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Activating proto-oncogene mutations in human cutaneous melanoma

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ABSTRACT

Cutaneous melanoma arises from melanocytes located in the basal layer of the epidermis. The disease is known to progress through well-defined steps: 1) common nevus; 2) dysplastic nevus; 3) radial growth phase (RGP) primary melanoma; 4) vertical growth phase (VGP) primary melanoma; and 5) metastatic melanoma. Whereas RGP melanoma is confined to the epidermis, VGP melanoma expands into the dermis and has acquired the capacity to metastasize. If detected at the early RGP stage, melanoma is easily cured by surgical excision. However, metastatic melanoma is highly resistant to existing therapies and has a poor prognosis. Therefore a better understanding of the mechanisms underlying melanoma initiation and progression is required to allow the development of more effective therapies. The aim of this thesis was to determine the frequency and timing of *CTNNB1*, *NRAS*, *BRAF* and *PIK3CA* mutations in sporadic cutaneous melanoma.

β-catenin (encoded by *CTNNB1*) is a molecule that plays central roles both in the Wnt/β-catenin signaling pathway and in cell-cell adhesion. Using PCR-single strand conformation polymorphism analysis, we identified *CTNNB1* mutations in 3% of melanoma tumors. Interestingly, cytoplasmic and nuclear β-catenin was observed not only in tumors that harbored *CTNNB1* mutations but also in about one third of primary tumors that were wild-type for *CTNNB1*. This suggests that there must exist other mechanisms of β-catenin stabilization in cutaneous melanoma.

The Ras GTPases transduce extracellular growth signals to a variety of different effector pathways, including the Raf-MEK-ERK and PI3K-Akt pathways. Signaling through these pathways is often deregulated in human cancers. Cutaneous melanomas are known to harbor *NRAS* and *BRAF* mutations at a high frequency. To determine the timing of these mutations in melanoma tumorigenesis we screened a large series of paired primary and metastatic melanomas from a total of 71 patients. *NRAS* mutations were found in 30% (21 of 71) and *BRAF* mutations in 59% (42 of 71) of melanoma patients. Interestingly, mutations in *NRAS* and *BRAF* were observed to be mutually exclusive and thus 89% (63 of 71) of the patients analyzed had mutations in either of these genes. We found that *NRAS* and *BRAF* mutations are present in early RGP lesions and preserved in later stage VGP and metastatic lesions. Only in rare cases did mutations arise at the metastatic stage. Together, these results indicate that *NRAS* and *BRAF* mutations correlate with melanoma initiation rather than progression, and that they are not responsible for the critical RGP to VGP transition. The finding that *NRAS* and *BRAF* mutations are preserved throughout melanoma progression suggests that they may be of importance for melanoma maintenance.

Furthermore, we found that the *PIK3CA* gene, which encodes the p110α catalytic subunit of class IA PI3Ks, is mutated in approximately 3% of melanoma metastases. Interestingly, *PIK3CA* missense mutations were observed only in metastases that did not carry *NRAS* mutations, suggesting that *PIK3CA* and *NRAS* mutations may be mutually exclusive in melanoma. Finally, we analyzed a large series of melanoma metastases with known *NRAS* and *BRAF* mutation status for expression of phosphorylated (active) ERK and phosphorylated (active) Akt. We found that ERK and Akt are activated in the majority of melanoma metastases, irrespective of *NRAS* and *BRAF* mutation status. Taken together, our results confirm the involvement of both the Raf-MEK-ERK and PI3K-Akt signaling pathways in melanoma tumorigenesis and suggest that these pathways can be activated through multiple mechanisms. The frequent activation of both the Raf-MEK-ERK and PI3K-Akt pathways in melanoma suggest that therapy targeting only one of these pathways may not be effective, but rather that both pathways should be targeted.

CONTENTS

LIST OF PUBLICATIONS

The thesis is based on the following papers. In the text the papers will be referred to by their roman numerals:

I **Omholt K**, Platz A, Ringborg U and Hansson J.
Cytoplasmic and nuclear accumulation of β-catenin is rarely caused by *CTNNB1* exon 3 mutations in cutaneous malignant melanoma.
International Journal of Cancer (2001), 92: 839-842.

II **Omholt K***, Karsberg S*, Platz A, Kanter L, Ringborg U and Hansson J.
Screening of *NRAS* codon 61 mutations in paired primary and metastatic cutaneous melanomas: mutations occur early and persist throughout tumor progression.
Clinical Cancer Research (2002), 8: 3468-3474.

III **Omholt K**, Platz A, Ringborg U and Hansson J.
NRAS and *BRAF* mutations arise early during melanoma pathogenesis and are preserved throughout tumor progression.
Clinical Cancer Research (2003), 9: 6483-6488.

IV **Omholt K**, Kröckel D and Hansson J.
PIK3CA mutations contribute to Akt activation in a minor subset of cutaneous melanomas.
Manuscript

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ABBREVIATIONS

ALM	Acral lentiginous melanoma
APC	Adenomatous polyposis coli
CKI	Casein kinase I
Dsh	Dishevelled
ERK	Extracellular signal regulated kinase
FTI	Farnesyl transferase inhibitor
Fz	Frizzled
GAP	GTPase-activating protein
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GRB2	Growth-factor-receptor-bound protein 2
GSK-3β	Glycogen synthase kinase-3β
GTP	Guanosine triphosphate
GTPases	Guanosine triphosphatases
LCM	Laser capture microdissection
LEF	Lymphoid enhancer factor
LMM	Lentigo maligna melanoma
LRP	LDL-receptor-related protein
LOH	Loss of heterozygosity
MEK	MAPK/ERK kinase
MMP	Matrix metalloproteinase
NM	Nodular melanoma
PDK	Phosphoinositide-dependent kinase
PH	Pleckstrin-homology domain
PI3K	Phosphatidylinositol 3-kinase
PLCε	Phospholipase Cε
PtdIns(4,5)P_2	Phosphatidylinositol-4,5-bisphosphate
PtdIns(3,4,5)P_3	Phosphatidylinositol-3,4,5-trisphosphate
PTEN	Phosphatase and tensin homolog on chromosome ten
RBD	Ras-binding domain
RGP	Radial growth phase
RSK	Ribosomal S6 kinase
RTK	Receptor tyrosine kinase
SH2	Src homology 2
siRNA	Small interfering RNA
SSCP	Single strand conformation polymorphism
SSM	Superficial spreading melanoma
TCF	T cell factor
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
VGP	Vertical growth phase

INTRODUCTION

Tumorigenesis

Tumorigenesis is a multistep process and it generally takes many years for a cancer to develop. In humans, it is believed that 3-6 genetic changes are required for a normal cell to become malignant (Vogelstein & Kinzler, 1993). These changes occur in a stepwise manner and include both activation of proto-oncogenes and inactivation of tumor suppressor genes. Proto-oncogenes encode proteins that act as positive regulators of cellular processes such as proliferation, growth and survival (Vogelstein & Kinzler, 2004). The proteins encoded by proto-oncogenes can be divided into broad groups such as: 1) growth factors and their receptors (e.g. epidermal growth factor receptor); 2) signal transducers (e.g. Ras, Raf and PI3K); and 3) nuclear transcription factors (e.g. β-catenin and Myc). Proto-oncogenes may be activated by a variety of mechanisms including point mutation, gene translocation, gene amplification, and overexpression. Mutations in proto-oncogenes may be described as "dominant", since an activating mutation in one allele of a proto-oncogene is sufficient to promote tumorigenesis. In contrast to proto-oncogenes, tumor suppressor genes encode proteins that function as negative regulators of cell proliferation, growth and survival (e.g. p53, p16, PTEN and APC) (Vogelstein & Kinzler, 2004). For a tumor suppressor gene to lose its activity, both alleles have to be inactivated. This frequently occurs through loss of one allele followed by a point mutation or deletion of the remaining allele. Inactivation of the second allele may also occur by epigenetic mechanisms, such as promoter methylation. There is also evidence that haplo-insufficiency (i.e. loss of one allele only) can contribute to tumorigenesis in some cases. Tumor cells have acquired a number of capabilities that make them very different from normal cells. These capabilities are thought to be shared by most tumor cell types and include self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, escape from apoptosis, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis (Hanahan & Weinberg, 2000). Metastasis is a complex process that involves a number of distinct steps, including detachment of cancer cells from the primary tumor, survival of the cells in the circulation, and arrest and extravasation of cells in a distant secondary site (Fidler, 2003).

This thesis investigates the role of the proto-oncogenes *CTNNB1*, *NRAS*, *BRAF* and *PIK3CA* in the development and progression of cutaneous melanoma.

The Wnt/β-catenin signaling pathway

β-catenin is a highly conserved protein that is involved both in the Wnt/β-catenin signaling pathway and in cell-cell adhesion. In the Wnt/β-catenin signaling pathway, β-catenin functions as a co-activator of T cell factor/lymphoid enhancer factor (TCF/LEF) regulated transcription. In cell-cell adhesion, β-catenin binds to the cytoplasmic domain of E-cadherin and acts together with α-catenin by linking E-cadherin to the actin cytoskeleton (Ozawa et al., 1990; Aberle et al., 1994). β-catenin consists of 781 amino acids and contains 12 armadillo repeats in its central region. These armadillo repeats mediate protein-protein interactions with cadherin, APC, axin, and TCF/LEF. The N-terminal region of β-catenin contains critical regulatory sites and the C-terminal region contains a TCF/LEF transactivation domain. The gene that encodes β-catenin (*CTNNB1*) resides on chromosome 3q21.

The Wnt/β-catenin signaling pathway plays central roles during embryogenesis and tissue homeostasis. Wnts are secreted glycoproteins that are thought to act mainly in a paracrine fashion (Miller, 2002). Nineteen different mammalian Wnt proteins have been described and they bind to the Frizzled family of seven-transmembrane receptors and to the LDL-receptor-related proteins 5 and 6 (LRP5/6). These latter receptors are believed to function as co-receptors. Wnts can activate at least three different signaling pathways, of which the Wnt/β-catenin pathway is the best understood (Miller, 2002).

As a co-activator of gene transcription, β-catenin is subjected to tight regulation. In the absence of Wnt signals, a multi-protein complex composed of the scaffold protein axin, the tumor suppressor protein adenomatous polyposis coli (APC), and the two serine/threonine kinases glycogen synthase kinase-3β (GSK-3β) and casein kinase Iα (CKIα) ensures that the cytosolic level of β-catenin is kept low (van Es et al., 2003). More specifically, the CKIα kinase phosphorylates the serine residue 45 in the N-terminal region of β-catenin (Liu et al., 2002). This phosphorylation has been described as a priming event that allows the GSK-3β kinase to sequentially phosphorylate three adjacent serine and threonine residues (Thr41, Ser37 and Ser33) (Yost et al., 1996). Phosphorylated β-catenin is recognized by the F-box protein β-TrCP, which through ubiquitination targets β-catenin for proteosomal degradation (Aberle et al., 1997; Orford et al., 1997). In the presence of Wnt signals, β-catenin phosphorylation is inhibited. The mechanism by which this occurs is complex and poorly understood. It has been suggested that a protein termed Dishevelled inhibits the activity of GSK-3β and that LRP recruits Axin to the cell membrane for degradation (He et al., 2004). As a result of GSK-3β inhibition β-catenin is no longer targeted for proteosomal degradation, but instead accumulates in the cytoplasm. Eventually β-catenin translocates to the nucleus, where it interacts with and activates members of the TCF/LEF family of transcription factors (Behrens et al., 1996; Molenaar et al., 1996). Several TCF/LEF target genes have been identified (Giles et al., 2003), two of which are those that encode c-MYC (He et al., 1998) and cyclin D1 (Tetsu & McCormick, 1999).

The Wnt/β-catenin signaling pathway and cancer

The Wnt/β-catenin signaling pathway is hyper-activated in a variety of different human cancers (Giles et al., 2003). A common mechanism of Wnt/β-catenin activation is through inactivation of the *APC* tumor suppressor gene. Mutations of this gene are found in approximately 80% of sporadic colorectal cancers and at lower frequencies in a number of other tumor types (Giles et al., 2003). The majority of *APC* mutations are nonsense mutations that result in truncated proteins without ability to regulate β-catenin. In 1997, it was shown that colon cancer cell lines that are wild-type for *APC*, often carry

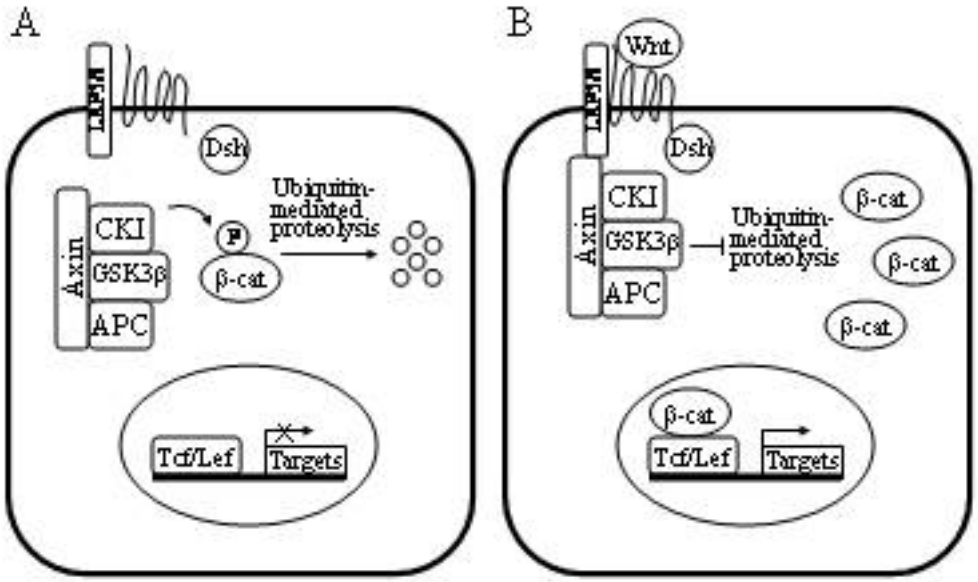

Figure 1. *The Wnt/β-catenin signaling pathway. In the absence of Wnt signals (A), cytosolic levels of β-catenin are low, due to proteosomal degradation of the molecule. In the presence of Wnt signals (B), degradation is inhibited and β-catenin enters the nucleus to activate TCF/LEF regulated transcription.*

mutations in *CTNNB1* (Morin et al., 1997). At the same time it was reported that *CTNNB1* mutations occur at a high frequency in melanoma cell lines (Rubinfeld et al., 1997). Since then *CTNNB1* mutations have been described in a number of different cancer types, including ovarian, hepatocellular, prostate, thyroid and gastric cancers (Giles et al., 2003). Most β-catenin mutations occur in exon 3 of the *CTNNB1* gene, affecting one of the four phosphorylation sites for GSK-3β and CKIα. Mutations that alter any of these sites render β-catenin insensitive to phosphorylation and proteasomal degradation. Both APC loss and *CTNNB1* exon 3 mutations result in nuclear accumulation of β-catenin and constitutive transcription of TCF/LEF target genes (Korinek et al., 1997; Morin et al., 1997). A third component of the Wnt/β-catenin pathway that has been found mutationally altered in human cancer is axin (Satoh et al., 2000).

Ras signaling

The Ras proteins belong to the Ras superfamily of small GTPases, which is divided into five sub families (Ras, Rho, Rab, Ran and Arf) (Wennerberg et al., 2005). In normal cells, Ras is transiently activated in response to extracellular growth signals and functions to transduce these signals to a number of different effector molecules. These effectors in turn regulate a variety of cellular functions, including proliferation, survival, differentiation, growth and motility (Shields et al., 2000). In contrast to normal cells, signaling through Ras is hyper-activated in many tumor cells. Hyper-activation may occur as a result of mutations in Ras itself, or of mutations in some other component up- or downstream of Ras. The Raf-MEK-ERK and PI3K-Akt pathways are the two best-characterized effector pathways downstream of Ras, both with well-established roles in Ras-mediated transformation (Repasky et al., 2004). But there are also other effector pathways downstream of Ras, and there is increasing evidence that activation of at least some of these pathways is also required for Ras induced tumorigenesis (Repasky et al., 2004; Rodriguez-Viciana & McCormick, 2005).

Ras

The Ras GTPases are located at the inner surface of the cell membrane where they function to transduce extracellular growth signals to a variety of different downstream effectors (Fig. 2). As GTPases, Ras proteins cycle between an inactive GDP-bound state and an active GTP-bound state (Campbell et al., 1998). The mechanism of Ras activation involves the following steps: 1) binding of extracellular growth factor to its receptor tyrosine kinase, resulting in receptor dimerization and activation; 2) SH2 mediated binding of the adaptor protein GRB2 to phosphotyrosines on the activated receptor; and 3) GRB2 mediated recruitment of a guanine-nucleotide exchange factor (GEF) into the vicinity of Ras. The function of GEFs is to displace GDP, allowing GTP to bind. Whereas activation of Ras is dependent on GEFs, termination of Ras signaling is dependent on GTPase activating proteins (GAPs), such as p120GAP and neurofibromin (Donovan et al., 2002). GAPs function to stimulate the hydrolysis of GTP to GDP.

There are three different mammalian *RAS* genes that encode four highly related proteins of 188 residues: H-Ras, N-Ras, K-Ras4A and K-Ras4B (Barbacid, 1987). The *HRAS*, *NRAS* and *KRAS* genes reside on chromosomes 11p15, 1p22 and 12p12, respectively. The four Ras proteins are identical in their first 85 amino acid residues. This part contains the effector domain (residues 32-40), through which Ras proteins interact with their effectors. The N-terminal part also contains two mobile regions known as the switch I (residues 30-40) and switch II (residues 60-76) regions, both of which undergo conformational changes upon GTP binding. The most C-terminal part of Ras contains a CAAX motif. This motif is subjected to a number of post-translational modifications, which are required for the proper anchoring of Ras to the cell membrane. One such modification involves the enzyme farnesyltransferase which catalyzes the attachment of a farnesyl group to the cysteine residue of the CAAX motif (Silvius, 2002).

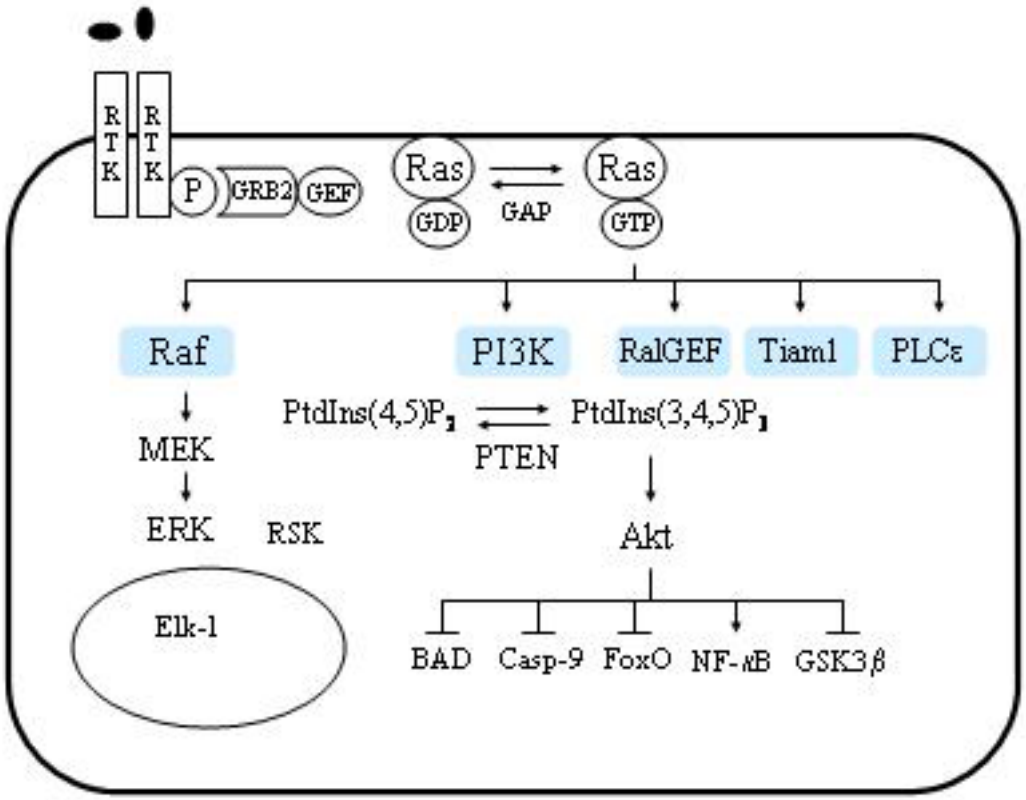

Figure 2. *Signaling upstream and downstream of Ras.*

Ras effectors

In their active GTP-bound state, Ras proteins bind to their effectors. To date 10 distinct classes of Ras effectors have been identified (Repasky et al., 2004). The two best-characterized effectors are the serine/threonine Raf kinases (Moodie et al., 1993) and the class I PI3Ks (Rodriguez-Viciana et al., 1994). A third group of Ras effectors include the GEFs (RalGDS, RGL, RGL2/Rlf and RGL3) for the RalA and RalB small GTPases (Wolthuis & Bos, 1999). A fourth Ras effector is Tiam1 (Lambert et al., 2002), which is a GEF for the Rac GTPase. Other Ras effectors include phospholipase Cε (PLCε), AF-6, RIN1, NORE1 and IMP (Repasky et al., 2004). All Ras effectors contain a Ras binding domain (RBD), through which they interact with Ras. Three different types of RBDs have been identified: the RBD of Raf and Tiam1, the PI3K-RBD and finally the RA domain, which is found in the majority of Ras effectors (Repasky et al., 2004).

It has been demonstrated that the different Ras proteins vary in their abilities to activate different effector molecules. For example, K-Ras and N-Ras have been shown to activate Raf more efficiently than H-Ras, while H-Ras has been shown to activate PI3K more efficiently than K-Ras and N-Ras (Yan et al., 1998; Li et al., 2004). In addition to the four Ras proteins, other members of the Ras-family, such as R-Ras, TC21 and Rit, are also able to activate various isoforms of Raf, PI3K and RalGEF (Reuther & Der, 2000; Rodriguez-Viciana et al., 2004).

There is increasing evidence that oncogenic Ras activates distinct effector pathways to induce transformation of different cell types (Shields et al., 2000). For example, whereas the Raf pathway can induce transformation of mouse NIH3T3 fibroblasts (Cowley et al., 1994), activation of this pathway alone is not sufficient to induce transformation of RIE-1 intestinal rat epithelial cells (Oldham et al., 1996). Several recent studies have demonstrated the importance of RalGEFs in Ras-mediated transformation. Hamad *et al.* have shown that activation of the RalGEF pathway alone is sufficient to induce transformation of hTERT/SV40T/t-immortalized human fibroblasts, human embryonic kidney epithelial (HEK) cells and human astrocytes, as determined by anchorage-independent growth (Hamad et al., 2002). In contrast, neither Raf nor PI3K alone were able to induce transformation of these cells. In another study on immortalized human cells it was shown that human fibroblasts require activation of Raf and RalGEF to form tumors in nude mice, while HEK cells require activation of PI3K and RalGEF and mammary epithelial cells require activation of Raf, PI3K and RalGEF (Rangarajan et al., 2004). Finally, a very recent study has shown that RalGDS deficient mice are resistant to H-Ras induced skin tumors (Gonzalez-Garcia et al., 2005). Two other Ras effectors that recently have been suggested to play a role in Ras mediated tumorigenesis are Tiam1 and PLCε. Like RalGDS deficient mice, Tiam1-deficient (Malliri et al., 2002) and PLCε-deficient (Bai et al., 2004) mice display resistance to H-Ras-induced skin tumors.

Raf

The best-characterized Ras effectors are the serine/threonine Raf kinases. There are three different human Raf kinases: A-Raf, B-Raf and C-Raf (also termed Raf-1). The *ARAF*, *BRAF* and *CRAF* genes reside on chromosomes Xp11, 7q34 and 3p25, respectively. The Raf proteins are structurally related and share three conserved regions (CR1, CR2 and CR3). The N-terminally located CR1 contains the RBD as well as a cysteine-rich domain (CRD), which also functions to bind Ras (Morrison & Cutler, 1997). The C-terminally located CR3 region contains the kinase domain. Whereas C-Raf is ubiquitously expressed, A-Raf and B-Raf display a more restricted expression pattern (Storm et al., 1990). B-Raf is expressed mainly in different neuronal tissues, but also in other cell types such as testis and heart (Barnier et al., 1995). B-Raf is also subjected to alternative splicing (Barnier et al., 1995). Knockout studies suggest that the different Raf proteins have non-redundant functions. Neither *b-raf*$^{-/-}$ (Wojnowski et al., 1997) nor *c-raf*$^{-/-}$ (Wojnowski et al., 1998) mice are viable, whereas mice that are *a-raf*$^{-/-}$ die soon after birth (Pritchard et al., 1996). Mice that are *b-raf*$^{-/-}$ die of vascular and neuronal defects (Wojnowski et al., 1997).

Inactive Raf resides in the cytoplasm, but is recruited to the cell membrane upon binding to Ras-GTP. Here, Raf is activated through a number of phosphorylation events. Two critical phosphorylation sites are located within the so-called activation segment of the kinase domain. These two sites are conserved in all three Raf proteins as well as through evolution, but the kinase(s) that phosphorylates these sites has not yet been identified. B-Raf requires phosphorylation only at these

two residues (Thr598 and Ser601) for activation and replacement of these residues by alanines has been shown to inhibit Ras induced activation of B-Raf (Zhang & Guan, 2000). In contrast, substitution of these two residues by acidic amino acids renders B-Raf constitutively active. This B-Raf mutant is able to transform NIH3T3 cells and to induce PC12 cell differentiation (Zhang & Guan, 2000). C-Raf and A-Raf differ somewhat from B-Raf in that they require phosphorylation at two additional sites (Ser338 and Tyr341 in C-Raf) for activation (Marais et al., 1997; Mason et al., 1999; Chong et al., 2001). These two residues are located within a region called the N-region (for negative-charge regulatory region). Tyr341 is phosphorylated by Src, whereas the kinase that phosphorylates Ser338 remains to be identified. In B-Raf the residue equivalent to Ser338, Ser445, is constitutively phosphorylated (Mason et al., 1999), whereas Tyr341 is replaced by an aspartic acid (Asp448). Because B-Raf requires less phosphorylation for activation and does not depend on Src, B-Raf has a higher basal kinase activity compared to C-Raf and A-Raf (Marais et al., 1997; Papin et al., 1998). It is thought that these differences explain why only B-Raf and not A-Raf and C-Raf is mutated in human cancers (Wellbrock et al., 2004).

The major substrates downstream of Raf are the MEK1 and MEK2 dual-specificity kinases. Raf activates these proteins by phosphorylating serine residues within their activation segment (Papin et al., 1995). MEKs in turn activate the serine/threonine kinases ERK1 and ERK2 by phosphorylating threonine and tyrosine residues within their activation segment. When activated, ERKs phosphorylate and activate a variety of substrates, some of which are located in the cytoplasm and others in the nucleus. An example of a cytoplasmic substrate is the 90 kDa ribosomal S6 kinase (RSK), whereas Jun and the transcription factor Elk-1 are examples of nuclear substrates (Campbell et al., 1998). A recent microarray study has shown that gene expression induced by C-Raf, can be blocked by the MEK inhibitor PD98059, indicating that C-Raf does not induce transcription via MEK independent pathways (Schulze et al., 2004).

The Raf-MEK-ERK pathway

The Raf-MEK-ERK pathway is a key regulator of cell proliferation. A major way by which ERK signaling promotes cell cycle progression is through transcriptional upregulation of cyclin D1 (Pruitt & Der, 2001). Cyclin D1 activates the cyclin-dependent kinases (CDKs) 4 and 6, which through phosphorylation and inactivation of the retinoblastoma protein allows cells to progress from the G1 to S phase of the cell cycle.

The Raf-MEK-ERK pathway is also involved in the regulation of cell survival. For example, the ERK substrate RSK, has been reported to phosphorylate and inactivate the pro-apoptotic protein BAD (Bonni et al., 1999).

Examples of genes that are transcriptionally induced in response to ERK activation include vascular endothelial growth factor (VEGF), a positive regulator of angiogenesis, and matrix metalloproteinase 1 (MMP-1), a collagenase involved in extracellular matrix degradation (Schulze et al., 2001). Sustained ERK activation has also been shown to induce expression of β3-integrin in certain cell types (Woods et al., 2001). Proteins such as VEGF, MMP-1 and β3-integrin are believed to play crucial roles in Ras-mediated tumor cell invasion and metastasis (Campbell & Der, 2004).

RAS and RAF mutations in human cancer

The *RAS* genes are mutated in approximately 30% of all human tumors (Bos, 1989). *KRAS* mutations are most common, followed by *NRAS* mutations, whereas *HRAS* mutations are relatively rare. Many tumor types appear to be associated with mutations in a specific *RAS* gene. For example, pancreatic and colorectal tumors harbor mainly *KRAS* mutations, whereas hematologic malignancies and melanomas mostly have *NRAS* mutations (Bos, 1989). An exception is thyroid carcinoma, where mutations in all three *RAS* genes are equally common. Most *RAS* mutations are point mutations affecting residues 12, 13 and 61. These mutations render Ras insensitive to GAP-stimulated GTP hydrolysis (Der et al., 1986; Polakis & McCormick, 1993). As a result mutant Ras is locked in an active GTP-bound state.

In 2002, a genome-wide screen for proto-oncogenes showed that the *BRAF* gene is mutated in a variety of different human cancers (Davies et al., 2002). The highest frequencies of *BRAF* mutations were identified in melanomas (67%), colorectal (18%) and ovarian (14%) cancers (Davies et al., 2002). A subsequent study showed that *BRAF* is also frequently mutated in thyroid carcinoma (Cohen et al., 2003). Most *BRAF* mutations cluster within the activation segment of the kinase domain, with a single T1796A transversion accounting for approximately 90% of mutations. This mutation leads to a glutamic acid for valine substitution at codon 599 (V599E). [Note: In 2003, an error in the NCBI gene sequence for *BRAF* was identified. The correct numbering of the mutated codon should be 600 (Garnett et al., 2004). To avoid confusion with published results the old numbering system is used in this thesis]. It is believed that the insertion of an acidic residue at position 599 mimics the phosphorylation of Thr598/Ser601 in wild-type B-Raf (Davies et al., 2002; Wan et al., 2004). A minority of *BRAF* mutations have also been found in the glycine-rich loop GXGXXG (G = glycine, X = variable), which is located in the N-terminal region of the kinase domain and is a highly conserved protein motif that is involved in the anchoring of ATP (Hanks & Hunter, 1995). Over 40 different *BRAF* mutations have been described in the literature (Garnett & Marais, 2004), approximately half of which have been functionally analyzed (Davies et al., 2002; Wan et al., 2004). Many of these *BRAF* mutants (including V599E) have elevated kinase activity, and are able to transform NIH3T3 fibroblasts, although less efficiently than mutant *RAS* (Davies et al., 2002; Wan et al., 2004). However, there are mutants that have decreased kinase activity compared to wild-type *BRAF*. Interestingly a majority of these mutants can still activate ERK, via activation of C-Raf (Wan et al., 2004). The mechanism by which this occurs is not known, although it has been shown that B-Raf and C-Raf do form heterodimers (Weber et al., 2001).

Drugs targeting Ras and Raf

Because the Ras-Raf-MEK-ERK pathway is so frequently activated in human cancers, there is much research focused on finding agents that specifically block components of this pathway. Farnesyltransferase inhibitors (FTIs) represent a group of drugs that were developed to block farnesylation of Ras (Cox & Der, 2002). When un-farnesylated, Ras proteins lose their ability to associate with the cell membrane, which is a prerequisite for their biological activity. Although very efficient in reducing *HRAS* induced tumor growth in mice, FTIs have shown no effect in clinical trials (Downward, 2003). This is because K-Ras and N-Ras, but not H-Ras, can be modified by an alternative mechanism when farnesylation is blocked. The enzyme geranylgeranyltransferase can

attach a geranylgeranyl group to Ras, and this group functions in the same way as the farnesyl group by anchoring Ras to the plasma membrane (Whyte et al., 1997).

Another drug that has been identified more recently is BAY43-9006 (Lyons et al., 2001). It was originally described as a C-Raf inhibitor, but has now been shown to target other kinases as well, including B-Raf (both wild-type B-Raf and V599E mutant B-Raf), VEGF receptor-2, VEGF receptor-3, platelet-derived growth factor receptor β and c-KIT (Wilhelm et al., 2004). BAY43-9006 has been shown to block ERK activation in a variety of different tumor cell lines (Wilhelm et al., 2004), including melanoma cell lines (Karasarides et al., 2004; Wellbrock et al., 2004). BAY43-9006 has also been shown to halt melanoma tumor growth in nude mice, but does not seem to induce tumor regression (Karasarides et al., 2004; Sharma et al., 2005). Preliminary clinical data suggest that the combination of BAY43-9006 with chemotherapy (carboplatin and paclitaxel) has antitumor activity in patients with metastatic melanoma (Flaherty et al., 2004)

PI3K and Akt

The class I PI3Ks are the second best-characterized Ras effectors (Rodriguez-Viciana et al., 1994). PI3Ks are lipid kinases that phosphorylate the 3'-OH position of the inositol ring of phosphoinositide lipids, creating secondary messengers that serve to recruit pleckstrin-homology (PH) domain-containing proteins to the cell membrane. There are three different classes of PI3Ks: class I (subclass A and B), II and III. Class IA PI3Ks (the only class that will be discussed here) are composed of a 110 kDa catalytic subunit and an 85 kDa regulatory subunit and are activated by Ras or by RTKs (Cantley, 2002). The p110 catalytic subunit contains the RBD, to which Ras-GTP binds. The catalytic subunit also contains a p85 binding domain and the kinase domain. There are three mammalian isoforms of the catalytic subunit (p110α, β, δ), encoded by three separate genes. The p85 regulatory subunit contains two SH2 domains that interact either directly or via an adaptor molecule with phosphotyrosines on activated RTKs. Seven isoforms of the regulatory subunit have been described that are generated by alternative splicing of three different genes. The primary substrate of class IA PI3Ks is the 3'-OH position of phosphatidylinositol 4,5-bisphosphates (PtdIns(4,5)P_2), phosphorylation of which results in increased levels of phosphatidylinositol 3,4,5-trisphosphates (PtdIns(3,4,5)P_3) (Cantley, 2002). A major downstream target of PI3K is the serine/threonine kinase Akt (also known as protein kinase B). In mammals, three different isoforms of Akt (Akt1, 2, 3) have been described. Upon PI3K activation, Akt is recruited to the plasma membrane by binding to PtdIns(3,4,5)P_3 via its PH domain. At the plasma membrane, Akt is activated through phosphorylation of critical residues (Thr308 and Ser473) (Scheid & Woodgett, 2003). Thr308 is phosphorylated by the 3-phosphoinositide-dependent protein kinase-1 (PDK-1), a kinase that like Akt contains a PH domain and binds to PtdIns(3,4,5)P_3. Ser473 is phosphorylated by a kinase often termed PDK-2, although the identity of this kinase is not completely clear. When activated, Akt phosphorylates numerous downstream substrates that control cellular processes such as survival, cell cycle progression and growth (Testa & Bellacosa, 2001).

The PI3K-Akt pathway

The PI3K-Akt pathway is a key regulator of cell survival. Akt promotes cell survival by phosphorylating and inactivating the pro-apoptotic proteins BAD (Datta et al., 1997) and caspase-9 (Cardone et al., 1998). Moreover, Akt has been shown to phosphorylate and inactivate members of the FoxO subfamily of forkhead transcription factors (Brunet et al., 1999). These transcription factors are known to induce expression of pro-apoptotic proteins such as Fas Ligand and Bim (Burgering & Medema, 2003). Finally, there is evidence that Akt promotes cell survival by indirectly activating NF-κB, a transcription factor that induces expression of various anti-apoptotic genes (Downward, 2004).

In addition to being a critical regulator of cell survival, the PI3K-Akt pathway is also involved in the regulation of cell cycle progression. For example, Akt contributes to stabilization of cyclin D1 levels, by phosphorylating and inactivating GSK-3β (Cross et al., 1995). Normally, GSK-3β targets cyclin D1 for proteasomal degradation (Diehl et al., 1998).

Activation of PI3K-Akt in human cancer

The PI3K-Akt pathway is believed to play a central role in tumorigenesis and is deregulated in a wide variety of human cancers. Deregulation may occur as a result of mutations in *RAS* or in some other component of the pathway (Vivanco & Sawyers, 2002). For example, activating mutations in the gene that encodes the p85α regulatory subunit of PI3K have been described in ovarian and colorectal cancers. Moreover, the *AKT1* and *AKT2* genes are amplified in various cancers (Testa & Bellacosa, 2001). And recently it has been demonstrated that the gene that encodes the p110α catalytic subunit of PI3K, *PIK3CA*, is mutated at a high frequency in colorectal cancers as well as in a number of other tumor types (Samuels et al., 2004). Most of the reported *PIK3CA* mutations cluster in small regions within the helical (exon 9) and kinase (exon 20) domains, with E545K (helical domain) and H1047R (kinase domain) constituting two mutational hot-spots (Samuels et al., 2004). Functional studies have shown that E545K and H1047R mutant PI3K display increased lipid kinase activity, induce constitutive activation of Akt, and are able to transform chicken embryo fibroblasts (Samuels et al., 2004; Kang et al., 2005).

PTEN

Yet another mechanism by which Akt is activated in human cancer is through inactivation of the tumor-suppressor gene *PTEN*. This gene is located on chromosome 10q23 and is frequently altered in glioblastomas, endometrial and prostate cancers (Parsons, 2004). The PTEN protein functions both as a lipid phosphatase (Maehama & Dixon, 1998) and as a dual-specificity protein phosphatase (Myers et al., 1997). The lipid phosphatase activity of PTEN dephosphorylates the 3' position of PtdIns(3,4,5)P_3, resulting in increased levels of PtdIns(4,5)P_2 (Maehama & Dixon, 1998). Thus, PTEN acts by antagonizing PI3K signaling. Loss of PTEN function results in increased levels of PtdIns(3,4,5)P_3 and in hyperactivation of Akt (Myers et al., 1998; Stambolic et al., 1998). Overexpression of wild-type PTEN in a variety of PTEN deficient cancer cell lines has been shown to result in either G1 arrest or apoptosis, depending on cell type (Di Cristofano & Pandolfi, 2000).

Cutaneous Malignant Melanoma

Incidence

Cutaneous malignant melanoma arises from the malignant transformation of melanocytes, located in the basal layer of the epidermis. The main function of these cells is to synthesize and transfer melanin pigment to surrounding keratinocytes, thereby protecting these cells from harmful exposure to ultraviolet (UV) radiation. The incidence of cutaneous melanoma is increasing more rapidly than that of most other cancers both in Sweden and other western countries. In Sweden, the annual increase during the past 20 years has been approximately 2% (Cancer Incidence in Sweden 2003). In 2003, almost 1900 new cases of invasive cutaneous melanoma were diagnosed in Sweden, and currently, cutaneous melanoma is the sixth and seventh most common cancer among females and males, respectively (Cancer Incidence in Sweden 2003). Unfortunately, the rising incidence of cutaneous melanomas has been accompanied by an increased mortality (Causes of death 2002).

Melanoma progression

In 1984, Clark *et al*. proposed a model for melanoma development and progression (Clark et al., 1984). Based on histopathological and clinical features this model describes five different progression steps: 1) common melanocytic nevus; 2) dysplastic nevus; 3) radial growth phase (RGP) primary melanoma; 4) vertical growth phase (VGP) primary melanoma; and 5) metastatic melanoma (Fig. 3). Melanocytic nevi are benign clusters of melanocytes that may be regarded as potential precursor lesions of melanoma (Strungs, 2004). However, only ~30% of invasive primary melanomas are associated with a pre-existing nevus (Sagebiel, 1993), suggesting that many melanomas do not arise from nevi. Most melanocytic nevi appear after birth (common acquired nevi) and, based on growth patterns, may be classified as junctional, compound or intradermal (Strungs, 2004). Melanocytic nevi may also be present at birth (congenital nevi). Dysplastic nevi differ from common nevi in that they display architectural disorder and cytological atypia (Strungs, 2004). In RGP melanomas tumor growth is confined to the epidermis (*in situ* melanoma) and the most superficial papillary dermis (microinvasive melanoma). RGP melanomas lack the potential to metastasize and are easily cured by surgical excision (Guerry et al., 1993). Moreover, RGP melanoma cells do not grow in soft agar and are non-tumorigenic in nude mice (Meier et al., 1998). In VGP melanoma tumor growth expands into the papillary and reticular dermis. Unlike RGP melanomas, VGP melanomas have acquired the capacity to metastasize. Melanoma may metastasize both by lymphatic and blood vessels, and the most common sites of melanoma metastases are skin, subcutaneous tissue and lymph nodes. Other sites include lung, liver, brain and bone (Chin et al., 1998).

The genetic changes responsible for the stepwise progression of cutaneous melanoma are only partly understood (Meier et al., 1998; Bennett, 2003). The recent discovery of *BRAF* mutations in a high proportion (~80%) of nevi has led to a better understanding of the early genetic events in melanoma development (Pollock et al., 2002). However, no genetic alterations have so far been associated with the critical RGP to VGP transition. Although the genetic changes responsible for the RGP to VGP transition are unknown, it is well established that expression of β3-integrin is increased in VGP melanomas compared with RGP melanomas (Van Belle et al., 1999). Moreover, an array study has shown that RhoC is overexpressed in melanoma metastases, suggesting a role for this protein in the metastatic process (Clark et al., 2000).

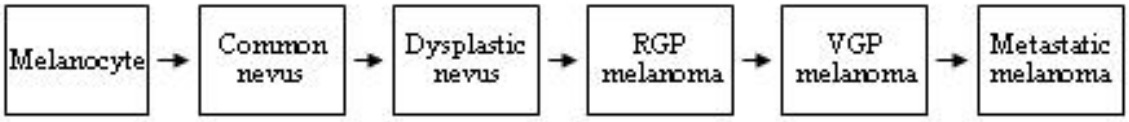

Figure 3. *Model of melanoma development and progression.*

Histological subtypes

There are four major histological subtypes of cutaneous melanoma; superficial spreading melanoma (SSM), nodular melanoma (NM), lentigo maligna melanoma (LMM) and acral lentiginous melanoma (ALM). SSM is the most common subtype accounting for approximately 70% of cases. NM represents about 15% of cutaneous melanoma. This subtype lacks RGP and therefore progresses in a more aggressive manner. LMM and ALM are relatively rare (5%). LMM usually arises on chronically sun-exposed sites in elderly people, whereas ALM arises on palms, soles and subungual sites.

Melanoma risk factors

Environmental

The only known environmental risk factor for cutaneous melanoma is sun exposure. It is believed that intermittent sun exposure is associated with a greater risk than cumulative exposure. Melanomas also occur more frequently on body sites that are intermittently exposed to the sun (e.g. the trunk) (Houghton & Polsky, 2002). The UV radiation that reaches the earth's surface comprises UVA (320-400 nm) and UVB (280-320 nm). UVB is known to induce DNA lesions at dipyrimidine sites. The most common lesions produced are cyclobutane pyrimidine dimers, followed by 6-4 photoproducts (Ichihashi et al., 2003). If not properly repaired these lesions may give rise to mutations such as C to T and CC to TT transitions. Another type of DNA damage induced by UV radiation is 8-oxo-deoxyguanosine (Ichihashi et al., 2003). This lesion can mispair with adenosine during DNA replication, giving rise to G to T transversions. *In vitro* studies have demonstrated that UV radiation can induce activating mutations in codons 12 and 61 of *NRAS* (Van der Lubbe et al., 1988).

Host

High numbers of common acquired nevi and the presence of dysplastic nevi are associated with an increased risk of melanoma (Tucker & Goldstein, 2003). Other host factors associated with melanoma risk include a family history of melanoma, fair skin, extensive freckling, and red hair. Finally, two susceptibility genes for melanoma, *CDKN2A* and *CDK4*, have been identified. Germline mutations in *CDKN2A* account for approximately 20% of familial melanoma cases, whereas mutations in *CDK4* are rare (Tucker & Goldstein, 2003).

Prognostic factors and survival

The most important prognostic factor is disease stage at diagnosis. The American Joint Committee on Cancer staging system is currently used (Balch et al., 2001). This system describes four different disease stages. Stage I and stage II melanomas are localized primary tumors with no signs of metastatic spread. Stage III melanoma is characterized by regional metastatic spread through lymphatic vessels and stage IV melanoma by distant metastatic spread.

For localized disease the major prognostic factor is tumor thickness according to Breslow, followed by ulceration and Clark level of invasion (Chin et al., 1998). Whereas Breslow thickness measures tumor thickness in millimeters, Clark levels (I-V) refer to the different anatomical levels of the skin, where level I represents epidermis, II papillary dermis, III border between papillary and reticular dermis, IV reticular dermis, and V subcutaneous fat (Fig. 4).

When diagnosed at an early stage, melanoma is highly curable by surgical excision. However, late-stage melanoma with distant metastatic spread has a very poor prognosis, with a 5-year survival of about 6% and a median survival of 7.5 months (Barth et al., 1995). A major clinical problem in the treatment of late stage melanoma is the low response rate to conventional chemotherapy.

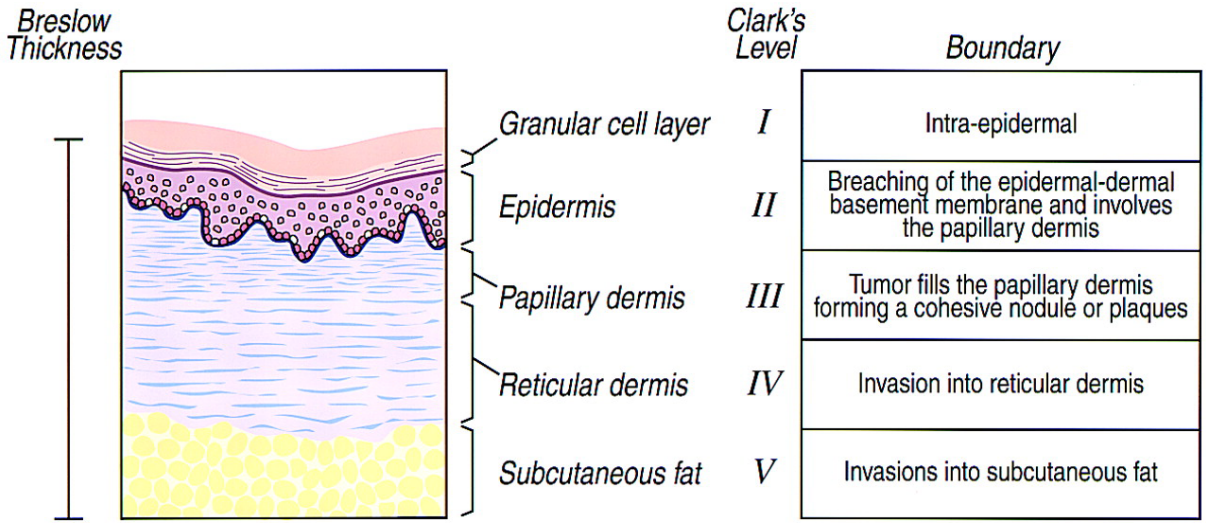

Figure 4. *Thickness of primary cutaneous melanoma according to Breslow and level of tumor invasion according to Clark [reprinted with permission from Genes & Development (Chin et al, 12: 3467-81) copyright Cold Spring Harbor Laboratory Press, 1998].*

Gene alterations in sporadic cutaneous melanomas

Proto-oncogenes

Ras signaling has long been implicated in sporadic cutaneous melanoma. *RAS* mutations are found in 5 to 36% of melanomas, with the majority of mutations affecting codon 61 of *NRAS* (Raybaud et al., 1988; van 't Veer et al., 1989; Albino et al., 1989; Ball et al., 1994; Platz et al., 1994). Mutations in *HRAS* and *KRAS* are rare in melanoma. The presence of *NRAS* mutations in tumor associated nevi and RGP lesions suggests that *NRAS* activation occurs at an early stage during melanoma development (Demunter et al., 2001; Omholt et al., 2002). *NRAS* mutations are also found in approximately 10% of common acquired nevi (Pollock et al., 2002; Kumar et al., 2004) and 28 to 56% of congenital nevi (Carr & Mackie, 1994; Papp et al., 1999). Several studies have reported that *NRAS* mutations occur more frequently in melanomas from chronically sun-exposed body sites than melanomas from intermittently exposed sites (van 't Veer et al., 1989; Ball et al., 1994; van Elsas et al., 1996; Jiveskog et al., 1998). Moreover, *NRAS* mutations have been described to be very rare in melanomas from sun-protected skin, suggesting that UV-radiation may play a role in the genesis of *NRAS* mutations in melanoma (Jiveskog et al., 1998).

In 2002 it was reported that the *BRAF* gene is mutated in about 60% of cutaneous melanomas (Davies et al., 2002). Shortly thereafter it was shown that *BRAF* mutations occur at a similar high frequency in nevi (both common acquired and dysplastic nevi), indicating not only that *BRAF* mutations occur at an early stage during melanoma development but also that activation of *BRAF* alone is insufficient to induce melanoma tumorigenesis (Pollock et al., 2002). In line with this, a recent study has shown that expression of V599E mutant B-Raf in zebrafish result in nevi but not melanoma formation. However, when V599E mutant B-Raf was expressed in fish deficient for p53 invasive melanomas readily developed (Patton et al., 2005). Further support for the involvement of B-Raf in melanoma tumorigenesis is that V599E B-Raf can transform immortalized mouse melanocytes (Wellbrock et al., 2004). Moreover, melanoma cells in which V599E B-Raf has been suppressed by small interfering RNA (siRNA) grow less efficiently in nude mice compared with control cells (Sumimoto et al., 2004; Sharma et al., 2005). *BRAF* mutations have been reported to occur more frequently in melanomas arising on intermittently sun-exposed skin than in tumors arising on chronically exposed skin (Maldonado et al., 2003). Moreover, *BRAF* mutations appear to be absent in melanomas from sun-protected skin (Edwards et al., 2004). This has led to the speculation that UV radiation plays a role in the genesis of *BRAF* mutations, although the mechanism by which this occurs is unclear. The *BRAF* gene resides at chromosome 7q34, a chromosomal region that is frequently amplified in melanoma tumors (Bastian et al., 1998). It has recently been shown that wild-type B-Raf is overexpressed in a subset of melanoma cell lines that lack activating *NRAS* and *BRAF* mutations, and that this overexpression results in activation of ERK and increased cell proliferation (Tanami et al., 2004).

Tumor suppressor genes

CDKN2A at chromosomal locus 9p21 is the most frequently inactivated tumor suppressor gene in cutaneous melanoma. This locus encodes two different proteins, p16 and $p14^{ARF}$, that act in the Rb and p53 pathways, respectively. LOH at 9p21 is observed in about 60% of melanomas. It is believed that 9p21 loss represents an early event in melanoma tumorigenesis, as it has been described both in dysplastic nevi and primary melanoma lesions. Homozygous *CDKN2A* deletions are found in approximately 40% of melanoma metastases (Grafstrom et al., 2005). Other less frequent mechanisms

of *CDKN2A* inactivation include point mutations and promoter methylation. There are several lines of evidence that Ras signaling and *CDKN2A* loss cooperate to promote melanoma development. For example, animal studies have shown that melanocytic specific expression of H-Ras^{V12G} in mice deficient for *CDKN2A* results in development of melanoma at a high frequency (Chin et al., 1997). In contrast, expression of H-Ras^{V12G} in mice that are wild-type for *CDKN2A* does not result in melanoma formation (Chin et al., 1997). Moreover, our group has shown that *NRAS* mutations occur at a very high frequency (95%) in primary melanomas from patients carrying *CDKN2A* germline mutations (Eskandarpour et al., 2003). Expression of oncogenic Ras in primary human cells carrying intact p16 is known to result in cellular senescence (Serrano et al., 1997). This fact together with the observation that most nevi remain stable despite the presence of activating *NRAS* and *BRAF* mutations (see above) has led to the suggestion that the melanocytes in nevi are senescent (Bennett, 2003). It has been speculated that disruption of the p16 pathway is necessary to overcome this senescence and that loss of p16 is critical for the progression of dysplastic nevi to RGP melanoma (Bennett, 2003).

A second tumor suppressor gene implicated in melanoma tumorigenesis is *PTEN* at chromosome 10q23. LOH at this chromosome is observed in approximately 30 to 50% of melanomas (Wu et al., 2003), and is believed to represent a relatively early event in melanoma development. *PTEN* deletions and mutations have been reported in 30 to 40% of melanoma cell lines (Guldberg et al., 1997; Tsao et al., 1998), but appear to be relatively rare (10%) in melanoma tumors (Tsao et al., 1998; Birck et al., 2000). However, immunohistochemical studies have shown that PTEN expression is reduced or completely lost in as many as 33 to 65% of melanoma tumors (Zhou et al., 2000; Whiteman et al., 2002; Tsao et al., 2003). This has led to the speculation that epigenetic mechanisms may contribute to *PTEN* silencing in melanoma (Zhou et al., 2000). Interestingly, *NRAS* and *PTEN* mutations appear to be mutually exclusive in melanoma (Tsao et al., 2000). This observation suggests that *NRAS* and *PTEN* mutations may have overlapping functions in melanoma (i.e. both *NRAS* activation and *PTEN* loss induce Akt activation). *BRAF* and *PTEN* mutations on the other hand, have been described to coexist in the same melanoma cell lines (Tsao et al., 2004). This suggests that B-Raf activation and PTEN loss can cooperate to activate ERK and Akt in melanoma.

Ras effector pathways involved in melanoma tumorigenesis

Raf-MEK-ERK

The Raf-MEK-ERK pathway is believed to play a central role in cutaneous melanoma. As a result of the frequent occurrence of *NRAS* and *BRAF* mutations, ERK is hyper-activated in the majority of melanomas (Satyamoorthy et al., 2003). Immunohistochemical studies have shown that phosphorylated (active) ERK is expressed in RGP melanomas and later stage lesions, but not in common nevi (Cohen et al., 2002; Jorgensen et al., 2003; Satyamoorthy et al., 2003). This indicates that ERK activation occurs early during melanoma progression. Numerous recent studies have investigated the role of V599E mutant B-Raf in melanoma tumorigenesis. Suppression of V599E B-Raf using siRNA in melanoma cell lines that express this oncogenic form of Raf result in reduced levels of activated ERK and decreased proliferation in most cell lines tested (Hingorani et al., 2003; Karasarides et al., 2004; Sumimoto et al., 2004). This indicates that V599E B-Raf is a critical regulator of melanoma cell proliferation. It has been shown that V599E B-Raf induces expression of cyclin D1 and down-regulates levels of p27 in normal human epidermal melanocytes, suggesting a mechanism by which V599E B-Raf promotes S phase entry in melanoma (Bhatt et al., 2005).

Suppression of V599E B-Raf has also been shown to induce apoptosis in some, but not all melanoma cell lines (Hingorani et al., 2003; Karasarides et al., 2004; Sumimoto et al., 2004). This suggests that ERK signaling is also critical for melanoma cell survival. It has been reported that ERK induces survival signals in melanoma by regulating RSK-mediated phosphorylation of the proapoptotic protein BAD (Eisenmann et al., 2003). In addition to regulating cell proliferation and survival, the ERK pathway is also believed to play an important role in melanoma invasion and metastasis. For example, ERK is known to induce expression of MMP-1 in certain melanoma cell lines, and when treated with the MEK inhibitor U0126 these melanoma cells lose their ability to digest type I collagen *in vitro* (Huntington et al., 2004). Moreover, melanoma cells in which V599E B-Raf has been suppressed by siRNA exhibit decreased MMP-2 activity and reduced invasive ability (Sumimoto et al., 2004). Finally, there is evidence that V599E B-Raf is involved in the regulation of angiogenesis in melanoma. A recent study has shown that siRNA mediated suppression of V599E B-Raf leads to decreased secretion of VEGF from melanoma cell lines and that the B-Raf inhibitor BAY43-9006 inhibits vascular development of melanoma tumors in nude mice (Sharma et al., 2005). Taken together, these studies demonstrate that B-Raf-ERK signaling regulates proliferation, survival, invasion and angiogenesis in melanoma cells.

PI3K-Akt

A second Ras effector pathway that is believed to play a central role in melanoma tumorigenesis is the PI3K-Akt pathway. A recent study of 292 melanocytic lesions at different progression stages has shown that expression of phosphorylated (active) Akt correlates with melanoma progression (Dai et al., 2005). Strong phospho-Akt expression was observed in 17% of common nevi, 43% of dysplastic nevi, 49% of primary melanomas, and 77% of metastatic melanomas. The same study also showed that strong phospho-Akt expression in primary melanomas is associated with worse patient survival (Dai et al., 2005). Another recent study has identified Akt3 as the major Akt isoform deregulated in sporadic cutaneous melanoma (Stahl et al., 2004). This study also demonstrated that Akt3 is overexpressed in a subset of metastatic melanomas, and speculated that this overexpression is due to gene amplification (Stahl et al., 2004). The *AKT3* gene resides on chromosome 1q43-44, a region that is amplified in approximately 25% of melanomas (Bastian et al., 1998). Inhibition of PI3K signaling in melanoma cells that express constitutive active Akt has been reported to induce apoptosis via activation of NF-κB (Dhawan et al., 2002). This suggests that Akt signaling is critical for melanoma cell survival. In support of this, ectopic expression of PTEN in melanoma cell lines deficient for PTEN has been reported to result in decreased levels of activated Akt and increased apoptosis (Stahl et al., 2003). Similarly, siRNA mediated suppression of Q61R mutant N-Ras in melanoma cells that carry this oncogenic form of Ras results in reduced levels of activated Akt and increased apoptosis (Eskandarpour et al., 2005). It appears that multiple mechanisms contribute to Akt activation in melanoma. In addition to *NRAS* activation, PTEN loss, and Akt3 overexpression, we have recently showed that *PIK3CA* mutations may contribute to Akt activation in a minor subset of melanomas (Paper IV of this thesis).

AIMS

The overall aim of this thesis was to investigate the role of a number of proto-oncogenes in cutaneous melanoma. The studies were performed on a large series of paired primary and metastatic melanomas. Specific aims were:

- To determine the frequency of *CTNNB1* exon 3 mutations in cutaneous melanoma (Paper I).
- To determine the frequency and timing of *NRAS* mutations in melanoma tumorigenesis (Paper II).
- To determine the frequency and timing of *BRAF* mutations in melanoma tumorigenesis (Paper III).
- To determine the frequency of *PIK3CA* mutations in cutaneous melanoma, and to correlate *NRAS*/*BRAF* mutation status with ERK and Akt activity in melanoma (Paper IV).

RESULTS AND DISCUSSION

Paper I

Cytoplasmic and nuclear accumulation of β-catenin is rarely caused by *CTNNB1* exon 3 mutations in cutaneous malignant melanoma.

Mutations that alter any of the GSK-3β and CKIα phosphorylation sites (Ser33, Ser37, Thr41 and Ser45) of β-catenin result in β-catenin stabilization and elevated transcription of TCF/LEF target genes. In 1997, Rubinfeld *et al.* reported that such mutations occur at a high frequency (6 of 26; 23%) in melanoma cell lines (Rubinfeld et al., 1997). To determine whether β-catenin mutations are as common in melanoma tumors, we screened a series of paired primary and metastatic melanomas from a total of 30 patients. Using PCR-single strand conformation polymorphism (SSCP) and nucleotide sequence analysis, we identified *CTNNB1* exon 3 mutations in tumors from 1 of 30 (3%) patients. The observed mutation consisted of a serine to phenylalanine substitution at codon 45 (S45F), thus affecting the CKIα phosphorylation site. This mutation was identified both in the primary tumor and a metastasis from the patient, suggesting that mutational activation of *CTNNB1* may represent an early event in melanoma tumorigenesis. Our results indicate that *CTNNB1* mutations are relatively rare in melanoma. Several other studies have also reported a low frequency of *CTNNB1* mutations in melanoma tumors (Rimm et al., 1999; Demunter et al., 2002; Reifenberger et al., 2002). Similarly, studies in melanoma cell lines have not confirmed the high frequency of *CTNNB1* mutations originally reported by Rubinfeld *et al*. (Pollock & Hayward, 2002; Worm et al., 2004).

Using immunohistochemistry we also determined the cellular localization of β-catenin in our series of primary and metastatic lesions. As expected, the primary tumor and metastasis that carried the S45F mutation both displayed a widespread cytoplasmic and nuclear expression pattern of β-catenin. Moreover, the majority of primary and metastatic melanomas that were wild-type for *CTNNB1* showed a membraneous staining pattern of β-catenin. However, approximately one third of the primary tumors that were wild-type for *CTNNB1* displayed a focal cytoplasmic and nuclear accumulation of β-catenin. This result is in agreement with a study by Rimm *et al*. demonstrating abnormal accumulation of β-catenin in about one third of melanomas that express wild-type *CTNNB1* (Rimm et al., 1999). Together, our study and the report by Rimm *et al*. indicate that there must exist alternative mechanisms of β-catenin stabilization in melanoma, such as mutations in some other component(s) of the Wnt/β-catenin pathway. It has been shown that the *APC* gene is mutated at a low frequency in melanoma cell lines, suggesting a role for this tumor suppressor gene in a subset of melanomas (Rubinfeld et al., 1997; Worm et al., 2004).

To summarize, our results demonstrate that β-catenin is abnormally accumulated in one third of primary melanomas, supporting a role for the Wnt/β-catenin pathway in melanoma development. The mechanism(s) behind this accumulation remains to be identified.

Paper II

Screening of *NRAS* codon 61 mutations in paired primary and metastatic cutaneous melanomas: mutations occur early and persist throughout tumor progression.

NRAS codon 61 mutations are found in 5 to 36% of sporadic cutaneous melanomas (Raybaud et al., 1988; van 't Veer et al., 1989; Albino et al., 1989; Ball et al., 1994; Platz et al., 1994). The aim of this study was to determine at what stage of melanoma tumorigenesis *NRAS* codon 61 mutations occur. To this end, we screened a large series of primary and metastatic melanomas from a total of 72 patients. Using PCR-SSCP and nucleotide sequence analysis, we identified *NRAS* codon 61 mutations in 21 of 72 patients (29%). The two most frequent mutations observed were a CAA to AAA transversion resulting in a Q61K change (10 patients) and a CAA to CGA transition resulting in a Q61R change (9 patients). Moreover a CAA to CAT transversion (Q61H) and a novel CAA to TTA tandem mutation (Q61L) was observed in one patient each. In the majority of patients both primary tumors and metastases were available for analysis. Interestingly, mutations that were observed in primary melanoma tumors were in the vast majority of cases preserved in the corresponding metastases. In no patient did mutations arise at the metastatic stage. Together these results suggest that *NRAS* mutations correlate with melanoma initiation rather than progression. To determine more accurately at what stage of primary melanomas *NRAS* mutations occur, we used laser capture microdissection (LCM) to isolate RGP and VGP cells. We could show that *NRAS* mutations are present in the early RGP cells and preserved in the corresponding VGP cells. These results confirm that *NRAS* codon 61 mutations represent early events in melanoma tumorigenesis and that they are not responsible for the critical RGP to VGP transition. Histopathological evaluation revealed that two of our primary tumors that harbored *NRAS* mutations were associated with a nevus (one compound and one dysplastic nevus). Microdissection and subsequent mutational analysis of these nevi showed that they contained the same *NRAS* mutations as that of the contiguous primary tumors. This clonal relationship indicates that the two nevi have served as precursor lesions. There have been conflicting results as to whether *NRAS* mutations arise early or late during melanoma tumorigenesis. Whereas some studies have suggested that *NRAS* mutations associate with melanoma initiation (van 't Veer et al., 1989; van Elsas et al., 1996), others have concluded that they correlate with melanoma progression (Albino et al., 1989; Ball et al., 1994). However, in neither of these studies has LCM been used to study the different growth phases of primary melanomas and the number of paired primary and metastatic lesions has been very limited. Our study and a report by Demunter *et al*., which also identifies *NRAS* mutations in tumor-associated nevi and RGP lesions by the use of LCM (Demunter et al., 2001), both support the hypothesis that *NRAS* mutations arise at an early stage during melanoma development.

The observation that *NRAS* mutations are preserved during melanoma progression suggests that Ras activity is important for melanoma maintenance. This result is consistent with a melanoma mouse model where persistent expression of H-Ras^{V12G} was found to be required for melanoma maintenance (Chin et al., 1999).

It has been suggested that UV radiation plays a role in the genesis of *NRAS* mutations in melanoma (van 't Veer et al., 1989; Ball et al., 1994; van Elsas et al., 1996; Jiveskog et al., 1998). The two most frequent mutations that we observed in our series of melanomas (a CAA to AAA transversion and a CAA to CGA transition, respectively) have both been shown to be induced by UV radiation *in vitro* (Van der Lubbe et al., 1988) and have also been described at high frequencies in other series of melanomas. The non-coding strand of codon 61 contains a TT dimer; a known target

for UVB induced DNA lesions. Whereas the CAA to CGA transition occurred opposite to this dipyrimidine site, the CAA to AAA transversion may have resulted from the mispairing of adenosine with 8-oxo-deoxyguanosine.

In summary, we show that *NRAS* codon 61 mutations are present in early RGP melanoma lesions and preserved in later stage VGP and metastatic lesions. This suggests not only that *NRAS* mutations occur early during melanoma development, but also that N-Ras activity is of importance for melanoma tumor maintenance.

Paper III

***NRAS* and *BRAF* mutations arise early during melanoma pathogenesis and are preserved throughout tumor progression.**

In 2002, Davies *et al*. reported that the *BRAF* gene is mutated at a high frequency in cutaneous melanoma, with a single V599E substitution accounting for 90% of mutations (Davies et al., 2002). To confirm the results by Davies *et al*. in a larger series of melanoma tumors and to determine the timing of *BRAF* mutations in melanoma tumorigenesis, we screened the same series of primary and metastatic lesions that we had previously analyzed for *NRAS* mutations. Using PCR-SSCP and nucleotide sequence analysis we identified *BRAF* mutations in 42 of 71 (59%) patients. Interestingly, *BRAF* mutations were observed only in tumors that did not carry *NRAS* mutations, indicating that mutations of these genes are mutually exclusive in melanoma. Overall, 63 of 71 (89%) patients had tumors with mutations in either *NRAS* or *BRAF*. The mutually exclusive occurrence of *NRAS* and *BRAF* mutations is not restricted to melanoma, but has been described in a number of other tumor types as well, including colorectal (Rajagopalan et al., 2002), ovarian (Singer et al., 2003) and thyroid (Kimura et al., 2003) cancers.

As illustrated in Fig. 5 we identified six different *BRAF* mutations. The T1796A transversion that results in the V599E substitution was observed in 37 patients, thus accounting for 88% (37 of 42) of the mutations. A V599R, V599K and K600E change was identified in one patient each. In addition, one patient had a novel 6-bp insertion between codons 598 and 599, encoding two threonine residues. These five mutations are all located within the activation segment of the kinase domain. Finally, a G468S substitution was observed in one patient. This substitution affects the third glycine residue of the glycine-rich loop located in the N-terminal of the kinase domain. Of these mutations, V599E, V599R, V599K and K600E have been functionally characterized (Wan et al., 2004). They all exhibit elevated kinase activity compared with wild-type B-Raf and induce activation of ERK (Wan et al., 2004), suggesting that they are oncogenic.

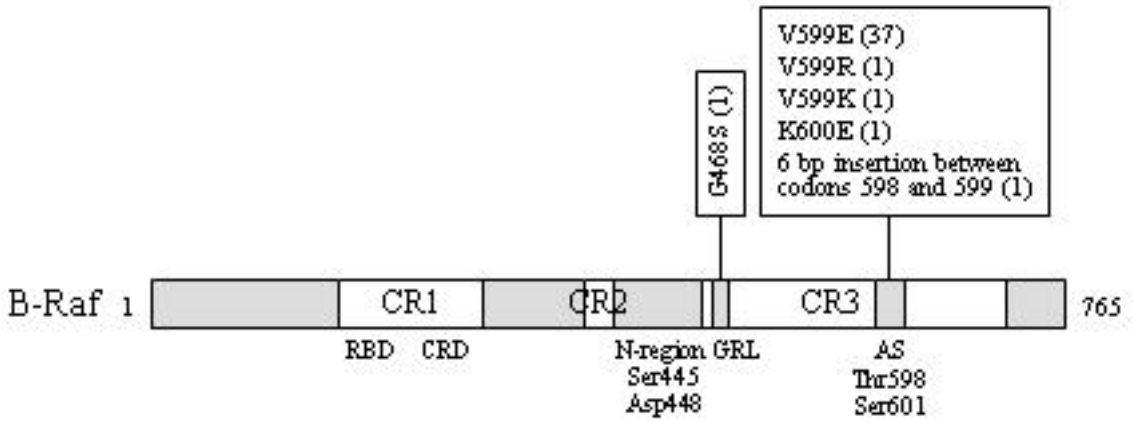

Figure 5. *BRAF mutations in cutaneous melanoma. The three conserved domains CR1, CR2 and CR3 are indicated. CR1 contains the Ras binding domain (RBD) and the cysteine-rich domain (CRD). The negative-charge regulatory region (N-region) is located upstream of CR3 and contains residues Ser445 and Asp448. The kinase domain is located within CR3 and contains the glycine-rich loop (GRL) and the activation segment (AS). The activation segment contains the two phosphorylation sites Thr598 and Ser601.*

In 51 of the 71 patients both primary tumors and metastases were available for analysis. We found that *BRAF* mutations in primary melanomas were always preserved in the corresponding metastatic tumors. Only in two patients did mutations arise at the metastatic stage. This suggests that *BRAF* mutations, like *NRAS* mutations, most likely represent early events in melanoma tumorigenesis and that they play no major role in initiation of melanoma metastasis. Using LCM, we demonstrated that *BRAF* mutations are present in RGP cells and preserved in the corresponding VGP cells. The presence of *BRAF* mutations in RGP cells confirms that *BRAF* mutations occur early and that they are not responsible for the critical RGP to VGP transition. The observation that *BRAF* mutations are preserved throughout melanoma tumor progression suggests that B-Raf signaling is critical for melanoma maintenance.

Our finding that *BRAF* mutations are present in the early RGP is consistent with the frequent occurrence of *BRAF* mutations in nevi (Pollock et al., 2002; Kumar et al., 2004). However, our results differ from a study by Dong *et al*., in which *BRAF* mutations were found at a significantly lower frequency in RGP melanomas compared with VGP melanomas (Dong et al., 2003). The reason for this difference is not known, but may reflect the fact that we have used different screening techniques or that we have analyzed melanoma lesions at different progression stages. Whereas Dong *et al*. investigated pure RGP melanomas we studied the RGP of tumors that had progressed to metastatic disease.

We also determined the relationship between *NRAS*/*BRAF* mutational status and known clinicopathological factors. No significant associations were found between *NRAS* or *BRAF* mutations and gender, age at diagnosis, histogenetic type, level of tumor invasion according to Clark, tumor thickness, ulceration, and site of first recurrence. Neither did *NRAS* and *BRAF* mutations have an impact on patient overall survival. Our results are consistent with results of other studies (Chang et al., 2004; Shinozaki et al., 2004). One study, however, has indicated that the presence of *NRAS*/*BRAF* mutations in melanoma metastases is associated with a shortened patient survival (Houben et al., 2004).

In summary, our results show that *BRAF* mutations are present in early RGP lesions and preserved in later stage VGP and metastatic lesions. This indicates not only that *BRAF* mutations arise early during melanoma development, but also that they may be of importance for melanoma tumor maintanence.

Paper IV

***PIK3CA* mutations contribute to Akt activation in a minor subset of cutaneous melanomas.**

It has recently been shown that the *PIK3CA* gene, which encodes the p110α catalytic subunit of class IA PI3Ks, is mutated at a high frequency in colorectal cancers as well as in a number of other tumor types (Samuels et al., 2004). Most of the observed *PIK3CA* mutations cluster in small regions within the p85 binding (exon 1), helical (exon 9) and kinase (exon 20) domains, with E545K (exon 9) and H1047R (exon 20) constituting two mutational "hot-spots". To determine whether *PIK3CA* is altered in cutaneous melanoma, we analyzed a series of 101 melanoma metastases and a panel of 6 melanoma cell lines with known *NRAS*/*BRAF* mutation status. Using PCR-SSCP and nucleotide sequence analysis, we screened for mutations in exons 1, 9 and 20 of *PIK3CA*. We identified *PIK3CA* missense mutations in 3 of 101 metastases (3%) (Fig. 6). A novel D538N mutation was observed in a metastasis that carried a *BRAF* mutation. The exon 9 hot-spot E545K mutation was found in two metastases that were wild-type for both *NRAS* and *BRAF*. These two metastases originated from the same patient, indicating that the E545K mutation most probably had occurred prior to tumor metastasis. Overall, *PIK3CA* missense mutations were observed in 2 of 87 (2.3%) patients, indicating that mutations in *PIK3CA* are relatively rare in melanoma. However, as we have screened only a limited number of *PIK3CA* exons, it cannot be excluded that there are mutations in other exons that remain unidentified. Interestingly, *PIK3CA* missense mutations were observed only in melanomas that did not carry *NRAS* mutations, suggesting that *PIK3CA* and *NRAS* mutations may be mutually exclusive in melanoma. The observation that *NRAS* and *PIK3CA* mutations do not overlap suggests that these mutations may have equivalent roles in melanoma tumorigenesis (i.e. both *NRAS* and *PIK3CA* mutations contribute to Akt activation). Our results also demonstrate that *BRAF* and *PIK3CA* mutations can coexist in the same tumor. Although such coexistence was observed only in one metastasis, it suggests that activating mutations in *BRAF* and *PIK3CA* may cooperate to induce ERK and Akt activation in melanoma.

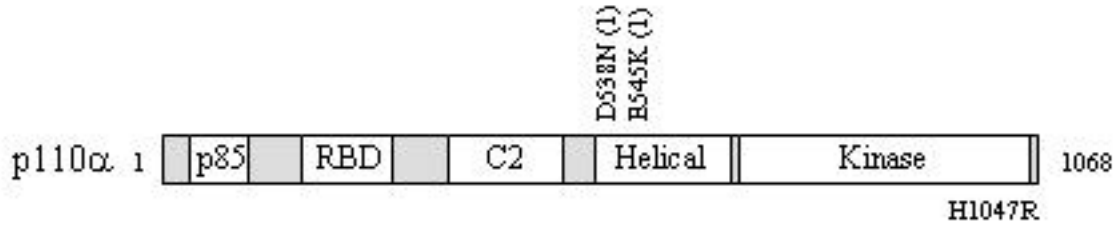

Figure 6. *PIK3CA mutations in cutaneous melanoma. The different functional domains of PIK3CA are indicated (P85BD, p85 binding domain; RBD, Ras binding domain; C2 domain; Helical domain; Kinase domain). The H1047R substitution that is frequently observed in other types of cancers is also shown.*

Both the Raf-MEK-ERK and PI3K-Akt signaling pathways are believed to play central roles in melanoma tumorigenesis. Although it is well established that *NRAS* and *BRAF* mutations contribute to ERK activation in melanoma cell lines, no study has correlated ERK activity with *NRAS* and *BRAF* mutation status in melanoma tumors (Satyamoorthy et al., 2003). To determine the relationship between *NRAS/BRAF* mutation status and ERK activity in melanoma tumors, we analyzed our metastases for expression of phosphorylated (active) ERK using immunohistochemistry. As expected, ERK was frequently activated in metastases that harbored *NRAS* or *BRAF* mutations. Moderate to strong phospho-ERK staining was observed in 70% (19 of 27) of *NRAS* mutated metastases and 74% (31 of 42) of *BRAF* mutated metastases. Interestingly, moderate to strong phospho-ERK expression was also observed in 78% (25 of 32) of metastases that were wild-type for both *NRAS* and *BRAF*. These results indicate that ERK activation in melanoma is not limited to tumors that carry *NRAS* or *BRAF* mutations, but is as common in the subset of melanomas that lack activating mutations of these genes. The presence of ERK activity in melanomas that are wild-type for *NRAS* and *BRAF* indicate that there must exist alternative mechanisms of ERK activation in melanoma. In melanoma cell lines, ERK activation has been observed also as a result of autocrine growth factor stimulation (Satyamoorthy et al., 2003) and overexpression of wild-type *BRAF* (Tanami et al., 2004).

Immunohistochemical studies have shown that Akt is activated in 70 to 80% of melanoma metastases (Dhawan et al., 2002; Stahl et al., 2003; Dai et al., 2005). However, no study has correlated Akt activity with *NRAS* mutation status in melanoma tumors, and it has therefore not been known whether *NRAS* mutations induce Akt activation. To determine the relationship between *NRAS* mutation status and Akt activity in melanoma tumors, we analyzed our metastases for expression of phosphorylated Akt (Ser473) using immunohistochemistry. Moderate to strong phospho-Akt expression was observed in 78% (21 of 27) of *NRAS* mutated metastases, 71% (30 of 42) of *BRAF* mutated metastases, and 78% (25 of 32) of metastases that were wild-type for both *NRAS* and *BRAF*. These results indicate that *NRAS* mutations contribute to Akt activation in melanoma metastases. These results also demonstrate the existence of *NRAS*-independent mechanisms of Akt activation in melanoma. Interestingly, the three metastases that harbored the *PIK3CA* D538N and E545K mutations all displayed a strong expression of phospho-Akt. Thus, our results suggest that *PIK3CA* mutations may contribute to Akt activation in a minor subset of melanomas that lack *NRAS* mutations. Another possible mechanism of Akt activation in melanomas without *NRAS* mutations is inactivation of *PTEN*. Although mutational inactivation of this tumor suppressor gene appears to be a

relatively rare event in melanoma tumors (10%) (Tsao et al., 1998; Birck et al., 2000), immunohistochemical studies suggest that PTEN expression is reduced or completely lost in up to 65% of melanomas (Zhou et al., 2000; Whiteman et al., 2002; Tsao et al., 2003). Yet another possible mechanism of Akt activation in melanoma includes overexpression of Akt3 (Stahl et al., 2004).

Our study confirms the involvement of both the Raf-MEK-ERK and PI3K-Akt pathways in melanoma tumorigenesis. We demonstrate two possible mechanisms by which these pathways can be activated in melanoma tumors: 1) *NRAS* mutation alone; or 2) *BRAF* mutation in combination with *PIK3CA* mutation. The finding that both the Raf-MEK-ERK and PI3K-Akt pathways are activated in the majority of melanomas is consistent with the hypothesis that activation of either pathway alone is insufficient to induce transformation of melanocytes. Recent studies have demonstrated the importance of RalGDS (Hamad et al., 2002; Rangarajan et al., 2004; Gonzalez-Garcia et al., 2005), Tiam1 (Malliri et al., 2002) and PLCε (Bai et al., 2004) in Ras-mediated tumorigenesis. It would be interesting to know whether signaling through any of these Ras effectors is required for melanocytic transformation. If so, it raises the possibility that some components of these pathways are mutationally altered in those melanomas that do not carry mutations in *NRAS*. The frequent involvement of both the Raf-MEK-ERK and PI3K-Akt pathways in melanoma suggests that therapy targeting only one of these pathways may not be effective, but rather that multiple Ras effector pathways must be targeted.

CONCLUSIONS

This thesis has focused on determining the role of *CTNNB1*, *NRAS*, *BRAF* and *PIK3CA* mutations in cutaneous melanoma. The main conclusions are:

- β-catenin mutations are found in approximately 3% of cutaneous melanomas.

- Abnormal accumulation of β-catenin is observed in 30% of primary melanoma tumors, indicating that the Wnt/β-catenin pathway plays a role in melanoma tumorigenesis.

- Activating *NRAS* codon 61 mutations are found in 30% of cutaneous melanomas and *BRAF* mutations in approximately 60% of cases. A single V599E substitution accounts for almost 90% of *BRAF* mutations. *NRAS* and *BRAF* mutations occur in a mutually exclusive fashion, and thus up to 90% of melanomas harbor mutations in either of these genes.

- *NRAS* and *BRAF* mutations represent early events in melanoma development.

- *NRAS* and *BRAF* mutations are preserved from RGP lesions to metastatic disease, indicating that they are important for melanoma tumor maintenance.

- *PIK3CA* mutations are found in 3% of melanoma metastases and appear to occur only in tumors that do not carry *NRAS* mutations. This suggests that mutations in *NRAS* and *PIK3CA* may be mutually exclusive in melanoma.

- ERK activation is observed in 74% of melanoma metastases. We show that ERK activation is not limited to tumors that carry *NRAS* or *BRAF* mutations, but is as common in the minority of melanomas that lack activating mutations of these genes.

- Akt activation is observed in 74% of melanoma metastases. We demonstrate that *NRAS* mutations and *PIK3CA* mutations contribute to Akt activation in melanoma metastases.

- Our results suggest that melanocytes require activation of both the Raf and PI3K pathways for transformation.

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