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The Brn-2 Transcription Factor Links Activated BRAF to Melanoma Proliferation

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Malignant melanoma, an aggressive and increasingly common cancer, is characterized by a strikingly high rate (70%) of mutations in BRAF, a key component of the mitogen-activated protein (MAP) kinase signaling pathway. How signaling events downstream from BRAF affect the underlying program of gene expression is poorly understood. We show that the Brn-2 POU domain transcription factor is highly expressed in melanoma cell lines but not in melanocytes or melanoblasts and that overexpression of Brn-2 in melanocytes results in increased proliferation. Expression of Brn-2 is strongly upregulated by Ras and MAP kinase signaling. Importantly, the *Brn-2* promoter is stimulated by kinase-activating BRAF mutants and endogenous Brn-2 expression is inhibited by RNA interference-mediated downregulation of BRAF. Moreover, silent interfering RNA-mediated depletion of Brn-2 in melanoma cells expressing activated BRAF leads to decreased proliferation. The results suggest that the high levels of Brn-2 expression observed in melanomas link BRAF signaling to increased proliferation.

The mitogen-activated protein (MAP) kinase signaling pathway, comprising the RAS-RAF-MEK-ERK cascade, plays a key role in the cellular response to extracellular signals, and deregulation of the pathway, for example, as a result of activating mutations in Ras, is a feature of many cancers. Three Raf genes have been described (ARAF, BRAF, and CRAF) that are regulated by interaction with Ras, and RAF protein can phosphorylate and activate the MAP kinase kinase MEK. The importance of this signaling pathway in cancer has recently been highlighted by the observation that almost 70% of melanomas and primary nevi exhibit kinase-activating mutations in BRAF (11, 26). Understanding how signaling by the MAP kinase pathway affects the underlying program of gene expression is therefore a particularly important issue for melanoma, and it is presumed that deregulation of MAP kinase signaling results in altered expression or activity of key transcription factors, leading to aberrant growth control. The identification of BRAF target genes currently represents a major goal in melanoma biology.

The POU domain transcription factor Brn-2 (also called N-Oct3 and POU3F2) (see reference 28 for a review) has been implicated in neuronal differentiation (16) and activation of the corticotropin-releasing hormone gene (22, 29). Targeted disruption of the *Brn-2* gene in the mouse results in loss of specific neuronal lineages in the hypothalamus and consequent loss of the posterior pituitary gland (25, 29). Brn-2-negative mice therefore die within 10 days after birth, although the specific cause of death is not apparent. Although *Brn-2* clearly plays a major role in neuronal development, evidence also implicates it in melanoma growth and survival. Thus, *Brn-2* mRNA is overexpressed in melanoma compared to normal melanocytes (15, 33, 34), and *Brn-2* expression is substantially downregulated by differentiating agents (32). Importantly, downregulation of Brn-2 with an antisense strategy resulted in a decreased growth rate and loss of tumorigenicity in SCID mice, while complete loss of Brn-2 led to cell death in culture (34). Thus, Brn-2 may represent a critical factor in melanoma growth and survival. Understanding the controls operating on *Brn-2* expression is therefore a key issue.

Given the overexpression of Brn-2 in melanoma cells and the deregulation of the MAP kinase signaling pathway arising from mutations in the BRAF gene, we asked whether Brn-2 expression was regulated by MAP kinase signaling in general and by BRAF in particular. The results indicate that Brn-2 expression is strongly upregulated by MAP kinase-dependent signaling and, significantly, that Brn-2 expression in melanoma cell lines could be substantially reduced by using small interfering RNAs (siRNAs) to specifically downregulate BRAF expression. We also show that overexpression of Brn-2 in melanocytes leads to increased proliferation, while silent interfering RNA (siRNA)-mediated downregulation of Brn-2 in melanoma cells leads to decreased proliferation. The results suggest that the high level of Brn-2 expression observed in melanomas links BRAF signaling to increased proliferation.

MATERIALS AND METHODS

***Brn-2* promoter reporters.** The *Brn-2* promoter-luciferase reporter used in this work was constructed as follows. The *Brn-2* upstream sequence (GenBank accession no. M88301) between the EcoRI site located at about −2.5 kb and the KpnI site at −520 with respect to the ATG initiator codon was cloned into pUC18; the EcoRI site was then converted to a SalI site by cutting with EcoRI, repairing with Klenow, and ligating an 8-bp SalI linker. The proximal *Brn-2* promoter and leader sequence between the upstream KpnI site and the ATG translation initiation codon was isolated by PCR with primers which maintained

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the 5′ KpnI site and placed a novel HindIII site at the 3′ end. This KpnI-HindIII fragment was then ligated to the engineered SalI-KpnI fragment in pUC18 so that the entire *Brn-2* promoter and leader sequence extending to but not including the ATG initiation codon could be excised by SalI and HindIII. The SalI-HindIII fragment was then cloned upstream from the luciferase reporter in pGL3. The original *Brn-2* genomic clone used to isolate the *Brn-2* promoter has been described elsewhere (20).

Cell lines and transfection assays. Melan-c cells are a highly differentiated melanocyte-derived cell line (2) derived from an albino mouse and were cultured in RPMI supplemented with 10% fetal calf serum and 200 ng of phorbol myristyl acetate per ml. All other cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Melan-a, melb, and melan-a Hm cells (35) and primary melanocytes were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 200 nM tetradecanoyl phorbol acetate, and 1.2 nM cholera toxin. For luciferase assays, cells were seeded at a density of 1.5×10^3 cells/2 cm^2 in a 24-well plate the day before transfection; 25 ng of promoter-reporter construct was transfected with increasing amounts of plasmid expressing activators with Fugene (Boehringer-Mannheim) according to the manufacturer's instructions. An equal total amount of DNA was maintained by compensation with empty expression vector DNA. Cells were harvested 48 h after transfection and assayed for firefly luciferase activity, and the results were normalized to either the *lacZ* activity of a cotransfected simian virus 40-*lacZ* reporter in the case of the *Brn-2*-luciferase test plasmids or to a simian virus 40-luciferase reporter in the case of the *Brn-2* promoter-*lacZ* reporters, to control for transfection efficiency for assays of promoter deletion mutants. All transfection assays were repeated multiple times and at least four times for the Ras and BRAF cotransfection studies. The β-catenin expression vectors have been described previously (21) and were provided by Rudi Grosschedl.

Reverse transcription-PCR was performed as described previously (4, 5) with aliquots of each reaction mixture taken for analysis every four cycles to determine the kinetics of the PCR process. Samples presented were those taken from the log phase of the reaction, and comparisons between different samples were made after the same number of PCR cycles. The results from the gel analysis were confirmed by real-time PCR and quantitated with a Light Cycler (Roche).

Retroviruses. The simian virus 5 (SV5) epitope was also introduced into the BamHI site of the pBabePuro retrovirus vector (24). Details of construction are available on request. The *Brn-2* cDNA was isolated by PCR from a genomic clone (20) with primers that place BamHI sites at each end. The *Brn-2* BamHI fragment was then inserted into the SV5 epitope vectors. The pBabePuro vector expressing the constitutively active version of MEK (MEK.EE) as a His-tagged protein was kindly provided by Chris Marshall. Viruses were produced after transfection of the pBabePuro DNA into the Psi2 packaging cell line, and infections into melan-a cells were performed as described previously (24).

Band shift assays. The band shift assays for Brn-2 were performed in a final volume of 20 μl containing 20 mM HEPES (pH 7.9), 10% glycerol, and 112 mM KCl; nuclear extracts were prepared as described previously (41).

Western blot analysis. Whole-cell extracts were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 10% polyacrylamide). Nitrocellulose membranes were used for transfer, and after being blocked with 10% skim milk (99% fat free)–0.1% Tween 20–phosphate buffer, membranes were probed with appropriate primary antibodies (for 1 h to overnight at 4°C). Proteins were detected with peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin secondary antibody and visualized with the ECL detection kit (Amersham). Note that for the results shown in Fig. 3, blots were blocked with bovine serum albumin, and consequently a nonspecific band was picked up by the anti-Brn-2 monoclonal antibody.

The primary antibodies used were the mouse monoclonal anti-Brn-2 antibody that we raised against bacterially expressed full-length mouse Brn-2 protein; anti-ERK2, anti-Rsk, and anti-BRAF antibodies purchased from Santa Cruz; anti-diphospho-Rsk from Biolabs; anti-CRAF monoclonal antibody and anti-diphospho-ERK antibody from Sigma; and anti-α-tubulin from Amersham.

siRNA-mediated downregulation of Brn-2 and BRAF. A 21-base Brn-2-specific siRNA was synthesized by Dharmacon. The sequence used was 5′-GCGCAGA GCCUGGUGCAGGUU-3′ and its complement, leaving a 3′ UU overhang on both strands. The siRNA control for Brn-2 was 5′-UUCUCCGAACGUGUCA CGUdTdT-3′ and its complement, leaving a dTdT 3′ overhang on both strands. The siRNA was transfected into cells with Oligofectamine (Invitrogen) as per the manufacturer's instructions, and cells were harvested after 3 days, by which time Brn-2 was effectively downregulated.

For siRNA-mediated downregulation of BRAF, WM266.4 cells were seeded at 2.5×10^3 cells per six-well plate the day before transfection. Cells were transfected in 1 ml of Optimem with 6 μl of 20 μM BRAF-specific siRNA (5′-AAGUGGCAUGGUGAUGUGGCA-3′) or 6 μl of 20 μM control siRNA (5′-AAGUCCAUGGUGACAGGAGAC-3′) with Oligofectamine. After 5 h, 1 ml of Dulbecco's modified Eagle's medium containing 10% fetal calf serum was added, and the cells were cultured overnight. Then the medium was replaced by Dulbecco's modified Eagle's medium with 1% fetal calf serum, and the cells were harvested and lysed after a further 60 h.

[^{3}H]thymidine incorporation. Cells were seeded at a density of 2×10^4 in 12-well plates and left overnight before transfection with specific or control siRNA. Three days later, cells were labeled by incubation for 4 h at 37°C with [^{3}H]thymidine with 10 μl of medium of the following thymidine stock: 24 μl of [^{3}H]thymidine (Amersham, TRK686; 87 Ci/mmol) in 600 μl of unlabeled 10 nM thymidine (final concentration, 0.4 μCi/ml). Cells were then trypsinized and harvested onto glass fiber filters (Millipore APFF02500) with the12-place sampling manifold from Millipore (Millipore XX 27 025 50). Filters were then washed twice with water and once with 70% ethanol and transferred to scintillation vials before being dried for 30 min at 80°C, followed by scintillation counting.

RESULTS

Overexpression of Brn-2 in melanoma cell lines. Previous work with polyadenylated mRNA and Northern blotting established that *Brn-2* mRNA is expressed to higher levels in some melanoma cell lines than in melanocytes (15, 34), and consistent with this, reverse transcription-PCR demonstrated at least 10- to 30-fold more *Brn-2* mRNA in two human (HMB2 and 501 mel) and one mouse (K1735) melanoma cell line compared to a melanocyte cell line, melan-c (Fig. 1A). No expression of *Brn-2* was detected in Cos7 cells. The elevated expression of *Brn-2* mRNA observed in melanoma cells was also reflected in the protein, which was highly expressed in all human melanoma cell lines tested with the exception of the uveal melanoma line VUP (Fig. 1B). The highest levels of Brn-2 protein were observed in the highly undifferentiated mouse melanoma line K1735, while the relatively differentiated mouse melanoma cell line B16 expressed Brn-2 at very low levels. Brn-2 protein was not readily detected in either the melanoblast cell line melb, the melanocyte cell line melan-c, or primary mouse melanocytes (Fig. 1C).

Brn-2 controls melanocyte proliferation. The high levels of Brn-2 observed in melanoma cell lines, coupled to the fact that members of the POU domain family regulate many aspects of development, raised the possibility that increased Brn-2 expression would contribute significantly to the transformed phenotype. To determine how elevated Brn-2 expression might influence melanocyte growth or differentiation, we infected the highly differentiated and pigmented melanocyte cell line melan-a with either a retrovirus expressing an SV5 epitope-tagged Brn-2 or control virus expressing an epitope only. Puromycin-resistant colonies were expanded for further analysis. The initial characterization of the clones (not shown) confirmed that they were expressing ectopic Brn-2, and three clones (cl.14, cl.11, and cl.5) exhibiting various levels of Brn-2 expression were chosen for further analysis.

Using an anti-Brn-2 antibody, we compared the total level of Brn-2 expressed in the three Brn-2-expressing clones to the parental cell line melan-a as well as to that observed in the A375 melanoma line. The result (Fig. 2A) revealed that the level of Brn-2 expression in clone 5 was similar to that observed in the A375 melanoma cell line, while the level of Brn-2 expressed in clones 11 and 14 was somewhat lower but nevertheless substantially higher than the background level of Brn-2 expression in melan-a cells or a clone of melan-a cells infected with an empty SV5-tagged retrovirus. In all assays, the melan-a

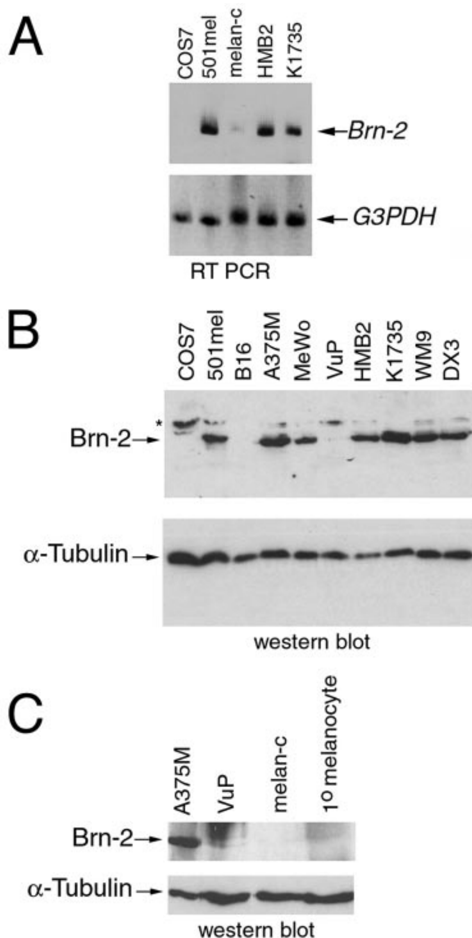

FIG. 1. *Brn-2* is expressed at high levels in melanoma cell lines. (A) Reverse transcription-PCR showing relative levels of *Brn-2* mRNA in the indicated cell lines compared to a glyceraldehyde-3-phosphate dehydrogenase (G3PDH) control. 501 mel and HMB2 are human melanoma cell lines, melan-c is a mouse melanocyte cell line, and K1735 is an undifferentiated mouse melanoma cell line. Cos7 cells were used as a negative control. (B and C) Western blots with an anti-Brn-2 monoclonal antibody, showing relative levels of Brn-2 protein expressed in the indicated cell lines compared to the expression of α-tubulin. Melan-c is a mouse melanocyte cell line, melb is a mouse melanoblast line, 1° melanocytes are primary mouse melanocytes, and B16 and K1735 represent relatively differentiated and undifferentiated mouse melanomas, respectively. Cos7 cells were used as a negative control. The remaining cell lines were derived from human melanomas, VUP representing a uveal melanoma.

cells infected with the empty retrovirus behaved identically to the parental melan-a cell line. The level of Brn-2 DNA-binding activity present in the most highly expressing clone was similar to that observed in a range of melanoma cell lines (not shown).

Visual examination of the cells revealed that, compared to the parental melan-a cells, the clones overexpressing the Brn-2 protein tended to be less dendritic and appeared less pigmented (Fig. 2B), with clone 14, which expressed the least Brn-2, exhibiting a phenotype more closely resembling that of melan-a cells than the other Brn-2-positive clones. Like the parental melan-a cell line, those overexpressing Brn-2 retained their tetradecanoyl phorbol acetate dependence (not shown). Importantly, however, the overexpression of Brn-2 in all clones tested led to an increase in [^{3}H]thymidine incorporation compared to the melan-a cells, with incorporation of [^{3}H]thymidine in the clone expressing the highest level of Brn-2 during a 20-min pulse being similar to that observed in the 501 mel melanoma cell.

Taken together, the results suggest that increasing the expression of Brn-2 confers a less differentiated phenotype on melanocytes. However, like the parental melan-a cells, those expressing ectopic Brn-2 were not able to form tumors in nude mice.

Brn-2 expression is elevated by MAP kinase signaling downstream from a melanoma-associated receptor tyrosine kinase. In melanoma cells, *Brn-2* mRNA levels are elevated about 10-fold compared to melanocytes (Fig. 1A) (15) but may be

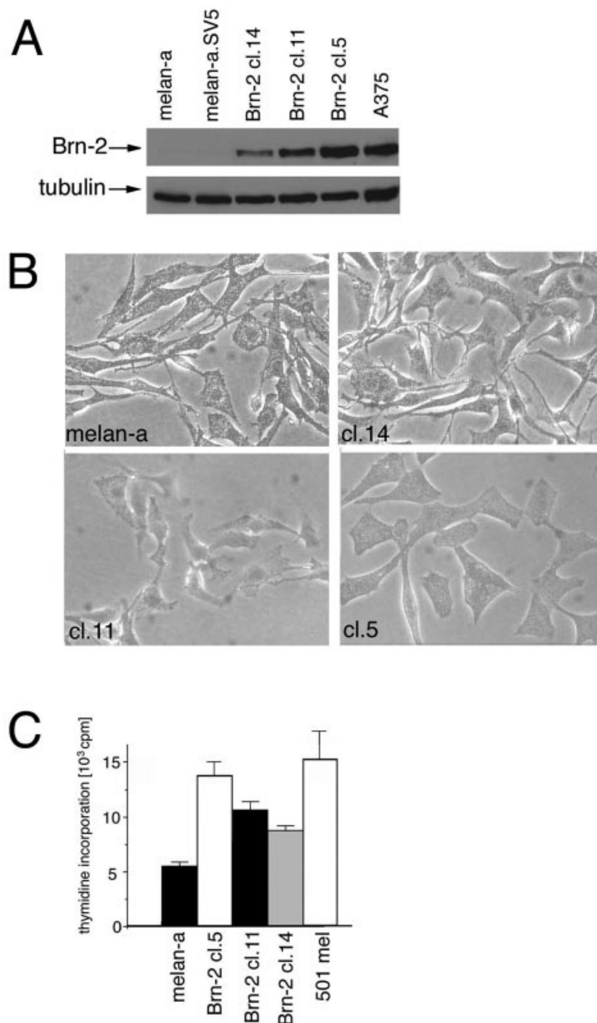

FIG. 2. Brn-2 overexpression results in increased [^{3}H]thymidine incorporation into melanocytes. (A) Western blot with anti-Brn-2 antibody of melan-a cells, melan-a cells infected with an empty retrovirus (SV5), or three independent clones of melan-a cells infected with a retrovirus expressing SV5 epitope-tagged Brn-2. The A375 melanoma cell line was used a positive control. The same blot was also probed for α-tubulin as a loading control. (B) Phase contrast image of melan-a cells and the indicated clones of melan-a cells stably overexpressing Brn-2 from the pBabePuro retrovirus. (C) [^{3}H]thymidine incorporation into the indicated cell lines.

reduced by treatment of cells with the differentiating agents butyric acid and dimethyl sulfoxide (32). In contrast, in the central nervous system, *Brn-2* expression appears to be upregulated in postmitotic neurons of the developing mouse neocortex (19). These observations would indicate that *Brn-2* expression is regulated, although the nature of the signal transduction pathways operating to target the *Brn-2* promoter is not known. One feature of melanoma cells is deregulation of the MAP kinase signaling pathway, either through expression of a variety of receptor tyrosine kinases (RTKs) which would normally be present in melanoblasts but not in melanocytes (6, 12, 13) or, as has been shown recently for a the majority of melanomas (70%) and nevi, by acquiring kinase-activating mutations in BRAF (11, 26).

We initially investigated the possibility that Brn-2 might represent a downstream target for RTKs implicated in melanoma formation or progression. One of the best-characterized and most potent melanoma-associated RTKs is the *Xiphophorus* melanoma receptor tyrosine kinase Xmrk (37). The Xmrk receptor is known to efficiently activate the MAP kinase pathway (35), and importantly, inheritance of Xmrk leads to melanoma. However, few downstream targets of Xmrk have been identified to date. The overexpression of Brn-2 in melanoma cell lines led us to suspect that Brn-2 expression would be strongly upregulated in response to Xmrk signaling via the MAP kinase pathway.

To test this, we made use of melan-a cells that were stably transfected with a vector expressing a chimeric receptor (EGFR-mrk) consisting of the intracellular domain of the Xmrk receptor fused to the extracellular ligand-binding domain of the human epidermal growth factor receptor (EGFR). In these cells, which do not express endogenous EGFR, signaling via the Xmrk intracellular domain, depicted in Fig. 3A, is EGF responsive, and stimulation with EGF leads to strong and prolonged upregulation of MAP kinase signaling that is inhibited by the U0126 MEK inhibitor (Fig. 3B) as well as by transformation characterized by increased growth and dedifferentiation (36). In the parental melan-a cells and melan-a Hm cells which expressed the EGFR-mrk chimera, very little endogenous Brn-2 could be detected when the cells were cultured in normal growth medium (Fig. 3C). In contrast, melan-a Hm cells grown in the presence of EGF were found to express Brn-2. Induction of Brn-2 expression by the RTK occurred during 24 h and increased with time, as substantially more Brn-2 protein was found in cells grown in EGF for 48 h (Fig. 3D). Significantly, the increase in Brn-2 expression resulting from activation of the EGFR-mrk receptor was completely inhibited in the presence of the U0126 inhibitor of the MAP kinase kinase MEK (Fig. 3E), providing a key indication that elevated Brn-2 expression in melanoma is likely to result from induced MAP kinase signaling.

Significantly, activation of MAP kinase signaling in melanocytes by targeted expression of activated Ras in conjunction with loss of the *INK4a* locus can lead to tumor formation in a mouse melanoma model (8). Given the important role of MAP kinase signaling downstream from Ras, we asked whether Ras signaling could upregulate *Brn-2* expression. Importantly, robust expression of endogenous Brn-2 protein was also detected in melan-a cells stably transformed with Ras, whereas Brn-2 protein was barely detected in the parental melan-a melanocyte cell line (Fig. 4A). Note, however, that the ability of Ras

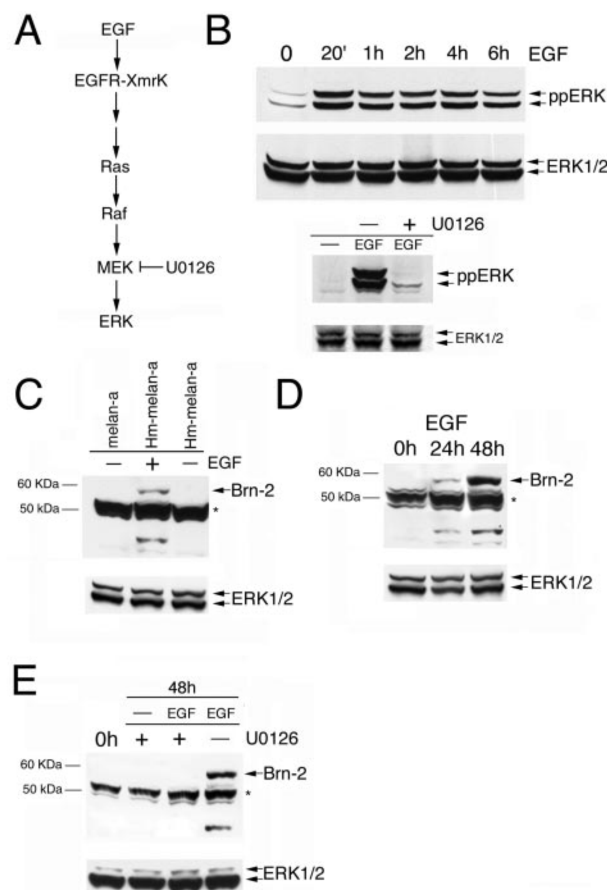

FIG. 3. Robust activation of *Brn-2* expression in response to a melanoma-associated RTK. (A) Schematic showing the MAP kinase signaling pathway downstream from the HER-mrk chimeric receptor in which the ligand binding domain of the EGFR is fused to the intracellular signaling domain of the Xmrk RTK. U0126 is a specific inhibitor of the MAP kinase kinase MEK. (B) Western blot showing expression of ERK in cells expressing the HER-mrk fusion protein (melan-a Hm) (36) grown in the presence of EGF (50 ng/ml). The smaller panel indicates that EGF-mediated stimulation of MAP kinase was inhibited by treatment with the MEK inhibitor U0126. (C) Western blot with anti-Brn-2 antibody, showing that Brn-2 expression is induced by treatment of cells expressing the HER-mrk fusion protein (melan-a Hm) with EGF (50 ng/ml) for 24 h. The location of Brn-2 is indicated, and the asterisk indicates a nonspecific band that cross-reacted with the antibody. This band was apparent when bovine serum albumin rather then skim milk was used as a blocking agent for the Western blot. Parental melan-a cells and melan-a Hm cells grown in the absence of EGF are shown as controls. The blot was reprobed with anti-ERK2 polyclonal antibody as a loading control. No expression of Brn-2 was observed in the absence of treatment. ERK2 was used as a loading control. (D) Western blot with anti-Brn-2 monoclonal antibody of melan-a Hm cells grown in either the absence or presence of EGF (50 ng/ml) for 24 or 48 h, as indicated. The blot was probed with anti-Brn-2 monoclonal or anti-ERK2 polyclonal antibodies. (E) Induction of Brn-2 expression by Xmrk requires MAP kinase signaling. Melan-a Hm cells were stimulated with EGF for 48 h in the presence or absence of 20 μM U0126. Cell extracts were Western blotted with anti-Brn-2 or anti-ERK2 antibody, as indicated.

to upregulate Brn-2 expression is cell type specific, as Ras-transformed NIH 3T3 cells do not express Brn-2 (not shown).

Although Ras can act through multiple signaling pathways, the most likely explanation of these results, given that the

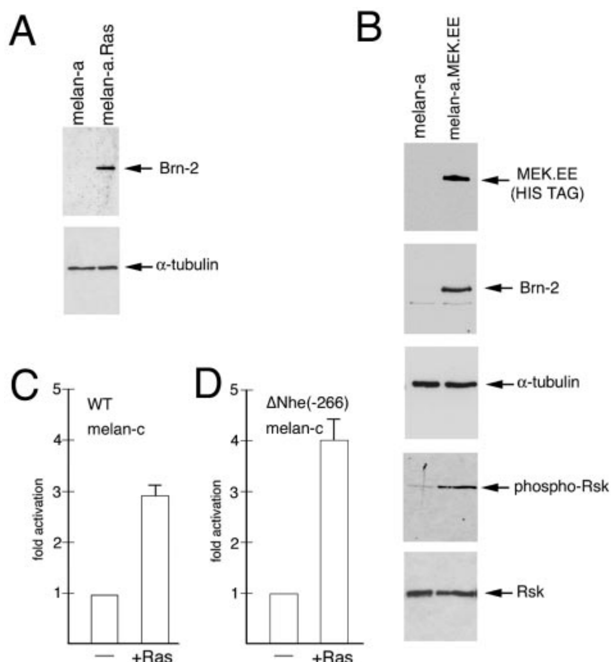

FIG. 4. Ras- and MAP kinase-induced expression of the *Brn-2* promoter. (A) Western blot with anti-Brn-2 or anti-α-tubulin antibodies and either melan-a cells or Ras-transformed melan-a cells, as indicated. (B) Western blot of melan-a cells and a melan-a cell line stably expressing constitutively activated MEK (MEK.EE). MEK.EE expression was determined with an anti-His tag antibody. Antibodies against Brn-2, Rsk, phospho-Rsk, and α-tubulin were used where indicated. (C and D) Regulation of the *Brn-2* promoter by Ras. The wild-type *Brn-2* promoter fused to a luciferase reporter extending to −2.3 kb or to −266 (ΔNhe) was transfected into the melan-c melanocyte cell line together with either an empty expression vector or a vector expressing activated Ras (RasN12), as indicated. The results presented are the averages of four experiments.

U0126 MEK inhibitor can block the induction of Brn-2 expression by the EGFR-mrk RTK, is that Ras-mediated activation of the MAP kinase pathway in melanocytes leads to upregulation of Brn-2 expression. To verify this, we infected melan-a cells with a retrovirus expressing a His-tagged, constitutively active form of the MAP kinase kinase (MEK.EE) and selected for expressing cells. The cell lines obtained were screened for expression of MEK.EE with an anti-His tag antibody and were also probed for expression of Brn-2. The results from a representative cell line are shown in Fig. 4B. The expression of activated MEK led to elevated levels of Brn-2 expression substantially higher that that of the parental melan-a cells. Tubulin expression was used as a loading control, and elevated activation of the MAP kinase pathway in the cells expressing MEK.EE was confirmed with antibodies against total RSK or diphosphorylated (activated) RSK.

The results from the melan-a cell lines expressing either Ras or constitutively activated MEK indicated that the expression of Brn-2 was upregulated by MAP kinase signaling. Consistent with MAP kinase activating transcription of the *Brn-2* gene, the expression of activated Ras in melan-c melanocytes raised the activity of a 2.3-kb *Brn-2* promoter by about threefold (Fig. 4C) and that of a *Brn-2* promoter deleted to −266 (*Nhe*I) by fourfold (Fig. 4D). Given that the −266 promoter retained its responsiveness to Ras signaling, it is likely that transcription factors binding downstream from −266 mediate the Ras effect. Within this region of the promoter, there are several full consensus binding sites for transcription factors such as SP1, NFY, and AP1, and DNA binding assays confirmed that SP1 and NFY bind the *Brn-2* promoter in vitro (not shown). Since these factors are known to be Ras and MAP kinase responsive (1, 23, 40, 42, 43), it is likely that the ability of Ras to activate Brn-2 expression is mediated at least in part by the combination of these factors acting in concert at the promoter.

Brn-2 is upregulated by activated BRAF. The results so far indicated that Brn-2 expression can be strongly activated by MAP kinase signaling downstream from a melanoma-associated RTK or Ras in engineered melanocytes. We next wished to determine whether Brn-2 expression is controlled by the same signaling pathway in melanomas and specifically whether signaling by BRAF is implicated. With two melanoma cell lines that have characterized BRAF kinase-activating mutations, A375M and WM266.4 (11), Brn-2 expression was measured by Western blotting of cells cultured in the presence or absence of the MEK (MAP kinase kinase) inhibitor U0126. In the presence of the inhibitor, the phosphorylated form of ERK2 was absent after U0126 treatment, while the total levels of ERK2 in the cell were unaffected (Fig. 5A). Significantly, inhibition of the MAP kinase pathway abolished Brn-2 expression. Thus, Brn-2 expression in melanoma cell lines requires MAP kinase signaling.

To ask whether kinase-activating mutations in BRAF could activate the Brn-2 promoter, B16 cells, which have low levels of constitutive MAP kinase activity (not shown), were cotransfected with a *Brn-2* promoter–luciferase reporter together with vectors expressing wild-type or mutated BRAF proteins. The results obtained (Fig. 5B) indicated that while expression of the wild-type BRAF protein had little effect on *Brn-2* promoter activity, the promoter was strongly activated, up to 20-fold, by the most common BRAF mutant, V599E, which is found in the majority of melanomas and nevi (11, 26), weakly by the G463V mutant, and to an intermediate level by the G468A mutant. The level of activation of the *Brn-2* promoter by these BRAF mutants corresponds well with their basal kinase activity (11).

We have recently shown that Brn-2 expression can also be activated by β-catenin signaling via the LEF1 site in the promoter (18). To determine whether BRAF and β-catenin can cooperate in activation of Brn-2 expression, we cotransfected cells with the *Brn-2* promoter–luciferase reporter either alone or together with low levels of the BRAF (V599E) or a β-catenin expression vector or both. Although the BRAF and β-catenin expression vectors alone can activate the *Brn-2* promoter up to 20-fold (Fig. 5B) (18), reducing the level of reporter used in this experiment limited the activation of the promoter to a maximum of 2-fold (Fig. 5C). In contrast, if BRAF and β-catenin were coexpressed, *Brn-2* promoter activity was induced about eightfold. These results suggest that the BRAF/MAP kinase and Wnt/β-catenin pathways may in some circumstances act cooperatively to activate Brn-2 expression.

To determine whether the expression of endogenous Brn-2 is also controlled by BRAF signaling in melanoma cells, we made use of siRNA to downregulate BRAF in the melanoma cell line WM266.4. These cells carry an activating V599D mutation in the BRAF gene (11). With these reagents, we estab-

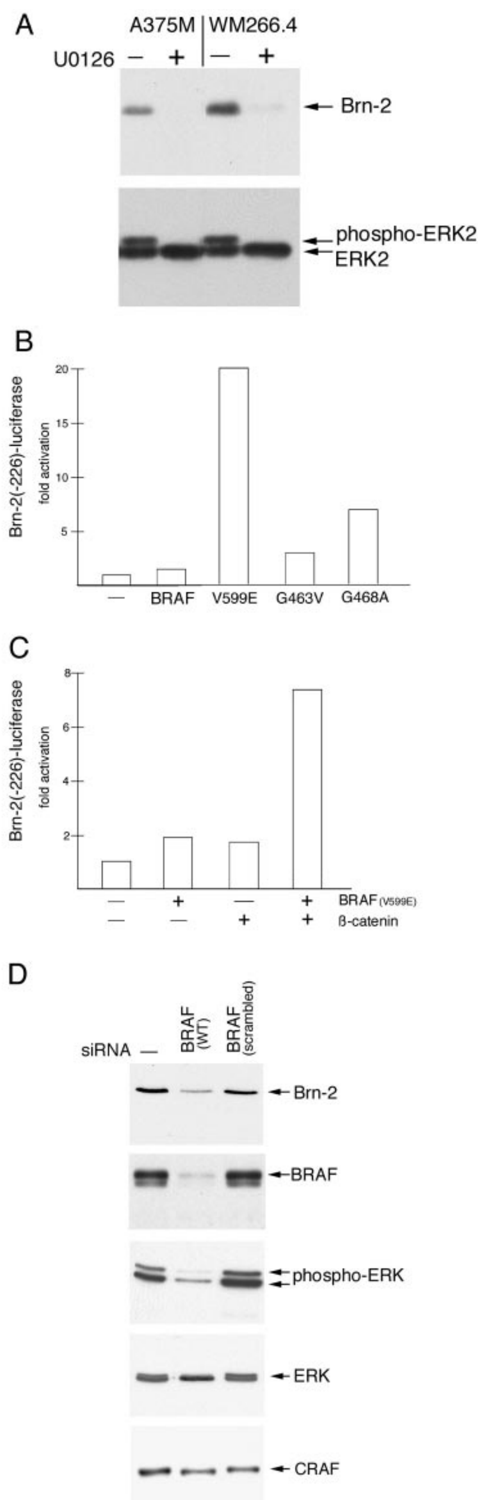

FIG. 5. *Brn-2* is a target for BRAF signaling. (A) Western blot of human melanoma cell lines A375M and WM266.4, which express constitutively active forms of BRAF (11), with either an anti-Brn-2 antibody or anti-ERK2 antibody. Where indicated, cells were treated for 24 h with the MEK inhibitor U0126 at a concentration of 10 μM. (B) BRAF activates the *Brn-2* promoter. B16 melanoma cells were transfected with the *Brn-2* promoter (−266)–luciferase reporter (500 ng) and either an empty vector or a vector expressing wild-type BRAF or the indicated BRAF mutants (200 ng). (C) BRAF and β-catenin can cooperate for activation of the *Brn-2* promoter. B16 melanoma cells were transfected with the *Brn-2* promoter (−266)-luciferase reporter (100 ng) and either an empty vector or a vector expressing wild-type BRAF, β-catenin, or both, as indicated. Luciferase activity was determined 48 h posttransfection. (D) siRNA-mediated inhibition of BRAF leads to loss of MAP kinase activity and Brn-2 expression. Shown is a Western blot of WM266.4 cells either untreated or transfected for 60 h with BRAF-specific siRNA or a scrambled oligonucleotide control with either anti-Brn2 monoclonal, anti-ERK2, or anti-phospho-ERK2 antibodies, where indicated.

lished that in these cells, BRAF is the primary RAF isoform responsible for signaling to ERK; CRAF does not appear to mediate ERK activity in these cells (Maria Karasarides and R. Marais, submitted for publication). Treatment of the WM266.4 cells with BRAF-specific siRNA leads to a reduction in BRAF expression of over 80% and as a consequence leads to a strong suppression in ERK activity (Fig. 5D). Similar results were obtained with A375M cells, a melanoma line that carries a V599E activating mutation in the BRAF gene (not shown). By contrast, treatment with a scrambled siRNA control did not affect BRAF expression, and ERK activity remained high (Fig. 5D). Importantly, the BRAF siRNA probe did not affect the expression of either CRAF or ERK. However, consistent with BRAF playing a key role in regulating Brn-2 expression, Brn-2 levels were substantially reduced following treatment with the BRAF-specific siRNA but not with the scrambled control.

Brn-2 expression enhances proliferation in melanoma cells. Overexpression of Brn-2 in melan-a melanocytes resulted in enhanced proliferation. To determine whether Brn-2 expression also contributes to proliferation in melanoma cells, we used a Brn-2-specific siRNA to downregulate Brn-2 in the WM266.4 cell line, which expresses high levels of Brn-2 as a result of constitutive BRAF signaling. The Brn-2 siRNA used is highly specific and does not affect the levels of the related Oct1 POU domain protein (18). Western blotting of Brn-2 showed that treatment with the Brn-2-specific siRNA but not a control siRNA resulted in a robust downregulation of Brn-2 expression after 48 h of treatment and that loss of Brn-2 expression persisted at least until 96 h (Fig. 6A). ERK expression was used as a loading control. Moreover, treatment with the Brn-2-specific siRNA led to the cells' adopting a more elongated shape than untreated cells or cells transfected with a control nonsilencing siRNA (Fig. 6B), a result consistent with that obtained by overexpressing Brn-2 in melanocytes, which led to their adopting a less dendritic phenotype (Fig. 2B). As a measure of proliferation, [^{3}H]thymidine incorporation was determined for untreated cells and cells treated with either the control siRNA or the Brn-2-specific siRNA for 72 or 96 h. The results (Fig. 6C) revealed that treatment with the Brn-2-specific siRNA but not the control siRNA led to a marked decrease in [^{3}H]thymidine incorporation. This result suggests that Brn-2 expression contributes substantially to proliferation in melanoma cells in which BRAF signaling is constitutively active.

DISCUSSION

Activating mutations in BRAF leading to constitutive activation of the MAP kinase signaling pathway are a key feature of melanoma (11). Importantly, acquisition of activating BRAF

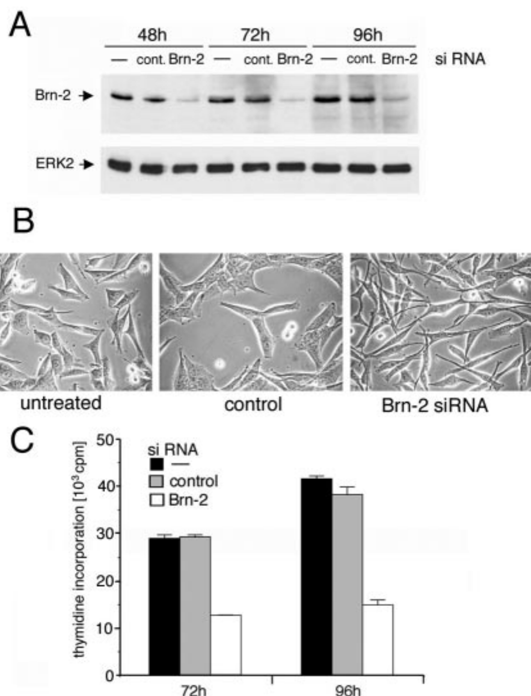

FIG. 6. siRNA-mediated downregulation of Brn-2 leads to decreased [^{3}H]thymidine incorporation in melanoma cells. (A) Western blot with anti-Brn-2 antibody of WM266.4 cell cells transfected with a Brn-2-specific siRNA or a control nonsilencing siRNA for the indicated times. Untransfected cells were used as an additional control. (B) Phase contrast image of WM266.4 cells transfected with a Brn-2-specific siRNA or a control nonsilencing siRNA, as indicated. (C) [^{3}H]thymidine incorporation into untransfected WM266.4 cells or cells transfected with a Brn-2-specific siRNA or a control nonsilencing siRNA, as indicated, either 72 or 96 h posttransfection.

mutations is likely to be involved in melanoma initiation, with over 80% of primary nevi bearing the V599E substitution (26). If the implications of BRAF activation for the control of proliferation and survival of melanoma cells are to be understood, it is essential to characterize key transcription factors that lie downstream from BRAF signaling. Here we provide several lines of evidence that point to Brn-2 as a BRAF effector downstream from the MAP kinase cascade: Ras and BRAF activate the *Brn-2* promoter in transfection assays; endogenous Brn-2 expression is elevated in melanocytes stably transformed with Ras or a constitutively active form of MEK; Brn-2 expression is induced by a melanoma-associated RTK; and Brn-2 expression in melanoma cell lines can be blocked by an inhibitor of the MAP kinase pathway.

Although it was evident from our results both with the ectopic expression of a melanoma-associated RTK in melanocytes and with the use of the U0126 MEK inhibitor that MAP kinase signaling was critical for expression of Brn-2, a key question arising from this observation was whether BRAF was specifically implicated. We addressed this question by transfecting two melanoma cell lines with characterized activating BRAF mutations with an siRNA specific for BRAF. Elimination of BRAF in these cells led to a complete suppression of ERK activity without affecting CRAF levels. Moreover, we detected a concomitant drop in Brn-2 expression with the BRAF-specific siRNA but not with a control nonsilencing siRNA. These results clearly implicate BRAF in the maintenance of Brn-2 expression.

BRAF mutations are found in the majority of primary nevi (26), and it seems likely that expression of activated BRAF may be necessary but not sufficient for the development of melanoma. Activated BRAF may therefore be important in the proliferation of melanocytes, leading to nevus formation, and most likely will also contribute to proliferation of melanoma cells. With Brn-2 expression being linked to BRAF-mediated MAP kinase signaling, a key question that arises is whether Brn-2 is in any way implicated in melanocyte proliferation.

Since BRAF signaling via the MAP kinase pathway will affect a wide range of downstream targets, such as the stability of the microphthalmia-associated transcription factor, which plays a key role in melanocyte development (17, 38, 39), it seemed unlikely that expression of Brn-2 alone would have a major impact on melanocyte growth or differentiation. Yet remarkably, the ectopic expression of Brn-2 in melanocytes to a level comparable to that seen in many melanoma cell lines led to increased [^{3}H]thymidine incorporation compared to the parental melanocyte cell line. Moreover, in a melanoma cell line in which Brn-2 expression is maintained by constitutive BRAF activity, siRNA-mediated downregulation of Brn-2 led to decreased [^{3}H]thymidine incorporation. These results, coupled with previous observations that also linked the elevated expression of Brn-2 in melanomas to tumorigenicity and proliferation (34), suggest that Brn-2 may represent a critical factor in melanoma growth and survival.

Given the link between BRAF signaling and Brn-2 expression, it therefore seems likely that Brn-2 plays an instrumental role in facilitating BRAF-mediated hyperproliferation of melanocytes. However, while overexpression of Brn-2 increases the proliferation of melanocytes, it does not render them tetradecanoyl phorbol acetate independent or confer an ability to form tumors in nude mice. Interestingly, targeted expression of activated Ras to the melanocyte lineage in mice also fails to generate tumors unless the host animal is also deficient in the *INK4a* locus (7, 8). Investigating the oncogenic potential of Brn-2 in the melanocyte lineage in different genetic backgrounds as well as understanding how Brn-2 controls proliferation are clearly important goals arising from this study.

The presence of activating BRAF mutations in the majority of melanomas (11) may account for the high levels of Brn-2 found in these cells compared to normal melanocytes. Indeed, of the melanoma cell lines for which BRAF and Brn-2 expression status has been determined, all 12 lines that are known to express activated BRAF also overexpress Brn-2. In contrast to cutaneous melanomas, uveal melanomas do not exhibit BRAF mutations (9, 10, 14, 27), and the uveal melanoma cell line VUP does not exhibit high levels of Brn-2. The observation that Brn-2 expression can also be induced by activation of an RTK in melanocytes also suggests that in some melanomas not bearing activating BRAF mutations, Brn-2 may still be expressed as a result of the aberrant expression of melanoma-associated RTKs normally expressed during melanocyte development (6, 12). Although our data implicate MAP kinase signaling downstream from RTKs and BRAF in Brn-2 expression, it will be important to determine whether Brn-2 expres-

sion can be detected in nevi bearing activated BRAF mutations or whether it will only be detected at later stages of the disease, when deregulation of additional signaling pathways may act cooperatively with the MAP kinase cascade to induce Brn-2 expression. Nevertheless, it is apparent that given an appropriate cellular environment, activation of the MAP kinase cascade, either by activation of RTKs or via activation of BRAF, is sufficient to trigger Brn-2 expression and is also necessary to maintain Brn-2 levels.

Finally, although this study focused on its expression in melanomas, *Brn-2* is expressed in both neuroblastomas and small cell lung carcinomas (30, 31) and T-cell lymphomas (3). Whether *Brn-2* expression in these cancers is also linked to activation of the MAP kinase pathway has yet to be determined but seems likely, given our results on melanoma. Similarly, our results are also likely to be relevant to development, where precise and coordinated activation of MAP kinase signaling downstream from key RTKs may make a major contribution to the cell type-specific expression of Brn-2 in neuronal lineages, where, for example, *Brn-2* plays a key role in the development of the hypothalamus (25, 29). The identification of the MAP kinase signaling pathway as a critically important control in its expression marks a significant step forwards in our understanding of both the regulation of *Brn-2*, a key developmental and cancer-related transcription factor, and the downstream events resulting from mutation of BRAF.

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