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ERK and PDE4 cooperate to induce RAF isoform switching in melanoma

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Melanocytes use BRAF to activate the MAP kinase (MAPK) pathway because CRAF is inhibited by the cyclic AMP (cAMP) pathway in these cells. By contrast, melanomas harboring Ras mutations use CRAF to activate the MAPK pathway. We describe the molecular mechanism of Raf isoform switching and cAMP pathway disruption, which take place during melanocyte transformation. We show that overactivation of the MAPK pathway, induced by the oncogenic Ras in melanoma, induces constitutive phosphorylation of BRAF on Ser151 by ERK, which inhibits NRAS-BRAF interaction . We also demonstrate that melanoma cells have elevated cAMP phosphodiesterase activity owing to overexpression of the cAMP-specific phosphodiesterase-4 enzymes; this activity inhibits cAMP signaling and allows CRAF reactivation in these cells. Reactivating the cAMP pathway inhibits proliferation and induces apoptosis of Ras-mutated melanoma cells, suggesting a new therapeutic approach for treating melanomas harboring Ras mutations.

Cutaneous melanoma is the deadliest form of skin cancer, and its incidence is increasing at a rate of 2–5% per year[1]. Melanoma arises from the malignant transformation of melanocytes, pigment-producing cells in the basal layer of the epidermis. Under physiological conditions, growth factors and hormones released into the local microenvironment simultaneously activate several signaling pathways in melanocytes that interact to regulate their proliferation, differentiation and migration[2]. The connections between these pathways are essential in regulating the fate of melanocytes. Two major signaling pathways that are activated simultaneously in melanocytes, leading to signaling 'cross-talk,' are the MAPK pathway and the cAMP pathway[3].

Under physiological conditions, the MAPK pathway is stimulated by the interaction of growth factors with their cell surface receptor tyrosine kinase (RTK) and the transmission of these signals through the small GTPase Ras[4]. When Ras is active in its GTP-bound state, it activates several downstream effectors, including the RAF family of serine/threonine kinases[5]. The three isoforms of RAF—ARAF, BRAF and CRAF (also called RAF1)—all activate MAPK-ERK kinase 1 (MEK1) and MEK2, which in turn activate extracellular signal–regulated kinase 1 (ERK1) and ERK2 (ref. 6). ERKs have many targets, which are primarily involved in the regulation of melanocyte proliferation[7]. The situation is different in melanoma cells, as most clinical melanoma specimens have continuous hyperactivity in the MAPK pathway[8], usually owing to acquisition of an activating oncogenic mutation in proteins involved in the MAPK pathway. Oncogenic mutations of RTK, such as in the *KIT* gene, have been described in a subset of melanomas and can continuously activate the MAPK pathway in these cells[9,10]. Oncogenic mutations in the *NRAS* gene have been identified in 15–20% of all melanomas and lead to inhibition of Ras GTPase activity and activation of the MAPK pathway[11,12]. About 50% of all melanomas harbor activating mutations in the *BRAF* gene, with the *BRAF* mutation that yields the V600E substitution being the most frequent[12,13]. This mutation strongly activates BRAF and leads to constitutive MAPK signaling. Oncogenic mutations of RTK, RAS or BRAF can transform immortalized melanocytes and stimulate survival and proliferation in melanoma cells[10,14,15]. These mutations are mutually exclusive in melanoma because their transforming activity is thought to result from the stimulation of the MAPK pathway[12].

cAMP is a second messenger produced in melanocytes downstream of melanocytic agonists such as α-melanocyte–stimulating hormone (α-Msh) acting through the seven-transmembrane G protein–coupled receptor MC1R[16]. cAMP is closely associated with melanocyte differentiation because it stimulates responses such as melanin synthesis. Through activation of the cAMP-dependent protein kinase A (PKA), cAMP stimulates phosphorylation and activation of the cAMP responsive element–binding protein (CREB) transcription factor, which in return stimulates transcription of the microphthalmia-associated transcription factor (*MITF*) gene[17]. MITF exerts a key role in melanocyte differentiation by directly controlling the transcription of many genes involved in melanin synthesis or melanosome functioning (*TYR*, *TYRP1*, *DCT*, *RAB27A* and *GPR143*)[18]. cAMP is regulated in both a spatial and a temporal manner by cAMP phosphodiesterases (PDEs), which provide the sole route for degradation of cAMP in cells. PDE activity is found not only in the cytosol, but also in a variety of membrane, nuclear and cytoskeletal locations[19–21]. There are 11 different PDE families, 8 of which generate >30 different isoforms that can hydrolyze cAMP[22–24]. Notably, particular isoforms can be regulated by other signaling pathways[19]. Thus PDEs can have a pivotal role in the cross-talk between cAMP signaling and various other intracellular signaling systems[19].

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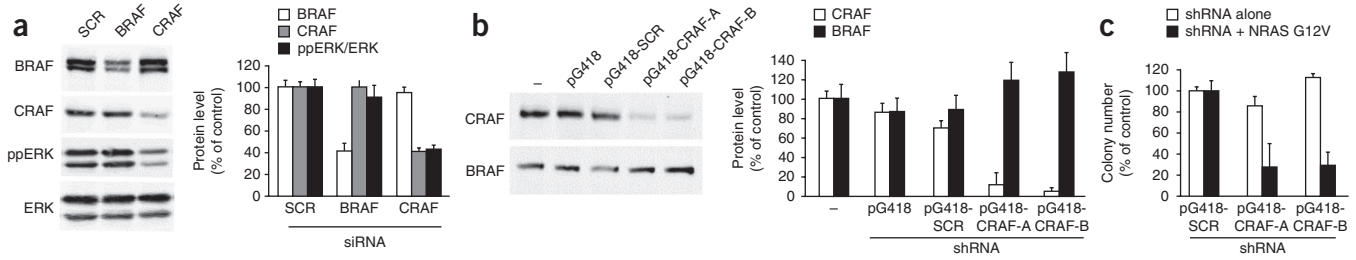

Figure 1 NRAS G12V is oncogenic in melanocytes and induces Raf isoform switching. (**a**) Immunoblots of BRAF, CRAF, phosphorylated ERK (ppERK) or total ERK (ERK) expression in control (SCR) and NRAS G21V Melan-a cells depleted of BRAF or CRAF with siRNA. (**b**) Immunoblots of BRAF or CRAF in Melan-a cells mock transfected (–) or transfected with the empty pG418 vector (pG418), pG418 expressing a shRNA control (pG418-SCR) or a shRNA targeting CRAF (pG418-CRAF-A and pG418-CRAF-B). Graphs on right in **a** and **b**, mean ± s.d. of three independent experiments. (**c**) Counting of colonies of Melan-a cells transfected with a vector expressing NRAS G12V in combination with the pG418 vectors described above and selected with G418 with (shRNA alone) or without (shRNA + NRAS) TPA. Graph, mean ± s.d. of three independent experiments.

Melanocytes provide a good model for studying cross-talk between the cAMP pathway and the MAPK pathway. In physiological conditions, RTK-induced activation of RAS in normal melanocytes leads to stimulation of the MAPK pathway via BRAF, whereas constitutive cAMP-induced PKA activity leads to the phosphorylation and inactivation of CRAF[15,25]. However, in melanomas with RAS mutations, the cells switch their signaling from BRAF to CRAF, deregulating cAMP signaling so that PKA no longer suppresses CRAF and allowing CRAF-mediated MAPK activation to occur[15]. In this study we clarify the molecular mechanism of BRAF activity downregulation and cAMP signaling disruption in melanoma.

RESULTS

Oncogenic Ras induces Raf isoform switching in melanocytes

To study the cross-talk between cAMP and RAF in melanocytes, we used the Melan-a cell line, a nontransformed mouse melanocyte line that retains many of the characteristics of normal melanocytes[26]. For *ex vivo* culture, Melan-a cells require ERK signaling to stimulate proliferation and cAMP signaling to maintain their differentiated phenotype[26]. Oncogenic NRAS G12V transformed Melan-a cells, indicated by the growth of colonies in medium without 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and by their ability to form colonies in soft agar; parental cells did not grow in these conditions[10] (data not shown). To study the roles of CRAF and BRAF in NRAS G12V Melan-a cell signaling, we used RNA interference (RNAi) to specifically inhibit CRAF or BRAF expression. CRAF depletion blocked ERK activation by NRAS G12V, whereas BRAF depletion had no effect (**Fig. 1a**). Thus, CRAF is essential for ERK activation by oncogenic RAS in transformed mouse melanocytes, whereas BRAF is not required. In comparison, as we have previously described, in melanocytes under normal growth conditions, the high levels of cAMP required to maintain their differentiated phenotype block CRAF activation. Consequently, BRAF alone is responsible for MEK activation downstream of growth-promoting agents[15]. To determine whether CRAF is necessary for melanocyte transformation by NRAS G12V, we used two different short hairpin RNAs (shRNAs), CRAF-A and CRAF-B, to inhibit CRAF expression in these cells. We first found that only CRAF-A and CRAF-B and not the controls inhibited expression of CRAF but not of BRAF (**Fig. 1b**). We then co-transfected melanocytes with these shRNAs and a vector expressing NRAS G12V, and selected either for the stable expression of NRAS G12V and shRNA, or for the stable expression of shRNA alone. Without NRAS G12V, we obtained clones expressing CRAF-A or CRAF-B shRNA, suggesting that in normal growing conditions Melan-a cells do not require CRAF to grow (**Fig. 1c**). In contrast, with NRAS G12V, we obtained fewer clones in the cells transfected with CRAF-A or CRAF-B shRNA than with the control, demonstrating that CRAF is necessary to transform melanocytes with oncogenic NRAS (**Fig. 1c**).

A feedback loop inhibits BRAF binding to oncogenic Ras

To understand the molecular mechanism leading to the inability of BRAF to activate the MAPK pathway in Ras-mutated melanoma, we studied the interaction of RAF kinases with RAS in these cells. RAS-RAF interaction is the first step in the activation of RAF kinases. We immunoprecipitated Flag-tagged NRAS G12V in melanocytes transformed by this oncogene and found that CRAF but not BRAF coimmunoprecipitated with NRAS G12V (**Fig. 2a**). To test whether NRAS G12V could bind BRAF in another cell type, we coexpressed NRAS G12V with CRAF or BRAF in COS cells. Both CRAF and BRAF bind NRAS G12V in COS cells (**Supplementary Fig. 1a**). Thus, the lack of interaction between NRAS G12V and BRAF is specific to transformed melanocytes and could cause the inability of BRAF to activate the MAPK pathway in these cells.

To investigate whether a feedback mechanism could inhibit RAS-BRAF interaction in melanoma, we treated NRAS G12V Melan-a cells with the MEK inhibitor U0126 or PD98059, immunoprecipitated NRAS G12V and then analyzed the presence of coimmunoprecipitated

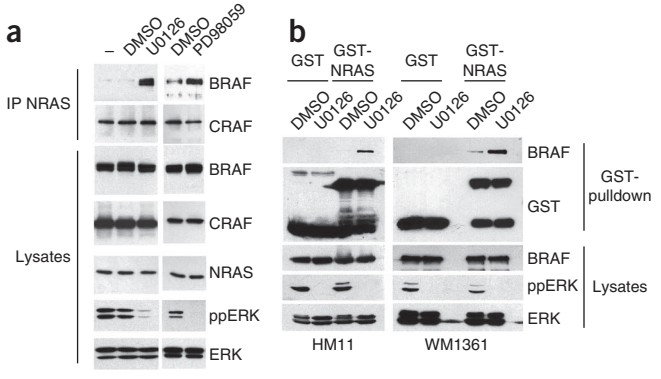

Figure 2 Mek inhibition rescues NRAS-BRAF binding in melanoma. (**a**) Melan-a cells expressing NRAS G12V were untreated (–) or treated with DMSO, U0126 (10 μM) or PD98059 (20 μM). NRAS G12V was immunoprecipitated (IP) and probed for BRAF or CRAF. Lysates were directly probed for BRAF, CRAF, phosphorylated ERK (ppERK) or total ERK (ERK). (**b**) GST pulldown assay with either GST or GST-NRAS on HM11 and WM1361 human melanoma cell lines treated with DMSO or U0126 (10 μM) and probed for BRAF or GST. Lysates were directly probed for BRAF, ppERK or ERK. See also **Supplementary Figure 2**.

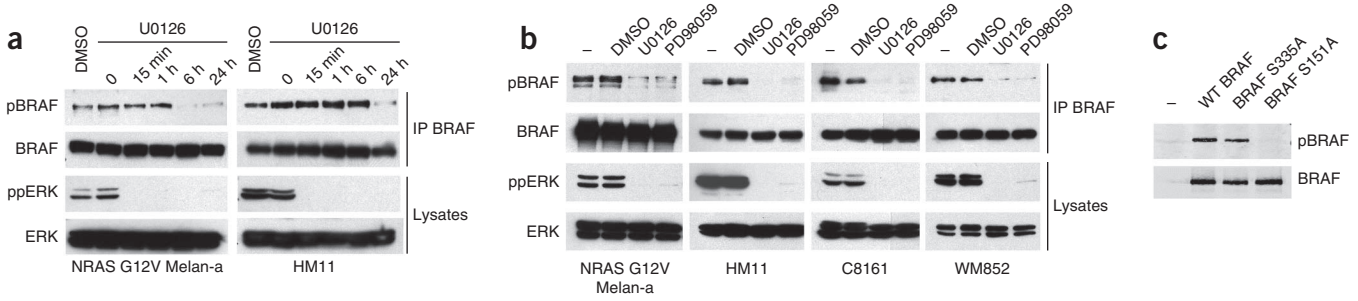

Figure 3 BRAF is phosphorylated on Ser151. (**a**,**b**) NRAS G12V Melan-a or human melanoma cell lines were treated with DMSO, U0126 (10 μM) or PD98059 (20 μM). BRAF was immunoprecipitated (IP) and probed with a phospho-serine/proline (pBRAF) or a BRAF antibody. Lysates were directly probed for phosphorylated ERK (ppERK) or total ERK (ERK). (**c**) NHEMs were transfected with an empty vector (–), Myc-tagged wild-type (WT) BRAF, BRAF S335A or BRAF S151A. BRAF was immunoprecipitated and probed with a phospho-SP (pBRAF) or a BRAF antibody.

CRAF or BRAF by western blotting. We found that the inhibition of MEK, measured by inhibition of ERK phosphorylation, enabled the interaction of BRAF with NRAS G12V but had no effect on RAS-CRAF interaction (**Fig. 2a**). To confirm this result in human melanoma cells, we studied RAS-BRAF interaction *in vitro* as there are no available antibodies to coimmunoprecipitate endogenous NRAS with endogenous RAF kinases. Glutathione *S*-transferase (GST)-tagged NRAS (wild type, G12V or S17N) produced in bacteria was incubated with extracts from HM11 or WM1361 cell lines that were treated or untreated with U0126. We found that inhibition of MEK or ERK by U0126 increased the *in vitro* interaction between NRAS (wild type and G12V but not S17N) and BRAF from HM11 and WM1361 melanoma cells (**Fig. 2b** and **Supplementary Fig. 2a**).

These results confirm that the interaction between NRAS and BRAF is negatively regulated by MEK or ERK in melanoma cell lines carrying a mutant RAS. To test whether MEK or ERK inhibition also increased BRAF activity, we measured its kinase activity in HM11 cells treated with MEK inhibitors U0126 and PD98059. We found that the activity of BRAF increased, in parallel with its interaction with RAS, 24 h after treatment with the MEK inhibitors (**Supplementary Fig. 3**). Together these data show that MEK or a downstream substrate negatively regulates BRAF by inhibiting its interaction with NRAS.

BRAF is constitutively phosphorylated on Ser151

One of the hypotheses that would explain the negative regulation of BRAF in melanoma is a direct phosphorylation of BRAF by ERK kinase, inhibiting its interaction with NRAS. To test this hypothesis, we used a phosphospecific antibody that recognizes certain serines phosphorylated by ERK (in the motif PXSP or SPXR/K). We carried out a time-course treatment with the MEK inhibitor U0126 and then immunoprecipitated BRAF and analyzed its phosphorylation by ERK. We found that BRAF was phosphorylated on an ERK motif and that this phosphorylation persisted for several hours (6–24 h) despite the complete inhibition of ERK after 15 min of treatment with the MEK inhibitor (**Fig. 3a**). In addition, we found that phosphorylation of BRAF on the ERK motif was lost after treatment with two different MEK inhibitors (U0126 or PD98059) in four different melanoma cell lines bearing a mutation in the Ras gene (NRAS G12V Melan-a, C8161, HM11 and WM852; **Fig. 3b**).

To determine whether phosphorylation of BRAF kinase on an ERK motif was specific to melanoma cell lines, we examined BRAF phosphorylation in COS cells. We found that BRAF was phosphorylated in COS cells and that treating these cells with U0126 prevented its phosphorylation (**Supplementary Fig. 1b**). We noticed a decrease in BRAF phosphorylation when it was coexpressed with NRAS G12V, despite the strong ERK activation (**Supplementary Fig. 1b**). These results show that BRAF is phosphorylated by ERK in different cell types but that the constitutive phosphorylation of BRAF by ERK in the presence of oncogenic RAS is specific to melanoma cell lines. By analyzing the amino acid sequence of BRAF, we identified two serines that could be recognized by the antibody used, Ser151 and Ser335. To determine which site was phosphorylated by ERK, we expressed Myc-tagged wild-type BRAF, BRAF S151A and BRAF S335A in normal human epidermal melanocytes (NHEMs). We found that BRAF S151A was not recognized by the phosphospecific antibody, whereas wild-type BRAF and BRAF S335A were recognized, indicating that BRAF is phosphorylated on Ser151 in melanoma cells (**Fig. 3c**).

Furthermore, we generated a rabbit polyclonal phosphospecific antibody to Ser151 (pS151 BRAF). This antibody bound strongly to wild-type BRAF expressed in C8161 melanoma cells, and binding was obliterated with an S151A mutation (BRAF S151A) (**Supplementary Fig. 4a**). Binding of this antibody was also blocked when it was preincubated with the phosphorylated peptide immunogen, but not with the corresponding unphosphorylated peptide (**Supplementary Fig. 4b**), confirming that the antibody is specific for phosphorylated Ser151. Using this antibody, we confirmed that BRAF is phosphorylated on Ser151 in an ERK-dependent manner in all melanoma cell lines tested (**Supplementary Fig. 4b** and data not shown). Moreover, we found that in melanocytes, only Ser151 became phosphorylated upon ERK activation, and that this phosphorylation was inhibited by the MEK inhibitor U0126 (**Supplementary Fig. 2b**). As we observed in melanoma, phosphorylation of Ser151 in melanocytes paralleled the inhibition of NRAS-BRAF interaction (**Supplementary Fig. 2c**). As Ser151 is situated near the Ras-binding domain of BRAF, we suspected that phosphorylation of Ser151 could inhibit the interaction between NRAS and BRAF. To test this hypothesis, we analyzed the interaction of RAS (wild type and G12V) with BRAF (wild type or mutated on Ser151). We found that the S151A mutation enhanced BRAF interaction with Ras (wild type and G12V) whereas a phosphomimetic mutant (BRAF S151D) interacted only weakly with Ras (**Supplementary Fig. 4c**), indicating that phosphorylation of Ser151 inhibits the interaction between NRAS and BRAF.

Oncogenic Ras inhibits cAMP signaling in melanocytes

Although the data presented above can explain the inability of BRAF to activate the MAPK pathway in RAS-mutated melanoma, they cannot explain the imbalance in the cAMP pathway that allows CRAF to be activated by oncogenic RAS in these cells. To analyze the molecular mechanism of cAMP signaling disruption in RAS-mutated melanoma, we first used RAS-transformed melanocytes. Compared with parental

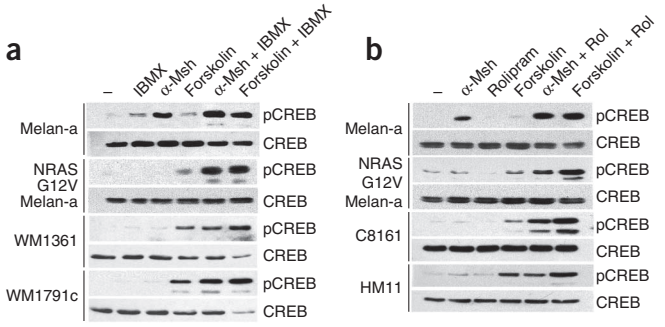

Figure 4 Disruption of the cAMP pathway in melanoma. (**a**) Immunoblots of phosphorylated CREB (pCREB) or total CREB (CREB) in Melan-a, NRAS G12V Melan-a, WM1361 and WM1791c human melanoma cell lines treated for 15 min with IBMX (100 μM), α-Msh (1 μM), forskolin (10 μM) or a combination of α-Msh (1 μM) + IBMX (100 μM) or forskolin (10 μM) + IBMX (100 μM). (**b**) Immunoblots of phosphorylated CREB (pCREB) or total CREB (CREB) in Melan-a, NRAS G12V Melan-a, C8161 and HM11 human melanoma cell lines treated as described above but using rolipram (Rol) (10 μM) instead of IBMX.

Melan-a cells, NRAS G12V Melan-a cells have lost their differentiation characteristics, such as formation of dendrites and expression of melanin (**Supplementary Fig. 5**). Because melanocyte differentiation is driven by the cAMP pathway, these data suggest that this pathway is inactivated in NRAS G12V Melan-a cells. To address this question, we investigated the cAMP pathway in RAS-transformed melanocytes through the analysis of the phosphorylation of a well-characterized PKA substrate, the transcription factor CREB. We found that although α-Msh stimulated CREB phosphorylation in parental Melan-a cells, it did not do so in transformed NRAS G12V Melan-a cells (**Fig. 4**). This confirms that cAMP signaling is uncoupled in transformed cells. However, treatment of NRAS G12V Melan-a cells with a combination of IBMX (a nonspecific phosphodiesterase inhibitor) and either α-Msh or forskolin (activator of adenylyl cyclase) induced CREB phosphorylation (**Fig. 4**).

We obtained similar results in human melanoma cell lines containing oncogenic RAS. α-Msh alone did not stimulate phosphorylation of CREB, but when it was combined with the PDE inhibitor IBMX, it led to a robust phosphorylation of CREB (**Fig. 4**). When PDE activity is blocked, α-Msh can stimulate CREB phosphorylation, demonstrating that melanoma cells have a functional MC1R but that cAMP signaling is inefficient, presumably because its degradation is increased. To address whether cAMP degradation was increased we determined the cAMP-PDE activity in protein extracts from NHEM and melanoma cell lines. Because the PDE family is large and complex, we used a range of PDE inhibitors with different specificities to evaluate the relative contribution of the different families of PDE to the total cAMP-PDE activity, including BRL50481 (specific for PDE7), dipyridamole (specific for PDE5, PDE6 and PDE9–PDE11), rolipram (specific for PDE4) and zaprinast (specific for PDE1, PDE5 and PDE6)[22]. We found that, in melanoma cell lines, cAMP-PDE activity was inhibited only by IBMX and rolipram, indicating that PDE4s are the primary contributor to cAMP-PDE activity in melanoma (**Fig. 5a**). Moreover, all RAS-mutated melanoma cell lines had a higher cAMP-PDE activity than melanocytes, and this activity was sensitive to rolipram (**Fig. 5b**).

To determine the effect of rolipram on the cAMP signaling pathway downstream of α-Msh, we evaluated CREB phosphorylation in response to α-Msh with or without rolipram in Melan-a, NRAS G12V Melan-a and human melanoma cell lines using forskolin as a positive control. As we showed above, α-Msh induced CREB phosphorylation in melanocytes but not in melanoma cells. Although rolipram alone did not induce CREB phosphorylation, it cooperated with α-Msh or forskolin to stimulate robust CREB phosphorylation in all cell lines (**Fig. 4**), suggesting that members of the PDE4 family are responsible for the loss of activation of cAMP in response to α-Msh in melanoma cell lines.

PDE4B2 and PDE4D5 are expressed in melanoma cell lines

We first analyzed the expression pattern of the four PDE4 genes (*PDE4A*, *PDE4B*, *PDE4C* and *PDE4D*)[19] by reverse transcription followed by real-time PCR. We found that NHEM and all melanoma cell lines studied (**Fig. 5c**) expressed the mRNA of all four genes, but that of *PDE4C* only very weakly. At the protein level, PDE4C was not detected in any melanoma cell lines, PDE4A was detected at a very low level in all but one cell line, and PDE4B and PDE4D isoforms were detected in all melanoma cell lines and in NHEMs (**Fig. 6a**). In mouse melanocytes, we detected only PDE4B isoforms, and transformation by NRAS G12V induced an increase in the 70-kDa isoform (which comigrates with PDE4B2) and the appearance of a slower-migrating form (which comigrates with PDE4B3; **Fig. 6b**). As several PDE4B and PDE4D isoforms can comigrate on a western blot[27], we used reverse transcription followed by real-time PCR to identify the PDE4B and PDE4D isoforms expressed in NHEM and human melanoma cells. Using specific probes, we found that human melanoma cell lines expressed mainly the PDE4B2 and PDE4D5 isoforms, whereas normal human melanocytes expressed the PDE4B2 and several PDE4D isoforms (**Supplementary Fig. 6a**,**b**).

To determine which PDE4 protein contributes to the PDE4 activity, we used siRNA to specifically inhibit PDE4A, PDE4B and PDE4D

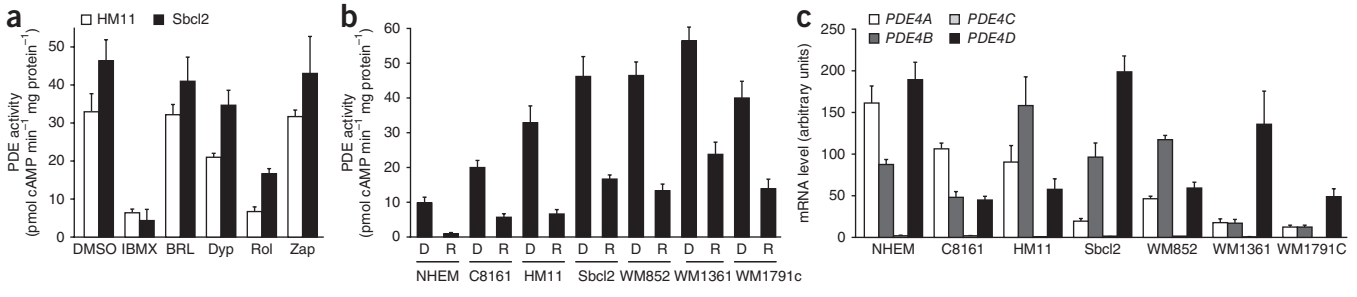

Figure 5 Increased PDE4 activity in melanoma compared with melanocytes. (**a**,**b**) Protein extracts from NHEM or melanoma cell lines were assayed for cAMP-PDE activity in the presence of DMSO (D) or PDE inhibitors as indicated: BRL50481 (BRL), dipyridamole (Dyp), rolipram (Rol or R) and zaprinast (Zap). Values are mean ± s.d. of two independent experiments in duplicate. (**c**) The mRNA level of *PDE4A*, *PDE4B*, *PDE4C* and *PDE4D* was evaluated in NHEM and melanoma cell lines by reverse transcription followed by real-time PCR using primers able to detect all species within a family. See also **Supplementary Figures 6** and **7**.

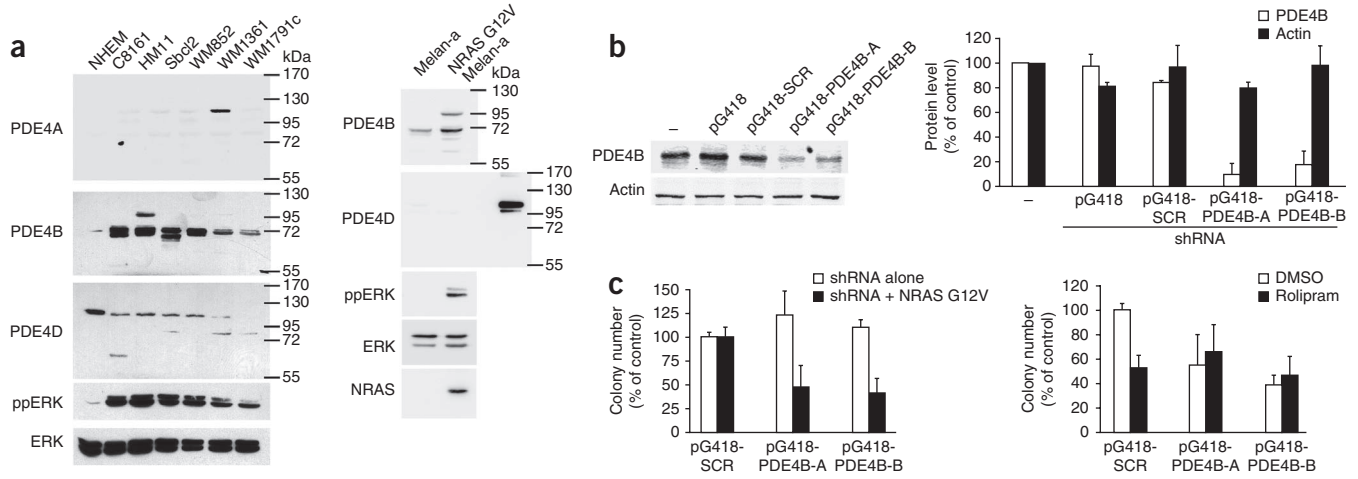

Figure 6 Increased PDE4B2 expression in melanoma compared with melanocytes. (**a**) Immunoblots of PDE4A, PDE4B, PDE4D, phosphorylated ERK (ppERK) and total ERK (ERK) in NHEM, Ras-mutated melanoma, Melan-a and NRAS G12V Melan-a cell lines. (**b**) Immunoblots of PDE4B and actin in Melan-a cells mock transfected (–) or transfected with the empty pG418 vector (pG418), pG418 expressing an shRNA control (pG418-SCR) or an shRNA targeting all splice variants of PDE4B (pG418-PDE4B-A and pG418-PDE4B-B). Graph on right, mean ± s.d. of three independent experiments. (**c**) Counting of colonies of Melan-a cells transfected with a vector expressing NRAS G12V in combination with the pG418 vectors described above and selected with G418 with (shRNA alone) or without (shRNA + NRAS) TPA. Right, cells transfected with NRAS G12V and the indicated shRNA were selected in the presence of DMSO or rolipram (10 μM). Values are mean ± s.d. of three independent experiments.

expression. We determined the effect of siRNA on PDE4 expression at the mRNA level for *PDE4A* (owing to the barely detectable level of protein) and at the protein level for PDE4B and PDE4D. Notably, the knockdown of one isoform was not compensated by an increase in another. This has been observed in other cellular systems and confirms the nonredundant roles of PDE4 isoforms[28]. PDE4 activity was measured as the component that can be selectively inhibited by rolipram[19]. We found that siRNA inhibition of both PDE4B and PDE4D, but not of PDE4A, decreased PDE4 activity, indicating that PDE4B and PDE4D both contribute to PDE4 activity in melanoma cell lines (**Supplementary Fig. 7**).

To ascertain whether expression of PDE4 isoforms was necessary to transform melanocytes with oncogenic Ras, we used Melan-a cells. As mouse melanocytes express only PDE4B2 and not PDE4D (**Fig. 6b**), we used two different shRNAs (PDE4B-A and PDE4B-B) to inhibit PDE4B expression in these cells. We first found that only PDE4B-A or PDE4B-B and not the controls inhibited expression of PDE4B2, but not of actin (**Fig. 6c**). We then co-transfected melanocytes with these shRNA and a vector expressing NRAS G12V, and selected for the stable expression either of NRAS G12V and shRNA or of shRNA alone. Without NRAS G12V, we obtained clones expressing PDE4B-A or PDE4B-B shRNA, suggesting that in normal growing conditions Melan-a cells do not require PDE4B (**Fig. 6c**). In contrast, with NRAS G12V, we obtained fewer clones in the cells transfected with PDE4B-A or PDE4B-B shRNA than with the control, demonstrating that PDE4B expression is necessary to transform melanocytes with oncogenic NRAS (**Fig. 6c**). We reproduced the experiment in the presence of rolipram, and obtained fewer transformed clones in the presence of this PDE4-selective inhibitor than in its absence with the control shRNA but not with the PDE4B shRNA (**Fig. 6c**). These data demonstrate that inhibition of PDE4B by shRNA or rolipram inhibits melanocyte transformation by oncogenic Ras.

The cAMP pathway inhibits ERK and induces apoptosis

As CRAF kinase is negatively regulated by PKA in melanocytes, overexpression of PDE4 isoforms, by inhibiting the cAMP pathway, could allow the reactivation of CRAF in these melanoma cell lines. To test this hypothesis, we inhibited all PDE4 isoforms with a saturating concentration of rolipram[19] and combined this treatment with a suboptimal dose of forskolin to reactivate the cAMP pathway. We found that CRAF activity was suppressed (**Fig. 7a**) and that ERK activity decreased accordingly (**Fig. 7b**). As all analyzed melanoma cells show an increased PDE4 activity (**Fig. 5b**), we investigated whether altered PDE4 activity had a functional effect on cell proliferation and differentiation in melanoma cell lines. We first measured the proliferation of Ras-mutated melanoma cell lines and NHEMs using tritiated thymidine incorporation after treatment of cells by rolipram or forskolin. We found that treatment of cells by rolipram or forskolin alone had little or no effect on the proliferation of all tested melanoma cell lines but that, in contrast, the combination of both compounds induced a marked decrease in proliferation of all melanoma cell lines but not NHEMs (**Fig. 7c**). Thus, under conditions of suboptimal adenylyl cyclase activity, inhibition of PDE4 can suppress the growth regulatory pathway in melanoma but not in melanocytes.

We wanted to determine whether this inhibition of proliferation was linked to induction of differentiation in melanoma cell lines, but we did not detect induction of differentiation markers with the combination of forskolin and rolipram (data not shown). However, we noticed that melanoma cells treated with forskolin and rolipram were dying, and therefore we analyzed the induction of apoptosis after reactivation of the cAMP pathway in these cells. We found that the combination of forskolin and rolipram for 24–48 h induced the cleavage of PARP, caspase 3 and caspase 9 in melanoma cell lines (**Fig. 7d**); these three proteins are markers of apoptosis. Moreover, we labeled the treated cells with annexin V and propidium iodide (PI) and found, 24 h after treatment, a strong increase in annexin$^+$ PI$^-$ cells, an early marker of apoptosis (**Supplementary Fig. 8**).

To compare the effect of PDE4B and PDE4D inhibition on apoptosis, we used siRNA to specifically inhibit expression of either PDE4B or PDE4D. We found that inhibition of either PDE4B or PDE4D sensitized the cells to apoptosis induced by a suboptimal dose of forskolin (**Supplementary Fig. 9**). We could not analyze the effect

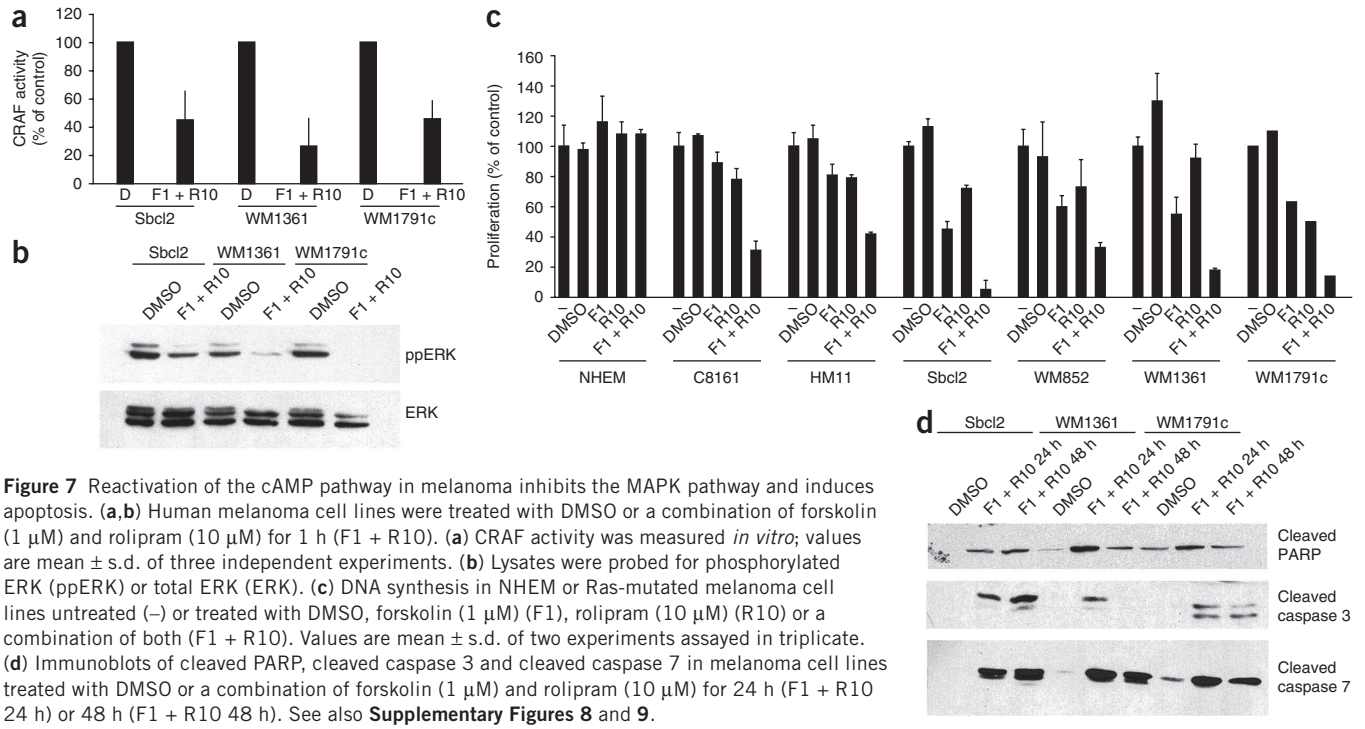

Figure 7 Reactivation of the cAMP pathway in melanoma inhibits the MAPK pathway and induces apoptosis. (**a**,**b**) Human melanoma cell lines were treated with DMSO or a combination of forskolin (1 μM) and rolipram (10 μM) for 1 h (F1 + R10). (**a**) CRAF activity was measured *in vitro*; values are mean ± s.d. of three independent experiments. (**b**) Lysates were probed for phosphorylated ERK (ppERK) or total ERK (ERK). (**c**) DNA synthesis in NHEM or Ras-mutated melanoma cell lines untreated (–) or treated with DMSO, forskolin (1 μM) (F1), rolipram (10 μM) (R10) or a combination of both (F1 + R10). Values are mean ± s.d. of two experiments assayed in triplicate. (**d**) Immunoblots of cleaved PARP, cleaved caspase 3 and cleaved caspase 7 in melanoma cell lines treated with DMSO or a combination of forskolin (1 μM) and rolipram (10 μM) for 24 h (F1 + R10 24 h) or 48 h (F1 + R10 48 h). See also **Supplementary Figures 8** and **9**.

of concomitant inhibition of both PDE4B and PDE4D by siRNA on apoptosis because the simultaneous knockdown of PDE4B and PDE4D was too toxic for the cells. Therefore, as shown for PDE4 activity (**Supplementary Fig. 7**), resistance to apoptosis is carried by both PDE4B and PDE4D in melanoma cells (**Supplementary Fig. 9**). These data indicate that reactivating the cAMP pathway in Ras-mutated melanoma cells induces their apoptosis rather than their differentiation.

DISCUSSION

In normal melanocytes, activation of the MAPK pathway works via BRAF, as constitutive cAMP-induced PKA activity leads to the phosphorylation and inactivation of the other Raf isoform, CRAF[15,25]. However, recent data, including ours, have shed light on the importance of CRAF in the activation of the MAPK pathway in melanoma under certain conditions: (i) in melanomas containing a mutation of Ras[15]; (ii) in melanomas containing low-activity BRAF mutants[29] and (iii) in the resistance of melanoma cells to BRAF inhibitors[30]. We have previously shown that in human melanoma cells harboring a Ras mutation, there is a switch in Raf isoforms to activate the MAPK pathway accompanied by deregulation of the cAMP pathway, meaning that PKA no longer suppresses CRAF in these cells[15]. We demonstrate here that modification of the cAMP metabolism and the switch in RAF isoform were concomitantly induced through transformation of melanocytes by an oncogenic RAS. We show that BRAF cannot activate the MAPK pathway in RAS-mutated melanoma because of a permanent negative-feedback regulation that prevents its association with Ras (**Fig. 2** and **Supplementary Figs. 2** and **3**).

ERK phosphorylates BRAF on Ser151 near its Ras-binding domain, preventing its interaction with RAS; this indicates that ERK-dependent feedback phosphorylation is responsible for the negative effect on the RAS–BRAF interaction (**Fig. 3** and **Supplementary Figs. 2 and 4**). Notably, BRAF does not contain a docking site for ERK but binds to the scaffolding protein KSR1, which has a DEF docking motif for activated ERK[31]. By docking phosphorylated ERK on its DEF motif, KSR1 allows ERK-dependent feedback phosphorylation on BRAF without a need for a direct binding of ERK onto BRAF[31]. BRAF is a substrate of activated ERK that phosphorylates it on four sites: Ser151, Thr401, Ser750 and Thr753. In accordance with our data, Ser151 phosphorylation promotes the dissociation of the BRAF–RAS complex, whereas Thr401, Ser750 and Thr753 phosphorylation control BRAF-CRAF heterodimerization[32,33]. The negative-feedback regulation of BRAF by ERK prevents BRAF overactivation after mitogen activation; the hyperphosphorylated BRAF is recycled to a signaling-competent state by the combined action of PP2A and Pin-1 prolyl-isomerase[33,34]. In accordance with this, we show that in melanocytes, Ser151 of BRAF only becomes phosphorylated after ERK activation to inhibit NRAS-BRAF interaction, thereby preventing overactivation of MEK or ERK (**Supplementary Fig. 2**). However, in melanomas bearing an oncogenic RAS, BRAF phosphorylation on Ser151 is constitutive and very stable, persisting for several hours despite the complete inhibition of ERK by U0126 or PD98059, suggesting that ERK-phosphorylated BRAF is not recycled. On the contrary, CRAF, which binds Ras and activates the MAPK pathway in these cells (**Figs. 1** and **2**), seems insensitive to feedback inhibition. It seems that PP2A and Pin1 target BRAF and CRAF differently in melanoma; we are currently testing this hypothesis. Notably, this phenomenon seems specific to melanoma cells: in COS cells, NRAS G12V expression induces BRAF dephosphorylation on Ser151 (**Supplementary Fig. 1a**), allowing it to bind RAS (**Supplementary Fig. 1a**).

To bypass the negative-feedback regulation of BRAF by the constitutive activation of the MAPK pathway, melanoma cells have developed two strategies: (i) in ~50% of melanomas, cells have acquired a mutation of BRAF (V600E) which renders this kinase independent of RAS binding: BRAF V600E is phosphorylated on Ser151 in melanoma cell lines (data not shown), but because it does not require binding to Ras to activate the MAPK pathway, BRAF V600E is resistant to ERK-dependent negative feedback; (ii) in melanomas bearing a RAS

mutation (~20% of melanoma), cells switch signaling from BRAF to CRAF and consequently, without CRAF, transformation by oncogenic RAS is strongly impaired (**Fig. 1c**). However, to use CRAF to activate the MAPK pathway, these melanoma cells need to inactivate the cAMP pathway, which, in melanocytes, constitutively inhibits CRAF.

We demonstrate here that the modification of the cAMP pathway is induced by oncogenic Ras transformation. We show that Ras-transformed melanocytes and melanoma cell lines harboring RAS mutations do not respond to α-Msh because their signaling is uncoupled at the level of cAMP metabolism, an effect that can be overcome when PDE activity is inhibited. These data highlight the role of PDEs in the constitutive inhibition of the cAMP pathway in melanoma. We show that cAMP-PDE activity in melanocytes and melanoma is due to the PDE4 family of enzymes and that PDE4 activity is increased in melanoma compared with melanocytes (**Fig. 5**). The four PDE4 genes generate >16 different isoforms, each of which are characterized by a unique N-terminal region and, for PDE4B, PDE4C and PDE4D, an ERK phosphorylation site on a C-terminal residue that modulates their activity. Functional PDE4 isoforms can be divided into three major categories: long, short and super-short[19]. Whereas ERK phosphorylation of PDE4 long forms leads to their inhibition, it activates the PDE4 super-short forms[35–37]. We show that melanocytes express several PDE4D isoforms (as recently described[38]), whereas melanoma cell lines express mainly the long PDE4D5 form and the super-short PDE4B2 form, both of which contribute to PDE4 activity and resistance to apoptosis (**Figs. 5** and **6** and **Supplementary Figs. 6**, **7** and **9**). As PDE4B2 is activated by ERK phosphorylation, this cross-talk could contribute to the inhibition of the cAMP signaling pathway, which has been observed in melanoma but not in melanocytes, and which allows reactivation of CRAF . This inhibition of the cAMP pathway also induces the loss of the differentiated characteristics of transformed melanocytes (**Supplementary Fig. 5**). The modification of the expression profile of PDE according to the state of cell differentiation has been described during differentiation of monocytes into macrophages[39]. In melanomas harboring an oncogenic Ras, inhibition of PDE4 by rolipram or siRNA reactivates the cAMP pathway, inhibits CRAF and consequently ERK (**Fig. 7a**,**b**) and impairs transformation by oncogenic RAS (**Fig. 6c**). Our study provides evidence, for the first time, of a physiological role for PDE4 in melanocyte transformation by oncogenic RAS.

Although both inhibition of BRAF and an increase in PDE4 activity are hallmarks of melanocyte transformation by RAS, we did not find a direct link between these events. Inhibition of PDE by a combination of rolipram and suboptimal forskolin did not activate BRAF (**Supplementary Fig. 10a**) and did not rescue BRAF binding to RAS (**Supplementary Fig. 10b**). However, this treatment inhibited CRAF binding to Ras and therefore MAPK activation (**Supplementary Fig. 10b**). Similarly, overexpression of PDE4B2 in melanocytes was not sufficient to induce RAF isoform switching in these cells (**Supplementary Fig. 11a**). Moreover, contrary to recent observations of PDE4D3 in melanocytes[38], we did not find that PDE4D5 or PDE4B2 expression was correlated with MITF expression in melanoma (data not shown). These data suggest that inactivation of BRAF by a feedback mechanism and inhibition of the cAMP pathway that allows CRAF activation are independent events that happen concomitantly to allow melanocyte transformation by oncogenic Ras.

Notably, melanomas harboring a Ras mutation require inhibition of the cAMP pathway to proliferate: reactivating the cAMP pathway with rolipram in combination with a relatively low dose of forskolin induced a marked decrease in the proliferation of melanoma cell lines, but not melanocytes, owing to an induction of apoptosis (**Fig. 7** and **Supplementary Figs. 8** and **9**). The induction of apoptosis by increasing cAMP levels with PDE4 inhibitors has been shown for colon cancer and chronic lymphoid leukemia[40,41], suggesting that the PDE4 inhibitors, in clinical development for different therapeutic indications, could be a new strategy for cancer treatment. Notably, activation of the cAMP pathway in melanocytes drives differentiation and contributes to their proliferation (**Fig. 7**), whereas transformed melanocytes require the cAMP pathway to be inhibited because a rise in cAMP inhibits their proliferation. These data suggest that PDE4 could be a new therapeutic target in melanomas harboring a RAS mutation that are resistant to the therapies currently being developed. Inhibitors developed to treat melanomas harboring the BRAF V600E mutation are ineffective and could even be detrimental in Ras-mutated melanomas in which BRAF is inactivated[42,43]. Moreover Ras-mutated melanomas are fairly resistant to MEK inhibitors[44]. There is currently much interest in the development of selective PDE4 inhibitors for the treatment of asthma, chronic obstructive pulmonary disease and rheumatoid arthritis[45]. Our data suggest that modulating intracellular cAMP by targeting PDE4 with these inhibitors could be a new way of inhibiting the proliferation of melanoma cells containing a Ras mutation.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/nsmb/.

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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AUTHOR CONTRIBUTIONS

A.M., J.A. and N.D. carried out research; M.B., A.B. and N.D. designed and directed the project; N.D. wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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1. MacKie, R.M., Hauschild, A. & Eggermont, A.M. Epidemiology of invasive cutaneous melanoma. *Ann. Oncol.* **20** Suppl 6, vi1–vi7 (2009).
2. Hirobe, T. Role of keratinocyte-derived factors involved in regulating the proliferation and differentiation of mammalian epidermal melanocytes. *Pigment Cell Res.* **18**, 2–12 (2005).
3. Dumaz, N. & Marais, R. Integrating signals between cAMP and the RAS/RAF/MEK/ERK signalling pathways. Based on the anniversary prize of the Gesellschaft fur Biochemie und Molekularbiologie Lecture delivered on 5 July 2003 at the Special FEBS Meeting in Brussels. *FEBS J.* **272**, 3491–3504 (2005).
4. McKay, M.M. & Morrison, D.K. Integrating signals from RTKs to ERK/MAPK. *Oncogene* **26**, 3113–3121 (2007).
5. Karnoub, A.E. & Weinberg, R.A. Ras oncogenes: split personalities. *Nat. Rev. Mol. Cell Biol.* **9**, 517–531 (2008).
6. Wellbrock, C., Karasarides, M. & Marais, R. The RAF proteins take centre stage. *Nat. Rev. Mol. Cell Biol.* **5**, 875–885 (2004).
7. Turjanski, A.G., Vaque, J.P. & Gutkind, J.S. MAP kinases and the control of nuclear events. *Oncogene* **26**, 3240–3253 (2007).
8. Cohen, C. *et al.* Mitogen-actived protein kinase activation is an early event in melanoma progression. *Clin. Cancer Res.* **8**, 3728–3733 (2002).
9. Satzger, I. *et al.* Anal mucosal melanoma with KIT-activating mutation and response to imatinib therapy—case report and review of the literature. *Dermatology* **220**, 77–81 (2010).

10. Monsel, G., Ortonne, N., Bagot, M., Bensussan, A. & Dumaz, N. c-Kit mutants require hypoxia-inducible factor 1α to transform melanocytes. *Oncogene* **29**, 227–236 (2010).
11. Brose, M.S. *et al.* BRAF and RAS mutations in human lung cancer and melanoma. *Cancer Res.* **62**, 6997–7000 (2002).
12. Davies, H. *et al.* Mutations of the BRAF gene in human cancer. *Nature* **417**, 949–954 (2002).
13. Garnett, M.J. & Marais, R. Guilty as charged; B-RAF is a human oncogene. *Cancer Cell* **6**, 313–319 (2004).
14. Wellbrock, C. *et al.* V599EB-RAF is an oncogene in melanocytes. *Cancer Res.* **64**, 2338–2342 (2004).
15. Dumaz, N. *et al.* In melanoma, RAS mutations are accompanied by switching signaling from BRAF to CRAF and disrupted cyclic AMP signaling. *Cancer Res.* **66**, 9483–9491 (2006).
16. Lee, H.J., Wall, B. & Chen, S. G-protein-coupled receptors and melanoma. *Pigment Cell Melanoma Res.* **21**, 415–428 (2008).
17. Lin, J.Y. & Fisher, D.E. Melanocyte biology and skin pigmentation. *Nature* **445**, 843–850 (2007).
18. Cheli, Y., Ohanna, M., Ballotti, R. & Bertolotto, C. 15-year quest in search for MITF target genes. *Pigment Cell Melanoma Res.* **23**, 27–40 (2009).
19. Houslay, M.D. & Adams, D.R. PDE4 cAMP phosphodiesterases: modular enzymes that orchestrate signalling cross-talk, desensitization and compartmentalization. *Biochem. J.* **370**, 1–18 (2003).
20. Baillie, G.S., Scott, J.D. & Houslay, M.D. Compartmentalisation of phosphodiesterases and protein kinase A: opposites attract. *FEBS Lett.* **579**, 3264–3270 (2005).
21. Houslay, M.D. Underpinning compartmentalised cAMP signalling through targeted cAMP breakdown. *Trends Biochem. Sci.* **35**, 91–100 (2010).
22. Lugnier, C. Cyclic nucleotide phosphodiesterase (PDE) superfamily: a new target for the development of specific therapeutic agents. *Pharmacol. Ther.* **109**, 366–398 (2006).
23. Conti, M. & Beavo, J. Biochemistry and physiology of cyclic nucleotide phosphodiesterases: essential components in cyclic nucleotide signaling. *Annu. Rev. Biochem.* **76**, 481–511 (2007).
24. Omori, K. & Kotera, J. Overview of PDEs and their regulation. *Circ. Res.* **100**, 309–327 (2007).
25. Buscà, R. *et al.* Ras mediates the cAMP-dependent activation of extracellular signal-regulated kinases (ERKs) in melanocytes. *EMBO J.* **19**, 2900–2910 (2000).
26. Bennett, D.C., Cooper, P.J. & Hart, I.R. A line of non-tumorigenic mouse melanocytes, syngeneic with the B16 melanoma and requiring a tumour promoter for growth. *Int. J. Cancer* **39**, 414–418 (1987).
27. Shepherd, M. *et al.* Molecular cloning and subcellular distribution of the novel PDE4B4 cAMP-specific phosphodiesterase isoform. *Biochem. J.* **370**, 429–438 (2003).
28. Lynch, M.J. *et al.* RNA silencing identifies PDE4D5 as the functionally relevant cAMP phosphodiesterase interacting with beta arrestin to control the protein kinase A/AKAP79-mediated switching of the beta2-adrenergic receptor to activation of ERK in HEK293B2 cells. *J. Biol. Chem.* **280**, 33178–33189 (2005).
29. Wan, P.T. *et al.* Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. *Cell* **116**, 855–867 (2004).
30. Montagut, C. *et al.* Elevated CRAF as a potential mechanism of acquired resistance to BRAF inhibition in melanoma. *Cancer Res.* **68**, 4853–4861 (2008).
31. McKay, M.M., Ritt, D.A. & Morrison, D.K. Signaling dynamics of the KSR1 scaffold complex. *Proc. Natl. Acad. Sci. USA* **106**, 11022–11027 (2009).
32. Brummer, T., Naegele, H., Reth, M. & Misawa, Y. Identification of novel ERK-mediated feedback phosphorylation sites at the C-terminus of B-Raf. *Oncogene* **22**, 8823–8834 (2003).
33. Ritt, D.A., Monson, D.M., Specht, S.I. & Morrison, D.K. Impact of feedback phosphorylation and Raf heterodimerization on normal and mutant B-Raf signaling. *Mol. Cell. Biol.* **30**, 806–819 (2010).
34. Dougherty, M.K. *et al.* Regulation of Raf-1 by direct feedback phosphorylation. *Mol. Cell* **17**, 215–224 (2005).
35. Hoffmann, R., Baillie, G.S., MacKenzie, S.J., Yarwood, S.J. & Houslay, M.D. The MAP kinase ERK2 inhibits the cyclic AMP-specific phosphodiesterase HSPDE4D3 by phosphorylating it at Ser579. *EMBO J.* **18**, 893–903 (1999).
36. MacKenzie, S.J., Baillie, G.S., McPhee, I., Bolger, G.B. & Houslay, M.D. ERK2 mitogen-activated protein kinase binding, phosphorylation, and regulation of the PDE4D cAMP-specific phosphodiesterases. The involvement of COOH-terminal docking sites and NH2-terminal UCR regions. *J. Biol. Chem.* **275**, 16609–16617 (2000).
37. Baillie, G.S., MacKenzie, S.J., McPhee, I. & Houslay, M.D. Sub-family selective actions in the ability of Erk2 MAP kinase to phosphorylate and regulate the activity of PDE4 cyclic AMP-specific phosphodiesterases. *Br. J. Pharmacol.* **131**, 811–819 (2000).
38. Khaled, M., Levy, C. & Fisher, D.E. Control of melanocyte differentiation by a MITF-PDE4D3 homeostatic circuit. *Genes Dev.* **24**, 2276–2281 (2010).
39. Shepherd, M.C., Baillie, G.S., Stirling, D.I. & Houslay, M.D. Remodelling of the PDE4 cAMP phosphodiesterase isoform profile upon monocyte-macrophage differentiation of human U937 cells. *Br. J. Pharmacol.* **142**, 339–351 (2004).
40. McEwan, D.G. *et al.* Chemoresistant KM12C colon cancer cells are addicted to low cyclic AMP levels in a phosphodiesterase 4-regulated compartment via effects on phosphoinositide 3-kinase. *Cancer Res.* **67**, 5248–5257 (2007).
41. Moon, E.Y. & Lerner, A. PDE4 inhibitors activate a mitochondrial apoptotic pathway in chronic lymphocytic leukemia cells that is regulated by protein phosphatase 2A. *Blood* **101**, 4122–4130 (2003).
42. Fisher, D.E. *et al.* Melanoma from bench to bedside: meeting report from the 6th international melanoma congress. *Pigment Cell Melanoma Res.* **23**, 14–26 (2009).
43. Heidorn, S.J. *et al.* Kinase-dead BRAF and oncogenic RAS cooperate to drive tumor progression through CRAF. *Cell* **140**, 209–221 (2010).
44. Solit, D.B. *et al.* BRAF mutation predicts sensitivity to MEK inhibition. *Nature* **439**, 358–362 (2006).
45. O'Byrne, P.M. & Gauvreau, G. Phosphodiesterase-4 inhibition in COPD. *Lancet* **374**, 665–667 (2009).

ONLINE METHODS

Cell culture. Melan-a cells were cultured in RPMI 1640 (Invitrogen) containing 10% (v/v) fetal calf serum (FCS; Perbio), antibiotics (penicillin-streptomycin; Invitrogen), 200 nM TPA (Sigma), and 300 pM cholera toxin (Sigma). NHEMs (Invitrogen) were cultured in medium 154 supplemented with Human Melanocyte Growth Supplement (Invitrogen). A375 and C8161 human melanoma cell lines were cultured in DMEM (Invitrogen) containing 10% (v/v) FCS. HM11, WM1361 and WM1791c human melanoma cell lines were cultured in RPMI containing 10% (v/v) FCS. Sbcl2 and WM852 human melanoma cell lines were cultured in MCDB153 (Sigma) and L15 medium (Invitrogen) (v/v; 4:1) supplemented with $CaCl_2$ (2 mmol l^{-1}), insulin (5 mg ml^{-1}, Sigma) and 2% (v/v) FCS. All melanoma cell lines used but A375 were mutated on Ras as indicated in **Supplementary Methods**. NHEMs were transfected by nucleofection according to the manufacturer's protocol (Lonza). Melan-a cells were transfected with JetPEI (Polyplus-transfection) according to the manufacturer's instructions and selected by G418 (50 µg ml^{-1}; PAA) with or without TPA (200 nM) and with or without rolipram (10 µM). After 2–3 weeks, clones were either stained with 0.5% (v/v) crystal violet or collected and grown up for further analysis. To measure proliferation, DNA synthesis was determined using [^{3}H]thymidine as described[15].

RNA interference. Transient RNAi was done as described[15]; sequences of the siRNA are in **Supplementary Methods**. For stable RNAi, oligonucleotides (see **Supplementary Methods** for sequences) were cloned in the pG418 vector[46], allowing the stable expression of shRNA and carrying resistance to G418.

Vector construction. Myc epitope–tagged BRAF, hemagglutinin (HA) epitope–tagged CRAF, Flag epitope–tagged NRAS G12V and HA epitope–tagged PDE4B2 were cloned in the PMCEF vectors[47]. Mutations were introduced by site-directed mutagenesis and confirmed by sequence analysis.

Protein expression. Before treatment, cells were maintained for 16 h in TPA-free medium (Melan-a cells) or serum-free medium (human melanoma cell lines). Cells were lysed in 50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% (v/v) NP-40, 5 mM NaF, 2 mM Na_3VO_4, 10 µg ml^{-1} leupeptin and 10 µg ml^{-1} aprotinin, except for in analysis of CREB phopshorylation, in which cells were lysed in Laemmli buffer and sonicated. For immunoprecipitation, samples were incubated with 1 µg Flag (M2; Sigma), Myc (9E10, Santa Cruz Biotechnology) or BRAF (clone F-7, Santa Cruz) antibodies for 1 h and immunoprecipitated by G-Sepharose (GE Healthcare) for 1 h at 4 °C. The immune complexes were washed three times with lysis buffer, proteins were separated by SDS-PAGE and western blot analysis was carried out according to standard protocols using the following antibodies: BRAF (F7); CRAF (C12); Myc (9E10; Santa Cruz); Flag (M2); phospho-ERK (MAPK-YT; Sigma); ERK; GST (Upstate/Millipore); phospho-MEK; phospho-MAPK/CDK substrates (34B2, no. 2325); phospho-CREB (Ser133); CREB; cleaved caspase 3; cleaved caspase 9; cleaved PARP (Cell Signaling Technology); PDE4A (ab14607 Abcam and H-7 Santa Cruz); PDE4B (provided by M. Houslay[48]); PDE4C (ab14608 Abcam); PDE4D (K-16; Everest Biotech and ab14613 Abcam). Antibodies to phosphorylated Ser151 of BRAF were raised in rabbits according to standard protocols by Eurogentec using the peptide ARSNPKpSPQKPI (where pS is phosphorylated serine) coupled to keyhole lymphocyte hemocyanin. The antisera were purified by affinity chromatography with the peptide immunogen. For competition analysis, the purified antibody was preincubated with peptides (10 µM, 2 h at 20 °C). Western blots were visualized and quantified by fluorescence on an Odyssey imaging system. GST pulldown assay was done as described[49].

Kinase assay. BRAF or CRAF was immunoprecipitated from cellular extract and their activity toward MEK was measured *in vitro*. Briefly, immunoprecipitated BRAF or CRAF was incubated with 5 µg purified GST-MEK in 30 mM Tris, pH 7.5, 100 µM EDTA, 10 mM $MgCl_2$, 0.1% (v/v) Triton 100, 5 mM NaF, 1 mM ATP, 0.3% (v/v) β-mercaptoethanol for 30 min at 30 °C. Reaction was stopped by adding Laemmli buffer and analyzed by SDS-PAGE with the phospho-MEK and BRAF antibodies. Phosphorylated MEK, BRAF and CRAF were visualized and quantified by fluorescence on an Odyssey imaging system.

cAMP-PDE activity assay. The PDE activity was assayed as described[50].

Apoptosis assay. Apoptotic cells were stained with by annexin V–fluorescein isothiocyanate and propidium iodide according to the manufacturer's instructions (Beckman Coulter) and analyzed by flow cytometry.

Reverse transcription and real-time PCR. Total RNA was extracted from NHEM and melanoma cell lines (RNeasy Mini Kit, QIAGEN) and treated with DNase (Invitrogen); cDNA was prepared using the Themoscript kit (Invitrogen). *PDE4A*, *PDE4B*, *PDE4C* and *PDE4D* mRNA levels were quantified by real-time PCR normalized to *GAPDH* using primers described in **Supplementary Methods**, which amplify either all known slice variants of each subtype (indicated as pan-primers) or specific variants as indicated in the figures.

46. Cahill, A.L., Herring, B.E. & Fox, A.P. Stable silencing of SNAP-25 in PC12 cells by RNA interference. *BMC Neurosci.* **7**, 9 (2006).
47. Marais, R., Light, Y., Paterson, H.F., Mason, C.S. & Marshall, C.J. Differential regulation of Raf-1, A-Raf, and B-Raf by oncogenic ras and tyrosine kinases. *J. Biol. Chem.* **272**, 4378–4383 (1997).
48. Huston, E. *et al.* Molecular cloning and transient expression in COS7 cells of a novel human PDE4B cAMP-specific phosphodiesterase, HSPDE4B3. *Biochem. J.* **328**, 549–558 (1997).
49. Moodie, S.A., Willumsen, B.M., Weber, M.J. & Wolfman, A. Complexes of Ras.GTP with Raf-1 and mitogen-activated protein kinase kinase. *Science* **260**, 1658–1661 (1993).
50. Marchmont, R.J. & Houslay, M.D. Insulin trigger, cyclic AMP-dependent activation and phosphorylation of a plasma membrane cyclic AMP phosphodiesterase. *Nature* **286**, 904–906 (1980).