder S., Stierner U., Olsson H., Ingvar C., Hansson J. and Borg Å. Haplotype analysis and age estimation of the 113insR *CDKN2A* founder mutation in Swedish melanoma families.
Genes, Chromosomes & Cancer, 31: 107-116, 2001.

III.   **Hashemi J*.**, Platz A*., Ueno T., Stiener U., Ringborg U. and Hansson J. *CDKN2A* germ-line mutations in individuals with multiple cutaneous melanomas.
Cancer Research, 60: 6864-6867, 2000.

IV.   **Hashemi J*.**, Lindström MS*., Asker CE., Platz A., Hansson J. and Wiman K. A melanoma-predisposing germline *CDKN2A* mutation with functional significance for both p16 and p14ARF.
Cancer Letters, In press, 2002.

V.   Zhou J-N., **Hashemi J.**, Helou K., Zhang A., Röhme D., Zetterberg A., Levan G. and Linder S. Analysis of mechanisms and frequency of *CDKN2A/B* gene loss during progression of *RAS*-transformed RAT embryo fibroblast clones.
Somatic Cell & Molecular Genetics, 24: 327-339, 1998.

* The first two authors contributed equally to these articles.
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ABBREVIATIONS

ARF	Alternative reading frame (p14 in human, p19 in mice)
bp	base pair(s)
BrdU	bromodeoxyuridine
CDKN2A	Cyclin-dependent kinase inhibitor 2A (in human) (p16, MTS1)
CDKN2B	Cyclin-dependent kinase inhibitor 2B (in human) (p15)
CDKN2C	Cyclin-dependent kinase inhibitor 2C (in human) (p18)
CDKN2D	Cyclin-dependent kinase inhibitor 2C (in human) (p19)
Cdkn2a	Cyclin-dependent kinase inhibitor 2a (in rat)
Cdkn2b	Cyclin-dependent kinase inhibitor 2b (in rat)
CDK4/6	cyclin-dependent kinase 4/6
CIP	CDK-inhibitory protein
CKI	CDK inhibitors
CMM	Cutaneous malignant melanoma
DEX	Dexamethasone
DN	Dysplastic nevus
DNS	Dysplastic nevus syndrome
INK4A	Inhibitors of kinase 4A
kDa	kilo-Dalton
KIP	Kinase inhibitory protein
LOH	Loss of heterozygosity
MDM2	Mouse double minute 2
MPM	Multiple primary melanoma
MTS1	Multiple tumor suppressor 1
NLS	Nuclear localization signal
NoLS	Nucleolar localization signal
p	Short arm of chromosome
PCR	Polymerase chain reaction
p14ARF	*CDKN2A* alternative gene product in human
p15	*CDKN2B* gene product
p16	*CDKN2A* gene product
p19Arf	*Cdkn2a* alternative gene product in mouse
p53	*p53* gene product
pRB	*RB* gene product
q	The long chromosome arm
RAS	Activated ras oncogene
RB	Retinoblastoma gene
REF	Rat embryo fibroblast
RNA	Ribonucleic acid
RNO5	Rat chromosome 5
SDS-PAGE	Sodium dodecyl sulfate- polyacrylamide gel electrophoresis
SSCP	Single strand conformation polymorphism
TSG	Tumor suppressor gene
UV	Ultraviolet radiation
wt	wild-type

The nomenclature used in this thesis, are all in capital letters when referring to human genes and encoded proteins (e.g. *RB* gene, RB protein), and initial capital letters when referring to murine homologues. The *CDKN2A* gene products are referred to as p16 and p14ARF proteins.

INTRODUCTION

Cutaneous Malignant Melanoma

### *Clinical, histological and biological aspects*

Cutaneous malignant melanoma (CMM) is a form of skin cancer, which results from the malignant transformation of melanocytes, the pigment-producing cells in the skin. The incidence of melanoma is increasing in white-skinned populations throughout the world (MacLennan *et al.,* 1992; Coleman *et al.,* 1993). The rate of increase has been greater than that of any other cancer in Sweden during recent decades. At present, the overall incidence of cutaneous melanoma in Sweden is approximately 1600 cases per year corresponding to an incidence of approximately 18 cases per 100 000 inhabitants and it is the eight most common cancer in Sweden (Cancer incidence in Sweden, 2001). The Swedish CMM incidence rate is similar to the rate in the United States but is higher than that of most other European countries. The highest incidence of cutaneous melanoma has been found in Australia and New Zealand (Garbe &Blum, 2001). CMM is the most dangerous type of human skin cancer since the tumors may metastasize and become resistant to conventional cancer therapy.

The definition of melanoma as a tumor disease was used for the first time in 1812 (Laennec, 1812). The term "melanosis" is derived from the Greek word meaning "black". The first description of melanoma in English literature was published in 1820 by Norris, who also suggested the familial occurrence of CMM since he had found a father and son case of melanoma (Norris, 1820).

Melanomas are divided into four subclasses according to clinical and histological features (Clark, 1967; Clark *et al.,* 1986). The most common form of the disease is superficial spreading melanoma (SSM), which accounts for approximately 70% of cases. SSM melanomas may switch from an initial radial growth phase without metastatic capacity into a later vertical growth phase, associated with a risk of metastasis. Nodular melanoma (NM) represents 10-15% of all tumors, lacks radial growth phase and develops more rapidly than other forms of melanoma. Lentigo maligna melanoma (LMM) develops in an indolent manner in chronically sun-exposed skin in elderly patients and represents 5% of melanoma cases. Acral lentiginous melanomas (ALM) are rare tumors, which arise in the skin of the palms of hands, soles of feet and under nails. In 1967, Clark described an inverse correlation between increasing levels of

microinvasion into the dermis or subcutaneous tissue and patient survival (Clark *et al.,* 1967). Invasion levels refer to the depth of invasion of melanoma cells in the skin and are defined as follows: Clark level I: epidermis, II: papillary dermis, III: border between papillary and reticular dermis, IV: reticular dermis and V: subcutaneous fat. Shortly after the description of the anatomic levels, Breslow in 1970, established a linear relationship between tumor thickness and patient survival (Breslow, 1970), (**Figure 1**).
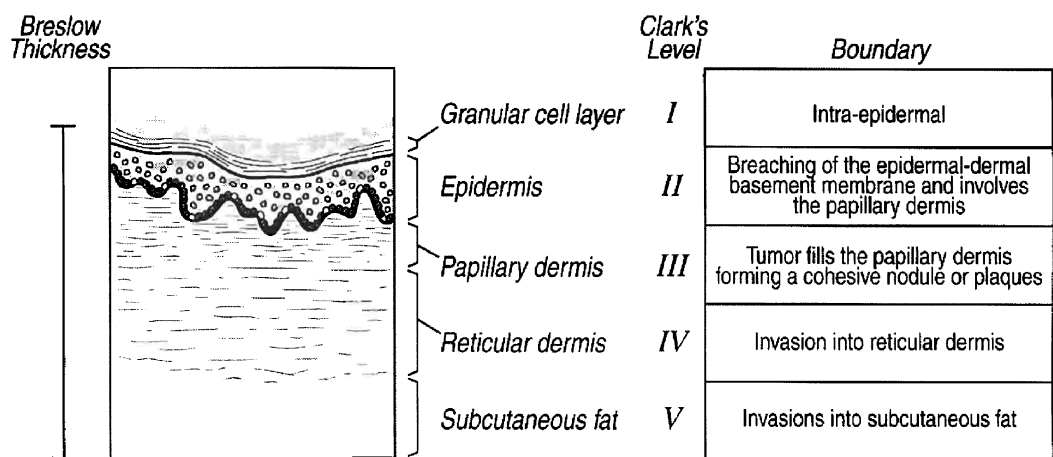
Figure 1. Tickness of primary cutaneous melanoma according to Breslow and level of invasion according to Clark [reprinted by permission of Genes & Development (Chin *et al.,* 12: 3467-81) copyright Cold Spring Harbor Laboratory Press, 1998].

Risk factors for CMM

Melanoma is a complex disease. Several risk factors, at genetic and environmental levels are known to be involved in its development.

Ultraviolet (UV) radiation and sun exposure: Epidemiological studies have shown that the major environmental risk factor for CMM and nevi is frequent exposure to solar ultraviolet (UV) radiation (Fraser, 1991; Goldstein *et al.,* 1993a; Armstrong & Kricker, 1993). The increase in CMM is probably, to a large extent, a consequence of increased exposure to UV through sunbathing (Green *et al.,* 1985; Rivers, 1996).

Hereditary factors: Approximately 10% of CMM arise in families with dysplastic nevus syndrome (DNS) with several affected members, likely as a result of inherited predisposition for the disease. The rare recessive human inherited disease, xeroderma pigmentosum (XP) caused by deficient nucleotide excision repair of UV-induced DNA damage, leads to a marked photosensitivity and the development of skin tumors including melanoma (Cleaver, 1989).

Melanocytic nevi (moles): Melanoma risk is also strongly associated with melanocytic nevi, including both common (Swerdlow et al., 1986; Holly et al., 1987) and dysplastic or atypical nevi (Clark et al., 1978; Greene et al., 1985a;b). Epidemiological studies have indicated the importance of the number of nevi present in an individual and their distribution on the skin in determining melanoma risk (Swerdlow et al., 1984; Swerdlow & Green, 1987; Grob et al., 1990; Bataille et al., 1996). Individuals with 50 or more nevi, which are larger than 2 mm in diameter have more than ten times, a higher risk of melanoma than the general population (Swerdlow et al., 1986). Individuals who belong to melanoma families often have large numbers of common and atypical nevi (Greene et al., 1985b; MacKie et al., 1993; Newton Bishop et al., 1994).

Additional genetically dependent host factors: Skin type, hair and eye color are implicated in both familial and sporadic melanoma. Melanoma is more common in individuals with fair skin, many freckles, blue or green eyes, red or blond hair, and in individuals who react to sunlight by burning rather than tanning (Greene et al., 1985b; Beitner et al., 1990; Bliss et al., 1995). Individuals with a history of basal skin cancer or squamous cell skin cancer also have an increased risk of melanoma (Kraemer, 1987a).

Hereditary cutaneous melanoma

Familial occurrence of CMM, first recognized by Norris (1820) was similarly observed by Cawley, 132 years later (Cawley, 1952). The presence of numerous nevi in melanoma families has been documented each by Norris and Cawley. However, research to prove the existence of a Mendelian basis for melanoma began in the late 1970s. Melanoma is inherited in a dominant Mendelian mode; and it was postulated that a single defective germline copy of a gene may predispose an individual to melanoma (Greene et al., 1983; Bale et al., 1986).

Approximately 10% of melanoma cases occur in patients with a family history of this disease (Greene, 1979), often in association with an accumulation of clinically dysplastic or atypical nevi (Tucker, 1988a; Tucker & Bale, 1988b). The association of familial melanoma with the inherited accumulation of atypical nevi was originally described as the 'B-K mole syndrome',

BK representing the initials of the family names of the kindreds first identified (Clark *et al.,* 1978). This syndrome later became known as 'dysplastic nevus syndrome' (DNS) (Greene *et al.,* 1980). Independently, the same syndrome was denoted as the familial atypical multiple mole- melanoma (FAMMM) syndrome by Lynch and co-workers (Lynch *et al.,* 1978). The same syndrome was later named atypical mole syndrome (AMS) (Newton Bishop *et al.,* 1994). The familial condition was defined by Kraemer *et al.,* (1987b), as D-2 type kindreds characterized by verified cutaneous melanomas and dysplastic nevi in two or more family members. The definition of familial melanoma varies and several groups require three or more affected members for a kindred to be classified as a melanoma family.

In general, tumors in familial melanoma cases are clinically and histologically indistinguishable from nonfamilial melanomas, however, several features are characteristic of familial melanoma, as opposed to its non-familial form (Barnhill *et al.,* 1992; Grange *et al.,* 1995a; Ang *et al.,* 1998;). Familial cases tend to have an earlier age of onset at first diagnosis of cutaneous melanoma (Kopf *et al.,* 1986) and thinner melanomas (Greene *et al.,* 1985b; Kopf *et al.,* 1986), this may be possibly due to surveillance programs. Furthermore, familial melanoma is associated with a greater frequency of multiple primary melanomas (Wallace *et al.,* 1973; Kefford *et al.,* 1991; Grange, 1995b), and varying numbers of dysplastic nevi (Greene *et al.,* 1985b; Aitken *et al.,* 1994).

The Dysplastic nevus (DN)

Dysplastic/atypical nevi are considered to be precursor lesions of melanoma (Reimer *et al.,* 1978; Greene *et al.,* 1985a; Tucker, 1988a; Tucker & Bale, 1988b; Halpern *et al.,* 1991). Discrepancies exist among different studies regarding the role of DN in familial melanoma (Greene, 1999). The relative risk of melanoma increases with the number of nevi. One twin study has suggested that the presence of a high number of nevi leads to genetic susceptibility (Easton *et al.,* 1991). The clinical characteristics of dysplastic or atypical nevi include: **a**symmetry, **b**order irregularity (irregular and indistinct margins), **c**olor variation (including tan, shades of brown ranging from light to very dark, pink and red), **d**iameter usually > 5 mm, abbreviated as the "ABCD-rules".

Tumorigenesis

Cancer development is the consequence of an imbalance between cell birth and cell death. A number of genes have been characterized, which maintain homeostasis in multicellular organisms. Genetic alterations in genes that are involved in cell cycle control, DNA replication and repair, and control of cell survival may lead to malignant transformation and tumor development. Several decades ago, it was estimated that at least five to seven successive mutations are necessary to convert a normal cell into an invasive carcinoma (Armitage, 1954). Today, recent evidence indicates that cancer cells contain large numbers of mutations (Loeb, 2001). Accumulation of multiple mutations and also expression of a mutator phenotype resulting from mutations in genes involved in genetic conservation (DNA replication and repair genes) have been suggested to be an early step in tumor progression (Hartwell, 1992; Loeb, 2001). Mutations in cancer related genes are found in all cancer types and include several changes in the genome such as point mutations, deletions, insertions, chromosomal translocations and amplifications. Such changes result in the growth advantage of an altered cell over surrounding normal cells leading to clonal expansion and consequently to tumor progression. The further progression of tumor cells also requires alterations in cell physiology such as, self-sufficiency in growth signals, escape from anti-growth signals, loss of ability to programmed cell death (apoptosis), replication without restriction, ability to induce angiogenesis (formation of new blood vessels), tissue invasion and metastasis, and development of therapy resistance (Hanahan & Weinberg, 2000). Epigenetic changes, such as hypermethylation of promoter CpG islands and global demethylation of the genome, can also play a role in cancer development (Jones & Laird, 1999; Costello *et al.*, 2000; Baylin *et al.*, 2001).

As to date, the main classes of cancer-related genes recognized are: oncogenes, tumor suppressor genes (TSGs), DNA repair and replication genes, and genes affecting cell survival such as anti- and pro-apoptotic genes (Mullauer *et al.*, 2001).

Oncogenes

Oncogenes, initially identified as genes carried by oncogenic viruses, are genes, which contribute to the neoplastic transformation of cells by activation or gain of function. A major class of the retroviral oncogenes has cellular counterparts called proto-oncogenes (Bishop, 1983). In a normal cell, proteins encoded by proto-oncogenes commonly participate in the transduction of

signals from the cell surface into the nucleus, or are directly involved in cell cycle regulation. They may be divided into four categories according to function and cellular localization; 1) growth factors, 2) growth factor receptors, 3) signal transducers, 4) nuclear transcription factors. Cellular oncogenes arise by alterations of proto-oncogenes that deregulate their normal activity and can in certain cases lead to tumor formation.

There are several mechanisms by which cellular proto-oncogenes can become oncogenically activated:

a) Point mutations leading to constitutive active protein products (e.g. *ras* genes).

b) Fusion of an oncogene with a second gene at a site of chromosomal translocation, or inversion of chromosomes generating a chimeric gene and a new protein product, (e.g. *bcr-abl* fusion gene on the Philadelphia chromosome).

c) Juxtaposition of an oncogene to regulatory elements in the immunoglobulin gene leading to an altered expression of the oncogene.

d) Chromosomal amplification in the form of homogeneously staining regions (HSR) or double minutes (DMs) (e.g. *c-myc* and *Mdm2*)

e) Insertion of a retrovirus into the vicinity of a gene leading to its increased expression (e.g. *c-myc*).

Oncogenes are genetically dominant i.e. an activating mutation in one allele of the gene overrides the activity of the remaining normal allele and contributes to tumorigenesis.

Tumor suppressor genes (TSGs)

Anti-oncogenes, or tumor suppressor genes (TSGs) are involved in preventing unrestricted cell growth. TSGs were identified initially through the study of somatic cell hybrids where the malignancy of tumor cells could be suppressed by fusion to normal cells (Harris *et al.,* 1969). The normal roles of TSGs are to control cell growth, apoptosis, differentiation, restriction of angiogenic and metastatic capabilites. The "two hit" model was first described by Knudson (Knudson, 1971). This model implies that in retinoblastoma, two independent genetic events are necessary for tumorigenesis. Even though predisposition is dominantly inherited, TSGs act in a recessive manner at the cellular level, i.e. both alleles of the gene need to be inactivated for complete loss of function. In familial cancer, a mutant allele of a TSG is inherited and present in every cell (the first hit, often a point mutation). The presence of such a germline mutation predisposes the carrier to cancer development since occurrence of a second mutational event in a somatic cell (the second hit) during the lifetime of the individual may eliminate the function of

the remaining normal allele, resulting in abnormal cell proliferation and clonal expansion. The second-hit can either be genetic (allelic loss or somatic mutation) or epigenetic (hypermethylation of CpG islands) (Esteller *et al.,* 2001). In sporadic cases, the inactivation of both alleles of a TSG (two hits) occurs through somatic alterations.

Loss of heterozygosity (LOH)

LOH refers to the loss of one of the parental alleles, which is present in the patient's normal cells (Cavenee *et al.,* 1983). LOH has been associated with the presence of TSGs (Cavenee *et al.,* 1985) in both sporadic and familial retinoblastoma. In sporadic cases constitutional genomic DNA isolated from blood is usually heterozygous for one or more genetic markers. However, the corresponding tumor cells are hemizygous or homozygous for the same allele. In tumors from patients with inherited disease the wild-type allele of TSG was often lost during tumorigenesis while the mutant allele was retained. LOH may involve a large region of a chromosome. There are cases in which TSGs are inactivated without an apparent second hit involving either LOH or hypermethylation. A dominant effect of the germline mutation may be present in such tumors. Cytogenetic analyses combined with marker studies, have suggested a number of possible mechanisms for allelic loss: a) loss of the whole wild-type chromosome by mitotic nondisjunction, resulting in hemizygosity at all loci on the chromosome b) loss of one chromosome followed by reduplication of the retained chromosome resulting in homozygosity at all loci on the chromosome, c) mitotic recombination, d) deletion and, e) gene conversion in which the wild-type allele is replaced by a duplicated copy of the homologous chromosome region that carries the mutant allele, resulting in two identically mutated alleles in the tumor **(Figure 7)**, (Cavenee *et al.,* 1983). Studies on chromosome deletion and LOH in familial cancers have facilitated the localization and identification of several tumor suppressor genes.

New perspectives on TSGs: Haplo-insufficiency

Knudson's 'two-hit' hypothesis to explain cancer predisposition still holds true for most TSGs. However, in some cases, deletion of a single copy of a TSG appears to be sufficient to potentially initiate or drive tumor development without any mutation or deletion of the second allele. In a few cases, haploid levels of wild-type TSG (e.g. p16, p53, p27, p14ARF) are not enough to maintain the normal phenotype and thus cannot exert their normal protective function. This is defined as haplo-insufficiency (Glendening *et al.,* 1995; Venkatachalam *et al.,* 1998; Fero *et al.,* 1998; Krimpenfort *et al.,* 2001). Haplo-insufficiency can be caused by transcriptional silencing of the wild-type allele resulting in reduction of the normal allele

expression (Shoemaker *et al.,* 1998) or by dominant negative effects blocking the activity of wild-type tumor suppressor protein, e.g. p53 (Venkatachalam *et al.,* 1998). Aberrant methylation of gene promoter regions is one mechanism to epigenetically silence and inactivate the second allele.

As a consequence of the growing number of TSG' (Macleod, 2000) and increasing knowledge on their function, the traditional definition of TSG has become too restrictive. TSGs have therefore been subdivided into three functional classes: 'gatekeepers', 'caretakers' and 'landscapers' (Kinzler & Vogelstein 1996; Lengauer et al. 1998).
The 'gatekeepers' TSGs, whose products act as critical bulwarks against the establishment of malignancy.
The 'caretaker' TSGs suppress growth indirectly by ensuring the fidelity of DNA replication through effective repair of DNA damage or prevention of genomic instability (Lengauer *et al.,* 1998; Macleod, 2000).
The 'landscaper' genes modulate the microenvironment in which tumor cells grow. These genes do not act on the tumor cell itself but rather upon their local environment (Kinzler & Vogelstein, 1998). The multistep development of tumors may involve mutations in members of all these different gene types.

The cell cycle and cancer
The process of cellular reproduction is known as the cell cycle. Cancer may be viewed as a disease of the cell cycle and indeed many types of human tumors suffer one or more defects that are related to the cell cycle machinery.

The eukaryotic mitotic cell cycle is divided into four phases: G1, S, G2 and M (**Figure 2**). During the S (DNA synthesis) phase, chromosomes are duplicated and they are then distributed equally to the two daughter cells during the M (mitosis) phase. The phases before and after the S phase are termed G1 (gap 1) and G2 (gap 2), respectively. During these phases, cells respond to proliferative and anti-proliferative signals. In addition, following mitosis, cells may rest temporarily in a quiescent (G0) state from which they exit in response to extracellular signals. Alternatively, cells may be induced to permanently exit the cell cycle and enter postmitotic states associated with terminal differentiation or senescence. The G1 phase is the interval in which cells prepare to synthesize DNA and respond to extracellular signals that ultimately

determine whether they will replicate their DNA and divide or exit the cell cycle into G0 phase. The G2 gap is a period in which preparations are made for cell division.

The eukaryotic cell cycle is controlled by a series of positive (engines) and negative regulators (brakes), which act at several checkpoints throughout the process of cell division. Different cyclin and cyclin-dependent kinase (CDK) complexes act as positive regulators, while the inhibitors of CDKs (CKIs) are negative regulators.
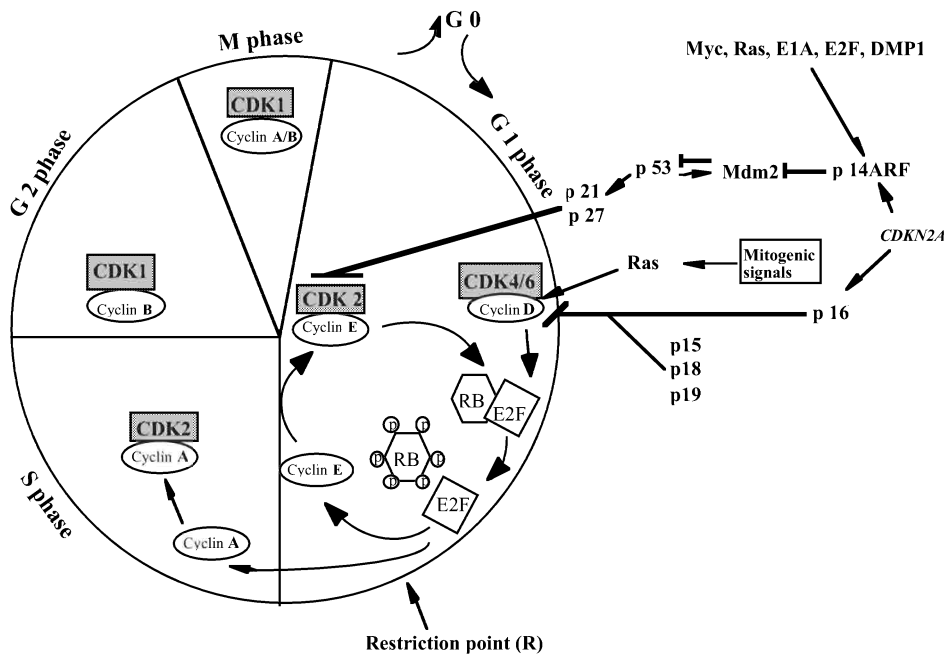

Figure 2. Schematic representation of the cell cycle and G1/S controlling elements.

——————| = inhibition;   ————▶ = activation

Cell cycle "checkpoints"

Cell cycle transition depends on an ordered series of molecular interactions in which the initiation of one event is dependent on the successful completion of an earlier event. Such order and timing of molecular events in the cell cycle are critical for the accurate transmission of genetic information to progeny cells. Errors in key cell cycle events such as DNA replication and mitosis can have fatal consequences for proliferating cells. Consequently, a number of surveillance pathways termed as checkpoint controls (Hartwell & Weinert, 1989) have evolved to ensure the correct order of cellular events during G1/S and G2/M transitions. Activation of these checkpoints halts cell cycle progression until either the problems are corrected or the cell goes into apoptosis. One important control point in mammalian cells occurs late in the G1 phase of the cell cycle and is named the "restriction point" (R). At this point, a cell decides whether or not to commence DNA replication. After passage through the R point, cells are committed to complete a cycle of cell division, without the requirement for extracellular growth factors (Pardee, 1974; Sherr, 1996).

"Cell cycle engines"

The eukaryotic cell cycle is composed of highly conserved protein complexes, called cyclin-dependent kinases (CDKs) due to their requirement of a cyclin to from active kinases. The activity of CDKs is regulated by both activating and inactivating phosphorylation events. These holoenzymes, thus contain both regulatory (cyclin) and catalytic (cdk) subunits. In mammalian cells, different cyclin/CDK complexes are assembled and activated at specific points during the cell cycle (Sherr *et al.,* 1994).

Cells respond to proliferative or antiproliferative signals through the cyclinD1-RB-CDK4/6 pathway or the p53-p14ARF pathway **(Figure 2)**. When quiescent cells in G0 are stimulated to enter the cell cycle, genes encoding D-type cyclins (D1, D2, or D3) (Sherr, 1995a) are induced in response to mitogenic signals **(Figure 2)**. These cyclins associate with either CDK4 or CDK6 subunits and the complex becomes activated by phosphorylation. Active cyclin/CDK complexes drive the cell cycle forward *via* phosphorylation of substrates such as RB in late G1 phase (Weinberg, 1995). RB is thereby inactivated, and its growth-repressive function abolished, resulting in release of a class of associated transcription factors known as E2Fs (Dyson, 1998). Released E2Fs (1-3) can drive quiescent cells into S phase by transcriptional activation of genes whose products are necessary for DNA synthesis (Nevins, 1998; Sherr,

1996; Sherr, 2001a). Cyclins E and A are two such E2F–regulated genes, which are both required for G1/S transition (Ohtsubo *et al.,* 1995; Strausfeld *et al.,* 1996).

Cyclin D-dependent kinases have a second non-catalytic role, namely sequestration of the CIP/KIP proteins, p21 [CIP], p27 [kIP] (see below), from cyclin E to cyclin D during G1 phase. Upon release of cyclinE-CDK2 from CIP/KIP inhibition, the active complex further phosphorylates and inactivates the RB protein. CyclinE-CDK2 cooperates with cyclinD-CDK4 in the G1/S transition to complete the RB inactivation, thereby creating a positive feedback loop **(Figure 2)** (Sherr & Roberts, 1999; Sherr & Weber, 2000). The activity of the cyclin E-CDK2 complex peaks at the G1-S transition, after which cyclin E normally is degraded and replaced by cyclin A.

"Cell cycle brakes": CDK inhibitors

Specific CDK inhibitors (CKI) inhibit the activity of CDKs by specifically binding to CDKs, thus preventing their association with the appropriate cyclins and/or blocking the catalytic activity of the kinase and thereby causing cell cycle arrest (Serrano *et al.,* 1993; Hannon & Beach, 1994; Hirai *et al.,* 1995). There are two classes of CKIs based on their structure and targets: the INK4 (named according to their ability to <u>in</u>hibit cd<u>k4</u>) and the CIP/KIP (<u>C</u>DK-interacting <u>p</u>rotein/<u>K</u>inase <u>i</u>nhibitory <u>p</u>rotein) families (Sherr & Roberts, 1995b). The first class, the INK4-proteins include p16 (Serrano *et al.,* 1993), p15, p18 and p19 (also referred to as INK4a, INK4b, INK4c and INK4d, respectively) (Roussel, 1999), which all contain multiple ankyrin repeats and bind only to CDK4/CDK6. These CKIs have the capacity to release the D-type cyclin moiety and to induce a G1 cell cycle arrest by preventing the phosphorylation of RB and thus, maintaining these pocket proteins in its active, anti-proliferative state. Binding of INK4 proteins to CDK4/CDK6 cause the release of the bound pool of CIP/KIP proteins from cyclinD–CDK4 complexes, resulting in cyclinE-CDK2 inhibition and further Rb hypophosphorylation and reactivation (Sherr, 2001a). The end result is that INK4 proteins prevent E2F–dependent transcription and blocks the cell cycle in G1. The ability of the INK4 proteins to arrest the cell cycle in the G1 phase depends upon the presence of a functional RB protein (Medema *et al.,* 1995; Lukas *et al.,* 1995). Although p16, cyclin D1, CDK4 and RB are often deregulated in cancer cells, p15, p18 and cyclin D2 have been implicated in fewer tumors.

In contrast to the INK4 proteins, the CIP/KIP class of proteins can bind to a variety of cyclins and CDKs (cyclin D-, E-, and A- dependent kinases), and at least in complex with CDK2 they

negatively regulate CDK activity (Sherr & Roberts, 1999). The CIP/KIP proteins (including p21^{CIP}, p27^{KIP}, p57^{KIP}) all contain characteristic structural motifs within their conserved amino-terminal regions that enable them to bind both to cyclin and CDK subunits (Chen *et al.,* 1995; Russo *et al.,* 1996).

The transcription factor p53 protein is mutated in almost 50% of human cancers. p53 accumulates in response to cellular stress from DNA damage, oncogene activation, and hypoxia (Levine, 1997). When p53 is stabilized and activated, it upregulates transcription of target genes including p21 (Harper *et al.,* 1993). Induction of p21 by p53 inhibits cyclin-E-CDK2 and returns Rb to its hypophosphorylated state (active inhibitory state), resulting in cell cycle arrest in late G1 phase.

p16, CDK4, RB and cyclin D1, all function in the same growth control pathway; the "RB pathway", which is involved in control of the cell cycle and cell proliferation. Functional disruption of the tumor suppressors p16, or RB, or overexpression of the proto-oncogene products cyclin D and CDK4 are common features in human cancers (Sherr, 1996; Ruas & Peters, 1998; Sherr & Roberts, 1999). Perturbation of any component of the "RB pathway", therefore, may have similar consequences: an uncontrolled progression from G1 to S phase.

The genetics of hereditary melanoma

A variety of approaches, including cytogenetic analysis, loss of heterozygosity (LOH), linkage studies and positional cloning have defined a melanoma susceptibility locus on chromosome 9p21.

Cytogenetic studies

The first step in the characterization of the genetic mechanisms responsible for development of melanoma involves the cytogenetic analysis of dysplastic nevi, primary and metastatic melanoma tumors.

A review of 31 studies comprising 97 cases of melanoma (Albino & Fountain, 1993) and a report of 158 cases of metastatic melanoma (Thompson *et al.*, 1995) showed that chromosomes 1, 6, 7, 9, and 11 had the highest frequencies of chromosomal aberration. Studies on a patient with eight primary cutaneous melanomas showed a *de novo* constitutional rearrangement involving chromosomes 5 and 9 (Petty *et al.*, 1993). A recent study on 15 melanoma cell lines using spectral karyotyping showed losses, gains and rearrangement of material on chromosomes 1, 4, 6, 9, 10, 12, 13 19 and 20 (Sargent *et al.*, 2001).

LOH studies

LOH studies of chromosomal regions surrounding putative tumor suppressor loci were first performed with restriction fragment polymorphism (RFLP) markers and later with microsatellite markers in order to localize the molecular alterations in familial melanoma. These studies demonstrated loss of chromosome 10 (Dracopoli *et al.*, 1985), 9p (Dracopoli *et al.*, 1987) and 1p (Dracopoli *et al.*, 1989) in melanoma tumor biopsies and cell lines. Fountain *et al.*, showed chromosome 9p21 LOH in 86% of melanoma cell lines and tumors. An important landmark was the demonstration of homozygous deletion of the marker D9S126 on 9p21 in 10% of melanoma cell lines using Southern blot analysis (Fountain *et al.*, 1992). Analysis of chromosome 9p21 in other tumor types demonstrated that this region is involved in a spectrum of cancers (reviewed by Haluska & Hodi,1998). These findings suggest that this region is a location for one or several gene(s), which are important in the development of melanoma and other human cancers.

Linkage studies

Cytogenetic and molecular genetic studies of melanoma tumors showed loss of genetic material on several chromosomes and provided the foundation for linkage studies. Several loci were initially studied for linkage to melanoma predisposition. Two important loci of potential linkage to hereditary melanoma were identified on chromosome 1p36 and chromosome 9p21 (Bale *et al.*, 1989). While linkage to chromosome 1p36 is controversial (Cannon-Albright, 1996; Haluska & Hodi, 1998), linkage was found between 9p21 loci (D9126 and IFNA) and familial melanoma in large pedigrees (Cannon-Albright *et al.*, 1992; Nancarrow *et al.*, 1993; Goldstein *et al.*, 1994). These studies have indicated that 9p21 is a melanoma predisposition locus containing candidate gene(s).

Molecular alterations involved in melanoma

Neoplasms of melanocytes evolve in a stepwise fashion starting from small benign nevi and progress to highly malignant and finally metastatic melanoma tumors. Melanoma development can be divided into five major steps, based on clinical observations (Clark *et al.*, 1984) **(Figure 3)**. The first step is represented by the genesis of a common nevus from normal melanocytes in the skin, followed by the development of a dysplastic (atypical) nevus; this may lead to a primary melanoma, which firstly exhibits a radial growth phase (RGP) and later switches to a vertical growth phase (VGP), and finally to metastasic melanoma. RGP melanoma includes all *in situ* melanomas (Clark level I) and 'microinvasive' melanomas. VGP or 'tumorigenic' melanomas are characterized by an expansive mass of melanoma cells in the dermis and have the potential to metastazise.
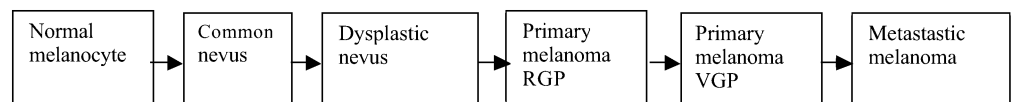
Figure 3. **Model for progression of melanoma.**

The underlying molecular steps leading to the development of human malignant melanoma and subsequent metastasis are not well known. It has been speculated that they are similar to other well-defined cancer types like human colorectal cancer that arise as a result of a number of stepwise occurring genetic events (Fearon & Vogelstein, 1990; Vogelstein & Kinzler, 1993; Lu & Kerbel, 1994). However, at present, it has not been convincingly possible to correlate the

different stages of melanoma neoplasia with individual genetic events. Cytogenetic studies have demonstrated numerous chromosomal alterations in malignant melanomas. The loss of chromosome 9p21 in both dysplastic nevi and primary melanoma lesions supports the hypothesis that mutation or deletion of at least one copy of this region is an early step in melanoma development (Dracopoli & Fountain, 1996; Bastian et al., 1998). Homozygous deletion of the 9p21 region in both familial and sporadic melanoma may be common (Hussussian et al., 1994; Kumar et al., 1998). The p16 protein encoded by the CDKN2A gene on 9p21 has been shown to be inactivated in subsets of familial and sporadic melanomas by a variety of mechanisms (Liggett & Sidransky, 1998). The frequencies of known CDKN2A alterations are extremely low in thin-intermediate thickness lesions (10% in lesions of <4mm), although p16 expression is lower than that of premalignant melanocytic lesions (Reed et al., 1995; Funk et al., 1998; Polsky et al., 2001). It has recently been demonstrated that p16 expression is decreased in early stage melanomas that are confined to a radial growth phase by Id1, a helix-loop-helix (HLH) transcriptional regulatory protein. This transcriptional inactivation of p16 expression may bypass cellular senescence. Subsequent epigenetic and genetic changes are then associated with tumor progression, invasion and metastasis (Polsky et al., 2001; Alani et al., 2001). There are in addition, possible epigenetic events such as hypermetylation of 5' CpG islands of the CDKN2A gene which take place during development of CMM (Merlo et al., 1995; Costello et al., 2000)

In addition to the alteration of chromosome 9p21, which is the most commonly targeted region for significant genomic loss, consistent amplifications and translocations of other chromosomes, may be involved in different steps of melanoma tumor progression, (see page 21), (Healy et al., 1996; Dracopoli & Fountain, 1996; Sargent et al., 2001). Such chromosomal alterations suggest the presence of genes that confer a growth advantage (in the case of amplification), and indicate that these regions contain candidate growth suppressor genes (genetic loss) or the possibility of formation of fusion genes or altered gene regulation (translocations).

Several studies have showed that mutational activation of the N-ras proto-oncogene is also of importance in both early melanomas as well as in advanced tumors by conferring a growth advantage during the vertical growth as well as in later phases of progression (Ball et al., 1994; Carr & Mackie, 1994; Platz et al., 1994; 1995; Jiveskog et al., 1998).
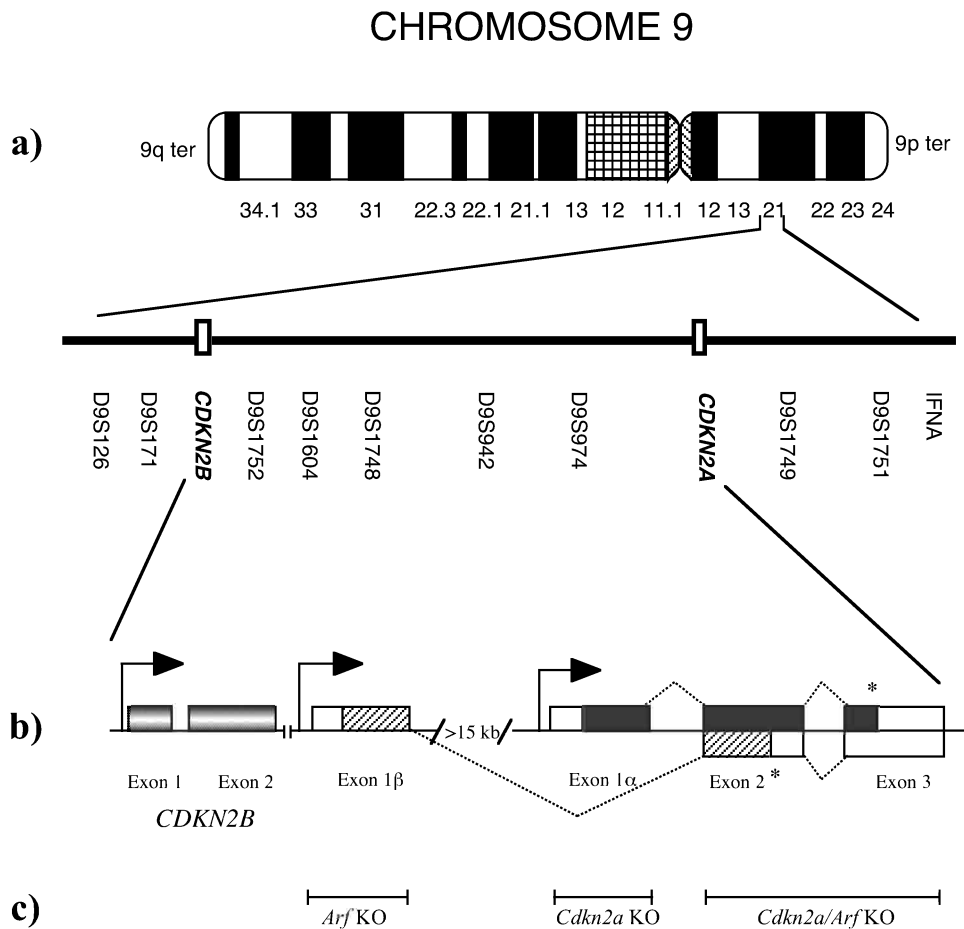
Isolation and localization of CDKN2A

After the establishment of melanoma linkage to 9p21 markers, efforts were made to isolate the "melanoma gene". Cell lines were used as the primary tools for gene localization. In a study of 100 melanoma cell lines by Weaver-Feldhaus *et al.*, (1994) and in another study of a panel of 290 human tumor cell lines by Kamb *et al.*, (Kamb *et al.*, 1994a) 60-75% of melanoma cell lines and 46% of the human tumor cell lines had homozygous deletions clustered around a locus in 9p21. This site contains two genes, *CDKN2A* and *CDKN2B*. The *CDKN2A* gene (Kamb *et al.*, 1994a; Nobori *et al.*, 1994) has a gene product, p16, which had been isolated independently by exploiting its interaction with CDK4 (Serrano *et al.*, 1993). The sequence of *CDKN2A* determined by Kamb *et al.*, 1994 was identical to that determined by Serrano *et al.*, 1993. Subsequent mutation analysis of *CDKN2A* uncovered the first germline mutations associated with disease predisposition in melanoma kindreds (Hussussian *et al.*, 1994; Kamb *et al.*, 1994b), linking melanoma development to aberrant cell cycle control. *CDKN2B* was the second cloned 9p21 gene, it encodes the cdk inhibitor p15 (Hannon & Beach, 1994). Additional homologues of *CDKN2A* have also been cloned, *CDKN2C* (p18) on chromosome 1p32 (Guan *et al.*, 1994; Hirai *et al.*, 1995) and *CDKN2D* (p19) on chromosome 19p13 (Hirai *et al.*, 1995; Chan *et al.*, 1995).

Unique gene structure of CDKN2A

The *CDKN2A* gene on human chromosome 9p21 consists of three exons and an alternative exon 1 (E1ß). The two exons 1 are driven by two separate promoter regions. One promoter produces a transcript (α), which is formed by exons 1α, 2 and 3 and encodes a 156 amino acid, 15.8 kD protein (p16) **(Figure 4)**. The second promoter produces a smaller transcript (ß) that contains the second and third coding exons of *CDKN2A*, but through alternative splicing incorporates exon1ß instead of exon 1α. Both transcripts thus use the common exon 2 and 3 sequences but utilize different reading frames, resulting in two totally unrelated proteins (Mao *et al.*, 1995; Quelle *et al.*, 1995; Stone *et al.*, 1995a). The open reading frame for the ß form transcript extends from exon 1ß to the 5' half of exon 2 of the *CDKN2A* gene. The product of the ß transcript, designated ARF (for alternative reading frame), encodes a 14 kD protein (p14) in humans and 169 amino acid 19kD protein (p19) in the mouse (Stott *et al.*, 1998). Both proteins encoded from this locus, p16 and p14ARF exert cell cycle control. While p16 is a component of the RB regulatory pathway (Serrano *et al.*, 1993; 1996), p14ARF acts mainly in the p53 pathway (Sherr, 1998; Stott *et al.*, 1998). Since the expression of p14ARF is regulated by RB through the E2F transcription-factor, the *CDKN2A* locus converges both RB and p53 pathways (DeGregori *et al.*, 1997; Bates *et al.*, 1998).

24

CHROMOSOME 9

a)

9q ter   9p ter

34.1 33   31   22.3 22.1 21.1 13 12 11.1 12 13 21   22 23 24

D9S126 D9S171 **CDKN2B** D9S1752 D9S1604 D9S1748 D9S942 D9S974 **CDKN2A** D9S1749 D9S1751 IFNA

b)

>15 kb

*

Exon 1   Exon 2   Exon 1β   Exon 1α   Exon 2 *   Exon 3

CDKN2B

c)

Arf KO   *Cdkn2a* KO   *Cdkn2a/Arf* KO

Figure 4. **Chromosome 9p21, the map order for markers on 9p21 and the genomic organization of *CDKN2A* and *CDKN2B*. a**) The ideogram of human chromosome 9 identifies band 9p21 and the linear map below shows the relative locations of several microsatellite markers. The positioning of the markers is based on Randerson-Moor *et al.*, (2001). **b**) The expanded map of the *CDKN2A* and *CDKN2B* loci shows the positions of the coding exons, the direction of transcription (arrows) and the location of the alternative first exon (exon 1β) of *CDKN2A*. Dashed lines indicate the alternative splice events that distinguish the p14ARF transcript from the p16 transcript. Protein coding sequences are showed by hatched (p14ARF) or closed bar (p16) and noncoding sequences by open bars.

*, stop codon. **c**) Knockout genotypes at the *Cdkn2a/Arf* locus.

The *CDKN2A/ARF* locus is regarded as one of the most important anti-tumoral defences in the mammalian organism (Serrano, 1997; Ruas & Peter, 1998; Sherr, 1998; Sharpless & DePinho, 1999). The dual coding capacity of this locus may account for its high disruption rate in human cancers. The reason for the colocalization of *CDKN2A* and *ARF* sequences is unknown. Human p14ARF shares only 49% amino acid identity with its mouse homologue, in comparison to the human p16 protein, which is 63% identical to mouse p16. The high degree of sequence diversity in *ARF* indicates a rapid evolution (Zhang & Xiong, 2001).

The expression of *CDKN2A* and *ARF* is co-regulated in a coordinated manner (Hara *et al.,* 1996; Rizos *et al.,* 1997). The normal levels of p16 and p14ARF expression are low in tissues and primary cells and are upregulated with *in vitro* senescence, ageing, and illegitimate oncogene activation (Zindy *et al.,* 1997; Nielsen *et al.,* 1998; Thullberg *et al.,* 2000).

The unique organization of this locus complicates the analysis of the role of p16 and p14ARF (Rocco & Sidransky, 2001). It is not always possible to distinguish the actual target for inactivation since homozygous and hemizygous deletions often include both *CDKN2A* and *ARF* and due to dual utilization of exon 2, some mutations in this exon alter both p16 and p14ARF amino acid sequences (Ruas & Peters, 1998). This fact, and the known distinctive biological role of p16 and p14ARF raised questions regarding the individual roles of these two proteins in tumor suppression. This debate appears to be resolved by the recent construction of two types of "pure" *Cdkn2a* knockout mice (reviewed by Sherr, 2001b). Initially, the disruption of exons 2 and 3 of *Cdkn2a* (*Cdkn2a*$^{-/-}$/*Arf* $^{-/-}$) in mice led to a cancer prone phenotype. Embryonic fibroblasts (MEFs) from these mice exhibited spontaneous immortalization and were transformed by activated H-ras alone (Serrano *et al.,* 1996). However, disruption of exon 1ß to generate *Arf* null (*Arf*$^{-/-}$) mice gave the same phenotype as *Cdkn2a*$^{-/-}$/*Arf*$^{-/-}$ (Kamijo *et al.,* 1997). However, neither *Cdkn2a*$^{-/-}$/*Arf* $^{-/-}$ nor *Arf* $^{-/-}$ mice spontaneously developed melanomas. In another mouse model, *Cdkn2a*$^{-/-}$/*Arf* $^{-/-}$ animals were crossed with mice carrying an activated tyrosinase-Ha-ras transgene, which led to a high frequency of melanoma development (Chin, *et al.*, 1997). These studies raised the question of what a "pure"*Cdkn2a* knockout mouse would be like and whether the loss of *Cdkn2a* or *Arf* was responsible for melanoma susceptibility in mice. Recently, two groups used different strategies to disable *Cdkn2a* alone but leaving *Arf* intact. Krimpenfort *et al.,* performed this by knocking in a point mutated *Cdkn2a* allele, and thus replaced the wild-type allele with one containing a termination codon at amino acid 101 resulting in a truncated unstable form of p16 protein (Krimpenfort *et al.,* 2001). Sharpless *et al.,* deleted

exon 1α by removing the translation initiation codon and 35 codons of *cdkn2a* but leaving the cis-regulatory sequence intact (Sharpless *et al.,* 2001)). The *Cdkn2a* null mice (with mutant *Cdkn2a* allele) showed a very mild phenotype confirming that the very strong phenotype of original *Cdkn2a$^{-/-}$/Arf$^{-/-}$* mice (Serrano *et al.,* 1996) was due mainly to loss of *Arf*. When these mice were crossed with heterozygous *Arf* animals, the progeny (*Cdkn2$^{-/-}$/Arf$^{+/-}$*) developed melanoma and other tumors to a greater extent than mice with *Cdkn2a* deficiency and two functional *Arf* alleles. This suggests a novel relationship between these two genes (Carnero *et al.,* 2000) and reflects a genetic interaction between *Arf*-p53 and the *Cdkn2a*-Rb pathway in melanoma development. In the absence of functional *Cdkn2a*, the reduced dosage of *Arf* was sufficient to contribute to melanoma development. *Arf* thus, appears to be haplo-insufficient for tumor suppression in this mouse melanoma model. Mice with a partial deletion of exon 1α were slightly more prone to spontaneous tumor development. Interestingly, both types of pure *Cdkn2a* null mice developed melanoma while *Cdkn2a$^{-/-}$/Arf$^{-/-}$* or *Arf$^{-/-}$* mice did not. However, more studies are required to confirm whether the knock-out models are applicable to melanoma risk in humans.

Recent studies of knockout mice (Krimpenfort *et al.,* 2001; Sharpless *et al.,* 2001) have not clarified completely whether the loss of *Arf* is responsible for melanoma susceptibility in mice. However, the effect of Arf haplo-insufficiency in *Cdkn2a$^{-/-}$* background mice and the high frequency of melanoma in *Cdkn2a$^{-/-}$/Arf$^{-/-}$/tyrosinase*-Ha-*ras* mice (Chin *et al.,* 1997) may provide us with a good model for melanoma development. This model is based on activation of the ras-MAP kinase pathway, and inactivation of Cdkn2a-Rb and Arf-p53 pathways (Walker & Hayward, 2002) **(Figure 5)**. Dual inactivation of *Cdkn2a* and *Arf* is a very efficient way to induce cancer in mice as both the Cdkn2a-Rb and Arf-p53 pathways are disrupted. The mechanism by which these two disrupted pathways co-operate in increasing cancer susceptibility is unclear.
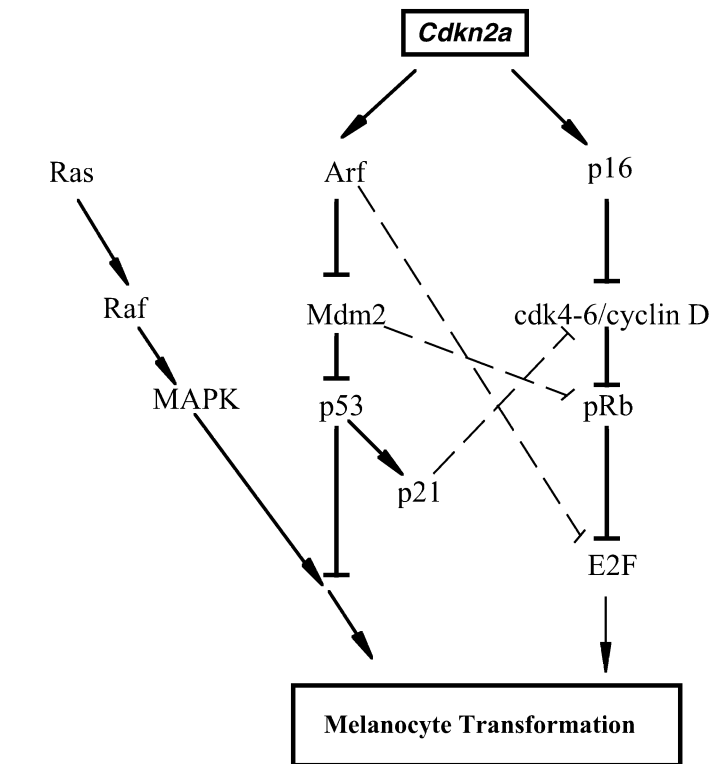
Figure 5. Pathways involved in melanoma development in mice. Cross talk between p16-Rb and Arf-p53 pathways are shown by dashed lines (adapted from Walker & Hayward 2002)

————| = inhibition;   ————▶ = activation

p14ARF

The p14 ARF protein interacts directly with MDM2 to stabilize and activate p53 (Zhang *et al.,* 1998; Stott *et al.,* 1998; Pomerantz *et al.,* 1998; Weber *et al.,* 2000a). MDM2, a p53-inducible gene product, binds to p53 and blocks its transcriptional activity (Momand *et al.,* 1992; Oliner *et al.,* 1993), as it acts as an E3 ubiquitin ligase to target p53 for degradation in cytoplasmic proteasomes (Honda *et al.,* 1997; Kubbutat *et al.,* 1997), and transports p53 from the nucleus to the cytoplasm for proteasomal degradation (Roth *et al.,* 1998; Tao & Levine, 1999).

p14ARF is localized predominantly in the nucleolus but is also present in the nucleoplasm, while p53 and MDM2 are nucleoplasmic proteins (Sherr 1998; Weber *et al.,* 1999; Zhang & Xiong 1999; Lindström *et al.,* 2000). p14ARF is an upstream key mediator of p53-dependent

growth suppression in response to activated oncogenes. Induction of p19Arf by oncogenes such as Myc, mutated Ras and E2F1 results in a p53-mediated cell cycle arrest or in apoptosis depending on cell type (Sherr, 2001a). p14ARF stabilizes and activates p53 by antagonizing the functions of MDM2. Several models have been proposed for this function of p14ARF (Zhang & Xiong, 2001). One model is based on the ability of p14ARF to bind to MDM2 (Pomerantz *et al.*, 1998; Kamijo *et al.*, 1998), in order to sequester it in nucleoli (Lohrum *et al.*, 2000; Weber *et al.*, 1999, 2000a) and to block MDM2-mediated ubiquitination and nuclear export of p53 (Tao & Levine 1999; Honda &Yasuda 1999; Zhang & Xiong 1999). Ectopic expression of p14ARF therefore stabilizes p53 and arrests cells in G1 and G2 (Stott *et al.*, 1998). Recent studies have demonstrated that p14ARF stabilizes p53 without relocalization of MDM2 from nucleoplasm to nucleoli (nucleoplasmic forms of p14ARF can stabilize p53), and that p53 stabilization is not essential for Arf-mediated p53 activation (Llanos *et al.*, 2001; Lin & Lowe, 2001; Korgaonkar *et al.*, 2002).

The p14ARF protein contains two nuclear and nucleolar localization signals (NLS, NoLS), partly overlapping with the MDM2 binding domain (Zhang & Xiong 1999; Weber *et al.*, 2000a; Rizos *et al.*, 2000; Lohrum *et al.*, 2000; Llanos *et al.*, 2001). The cell cycle inhibitory function of the p14ARF protein as well as a nuclear localization signal has been localized to the N-terminal region encoded by exon 1ß (Weber *et al.*, 2000a; Rizos *et al.*, 2000). The highly basic C-terminal nucleolar localization domain encoded by exon 2 is not strictly essential for p53 stabilization but appears to potentiate the activity of human p14ARF (Zhang & Xiong 1999; Llanos *et al.*, 2001; Rizos *et al.*, 2001a).

Melanoma predisposing gene mutations

To date germline mutations in only two melanoma predisposing genes *CDKN2A* including *ARF*, and *CDK4* genes have been identified (reviewed by Platz *et al.*, 2000).

Several distinct *CDKN2A* germline mutations (>50) have been reported in many melanoma kindreds from Australia, North America and Europe (Foulkes *et al.*, 1997; Liggett and Sidransky 1998; Ruas & Peters, 1998; Holland *et al.*, 1999). Most mutations described are point mutations, but also include deletions, insertions, and duplications. While approximately 50% of all melanoma kindreds show genetic linkage to markers within the 9p21 region (Cannon-Albright *et al.*, 1992; Goldstein *et al.*, 1994; Holland *et al.*, 1995; Hayward, 1996; Dracopoli, 1996; Foulkes, 1997), not all of these families show evidence of *CDKN2A* alterations. The reported germline mutation frequency of *CDKN2A* in familial melanoma varies from 8% (5/64) in Sweden

(Platz *et al.,* 1997), to 50% (9/18) of familial cases in an American study (Hussussian *et al.,* 1994). These frequency differences may be explained by a selection of melanoma kindreds with genetic linkage to 9p21 in some studies but not in others (FitzGerald *et al.,* 1996; Haluska & Hodi, 1998). Variation in the criteria used to define kindreds with hereditary melanoma selection and the size of the family material studied constitute other possible explanations for these differences. Overall, germline alterations in the *CDKN2A* gene have on average been detected in 20% of families with hereditary CMM and 3 or more affected members worldwide. The frequency of detected *CDKN2A* germline mutations increases with the number of affected individuals in a family (Kefford *et al.,* 1999; Goldstein & Tucker, 2001a). *CDKN2A* germline mutations involved in familial melanoma segregate with the disease and encode functionally aberrant p16 protein.

The occurrence of 9p21 linkage in the absence of mutation in the coding regions of *CDKN2A* may be explained in several ways. Such explanations include:

a) The possibility of mutations occurring in other candidate melanoma susceptibility genes at 9p21, such as *CDKN2B* and exon 1β (*ARF*). However, no germline mutations in *CDKN2B* have been found in melanoma kindreds (Stone *et al.,* 1995b; Liu *et al.,* 1997; Platz *et al.,* 1997; Flores *et al.,* 1997; Fargnoli *et al.,* 1998;). To date only two germline alterations in *ARF* have been reported. One is a germline deletion of the *ARF*-specific exon 1β in a family characterized by multiple melanomas and neural system tumors (Randerson-Moor *et al.,* 2001) and the other is a 16 bp insertion in a family with multiple primary melanomas (Rizos *et al.,* 2001b).

b) An alternative explanation for the low incidence of *CDKN2A* mutations in 9p21 linked families is the presence of another unknown tumor suppressor gene(s) on chromosome 9p (Holland *et al.,* 1995; Puig *et al.,* 1995; Otha *et al.,* 1996; Flores *et al.,* 1996; Morita *et al.,* 1998; Ruiz *et al.,* 1998; van der Velden *et al.,* 1999; Parris *et al.,* 1999; Fujimoto *et al.,* 1999; Rubben *et al.,* 2000). Cytogenetic and LOH studies have suggested the possibility of additional TSGs on 9p (Pollock *et al.,* 2001a).

c) Yet to be recognized large genomic deletions (Bahuau *et al.,* 1998), is another possibility.

d) Mutations altering gene expression have also been reported for *CDKN2A* (Rizos *et al.,* 1997), however, the possible importance of this in melanoma predisposition is unknown.

e) It has been hypothesized that mutations in non-coding segments of the *CDKN2A* locus could be another possible reason for the low frequency of germline mutation. Several Canadian families have been reported to carry a mutation in the 5'-untranslated region (UTR) at base

–34 of *CDKN2A*, which creates a novel translation initiation site and markedly decreased translation from the wild-type site (Liu *et al.,* 1999). Although mutations in the 5'-UTR account for a small proportion of 9p21-linked families, no true promoter mutations have been found. This suggests that mutations in the promoter and 5' UTR of *CDKN2A* gene play a minor role in melanoma predisposition (Soufir *et al.,* 1998; Harland *et al.,* 2000; Pollock *et al.,* 2001b).

f) A deep intronic mutation in *CDKN2A*, IVS2-105, has been reported in 15% of UK melanoma families who do not have previously reported coding or promoter mutations (Harland *et al.,* 2001). Germline splicing mutations have been identified in *CDKN2A*; IVS1-1 and IVS2+1 (MacKie *et al.,* 1998; Petronzelli *et al.,* 2001). *CDKN2A* mutations, in particular intronic mutations may go undetected due to methodological difficulties.

g) No evidence of germline inactivation by methylation of CpG islands within the promoter, as an alternative mechanism of *CDKN2A* inactivation has been reported in familial melanoma.

Familial melanoma patients who have inherited two mutated copies of the *CDKN2A* gene develop normally and, surprisingly, are not more severely affected than their heterozygous counterparts (Gruis *et al.,* 1995a), which again raises questions concerning other genes or exogenous factors that may be involved in the phenotypic expression of melanoma. Melanoma susceptibility thus, may have polygenic cause. Evidence of locus heterogeneity has been reported in genetic linkage studies for familial melanoma (Goldstein *et al.,* 1994; MacGeoch *et al.,* 1994; Hussussian *et al.,* 1994)

Predisposition to melanoma has also been linked to germline mutations within a second gene, *CDK4*, located on chromosome 12q14 (Demetrick *et al.,* 1994; Wolfel *et al.,* 1995), providing evidence that more than one melanoma predisposing gene exists within the genome and indicates that the RB pathway is pivotal in melanoma development. However, as of to date only three kindreds carrying *CDK4* germline mutations have been documented (Zuo *et al.,* 1996; Soufir *et al.,* 1998). The *CDK4* germline mutation identified, abolishes the binding of cdk4 to p16 but preserves its ability to interact with cyclin D (Zuo *et al.,* 1996), providing further evidence that impaired p16-mediated cell cycle regulation may predispose carriers to melanoma development. Despite the fact that the dominant oncogene *CDK4* and the tumor suppressor *CDKN2A* have different modes of action, no obvious differences in clinical characteristics such as the age of onset, tumor and nevi number observed between melanoma patients carrying *CDKN2A* or *CDK4* mutations have been observed (Zuo *et al.,* 1996).

The penetrance of the *CDKN2A* gene for melanoma development has been estimated to be 53% by the age of 80 years (Zuo *et al.*, 1996; Cannon-Albright *et al.*, 1994). In a study from the Melanoma Genetics Consortium, the age-specific penetrance has been estimated in *CDKN2A* mutation carriers from 80 melanoma prone kindreds. The overall penetrance is 0.30 by age 50 and 0.67 by the age of 80 years, but the risk varies significantly with geographical region suggesting a gene-environment interaction (Bishop *et al.*, 2002). This risk is not modified by gender or whether the *CDKN2A* mutation alters or does not alter p14ARF protein.

CDKN2A mutations have been analyzed in a large population-based cancer registry study, which included 481 families with melanoma from Australia (Aitken *et al.*, 1999). These families were stratified into three groups, (high-, intermediate-, and low-risk according to the total number of melanoma cases). Germline *CDKN2A* mutations were found only in the high-risk group where the frequency was 10.3%, (9 of 87). The frequency of germline *CDKN2A* mutations was estimated to be approximately 0.2% in all melanoma cases in Queensland (Atiken *et al.*, 1999).

In addition to mutations, several common polymorphisms e.g., 500 C>G; A148T (substitution of alanine to threonine), in the *CDKN2A* gene have been reported (Kumar *et al.*, 2001; Atiken *et al.*, 1999). However, the potential effect of this polymorphism on p16 function is unknown and the association between this *CDKN2A* polymorphism and melanoma risk is weak (Atiken *et al.*, 1999).

CDKN2A founder mutations
Some mutations in the *CDKN2A* gene have been observed only in one family while others were repeatedly identified in independent families of diverse geographical origins (e.g., M53I, 23ins24, G101W) or from the same geographic areas (e.g., 225del119 from the Netherlands, 113insR from Sweden, G34T from Canada; IVS2-105 from England). Haplotype analyses of such families have revealed that most of these mutations arose from common founders (single genetic origins), rather than from independent mutational events in several kindreds (Gruis *et al.*, 1995b; Pollock *et al.*, 1998; Liu *et al.*, 1999; Ciotti *et al.*, 2000; Goldstein *et al.*, 2000; 2001a,b; Auroy *et al.*, 2001; Harland *et al.*, 2001; **paper II**). Indeed, only the 23ins24, a 24-base pair duplication, has been shown to have arisen independently at least 3 times, probably due to instability of the 5' tandem repeat region (Pollock *et al.*, 1998). The age of some founder mutations, have been approximated, the G101W, and 113insR seem to have arisen

approximately 100 generations (2000 years) ago (Ciotti *et al.,* 2000; **paper II**), while V126D mutation, found in North American families originated approximately 34-52 generation ago (Goldstein *et al.,* 2001b).

Modifier (low risk) genes in CMM

Allelic variants of low risk genes may also confer susceptibility to CMM by acting as modifiers of high-risk genes (*CDKN2A*, *CDK4*) in familial melanoma.

The melanocortin 1 receptor-gene (*MC1R*), located on 16q24, involved in the regulation of melanin production, both eumelanin (brown-black) and pheomelanin (red-yellow) by melanocytes, is a low-risk susceptibility gene. Polymorphic variants of *MC1R* are associated with skin type, tanning ability and hair colour. It has been shown recently that *MC1R* is also associated with the risk of developing both sporadic and hereditary melanoma and acts as a risk modifier in *CDKN2A* germline mutation carriers. (Palmer *et al.,* 2000; Box *et al.,* 2001; van der Velden *et al.,* 2001a; Kennedy *et al.,* 2001). Some *MC1R* genotypes increased the melanoma penetrance in *CDKN2A* gene carriers from 18 to 55% in Dutch melanoma families (van der Velden *et al.,* 2001a).

The individual melanoma risk may thus be modified through a variety of polymorphisms (SNPs, single nucleotide polymorphism) in several genes. Among the risk modifiers one could expect to find variants of genes involved in DNA repair capacity (*DRC*). It has been shown that lowered DRC in combination with low tanning ability and the presence of dysplastic nevi may increase the melanoma risk (Landi *et al.,* 2002).

CDKN2A germline mutations in patients with multiple primary melanomas (MPM)

Patients with hereditary melanomas are particularly prone to develop MPM. Patients with a family history of melanoma have a 14% risk of a developing second primary lesion during the first 10 years after diagnosis of a first melanoma, while this risk is 5% in sporadic melanomas (Slingluff *et al.,* 1993). In principle MPM may be related both to the genetic background such as a predisposing germline mutation and to environmental factors such as high sun exposure. It has been reported that the occurrence of MPM in an individual increases the chance of finding *CDKN2A* germline mutations. Approximately 10% of individuals with MPM but without known family history of melanoma have been shown to carry *CDKN2A* germline mutation (Monzon *et al.,* 1998; MacKie *et al.,* 1998; Kefford *et al.,* 1999; Auroy *et al.,* 2001; **paper III**).

CDKN2A germline mutations are more frequent among patients with MPM and with a family history of melanoma (Monzon *et al.,* 1998; MacKie *et al.,* 1998; Holland *et al.,* 1999).

Functional analysis of mutant p16 proteins

To date, at least 50 *CDKN2A* germ-line mutations have been identified in over 100 melanoma kindreds world-wide. Although most of these mutations co-segregate with the disease in melanoma pedigrees, functional analyses of mutants are also required to distinguish disease-associated mutations from population polymorphisms. The major biochemical function of p16 is to bind to the catalytic subunit of cyclin dependent kinases, CDK4 and CDK6 (Serrano *et al.,* 93), thereby blocking their ability to form catalytic complexes with cyclin D. The normal p16 protein thus maintains cell cycle arrest. A non-functional p16 protein has lost its regulatory ability and cannot prevent cells from passing through the cell cycle (Ruas & Peter 1998; Chin *et al.,* 1998b). The p16 protein is composed of four ankyrin repeats that facilitate protein–protein interactions. Some residues in these repeats are conserved (consensus sequences). Thus, mutations of these amino acids are likely to affect protein function (Serrano *et al.,* 1993). A variety of functional assays have been applied which reflect properties of p16 protein, including CDK4/CDK6 binding, kinase inhibition, growth inhibition (G1 arrest), and p16 folding and stability assays (Koh *et al.,* 1995; Lukas *et al.,* 1995; Ranade *et al.,* 1995; Yang *et al.,* 1995; Parry & Peters, 1996; Zhang & Peng, 1996; Lilischkis *et al.,* 1996; Arap *et al.,* 1997; Sun *et al.,* 1997; Monzon *et al.,* 1998; Ruas *et al.,* 1999). The functional effect of *CDKN2A* mutations may also be investigated by comparing differences in the cellular localization of wild-type with mutant *CDKN2A* (Walker *et al.,* 1999).

However, different groups have reported inconsistent results for the identical mutant proteins, this may be a reflection of different assays used (Ruas *et al.,* 1999). Differences in the estimated degree of functional loss could be due to protein aggregation during production of recombinant p16 protein in bacteria. Furthermore, *in vitro* assays may not be a true reflection of *in vivo* conditions, due to variation in the level of p16 expression, ectopic expression of p16 in eukaryotic cells, the type of fusion proteins used and the assay conditions applied.

Germline CDKN2A mutations in Swedish melanoma kindreds

Initial screening of Swedish families with two or more relatives with CMM resulted in the identification of two different germline mutations in the *CDKN2A* gene; one, a single point mutation in exon 1, at codon 48 leading to a substitution of leucine for proline (P48L) observed

in one family (**Table 1**). The other mutation, a 3bp insertion in exon 2, leads to insertion of an extra arginine at codon 113 (113insR) and was observed in 4 families from Stockholm and 2 families from Southern Sweden (Borg *et al.,* 1996; Platz et al 1997). Further screening has resulted in the identification of an additional eleven families carrying the same mutation, which represents a founder mutation in the Swedish population (**paper II**). A third mutation a 24 bp deletion (Δ62-69) was identified in a patient with MPM and melanoma heredity (**paper III, IV**). This 24 bp deletion causes an in-frame deletion of residues 62-69 in p16 and also of residues 77-84 in p14ARF. Finally, a missense mutation, leading to substitution of glycine for valine at codon 115 has been detected in one family (Borg *et al.,* 2000). The estimated germline *CDKN2A* mutation frequency in two different studies in Sweden was 7.8% of 64 (Platz *et al.,* 1997) and 19% of 52 families (Borg *et al.,* 2000).

Table 1: Germine *CDKN2A* alterations in Swedish melanoma families and patients with MPM

Exon	Base change	AA change	No. of families carry mutation	ARF change	Function defect
1α	CCG>CTG	Pro48Leu	1		Yes
1α	-14C>T	Noncoding	-*		Unknown
1α	Ins 1 bp T	28insT	-*		Yes(?)
2	Ins bp GTC	113insArg	17	128insSer	Yes
2	Del 24 bp	△62-69	1	△77-84	Yes
2	GTG>GGG	Val115Gly	1		No

(*) Mutation in MPM patient without known family history.

Genetic susceptibility to melanoma and DNS

Atypical / dysplastic nevi play an important role in the etiology of CMM (Clark *et al.,* 1978; Bergman *et al.,* 1994), and their presence is known to be one of the strongest epidemiologic risk factors for melanoma (Swerdlow & Green, 1987). *CDKN2A* mutations have been suggested to be an early genetic event in the development of dysplastic nevi (Park *et al.,* 1998). Since both melanoma and dysplastic nevus syndrome (DNS) appear to have a hereditary basis, the hypothesis that the same gene(s) predispose to both conditions has been made. Although segregation analysis of the transmission of melanoma alone has suggested a simple Mendelian autosomal dominant mode of inheritance, the analysis of melanoma and DNS together, have

yielded conflicting results (Greene *et al.,* 1983; Bale *et al.,* 1986; Gruis *et al.,* 1993; Goldstein *et al.,* 1996; Cannon-Albright, 1996). Some evidence for linkage of normal nevus count with *CDKN2A* mutation has been found in three *CDKN2A*-linked families (Cannon-Albright *et al.,* 1994). Therefore the segregation of DNS phenotype with *CDKN2A* remains controversial.

Some studies suggest that the DNS phenotype may not be directly related to *CDKN2A* mutations (Ciotti *et al.,* 1996; Healy *et al.,* 1996; Puig *et al.,* 1997; Soufir *et al.,*1998; Kefford *et al.,* 1999). Among five melanoma families in the U.K., *CDKN2A* mutation carriers were reported to have a higher nevus count in comparison to non-carriers (Wachsmuth *et al.,* 1998). Finally, Sib-pair linkage analysis has showed that 30% of variance in nevus counts is due to genetic variations in the 9p21 region, but outside the sequence encoding *CDKN2A* (Zhu *et al.,* 1999). Thus, the role of this melanoma susceptibility gene in the development of dysplastic nevi still remains unclear, and further analysis is required to elucidate its precise role.

Mechanisms of CDKN2A inactivation in human cancer

There are three main mechanisms underlying the genetic inactivation of *CDKN2A* in human tumor cells: (1) deletion of both alleles, (2) deletion of one allele and mutation of the remaining allele, and (3) deletion or mutation of one allele and hypermethylation of the remaining allele (Myohanen *et al.,* 1998; Esteller *et al.,* 2001). Unlike other tumor-suppressor genes that are commonly inactivated by point mutations, homozygous deletion represents the major mechanism of *CDKN2A* inactivation. p15 and exon1ß of *CDKN2A* are often co-deleted with *CDKN2A* in tumors. A Homozygous deletion of *CDKN2A* has been found in a wide range of cell lines derived from a variety of human tumors (Kamb *et al.,* 1994a; Hall and Peters, 1996; Foulkes *et al.,* 1997; Liggett & Sidransky, 1998; Ruas & Peters, 1998). The high rate of *CDKN2A* deletions in human cancer may be explained by its unique genomic organization, since the two proteins p16 and p14ARF are important regulators of the two central growth control pathways, RB and p53, respectively. Deletion of this locus may, thus, cause loss of control of proliferation by targeting both of these pathways simultaneously.

The frequency of homozygous *CDKN2A* deletions is roughly 60% in melanoma cell lines (Castellano *et al.,* 1997), and 25% in uncultured tumors (depending upon the tumor type) (Flores *et al.,* 1997). *CDKN2A* has a role in establishing G1 arrest associated with replicative senescence (Alcorta *et al.,* 1996; Hara *et al.,* 1996). A role for the *CDKN2A* gene in replicative senescence may explain the higher rate of loss of gene expression in immortalized cell lines as

opposed to primary tumors, suggesting selection for the loss of *CDKN2A* function during the process of immortalization (Okamoto *et al.,* 1994a,b).

Hypermethylation of 5'CpG islands upstream of *CDKN2A* associated with transcriptional silencing is another mechanism for gene inactivation, which releases the cell from a potent cell cycle inhibitor and occurs in a wide range of malignancies (Gonzalez-Zulueta *et al.,* 1995; Herman *et al.,* 1995; Merlo *et al.,* 1995; Jones & Laird, 1999; Baylin *et al.,* 2001; Rocco & Sidransky, 2001; Soengas *et al.,* 2001; Esteller *et al.,* 2001). *CDKN2A* inactivation by promoter methylation is uncommon in sporadic melanoma but has been reported to occur in 32% of primary uveal melanomas (van der Velden *et al.,* 2001b).

Fifteen to twenty percent of melanoma cell lines and 10-30% of primary melanomas carry point mutations in the *CDKN2A* gene (Piccinin *et al.,* 1997; Kumar *et al.,* 1998; Ruas & Peters, 1998). In addition, known polymorphisms have been identified in the 3'UTR of *CDKN2A* gene (Kumar *et al.,* 2001). The role of *ARF* in sporadic melanoma is not clear. In sporadic melanomas, the frequency of small deletions and mutations in the *CDKN2A* gene are generally lower than allelic losses (LOH) at chromosome 9p21, suggesting the presence of other TSG(s) in 9p locus (Flores *et al.,* 1996; Ohta *et al.,* 1996; Fujimoto *et al.,* 1999; Kumar *et al.,* 1999). A decreased level of p16 expression in early melanoma has been associated with progression of disease (Reed *et al.,* 1995; Fujimoto *et al.,* 1999).

Germline CDKN2A mutations in other tumors

An increased risk of cancers other than melanoma in melanoma-prone families has been reported in some studies (Lynch *et al.,* 1981; Bergman *et al.,* 1990). Schenk *et al.,* reported a two-fold risk of pancreatic cancer, especially in patients with a first diagnosis of melanoma before 50 years of age (Schenk *et al.,* 1998). Some melanoma kindreds with mutations in the *CDKN2A* gene show an association with pancreatic cancer (Goldstein *et al.,* 1995; Whelan *et al.,* 1995; Bergman & Gruis, 1996; Ghiorzo *et al.,* 1999; Borg *et al.,* 2000), but the precise relationship between pancreatic cancer and *CDKN2A* mutation has remained unresolved.

Certain studies suggest that constitutive abnormalities in *CDKN2A* may be associated with squamous cell carcinoma of the head/neck region, although these results were based on small numbers of pedigrees (Whelan *et al.,* 1995; Yarbrough *et al.,* 1996; Sun *et al.,* 1997).

Additionally, evidence has been presented for a role of *CDKN2A* alterations in susceptibility to breast cancer (Borg *et al.,* 2000; Plna & Hemminki, 2001).

A combined predisposition to malignant melanoma and nervous system tumors has been reported (Bahuau *et al.,* 1998; Tachibana *et al.,* 2000; Petronzelli *et al.,* 2001).

On the whole, data relating the presence of additional cancer types in melanoma prone families indicate a possible role of *CDKN2A* which may be modified by the influence of other gene/s or environmental factors. We have observed a number of other malignancies, including pancreatic carcinoma and squamous cell carcinoma of the oral cavity, in Swedish melanoma families with germline *CDKN2A* mutations (Platz *et al.,* 1997, unpublished data).

AIMS OF THE STUDY

- To clarify the role of germline *CDKN2A* mutations in the development of melanoma tumors in kindreds with hereditary melanoma.

- To study the relationships between germline *CDKN2A* mutations and melanoma, and between DNS phenotype and melanoma, in kindreds with hereditary melanoma.

- To investigate the role of germline *CDKN2A* mutations in Swedish patients with multiple primary melanomas.

- To elucidate the ancestral background of the *CDKN2A* 113insR mutation which occurs in several Swedish melanoma families, and to estimate the age of a putative founder mutation.

- To clarify the possible role of *CDKN2A* exon 2 mutation in melanoma development especially with respect to *ARF*.

- To understand the mechanisms and frequency of *Cdkn2a/b* gene loss during progression by using a model system consisting of RAS-transformed rat embryo fibroblast clones.

MATERIALS AND METHODS

For detailed descriptions, the reader is referred to papers I-V

Patients and DNA samples

The families were identified in a national screening program aimed at detecting melanoma kindreds established in 1987 by the Swedish Melanoma Study Group or recruited from the Oncogenetic Clinic at the University Hospital in Lund. All diagnoses of melanoma were confirmed by pathologists specialized in dermatology as part of the clinical program for melanoma care. Each patient was questioned regarding melanoma heredity.

In the investigation of the relationships between germline *CDKN2A* mutations, DNS phenotype and melanoma, five melanoma families from Stockholm including 19 individuals with invasive cutaneous melanoma and 3 with *in situ* melanomas were studied **(paper I)**.

To study the role of germline *CDKN2A* mutation in MPM, 79 living patients were screened for *CDKN2A* mutations. These patients had been treated for two or more histologically verified cutaneous melanomas and identified in the Regional Cancer Registry of Stockholm-Gotland area **(paper III)**.

In the investigation of the ancestral background of *CDKN2A* 113insR, 17 melanoma families carrying the same mutation were haplotyped. Eight of these families were identified in Stockholm, originating from the County of Stockholm and Gothenburg and nine families who were identified in Lund, originated from the southern health care region of Sweden. Blood samples were obtained from 45 mutation carriers and four non-carriers belonging to the 17 melanoma families. Archival tissue was obtained from four additional mutation carriers. Leukocytic DNA was obtained from blood and/or tumor samples belonging to members of melanoma kindreds and individuals with multiple primary cutaneous melanoma **(papers I, II, III)**. Blood samples from 46 control individuals were used to estimate allele frequencies **(paper II)**. DNA was isolated from 19 rat embryo fibroblast (REF) established cell lines to investigate the frequency of *Cdkn2a*/2b gene loss **(paper V)**.

Mutational analysis (papers I, II, III)

SSCP analysis and nucleotide sequencing

Single strand conformation polymorphism (SSCP) is a screening technique for the detection of point mutations and small deletions. It is based on the principle that single-stranded DNA molecules have a sequence dependent secondary structure which determine the molecules' electrophoretic mobility in a nondenaturing gel matrix (Orita *et al.,* 1989). Mutational alterations in single-stranded DNA molecules change the secondary structure (conformation) of DNA and therefore different electrophoretic mobilities will arise in non-denaturing gel electrophoresis conditions. The detection sensitivity of SSCP is 80-90% in fragments of 100 to 250 bp lengths and depends on electrophoresis temperature, GC content of the fragment and gel matrix composition (Hayashi, 1992). SSCP does neither reveal the nature nor the position of mutations/polymorphisms. Therefore sequencing is always required after SSCP, when previously unknown mutations are studied. In our study, each of the three *CDKN2A* exons were amplified by PCR from genomic DNA using radiolabelled dCTP. PCR products were denatured by heating and the single-stranded molecules were analyzed both in the presence and absence of 10% glycerol at 18° C and in the cold on 7% polyacrylamide gels. PCR conditions and SSCP analysis have been described in detail in **(paper III)** and by Platz *et al.,* (1997).


Direct DNA sequencing

Direct sequencing was performed using a dye terminator kit (BigDye, Applied Biosystems) and an ABI 310 capillary sequencing system (Applied Biosystems)

Microsatellite analysis in LOH studies and genotyping (paper I, II, III, V)

An individual with two different alleles of a gene is decribed as heterozygous for that gene. Loss of heterozygosity (LOH) signifies the loss of one of the alleles. In tumors, the wild-type allele of TSG is frequently lost during tumorigenesis. Haplotype refers to a particular combination of alleles in a defined region of a chromosome. LOH and haplotype analysis are based on variations in microsatellite polymorphic markers. Microsatellites are short tandem repeat sequences (< 0.1 kb) spaced across the genome, and can be composed of di-, tri-, tetra-, or penta-nucleotide repeats (Edwards *et al.,* 1991), e.g. CA dinucleotide repeats. The number of oligonucleotide repeats in each repeat (n) is polymorphic in the normal population. These normal differences in the DNA sequence are present at specific loci of chromosomes and therefore are useful for analysis of chromosome segregation in different generations. The nucleotide sequences flanking

the location of the polymorphic repeat are used to design PCR primers. PCR amplification of alleles whose repeat sizes differ permit us to distinguish between paternal and maternal alleles (**Figure 6**). Microsatellite polymorphic markers located around the *CDKN2A* gene (**Figure 4**) were used for LOH analysis in **paper I** and for genotyping in **paper III**. DNA from normal or tumor tissues was amplified using radiolabelled primers of microsatellite markers flanking the *CDKN2A* gene on chromosome 9p21. PCR products were separated on denaturing polyacrylamide gels followed by autoradiography. Autoradiograms were scanned using a laser densitometer for quantitation.

In **paper I & V**, loss of an allele in tumor DNA was scored when the signal for one of the alleles was reduced by at least 50% in intensity when compared to the signal from DNA from normal tissue. In **paper III** haplotypes were studied using 9p21 markers.
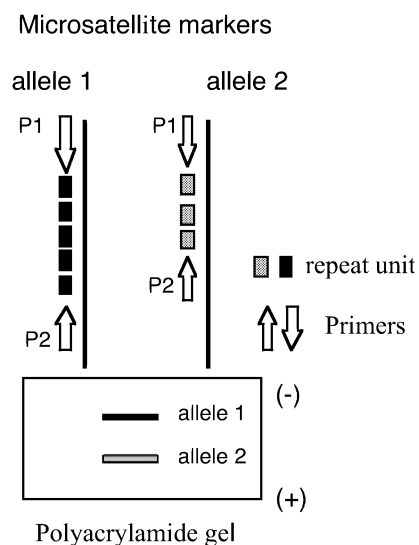
Figure 6: Detection of microsatellite polymorphisms. Allele 1 and allele 2 (one paternal and the other maternal with different unit lengths) are amplified with PCR primers (P1 & P2). The PCR products are separated on a polyacrylamide gel.

Functional studies (papers I, III, IV)

Although germline *CDKN2A* mutations may segregate closely with the disease in melanoma families, demonstration of functional defects in the p16 protein is important to distinguish disease-associated mutations from population polymorphisms. To test the effect of *CDKN2A*

germline mutations on protein function, the known properties of p16 have been exploited (Ruas *et al.,* 1999). As the main role of p16 is lies in its ability to bind directly to and inhibit CDK4 and CDK6, we have used this ability of p16 in our study to test its functionality.

In vitro mutagenesis of the CDKN2A gene

Site-directed *in vitro* mutagenesis is a procedure by which a mutation is introduced in a specific sequence of a gene. *CDKN2A* cDNA corresponding to the P48L mutation (a leucine to proline substitution at codon 48) was constructed using the Altered Sites II *in vitro* mutagenesis system (Promega) **(Paper I)**. In order to construct a *CDKN2A* cDNA containing the codon 62-69 24bp deletion, we used the PCR-based overlap extension approach of Ho *et al.,* (1989) **(paper III)**. The mutated *CDKN2A* cDNA inserts were excised by restriction enzyme cleavage and recloned in a plasmid vector. Direct sequencing of both strands of the plasmid DNA (from isolates) was performed to confirm that they only contained the desired mutation.

Protein binding assay (papers I, III)

(^{35}S)-methionine-labelled wild-type and mutant p16 proteins, human CDK4, CDK6 and CDK2 proteins, were synthesized by coupled transcription/translation of corresponding plasmid DNA constructs in rabbit reticulocyte lysates. Aliquots of a single protein or mixtures of two proteins were incubated for 30 min at either 30°C or 42°C, diluted to 1 ml, and precipitated with polyclonal antibody against either p16 or CDK4. The immune complexes were collected on protein A-Sepharose beads and electrophoresed in a polyacrylamide gel. The labeled proteins were visualized by autoradiography.

Cell culture and transfection (papers IV, V)

Cell-lines used were grown in Iscoves Modified Dulbeccos Medium (IMDM) in the presence of 10% fetal calf serum (FCS). Cells were transfected with pcDNA3 expression vectors encoding wild-type or mutant p16 or p14ARF using Lipofectamine Plus or Lipofectamine 2000 according to the manufacturer´s instructions (Invitrogene, MD). In **paper V** REF cells were prepared from 16 day old (BN/Mol x LEW/Mol) F1 rat embryos. Cells were plated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% fetal calf serum. The T24-*RAS* (12V-*H-RAS*) gene was cloned into a vector encoding the neomycin resistance gene. Cells were transfected with this vector, grown in medium containing dexamethasone and selected with G418.

Immunofluorescence staining **(papers IV, V)**

Immunostaining can be used to determine the subcellular locations of proteins.

Twenty four hours after transfection, cells were fixed in methanol/ acetone at -20°C and rehydrated in PBS. Cells were incubated with the primary antibody for 60 min at room temperature followed by three washes in PBS. The cells were incubated with the secondary, fluorochrome-conjugated antibody for an additional 30-45 min. Slides were examined with a Leitz DMRB fluorescence microscope.

BrdU incorporation assay **(paper IV)**

5-bromodeoxyuridine (BrdU) incorporation assay was used to measure DNA replication. Cells were seeded at equal cell density in 100 mm diameter dishes and transfected. Cells were trypsinized, washed and split equally onto glass coverslips in 6-well chambers (duplicates). BrdU was added to the culture medium 24 hours after transfection. Cells were fixed in methanol-acetone 24 hours after the addition of BrdU, stained with antibody and then treated for 15 min with 1.5M HCl. Finally, cells were stained for 1 hour with a mouse monoclonal anti-BrdU antibody followed by a FITC-conjugated anti-mouse antibody. Nuclei were visualized using Hoechst staining.

Colony formation assay **(paper IV)**

Cells were plated at an equal density in 6-well format dish and transfected overnight with linearized vector. Colony formation assays were performed by passaging trypsinized cells 24 hours after transfection at an equal low density into 100 mm petri dishes or alternatively in 6-well cell chambers containing media supplemented with G418. Colonies were stained with Giemsa 2 weeks later and scored using a Colony Counter 2000.

Immunoblotting *(Western blotting)* **(paper IV)**

Immunoblotting is used to study protein expression levels in cell extracts by electrophoretic separation and immunological recognition. Protein samples are solubilized, usually with sodium dodecyl sulfate (SDS) and separated by SDS-PAGE (polyacrylamide gel electrophoresis in the presence of SDS. SDS binds to hydrophobic regions of the protein molecules causing them to unfold and migrate at a rate that depends on their net charge, size and shape. The fractionated proteins are then transferred electrophoretically to a nitrocellulose, PVDF, or nylon membrane. The transferred proteins are bound to the surface of the membrane, providing access for reactions with immunodetection reagents. After probing with a primary antibody, the membrane

is washed and the antibody-protein complexes are identified with a secondary antibody, which is conjugated to a reporter, horseradish peroxidase (HRP). In the presence of chemical enhancers, HRP activity results in the oxidation of luminol, which then emits light, which can be detected.

Analysis of Cdkn2a/b genes in rat (paper V)

Using nested PCR, rat *Cdkn2a/b* was amplified from genomic DNA isolated from established cell lines. Rat *Jun* and *Ifn-a* were amplified using mouse primers. PCR products were separated by electrophoresis in 2% agarose gels.

Karyotyping (paper V)

Cultures were treated with colcemid and metaphase spreads slides were G-banded by trypsin-Giemsa treatment.

Reverse chromosome painting (paper V)

Total genomic DNA from cell line A14 was labelled with digoxigenin-11-dUTP by nick-translation. The DNA probe was applied to normal rat fibroblast metaphase chromosome slides. After hybridization, the slides were washed. The labelled DNA was detected with FITC-antidigoxigenin and the slides were then counterstained by DAPI.

p16 expression

Northern blotting for mRNA detection was performed by hybridization of total cellular RNA to a specific probe derived from the human *CDKN2A* and *GAPDH* genes.

Statistical analysis (papers I, II, III, IV)

A p-value for rejection of the null hypothesis was set at <0.05. The Chi^2 test was used to test possible differences among the groups and student's t-test was used to test for mean differences. Maximum likelihood approach was used to calculate the age of the 113insR founder mutation (Guo, 1997; Neuhausen *et al.,* 1996).

RESULTS AND DISCUSSION

Paper I

The association between CDKN2A mutations, melanoma development, and DNS

To clarify the role of *CDKN2A* mutations in melanoma predisposition, we analyzed five Swedish melanoma families with germline *CDKN2A* mutations, to determine the relationships between mutation carrier status, DNS and melanoma occurrence.

There was a highly significant correlation between the presence of germline *CDKN2A* mutations and development of cutaneous melanoma regardless of DNS status: 55% of mutation carriers had been diagnosed with melanoma, while 12.5% of individuals without mutations had melanoma ($p < 0.0002$). These results confirmed that *CDKN2A* germline mutations predispose an individual to the development of cutaneous melanoma.

There was also a significant correlation between the diagnosis of the DNS phenotype and melanoma, regardless of *CDKN2A* mutation status: 45% of individuals with DNS had melanoma, compared to 8% of those without DNS phenotype ($p < 0.01$). These results confirmed the association of DNS with familial melanoma.

We also found a significant correlation between the presence of *CDKN2A* mutations and the DNS phenotype, although the correlation was weaker than that for *CDKN2A* mutations and melanoma: 86% of mutation carriers exhibited DNS, while 57% of individuals without mutations had DNS ($p < 0.03$). Our results differ somewhat from those of some other groups who suggest that the DNS phenotype is not directly related to *CDKN2A* mutations (Gruis *et al.,* 1995b; Puig *et al.,* 1997; Soufir *et al.,* 1998). These differences may indicate a genetic heterogeneity with respect to the DNS phenotype (Goldstein *et al.,* 1993b, 1996; MacGeoch *et al.,* 1994; Hussussian *et al.,* 1994), and a differential effect of environmental or life style factors.

Our investigation of the possible effect of DNS status on the age of melanoma diagnosis in *CDKN2A* mutation carriers showed that all cases of early onset melanoma (<35 years) occurred in individuals with the DNS phenotype. We suggested that *CDKN2A* germline mutations and

DNS phenotype might both be related to a predisposition to develop melanoma at a younger age.

In addition to cutaneous melanoma, a number of other malignancies have been found in our studied melanoma kindreds with *CDKN2A* germline mutations. We suggested an association between inherited *CDKN2A* mutations and other malignancies.

Our results confirm that members of melanoma families who have the DNS phenotype should be under surveillance irrespective of *CDKN2A* germline mutation status, since there is a well-documented association between DNS and melanoma.

Functional analysis of the protein product of the previously reported P48L germline mutation in exon 1 of the *CDKN2A* gene present in one kindred (family 41) (Platz *et al.,* 1997) was performed using *in vitro* mutagenesis, coupled transcription/translation and an *in vitro* binding assay. The results showed that the mutant *CDKN2A* product does not bind to cdk4 and cdk6. We also showed LOH of the *CDKN2A* gene, with loss of the wild-type allele in both a primary and a metastatic melanoma in one P48L carrier. This suggests that loss of the wild type allele of the *CDKN2A* tumor suppressor gene may be a relatively early event in the development of melanoma in germline mutation carriers.

Paper II

A common founder for the 113insR CDKN2A mutation in 17 Swedish melanoma families
The 113insR germline mutation in exon 2 of *CDKN2A* was found in 17 Swedish melanoma families from Stockholm, Gothenburg, and the southern region of Sweden. Given the high frequency of this mutation, we wanted to determine whether the repetitive observation of 113insR is a result of independent mutational events or due to a common ancestor. We were also interested in estimating the age of a putative founder mutation to evaluate its potential geographic distribution.

Haplotype analysis of members of the 17 kindreds using ten microsatellite markers flanking the *CDKN2A* region showed that the majority of these families share a common allele at markers close to *CDKN2A*. This suggests that the mutation originates from a common ancestor.

To estimate when the 113insR mutation originated, we used the maximum likelihood (MLE) method, which is particulary sensitive to marker mutation rates (Neuhausen *et al.,* 1996; 1998). This statistical analysis of recombinant events in the 9p21 region, indicated that the mutation has arisen 98 generations (90% confidence interval, 52-167 generations), or approximately 2000 years ago (assuming 20 years per generation). We also used the Q model (Risch *et al.,* 1995), which is critical for the estimated distance between the disease and marker loci. The results were consistent with the MLE approach. With regard to the probable ancient origin of the 113insR mutation, we expect that it could be found also in other populations with historical connections to Sweden. A G101W founder mutation has arisen almost at the same time period as 113insR (Ciotti *et al.,* 2000) but the latter has also been found in Australia, North America, and Europe especially with a high frequency in Italy (Mantelli *et al.,* 2002). However, 113insR has so far been exclusively reported in Swedish kindreds. *CDKN2A* may lie in a recombination hot spot since many meiotic recombinations were found within a 1-cM region at 9p21. The number of generations that have passed from the original founder thus may be overestimated.

The results of this study are in agreement with other reported recurrent *CDKN2A* mutations showing that most of these mutations result from a single origin and have a common founder, suggesting that *CDKN2A* is relatively stable. The presence of a founder effect can facilitate carrier detection and provide unique opportunities to study the effect of other genetic and environmental factors on penetrance and disease phenotype.

Paper III

Screening for CDKN2A mutations in individuals with MPM

In some cases, the occurrence of multiple primary melanomas (MPM) may have a genetic background, and may be associated with a family history of melanoma. To investigate the role of specific genetic mechanisms in the etiology of MPM, we selected 80 individuals, followed-up in Stockholm and Gothenburg with at least two primary cutaneous melanomas and screened them for the presence of germline *CDKN2A* mutations, using PCR-SSCP analysis and nucleotide sequencing. *CDKN2A* mutations were detected in nine (11%) of these patients and the majority of these individuals had a family history of melanoma. Thus, seven individuals with a family history of melanoma carried the identical 113insR (insertion of argenine at position 113) founder mutation. In addition, one patient, also with melanoma heredity, had a 24 base pair deletion,

which included codons 62-69 (Δ62-69). Two patients without a known family history of melanoma had *CDKN2A* alterations: one had a mutation in 5'UTR (-14C/T); and the other carried an insertion of an extra T in codon 28 which results in a stop signal in codon 43.

We demonstrated that the (Δ62-69) mutant protein is unable to bind to CDK4 and CDK6 in an *in vitro* binding assay and thus most likely predisposes to melanoma. Functional analysis of 113insR has showed that the mutant protein is abnormal and does not bind to CDK4 and CDK6 (Ruas *et al.,* 1999; Borg *et al.,* 2000).

Our results are in agreement with other published reports, in which 15% (5/33), 12% (2/17), and 9% (9/100) of sporadic MPMs were carriers of *CDKN2A* germline mutations (Monzon *et al.,* 1998; MacKie *et al.,* 1998; Auroy *et al.,* 2001, respectively).

In addition, we found that: a) patients with ≥3 melanomas more frequently have *CDKN2A* mutations than patients with 2 melanomas (p<0.001), b) patients with *CDKN2A* mutations are younger at diagnosis of the first melanoma compared to patients without mutations (p = 0.023), c) *CDKN2A* mutation carriers, more frequently have relatives with melanoma than mutation non-carriers (p<0.001). In one study, female carriers of *CDKN2A* mutation were more prone to develop MPM than men with this mutation (Borg *et al.,* 2000). In another study, 91% of patients with MPM had clinically or histologically diagnosed dysplastic nevi (Monzon *et al.,* 1998). The presence of dysplastic nevi and the relatively young age at diagnosis of the first melanoma are typical for MPM (Kang *et al.,* 1992; Frank & Rogers, 1993). However, in our study there were no significant correlations between *CDKN2A* mutation carrier status and gender, or the presence of dysplastic nevi.

We conclude that screening of patients with MPM is a useful strategy to identify kindreds with germline *CDKN2A* mutations. Members of such families may benefit from preventive clinical programs aimed at reducing melanoma risk.

Paper IV

Functional analysis of a germline 24bp deletion on both p16 and p14ARF

Due to overlapping open reading frames in exon 2 of the *CDKN2A* gene, some mutations in this exon affect both p16 and p14ARF. The 24 bp deletion found in *CDKN2A* exon 2 in a patient with multiple primary melanomas and melanoma heredity also causes an in frame deletion of

residues 77-84 in p14ARF. This deletion disrupts an arginine-rich cluster motif (residues 80-90) in the C-terminus of p14ARF that confers nuclear and nucleolar localization (Rizos *et al.,* 2000). To further clarify the possible role of the 24 bp deletion in *CDKN2A* exon 2 for melanoma development, especially with respect to p14ARF, we have studied the cellular distribution and function of the resulting p14ARFΔ77-84 and p16Δ62-69 mutant proteins.

We found that p14ARFΔ77-84 exhibited decreased nucleolar localization using immunofluorescence staining. This is most likely due to the deletion of two arginine residues (Arg 81 and Arg 82) within the C-terminal nucleolar localization domain. The p14ARFΔ77-84 was less efficient than wt p14ARF in stabilizing p53, inducing G1 cell cycle arrest and inhibiting colony formation. One possible explanation for this is that the mutant protein has reduced nucleolar localization and thus may be unable to sequester MDM2 and to prevent MDM2-mediated nuclear export of p53. The p14ARFΔ77-84 may also bind to MDM2 less efficiently. ARF, a highly basic protein, binds to the central acidic domain of MDM2 (Weber *et al.,* 2000a; Lohrum *et al.,* 2000) and the elimination of two arginine residues in p14ARFΔ77-84 could reduce its electrostatic interaction with this MDM2 domain.

p14ARF has been shown to have p53 independent functions (Weber *et al.,* 2000b) and it remains possible that these activities of ARF also could be, at least in part, affected by this mutation.

We showed in **paper III** that the p16Δ62-69 mutant protein is unable to bind CDK4 and CDK6 *in vitro*. In this study, we have also shown that the p16Δ62-69 mutant protein localizes predominantly to the cytoplasm, does not induce G1 cell cycle arrest, and fails to suppress colony formation, thus confirming the previously reported functional defect of this mutant. This is in agreement with other reports, showing that mutant p16 proteins that fail to bind CDK4 and CDK6 *in vitro* are generally also impaired in all other functional tests (Ruas *et al.,* 1999).

In conclusion, our data indicate that the p14ARFΔ77-84 protein has a partial functional defect that may complement the effect of the clearly defective mutant p16 protein during melanoma development.

Germline alterations that specifically target p14ARF have been reported (Randerson-Moor *et al.,* 2001; Rizos *et al.,* 2001b). In addition, mutations in exon 2 of *CDKN2A* dually affect both

p16 and p14ARF, and implicate a role for p14ARF in melanoma predisposition. However, more investigations are required to define the role of p14ARF in human melanoma.

Paper V

Analysis of mechanisms and frequency of CDKN2A/B gene loss using an animal model
LOH is the most common molecular genetic alteration in human cancer. The study of mechanisms involved in the generation of LOH is important and may have significant implications for understanding the pathogenesis of cancer. The aim of this study was to address the mechanism and frequency of *Cdkn2a/b* gene loss in REF (Rat embryo fibroblast) cells transfected with a mutant *ras* gene. REFs were prepared from F1 (BNxLEW) rat embryos and transfected with a plasmid containing 12V-Hras sequences. Cells were initially grown in medium containing glucocorticoid hormone dexamethasone (DEX), previously found to stimulate the growth of RAS oncogene transfected REF cells (Martens *et al.,* 1988; Marshall *et al.,* 1993). We examined the mechanisms of progression into hormone-independent cell lines. Sixty-two days after transfection, 22 clones were established as cell lines and 13 of these were studied together with subclones, (in total 21 cell lines). All lines studied showed homozygous deletions at *Cdkn2* loci (*Cdkn2a*/*Cdkn2b)* on RNO5 (Rat chromosome 5) by PCR analysis of genomic DNA at 6 months after transfection.

To examine the mechanism of gene deletion, we used PCR amplification of microsatellite markers on RNO5. LOH was found for all of the genetic markers examined in 7 of 21 cell lines **(Figure 7)**. The most likely mechanism for LOH in these cell lines was mitotic non-disjunction, with loss of the wild-type chromosome leading to hemizygosity. Non-disjunction followed by reduplication leading to homozygosity was the likely mechanism for LOH in 2 clones of these seven cell lines in which two copies of RNO5 were present. Of the remaining 14 cell lines, 3 cell lines showed no or minimal LOH on RNO5. Eleven cell lines showed partial LOH and were heterozygous for markers on RNO5p but hemizygous for markers on RNO5q. Mitotic recombination was suggested as the mechanism for LOH in the SA12-D cell line with regard to karyotype analysis and possibly the SA24S-D line. Deletions/rearrangements were the underlying mechanism for LOH in the other remaining 9 cell lines. Thus, homozygous deletion of the *Cdkn2* locus occurs by deletion of one copy of the locus followed by nondisjunction, deletion/rearrangement and, rarely, mitotic recombination of another copy. These finding are in

excellent agreement with the report by Thiaglingam *et al.*, (2001) who analysed the mechanisms of LOH in human colorectal cancer. The frequency of *Cdkn2a* gene deletions was estimated to be 2×10^{-8}/ cell/generation based on calculation of the number of cells and number of passages. It was previously observed that DEX enhances REF transformation by *RAS* and facilitates the isolation of transformed and immortal cell lines, but the mechanism for hormone promotion was not clear. Here, we found that DEX-induced cell proliferation was paralleled by a decrease in *Cdkn2a* gene transcripts, as shown by Northern blotting.
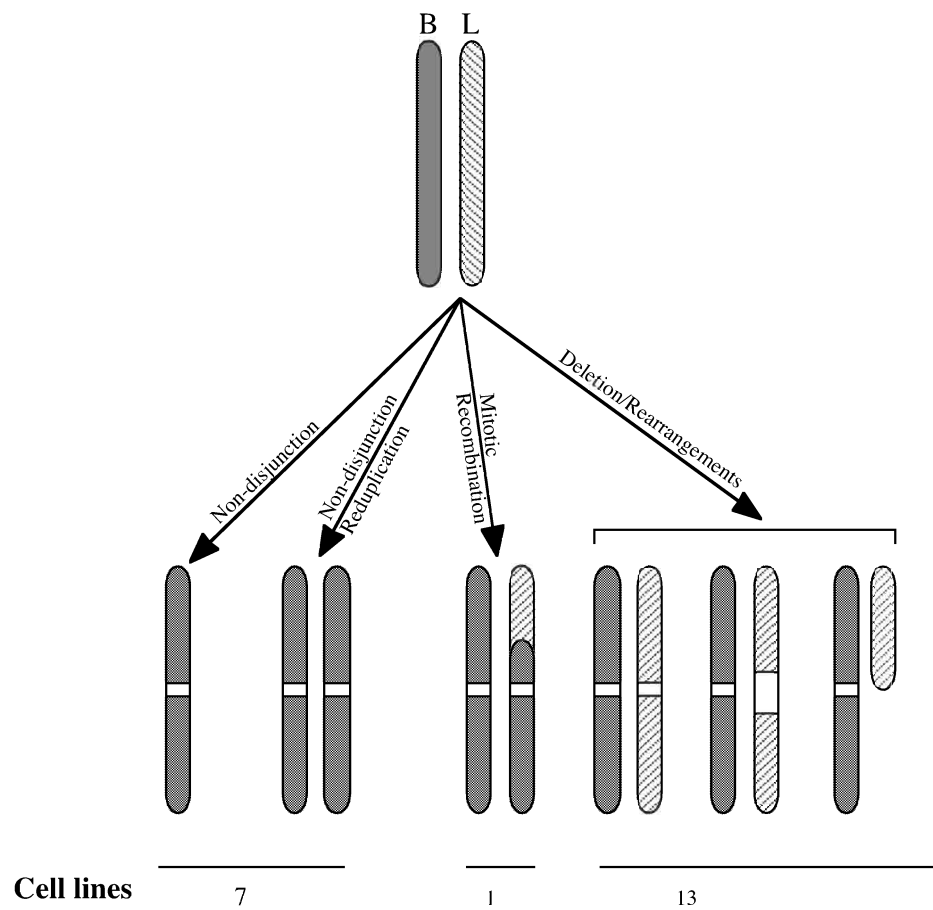
Figure 7: Summary of 21 REF cell lines studied showing the mechanism of loss of heterozygosity (LOH).

CONCLUSIONS

- *CDKN2A* germline mutations:
  - predispose to development of cutaneous melanoma.
  - have a weak correlation to DNS.
  - together with the DNS phenotype contribute to melanoma development at a young age.
  - are associated with other malignancies.

- The P48L and 24bp deletion mutations in the *CDKN2A* gene yield mutant p16 proteins, which do not bind to CDK4 and CDK6.

- 113insR is the dominating *CDKN2A* mutation in Swedish melanoma kindreds.

- 113insR is a Swedish founder mutation that has arisen approximately 2000 years ago.

- *CDKN2A* germline mutations are present in 11% of Swedish patients with multiple primary melanoma (MPM).

- Screening for germline mutations among individuals with MPM identifies high risk families and allow earlier detection and successful treatment of melanoma.

- p14 ARF mutations may complement the effect of defective p16 mutant proteins in melanoma development.

- In the rat embryo fibroblast system, homozygous deletion of *Cdkn2a/b* was estimated to occur at a frequency of 2 x 10^{-8}/cell/generation or higher.

- Deletion/rearrangements and non-disjunction appear to be the main mechanisms leading to deletion of *Cdkn2a/b* in REF cells.

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