

## Brn-2 Expression Controls Melanoma Proliferation and Is Directly Regulated by $\beta$ -Catenin

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**Constitutive activation of the Wnt/ $\beta$ -catenin signaling pathway is a notable feature of a large minority of cases of malignant melanoma, an aggressive and increasingly common cancer. The identification of target genes downstream from this pathway is therefore crucial to our understanding of the disease. The POU domain transcription factor Brn-2 has been implicated in control of proliferation and melanoma survival, and its expression is strongly upregulated in melanoma. We show here that *in vivo* Brn-2 is expressed in melanocytes but not in embryonic day 11.5 melanoblasts and that its expression is directly controlled by the Wnt/ $\beta$ -catenin signaling pathway in melanoma cell lines and in transgenic mice. Moreover, silent interfering RNA-mediated inhibition of Brn-2 expression in melanoma cells overexpressing  $\beta$ -catenin results in significantly decreased proliferation. These results, together with the observation that BRAF signaling also induces Brn-2 expression, reveal that Brn-2 is a focus for the convergence of two key melanoma-associated signaling pathways that are linked to cell proliferation.**

Melanocytes originate in the neural crest as undifferentiated nonpigmented melanoblasts that migrate to the epidermis and hair follicles, where they differentiate and are responsible for skin and hair color. As melanocytes are not essential for survival and as mutations affecting the survival or differentiation of the melanocyte lineage are reflected in an obvious pigmentation phenotype, the melanocyte system represents an excellent model for understanding how signal transduction pathways coordinate the program of gene regulation underlying the genesis of a specific cell type. Importantly, constitutive activation of signaling pathways normally operating during melanocyte development is linked to the transformation of a melanocyte to a malignant melanoma (5, 16), a highly aggressive cancer, the incidence of which is increasing at an alarming rate (33).

The Wnt signaling pathway (for reviews of Wnt signaling, see references 3 and 12) is critically required for development of the melanocyte lineage; in both zebrafish and mice, overexpression of components of the Wnt signaling pathway result in an increase in the number of melanocytes at the expense of neurons and glia (9, 11), and disruption of the *Wnt-1* and *Wnt-3a* genes leads to complete loss of melanoblasts (24). Wnt proteins interact with frizzled receptors and lead to the inhibition of serine-threonine kinase glycogen synthase kinase 3 $\beta$ . Phosphorylation of  $\beta$ -catenin by glycogen synthase kinase 3 $\beta$  is associated with the destabilization of  $\beta$ -catenin. Thus, increased Wnt signaling leads to stabilization of  $\beta$ -catenin and its translocation from the cytoplasm to the nucleus, where it can

activate transcription via association with the Lef1 and Tcf transcription factors (1, 22, 29). A key role for Wnt signaling in melanocyte development is the activation of the promoter for the gene encoding the microphthalmia-associated transcription factor Mitf (10, 43). Mitf (19, 23) is essential for the development of the melanocyte lineage and has two key functions: in control of cell proliferation and survival and in differentiation (17).

The link between melanocyte development and melanoma is underscored by the fact that many melanomas exhibit constitutively elevated levels of nuclear  $\beta$ -catenin (34, 36). Given the key role played by  $\beta$ -catenin in development, it is likely that the elevated level of this protein observed in melanomas makes a substantial contribution to their transformed phenotype, and indeed, activation of the Wnt pathway has been linked to a metastatic or migratory phenotype (30). The identification of  $\beta$ -catenin target genes therefore represents an important goal if the link between melanocyte development and malignant melanoma is to be understood.

Using both cell lines and transgenic mice, we show here that the promoter for the gene encoding the POU domain transcription factor Brn-2 (also called N-Oct3 and POU3F2) that has been implicated in control of proliferation and survival of melanoma cells is a direct target for  $\beta$ -catenin/Lef1 and that reduction of Brn-2 expression by silent interfering RNA (siRNA) results in a decrease in proliferation. The results provide a novel insight into the link between melanocyte development and melanoma and  $\beta$ -catenin and regulation of proliferation.

### MATERIALS AND METHODS

**Cell lines and transfection assays.** The mouse melanocyte cell line melan-a was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (Gibco-BRL) and 200 nM tetradecanoyl phorbol acetate (Sigma), as was the melan-c melanocyte cell line. Mouse melanoma cell line B16 and human melanoma cell lines 501 mel and VUP were grown in RPMI 1640 medium supple-

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mented with 10% fetal calf serum. COS7, 3T3, and XB2 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. All cells were grown at 37°C with 10% CO<sub>2</sub>, and lysates for Western blotting or band shift assays were prepared from subconfluent cultures.

Brn-2-luciferase assays were performed as described previously (15). Cells were seeded at a density of  $1.5 \times 10^3$  cells/2 cm<sup>2</sup> 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. For cotransfections, 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 those with a cotransfected simian virus 40-*lacZ* reporter.

Brn-2-*lacZ* assays were performed as described previously (27). Cell lines were seeded into 2.5-cm dishes and transfected when 40% confluent with 1 μg of plasmid DNA with Fugene. A cotransfected simian virus 40-luciferase reporter was used to control for transfection efficiency. All transfection assays were repeated multiple times.

**siRNA-mediated downregulation of Brn-2 and β-catenin.** A 21-base Brn-2-specific siRNA was synthesized by Dharmacon. The sequence used was 5'-GC GCAGAGCCUGGUGCAGGUU-3' and its complement, leaving a 3' UU overhang on both strands.

For β-catenin we used the siRNA described previously (47), 5'-AAAGCU GAUAUUGAUGGACAGdTdT-3' and its complement, leaving a dTdT 3' overhang on both strands. The siRNA control was 5'-UUCUCCGAACGUGU CACGUdTdT-3' and its complement, leaving a dTdT 3' overhang on both strands. 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 or β-catenin was effectively downregulated.

**Immunofluorescence and bromodeoxyuridine incorporation.** Cells treated with siRNA specific for Brn-2 or a control siRNA were incubated with medium containing bromodeoxyuridine labeling reagent (Roche) for 1 h before fixation with 4% paraformaldehyde, washed three times with phosphate-buffered saline (PBS), and permeabilized with 0.2% Triton X-100 in PBS. After washing four times with PBS, treatment for 30 min with 2 M HCl, washing again four times with PBS, and blocking for 1 h with 10% fetal bovine serum, bromodeoxyuridine incorporation was detected with a mouse monoclonal antibromodeoxyuridine primary antibody (Becton Dickinson) and a Texas Red-conjugated anti-mouse immunoglobulin secondary antibody. After being washed four times in PBS, cells were mounted in Vectashield (Vector Laboratories) containing 4',6'-diamidino-2-phenylindole (DAPI) to visualize DNA.

**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, the 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).

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-β-catenin antibody from Santa Cruz (C-18); an anti-α-tubulin antibody (Amersham); and mouse anti-bromodeoxyuridine monoclonal antibody from Becton Dickinson Biosciences. Rabbit anti-Oct1 antibody was a gift from Peter O'Hare.

**Chromatin immunoprecipitation assays.** Chromatin immunoprecipitation assays were performed as described previously (15) with goat polyclonal anti-Lef1 antibody (Santa Cruz C-19), goat polyclonal anti-β-catenin antibody (Santa Cruz C-18), or 10 μl of nonspecific immunoglobulin G (IgG) (Bio-Rad). After immunoprecipitation, samples were analyzed by quantitative PCR for 25 cycles, taking care that the PCR was in the log phase of amplification. For the HSP70 high-cycle control, the PCRs were allowed to proceed for a further five cycles.

The primers used for the PCR were 5'-GAGGAGGGCTAGGAGGACTC C-3' and 5'-CGCGTAACTGTCAATGAAAAA-3' for the *Brn-2* promoter; 5'-TCGGAAGTGGCAGTATTCCGG-3' and 5'-TTTAGGTGGCACCACCAATC C-3' for the *MITF* promoter; and 5'-CCTCCAGTGAATCCCAGAAGACTC T-3' and 5'-TGGGACAACGGGAGTCACTCTC-3' for the *HSP70* promoter.

**Plasmids.** The Brn-2 promoter-luciferase reporter has been described (18). The point mutations in the Lef1 binding site were introduced by PCR with the appropriate primers.

The β-catenin expression vector has been described previously (21) and was provided by Rudi Grosschedl.

**Whole-mount, histological in situ hybridizations and X-Gal staining of mouse**

**embryos.** Riboprobes were prepared by in vitro transcription with T7 and T3 RNA polymerases from pBS-Brn2 and with digoxigenin-11-UTP (Roche) according to the manufacturer's instructions. A 403-bp PCR fragment, corresponding to nucleotides 314 to 717 starting from the ATG of mouse Brn-2, was subcloned into pBluescript II and used as a template for the Brn-2 riboprobes. Timed matings were set up to obtain staged mouse embryos, designating the noon of plug formation as embryonic day 0.5 (E0.5). E11.5 embryos and postnatal day 1 (P1) newborn mice were fixed for 4 h room temperature and overnight in 4% paraformaldehyde in PBS at 4°C, respectively. Thereafter they were either dehydrated and stored in 100% methanol at -20°C or equilibrated in 15% sucrose for 2 days at 4°C, embedded in 15% (wt/vol) sucrose-7.5% (wt/vol) gelatin in PBS and frozen at -80°C and cryosectioned (16 μm). Whole-mount and histological in situ hybridizations were performed according to a procedure described by Wehrle-Haller et al. (48). The hybridization of digoxigenin-labeled riboprobes was detected with antidigoxigenin antibodies coupled to alkaline phosphatase. Details of the protocol will be provided upon request. For detection of β-galactosidase activity in whole mounts, *DCT-lacZ* transgenic embryos were fixed in 0.25% glutaraldehyde in PBS for 30 min at 4°C and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) as previously described (20, 28).

**Band shift assays.** The band shift assays for Lef1 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 (51). Lef1 protein was expressed as a glutathione *S*-transferase (GST) fusion, purified, and then removed from the GST by thrombin cleavage.

## RESULTS

**Expression of Brn-2 is β-catenin responsive.** The POU domain transcription factor Brn-2 is overexpressed in a number of cancers, including Merkel cell carcinoma (26), small cell lung carcinoma (39), neuroblastoma (38, 40), and T-cell lymphoma (2), and we and others have also shown that *Brn-2* mRNA expression is upregulated in many melanomas compared to melanocyte cell lines (14, 45). Since the majority of cutaneous melanomas (8), though not uveal melanomas (6, 7, 13, 35), bear activating mutations in BRAF, the elevated expression of Brn-2 observed in many melanomas may be accounted for by the observation that Brn-2 expression is strongly upregulated by BRAF-mediated activation of the mitogen-activated protein kinase signaling cascade (18). In addition to BRAF mutations, a subset of melanomas, typified by the 501 mel human melanoma cell line, are characterized by elevated levels of β-catenin (34, 36). Since Brn-2 expression is high in the 501 mel melanoma line that expresses high levels of β-catenin (Fig. 1A) but low in a human uveal melanoma line, VUP, and in the mouse B16 melanoma and melan-a melanocyte cell lines, which exhibit low β-catenin levels, we wished to determine whether Brn-2 expression was controlled by the β-catenin signaling pathway. Note that a low level of β-catenin can be detected in the other cell lines on longer exposure (data not shown).

As a first step towards understanding whether the control of *Brn-2* expression might be responsive to β-catenin, we asked whether the *Brn-2* promoter was able to direct cell type-specific expression in transfected cells. To this end, a *Brn-2* promoter fragment extending to about -2.5 kb with respect to the ATG initiation codon, or -2.3 kb upstream from the transcription start site, was cloned upstream from a *lacZ* reporter, and its activity was assayed after transfection. Similar results were obtained irrespective of whether a *lacZ* or luciferase (not shown) reporter was used. Several cell lines were used for transfection: melan-c, an albino melanocyte cell line, which expresses *Brn-2* mRNA at a low level (18); the human 501 mel

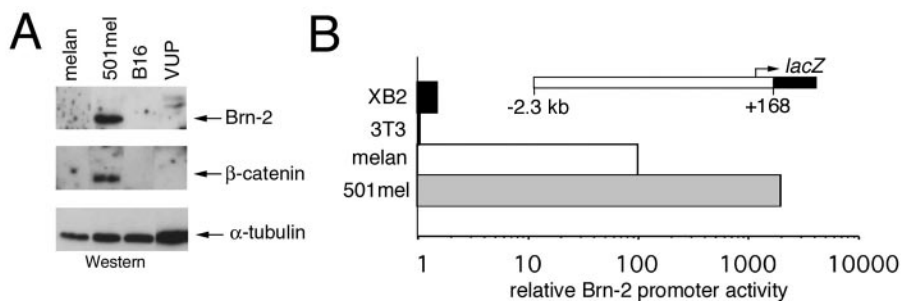


FIG. 1. Brn-2 and  $\beta$ -catenin expression in melanocyte and melanoma cell lines. (A) Extracts from the indicated melanocyte (melan-a) and melanoma (B16, 501 mel, and VUP) cell lines were subjected to Western blotting with the indicated antibodies. Note that a low level of  $\beta$ -catenin could be detected in all cell lines on longer exposure. (B) The *Brn-2* promoter is highly active in the 501 mel cell line. The melanocyte cell line melan-c, the human melanoma cell line 501 mel, NIH 3T3 cells, and the keratinocyte cell line XB2 were transfected with the indicated promoter construct driving expression of a *lacZ* reporter, and  $\beta$ -galactosidase activity was assayed 48 h posttransfection. All results are presented relative to those obtained with a cotransfected simian virus 40 promoter-luciferase reporter.

melanoma cell line, which expresses *Brn-2* RNA and protein at a high level; and NIH 3T3 cells and XB2 keratinocytes, which do not express *Brn-2*, as assessed by Northern blotting (not shown). The results from the transfection assays (Fig. 1B), normalized for the expression of a cotransfected simian virus 40 promoter-reporter plasmid, revealed that the *Brn-2* promoter is about 100-fold more efficiently expressed in the melanocyte cell line melan-c than in NIH 3T3 or XB2 cells but is also 20-fold more active in the 501 mel melanoma cell line compared to melan-c cells. Thus, 2.3 kb of the *Brn-2* promoter sequence contains sufficient information to direct differential expression in cells in culture, and the expression pattern appears to reflect that of the endogenous *Brn-2* gene.

The fact that the *Brn-2* promoter was so active in the 501 mel melanoma cells raised the possibility that *Brn-2* expression might be regulated by  $\beta$ -catenin's targeting the *Brn-2* promoter. Consistent with this, examination of the *Brn-2* promoter revealed a consensus potential high-affinity binding site for the Lef1 and Tcf transcription factors, ATCAAAG (Fig. 2A). That this element was a potential  $\beta$ -catenin response element was confirmed with an in vitro DNA-binding assay (Fig. 2B). With sequences spanning the putative Lef1 binding site as a probe, this element bound Lef1 as efficiently as a control consensus binding site but did not bind Lef1 if the core sequence was mutated to ATtcAAG (Lef1.m1).

To test whether this sequence was functional, we used a *Brn-2* promoter-luciferase reporter together with vectors expressing  $\beta$ -catenin in cotransfection assays in 501 mel cells. The *Brn-2* promoter used extends upstream to -2.3 kb, and the results obtained (Fig. 2C) indicate that the *Brn-2* promoter can be activated by  $\beta$ -catenin in this assay. Deletion analysis revealed that a promoter deletion mutant lacking sequences 5' to -266 retained its ability to respond to  $\beta$ -catenin but that removal of additional sequences to -184 or -111 eliminated the response, consistent with the location of the putative  $\beta$ -catenin-responsive element at -247 to -241. The ability of  $\beta$ -catenin to activate the *Brn-2* promoter was also abolished if the Lef/Tcf binding site were mutated (-266.LEF.m1), confirming the critical role of this element in the response of the *Brn-2* promoter to  $\beta$ -catenin signaling.

To verify that Lef1 and  $\beta$ -catenin could bind the *Brn-2* promoter in melanoma cells, we next performed a chromatin im-

muno-precipitation assay. In this assay, chromatin was prepared from cross-linked 501 mel cells and sheared to an average size of about 750 bp before being subjected to immunoprecipitation with either an anti-Lef1 or an anti- $\beta$ -catenin antibody. The DNA recovered after the immunoprecipitation was then used in a PCR together with primers specific for the *Brn-2* promoter. As a negative control, we used primers specific for the *HSP70* promoter, which is not regulated by  $\beta$ -catenin, and as a positive control for the Lef1 chromatin immunoprecipitation, we used primers specific for the *MITF* promoter, a known Lef1/ $\beta$ -catenin-target. The results, presented in Fig. 3A, reveal that a strong band corresponding to the *Brn-2* promoter was obtained with both the anti-Lef1 and anti- $\beta$ -catenin antibodies, similar to that obtained for the *MITF* promoter with the anti-Lef1 antibody. With nonspecific IgG in the immunoprecipitation, no PCR product above the level of the background was observed. For the *HSP70* negative control, no PCR product was observed if a similar number of PCR cycles were used for the *MITF* and *Brn-2* promoters, and if the PCR was allowed to proceed (*HSP70* high cycles), an equivalent level of product was obtained irrespective of whether a specific antibody against either Lef1 or  $\beta$ -catenin or nonspecific IgG was used.

To confirm that  $\beta$ -catenin was indeed regulating *Brn-2* expression in melanoma cells, we transfected the 501 mel melanoma cell line with an siRNA specific to  $\beta$ -catenin and examined the effect of loss of  $\beta$ -catenin expression on *Brn-2* levels. The results (Fig. 3B) indicated that the siRNA used, which has been shown previously to be both effective and specific for  $\beta$ -catenin (47), largely abolished  $\beta$ -catenin expression and moreover also resulted in a substantial downregulation of *Brn-2* compared to that in cells transfected with a control nonsilencing RNA. No effect was observed on tubulin levels. These results, together with those of the chromatin immunoprecipitation assay, are consistent with the *Brn-2* promoter's being bound and regulated by a Lef1/ $\beta$ -catenin complex in vivo.

**Brn-2 is required for proliferation.** Although the results obtained so far implicate  $\beta$ -catenin in the regulation of *Brn-2* expression, a key issue is whether *Brn-2* expression contributes to proliferation. To address this question, we designed a *Brn-2*-specific siRNA and used it together with a control nonsilencing RNA to transfect the 501 mel cell line, in which we have

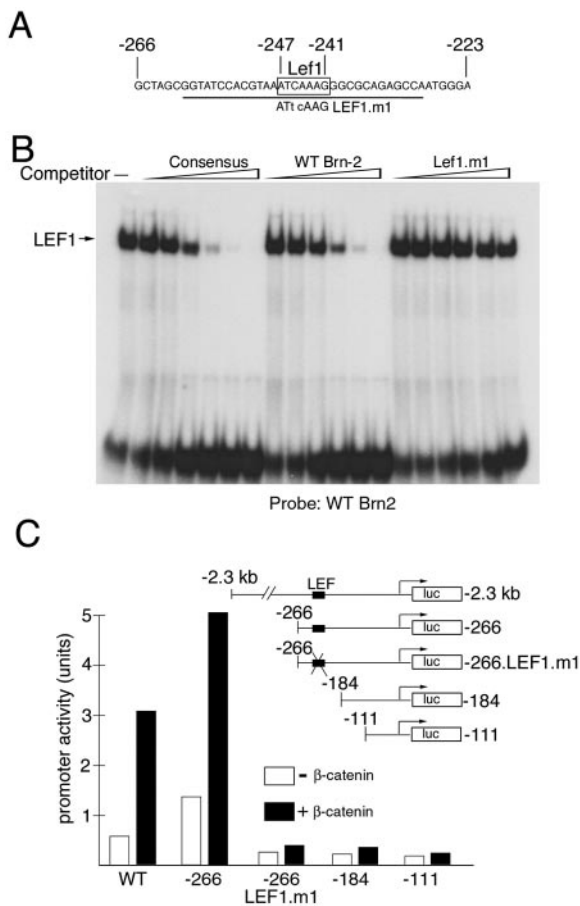


FIG. 2. *Brn-2* expression is activated by  $\beta$ -catenin in vitro and in vivo. (A) Sequence of a region of the *Brn-2* promoter required for activity in melanocytes and melanoma, with the consensus Lef1/Tcf factor binding site indicated ( $-241$  to  $-247$ ). The sequence of the mutation in the binding site is indicated, and the underlined region was used as a probe for the DNA-binding assays presented below. (B) Lef1 binds the *Brn-2* promoter in vitro. Band shift assay with a probe corresponding to the sequence underlined in panel A together with bacterially expressed Lef1. The indicated competitor oligonucleotides were used at 2, 5, 10, 20, 50, and 250 ng. The sequence of the Lef1 consensus oligonucleotide was 5'-CTAGAAGGGCACCCTTTGAAGCTCT-3'. (C)  $\beta$ -Catenin activates the *Brn-2* promoter. The 501 mel cells were transfected with a *Brn-2* promoter-luciferase reporter construct extending to either  $-2.3$  kb (wild type) or deleted to  $-266$ ,  $-184$ , or  $-111$  or a reporter,  $-266$ /Lef1.m1, in which the Lef1/Tcf site has been mutated, as illustrated in the inset. The indicated reporters were transfected alone or together with a  $\beta$ -catenin expression vector, and luciferase activity was determined.

established that *Brn-2* expression is controlled by  $\beta$ -catenin. The results obtained by Western blotting (Fig. 4A) and immunofluorescence (not shown) indicate that the *Brn-2* siRNA was highly specific and effective, decreasing *Brn-2* expression by up to 90% while not affecting expression of the related POU domain transcription factor Oct-1 or tubulin. Significantly, transfection of the 501 mel cell line with the *Brn-2* siRNA also resulted in up to an eightfold decrease in the proportion of cells incorporating bromodeoxyuridine compared to that in cells transfected with the control siRNA (Fig. 4B), although in most experiments (not shown) the bromodeoxyuridine-positive population was reduced by about fourfold. Consistent with

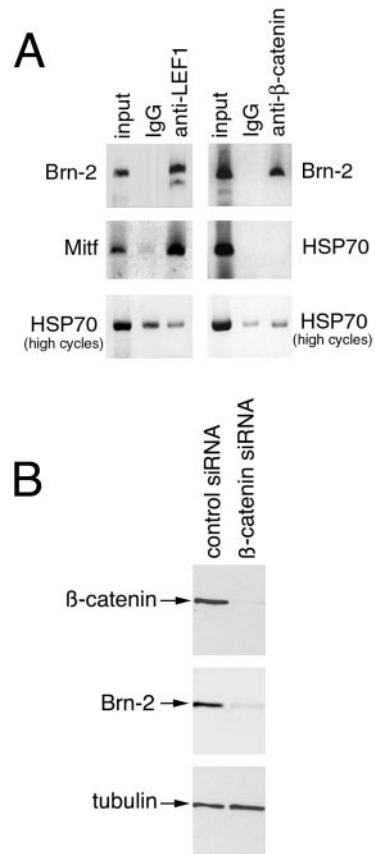


FIG. 3. *Brn-2* promoter is a target for Lef1 and  $\beta$ -catenin in vivo. (A) Chromatin immunoprecipitated from 501 mel cells with either nonspecific IgG or anti- $\beta$ -catenin or anti-Lef1 antibodies was subjected to PCR with primers specific for the *Brn-2* and *Mitf* promoters. Primers for the *HSP70* promoter were used as a negative control. For the *HSP70* promoter, PCR for the same number of cycles (25) used to generate a signal for the *Brn-2* or *Mitf* promoter immunoprecipitated with anti-Lef1 or anti- $\beta$ -catenin antibodies failed to reveal a signal. The PCR cycles were therefore increased to 30 so that a product was evident (high cycles). (B) siRNA-mediated downregulation of  $\beta$ -catenin results in decreased *Brn-2* expression; 501 mel cells were transfected with either a control siRNA or an siRNA specific for  $\beta$ -catenin (47), harvested after 3 days, and subjected to Western blotting with the indicated antibodies.

this, treatment of the 501 mel cells with the *Brn-2*-specific siRNA also led to substantially reduced [ $^3$ H]thymidine incorporation (not shown). However, while there was a significant decrease in the S-phase population in *Brn-2*-depleted 501 mel cells, we did not detect any obvious signs of increased apoptosis (not shown), suggesting that *Brn-2* expression is required for proliferation but not survival of these cells.

**$\beta$ -Catenin induces *Brn-2* expression in transgenic mice.** Although *Brn-2* is expressed in melanoma cell lines in culture, its expression in the melanocyte lineage in vivo has not been investigated previously. To determine whether *Brn-2* is expressed in melanoblasts in vivo, we performed whole-mount in situ hybridization with a sense or antisense *Brn-2*-specific probe and examined expression in a mouse embryo at E11.5, a time when melanoblasts are proliferating and migrating from the neural crest. As a control, we used an E11.5 embryo ex-

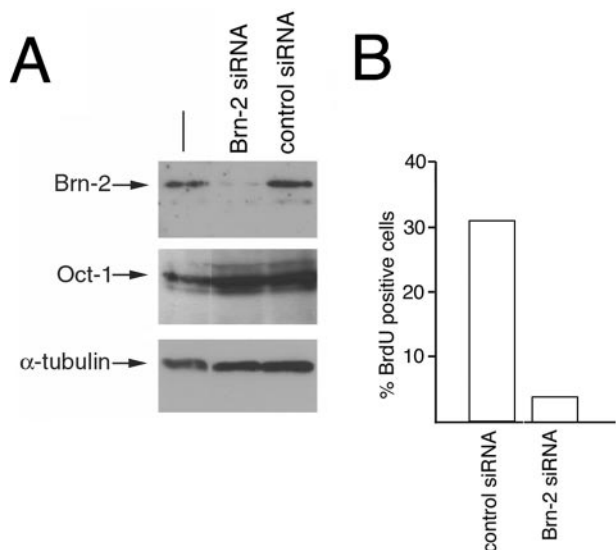


FIG. 4. *Brn-2* expression is required for proliferation in 501 mel cells. 501 mel cells were transfected with siRNA specific for *Brn-2* or a control siRNA. After 3 days, cells were grown in the presence of bromodeoxyuridine for 1 h before being subjected to either Western blotting or immunofluorescence. (A) Results of Western blotting with the indicated antibodies after *Brn-2* or control siRNA treatment. (B) Quantification of the immunofluorescence results obtained by counting 300 control or *Brn-2* siRNA-transfected cells stained for DNA with DAPI and for bromodeoxyuridine (BrdU) incorporation.

pressing LacZ from the *DCT* promoter, which directs expression in melanoblasts (28) and enables the neural crest-derived melanoblast population to be clearly visible (Fig. 5A and B). In these experiments, with an antisense probe, *Brn-2* expression was clearly evident in the neural tube (Fig. 5C and D), as has been seen previously (37), and no signal was generated with a sense probe (Fig. 5E and F) as a control. However, no detectable *Brn-2* expression was detected in melanoblasts or the neural crest. Although we failed to detect *Brn-2* expression at this time of development, we also examined the potential for *Brn-2* expression in postnatal melanocytes in the hair follicle. Here, in contrast to melanoblasts, a clear signal was obtained with the antisense *Brn-2* probe in the hair follicles, consistent with the localization of wild-type melanocytes (Fig. 5G). No *Brn-2* signal was present in *KitW-lacZ/KitW* mice, which lack the neural crest-derived melanocyte population (Fig. 5H), or if a sense probe was used on the wild-type mice (Fig. 5I).

To determine whether expression of *Brn-2* in mice could be upregulated in vivo by β-catenin, as suggested by the results from the cell lines, we examined *Brn-2* expression by reverse transcription (RT)-PCR in mouse skin from a wild-type mouse or one in which β-catenin fused to green fluorescent protein (GFP) is overexpressed in the melanocyte lineage from a tyrosinase promoter-enhancer expression cassette that specifically targets expression to the melanocyte lineage. The production of these mice and a detailed analysis of their phenotypes and the properties of their melanocytes will be described in detail elsewhere (S. Martinuzzi et al., unpublished data). Consistent with the results obtained in cell lines, RT-PCR analysis of newborn skin demonstrated that *Brn-2* was significantly overexpressed in the skin of the transgenic mice (GFP RNA

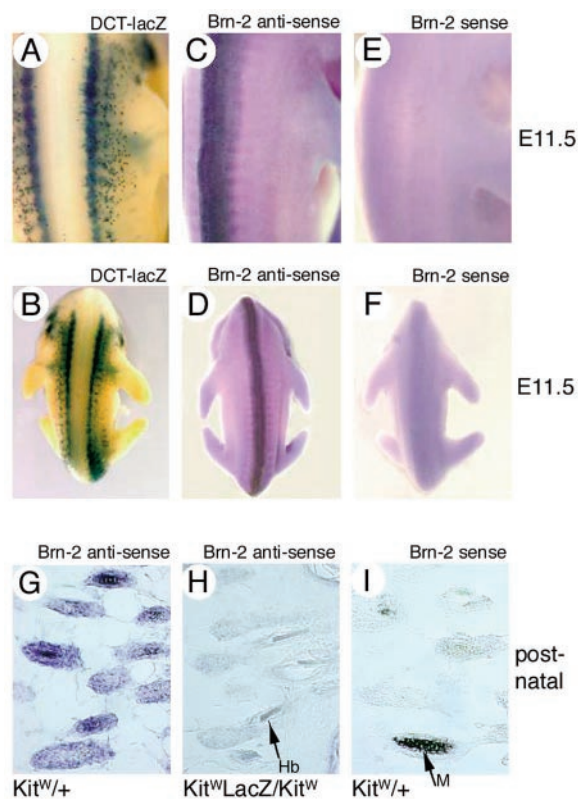


FIG. 5. *Brn-2* is not expressed in melanoblasts in vivo but is expressed in hair follicle melanocytes. (A and B) E11.5 mouse embryo bearing a *DCT-lacZ* reporter transgene stained for *lacZ* expression as a marker of melanoblasts. (C and D) In situ hybridization of an E11.5 embryo with a *Brn-2* antisense probe. (E and F) In situ hybridization of an E11.5 embryo with a *Brn-2* sense probe. Note that *Brn-2* expression is not detected in melanoblasts. (G to I) In situ hybridization of a cryosection of newborn mouse skin with the antisense (G and H) and sense (I) *Brn-2* probes with wild-type and *KitW-lacZ/KitW* mice. The *KitW-lacZ/KitW* mice lack neural crest-derived melanocytes. The arrow in panel I indicates melanin (M), and the arrow in panel H indicates a hair bulb (hb).

positive) compared to their nontransgenic littermates (Fig. 6). No change was observed in the total levels of cyclin D1 or hypoxanthine phosphoribosyltransferase RNA, which were used as internal controls. Note that although the cyclin D1 gene has been reported to be a target for β-catenin signaling (44), unlike *Brn-2* it is expressed in both melanocytes and keratinocytes, and as melanocytes represent only a minor population of skin cells, any elevation in cyclin D1 RNA levels in the melanocyte population is unlikely to be detected. In summary, the activation of *Brn-2* expression by Wnt/β-catenin observed in cells in culture was accurately reproduced in vivo in mouse skin and provides substantial evidence to support the idea that *Brn-2* expression is directly regulated by the Wnt signaling pathway.

DISCUSSION

In this paper we provide several lines of evidence to suggest that Wnt/β-catenin signaling controls *Brn-2* expression. The *Brn-2* promoter contains a functional Lef1 binding site, is re-

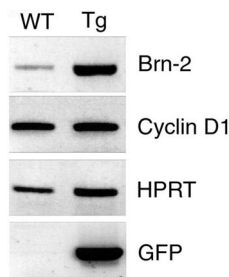


FIG. 6. *Brn-2* is upregulated by  $\beta$ -catenin in vivo.  $\beta$ -Catenin-GFP was overexpressed in the melanocyte lineage from a *tyrosinase* promoter including an upstream *tyrosinase* locus control region (Martinuzzi et al., unpublished data). RNA derived from the skin of transgenic (Tg) and nontransgenic wild-type (WT) littermates were subjected to RT-PCR with primers for the indicated genes. Primers specific for GFP were used to detect the transgene, and cyclin D1 and hypoxanthine phosphoribosyltransferase (HPRT) were used as controls.

sponsive to Wnt/ $\beta$ -catenin signaling, and, importantly, is recognized by Lef1 and  $\beta$ -catenin in vivo, as determined with a chromatin immunoprecipitation assay, while siRNA-mediated downregulation of  $\beta$ -catenin in melanoma cells also results in decreased *Brn-2* expression. Moreover, *Brn-2* expression is upregulated in mice expressing activated  $\beta$ -catenin in melanocytes. The ability of  $\beta$ -catenin to upregulate *Brn-2* expression will no doubt contribute to the elevated levels of Brn-2 observed in many melanomas and may also contribute to the highly specific pattern of Brn-2 expression observed during development (31, 37).

Strikingly, expression of Brn-2 is not only upregulated by  $\beta$ -catenin but is also elevated in response to mitogen-activated protein kinase signaling downstream from reporter tyrosine kinases (RTKs) and in particular downstream from BRAF (18), which is known to be activated by mutation in about 70% of melanomas and nevi (8, 32). Thus, the overexpression of *Brn-2* is a feature of melanoma irrespective of which pathway,  $\beta$ -catenin or mitogen-activated protein kinase, is constitutively activated. Moreover, we demonstrate here that siRNA-mediated downregulation of Brn-2 expression dramatically reduced the S-phase population of the 501 mel cells, strongly pointing to a role for *Brn-2* in proliferation. Consistent with this, we have also shown that overexpression of *Brn-2* in melanocytes leads to increased proliferation, while siRNA-mediated inhibition of *Brn-2* expression in melanoma cell lines expressing a constitutively activated BRAF leads to decreased [ $^3$ H]thymidine incorporation (18). Previous work has also linked expression of Brn-2 in melanoma cell lines tumorigenicity and proliferation (45). Indeed, we have found that of 16 melanoma cell lines for which we have information on the status of Brn-2 expression as well as BRAF and  $\beta$ -catenin mutations, only three, which are apparently wild type for both BRAF and  $\beta$ -catenin, do not express Brn-2. Of the remaining cell lines, only one exhibits both an activating mutation on BRAF and a mutated form of  $\beta$ -catenin.

There is now a substantial body of evidence indicating that Brn-2 plays a key role in controlling the proliferation of many melanoma cell lines. Moreover, since *Brn-2* expression lies downstream of the two genetically defined signaling pathways

that are constitutively activated in melanoma, it is possible that overexpression of *Brn-2* may be a necessary step in the formation of many melanomas and will confer a strong selective growth advantage. Consistent with this, expression of  $\beta$ -catenin in B16 melanoma cells results in increased proliferation, while expression of a dominant-negative Tcf slows melanoma cell growth (49). In this respect it is notable that in other systems,  $\beta$ -catenin signaling is implicated in growth control. For example, in colorectal cancer cells, expression of a dominant-negative Tcf4 leads to a G<sub>1</sub> arrest via the induction of the p21 cyclin-dependent kinase inhibitor (46), and siRNA-mediated downregulation of  $\beta$ -catenin in colon cancer cells also inhibited proliferation (47). Similarly, in the pituitary, expression of the PitX1 transcription factor is induced by Wnt/ $\beta$ -catenin signaling and is required for proliferation (25). The fact that  $\beta$ -catenin can induce the expression of *Brn-2* in melanoma appears to indicate that the control of proliferation by the Wnt/ $\beta$ -catenin signaling pathway may be a general theme.

In the melanocyte lineage, Wnt/ $\beta$ -catenin signaling is required to turn on expression of the Mitf transcription factor (10, 43), which plays a crucial role in melanoblast survival and is most likely a key factor in determining the identity of a melanoblast (17). Intriguingly, we found no evidence for *Brn-2* expression in the neural crest in melanoblasts at E11.5, a time when these cells are both proliferating and migrating. It is possible that at this stage in development, the chromatin across the *Brn-2* promoter is in a conformation that is incompatible with activation by Wnt/ $\beta$ -catenin signaling or that Wnt/ $\beta$ -catenin signaling in the melanocyte lineage operates only early in development, turns on Mitf expression, and then is no longer required. Alternatively, the levels of  $\beta$ -catenin expressed in melanoblasts at E11.5, while capable of activating Mitf expression, may be insufficient for activation of the *Brn-2* promoter, which would only respond to the increased  $\beta$ -catenin expression occurring when the migrating melanoblast population arrives in the epidermis. A further possibility is that melanoblasts have the potential to express *Brn-2* but that its expression can only be induced by the cooperative effect of multiple signal transduction pathways that are not simultaneously active in melanoblasts. Currently we know little about the timing of activation of signal transduction pathways in melanocyte development.

Despite the fact that *Brn-2* does not appear to be expressed in melanoblasts at E11.5, it is nevertheless present in melanocytes in postnatal hair follicles, where evidently the signals required to induce *Brn-2* expression are active. In these cells *Brn-2* expression can be further induced by the elevated levels of  $\beta$ -catenin expressed in the transgenic animals, confirming the results indicating that *Brn-2* expression is activated by  $\beta$ -catenin that were obtained by using cell lines. However, although *Brn-2* may be expressed in hair follicle melanocytes, whether it plays a role in the proliferation of these cells is likely to depend on several factors, including the absolute level of Brn-2 protein expressed, which will in turn depend on the activity of the mitogen-activated protein kinase pathway downstream of RTKs and BRAF as well as the  $\beta$ -catenin signaling pathway. In addition, the DNA-binding activity of the Brn-2 protein in regulating gene expression will, like that of other POU domain proteins (4, 41), be controlled by phosphorylation, and it is likely that the ability of Brn-2 to cooperate or

interact with other DNA-binding proteins such as Sox11, as has been seen in other cell types (50), will also be regulated. An additional level of control of Brn-2 activity may also be provided by the regulation of its ability to interact with specific transcriptional cofactors (42). Dissecting the signal transduction pathways that regulate Brn-2 and its ability to control transcription and proliferation as well as its oncogenic potential will lead to a better understanding of how mitogen-activated protein kinase and  $\beta$ -catenin signaling are integrated with cell cycle control.

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