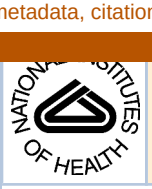

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Understanding Signaling Cascades in Melanoma†

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Abstract

Understanding regulatory pathways involved in melanoma development and progression has advanced significantly in recent years. It is now appreciated that melanoma is the result of complex changes in multiple signaling pathways that affect growth control, metabolism, motility and the ability to escape cell death programs. Here we review the major signaling pathways currently known to be deregulated in melanoma with an implication to its development and progression. Among these pathways are Ras, B-Raf, MEK, PTEN, phosphatidylinositol-3 kinase (PI3Ks) and Akt which are constitutively activated in a significant number of melanoma tumors, in most cases due to genomic change. Other pathways discussed in this review include the [Janus kinase/signal transducer and activator of transcription (JAK/STAT), transforming growth factor-β pathways which are also activated in melanoma, although the underlying mechanism is not yet clear. As a paradigm for remodeled signaling pathways, melanoma also offers a unique opportunity for targeted drug development.

THE RAS–RAF–MEK–ERK PATHWAY

The breakthrough finding in 2002 that B-Raf is mutated in a large percentage of melanomas (1) triggered a substantial number of new studies that focused on mitogen-activated protein kinase (MAPK) signaling in melanoma. These studies established the notion that constitutive activation of the extracellular signal-regulated protein kinase (Ras–Raf–MEK–ERK) signaling cascade is a hallmark of cutaneous malignant melanoma (Fig. 1). Alterations in other components within this pathway were known beforehand, and are best represented by the finding that Ras is mutated in approximately 15–20% of human melanomas (2,3). The Ras proteins regulate cell proliferation, survival and differentiation by activating a number of effector proteins, including the Ral guanine nucleotide dissociation stimulator (GDS) exchange factors, the phosphatidylinositol-3 kinase (PI3Ks), and the three Raf protein kinases (A-Raf, B-Raf and C-Raf) (4). Most Ras mutations are present in codon 61 of N-Ras, with K-Ras and H-Ras mutations being relatively rare (2,5).

B-Raf was found to be mutated in up to 82% of cutaneous melanocyte nevi (6), 66% of primary melanomas (1) and 40–68% of metastatic melanomas (7,8). More than 80% of the oncogenic B-Raf alleles described to date consist of the missense exchange from valine to glutamic acid in residue 599 (V599E). The mutation engenders constitutive and maximal activation of B-Raf kinase activity, likely by mimicking phosphorylation of S598/T601 in native B-Raf (1). *In vitro* studies demonstrated that transfection of V599E B-Raf resulted in a several fold induction of both MEK-ERK and transforming activity (1). Interestingly, B-Raf and N-Ras mutations are mutually exclusive (1,3,9), which is consistent with the finding that active ERK is found in almost all late-stage melanoma cell lines and in tumor tissues. This is in contrast to normal

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melanocytes and several early-stage radial growth phase melanoma cell lines (10). Constitutive activation of the Ras–Raf–MEK–ERK signaling cascade has been shown to contribute to melanoma tumorigenesis by increasing cell proliferation, tumor invasion and metastasis, and by inhibiting apoptosis (11). The importance of constitutive activation of this pathway for the maintenance of melanoma phenotypes has been demonstrated by specific targeting of the B-Raf and MEK pathways using kinase inhibitors such as CI1040, U0126 and BAY43-9006 (12,13) or B-Raf siRNA (13–15) in *in vitro* and xenotransplantation models. In all cases perturbation of these pathways suffice to significantly impact growth of melanoma tumors in xenograft mouse models.

Earlier studies revealed that the presence of B-Raf/N-Ras mutations can be associated with a poorer prognosis of melanoma (8,16). However, more recent studies raised several questions regarding the significance of B-Raf and N-Ras mutations in this disease. For example, Akslen *et al*. found no association between mutations and tumor cell proliferation, tumor thickness, microvessel density, vascular invasion or patient survival (17). In another study, Chang *et al.* compared patients with and without B-Raf mutations and found no significant differences in age, gender, location of primary melanoma, stage at diagnosis and depth of primary tumor. Interestingly melanomas harboring B-Raf mutations were more likely to metastasize to liver and multiple organs, although there was no clear association with survival (18). The finding that the V599E B-Raf allele could be detected in as many as 80% of benign nevi pointed to a possible role of oncogenic B-Raf in nevus formation and melanoma initiation (6,19). However, to date, no evidence exists to directly support the possibility that benign nevi harboring V599E B-Raf actually progresses to a malignant tumor. In fact, most nevi may represent nonprogressing terminally differentiated lesions (20,21) formed by senescent cells characterized by p16(INK4a) expression (22). Moreover, it has been suggested that this oncogene-induced senescence represents a genuine protective physiologic process (22). These data suggest that although B-Raf and N-Ras mutations are likely to be important for the initiation and maintenance of most melanomas, additional mutations or modifications are required to support melanoma progression to the invasive type. Along these lines, two studies identified association of mutated V599E B-Raf with p16/ARF loss and TP53 and PTEN (phosphatase and tensin homolog deleted on chromosome 10) mutations (23,24). It has been proposed that a possible cooperation between B-Raf activation and loss of either p16/ARF or PTEN contributes to melanoma development (23). Another line of evidence suggests that B-Raf mutation may not be essential in all forms of melanocyte neoplasia, and that distinct pathways of melanoma formation exist (25–27). In agreement with such a possibility, B-Raf mutations were not detected in mucosal or vulvar melanomas, or in more than 90% of sinonasal and uveal melanomas (28–31). Along these lines, through analysis of RNA expression profiles, Shields *et al*. found a molecularly distinct melanoma subtype characterized by lack of mutations of N-Ras and B-Raf, p53 inactivation, reduced ERK activity and increased expression of epithelial markers (32). These data suggest that despite sharing a common progenitor cell (the neural crest-derived melanocytes), different genetic, and possibly epigenetic, programs impact the diversity of melanoma forms that are formed, explaining their distinct characteristics (25, 26).

How does activation of B-Raf affect the oncogenic behavior of melanoma tumors? Alterations in MAPK signaling appear to play a major role in the pathogenesis of most melanomas. The melanoma-relevant effectors of ERK activation however, are largely unknown. Mechanistically, these effects are mediated both by posttranscriptional modification of proteins and by an increase in the transcription of specific genes. With the advent of microarray technology, genes whose expression is governed by MAPK signaling are being identified. More so, specific gene-expression signatures in addition to genes expressed by common MAPK activation are being uncovered for Q61K N-Ras and B-Raf V599E (33,34). Recently through supervised analysis of RNA expression profiles, 82 transcriptional targets in melanoma

were identified (including TWIST1, hypoxia-inducible factor-1α [HIF-1α] and interleukin-8 [IL-8]; 33). Activated ERK plays a pivotal role in cell proliferation by controlling the G1-phase to S-phase transition by negative regulation of the p27/Kip1 inhibitor (35,36) and upregulation of c-Myc activity (36). Inhibition of ERK activity is associated with reduced proliferation (36) and G1-phase cell cycle arrest, mediated by upregulation of p27/Kip1 (cyclin-dependent kinase inhibitor) mRNA and hypophosphorylation of retinoblastoma protein (35,37). Additional regulation of p27/Kip1 is provided by B-Raf- and cyclin D1-dependent Skp2 (S-phase kinase-associated protein) proteolysis (37). Interestingly, the combination of B-Raf (V599E) and Skp-2 siRNA resulted in a greater inhibition of matrigel invasive ability than the single suppression of each protein (38).

Another target of ERK is the Brn-2 POU domain transcription factor, which is highly expressed in melanoma cell lines but not in melanocytes or melanoblasts. Expression of Brn-2 is positively regulated by B-Raf and MAPK signaling. Overexpression of Brn-2 in melanocytes results in increased proliferation; depletion of Brn-2 in melanoma cells expressing activated B-Raf leads to decreased proliferation (39). Another mechanism by which constitutive active ERK stimulates cell proliferation is *via* its regulation of c-Jun, increasing both c-Jun transcription and stability, which are mediated by cyclic adenosine monophosphate response element-binding (CREB) and glycogen synthase kinase 3 (GSK3), respectively (40).

ERK is believed to also play a role in increased proliferation by inhibiting differentiation. During differentiation of melanocytes, an increase in intracellular cAMP leads to stimulation of the Ras–MEK–ERK pathway and expression of microphthalmia-associated transcription factor (MITF). MITF induces expression of the melanogenic enzyme tyrosinase, among other targets. Conversely, constitutively active ERK limits differentiation in melanoma by targeting MITF for degradation (41–43). The constitutively activated ERK pathway mediates melanoma-specific survival signaling by differentially regulating RSK-mediated phosphorylation and inactivation of the proapoptotic protein Bad (44). ERK-mediated inhibition of JAK–STAT (45) is another mechanism by which MAPK affects melanoma cell survival.

ERK contributes to tumor invasion and metastasis by regulating expression of proteins, such as matrix metalloproteinases (MMPs) and integrins. A critical step in the process of metastasis is degradation of the extracellular matrix to allow extravasation and migration of the metastatic cells. Two families of proteases are secreted by the invading cells, the urokinase plasminogen activation and MMPs, which are involved in matrix remodeling. The expression and activity of MMPs and urokinase are tightly controlled by MAPKs (46–48). The Ras–Raf–MEK–ERK pathway is constitutively active in melanoma and is the dominant pathway driving the production of collagenase-1 (MMP-1) (49–51). Furthermore, blocking MEK–ERK activity inhibits melanoma cell proliferation and abrogates collagen degradation, decreasing their metastatic potential (52). Constitutive activation of this MAPK pathway not only promotes increased proliferation of melanoma cells but is also important in the acquisition of an invasive phenotype (52). It has been demonstrated that sustained, and not transient, activation of the Raf–MEK–ERK signaling pathway specifically controls the expression of integrin subunits and may participate in changes in cell adhesion and migration that accompany the process of oncogenic transformation (53). In addition, novel functions for activated MAPK pathway are being discovered. For example, ERK activity was found to play a role in immune evasion by melanoma cells, since targeting of B-Raf and MEK decreased production of the immunosuppressive soluble factors IL-10, vascular endothelial growth factor (VEGF) or IL-6 (54).

Due to its important role in melanoma tumorigenesis, supported by extensive preclinical validation and epidemiologic studies, the B-Raf/MEK pathway represents an attractive

therapeutic target (55,56). Consistent with these expectations, promising results were obtained in mouse models (12,13,57) and several new small-molecule inhibitors of B-Raf kinase are currently undergoing clinical evaluation, with others due to enter clinical assessment in the near future. Clinical trials of these inhibitors are also expected to include a combination of drugs that affect different signaling pathways (*i.e.* in combination with PI3K inhibitors) to maximize the impact on diverse signaling pathways that are deregulated in this tumor type (58,59).

In any case, more original and interesting preclinical data is being generated to help rationale drug development. Sharma *et al.* found significant differences between the effects of B-Raf and MEK inhibitors. The B-Raf inhibitor BAY 43-9006 (which was found ineffective in clinical trials) did not decrease metastasis in a mouse model, whereas inhibition of MEK using U0126 decreased cellular proliferative capacity, thereby effectively reducing the number and size of lung metastases (60). Inhibition of metastasis was mediated through reduction in melanoma cell extravasation through the endothelium and decreased proliferative capacity (60). The latter is consistent with the growing notion that treatment of melanoma would require combined targeting of distinct signaling pathways (61,62). Altogether, these data show that advances made during the recent few years allow us to better understand the complexity of metastatic melanoma. While devoting substantial efforts to further understand changes underlying the development and progression of different subtypes of melanomas, progress is being made in developing therapeutic modalities to treat this tumor type.

THE PI3K/AKT PATHWAY

The PI3K/Akt pathway was shown to be activated in various cancers, mostly due to mutations in the tumor suppressor gene PTEN (63). In melanoma, the loss of chromosome 10 was first reported by Parmiter *et al.* (64) and since then has been studied extensively (65,66). The PTEN gene encodes a phosphatase whose primary function is to degrade the products of PI3K by dephosphorylating phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 3,4-bisphosphate at the 3 position (67). Loss of functional PTEN from tumor cells causes accumulation of these critical second messenger lipids, which in turn increase Akt phosphorylation and activity, leading to decreased apoptosis and/or increased mitogenic signaling (68). A PTEN mutation rate of 30–50% in melanoma cell lines has been reported by several groups. Among cell lines with a PTEN mutation, 57–70% showed homozygous deletion of the PTEN gene (69,70). On the other hand, PTEN mutations in metastatic melanoma samples are rare (5–20%) (71–73). Importantly, PTEN protein levels were found to be altered in metastatic melanoma in the absence of genetic alterations. Zhou *et al.* found no PTEN protein expression in 15% (5/34) and low expression in 50% (17/34) of melanoma samples (4 primary and 30 metastatic) (74). Surprisingly, among the five melanomas with no PTEN protein expression, four showed no deletion or mutation of the PTEN gene, indicating the action of an epigenetic mechanism of biallelic functional inactivation of PTEN (74). These observations have led to the conclusion that in addition to PTEN mutation, other mechanisms, such as epigenetic silencing (75–78), altered subcellular localization (79) or ubiquitination (80) are important in PTEN inactivation; collectively these changes could be as frequent as in 40–50% of sporadic melanomas (74). The role of PTEN in melanoma was confirmed by functional studies. Ectopic expression of PTEN was demonstrated to suppress melanoma cell growth (81) and melanoma tumorigenicity and metastasis (82). Moreover, the PI3K inhibitors wortmannin and LY294002 have antitumor activity *in vitro*, inhibiting proliferation and sensitizing cell lines to chemotherapy and radiation treatment (83). Collectively, these data suggest that PTEN and PI3K play an important role in melanoma tumorigenesis.

Because PTEN functions as an antagonist of PI3K-mediated signaling, a consequence of PTEN loss is the increase in Akt activity. Akt/protein kinase B (PKB), a serine/threonine kinase, is a

core component of the PI3K-signaling pathway activated through phosphorylation of Ser-473/474 and Thr-308/309 (84,85). Several studies have shown that Akt/PKB activates the transcription of a wide range of genes, especially those involved in immune activation, cell proliferation, apoptosis and cell survival (85). Considering the frequency of PTEN inactivation in melanoma, dysregulation of Akt activity is expected. Accordingly, several studies documented Akt activation in melanoma. Using an antibody developed against phospho-Akt Ser-473 Dhawan *et al.* found no significant pAkt levels in normal and slightly dysplastic nevi in marked contrast to the dramatic pAkt immunoreactivity seen in severely dysplastic nevi and melanomas (66.3% positive) (86). More recently, in a 292 sample study, Dai *et al.* analyzed pAkt levels using tissue microarray and immunohistochemistry. Strong pAkt expression was observed in 17%, 43%, 49% and 77% of the biopsies in normal nevi, dysplastic nevi, primary melanoma and melanoma metastases, respectively. Increasing pAkt expression was inversely correlated with both overall and disease-free survival, and it was a poor prognostic factor for patients with melanomas less than 1.5 mm in thickness (87). Similar results were obtained by Slipicevic *et al.*, except for a higher incidence of pAkt (54%) in benign nevi (88). Activation of Akt was also observed in Spitz nevi (89).

The finding that Akt is activated in melanomas without genetic aberrations in PTEN or Ras prompted several groups to search for possible mutations in other components of the pathway. A study by Samuels *et al.* showed that the PIK3CA gene, which encodes the p110α catalytic subunit of PI3Ks, is mutated in human cancers (90). However, from a series of 101 melanoma metastases, only three were identified to carry missense mutations in PIK3CA (91). Other studies failed to observe protein overexpression of PI3K (92) or amplification of PI3K genes in melanoma (93). An alternative mechanism for activation of the PI3K/Akt pathway is activation of Akt itself. Screening of the pleckstrin homology domain (94) and codons 308 and 473 (95) of Akt did not identify mutations. However, selective activation of Akt3 was shown in 43–60% of sporadic melanomas, occurring as a result of a combination of increased Akt3 expression accompanying copy number increases in the Akt3 gene and decreased PTEN protein activity caused by loss or haploinsufficiency of the PTEN gene (96). Consistent with these observations, targeted decrease in Akt3 activity using siRNA stimulated apoptotic signaling, which reduced cell survival and inhibited melanoma tumor development (96).

The positive effect of PI3K/Akt pathway on melanoma development was shown to be mediated by several mechanisms including inhibition of apoptosis, increase in cell survival and cell cycle regulation. Recently, a study by Gomez-Gutierrez showed that a triple mutant of FKHRL1, which cannot be phosphorylated by Akt, induced apoptosis in melanoma cells (97). Active Akt was shown to upregulate the cell adhesion protein MelCAM which plays a critical role in cell–cell interactions during melanoma development and whose increased expression has been associated with acquisition of malignancy by human melanoma (98,99). Similarly, PI3K and Akt have recently been shown to induce expression of MMP-2 and MMP-9 by a mechanism involving Akt activation of nuclear factor-kappa B (NF-κB) binding to the MMP promoter (100,101). Overexpression of an active form of Akt led to upregulation of VEGF, increased production of superoxide ROS, and made the switch to a more pronounced glycolytic metabolism. Moreover, subcutaneous implantation of WM35 cells overexpressing Akt led to rapidly growing tumors *in vivo*, while vector control cells did not form tumors (102). PI3K/Akt can also contribute to tumorigenesis by positively regulating cyclin D3 which contributes to G1-S progression (103). The mechanisms associated with the ability of Akt to suppress apoptosis include phosphorylation and inactivation of many proapoptosis proteins such as Bad (104) and caspase-9 (105). Downstream effects of Akt activation are also mediated by inactivation of the forkhead family of transcription factors (106), and activation of NF-κB (107). Overall, the PI3K pathway emerges as a central axis which is deregulated in melanoma, and in conjunction with the constitutively active MAPK signaling cascade, makes key contributions to melanoma development and progression. Targeting PI3K signaling in

conjunction with MAPK is expected to offer an important therapeutic modality for the treatment of this tumor type.

THE WNT PATHWAY

Wnts are secreted glycoproteins that act as ligands to stimulate receptor-mediated signal transduction pathways involved in cell proliferation, survival, behavior and fate. Wnt proteins activate at least three different intracellular signaling pathways—the Wnt/β-catenin, the Wnt/Ca^{2+} and the Wnt/planar polarity pathways (108). The first, termed canonical, involves stabilization of β-catenin; the other two involve activation of protein kinase C (PKC) and c-Jun N-terminal kinases (JNKs), respectively. In the canonical Wnt pathway, in the absence of a Wnt signal, cytoplasmic β-catenin is phosphorylated at serine and threonine residues through the action of casein kinase Iα and GSK3β and degraded in a complex that also includes adenomas polyposis coli (APC) and axin (109). The pathway becomes activated when Wnt binds to its receptor Frizzled and to a low-density lipoprotein receptor-related protein-5 or -6 (LRP5 or 6) coreceptors. This ternary complex ultimately leads to activation of the cytoplasmic phosphoprotein Dishevelled, which blocks the degradation of β-catenin. This is followed by nuclear translocation of β-catenin where it interacts with specific transcription factors T cell factor/lymphoid-enhancing factor (TCF/LEF) leading to regulation of target genes (110,111). Among the Wnt family members, Wnt 1, 2, 3, 3a, 7a and 8 are involved in the Wnt/β-catenin pathway (112).

Mutations of genes encoding members of the Wnt-signaling cascade, in particular *CTNNB1* and *APC*, are frequent in various types of human cancer. This includes, among others, colorectal carcinoma, hepatocellular carcinoma and hepatoblastoma, as well as primitive neuroectodermal tumors (113). Because activation of β-catenin appeared to be frequent in melanoma (114), in recent years several groups studied the occurrence of genetic modifications and changes in expression of *CTNNB1* and *APC*. *APC* mutations were found in sporadic cases of primary melanoma (114–116) whereas hypermethylation of APC promoter 1A was present in 13% of cell lines and in 17% of melanoma biopsies (116).

Oncogenic activation of β-catenin by amino acid substitutions or deletions has been demonstrated in 23% of melanoma cell lines (115). Conversely, β-catenin mutations are rare in primary melanoma (114,117). Nonetheless, almost one third of primary human melanoma specimens display aberrant nuclear accumulation of β-catenin, although generally without evidence of direct mutations within the β-catenin or *APC* gene (114,117,118). Metastatic melanomas were found to contain nuclear and cytoplasmic accumulation of β-catenin and increased TCF/LEF-dependent transcription (119). These observations are consistent with the hypothesis that the Wnt pathway contributes to the behavior of melanoma cells and might be inappropriately deregulated in the genesis of this disease. Moreover, evaluation of activated β-catenin using phosphoantibodies revealed that nuclear phospho β-catenin was more common in metastatic lesions and that high levels of nuclear phospho β-catenin are associated with significantly worse overall survival (120). The finding that more melanomas have nuclear and/or cytoplasmic β-catenin accumulation than those carrying detectable mutations in *CTNNB1* or *APC* suggests that the pathway may be activated in such tumors through aberrations in other genes. *ICAT* (inhibitor of β-catenin and T cell factor) was identified as a gene that negatively regulates the Wnt-signaling pathway by inhibiting the association of β-catenin with TCF-4 in the cell nucleus and represses transactivation of β-catenin–TCF-4 target genes (121). Messenger RNA expression analyses revealed ICAT transcript levels reduced to 20% or less relative to normal skin and benign nevi in more than two-thirds of melanomas. This suggests that loss of ICAT expression may contribute to melanoma progression and metastasis by virtue of altered β-catenin–TCF-4 regulation in the cell nucleus (118). The mechanism underlying the markedly reduced *ICAT* mRNA levels in melanomas is unclear at present.

Regardless of the underlying mechanism, constitutive activation of the Wnt/β-catenin signaling pathway is a notable feature of malignant melanoma. The identification of target genes downstream from this pathway is therefore crucial to our understanding of the disease. The POU domain transcription factor, Brn-2, has been found to be directly controlled by the Wnt/β-catenin signaling pathway in melanoma cell lines and in transgenic mice (122). Strikingly, expression of Brn-2 is not only upregulated by β-catenin but is also elevated in response to MAPK (39). Consistent with upregulation of these two pathways, Brn-2 expression is strongly upregulated in melanoma. Overexpression of this gene has been associated with increased proliferation and tumorigenicity in melanoma (122,123). A key role of Wnt signaling in melanocyte development is the activation of the promoter for the gene encoding MITF (124, 125). MITF (126,127) is essential for development of the melanocyte lineage and has key functions in control of cell proliferation and survival and in differentiation (41). It was also demonstrated that β-catenin's contribution to growth of melanoma cells depends on its downstream target, MITF. Moreover, suppression of melanoma clonogenic growth by disruption of the β-catenin–TCF/LEF complex is rescued by constitutive MITF. β-catenin regulation of MITF expression thus represents a tissue-restricted pathway that significantly influences the growth and survival behavior of this notoriously treatment-resistant neoplasm (128). Interestingly, it was recently shown that MITF can interact directly with β-catenin and redirect its transcriptional activity away from canonical Wnt signaling-regulated MITF-specific target genes (129).

Wnt induction is blocked by five classes of proteins—Dkk, Wise, Sfrp, Wif and Cerberus—competing for the Wnt ligand or for the Lrp-Frz-receptor (130). Interestingly, whereas Dickkopf-1 protein secretion was documented in breast, prostate and lung cancer lines, it was negligible in melanoma (131). Kuphal *et al.* found that DKK-1, -2 and -3 were downregulated or lost in all cell lines and in most of the melanoma tumor samples analyzed (132). Overexpression of Dkk-1 (133) and WIF-1 (134) inhibited melanoma tumor growth in a xenograft mouse model. Similarly, it has been recently shown that an anti-Wnt-2 monoclonal antibody induced apoptosis in malignant melanoma cells and inhibited tumor growth (135).

In recent years it has become clear that Wnt signaling can also function *via* β-catenin-independent pathways. These noncanonical pathways include: (1) calcium/calmodulin-dependent kinase II (CAMKII), and PKC, (2) phospholipase C (PLC) and phosphodiesterase (PDE), and (3) a pathway termed convergent extension, which is similar to the planar polarity in Drosophila that activates the Jun-N-terminal kinase (136). Activation of these noncanonical Wnt pathways is mediated by Wnt 4, Wnt11 and mainly Wnt 5a. Wnt signaling has been shown to be important not only in development but also in tumorigenesis. Wnt5a is upregulated in cancers of the lung, breast and prostate, and is downregulated in pancreatic cancer (137,138). Expression profiling studies aimed at identifying molecular subclasses of tumors found a series of genes whose expression differed in cutaneous melanomas with differing invasive phenotypes (139,140). Among genes that created the distinct classes were those important in cell motility and invasive ability. *WNT5a* was identified as a particularly robust marker of highly aggressive behavior (139). Unlike that of other Wnt family members (*e.g.* Wnt1 and Wnt8), Wnt5a expression does not profoundly affect β-catenin stabilization. Instead, Wnt5a stimulates intracellular Ca^{2+} release and activates CAMKII and PKC in a G-protein-dependent manner (141). The study by Bittner *et al.* confirmed early studies of Wnt5a RNA expression in tumors and indicated that overall, many tumors showed increased Wnt5a expression relative to their normal tissue of origin, and accordingly that melanomas showed increased Wnt5a expression relative to skin (139). Furthermore, Wnt5a protein expression in human melanoma biopsies directly correlates with increasing tumor grade, cell motility and invasion of metastatic melanoma (142), and inversely correlates with patient survival (139). Importance of the Wnt5a–Frizzled pathway in melanoma was confirmed by analysis of serial analysis of gene expression (SAGE) libraries from melanoma tissues. This study allowed identification of

several genes associated with changes in calcium flux and PKC signaling (PLC gamma, inositol-1,4,5-triphosphate-3 kinase B and C, PKC θ) (143), consistent with increased PKCα/βII and PKCμ activity and increased motility and invasiveness of melanoma cells expressing Wnt5a (142). As additional support for the notion that this pathway contributes to the invasive phenotype of the melanoma cells, the authors demonstrated that inhibition of this pathway by desensitization of the Wnt5a receptor, Frizzled5, by an antibody that interfered with activation by Wnt5a resulted in decreased activation of the PKC pathway and inhibition of *in vitro* motility and invasion phenotype of the melanoma cells (142). By using overexpression and downregulation of Wnt5a, it was recently found that Wnt5a/PKC stimulates melanoma cell motility *via* induction of genes involved in the epithelial to mesenchymal transition (EMT) of carcinomas including upregulation of vimentin and Snail (a repressor of E-cadherin), and downregulation of E-cadherin (144). Increased expression of Wnt5a in melanoma tumors is localized, occurring in cells at the site of active invasion and in cells showing morphologic features associated with aggressive tumor behavior. In connection with Wnt5a, it is noteworthy that PKC has been identified as a contributing factor in skin tumorigenesis. In models of melanoma, PKC-α activation is typically associated with increased tumor cell proliferation and invasiveness, and decreased differentiation (145). Of interest, it has also been shown that increase or inhibition of PKC activity results in corresponding changes in Wnt5a expression (146). These observations suggest the possibility that the activities of Wnt5a and PKC drive a positive feedback loop, perhaps a Wnt5a autocrine loop, and that increase in the activity of either may result in increased melanoma motility.

THE JNK/c-JUN PATHWAY

Investigation into the JNKs has focused typically on their activation in response to diverse stresses. In more recent studies these kinases are recognized for their importance in the regulation of mammalian physiology, including: cell proliferation, cell survival, cell death, DNA repair and metabolism. Activation of JNK is induced by a variety of extracellular stimuli, growth factors, cytokines, tumor promoters, UV radiation and hormones (147). JNK is activated by sequential protein phosphorylation through a MAP kinase module, *i.e.* MAP3K-MAP2K-MAPK (148). Two MAP2Ks (JNKK1/MKK4/SEK1 and JNKK2/MKK7) have been identified for JNK. Phosphorylation of JNK by these dual-specificity protein kinases on Thr183 and Tyr185 is necessary for its activation (147). Several MAP3Ks, including members of the map-erk kinase kinase (MEKK) family, activator of S-phase kinase 1 (ASK1), mixed lineage kinase (MLK), TGF-beta-activated kinase 1 (TAK1) and Tumor progression loci-2 (TPL 2), have been reported to act as MAP3Ks for JNK (149).

JNK has been shown to elicit both positive and negative effects on tumor development depending upon the cellular context (150). JNK activation is required for Ras-mediated transformation (151) and has been found to mediate proliferation and tumor growth (152, 153). These observations are consistent with the finding of constitutively active JNK in tumor samples and derived cell lines (40,150,154). On the other hand, studies using JNK−/− fibroblasts revealed that JNK is not required for Ras-dependent tumor development *in vivo* and that JNK1−/− JNK2−/− fibroblasts are more tumorigenic than wild-type cells (155). The role of JNK in tumorigenesis was also studied in the fly using three different tumor models of increasing malignancy. Similar to that found in mammals, JNK was found to either promote or eradicate tumors depending on the genetic context (156).

Among the many proteins phosphorylated following JNK activation, the one which has been better studied in context of cancer development is the transcription factor c-Jun. JNK-mediated phosphorylation at serines 63 and 73 enhances the ability of c-Jun, a component of the AP-1 transcription complex, to activate transcription in response to a plethora of extracellular stimuli (157). The JNK activation leads to induction of AP-1-dependent target genes involved in cell

proliferation, cell death and inflammation. Members of the AP-1 transcriptional complex include c-Jun, JunB, JunD, c-Fos, FosB, Fra-1 and Fra-2, all of which contain a leucine zipper and form either homodimers or heterodimers through this domain. The different dimer combinations recognize different response elements in the promoters and enhancers of target genes (158,159). AP-1 target genes are differentially regulated by distinct AP-1 dimers. The dynamic changes in AP1 composition after stress-stimuli balance discrete signals that play a key role in determining whether cells undergo apoptosis, survival or senescence (160). Among other c-Jun heterodimeric partners that influence the AP1 transcriptional readout is ATF2, which has been shown to play a key role in melanoma development (161).

Whereas the role of JNK in oncogenesis is emerging, c-Jun is a well-defined oncogene in several malignancies. c-Jun is highly amplified and overexpressed in undifferentiated and aggressive human sarcomas (162), and has been associated with proliferation and angiogenesis in invasive breast (163) and lung cancer (164). Moreover, c-Jun phosphorylation is required for Ras-induced transformation of fibroblasts *in vitro* and Ras-induced skin tumorigenesis *in vivo* (165). c-Jun has been shown to contribute to the early stages of carcinogen-induced hepatocellular carcinoma by antagonizing the action of p53 (166). Along these lines, a recent article by Das *et al.* showed that JNK negatively regulates p53-dependent senescence in MEF (167). These studies suggest that the JNK/c-Jun pathway may contribute to cellular transformation by downregulating the p53 tumor suppressor. In the last several years, the JNK/c-Jun pathway has been shown to play an important role in melanoma development. Of note, the relevance of this pathway in melanoma was already recognized in the early 1990s (168, 169). *c-Jun*, *Jun-B* and *c-fos* genes have been shown to play a role in the transformation of melanocytes into malignant melanoma (168). Further changes in the composition of AP-1 components studied during progression of melanoma were revealed in mouse melanoma B16 tumor models (169). These data suggest a potential role for AP-1 in the transformation of melanocytes into malignant melanoma. Constitutive activation of JNK in melanoma cell lines and melanoma tumor samples was recently described by our laboratory (40), and by Jørgensen *et al.* (170). Interestingly, activation of JNK during tumor progression is associated with cell proliferation and shorter relapse-free period for patients with superficial spreading melanomas (170).

The possible role of the JNK/c-Jun pathway in tumorigenesis has led several groups to study the potential clinical relevance of interfering with this pathway. Inhibition of JNK signaling by chemical inhibitors or siRNA inhibited proliferation in non-small cell lung cancer (NSCLC) (154) and breast (171) cell lines and induced apoptosis in prostate cancer cells (172). c-Jun and JunB knockdown in B16-F10 melanoma cells by short hairpin RNA resulted in cell cycle arrest and apoptosis mediated by apoptosis inducing factor and extended survival of mice inoculated with these modified tumor cells (173). These results suggest that in the absence of c-Jun, JunB can act as a tumor promoter; therefore, inactivation of both c-Jun and JunB may provide a valuable strategy for antitumor intervention (173).

Despite an increasing body of evidence implicating the JNK/c-Jun pathway in cancer, little is known about the genetic and mechanistic basis for these findings. Genetic alterations have not been described in JNK or in its upstream kinases. Worthy of mention is that Ras, which requires JNK and c-Jun for transformation, is activated in 30% of human cancers (174). A possible mechanism explaining JNK activation involves the tumor suppressor p16(INK4a) which is frequently deleted in melanoma (175). It was shown that p16(INK4a) can bind to the glycine-rich loop of the N-terminal domain of JNK, inhibiting c-Jun phosphorylation, thus interfering with cell transformation promoted by the H-Ras-JNK-c-Jun-AP-1 signaling axis (176). Recently, a new link between the constitutively active MEK/ERK and JNK pathway was demonstrated. Through its positive effect on c-Jun, ERK enforces a feed-forward mechanism by which c-Jun partially contribute to increased JNK activity (40). Tumor necrosis factor (TNF)

receptor associated factor 2 (TRAF2), as well as other members of the TRAF family (TNF receptor [TNFR]-associated factors), is upregulated in various tumors including melanomas (177), and through its effect on MEKK1–MKK4/7 can efficiently activate JNK-c-Jun. Stimulation of TNFR results in receptor trimerization and the recruitment of TNFR associated factors (TRAFs) and/or TNFR-associated death domain protein to the cytoplasmic regions of the receptors (178). TRAF2 plays a critical role in the regulation of most stress kinases, including ASK1, MEKK1 and I-kappa-B kinase (IKK) (179–181). Indeed, expression of a RING finger-deleted TRAF2, which serves as a dominant negative, resulted in sensitization of metastatic melanoma to apoptosis and coincided with upregulation of p38 and tumor necrosis factor-α (TNF-α) and downregulation of NF-κB activities (182).

JNK/c-Jun and PKC

PKC has long been identified as a contributing factor in skin tumorigenesis. In models of melanoma, PKCα expression and activation was associated with increased tumor cell proliferation, invasiveness and metastasis (145,183–187). This is in agreement with the upregulation of several PKC isoforms seen in melanoma cells compared to melanocytes (188). A classic effect of PKC activation is the transcriptional activation of AP-1 target genes (189). Recently it has been described in melanoma cultures that PKC can phosphorylate JNK and enhance JNK activation by MKK4/MKK7 (190). Similar to that described for JNK, the upstream events involved in PKC activation in melanoma are not clear. One interesting possibility is that PKC is activated in response to activation of the noncanonical Wnt pathway (142, see also The Wnt Pathway section). The recently established link between PKC and JNK raises the possibility that the noncanonical Wnt pathway might be also partially involved in the activation of JNK. JNK activation downstream of Wnt is firmly established as part of the planar cell polarity pathway (see Wnt section) but whether or not these pathways are connected in melanoma will require further studies.

JNK/ATF2

Another JNK target also implicated in melanoma is ATF2. ATF2 is activated by JNK and p38 which phosphorylates residues 69 and 71 on ATF2. p38 is a member of the MAPK signaling network and is activated by TRAF2/ASK1/MKK3/6 signaling. ATF2 is a member of the bZIP family of transcription factors, which elicits its transcriptional activities after heterodimerization with c-Jun, as with Rb, CREB and p65/NF-κB following its phosphorylation by the stress kinases, JNK or p38-MAPK (191,192). ATF2 has been implicated in the regulation of TNF-α, transforming growth factor-β (TGF-β), IL-6, cyclin A and E-selectin (192–194). ATF2 activities have also been associated with tumor development and progression (195). Activation of ATF2 by hepatocyte growth factor/scatter factor (HGF/SF) through p38-MAPK and SAPK/JNK mediates proliferation signals in melanoma cells (196). ATF2 plays an important role in the acquisition of resistance to chemotherapy and radiation therapy in human melanoma (197,198). As a transcription factor, ATF2 is active within the nuclear compartment, where it elicits its transcriptional activities. Of particular interest is the finding that nuclear ATF2 expression is more frequently found in metastatic sites (lymph nodes, bone metastases or visceral metastases) than in primary cutaneous specimens, and that it correlates with poor prognosis (199). Strong nuclear staining in a melanoma tumor specimen (199) suggests that ATF2 is subject to constitutive activity. Of importance, ATF2 activities are not limited to transcription control as it was also shown to play an important role in DNA damage response upon its phosphorylation by ataxia telangiectasia mutated (ATM)/ATM and Rad3-related (ATR) (200). ATF2 that is phosphorylated by ATM is found within DNA damage repair foci and contributes to the intra-S phase checkpoint response following the formation of double strand breaks (200). Inhibition of ATF2 activities, either by expression of its dominant negative forms or *via* short ATF2-driven peptides that out-compete the endogenous protein, has been found to be efficient in sensitizing human and mouse melanoma

cells to apoptosis (161,197,201). Significantly, expression of the ATF2-driven peptide in mouse melanoma models prone to metastasis (B16F10 and SW1) resulted in inhibition of melanoma growth and metastasis in the corresponding syngeneic mouse model (202). Mechanistically, when ATF2 is inhibited, JunD was found to cooperate with c-Jun and increase AP1 activities, which elicited a proapoptotic signaling in melanoma (203). Screening for natural compounds that could mimic the ATF2 peptide has led to the identification of Celasterol and gambogic acid that were found to efficiently inhibit melanoma both in culture and in mouse tumor models (204). Intriguingly, while ATF2 elicits oncogenic activities in melanoma, it appears to elicit tumor suppressor activities in nonmalignant skin tumors (205). The nature of tissue-specific differences is currently being elucidated. In all, ATF2 appears to serve one of the important functional arms for the ERK and JNK pathways in melanoma, through which it contributes to tumor development and progression.

THE NF-κB Pathway

The mammalian NF-κB family contains five members—p105/p50 (NF-κB1), p100/p52 (NF-κB2), RelA (p65), RelB and cRel (206,207). NF-κB1 and NF-κB2 are synthesized as the inactive cytosolic precursors p105 and p100, respectively. The canonical activation of NF-κB pathway involves TNF-α stimuli resulting in the activation of TNFR and association of TRAF2/MAP3K module with subsequent phosphorylation/activation of IKK. In turn, IKK-mediated phosphorylation of IκB leads to IκB ubiquitination and proteasomal degradation, releasing an active NF-κB complex (208,209). The composition of activated NF-κB complexes will determine the type of genes that will be *trans*-activated. For example, NF-κB complexes containing cRel generally activate proapoptotic genes such as DR4/DR5 and Bcl-X, and inhibit anti-apoptotic genes such as cellular inhibitor of apoptosis (cIAP)1, cIAP2 and survivin after TNF-related apoptosis-inducing ligand treatment. Conversely, RelA inhibits expression of DR4/DR5, and up-regulates caspase-8, cIAP1 and cIAP2 (210).

NF-κB is often activated in tumors including melanomas (211). Numerous distinct mechanisms were proposed to be responsible for the elevated level of NF-κB activity in malignant melanoma. For example, sustained NF-κB activation results in induction of chemokines CXCL1 and CXCL8. CXCL1, in turn, is capable of activating IKK and NF-κB demonstrating the presence of a feed-forward mechanism that could contribute to the constitutive activation of NF-κB in melanoma cells (212). The CXC chemokine MGSA/GROα, which is constitutively expressed in melanoma, is also able to induce NF-κB (RelA) activation in a manner dependent on Ras-MEKK1-MEK3/6-p38 pathway (213). Activation of NF-κB activity in melanoma was also linked to loss of E-cadherin expression seen frequently during malignant transformation of melanocytes (214) and was also associated with increased cytoplasmic β-catenin coupled with p38-dependent NF-κB activation (215). In contrast, UV-induced activation of ASK1-p38 disrupts IκBα phosphorylation and decreases transcriptional activity of NF-κB (216), suggesting that in melanoma multiple factors are cooperating, in concert, in the activation of NF-κB.

Consistent with the notion that multiple signaling can contribute to the activation of NF-κB in melanoma, several studies had pointed to the role of the Ras–Raf–MEK–ERK cascade in activation of NF-κB. NIK, an activator of IKK, is highly expressed in melanoma cells, and IKK-associated NIK activity is enhanced in these cells compared with normal cells. It was shown that expression of kinase-dead NIK blocked constitutive NF-κB promoter activity in melanoma cells, but not in control normal human melanocytes. Importantly, overexpression of wild-type NIK results in increased phosphorylation of ERK1/2, and overexpression of a dominant negative ERK construct causes decreased NF-κB promoter activity. These data suggest that ERK acts upstream of NF-κB and regulates NF-κB DNA binding activity (217). Activation of NF-κB by TNF-α prevented the induction of apoptosis following inhibition of

B-Raf signaling, further supporting a regulatory link between ERK and TRAF2 signaling (218).

Another important variable in the control of NF-κB availability in melanoma, but also other tumors, is the level and activity of the IκB ligase. Inhibition of β-Trcp (aka HOS), the ubiquitin ligase which efficiently targets IκB for ubiquitination-dependent degradation (219), efficiently sensitizes melanoma cells to apoptosis in response to treatment (220). Interestingly, melanocytes expressing the oncogenic form B-Raf^{V600E} exhibit enhanced expression of β-Trcp (221), thereby pointing to the possible use of this ligase as a selective target in melanoma.

The PI3K/Akt pathway was also implicated in activation of NF-κB through different mechanisms. Thus, full transcriptional activation of RelA requires phosphorylation in the transactivation domain, which might be mediated by Akt (222,223). Along these lines, PI3K/Akt pathway-dependent phosphorylation of p50 was shown to increase the binding of NF-κB to DNA (224). Of note, in melanoma cells, inhibitors of PI3K blocked the transcriptional activity of the endogenous NF-κB, without attenuating IKK-mediated phosphorylation of IkBα (86), indicating that PI3K/Akt may affect NF-κB independent of IKK/IκBα (222). Overall, signaling mediated by constitutively active Akt kinase in melanoma could contribute to high transcriptional activity of NF-κB dimers that specifically include RelA, and, thus to enhance the anti-apoptotic properties of melanoma.

It is generally accepted that a major contribution of NF-κB in the development and progression of melanoma relates to its function as a regulator of survival and apoptosis. Meyskens *et al.* showed that in metastatic melanoma cells, increase in DNA-binding activity of NF-κB is paralleled by increased expression of p50 and RelA—antiapoptotic regulators—and its inhibitor IkBα. However, expression of cRel—transcriptional activator of proapoptotic genes—is markedly decreased in melanoma cells compared with normal melanocytes (225). Consistent with these observations, strong p50 nuclear staining correlated with poor prognosis in melanoma patients (226). In addition to eliciting its antiapoptotic activities NF-κB mediates transcription of MMP2 (227,228) and MMP9 (229,230) whose overexpression was associated with tumor invasion, angiogenesis and metastasis.

Taken together, the emerging theme is that independent pathways which are activated in melanoma (Ras/Raf/MEK/ERK, PI3K/Akt, p38, p16INK4a) contribute not only to increased NF-κB activity, thereby assuring its role in proliferation and survival, but also to tumor progression and invasiveness.

THE JAK/STAT PATHWAY

STAT proteins include a family of transcription factors involved in the activation of target genes in response to cytokines and growth factors (231,232). Tyrosine-phosphorylated STATs undergo homodimerization or heterodimerization, followed by translocation to the nucleus where they contribute to gene transcription (233). Four mammalian JAKs (234) and seven STAT members (235) provide different patterns of gene transcription upon specific stimulation. A variety of mechanisms controlling the level and duration of STAT activation contributes to the complexity of the pathway. These include dephosphorylation of the receptor complex or nuclear STAT dimers by PTPases, interaction of activated STATs with inhibitory molecules from the protein inhibitor of activated STAT family, and feedback inhibition of the pathway by suppressor of cytokine signaling (SOCS) proteins through inhibition and/or degradation of JAKs (236,237). Also, different kinases are able to regulate STAT activity (45,238,239).

Two mechanisms have been suggested to mediate STAT's effect on carcinogenesis: their impact on immune surveillance (240) and control of growth factor signaling, apoptosis and

angiogenesis (241); both are likely to also impact melanoma development and progression. Among the different STATs, STAT3 was shown to play an important role in melanoma development. Expression of a STAT3 dominant-negative variant, STAT3β, was found to induce cell death in murine B16 melanoma cells and to cause inhibition of tumor growth and tumor regression associated with increased apoptosis (242). STAT3 mediates IL-6-induced growth inhibition of normal melanocytes and early-stage melanoma cells, and promotes growth of advanced melanomas (243). In addition to regulation of apoptosis and proliferation STAT3 directly interacts with MMP2 promoter in melanoma cells, causing overexpression of the MMP2 protein in metastatic melanomas. Blockage of activated STAT3 in metastatic cells suppresses invasiveness of the tumor cells, inhibits tumor growth and prevents metastasis in nude mice (244). Active STAT3 upregulates the activity of basic fibroblast growth factor and VEGF promoters in melanoma cells, suggesting its possible role in angiogenesis (245).

Interestingly, overexpression of mutated STAT3 which cannot be phosphorylated/activated increases expression of oncogenes N-Ras and c-MET (246), suggesting the possible existence of a feedback inhibition loop between those two pathways. Combined activity of STAT3 and c-Jun in human melanoma cells mediates suppression of Fas transcription, suggesting that STAT3 oncogenic activities could be mediated through its cooperation with c-Jun, resulting in downregulation of Fas expression, which is implicated in melanoma's ability to resist apoptosis (247). Interestingly, inhibition of PI3K/Akt signaling disrupts cooperation between c-Jun and STAT3, which is required for silencing of the FasR promoter, resulting in increased expression of FasR and concomitant sensitization to FasL-mediated apoptosis (239).

In addition, constitutive Src kinase activity, but not EGFR or JAK activity, was shown to contribute to increased activation of STAT3 in human melanoma cells (248). Inhibition of Src or STAT3 activity caused downregulation of the antiapoptotic genes Bcl-XL and Mcl-1, and increased apoptosis (248). Src and JAK1 kinases were implicated in STAT5 phosphorylation in malignant melanoma contributing to its survival (249). Another component important in the regulation of STATs is SOCS3, a member of the SOCS family of endogenous inhibitors of STATs. SOCS3's promoter region was found to be aberrantly methylated in three out of five malignant melanomas, which correlated with low expression of SOCS3 protein (250). In agreement with this observation Fojtova *et al.* (251) reported that progression of melanoma cells from interferon (IFN) sensitivity to IFN insensitivity associates with increase in SOCS3 expression, lower SOCS1–3 activation following IFN treatment and increased STAT1 activation. Taken together, these data suggest that the highly metastatic, resistant to IFN therapy phenotype of malignant melanoma can be attributed at least in part to changes in SOCS expression.

The contribution of JAK/STAT signaling to IFN resistance of melanoma cells is widely recognized, however the mechanism is not well understood. Wong *et al.* found low expression of STAT1 and STAT2 in resistant cells. Transfection of IFN-resistant cells STAT1 partially restored IFN responsiveness (252) and it was possible to abrogate IFN-γ-induced growth inhibition by overexpression of dominant negative STAT1 (253), suggesting that STAT1 plays a role in the antiproliferative response. Interestingly, IFN-α activation of STAT5 in melanoma cells attenuates the activation of STAT1. In a separate study, IFN-α was found to upregulate pSTAT1, whereas it downregulated pSTAT3 and total STAT3 levels in tumor cells (254). Thus the balance between STAT5, STAT3 and STAT1 is expected to impact their heterodimerization and transcriptional programs (255).

As outlined in this section, members of the STAT protein family are directly involved in distinct cellular mechanisms resulting in increased melanoma cell proliferation, its resistance to apoptotic stimuli, enhanced invasiveness and angiogenesis. Although attractive (243), further

clarification of the precise STAT members which elicits these activities is required before consideration for their selective targeting can be considered.

EMERGING PATHWAYS

HIF-1α

An important factor in tumor development and progression is its surrounding microenvironment. Among key microenvironmental factors is oxygen tension. The skin has been shown to be mildly hypoxic (256), which requires cells to activate programs to enable their survival under hypoxia. HIF-1 is among the key regulatory components of the cellular ability to adapt to hypoxia. HIF-1, which is stabilized under hypoxia, serves as a potent transcription factor which activates a multitude of O_2-responsive genes involved in survival, apoptosis, glucose metabolism and angiogenesis (257). This genetic reprogramming was shown to play major roles in tumor development and progression (258).

Nuclear HIF-1α staining has been shown in normal skin (259), suggesting HIF-1α activity in melanocytes. This finding is consistent with the finding that HIF-1α is a MITF transcriptional target in melanocytes (260). Moreover, HIF-1α transcription increases as a consequence of ERK pathway activation (261). A role for hypoxia and HIF-1α expression in melanoma has also been recently suggested. Bedogni *et al.* showed that the hypoxic microenvironment in the skin contributes to melanocyte transformation and tumor growth induced by Akt (262). Along this line, inhibition of HIF-1α decreases Akt transformation capacity under hypoxia and tumor growth *in vivo* (262).

The angiogenic factor VEGF is among HIF-1α targets and is an important mediator of HIF-1α effects in tumor growth and metastatic capacity. Production of VEGF has been shown to be induced in melanoma following hypoxia (263). As shown in other tumor models, expression of VEGF was shown to increase tumor growth, angiogenesis, invasiveness and metastasis of melanoma cells (264,265). Work from our laboratory has identified the role of the ubiquitin ligase Siah2 in the regulation of HIF-1α availability, through its ubiquitination-dependent degradation of prolyl hydroxylase 3 (PHD3; 266). PHD3 modification of HIF-1α is required for its recognition by the ubiquitin ligase pVHL, leading to its degradation under both normoxia and physiologic hypoxic conditions (267). More recent studies using melanoma cell lines have identified the role of Akt in the regulation of Siah2 transcription and HIF-1 stability (N. Singha, G. Hogg, P. Hu, P. Lopez-Bergami, K. Liu, G. Boyi, J.H. Paik, K. Nakayama, S.H. Lecker, R.A. DePinho, D. Bowtell, C. Hauser, R. Bodmer and Z. Ronai, unpublished data). Further, inhibition of Siah2 activity effectively blocks melanoma tumorigenesis and metastasis *via* two distinct pathways—HIF and Ras signaling (J. Qi, K. Nakayama, S. Gaitonde, J. Goydos, S. Krajewski, R. Cardiff, A. Eroshkin, D. Bar-Sagi, D. Bowtell and Z. Ronai, unpublished data). These observations link Akt and HIF signaling and the role of the ubiquitin ligase Siah in control of both HIF and Ras pathways, which are subject to major changes in melanoma.

While the emerging evidence clearly points to the important role of HIF-1a in melanoma development and progression and as an attractive target for development of novel cancer therapeutics (266), additional studies are required to better understand the impact of rewired signaling on the hypoxia response in this tumor type.

The TGF-β pathway

The TGF-β family includes multiple factors that have dual tumor suppressor and oncogenic effects (268). TGF-β binds to membrane receptors that have a cytoplasmic serine/threonine kinase domain. Binding of the ligand causes the assembly of a receptor complex that phosphorylates proteins of the SMAD family that bind DNA and regulates transcription of

several genes resulting in diverse effects (269). TGF-β1 is overexpressed in human melanoma cells and stimulates the neighboring stroma cells through increased production and deposition of extracellular matrix proteins. The activation of stroma leads to a tumor cell survival advantage and increased metastasis (270).

Although melanoma cells are resistant to the tumor-suppressive effects of TGF-β, there are no detectable defects at the receptor/SMAD level as seen in other tumors (271). Instead, inhibition of the TGF-β pathway in melanoma is mediated by expression of related genes such as SKI, filamin, endoglin and follistatin (272). For example, SKI inhibits TGF-β signaling through its association with the Smad proteins. However, recently identified novel functions imply that SKI can act as oncogen (273).

The Notch pathway

Notch is an evolutionarily conserved signaling mechanism that participates in a variety of cellular processes—cell fate specification, differentiation, proliferation, apoptosis, adhesion, EMT, migration and angiogenesis. Both Notch and its ligands, Delta and Jagged, are transmembrane proteins. The ligand is expressed on an adjacent cell and activates Notch signaling through a direct cell–cell interaction. Upon ligand binding, Notch intracellular domain is cleaved and translocated to the nucleus, with subsequent activation of target gene transcription (274).

Involvement of Notch signaling in several cancers is well known (275). Its participation in melanoma was first described by Hoek *et al.* following comparison of gene expression patterns in normal human melanocytes and melanoma cell lines using microarrays (276). Later, expression of Notch-1 and Notch-2, as well as Notch ligands, was found to be upregulated in “dysplastic nevi” and melanomas compared with common melanocytic nevi (277). Notch inhibitor studies implicate Notch signaling as a requirement for melanoma survival. Inhibition of γ-secretase, an enzymatic complex involved in cleavage, and release of Notch induced apoptosis in melanoma cell lines but not in control melanocytes. Apoptosis was mediated by upregulation of BH3-only members Bim and Noxa and was independent of p53 (278). The oncogenic effect of Notch-1 were described to be also mediated by β-catenin, which was upregulated following Notch-1 activation (279) and by activation of the MAPK and Akt pathways (280). However, a more complex signaling is foreseen as Notch signaling was shown to cross-talk with other signaling molecules including Sonic hedgehog (Shh) and p63 (281).

CONCLUDING REMARKS

In this review, we have summarized some of the major changes in signal transduction pathways that contribute to melanoma development and progression. As we better understand the relative contribution of each signaling pathway, we also appreciate the cross-talk among the pathways which is responsible for the modified blueprint of signal transduction cascades in this tumor type. Our better understanding of key signaling pathways that underwent modification in melanoma has spurred the development of drugs that selectively target these pathways. Along these lines several inhibitors of the B-Raf/MEK/ERK pathway have been produced, including the MEK1/2 inhibitors AZD6244, PD0325901 or CI-1040 (282,283) and the B-Raf inhibitor 43-9006 (Sorafenib) (284). The latter is actually a multikinase inhibitor as it also inhibits receptor tyrosine kinases. Currently, only preliminary results of small to medium-sized Phase II clinical trials are available for metastatic melanoma, and initial promising results have been reported for the combination of sorafenib and chemotherapy (285,286). It is expected that ongoing trials in combination with inhibitors to other pathways (*i.e.* PI3K/AKT; 83) will have a significant impact in the treatment of melanoma. Overall, exciting development in understanding melanoma biology over the past few years combined with growing efforts to further decipher the complexity of the genetic and epigenetic changes is expected to result in

better therapeutic modalities that would help treat malignant melanoma, while providing a paradigm for other tumor types in which rewired signaling is so predominant.

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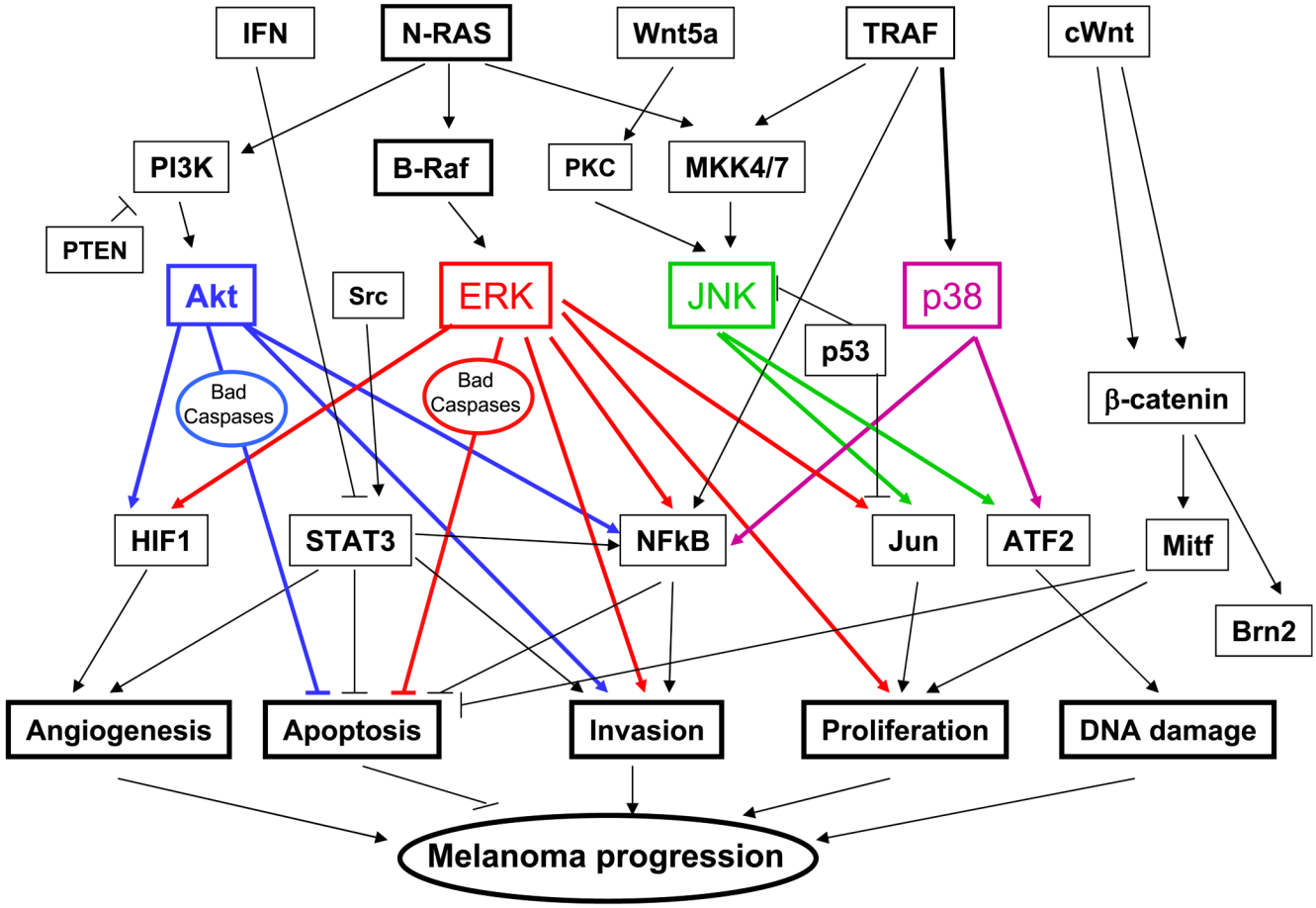

Figure 1.
Outline depicting the major signaling pathways that are deregulated in melanoma.