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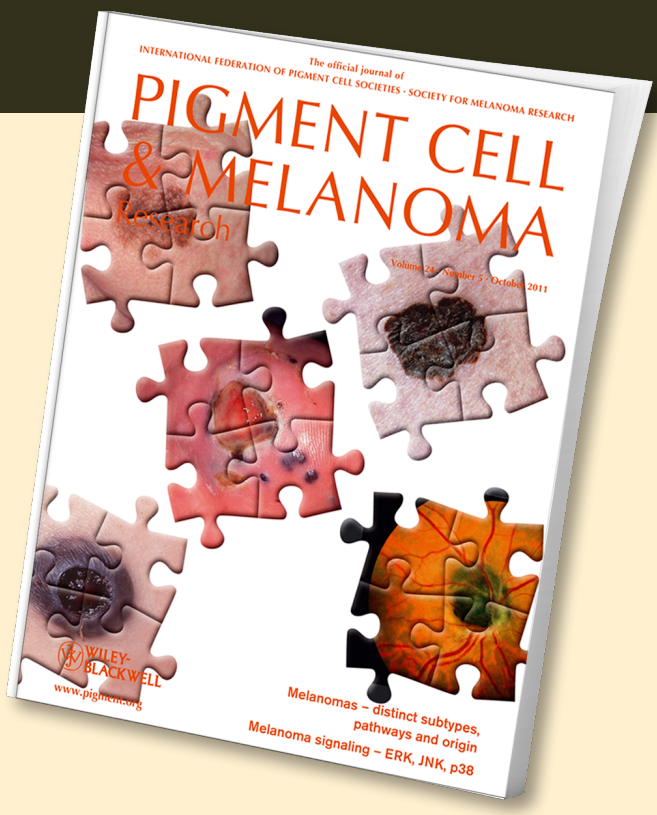

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Summary

Recent discoveries have increased our comprehension of the molecular signaling events critical for melanoma development and progression. Many oncogenes driving melanoma have been identified, and most of them exert their oncogenic effects through the activation of the RAF/MEK/ERK mitogen-activated protein kinase (MAPK) pathway. The c-Jun *N*-terminal kinase (JNK) and p38 MAPK pathways are also important in melanoma, but their precise role is not clear yet. This review summarizes our current knowledge on the role of the three main MAPK pathways, extracellular regulated kinase (ERK), JNK, and p38, and their impact on melanoma biology. Although the results obtained with BRAF inhibitors in melanoma patients are impressive, several mechanisms of acquired resistance have emerged. To overcome this obstacle constitutes the new challenge in melanoma therapy. Given the major role that MAPKs play in melanoma, understanding their functions and the interconnection among them and with other signaling pathways represents a step forward toward this goal.

Introduction

The discovery of activating mutations in BRAF in the majority of melanomas has stimulated the development of therapeutic strategies aimed at blocking its effect on disease progression. Mechanistically, mutant BRAF exerts its oncogenic effects through the activation of the RAF/MEK/ERK mitogen-activated protein kinase (MAPK) pathway. The MAPK family is a group of protein kinases that regulate cellular programs related to proliferation, differentiation, and survival in response to changes in the cell environment. The MAPK family is composed of three major groups: the extracellular regulated kinases (ERKs), the c-Jun *N*-terminal kinases (JNKs), and the p38 MAPKs. The ERK pathway primarily directs proliferation and survival programs, whereas the JNK pathway promotes either proliferation or apoptosis (Kennedy and Davis, 2003). Conversely, the p38 pathway is activated upon cellular stress and often engages pathways that block proliferation or promote apoptosis (Bulavin and Fornace, 2004). The importance of MAPK pathways is highlighted by the observation that their constitutive activation is a frequent event in multiple human cancers. Extracellular regulated kinase and JNK pathways support tumor growth although loss of JNK in some instances may also promote tumorigenesis (Kennedy and Davis, 2003). In contrast, the p38 MAPK pathway is generally implicated in suppression of tumorigenesis.

Each MAPK signaling axis comprises three components: a MAPK Kinase Kinase (MAP3K), a MAPK Kinase (MAP2K), and the MAPK itself (Figure 1). In humans, there are at least 19 MAP3K genes that phosphorylate and activate the MAP2Ks, which in turn phosphorylate and activate the MAPKs. The limited number of MAP2K proteins and their remarkable substrate selectivity contrasts with the more promiscuous nature of the MAP3Ks. However, each of the MAPKs has its preferred MAP2Ks and MAP3Ks. This, together with the action of scaffold proteins that insulate the MAPK pathways from one another, contributes to define the three distinctive MAPK pathways (Morrison and Davis,

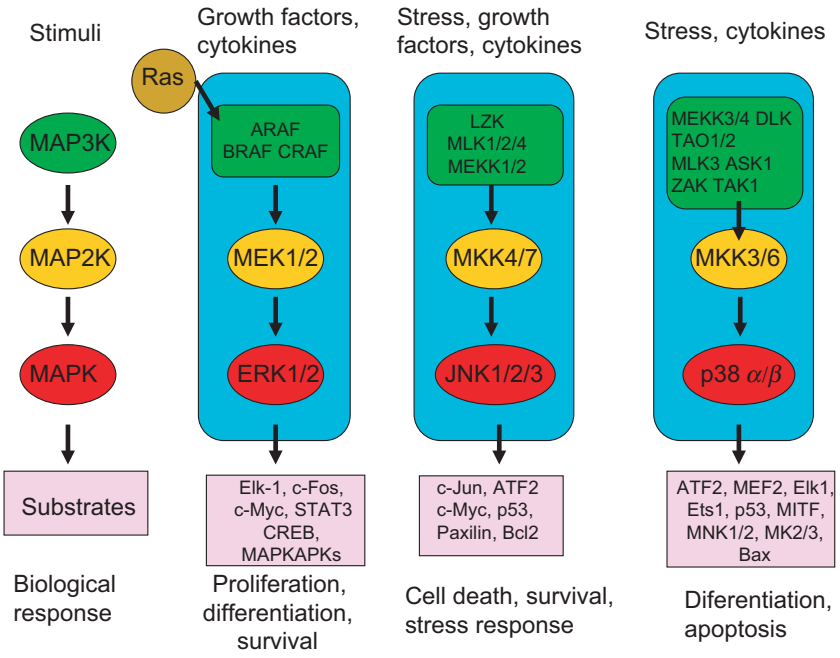

Figure 1. Architecture of the mitogen-activated protein kinase (MAPK) pathways. The three major MAPK pathways, extracellular regulated kinase, c-Jun *N*-terminal kinase, and p38, are shown. Extracellular stimuli activate the MAP3K through cell surface receptors and intracellular mediators (not depicted). The signal is transduced to MAP2K and MAPK. Activated MAPKs phosphorylate various substrate proteins including transcription factors, resulting in regulation of a variety of cellular activities including cell proliferation, differentiation, migration, inflammatory responses, and death. Interactions between components of each module are mediated by scaffold proteins (in blue).

2003). This arrangement is thought to confer specificity and appropriate kinetic properties to the activation of MAPKs by various stimuli. Activated MAPKs phosphorylate several substrate proteins including transcription factors such as Elk-1, c-Jun, activating transcription factor 2 (ATF2), microphthalmia-associated transcription factor (MITF), and p53 that direct specific gene expression programs in response to the stimuli.

As will be discussed later, MAPK pathways are implicated in diverse biological processes that vary greatly depending on the cellular context and the characteristics of the stimuli. However, even within the same cell type, an individual MAPK cascade can exhibit different responses leading to distinct cell fates (Marshall, 1995; O'Shaughnessy et al., 2011). How can the same pathway determine, for instance, mitogenesis versus differentiation? Several features of MAPK pathways (i.e., the three-tier architecture, the requirement of dual phosphorylation, etc) provide them with systems-level properties that allow a great plasticity in the response. For instance, upon a graded stimuli such as a growth factor, the MAPK pathways can show either a graded or a switch-like response (Ferrell, 1996; Huang and Ferrell, 1996; Mackeigan et al., 2005; Marshall, 1995). Whereas a graded, linear output allows to adjust the response to subtle changes in the environment, a switch-like or ultrasensitive response can filter-out noise (sub-optimal stimuli) and trigger all-or-none signals when the stimuli increase above a certain threshold. Ultrasensitive MAPK activation requires positive cooperativity at all or most steps of the phosphorylation cascade and can serve to achieve stable, potentially irreversible cellular decisions. The following sections discuss the mechanisms of activation of each of the three MAPK pathways and their role in melanoma.

The ERK pathway

The RAF/MEK/ERK pathway was the first MAPK cascade to be characterized. It couples growth factors, mitogenic and extracellular matrix signals to cell fate decisions such as growth, proliferation, migration, differentiation, and survival. RAF/MEK/ERK displays the characteristic three-tier MAPK architecture. The downstream MAPK tier – ERK1 and ERK2 (encoded by *MAPK3* and *MAPK1*, respectively) – is activated by phosphorylation on threonine and tyrosine residues within the conserved TEY motif in the activation loop. This phosphorylation is catalized by the dual specificity kinases MEK1 and MEK2 (the MAPKK tier), which are in turn activated by the upstream MAP3K tier that includes the serine/threonine kinases from the RAF family (ARAF, BRAF, and CRAF; Krishna and Narang, 2008). The activation of this cascade is usually triggered by cell surface receptors such as receptor tyrosine kinases (RTKs) and G-protein-coupled receptors (GPCRs) (Gutkind, 2000; McKay and Morrison, 2007). The signal is subsequently transduced to the MAPK module by small GTPases of the Ras family (N-RAS, K-RAS, and H-RAS). Upon receptor activation, Ras assumes an activated, GTP-bound state and recruits the RAF proteins to the plasma membrane where, through a series of phosphorylation and dephosphorylation events, they become activated. Activation of Ras also results in the activation of the PI3K/Akt pathway (Vojtek and Der, 1998).

ERK activation

Extracellular regulated kinase 1 and ERK2 are 44- and 42-kDa ubiquitously expressed proteins with nearly 85% amino acid identity. Extracellular regulated kinase 1 and ERK2 are the effector proteins of the MAPK cascade and transduce the upstream signal by phosphorylating a multitude of protein substrates. Because both proteins share similar substrate specificities, they are often referred to together as ERK1/2 or just ERK. In resting conditions, ERK is anchored to the cytoplasm by its association with MEKs, the microtubule network, or with phosphatases. Upon stimulation (i.e., by mitogens), ERK is strongly phosphorylated and activated within 10 min and translocates into the nucleus (Zehorai et al., 2010). Extracellular regulated kinase activity can persist until late G1 (up to 6 h, although much less vigorously) to drive entry into S-phase of the cell cycle. Extracellular regulated kinase is inactivated at the G1/S transition or upon disappearance of the stimulus by the serine/threonine phosphatase PP2A, the tyrosine phosphatase PTP-SL, or by dual specificity phosphatases of the MAP kinase phosphatase (MKP) subfamily, among others (Boutros et al., 2008). The phosphatases from the later group that are specific for ERK are MKP-3, MKP-X, and MKP-4 (Junttila et al., 2008).

It has been demonstrated that ERK can phosphorylate over 160 proteins (Roberts and Der, 2007; Yoon and Seger, 2006), including transcription factors, cytoskeletal proteins, enzymes that regulate cellular metabolism and protein kinases important in signal transduction. Among the formers, ERK phosphorylates NFAT, Elk-1, MEF-2, c-Fos, c-Jun, c-Myc, STAT3, and CREB that transactivate genes implicated in cell proliferation, differentiation, and survival (Gray-Schopfer et al., 2005; Figure 1). The MAPKs, but particularly ERK, phosphorylate and activate members of a family of Ser/Thr kinases termed mitogen-activated protein kinase-activated protein kinases (MAPKAPKs) that include the p90 ribosomal S6 kinase (RSK), the mitogen- and stress-activated kinase (MSK), the MAPK-interacting kinase (MNK), and the MAPK-activated protein kinase 2/3 (MK2/3). The MAPKAPKs in turn phosphorylate various transcription factors, amplifying the effect of ERK in cell proliferation and survival (Cargnello and Roux, 2011).

Dysregulation of the ERK pathway has been long connected to cancer development given its role as a major regulator of cell proliferation and its strategic position downstream of several oncogenes. In fact, activation of RAF/MEK/ERK pathway plays a key role in the pathogenesis of approximately one-third of all human cancers (Malumbres and Barbacid, 2003). Extracellular regulated kinase has been also linked to many other processes that contribute to oncogenesis such as cell migration and invasion, survival and angiogenesis. Because most of these processes are regulated by ERK in melanoma, they will be discussed in more detail later, in the context of this disease.

Genetic basis of ERK pathway activation in melanoma

The ERK pathway plays an important role in melanoma, with ERK being constitutively activated in up to 90% of melanomas (Oba et al., 2011; Zhuang et al., 2005). The level of activated ERK in melanocytic lesions has been shown to increase from early- to advanced-stage disease (Satyamoorthy et al., 2003), and increased levels of phosphorylated ERK (p-ERK) were observed at the deeper margins of primary melanoma where the tumor was invading into the dermis (Zhuang et al., 2005). Along this line, it seems that ERK localization is critical because shorter overall survival was associated with the absence of cytoplasmic p-ERK (Jovanovic et al., 2008).

The underlying mechanism for ERK activation was attributed initially to mutations in N-Ras, which are observed in 15–30% (van Elsas et al., 1995) of melanomas, or to autocrine loops triggered by growth factors. The major culprit was identified in 2002 when BRAF was found to be mutated in up to 82% of cutaneous melanocyte nevi, 66% of primary melanomas and 40–68% of metastatic melanomas (Davies et al., 2002; Gorden et al., 2003; Kumar et al., 2003). More than 90% of the mutant BRAF alleles described to date consist of the missense exchange from valine to glutamic acid in residue 600 (V600E BRAF). The mutation engenders a constitutive activation of the BRAF kinase activity, likely by mimicking the phosphorylation at T598 and S601 in the activation loop of BRAF (Davies et al., 2002). In vitro studies demonstrated that transfection of V600E BRAF resulted in a several fold induction of both MEK-ERK activity and cellular transforming activity. It should be noted that the cellular effects of BRAF mutations are attributed exclusively to ERK function because MEK1 and MEK2 are the only known substrates of BRAF. However, given the numerous regulatory feedback loops in this pathway (McCormick, 2010), in some instances, there is not a complete correspondence between BRAF mutations and p-ERK levels (Smalley et al., 2007; Yazdi et al., 2010). Interestingly, BRAF and N-Ras mutations are mutually exclusive (Davies et al., 2002; Omholt et al., 2003), explaining why almost all late-stage melanoma cell lines and tumor samples display ERK activation. BRAF mutation is often accompanied by loss of the tumor suppressor gene Phosphatase and Tensin homolog deleted on chromosome Ten (PTEN), a negative regulator of the PI3K/Akt pathway, that controls cell survival and apoptosis (Meier et al., 2005). Therefore, simultaneous activation of both ERK and PI3K/Akt pathways is commonly seen in melanoma.

ERK pathway in benign melanocytic lesions

The discovery of BRAF mutation and ERK activation not only paved the way to improve therapy (see below) but also allowed a better understanding of melanoma development. Extracellular regulated kinase activity is

not normally observed in melanocytes (Omholt et al., 2003), and its activation at this stage requires primary growth factors such as stem-cell factor (SCF), hepatocyte growth factor (HGF), and fibroblast growth factor (FGF; Gray-Schopfer et al., 2007). BRAF mutations are found in 52–82% of nevi and benign melanocytic skin lesions and are functionally preserved throughout melanoma progression, rarely arising at later stages (Michaloglou et al., 2005). Along these lines, the expression of V600E BRAF in mouse melanocytes induced widespread benign melanocytic hyperplasia with histological features of nevi (Goel et al., 2009). These and other evidences strongly suggest that ERK activation is a critical step in nevus formation and melanoma initiation (Dhomen et al., 2009; Hoeflich et al., 2006; Omholt et al., 2003; Pollock et al., 2003). Both human and mouse benign melanocytic lesions present biochemical markers of oncogene-induced senescence, a potent mechanism of tumor suppression that involves cell cycle arrest (Mooi and Peeper, 2006). It was shown that ERK pathway-induced senescence correlates with induction of classical senescence-associated genes, such as *p53* (via dephosphorylation of Mdm2), *p16* (*INK4A*), *p21*, and the p53 activator *p19/ARF* (Bansal and Nikiforov, 2010; Cagnol and Chambard, 2010; Dankort et al., 2007). Melanocyte senescence is also associated with downregulation of CDK2 and CDK4 kinase activities, the dephosphorylation of the retinoblastoma protein RB, and subsequent repression of genes under the control of E2F1 (Giuliano et al., 2011). Thus, upon BRAF mutation in melanocytes, ERK induces a biphasic cellular response initiated by a proliferative burst leading to nevi formation followed by senescence (Dankort et al., 2007; Michaloglou et al., 2008). Interestingly, different senescent phenotypes have been associated with different mutations in the MAPK pathway. It was demonstrated that senescence induced by oncogenic forms of H-RAS (G12V H-RAS) but not V600E BRAF was mediated by the ER-associated unfolded protein response (UPR; Denoyelle et al., 2006). Because melanomas commonly arise from nevi, melanocytes need additional genetic changes (i.e., disruption of *p53* or inactivation of *p16/INK4A*) to bypass the senescent response and develop melanoma (Delmas et al., 2007; Yu et al., 2009).

Mechanisms of ERK-mediated melanomagenesis

Most aspects of melanoma cell biology are regulated by mutant BRAF and the ensuing constitutive activity of MEK and ERK. Activated ERK plays a pivotal role in cell proliferation via a number of mechanisms (Lopez-Bergami et al., 2008; Sharma et al., 2005; Figure 2). For instance, it induces cyclin D1 expression, which is necessary for cell cycle progression (Bhatt et al., 2005; Smalley et al., 2008). In addition, BRAF controls the G1/S-phase transition by negative regulation of the tumor suppressor p27Kip1 (Bhatt et al., 2005; Kortylewski et al., 2001) and upregulation of c-Myc activity (Lefevre et al., 2003). Additional inhibition of p27Kip1 function is provided by MITF-dependent S-phase kinase-associated protein (SKP2) repression (Carreira et al., 2005) and BRAF- and cyclin D1-dependent SKP2 proteolysis (Bhatt et al., 2007). BRAF also finely tunes MITF expression and degradation to stimulate cell survival and proliferation and avoid MITF-induced differentiation and apoptosis (Kim et al., 2003; Levy et al., 2006; Wellbrock et al., 2008; Wu et al., 2000). BRAF and ERK negatively regulate at different levels the LKB1/AMPK/TSC1/2 pathway that drives cell proliferation and growth mainly by activation of mTORC1 signaling (Lopez-Bergami, 2009; Zheng et al., 2009). Another mechanism by which constitutive active ERK stimulates cell proliferation is through 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 (Lopez-Bergami et al., 2007). Extracellular regulated kinase increases expression of Brn-2 (Goodall et al., 2004), a protein that has been associated to enhanced invasive and metastatic capacity (Cook and Sturm, 2008).

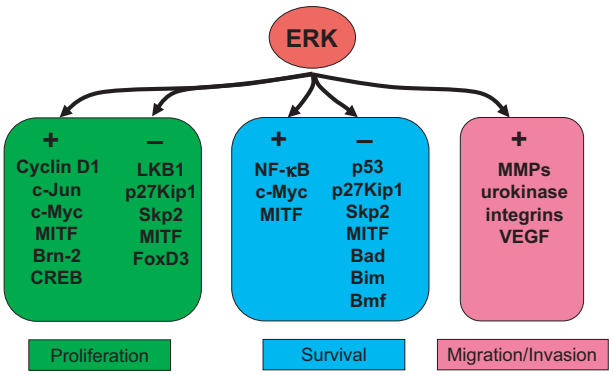

Figure 2. Mechanisms of extracellular regulated kinase (ERK)-mediated melanomagenesis. Constitutive activation of ERK contributes to proliferation, survival, and migration/invasion of melanoma cells by regulating positively (+) or negatively (–) the indicated proteins. See text for details.

Activation of the ERK pathway was shown to inhibit apoptosis by phosphorylating the proapoptotic Bcl-2 family members Bad and Bim (Eisenmann et al., 2003; Sheridan et al., 2008; Figure 2). Whereas Bad phosphorylation triggers its cytosolic sequestration by 14-3-3 proteins, Bim phosphorylation induces its proteasomal-dependent degradation. Phosphorylation of Bad disrupts its interaction with the antiapoptotic Bcl-2 proteins at the outer mitochondrial membrane allowing these proteins to promote survival. Bim degradation prevents its association with Bax and mitochondrial pore formation. However, siRNA-mediated silencing of Bim and Bad suggested that these proteins only partly contribute to the anti-apoptotic activities of BRAF (Sheridan et al.,

2008). In agreement with this observation, VanBrocklin et al. (2009) showed that induction of apoptosis by MAPK inhibition required mitochondrial translocation of Bmf. Mutant BRAF also contributes to melanoma survival and apoptosis resistance by activating the anti-apoptotic transcription factor NF-κB (Liu et al., 2007) and inhibiting p53-induced apoptosis (Kohno and Pouyssegur, 2003) and the JAK-STAT pathway (Krasilnikov et al., 2003).

Extracellular regulated kinase contributes to tumor invasion and metastasis by regulating the expression of proteins involved in matrix remodeling such as matrix metalloproteinases (MMPs), urokinase, and integrins, contributing to the acquisition of an invasive phenotype (Aguirre Ghiso et al., 1999; Estrada et al., 2009; Gener-sch et al., 2000; Santibanez et al., 2000; Tower et al., 2002; Woods et al., 2001; Figure 2). V600E BRAF also plays an important role in angiogenesis by promoting vascular development by stimulating autocrine vascular endothelial growth factor (VEGF) secretion (Liu et al., 2006). Mechanistically, these effects are mediated both by phosphorylation of proteins that alter their activity, stability, and localization and by an increase in gene transcription. In the last years, the gene expression signature associated with BRAF mutations in human melanomas has been delineated (Johansson et al., 2007; Kannengiesser et al., 2008; Packer et al., 2009).

ERK is the major tumorigenic pathway in melanoma

Besides the high incidence of BRAF mutation, many other oncogenes are implicated in melanoma development and progression. Interestingly, most of them such as N-Ras, c-Kit, GNA11, GNAQ, and ERBB4 activate the RAF/MEK/ERK pathway (Figure 3). Mutations in these genes (including BRAF) are very rarely seen together in the same tumor. Moreover, their occurrence strongly varies by site of origin and by the absence or presence of chronic solar damage. Thus, regardless of the underlying genetic alteration, the ERK pathway is almost invariably implicated in melanoma, reflecting the marked predilection of this cell type for this pathway as a vehicle to develop a tumor.

N-Ras was the first oncogene identified in melanoma back in the 1980s and affects around 10–30% of all malignant melanomas. N-Ras mutations are found in all melanoma subtypes, but may be slightly more common in melanomas derived from chronic sun-damaged skin. K-Ras and H-Ras mutations are relatively rare (van Elsas et al., 1995). In most cases, the mutation is a substitution of leucine for glutamine at residue 61, which impairs GTP hydrolysis and maintains the GTP-bound form in a state of constitutive activation that results in a sustained proliferative signal. In 2006, Bastian and colleagues found c-Kit mutations and/or copy number increases in 39% of mucosal, 36% of acral, and 28% of

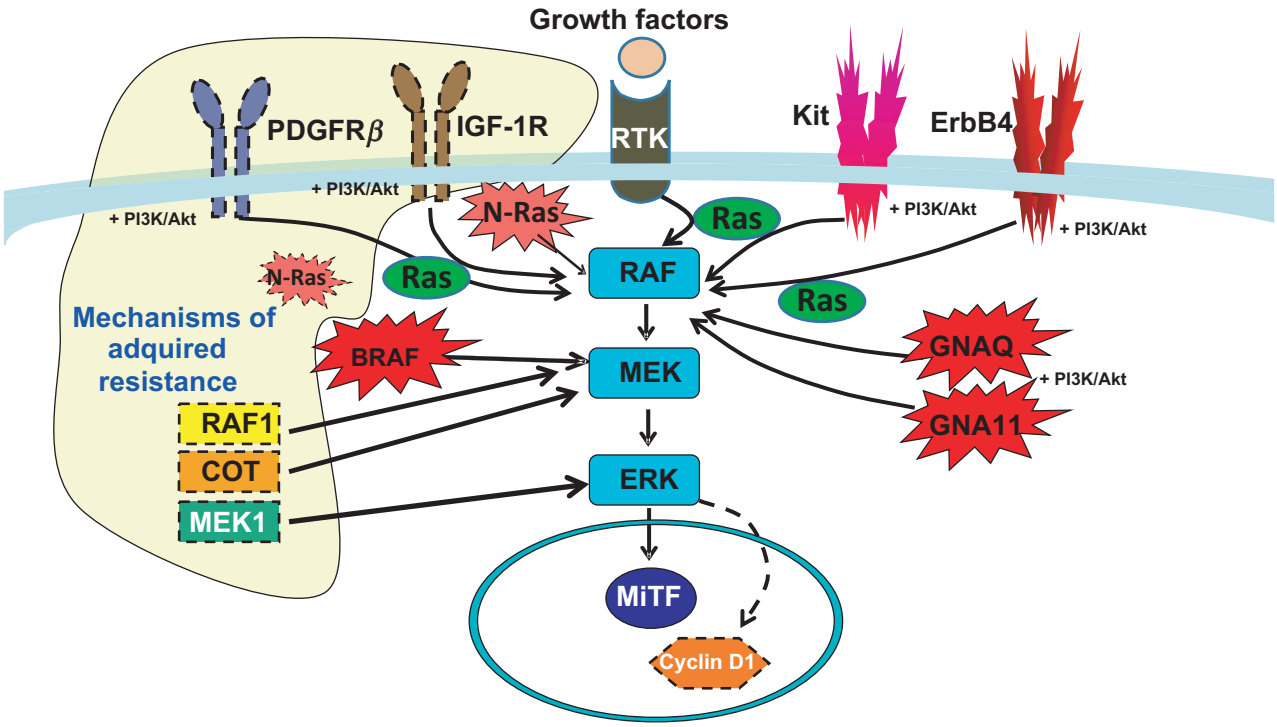

Figure 3. An 'ERK-centric' view of melanoma. Most melanoma oncogenes activate the RAF/MEK/ERK pathway. Oncogenes are depicted as red-pointed stars (mutations). Mechanisms of acquired resistance to BRAF inhibitors are shown on the left. They are depicted as red-pointed stars (N-Ras) or with broken lines to represent mutations or upregulation, respectively, of the genes involved. Concomitant activation of the PI3K/Akt pathway is indicated. See text for details.

melanomas on chronically sun-damaged skin, but not in melanomas on skin without chronic sun damage (Curtin et al., 2006). c-Kit is a RTK specific for SCF, and the somatic point mutations tend to occur in the juxtamembrane or the kinase domain and induce ligand-independent receptor activation (Growney et al., 2005). Receptor activation positively regulates downstream signal transduction pathways including the ERK and the PI3K/Akt pathways. More recently, frequent mutations in two G-protein α subunits involved in GPCR signaling were found in uveal melanoma. Mutations in GNAQ and GNA11 are mutually exclusive and together affect 82% of all primary uveal melanomas, and 90% of all uveal melanoma metastases (Van Raamsdonk et al., 2009). Most mutations occur at codon 209 within the catalytic (GTPase) domain. Similar to N-Ras, mutation at this site inactivates the GTPase domain, resulting in a constitutively active Gα subunit and increased signaling through the ERK pathway (Van Raamsdonk et al., 2010). High-throughput gene sequencing of RTK in melanoma revealed mutations in ErbB4 in 19% of a cohort of 79 uncultured melanoma tumors (Prickett et al., 2009). ErbB4 is a growth factor receptor that has been shown to contribute to the proliferation of malignant melanoma cells (Djerf et al., 2009). Unlike BRAF, N-RAS, and GNAQ, a mutation hotspot was not found. Six of the mutations identified were associated with hyperphosphorylation of the receptor and transformation of transfected fibroblasts. Treatment of mutant cells with the RTK inhibitor gefitinib inhibited growth of melanoma cell lines and reduced the phosphorylation of ERK (Djerf et al., 2009). Oncogene activation is not the only strategy used by melanoma cells to ensure high activity of the ERK pathway. Melanomas frequently fail to express proteins that negatively regulate ERK phosphorylation such as Raf-1 kinase inhibitory protein (Schuierer et al., 2004). Besides activating the ERK pathway, all the above-mentioned mutations also activate the PI3K/Akt pathway, implicated in cell growth and survival.

Interestingly, two other melanoma oncogenes, MITF, the master regulator of melanocyte development, and Cyclin D1, are ERK effectors (Oba et al., 2011; Wellbrock et al., 2008), suggesting that some of the mechanisms of tumorigenesis mediated by ERK need to be activated even when mutations in this pathway are not present. Microphthalmia-associated transcription factor was found to be amplified and mutated in melanoma (Cronin et al., 2009; Garraway et al., 2005), whereas genomic amplifications of Cyclin D1, a protein implicated in the G1/S transition, are primarily found in 44% of acral lentiginous melanoma (Sauter et al., 2002), a melanoma subtype with low rate of BRAF mutations (Saldanha et al., 2006).

The ERK pathway is a primary therapeutic target in melanoma

The hyperactivity of the RAF/MEK/ERK signaling cascade has been shown to contribute to melanoma tumorigenesis by increasing cell proliferation and survival, tumor invasion and metastasis, and by inhibiting apoptosis (Heath et al., 2011; Smalley, 2003). The importance of this pathway for the maintenance of melanoma phenotypes has been demonstrated by specific targeting of BRAF and MEK using kinase inhibitors such as CI1040, U0126, and BAY43-9006 (Collisson et al., 2003; Karasarides et al., 2004) or BRAF siRNA in in vitro experiments and xenotransplantation models (Sumimoto et al., 2004). The critical role of BRAF mutation in melanoma was definitively established when newly developed compounds against BRAF demonstrated clinical benefits in melanoma patients. One of these compounds, PLX4032, is a potent and exquisitely selective BRAF inhibitor with more than 10-fold greater selectivity for V600E BRAF compared with the wild-type protein and virtual no activity against other 200 kinases (Bollag et al., 2010). After satisfactory in vitro and in vivo testing, clinical trials began in 2007 and showed a dramatic and unprecedented success. A phase I dose escalation study with PLX4032 revealed promising activity in patients with V600E BRAF, but not in wild-type BRAF-expressing melanoma. Eleven of 16 patients bearing V600E BRAF showed positive responses leading to a progression-free survival of 8–9 months (Flaherty et al., 2010). This was followed by a phase 3 randomized clinical trial comparing vemurafenib (PLX-4032) with dacarbazine (the standard treatment for advanced melanoma) in 675 patients with previously untreated, metastatic melanoma with the V600E BRAF mutation. The response rates were 48% for vemurafenib and 5% for dacarbazine with overall survival of 84% and 64%, respectively (Chapman et al., 2011). These results prompted the recent approval of this drug by the FDA. However, some problems had emerged, including the appearance of drug resistance (see below) and the termination of the clinical benefit within 1 yr of treatment. The reader is referred to two excellent reviews describing current and future directions in melanoma treatment (Friedlander and Hodi, 2010; Sondak and Flaherty, 2011).

Mechanisms of acquired resistance to BRAF inhibitors

The addiction of melanoma cells to the ERK pathway is further illustrated by the fact that the mechanisms of resistance developed by melanoma cells upon treatment with BRAF inhibitors involve upregulation of proteins that will re-activate the ERK pathway. Indeed, recovery of ERK phosphorylation is a good indicator of resistance to BRAF inhibitors (Paraiso et al., 2010). The mechanisms of acquired resistance described so far are (i) the upregulation of the protooncogen COT, a MAP3K that activates ERK through RAF-independent and MEK-dependent mechanisms (Johannessen et al., 2010); (ii) CRAF upregulation (Johannessen et al., 2010); (iii) MEK1 mutations that confer resistance to MEK and

BRAF inhibition (Emery et al., 2009; Wagle et al., 2011); (iv) platelet-derived growth factor receptor β (PDGFRβ)-mediated activation of alternative survival pathways; (v) N-Ras mutations (Nazarian et al., 2010); (vi) insulin-like growth factor 1 receptor (IGF-1R) activation (Villanueva et al., 2010); (vii) BRAF amplifications (Corcoran et al., 2010; Figure 3).

Very interestingly, the patients that acquire resistance to treatment did not display gatekeeper mutations in BRAF (Nazarian et al., 2010). Gatekeeper mutations consist of secondary mutations in the target kinase that prevents the drug from binding its target. This is in marked contrast with gatekeeper mutations being the most common cause of resistance to ABL, EGFR, and KIT inhibitors used in other tumor types. Instead, melanoma cells develop mechanisms that by-pass BRAF but that retain dependence on RAF activation (i.e., N-RAS mutation, CRAF overexpression, and BRAF amplification). In contrast, COT activation causes RAF-independent and MEK-dependent ERK kinase signaling. Based on our current knowledge of the ERK pathway, it is possible to speculate that combined therapy using PLX4032 and a MEK inhibitor could be used successfully in patients with these genetic profiles to either prevent or delay resistance. This strategy would not be efficacious in patients developing MEK1 mutations. A third type of resistance mechanism found in melanoma patients depends on the activation of pathways other than MAPK. Indeed, activation of receptors such as PDGFRβ and IGF1R results in the activation of both ERK and PI3K/Akt pathway. Because the two pathways regulate both overlapping and distinct mechanism of cell proliferation and survival, both PI3K/Akt and ERK cascades must be inhibited to efficiently inhibit growth (Engelman, 2009; Smalley et al., 2006). It is important to note that in almost all patients to date, the underlying genetic lesion responsible for the resistance mechanisms has not been identified. Because patients respond for several months to the BRAF inhibitor, resistance likely arises from a yet unidentified mutation(s) in a single (or a few) tumor cell(s), that will make the whole tumor resistant after growing for several months. If this is the case, we cannot rule out the possibility that several pathways become upregulated simultaneously in the same tumor as a consequence of such mutation. If this is true, inhibiting one of the receptors found to be upregulated (i.e., PDGFRβ) might not be helpful as found by Nazarian et al. (2010). This discussion exposes how important it is to understand the signaling pathways involved in melanoma to develop therapeutical strategies based on the rational combination of drugs.

Complexity of the pathway – rewiring and crosstalk

Whereas the RAF/MEK/ERK pathway is typically represented as a simple linear cascade, it is important to note that its regulation is exceedingly complex. Crosstalk between the ERK pathway and other signaling cascades adds a great deal of complexity. Recent investigations have shown that in melanoma, the ERK pathway exerts its effects by also engaging some pathways not usually related to MAPK. A recent article by Marquette et al. (2011) describes the crosstalk between ERK and the cAMP pathway. They found that, in cells expressing mutant N-RAS, ERK mediates BRAF inactivation and the concomitant activation of CRAF by overexpression of the phosphodiesterase PDE4B2. This mechanism explains which RAF isoform is used to activate the ERK pathway in wild-type versus mutant N-Ras cells (Marquette et al., 2011). Another apparently melanoma-specific feature is the downregulation of the cGMP-specific phosphodiesterase PDE5A by mutant BRAF that increases melanoma cell invasion as a consequence of increased cytosolic Ca^{2+} (Arozarena et al., 2011). The LKB1-AMPK signaling pathway couples energy metabolism to cell growth, proliferation, and survival and needs to be inhibited for the tumor cell to grow. In melanoma cells with the BRAF V600E mutation, LKB1 is phosphorylated by ERK and Rsk affecting the ability of LKB1 to bind and activate AMPK. These and previous findings demonstrate that the tight control that RAF/MEK/ERK exert over the LKB1-AMPK pathway is an important mechanism of V600E BRAF-driven tumorigenesis (Lopez-Bergami, 2009; Zheng et al., 2009). The NF-κB pathway was also found to be regulated by ERK in melanoma, contributing to its activation (Dhawan and Richmond, 2002; Uffort et al., 2009). Extracellular regulated kinase was also shown to crosstalk with the PI3K/Akt through its ability to phosphorylate and stimulate FOXO degradation.

An important aspect of MAPK activity is their regulation by phosphatases. All three layers of the MAPK cascade can be regulated by different families of protein phosphatases controlling both signal amplitude and duration (Junttila et al., 2008). The MKP is a subgroup of ten specialized phosphatases that specifically dephosphorylate the MAPKs with different substrate preference. The expression of several MKPs is induced by the same stimuli that activate MAPKs. Moreover, MAPKs are directly implicated in increasing MKPs protein stabilization. This is the case for example of MKP-1 that is phosphorylated by its substrate ERK at Ser359 and Ser364, thereby increasing its half-life (Brondello et al., 1999). These negative feedback mechanisms – that contribute to turn-off MAPK signaling upon external stimuli – are sometimes altered in cancer, allowing constitutive MAPK signaling (Keyse, 2008). It has been shown that numerous crosstalks between the different MAPK cascades are dependent on increases in activity and expression of protein phosphatases. For instance, activation of JNK and p38 signaling has been shown to inhibit ERK pathway via the activation of phosphatases targeting ERK (Junttila et al., 2008). These crosstalks are complex, and different connections between the

phosphatases and MAPK components were established in different cellular systems. Unfortunately, little is known about the expression and activity of MAPK phosphatases in melanoma and how they regulate MAPK signaling.

The JNK pathway

The JNK MAPK pathway is involved in various cellular events, including cellular stress, apoptosis, survival, transformation, embryonic morphogenesis, and differentiation (Davis, 2000). The JNK proteins consist of JNK1, JNK2, and JNK3, encoded by three separate genes: *MAPK8*, *MAPK9*, and *MAPK10*, respectively. They are alternatively spliced giving rise to at least ten isoforms (four JNK1, four JNK2, and two JNK3 splice variants) whose functional significance remains unclear (Davis, 2000). c-Jun *N*-terminal kinase 1 and JNK2 are ubiquitously expressed, whereas JNK3 is mainly found in the brain, heart, and testis (Weston and Davis, 2007). c-Jun *N*-terminal kinase 1 and JNK2 are approximately 46 and 55 kDa in size, respectively, differing in the length of their carboxyl terminus and in their ability to bind to their substrates such as c-Jun and ATF2 (Figure 1).

JNK Activation

The JNK signaling pathway is activated by a variety of extracellular stimuli including growth factors, tumor promoters, hormones, and proinflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β. c-Jun *N*-terminal kinase is also strongly activated in response to cellular stresses including genotoxic, osmotic, hypoxic, or oxidative stress (Karin, 1995; Weston and Davis, 2007). c-Jun *N*-terminal kinase lies downstream of a typical MAPK module, i.e., MAP3K-MAP2K-MAPK (Karin, 1995; Weston and Davis, 2007) and is activated by phosphorylation at Thr183 and Tyr185 on a conserved TPY (threonine–proline–tyrosine) motif. Two MAP2Ks (MKK4 and MKK7) have been identified for JNK. Several MAP3Ks, including members of the MEKK family, ASK1, MLK, TAK1, and TPL-2, have been reported to act as MAP3Ks for JNK (Davis, 2000; Figure 1). Numerous phosphatases have been shown to inactivate JNK including MKP-1, MKP-5, and MKP-7 (Boutros et al., 2008).

c-Jun *N*-terminal kinase transmits signals via phosphorylation of specific substrate proteins, thereby altering its function or availability by, for example, changing its conformation, activity, or stability. c-Jun *N*-terminal kinase is a serine/threonine kinase, and the consensus JNK phosphorylation site is serine or threonine followed by proline (SP or TP). This sequence is, however, quite common in proteins and cannot be used by itself to predict a JNK substrate. The protooncogene c-Jun is its first described and most studied substrate and is also believed to mediate many of its cellular effects. c-Jun *N*-terminal kinase-mediated phosphorylation at serines 63 and 73 increases c-Jun stability and its ability to form transcriptionally active AP-1 complexes with c-Fos and ATF2 (among other AP-1 proteins) resulting in the activation of a wide set of target genes that control the cell cycle as well as cell proliferation, differentiation, and death (Lopez-Bergami et al., 2010b; Vogt, 2001). In addition to its kinase activity, association of inactive JNK with some of its substrates (i.e., c-Jun, ATF2 and p53) marks them for degradation (Fuchs et al., 1998). Upon its activation by stress, JNK phosphorylates these proteins resulting in their stabilization and activation. c-Jun *N*-terminal kinase also phosphorylates transcription factors from the c-Jun, NFAT, c-Myc, and STAT families, among others. c-Jun *N*-terminal kinase also regulates cell movement and migration by phosphorylating cytoskeletal proteins such as paxillin, microtubule-associated protein 2 and 1B, Spir and DCX. The pro-apoptotic effects of JNK are mediated by phosphorylation of mitochondrial proteins such as Bcl-2, Bcl-xl, Bad, Bim, and Bax. A comprehensive description of more than 50 JNK targets has been published (Bogoyevitch and Kobe, 2006).

The role of JNK in human cancer

c-Jun *N*-terminal kinase has been shown to elicit both positive and negative effects on tumor development depending upon the cellular context (Kennedy and Davis, 2003). In many tumor types, c-Jun, which requires JNK-dependent phosphorylation to be transcriptionally active, has been identified as a bona fide oncogene (Lopez-Bergami et al., 2010b). 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 of cells derived from non-small cell lung carcinoma (Khatlani et al., 2007) and breast tumors (Mingo-Sion et al., 2004) and induced apoptosis in prostate cancer cells (Uzgare and Isaacs, 2004). These observations are consistent with the presence of constitutively phosphorylated JNK and c-Jun in various tumor samples and tumor cell lines (Jorgensen et al., 2006; Kennedy and Davis, 2003; Khatlani et al., 2007; Lopez-Bergami et al., 2007, 2010b). Despite the increasing body of evidence implicating the JNK/c-Jun pathway in cancer, little is known about the genetic and mechanistic basis for these findings. Because mutant Ras alleles require JNK and c-Jun for transformation, the activating mutations in Ras found in several tumor types (Adjei, 2001) might account in some cases for JNK activation (Behrens et al., 2000). More recently, large-scale sequencing analyses of tumor samples of different origins have identified genetic alterations in JNK1/2 and in components of the JNK pathway that presumably activate JNK signaling (Greenman et al., 2007; Jones et al., 2008; Kan et al., 2010). Among JNK regulators, attention has been focused on MKK4, which was shown to have both tumor suppressor and tumor promoting activities

(Whitmarsh and Davis, 2007). Loss-of-function alleles of MKK4 are found in approximately 5% of colorectal, prostate, breast, pancreatic, and lung carcinomas, suggesting that it may have a tumor suppression function (Su et al., 1998; Yamada et al., 2002). Furthermore, MKK4 has been identified as a suppressor of metastasis in prostate and ovarian cancers (Su et al., 1998; Yamada et al., 2002). Kan et al. (2010) recently attempted to better understand MKK4's dual role by stably expressing cancer-derived mutant MKK4 cDNAs in mammalian cells. However, they failed to find a correlation between the kinase activity and the ability to promote anchorage-independent growth (Kan et al., 2010).

JNK in melanoma: oncogen or 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 (Rutberg et al., 1994; Yamanishi et al., 1991). However, as in other tumor types, JNK has been shown to elicit both positive and negative effects on melanoma development. Thus, its status as oncogene is still under debate. Although JNK has been much less studied than ERK in melanoma, from more than a hundred articles published in the field a recurrent subject emerges. In agreement with one of its known roles, JNK becomes activated and triggers an apoptotic response following treatment with various compounds. A large and wide range of antitumoral, cytotoxic, psychoactive drugs, poisons, toxins, and antioxidants behave in this manner, including doxycycline (Shieh et al., 2010), docetaxel (Mhaidat et al., 2007), vincristine (Zhu et al., 2008), paclitaxel (Selimovic et al., 2008), benzodiazepines (Hu et al., 2008), 2-acetyl furanonaphthoquinone (Rieber and Rieber, 2008), plumbagin (Wang et al., 2008), celastrol (Abbas et al., 2007; Kannaiyan et al., 2011), guggulsterone (Shishodia et al., 2007), norcantharidin (An et al., 2005), and aspirin (Ordan et al., 2003), just to name a few. As a whole, these articles show the ubiquitous nature of the JNK response. The straightforward conclusion has been that JNK is an essential component of the stress-activated apoptotic response.

Translating these findings to a clinical setting allows us to assume that JNK actively participates on the killing of melanoma cells in patients under treatment for melanoma (i.e., with dacarbazine or IFN-α). Moreover, JNK is also activated and might cooperate to cell death induced by PLX-4032 through an unexplored mechanism (Halaban et al., 2010). These findings have implications in the event of considering using JNK inhibitors as a companion drug in combination therapies. Regardless of the still unknown antitumor potential of JNK inhibitors, they would strongly affect the tumor cell's ability to undergo apoptosis and therefore would compromise the antitumoral activity of the co-administered compound (i.e., a BRAF inhibitor).

Clearly, the findings described earlier reveal JNK function under acute stress and should not be used to draw conclusions on JNK's role in melanoma development and progression. The difference between these two scenarios is not trivial. Whereas stress induces a potent and transient JNK activation, tumor cells usually show a more subtle and constitutive JNK activity. Thus, to understand the role of JNK in melanoma, we must look at models based on the analysis of tumor samples or unstressed cells in culture. Interestingly, under such circumstances, JNK acted as an oncogene in most cases. Alexaki et al. (2008) showed that inhibition of JNK with the small molecule inhibitor SP600125 induces either cell cycle arrest or apoptosis of human melanoma cells. These results were confirmed in knockdown experiments using JNK1 siRNA (Alexaki et al., 2008). Along this line, Gao et al. (2009) showed that a selective and cell-permeable peptide inhibitor of JNK suppressed tumor growth in vivo and cell proliferation in vitro. Inactivation of JNK/c-Jun in combination with JunB knockdown was shown to induce cell cycle arrest and apoptosis in B16-F10 cells (Gurzov et al., 2008). Another evidence for a protumorigenic role of JNK is provided by Choi et al. (2005) who found that the tumor suppressor p16/INK4a exerts its inhibitory role on tumor cells by binding to and suppressing the activity of JNK. In addition, tetraspanin CD9, a transmembrane protein involved in transendothelial invasion of melanoma cells (Longo et al., 2001), was shown to induce JNK activation and JNK-dependent synthesis of MMP-2 in human melanoma cells (Hong et al., 2005). These results are in agreement with data showing constitutive phosphorylation of JNK in melanoma cell lines and tumor samples (Jorgensen et al., 2006; Lopez-Bergami et al., 2007; Alexaki et al., 2008). Immunohistochemical analysis of 381 samples from primary, superficial spreading, nodular and melanoma metastasis revealed 126 samples with phosphorylated JNK (p-JNK) with percentages ranging from 25 to 41% for the above-mentioned subtypes. For patients with superficial spreading melanomas, high level of cytoplasmic p-JNK was associated with tumor thickness and shorter disease-free survival as well as with markers of cell proliferation such as cyclin A and p21 (Jorgensen et al., 2006). A similar prevalence of p-JNK was observed in another report studying melanoma cell lines and a smaller cohort of melanoma metastasis (Lopez-Bergami et al., 2007). Fittingly, an unbiased proteomic analysis suggested the participation of JNK pathway in melanoma metastasis. Han et al. (2010) compared cell lysates from a primary human melanoma cell line (WM793) and a highly metastatic variant of the parental cell line (1205Lu) to identify proteins involved in tumor progression. The 110 unique proteins with significant abundance changes were mapped to six cellular networks using protein network analyses (Han et al., 2010). The top network included JNK as a central protein and exhibited metastatic potential associated with abundance changes for at least 27 of the 35 proteins in the

network. In particular, JNK expression and phosphorylation increased by 2.9- and 1.5-fold in metastatic 1205Lu cells as compared with the primary cell (Han et al., 2010).

Do dual JNK functions depend on differential roles of its isoforms?

Little is known about the role of individual JNK family members on phosphorylation of their substrates. Because JNK2 has a much greater affinity for c-Jun than JNK1, several laboratories have tried to determine differential roles for JNK1 and JNK2 using mice lacking either gene and cells derived from them. These studies concluded that JNK2 mainly targets c-Jun for ubiquitylation and degradation, whereas JNK1 is the isoform that activates and stabilizes c-Jun leading to transcriptional activation (Sabapathy et al., 2004). This approach also provided substantial information about the role of JNK in differentiation and tumorigenesis (Eferl et al., 2003; Sabapathy et al., 2004). However, experiments using a chemical genetic approach indicated that both JNK1 and JNK2 are positive regulators of c-Jun expression as well as cell proliferation (Jaeschke et al., 2006). This observation indicates that data originated from deficient mice should be interpreted with extreme caution because of compensatory increases in JNK function (Jaeschke et al., 2006; Sabapathy et al., 2004).

Given the speculation that JNK1 and JNK2 might have different functions, it is important to identify which isoform is active in melanoma. p-JNK2 was slightly more prevalent than p-JNK1 in both melanoma cell lines and tumors although this observation needs to be reproduced in a greater number of samples (Lopez-Bergami et al., 2007). Interestingly, simultaneous activation of both JNK1 and JNK2 was very rare (Alexaki et al., 2008; Lopez-Bergami et al., 2007). Hence, Alexaki et al. (2008) identified cell lines characterized by activation of either JNK1 (sk28, WM983B, WM852, WM793, and 1205Lu) or JNK2 (Gerlach, 888mel, and WM983A) and determined that both JNK isoforms were able to support AP1-dependent transcriptional activity (Alexaki et al., 2008). Genetic and chemical inhibition of JNK in some of these cell lines revealed a much greater participation of JNK1 (over JNK2) in melanoma cell growth (Alexaki et al., 2008). This possibility needs to be further explored because previous reports in other cell types describe the participation of JNK2 in tumorigenesis (Ahmed and Milner, 2009; Du et al., 2004; Ke et al., 2010; MacCorkle and Tan, 2004; Nitta et al., 2011; Tsuiki et al., 2003; Whitmarsh and Davis, 2007).

Molecular basis of JNK activation

Little is known about the genetic and/or mechanistic events underlying JNK activation. Unlike ERK, genetic alterations have not been described in JNK's upstream kinases in melanoma. However, Ras (which is mutated in 10–30% of melanomas) can activate the JNK pathway (Adjei, 2001). A possible mechanism explaining JNK activation involves the tumor suppressor p16 (INK4a) that is frequently deleted in melanoma (Graf-strom et al., 2005). When expressed, p16 (INK4a) can bind and inhibit JNK (Choi et al., 2005). 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 would partially contribute to increased JNK activity (Lopez-Bergami et al., 2007). TRAF2, as well as other members of the TRAF family (TNFR-associated factors), is upregulated in various tumors including melanomas (Ivanov et al., 2000) and through its effect on MEKK1–MKK4/7 can efficiently activate JNK/c-Jun. Protein kinase C (PKC) has long been identified as a contributing factor in skin tumorigenesis, and several PKC isoforms were found to be upregulated in melanoma cells (Oka and Kikkawa, 2005). A classic effect of PKC activation is the transcriptional activation of AP-1 target genes (Angel et al., 1987). It has been described that PKC can phosphorylate JNK in melanoma cell lines and enhance JNK activation by MKK4/MKK7 (Lopez-Bergami et al., 2005). Another possibility is that the non-canonical Wnt pathway might be partially involved in the activation of JNK (Weeraratna et al., 2002). c-Jun *N*-terminal kinase activation downstream of Wnt is firmly established as part of the planar cell polarity pathway but whether or not these pathways are connected in melanoma will require further studies.

The p38 pathway

The p38 MAPK group consists of four members: p38α, p38β, p38γ, and p38δ encoded by *MAPK14*, *MAPK11*, *MAPK12*, and *MAPK13*, respectively. The p38 isoforms share more than 60% homology and differ in expression patterns, substrate specificities, sensitivities to pharmacological inhibitors, regulation by upstream stimuli, and selectivity for upstream regulatory kinases and phosphatases. While p38α and p38β are universally expressed, p38γ and p38δ appear to have a more tissue-specific expression pattern. Except for p38δ, all other isoforms are expressed in melanoma. p38α and p38β could have overlapping functions although disruption of the p38α gene results in embryonic death demonstrating its specialized functions. The other isoforms are not essential for normal development indicating some redundancy in their functions (Cuenda and Rousseau, 2007).

p38 activation

In mammalian cells, the four p38 isoforms are strongly and rapidly activated by a variety of stresses including proinflammatory cytokines, heat shock, ultraviolet (UV) light, hypoxia, ischemia, and cellular poisons (Cargnello and Roux, 2011). TNF-α and IL-1 activate p38 by recruiting TRAF proteins and subsequently activating the corresponding MAP3Ks. The p38 isoforms are also activated

by GPCRs, as well as by the Rho family GTPases Rac and Cdc42. Like the others MAPK pathways, the p38 signaling cascade involves sequential activation of MAP3K and MAP2K. MKK3 and MKK6 show a high degree of specificity for p38 and are thought to be their major MAP2Ks. MKK3 and MKK6 directly activate p38 through phosphorylation of the Thr-Gly-Tyr (TGY) dual phosphorylation motif in a cell-type- and stimulus-dependent manner. MKK3/6 are activated by a plethora of MAP3Ks, including MEKK1 to 3, MLK2/3, ASK1, Tpl2, TAK1, and TAO1/2 (Cargnello and Roux, 2011; Cuadrado and Nebreda, 2010; Figure 1). In response to certain stimuli, MKK4 has also been shown to activate p38, suggesting that MKK4 represents a site of integration for the p38 and JNK pathways (Brancho et al., 2003). p38 is also activated independently of MAP2Ks by autophosphorylation (Ashwell, 2006). p38 activity is downregulated by MKP-1, -4 or -5, or other phosphatases such as protein phosphatases 2C and wild-type p53-induced phosphatase 1 (Wip1; Owens and Keyse, 2007). Interestingly, MKP-1 was shown to be induced by Notch (an emerging pathway in melanoma) in myoblasts (Kondoh et al., 2007).

Most stimuli that activate p38 also stimulate JNK isoforms, and many MAP3Ks in the p38 module are shared by the JNK module. For these reasons, the identification of the anti-inflammatory drug SB203580, a p38 inhibitor, has been extremely useful in delineating the specific functions of p38. This drug specifically targets and inhibits p38α and p38β, by acting as competitive inhibitors of ATP binding (Young et al., 1997). However, p38γ and p38δ activities can be inhibited as well. Because p38α is generally more highly expressed than p38β, most of the published literature on p38 refers to the former. p38 isoforms are present in the nuclei and cytoplasm of quiescent cells. The MAPKAPKs MK2, MK3, and MK5 have been shown to anchor p38 to the cytoplasm. Upon stimulation, p38 accumulates in the nuclei of the cells where it phosphorylates a large number of transcription factors, including ATF1/2/6, MEF2, Elk-1, GADD153, Ets1, p53, and MITF. Although more than half of p38 targets identified so far are transcription factors, p38 also phosphorylates cytoplasmic proteins such as cPLA2, MNK1/2, MK2/3, HuR, Bax, and Tau (Cargnello and Roux, 2011; Cuadrado and Nebreda, 2010). All p38 isoforms phosphorylate the Ser-Pro or Thr-Pro MAPK consensus motifs, but some substrate selectivity has been reported. A comprehensive list of proteins phosphorylated by p38 can be found at http://www.kinasource.co.uk/Database/S_Substrates/SAPK2a_substrates.html.

Cellular functions of p38 and its role in human cancer

The p38 pathway has been most frequently associated with a tumor suppressor function by negatively regulating cell survival and proliferation (Han and Sun, 2007). Most evidence has come from studies using cell lines and mouse knockout models, where inactivation of the p38 pathway enhances cellular transformation (Bulavin and Fornace, 2004; Han and Sun, 2007). Similarly, chemical inhibition of p38 activity is key for Ras-mediated transformation. Along these lines, expression of the p38 activators, MKK3 and MKK6, inhibits transformation of fibroblasts and tumor formation in animals (Bulavin and Fornace, 2004; Han and Sun, 2007). Likewise, forced expression of active p38 in rhabdomyosarcoma cells inhibits proliferation and induces terminal differentiation (Puri et al., 2000). This tumor-suppressive activity has been associated with a negative regulation of cell cycle progression at both the G1/S and G2/M transitions by a number of mechanisms, including the downregulation of cyclins, upregulation of cyclin-dependent kinase (CDK) inhibitors, and modulation of the tumor suppressor p53.

The p38 isoforms have also been shown to play a role in cell survival. Induction of apoptosis by several chemotherapeutics is mediated in part through activation of p38 (Bradham and McClay, 2006). This effect is mediated by transcriptional and post-translational mechanisms, which affect either death receptors, survival pathways, caspases or Bcl-2 proteins (Wagner and Nebreda, 2009). However, the ability of p38 to suppress tumor-forming capacity does not always correlate with decreased cell proliferation or induction of apoptosis (Timofeev et al., 2005), consistent with alternative anti-tumorigenic roles for p38 (Estrada et al., 2009; Junttila et al., 2007).

A recent attempt to identify cancer-associated somatic mutations in protein kinase genes revealed that several components of the p38 pathway, including p38α, p38β and p38δ, are mutated in human tumors, although the importance of these mutations needs to be elucidated (Greenman et al., 2007). These evidences are in agreement with the downregulation of p38α observed in lung tumors (Ventura et al., 2007), the relative decrease of p38 activity in hepatocellular carcinomas compared to non-tumorigenic tissues, and the observed negative correlation between p38 activity and tumor size (Iyoda et al., 2003; Ventura et al., 2007). Similar observations were made in other tumor types (Aguirre-Ghiso et al., 2003; Puri et al., 2000). Supporting a role of p38 as tumor suppressor, several negative regulators of p38 signaling were found to be overexpressed in human tumors and cancer cell lines. The p38 phosphatase Wip1 is frequently activated through amplification in human breast cancer, resulting in impaired p38 activation (Bulavin et al., 2002). Other phosphatases such as PPM1D (Bulavin et al., 2002) and DUSP26 (Yu et al., 2007) and the inhibitor of ASK1, glutathione S-transferase Mu 1 (GSTM1) were also found to be upregulated (Dolado et al., 2007).

On the other hand, increased levels of phosphorylated p38α (p-p38) have been correlated with malignancy in various cancers, supporting a pro-oncogenic

role of p38 (Wagner and Nebreda, 2009). Along these lines, p38 signaling can increase transcription of VEGF and hypoxia inducible factor 1 (HIF1), suggesting that p38 might contribute to tumor invasion and angiogenesis (Shemirani and Crowe, 2002). In sum, these observations suggest that regardless of p38's emerging role as a tumor suppressor, different biological effects can be observed depending on the stimulus and the cellular context.

Cellular functions regulated by p38 in melanoma

The degree of activation of p38 in melanoma has been studied by Jorgensen et al. (2006) in 152 primary tumors. Active p38 was found in 38 samples (25%), and p-p38 levels did not correlate with any clinical parameter. They also found that just six of 68 (9%) metastasis showed positive p-p38 immunostaining (Jorgensen et al., 2006). These findings suggest that p38 does not play a major role in melanoma. In contrast with these findings, two independent reports showed activation of p38 in cell lines and melanoma tumor samples (Estrada et al., 2009; Huangfu et al., 2011). It would be important to resolve this controversy to correctly interpret the evidences describing the participation of p38 on different aspects of melanoma biology.

A cellular function that has been consistently associated with p38 is melanoma differentiation. p38 was shown to be involved in regulating the synthesis of melanin pigments. Several melanogenic stimuli such as α-melanocyte-specific hormone (α-MSH), UV irradiation, or lipopolysaccharide promote a sustained increase of p-p38 (Ahn et al., 2008; Corre et al., 2004; Newton et al., 2007; Smalley and Eisen, 2000). This, in turn, mediates both an increase in tyrosinase activity and transcriptional upregulation of melanogenic enzymes such as MITF, tyrosinase, and tyrosinase-related protein (TRP). However, it was shown that p38 also promotes the degradation of tyrosinase and related proteins although it was proposed that this would be a mechanism to prevent the excessive production of melanin synthesis driven by the cAMP/PKA pathway (Bellei et al., 2010).

Microphthalmia-associated transcription factor plays a key role in melanoma by modulating various differentiation and cell cycle progression genes (Saha et al., 2006). Transcriptional upregulation of MITF by p38 might be mediated by activation of CREB that is phosphorylated in response to stress and UV irradiation by a number of kinases including MAPKAPK2, a p38 target (Saha et al., 2006). Active CREB binds and activates the MITF promoter via the cyclic adenosine monophosphate (cAMP) response element. ATF2, another p38 substrate, likely cooperates with CREB in this task (Shah et al., 2010). Regulation of MITF also occurs through post-translational modifications, particularly phosphorylation by several protein kinases. Mansky et al. (2002) determined that p38 phosphorylates MITF at Ser307 in osteoclasts, thereby increasing its transcriptional activity. Moreover, genetic or chemical inhibition of p38 inhibited MITF-mediated changes in gene expression (Mansky et al., 2002).

p38 also regulates melanogenesis independently of MITF by upregulating two key upstream components of the melanin synthesis cascade such as melanocortin 1 receptor (MC1R) and α-MSH (after cleavage of proopiomelanocortin) (Abdel-Malek et al., 1995) via the transcription factor upstream stimulating factor-1 (USF-1; Corre et al., 2004). This establishes a positive feedback loop because activation of MC1R by α-MSH induces activation of p38, melanogenesis, and differentiation (Smalley and Eisen, 2000). The α-MSH- and p38-mediated differentiation was associated with decreased retinoblastoma phosphorylation and accumulation of cells in the G1 phase (Smalley and Eisen, 2000). Consistent with this role of p38, MKK6 was found to be a positive regulator of melanocyte dendricity through the modulation of Rho family GTPases (Kim et al., 2010). Interestingly, the mRNA levels of MITF and p38 were decreased in lesional skin of vitiligo patients compared with skin of healthy subjects (Kingo et al., 2008).

The role of p38 in apoptosis of melanoma cells is controversial. Several cytokines, notably IFN, TNFα, IL-1, and IL-24, have an antiproliferative role in melanoma mediated by activation of p38 (Gollob et al., 2005; Hattori et al., 2001; Itoh et al., 1999; Sarkar et al., 2002). However, some late-stage melanomas become resistant to some of these cytokines (i.e., TNFα). It needs to be further explored whether the deactivation of p38 observed in melanoma metastases (Jorgensen et al., 2006) contributes to this process. Similar to JNK, p38 activation also mediates apoptotic cell death upon treatment with citotoxic compounds. However, little is known about the mechanisms involved. It is well accepted that p38 is activated by reactive oxygen species (ROS) generated by some stressors. In turn, p38 contributes to amplify ROS production and the onset of apoptosis (Posen et al., 2005; Selimovic et al., 2008). It has been shown that the apoptotic role of p38 is mediated by PUMA (Keuling et al., 2010) and NOXA (Hassan et al., 2008) and that p38 is essential for RASSF1A-induced mitochondrial apoptosis (Yi et al., 2010). On the other hand, inhibition of p38 synergistically induces apoptosis in melanoma cells in combination with ABT-737, a Bcl-2 family inhibitor (Keuling et al., 2010). A substantial part of p38's response to stress is mediated by changes in gene expression. It was recently found that more than 60% of the up-regulated genes induced by osmostress, TNFα, and the protein synthesis inhibitor anisomycin are under the transcriptional control of p38 (Ferreiro et al., 2010).

It is important to stress the fact that p38's responses are context-specific and that opposing responses are frequently observed. For instance, p38-dependent activation of ATF2 can mediate proliferation signals in tumor cells through transcriptional activation of key cell cycle regulators (Recio and Merlino, 2002). p38 can positively

regulate cell adhesion, invasion, and metastasis. Garcia et al. (2009) showed that p38 is required for cell adhesion induced by arachidonic acid and mediated by RhoA. As described for other tumor types, activation of p38 mediates transcriptional activation of MMP-2 and MMP-9 following treatment with mda-9/syntenin, tetraspanin CD9, and platelet-activating factor (Boukerche et al., 2007; Denkert et al., 2002; Hong et al., 2005; Melnikova et al., 2006). As in other tumor types, p38 was found to increase the expression of VEGF in response to basic fibroblast growth factor (Fontijn et al., 2009). Overexpression of these proteins has been observed in human cancers, and they were suggested as important factors in metastasis. p38 was shown to be involved during in vitro invasion of malignant melanoma cells (Denkert et al., 2002) through regulation of VE-cadherin junction disassembly, facilitating melanoma migration across endothelial cells (Khanna et al., 2010). It has been suggested that p38 positively regulates NF-κB signaling which would further support a role of p38 in both cell growth and migration (Boukerche et al., 2008, 2010; Kuphal et al., 2004; Shah et al., 2009). Among other targets, NF-κB activates IL-8, a chemokine tightly linked to melanoma progression (Ueda and Richmond, 2006). Along these lines, it was shown that p38 inhibitors block IL-8 production and reduce proliferation and migration (Estrada et al., 2009). However, this needs further clarification because it was demonstrated that p38 suppresses Fas expression by limiting NF-κB activity (Ivanov et al., 2001).

Interplay among ERK, JNK, and p38 pathways

The three MAPK pathways are usually represented in the literature as independent signaling cascades with few points of contact among them. However, several positive and negative regulatory circuits have been described in normal and transformed cells. A lengthy description of these observations is beyond the scope of this review, and the discussion will be limited to data obtained in melanoma cells. It has been proposed that the balance between ERK and p38 activity is important for cell proliferation. Whereas high ERK/p38 ratio favors tumor growth, high p38/ERK ratio is associated with tumor growth arrest (dormancy). This balance is achieved by a negative regulation of ERK by p38 and has been described in several tumor types (Aguirre-Ghiso et al., 2003; Ding and Adrian, 2001; Shimo et al., 2007). Estrada et al. (2009) showed that such regulation is lost in melanoma cell lines and suggested that the p38 pathway may be altered. Along with this hypothesis, melanoma cells display a novel circuit by which ERK drives high expression of $\alpha V\beta 3$, an integrin involved in p38 activation in melanoma cells through vitronectin signaling (Estrada et al., 2009). However, these observations still need to be confirmed in melanoma tissue. Interestingly, it was shown that osteopontin induces $\alpha V\beta 3$ integrin-mediated MEKK1-dependent JNK1 phosphorylation and c-Jun expression. In turn, JNK1 activation leads to downregulation of ERK1/2 activation (Rangaswami and Kundu, 2007).

Another MAPK aberrant mechanism links ERK with JNK signaling in human melanoma. Constitutively active ERK increases c-Jun transcription and stability, which are mediated by CREB and GSK3, respectively. Subsequently, upregulation of c-Jun target genes such as RACK1 and PDK1 results in an increase in JNK activity, enforcing a feed-forward mechanism of the JNK/c-Jun pathway (Lopez-Bergami et al., 2007, 2010a). These evidences highlight the importance of rewired signaling in human cancer and show how constitutively active ERK can provide signals to increase the activity of JNK and p38. This model is in agreement with the observation of a positive correlation between cytoplasmic p-JNK, pERK, and p-p38 (Jorgensen et al., 2006).

Conclusions and perspectives

Almost a decade after the finding of mutations in BRAF in melanoma, we have a clearer picture with precise details of many aspects of the RAF/MEK/ERK pathway. There is no doubt that this pathway is critical for melanoma development and progression and a primary therapeutic target. The study of JNK and p38 pathways has been more superficial, and therefore, many important questions are still open. Most evidences show that the JNK pathway can mediate opposing biological effects in cancer, depending largely on cell type, environmental stimuli, and the genetic background. The studies that analyzed JNK function in the context of different stresses revealed the critical role of this pathway in triggering apoptosis. On the other hand, studies that focus on the role of JNK in tumor samples or in non-stressed cells revealed that JNK signal would importantly contribute to different aspects of melanoma. These findings need to be further studied, but in principle, they imply a paradox; whereas several evidences expose JNK as a contributing factor to melanoma, the inclusion of JNK inhibitors in mono or combination therapies might be counterproductive. Most of the evidences obtained in melanoma cells are in agreement with a tumor-suppressive role of p38. However, further studies are needed to identify precise circumstances where the pathway behaves differently.

Thirteen years after the last drug was approved for the treatment of melanoma (interleukin 2 in 1998), the FDA approved this year two new drugs – ipilimumab and zelboraf (based in PLX-4032) – for the treatment of advanced melanoma. The last one is also (together with imatinib) the first approved drug to treat melanoma by targeting a specific gene mutation, demonstrating the tremendous potential of targeted therapies. Although far from a cure, these drugs are expected to help patients with advanced melanoma. This achievement would

have been impossible without the significant advances in the understanding of melanoma biology that have been made in the recent years. The current challenge is to overcome the acquisition of drug resistance in patients treated with BRAF inhibitors. Given the central role of MAPK pathways in melanoma, to acquire a better comprehension of the intricacies of these pathways will be crucial toward this goal.

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