In Melanoma, *RAS* Mutations Are Accompanied by Switching Signaling from BRAF to CRAF and Disrupted Cyclic AMP Signaling

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Abstract

Melanocytes require the RAS/RAF/MEK/ERK and the cyclic AMP (cAMP) signaling pathways to maintain the fine balance between proliferation and differentiation. We have investigated how cross-talk between these pathways affects melanoma progression. We show that cAMP suppresses CRAF activity in melanocytes and that this is essential to suppress the oncogenic potential of CRAF in these cells. As a consequence, BRAF alone is responsible for signaling to MEK. However, when RAS is mutated in melanoma, the cells switch their signaling from BRAF to CRAF. This switch is accompanied by dysregulated cAMP signaling, a step that is necessary to allow CRAF to signal to MEK. Thus, a fundamental switch in RAF isoform usage occurs when RAS is mutated in melanoma, and this occurs in the context of disrupted cAMP signaling. These data have important implications for the development of therapeutic strategies to treat this life-threatening disease. (Cancer Res 2006; 66(19): 9483-91)

Introduction

Melanocytes are specialized pigment-producing cells in the skin that protect us from the damaging effects of UV light. They are also the precursors of melanoma, a skin cancer with poor prognosis that fails to respond to currently available therapies and whose incidence is rising in Western populations. Melanocytes reside in the epidermis, a specialized microenvironment in which their biological functions are regulated by several autocrine and paracrine factors (1). These factors stimulate intracellular signaling pathways that regulate cytoskeletal rearrangements, metabolism, and gene expression, thereby regulating cell function and fate. Due to the richness of their microenvironment, multiple interacting pathways can be activated simultaneously in melanocytes, resulting in signaling "cross-talk" that can alter cell fate decisions (2–4). Two such pathways are the RAS/RAF/MEK/ERK and cyclic AMP (cAMP) signaling pathways (5). Here, we investigate how these pathways interact to regulate melanocyte transformation.

RAS proteins are membrane-bound small G proteins, whereas RAF, MEK, and ERK are cytosolic protein kinases that form a tiered protein kinase cascade downstream of RAS (6). Signaling is initiated when active RAS recruits RAF to the plasma membrane for activation through a complex process requiring lipid and protein binding, conformational changes, and regulatory phosphorylation and dephosphorylation events. There are three RAF proteins in mammals, ARAF, BRAF, and CRAF, and they can all activate MEK but they clearly perform distinct functions *in vivo* as shown by the phenotypic differences between *araf*, *braf*, and *craf* null mice (7). They also serve distinct functions in cancer. BRAF is mutated in ~50% to 70% of human melanomas, whereas ARAF and CRAF are not mutated because their regulation is fundamentally different from that of BRAF (8). The most common (90% of cases) mutation in BRAF is a glutamic acid for valine substitution at position 600 (V600E), which activates BRAF 500-fold and stimulates constitutive MEK-ERK signaling in cells (9–11). Furthermore, ^{V600E}BRAF transforms immortalized melanocytes and stimulates survival and proliferation in melanoma cells (12–16).

cAMP is a second messenger that is produced following hormone stimulation of seven-transmembrane G protein coupled receptors. These receptors stimulate membrane-associated adenylyl cyclases to convert ATP into cAMP. This signaling is terminated when cAMP is degraded by one of a large family of enzymes called the cAMP-phosphodiesterases (17). Four cAMP effectors are known: the cAMP-activated exchange factors (Epac1 and Epac2), cAMP-gated ion channels, the cAMP-regulated phosphodiesterases, and the cAMP-dependent protein kinases (protein kinase A; PKA). PKA is a heterotetramer consisting of two catalytic and two regulatory subunits. The catalytic subunits are released in an active form when cAMP binds to the regulatory subunits (18), and the biological consequences of PKA activation are wide-ranging and depend on cell type and context.

Intriguingly, cAMP can augment or suppress ERK activity, depending on the cell type (19). In some cells, cAMP activates BRAF (5), but the underlying mechanisms are not understood. In contrast, CRAF is inhibited by PKA, which directly phosphorylates serine 43, serine 233, and serine 259 (S43, S233, and S259; refs. 20–24). S43 phosphorylation blocks CRAF binding to RAS through steric hindrance, whereas S233 and S259 phosphorylation recruits 14-3-3 adaptor proteins to the NH_2 terminus of CRAF, thus, preventing membrane recruitment (23, 25). Importantly, these sites work independently to uncouple CRAF from RAS and ensure that CRAF cannot be activated when cAMP signaling is stimulated.

Unlike other skin cells, melanocytes very rarely proliferate and only in response to specific environmental conditions that can be reproduced *ex vivo* by a mixture of growth factors that stimulate

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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several intracellular pathways. The RAS/RAF/MEK/ERK pathway regulates proliferation downstream of agonists such as stem cell factor and basic fibroblast growth factor (1). cAMP is produced downstream of melanocytic agonists such as α-melanocyte-stimulating hormone (α-Msh) acting through the melanocortin 1 receptor (MC1R; ref. 26), and although this weakly stimulates proliferation (27), cAMP is more closely associated with melanocyte differentiation because it stimulates responses such as melanin synthesis.

In this work, we examine cross-talk between RAS/RAF/MEK/ERK and cAMP in melanocytes. We show that cAMP blocks CRAF activity in these cells, and consequently, BRAF alone is responsible for coupling RAS signals to MEK. The inhibition of CRAF by PKA is essential to suppress the oncogenic activity of CRAF in these cells. However, when RAS is mutated in melanoma, there is a switch from BRAF to CRAF, and this is accompanied by a disruption to cAMP signaling, a step that is essential to allow CRAF signaling. These findings have important implications for treatment strategies.

Materials and Methods

Cell culture and protein expression. Expression vectors for mutant myc-epitope tagged BRAF (pEF/mBRAF) and CRAF (pEF/mCRAF) have previously been described (28). Stable cell lines were selected using the vector pMCEF- and G418 selection (29). Melan-a cells were cultured in RPMI 1640 (Invitrogen, Paisley, United Kingdom) containing 10% fetal calf serum (FCS; Invitrogen), 200 nmol/L of 12-*O*-tetradecanoylphorbol-13-acetate (TPA; Sigma, Gillingham, United Kingdom), and 300 pmol/L of cholera toxin (CT; Sigma) as previously described (13). Normal neonatal human epidermal melanocytes (NHM; Cascade Biologics, Nottinghamshire, United Kingdom) were cultured in medium 154 supplemented with human melanocyte growth supplement (Cascade Biologics). A375, SKMel2, SKMel28, and WM266.4 human melanoma cell lines were cultured in DMEM (Invitrogen) containing 10% FCS. Colo829, WM1361, and WM1791c human melanoma cell lines were cultured in RPMI containing 10% FCS. Sbcl2, WM852, and WM1366 human melanoma cell lines were cultured in MCDB153 (Sigma)/L15 medium (Invitrogen; v/v:4/1) supplemented with $CaCl_2$ (2 mmol/L), insulin (5 μg/mL, Sigma), and 2% FCS. Melan-a cells were transfected with LipofectAMINE in OptiMEM I reduced serum medium according to the manufacturer's instructions (Invitrogen). RNA interference, cell extraction, and CRAF kinase assays were done as previously described (13, 28). DNA synthesis was determined using [^{3}H]thymidine (0.4 μCi/mL) following 16 hours of incubation.

The following antibodies were used: mouse anti-9E10 (ICR hybridoma unit), phospho-ERK (Sigma), ERK2 (Santa Cruz Biotechnology, Calne, United Kingdom), BRAF (Santa Cruz Biotechnology), monoclonal CRAF (Becton-Dickinson, Cowley, United Kingdom), polyclonal CRAF (Sigma and Santa Cruz), phospho-serine 43 (p43), phospho-serine 233 (p233; ref. 20), phospho-serine 259 (p259; New England Biolabs, Hitchin, United Kingdom), phospho–cAMP-responsive element binding protein (CREB; Ser^{133}; New England Biolabs), and CREB (New England Biolabs).

Immunofluorescence. NHM were transfected with 5 μg of DNA by Nucleofection according to the manufacturer's protocol (Amaxa GmbH, Cologne, Germany). The cells were fixed in methanol/acetone, blocked with 1% bovine serum albumin/PBS, and incubated with rabbit anti-myc (Abcam, Cambridge, United Kingdom) and phospho-ERK. Staining was revealed using secondary Cy2- or Cy3-conjugated antibodies (Dianova, Helsingborg, Sweden) with mounting in DABCO-glycerol.

***In vivo* studies.** Female CD1 nude mice (Charles River, Margate, United Kingdom) weighing 19 to 32 g were used. Cell suspensions were inoculated s.c. in a volume of 0.2 mL into age-matched mice to give groups of seven mice; each mouse received an injection with 1×10^7 cells of either ^{WT}CRAF, ^{259A}CRAF, or $^{43A/233A/259A}$CRAF. Experiments were conducted in accordance with the United Kingdom Home Office regulations and United Kingdom Coordinating Committee on Cancer Research Guidelines.

CRAF sequencing. Genomic DNA sequencing of exon 7 of CRAF was done using established techniques (30) and 5′-AGCCTAAGTGCCAATCATGG-3′ and 5′-CAGAGACCTGAGAAAGTGTTGC-3′ primers.

Results

CRAF is not required for ERK signaling in melanocytes. To study the cross-talk between cAMP and RAF in melanocytes, we used melan-a cells, a nontransformed mouse melanocyte line that retains many of the characteristics of normal melanocytes (31). For *ex vivo* culture, melan-a cells require ERK signaling to stimulate proliferation and cAMP signaling to maintain their differentiated phenotype (32, 33). ERK signaling can be stimulated by the phorbol ester TPA and cAMP signaling can be stimulated by α-Msh through MC1R or by CT, which directly activates the stimulatory heterotrimeric G protein (G_s).

First, we examined if cAMP stimulates CRAF phosphorylation in melan-a cells. There are three PKA phosphorylation sites reported in CRAF: S43, S233, and S259. In the presence of CT, S43, S233, and S259 of CRAF were all phosphorylated and their levels of phosphorylation were not elevated when the cells were treated with α-Msh (Fig. 1*A*). However, in the absence of CT, S259 remained phosphorylated, whereas S43 and S233 became dephosphorylated and were rephosphorylated when the cells were treated with α-Msh (Fig. 1*A*). Thus, under normal growth conditions, the high levels of cAMP required to maintain melan-a cell differentiation stimulate constitutive phosphorylation of CRAF on S43 and S233. S259 is also constitutively phosphorylated, but the phosphorylation of this site seems to be independent of cAMP signaling.

We next examined CRAF kinase activity. TPA did not stimulate CRAF kinase activity when melan-a cells were grown in the presence of CT, but robust activation was seen when CT was omitted (Fig. 1*B*). Nevertheless the presence of CT did not prevent ERK activation by TPA (Fig. 1*B*). Similarly, when melan-a cells were treated with α-Msh, TPA could not activate CRAF, but it could activate CRAF when α-Msh was not present (Fig. 1*C*). Once again, despite the lack of CRAF activation, ERK activation by TPA was unaffected by α-Msh, suggesting that another RAF isoform might be responsible for coupling TPA to ERK activation. We therefore used RNA interference to study the roles of CRAF and BRAF in melan-a cell signaling.

BRAF depletion blocked ERK activation by TPA and also by α-Msh, whereas CRAF depletion had no effect (Fig. 1*D*). Thus, BRAF is essential for ERK activation by growth-promoting agents in these mouse melanocytes, whereas CRAF is not required. We extended these findings to normal diploid human melanocytes (NHM), which can be cultured *ex vivo* by provision of a cocktail of growth factors that includes pituitary gland extract as a convenient source of α-Msh. However, the proliferative potential of NHM is limited to 15 to 20 passages, after which the cells progressively senesce. As in melan-a cells, in NHM, S43, S233, and S259 were phosphorylated in the presence of pituitary gland extract and this phosphorylation was not affected by additional α-Msh (Fig. 2*A*). When the pituitary gland extract was omitted, S43 and S233 became dephosphorylated and their phosphorylation could be stimulated by α-Msh (Fig. 2*A*). Furthermore, CRAF kinase could only be activated by growth factors when the pituitary gland extract was omitted from the culture medium (Fig. 2*B*). Thus, under normal growth conditions, the high levels of cAMP required to maintain the differentiated phenotype of mouse and human melanocytes blocks CRAF activation, and consequently, BRAF alone is responsible for MEK activation downstream of the growth-promoting agents.

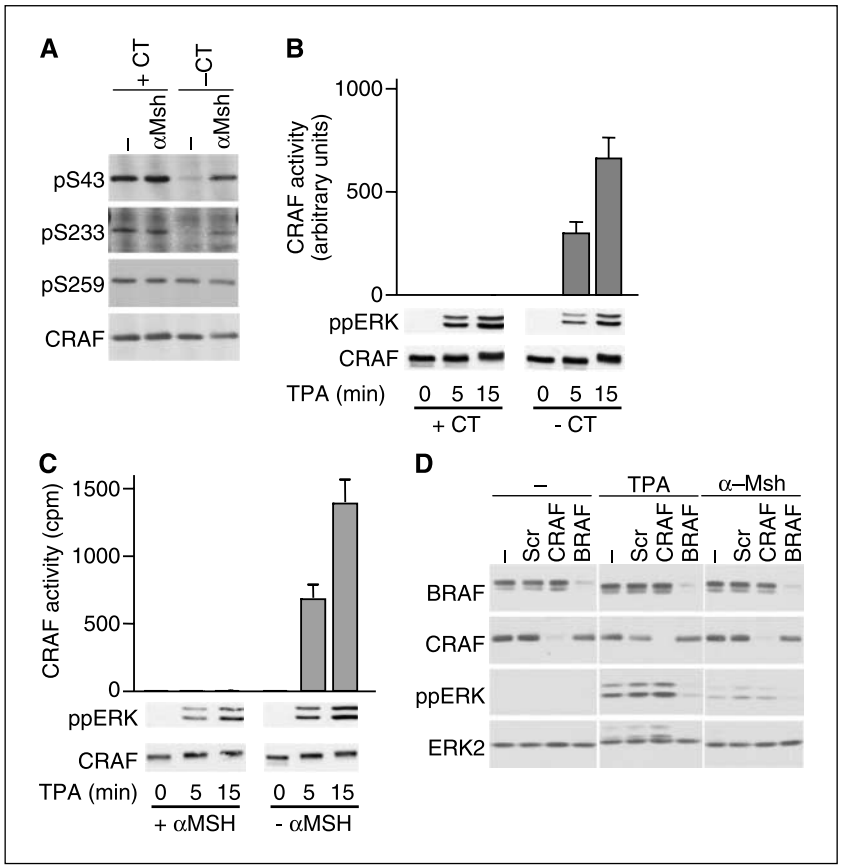

Figure 1. BRAF activates MEK in melanocytes whereas CRAF does not. *A,* phosphorylation of CRAF on S43 (*pS43*), S233 (*pS233*), and S259 (*pS259*), and total CRAF levels in melan-a cells incubated with (+*CT*) or without (−*CT*) cholera toxin for 24 hours and then treated with α-Msh (1 μmol/L, 15 minutes). *B,* CRAF kinase activity in melan-a grown in the presence (+*CT*) or in the absence (−*CT*) of cholera toxin, starved of TPA for 24 hours, and then stimulated with TPA (200 nmol/L) for the indicated times. *Columns,* means of one experiment assayed in triplicate; *bars,* SD. *C,* CRAF kinase activity in Melan-a grown in the presence (+ αMSH) or absence (-α MSH) of αMSH, starved of TPA for 24 hours and then stimulated with TPA (200 nmol/L) for the indicated time. Results are for one experiment assayed in triplicate. *Error bars*, standard deviations from the mean. *D,* Western blot for BRAF, CRAF, total ERK2, and phospho-ERK (*ppERK*) in melan-a cells that were treated with short interfering RNA for CRAF, BRAF, or the scrambled (*Scr*) control, starved of TPA and CT (24 hours), and then treated with TPA (200 nmol/L, 15 minutes) or α-Msh (1 μmol/L, 5 minutes).

cAMP-insensitive CRAF is an oncogene in melanocytes. To investigate why CRAF is inactivated in melanocytes, mutant forms of CRAF in which S43, S233, or S259 were substituted with alanine were expressed in melan-a cells. When these sites were mutated individually, weak ERK activation was observed, most strongly with ^{S259A}CRAF (Fig. 2*C*). However, when all three sites were mutated, ERK was strongly activated (Fig. 2*C*). The triple mutant ($^{S43A/S233A/S259A}$CRAF) stimulated similar levels of ERK activity such as that seen with the potent melanocyte oncogene ^{V600E}BRAF (Fig. 2*C*) and importantly, ^{S259A}CRAF and $^{S43A/S233A/S259A}$CRAF also activate ERK in NHM (Fig. 2*D*).

We have previously shown that ^{V600E}BRAF induces TPA-independent growth in melan-a cells (13); hence, we investigated if ^{S259A}CRAF and $^{S43A/S233A/S259A}$CRAF were also oncogenic in these cells. Like parental melan-a cells (13), clonal lines expressing ^{WT}CRAF did not proliferate without TPA (Fig. 3*A*). However, the clones expressing ^{S259A}CRAF or $^{S43A/S233A/S259A}$CRAF both proliferated without TPA (Fig. 3*A*). Proliferation was higher in the clones expressing the triple mutant than in the ^{S259A}CRAF-expressing clones (Fig. 3*A*). Furthermore, whereas the proliferation of the clones expressing ^{S259A}CRAF was still augmented by TPA, TPA did not augment the proliferation of the clones expressing $^{S43A/S233A/S259A}$CRAF. Finally, CT did not affect $^{S43A/S233A/S259A}$CRAF-stimulated proliferation, but it strongly antagonized ^{S259A}CRAF-stimulated proliferation (Fig. 3*A*). The inhibition of proliferation by CT in the ^{S259A}CRAF-expressing clones was accompanied by S43 and S233 phosphorylation (data not shown). Thus, unlike TPA-stimulated proliferation, which is augmented by CT, CT antagonizes ^{S259A}CRAF-stimulated proliferation because PKA can phosphorylate this mutant.

ERK signaling is essential for melan-a cell proliferation and there was a strong correlation between ERK activity and proliferation. ^{S259A}CRAF and $^{S43A/S233A/S259A}$CRAF both activated ERK in the absence of TPA, with the triple mutant being the stronger activator (Fig. 3*B*). Furthermore, whereas TPA augmented ERK activity in the ^{S259A}CRAF-expressing clones, it did not affect ERK activity in the $^{S43A/S233A/S259A}$CRAF-expressing clones, presumably because signaling was already maximal (Fig. 3*B*). Finally, whereas CT suppressed ^{S259A}CRAF-induced ERK activity, it did not suppress the TPA-independent ERK activity stimulated by $^{S43A/S233A/S259A}$CRAF (Fig. 3*B*). Together these data show that when S259 alone is mutated, CRAF can stimulate proliferation, but this is still further enhanced by TPA and can still be suppressed by CT. In contrast, $^{S43A/S233A/S259A}$CRAF stimulates maximal proliferation that cannot be further stimulated by TPA and which is insensitive to CT because PKA can still phosphorylate and inactivate ^{S259A}CRAF. Finally, both the ^{S259A}CRAF-expressing and the $^{S43A/S233A/S259A}$CRAF-expressing cells adopted a transformed morphology, ceasing melanin production, losing their dendritic appearance, and taking on a more epithelial form (data not shown). Importantly, clones expressing either version of CRAF grew as tumors in nude mice (Fig. 3*C*).

When RAS is mutated in melanoma, CRAF signals to MEK and cAMP signaling are dysregulated. Next, we investigated the role played by CRAF in ERK signaling in five melanoma lines (Sbcl2, WM852, WM1361, WM1366, and WM1791c) which carry activating mutations in RAS, but in which BRAF is wild-type (Table 1). These cells still require MEK-ERK signaling for proliferation because the small molecule MEK inhibitors, U0126 and PD184352, blocked their DNA synthesis (Fig. 4*A*). Surprisingly, however, when BRAF was

depleted in these cells, ERK activity was unaffected (Fig. 4*B*). Further examination revealed that it is CRAF, rather than BRAF, which is required for ERK activity in these cells (Fig. 4*B*). Thus, in contrast with melanocytes (shown above; Fig. 1) and melanoma cells harboring mutant BRAF (ref. 15; see Colo829 cells in Fig. 4*B*), when RAS is mutated, the cells stop signaling through BRAF and switch their signaling to CRAF. However, we have already shown that in melanocytes, CRAF cannot signal because cAMP signaling is elevated, therefore, we examined CRAF and cAMP in these cells in more detail.

First, we sequenced exon 7 (which encodes S233 and S259) of the *CRAF* gene in these five cell lines, but did not find any mutations that compromised these PKA sites in any of the lines (Table 1). We also sequenced exon 7 of *CRAF* in 82 primary melanoma samples that were wild-type for BRAF and which came from the palms and soles (n = 26), the mucosa (n = 15), skin with chronic sun–damage (n = 17), and skin without chronic sun–damage (n = 24). Again, we did not find any mutations, demonstrating that the PKA sites on CRAF are not commonly mutated in melanoma.

We therefore directly examined the PKA phosphorylation sites on CRAF in Sbcl2, WM852, WM1361, WM1366, and WM1791c cells. As in melanocytes (Fig. 1), S259 was constitutively phosphorylated in all of the cell lines (Fig. 5*A*). However, because these cells are grown in the absence of CT, S43 and S233 were not phosphorylated (Fig. 5*A*). Moreover, in contrast with melanocytes, α-Msh did not induce S43 or S233 phosphorylation in any of these lines (Fig. 5*A*). Because S43 can be phosphorylated as part of an ERK feedback loop downstream of growth factors (34), we also treated the cells with FCS, but did not find any evidence for this ERK-dependent feedback phosphorylation of S43 in these cells (Fig. 5*A*). However, when cAMP production was stimulated directly using forskolin (which activates adenylyl cyclase) and 3-isobutyl-1-methylxanthine (IBMX; which inhibits phosphodiesterase), both S43 and S233 were phosphorylated, and importantly, this was accompanied by a suppression of ERK activity in all five cell lines (Fig. 5*A*). The forskolin/IBMX-induced phosphorylation of CRAF persisted for several hours, whereas α-Msh did not induce CRAF phosphorylation at any time (Fig. 5*B*).

We performed a similar analysis in melanoma cell lines that harbor a mutation in BRAF. We used A375, WM266.4, Colo829, and SkMel28 cells (Table 1) and found that in these cells, the responses to α-Msh were variable. S43 was constitutively phosphorylated in A375 cells, but not in the other lines, and S233 was not phosphorylated in any of the lines (Fig. 5*C*). α-Msh stimulated S43 and S233 phosphorylation in WM266.4 and Colo829 cells, but not in SkMel28 or A375 cells, whereas forskolin/IBMX stimulated S233 phosphorylation in all four lines and S43 phosphorylation in WM266.4, Colo829, and SkMel28 cells (Fig. 5*C*). Thus, cAMP signaling is intact in all of the cell lines, but unlike the Ras mutant lines, half of the BRAF mutant lines still respond to α-Msh.

To investigate why α-Msh/MC1R signaling is defective in the RAS mutant cells, we sequenced the *MC1R* gene in these cells (Table 1). *MC1R* is a highly polymorphic human gene with "strong" and "weak" alleles (35), but we did not find any correlation between the type of *MC1R* variant and α-Msh responsiveness. We therefore used the phosphorylation of a well-characterized PKA substrate, the transcription factor, CREB, to further investigate cAMP signaling in the RAS mutant cells. Forskolin and IBMX stimulated robust phosphorylation of serine 133 (S133) of CREB in WM1361 or WM1791c cells (Fig. 5*D*), demonstrating that the PKA pathway is intact. Surprisingly, however, α-Msh did not stimulate CREB phosphorylation in these cells, confirming that α-Msh signaling

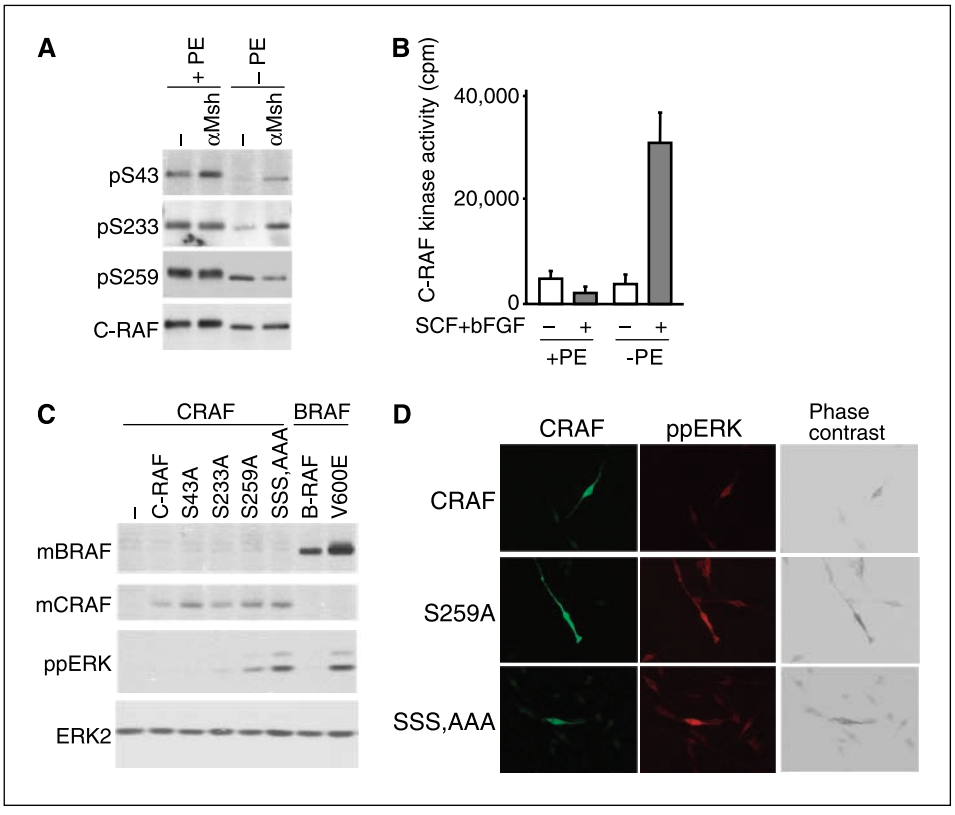

Figure 2. cAMP-insensitive CRAF induces constitutive ERK activation in human melanocytes. *A,* phosphorylation of CRAF on S43 (*pS43*), S233 (*pS233*), and S259 (*pS259*), and total CRAF levels in NHM that were incubated with (+*PE*) or without (−*PE*) pituitary gland extract (24 hours), then treated with α-Msh (1 μmol/L, 15 minutes). *B,* CRAF kinase activity in NHM incubated with (+*PE*) or without (−*PE*) pituitary gland extract (24 hours), then stimulated with stem cell factor (10 ng/mL) plus basic fibroblast growth factor (25 ng/mL) for 3 minutes. *Columns,* means of one experiment assayed in triplicate and similar results were obtained in two independent assays; *bars,* SD. *C,* Western blot for myc-tagged BRAF (*mBRAF*), myc-tagged CRAF (*mCRAF*), endogenous ERK2 and phosphorylated ERK (*ppERK*) in TPA and CT starved (24 hours) melan-a cells transiently expressing wild-type CRAF (*C-RAF*), ^{S43A}CRAF (*S43A*), ^{S233A}CRAF (*S233A*), ^{S259A}CRAF (*S259A*), $^{S43A/S233A/S259A}$CRAF (*SSS,AAA*), wild-type BRAF (*B-RAF*), or ^{V600E}BRAF (*V600E*). *D,* immunofluorescence for myc-tagged CRAF and phosphorylated ERK (*ppERK*) in NHM transiently expressing myc-tagged CRAF (*CRAF*), ^{S259A}CRAF (*S259A*) or $^{S43A/S233A/S259A}$CRAF (*SSS,AAA*). A phase image of the cells (*right*).

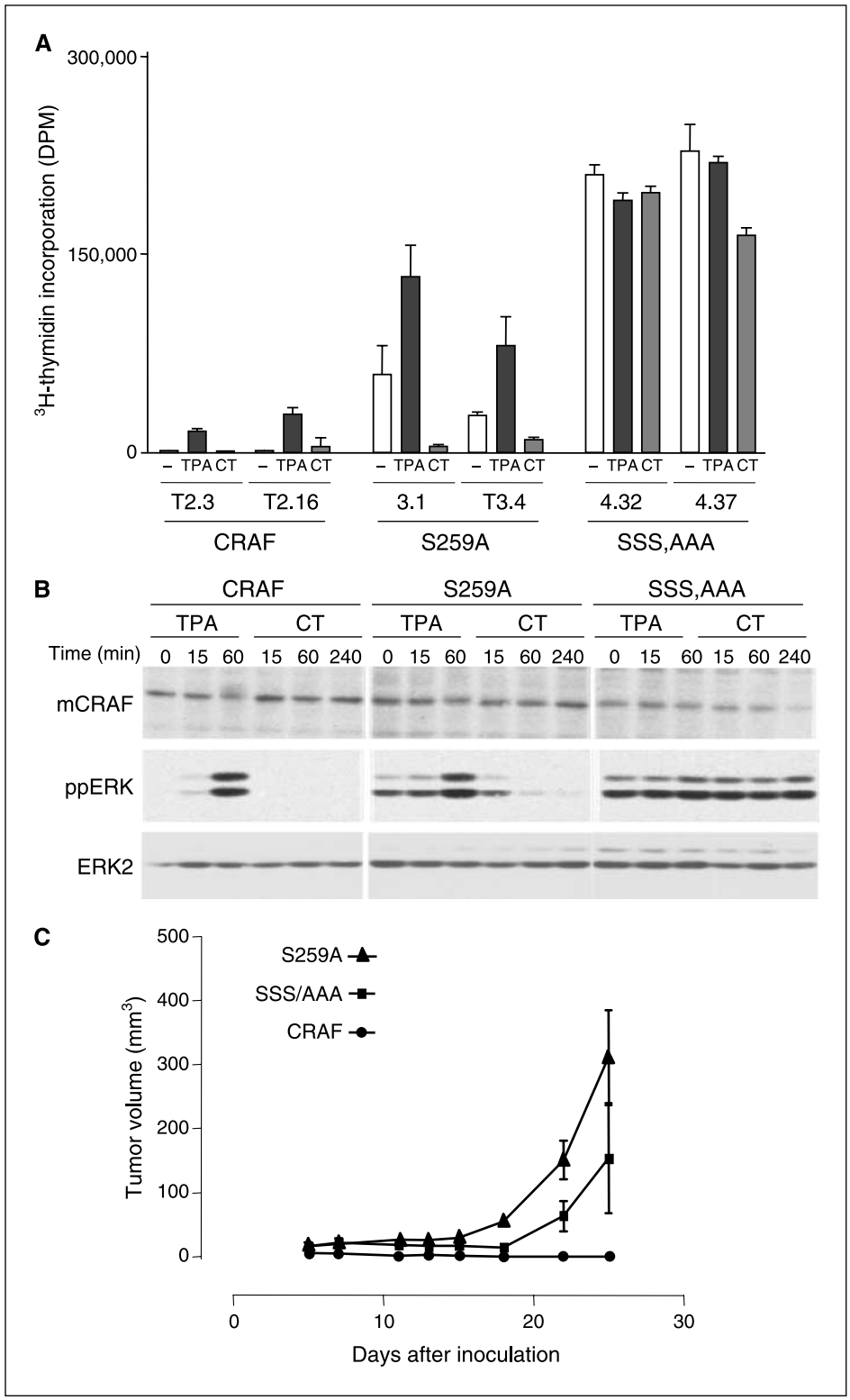

Figure 3. cAMP-insensitive CRAF is oncogenic in melanocytes. *A,* DNA synthesis in melan-a cells stably expressing myc-tagged CRAF (*CRAF*), ^{S259A}CRAF (*S259A*), or $^{S43A/S233A/S259A}$CRAF (*SSS,AAA*) in the absence (−) or presence of TPA or CT as indicated. *Columns,* means of two independent clones for each construct assayed in triplicate (similar results were seen in at least three other individual clones of each); *bars,* SD. *B,* Western blot for myc-tagged CRAF (*mCRAF*), total ERK2 (*ERK2*), and phosphorylated ERK (*ppERK*) in melan-a cells stably expressing myc-tagged CRAF (*CRAF*), ^{S259A}CRAF (*S259A*) or $^{S43A/S233A/S259A}$CRAF (*SSS,AAA*). The cells were starved of TPA and CT (24 hours) and then treated with TPA (200 nmol/L) or CT (300 pmol/L) for the times indicated (in minutes). Similar results were obtained in at least five individual clones for each CRAF construct. *C,* growth of melan-a cells expressing myc-tagged CRAF (*CRAF*), ^{S259A}CRAF (*S259A*), or $^{S43A/S233A/S259A}$CRAF (*SSS,AAA*) in nude mice.

is uncoupled in these cells. To determine if this is because the cells lack the MC1R receptor, we treated the cells with α-Msh and the phosphodiesterase inhibitor, IBMX, and found that this led to robust phosphorylation of S133 (Fig. 5*D*). These studies show that these cells do not fail to respond to α-Msh because they lack a functional MC1R, but rather, they do not respond because their signaling is uncoupled at the level of cAMP metabolism, an effect that can be overcome when phosphodiesterase activity is inhibited.

Discussion

The regulation of RAF/MEK/ERK signaling in melanoma is an area of great interest because this pathway regulates the

Table 1. Genomic mutations in melanoma cell lines

Cell line	BRAF* (exons 11 and 15)	CRAF* (exon 7)	K-RAS* (exons 2 and 3)	N-RAS* (exons 2 and 3)	MC1R*
Sbcl2	WT	WT	WT	Q61K	V60L
WM852	WT	WT	WT	Q61R	R160W †
WM1361	WT	WT	WT	Q61K	V60L
WM1366	WT	WT	WT	Q61L	V60L/R160W/W169stop †
WM1791c	WT	WT	T50I/Q61H	WT	WT
SKMel2	WT	WT	Q61R	WT	R142H
A375	V600E	WT	WT	WT	R151C
Colo829	V600E	WT	WT	WT	V92M
SkMel28	V600E	WT	WT	WT	S83P/I155T †
WM266.4	V600D	WT	WT	WT	R160W †

*Wild-type.
†Heterozygote.

proliferation and survival of melanoma cells. BRAF is mutated in 50% to 70% of melanomas and RAS is mutated in 15% to 20%. CRAF is not required for MEK/ERK signaling in melanoma cells when BRAF is mutated (15, 16), but RAF isoform usage has not been investigated when RAS is mutated. Here, we have shown that there is a switch in RAF isoform usage depending on whether BRAF or RAS is mutated. We show that in melanocytes or melanoma in which BRAF is mutated, it is BRAF that is primarily responsible for signaling to MEK and ERK. However, when RAS is mutated in melanoma, the cells switch to CRAF, findings that have implications for drug discovery programs.

We initiated our studies by examining the PKA phosphorylation sites in CRAF in melanocytes. S259 regulates 14-3-3 binding to the CRAF NH_2 terminus, preventing CRAF activation by RAS-related proteins such TC21 and R-Ras, and thereby maintaining CRAF signaling fidelity (34, 36–39). CRAF activation requires S259 dephosphorylation, which allows CRAF recruitment to the plasma membrane. Our data suggests that, as in other cells, S259 is not a PKA site in melanocytes, although it is possible that S259 is a PKA site that is dephosphorylated very slowly because 14-3-3 binding protects it from phosphatases. Presumably, S259 serves a similar function in melanocytes as it does in other cells, suppressing

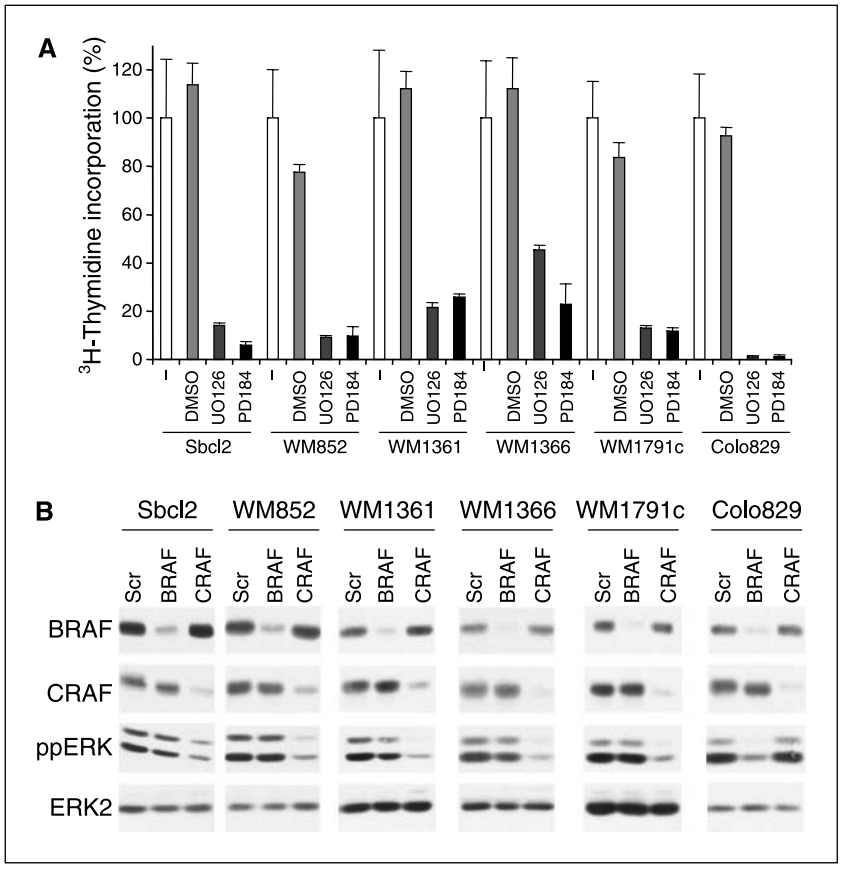

Figure 4. Melanoma cells that harbor mutations in RAS require CRAF and not BRAF for MEK activation. *A,* DNA synthesis in melanoma cell lines treated with DMSO, UO126 (10 μmol/L), or PD184352 (5 μmol/L). *Columns,* means of one experiment assayed in triplicate (similar results were seen in three independent experiments); *bars,* SD. *B,* Western blot for endogenous BRAF, CRAF, ERK2, and phosphorylated ERK (*ppERK*) in melanoma cells lines treated with short interfering RNA to CRAF, BRAF, or the scrambled control (*Scr*).

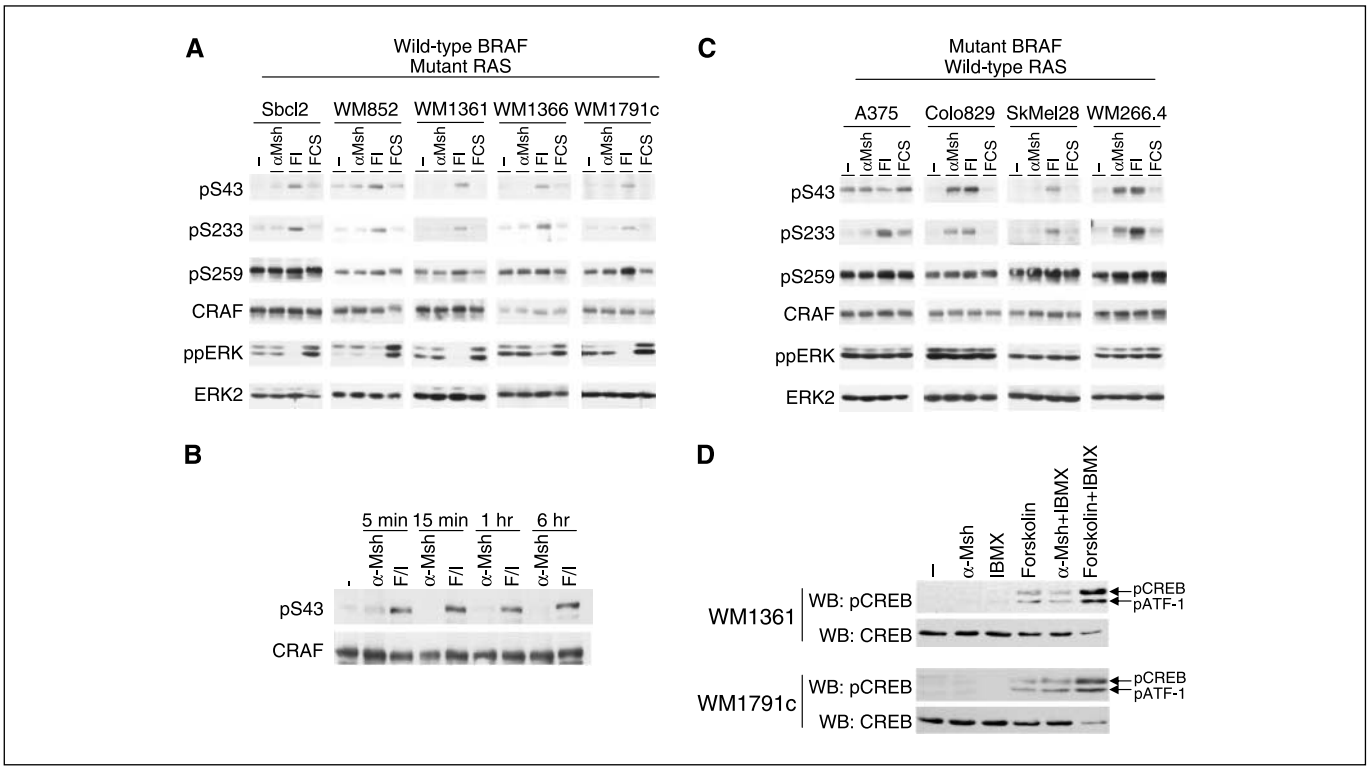

Figure 5. The cAMP signaling pathway is disrupted in melanoma cells that have mutations in RAS. *A,* Western blot of CRAF phosphorylated on S43 (*pS43*), S233 (*pS233*), or S259 (*pS259*) and for total CRAF, total ERK2, and phosphorylated ERK (*ppERK*) in untreated cells, or cells treated with α-Msh (1 μmol/L), forskolin (10 μmol/L) plus IBMX (100 μmol/L; *F/I*) or 10% FCS for 15 minutes. *B,* Western blot of CRAF phosphorylated on S43 (*pS43*) and for total CRAF in WM1361 cells untreated or treated with α-Msh (1 μmol/L) or forskolin (10 μmol/L) plus IBMX (100 μmol/L; *F/I*) for the times indicated. *C,* similar to (*A*) for cells with mutations in BRAF. *D,* Western blot for phospho-CREB (*pCREB*) and total CREB in WM1361 and WM1791c cell lines treated with α-Msh (1 μmol/L), IBMX (100 μmol/L), forskolin (10 μmol/L), or a combination of those for 15 minutes.

CRAF activity under resting conditions and maintaining signaling fidelity.

In cells such as fibroblasts, S43 and S233 are not normally phosphorylated, although S43 does become phosphorylated as part of a negative feedback loop from ERK that prevents persistent CRAF activation by growth factors (34). However, both sites are PKA substrates and their phosphorylation prevents CRAF activation when cAMP signaling is elevated (20, 23). Until now, the physiologic significance of these phosphorylation sites has been unclear. Here, we show that S43, S233, and S259 are constitutively phosphorylated in melanocytes under normal growing conditions and this maintains CRAF in a latent state from which it cannot be activated. As a consequence, BRAF alone is responsible for coupling growth-promoting signals from RAS to MEK. When cAMP signaling is blocked, conditions that favor melanocyte dedifferentiation, S43 and S233 become dephosphorylated and this then allows CRAF to be activated by growth factors.

There is clearly a fundamental difference in the regulation of proliferation in fibroblasts and melanocytes by cAMP. Whereas cAMP blocks the proliferation of fibroblasts, it weakly stimulates melanocyte proliferation (for review, see ref. 19). Curiously, however, the growth of melanoma cells harboring a mutant RAS is regulated in a fashion that is more similar to that of fibroblasts than melanocytes because their proliferation is blocked when their intracellular cAMP levels are elevated (Supplemental Fig. S1). Critically, when fibroblasts express the PKA-resistant CRAF mutant, $^{S43A/S233A/S259A}$CRAF, their proliferation is still blocked when cAMP levels increase (20). In contrast, when this CRAF mutant is expressed in melanocytes, agents that activate cAMP production do not block their proliferation (Fig. 3*A*).

These data suggest that CRAF is the primary growth-regulatory target of PKA in melanocytes and its activity must be suppressed in order to mask its oncogenic activity. In contrast, in fibroblasts, or in melanoma cells with mutant RAS, the effects of cAMP on proliferation are pleiotropic and CRAF seems to be only one of the many growth-regulatory targets of PKA. Thus, even if PKA cannot target CRAF in fibroblasts, and possibly in melanoma cells with mutant RAS, it targets other pathways to ensure that proliferation is suppressed when cAMP levels are elevated. In line with this, we note that CRAF plays a more important role in regulating the proliferation of fibroblasts and melanoma cells in which RAS is mutated than it does in regulating the proliferation of melanocytes. Our data shows that the interplay of signaling between these pathways in different cell types is biochemically distinct and has different biological consequences.

Taken together, our data suggests that S259 functions in melanocytes as in other cells, whereas S43 and S233 play an important role in keeping CRAF inactive under normal growth conditions. Support for this hypothesis comes from our observation that ^{S259A}CRAF allows melanocytes to grow as tumors when injected s.c. into nude mice, but importantly, cAMP can still suppress ERK activation and cell proliferation in the cells expressing this mutant. Presumably, in the correct microenvironment, melanocytic hormones such as α-Msh would still be able to block ERK activation and proliferation stimulated by ^{S259A}CRAF, and although nude mice are a convenient model to test if cells can

grow as tumors, the s.c. site does not mimic the rich microenvironment found in the dermus of human skin. We propose that whereas ^{S259A}CRAF expressing melanocytes can grow in nude mice, they would be unlikely to grow in human skin because α-Msh would induce S43 and S233 phosphorylation, thereby blocking signaling from this mutant.

Based on our findings, we propose the following model of cAMP-RAF cross-talk in melanocytes. We posit that cAMP levels are elevated in melanocytes due to signaling downstream of key factors such as α-Msh, resulting in PKA-mediated phosphorylation of CRAF, thereby preventing its activation and routing signaling through BRAF. Our data reveal that when PKA cannot block CRAF activity, CRAF becomes a melanocytic oncogene. However, in the normal melanocyte environment, all three sites must be mutated to allow this to occur and this would presumably be a very rare event. In agreement with this, we failed to find any mutations in the PKA sites in almost 100 samples that we tested. This inherent redundancy presumably protects melanocytes from the oncogenic potential of CRAF.

As a consequence of cAMP signaling, BRAF alone is responsible for coupling RAS signals to MEK. Surprisingly, however, when melanoma cells acquire a mutation in RAS, cells seem to switch their signaling from BRAF to CRAF. It is unclear why they should do this, but several studies have shown that excessive ERK signaling induces cell cycle arrest through transcriptional up-regulation of low molecular weight inhibitory proteins such as p21, p27, and p16^{INK4A} (40–42). We have shown that RAS activates BRAF significantly more strongly than it activates CRAF (28), so it is possible that oncogenic RAS cannot signal through BRAF because this leads to excessive ERK signaling and the induction of cell cycle arrest or senescence. To avoid this, the cells switch to CRAF, which provides weaker signaling and is compatible with tumor progression. This model predicts that BRAF cannot be activated when RAS is mutated, and this is supported by our RNA interference studies showing that BRAF is not required for ERK activation in these cells. An alternative explanation is that the target cell for melanoma is not the melanocyte, but an earlier melanoblast or stem cell that uses CRAF and not BRAF as part of its normal signaling. It is only later in development that the cells switch to BRAF, but if these cells acquire a mutation in RAS, they do not switch to BRAF and instead use their original signaling pathway.

Our model also requires that CRAF escapes the suppression normally mediated by cAMP in the cells in which RAS is mutated, and we show that these cells fail to respond to α-Msh. This seems to be because there is an imbalance in cAMP metabolism. We find that when phosphodiesterase activity is blocked, α-Msh can stimulate CREB phosphorylation, demonstrating that the cells have a functional MC1R, but that cAMP signaling is inefficient, presumably either because its synthesis is reduced, or its degradation is increased. This could be due to reduced activity or expression of an essential biosynthetic pathway component such as MC1R, the heterotrimeric G protein or adenylyl cyclase, or due to elevated phosphodiesterase expression or activity, and is the subject of ongoing studies. That half of the BRAF mutant lines still respond to α-Msh, shows that dysregulated cAMP signaling is not essential for melanoma progression, but that it only becomes important when RAS is mutated, an observation that is consistent with our model.

We do not know if RAS is mutated before the cAMP metabolism is disrupted. However, RAS mutations do occur early in melanoma development (43) and we have previously reported the unusual finding that both BRAF and CRAF contribute to MEK signaling in SkMel2 cells (44), a melanoma cell line that harbors a mutation in K-RAS (Table 1). Importantly, in contrast to the other RAS mutant lines, α-Msh still stimulates CRAF phosphorylation in SKMel2 cells, but this does not lead to suppression of ERK activity, presumably because BRAF can compensate for the inhibition of CRAF (Supplemental Fig. S2). This line seems to be an exception, because it is the only line of the six we tested with RAS mutations that behaves in this manner. It is possible that SKMel2 cells are at an intermediate stage of development and have not completed the process of switching from BRAF to CRAF, or that it arises from an earlier cell compartment. This may be because cAMP signaling is not fully suppressed in these cells and is entirely consistent with our model. It also argues that RAS mutations occur before cAMP signaling becomes disrupted. Our data may also provide a rational explanation of why BRAF mutations are more common than RAS mutations in melanoma. Melanomas that acquire RAS mutations will also need to disrupt their cAMP signaling, a step that is not required when BRAF is mutated, thus providing a more direct route to transformation when BRAF is mutated.

We have shown an intriguing difference in RAF isoform usage in melanoma depending on whether RAS or BRAF is mutated, and in addition, we show that RAS mutations in melanoma occur coincident with disrupted cAMP signaling. These findings have important therapeutic implications because they suggest that both BRAF and CRAF are valid targets in melanoma, but that this depends on the cell context. Thus, drugs that target both isoforms could be more useful than agents that selectively inhibit only oncogenic BRAF. Further studies are required to determine how tumors with different genetic backgrounds will respond to specific inhibitors. Finally, these findings suggest that drugs that reactivate cAMP signaling in the melanomas in which RAS is mutated could provide an attractive alternative therapeutic approach to treating this disease.

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