β-Catenin induces immortalization of melanocytes by suppressing *p16^{INK4a}* expression and cooperates with N-Ras in melanoma development

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Tumor progression is a multistep process in which proproliferation mutations must be accompanied by suppression of senescence. In melanoma, proproliferative signals are provided by activating mutations in NRAS and BRAF, whereas senescence is bypassed by inactivation of the *p16^{Ink4a}* gene. Melanomas also frequently exhibit constitutive activation of the Wnt/β-catenin pathway that is presumed to induce proliferation, as it does in carcinomas. We show here that, contrary to expectations, stabilized β-catenin reduces the number of melanoblasts in vivo and immortalizes primary skin melanocytes by silencing the *p16^{Ink4a}* promoter. Significantly, in a novel mouse model for melanoma, stabilized β-catenin bypasses the requirement for *p16^{Ink4a}* mutations and, together with an activated N-Ras oncogene, leads to melanoma with high penetrance and short latency. The results reveal that synergy between the Wnt and mitogen-activated protein (MAP) kinase pathways may represent an important mechanism underpinning the genesis of melanoma, a highly aggressive and increasingly common disease.

[*Keywords*: Mitf; Wnt; senescence; development; tumor suppressor; oncogene]

Supplemental material is available at http://www.genesdev.org.

Received July 23, 2007; revised version accepted September 27, 2007.

Increasing evidence has highlighted the role of senescence bypass as a critical event in cancer progression (Braig et al. 2005; Chen et al. 2005; Collado et al. 2005; Xue et al. 2007). If not accompanied by additional genetic events, such as inactivation of the Rb pathway, oncogenes, including RAS and RAF, promote senescence (Serrano et al. 1997; Zhu et al. 1998).

Malignant melanoma is a highly metastatic and increasingly common cancer and represents a valuable model for understanding senescence bypass in transformation. After an initial burst of cell division, benign melanocytic neoplasms—melanocytic nevi—bearing activating NRAS or BRAF mutations cease proliferating and the melanocyte population becomes senescent (Papp et al. 1999; Bastian et al. 2003; Bennett 2003; Michaloglou et al. 2005; Gray-Schopfer et al. 2006). Senescence bypass in the melanocyte lineage is usually achieved by loss of expression of *p16^{INK4a}*, generally a consequence of deletions of the *CDKN2A* locus on chromosome 9p21, and is one of the key events during melanoma progression (Chin et al. 1997; Ackermann et al. 2005). However, not all melanomas show genetic alterations affecting the CDKN2A locus, and there are presumably other mechanisms leading to loss of *p16^{INK4a}* expression.

Melanoma, like other cancers, often presents constitutive activation of the Wnt signaling pathway (Rimm et al. 1999; Omholt et al. 2001; Giles et al. 2003) as evidenced by nuclear accumulation of β-catenin. In most cells, β-catenin is primarily found associated with cadherins at the membrane where it plays a role in cell–cell adhesion (Butz and Larue 1995). The β-catenin pool not associated with cadherins can be phosphorylated by gly-

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Article is online at http://www.genesdev.org/cgi/doi/10.1101/gad.450107.

cogen synthase kinase-3β (GSK-3β) on serine (S) and threonine (T) residues and is consequently ubiquitinated and degraded (for review, see Kimelman and Xu 2006). The phosphorylation of the ST residues (S45, T41, S37, and S33) is processive, the initiating phosphorylation event being performed by CK1α on S45; then GSK-3β sequentially phosphorylates T41, S37, and S33. The F-box protein β-TRCP1 of the ubiquitin ligase complex then recognizes the two N-terminal phosphorylated serines in β-catenin (S37 and S33) that is subsequently degraded. The processivity means that a single mutation of any of the ST residues leads to a higher stability of β-catenin in cell culture (Liu et al. 2002). After exposure of cells to Wnt factors, GSK-3β is inhibited and the stabilized β-catenin translocates to the nucleus, where it interacts with Lef/Tcf factors to regulate target genes. Mutations in β-catenin that mimic its activation by Wnt and lead to its stabilization and translocation from the plasma membrane to the nucleus are found in several cancers, including melanoma (Giles et al. 2003). For example, the S45P/Y/F and S37F mutations were identified in β-catenin in melanomas (Rubinfeld et al. 1997; Rimm et al. 1999; Omholt et al. 2001), and their capacity to stabilize β-catenin is similar to S37F and S37A mutants (Rubinfeld et al. 1997), suggesting that the main effect of these mutations is to prevent degradation. Transgenic mice expressing a stabilized form of β-catenin have been established using two main types of constructs based on the findings of previous work (Yost et al. 1996): the absence of exon 3 encoding the region containing the ST residues and the substitution of the ST residues by A (S45, T41, S37, and S33 by alanines). In the absence of exon 3, the mice develop various tumors affecting the hair follicle, intestine, and mammary gland (Gat et al. 1998; Harada et al. 1999; Romagnolo et al. 1999; Imbert et al. 2001). Substitution of the ST residues by A resulted in aggressive fibromatosis and gastrointestinal tumors (Cheon et al. 2002). These in vivo studies revealed that stabilized β-catenin contributes to the malignant transformation of a variety of cell types by promoting cell proliferation.

β-Catenin is produced in developing melanocytes, in mature melanocytes, and in melanomas (Jouneau et al. 2000). During early development, β-catenin is crucial in determining the fate of melanoblasts (Hari et al. 2002; Lee et al. 2004), most likely via its capacity to activate the expression of the Microphthalima-associated transcription factor Mitf that plays a critical role in melanoblast survival and differentiation (Dunn et al. 2000; Larue et al. 2003; Steingrimsson et al. 2004; Larue and Delmas 2006).

To determine the contribution of β-catenin to melanocyte proliferation, immortalization, and transformation in vivo, we generated transgenic mice producing a stabilized form of β-catenin in the committed cells of the melanocyte lineage. In contrast to other cell types, our results reveal that in melanocytes β-catenin does not induce proliferation and that unexpectedly, by repressing the expression from the $p16^{Ink4a}$ promoter, β-catenin promotes immortalization. Strikingly, we show, using a novel mouse melanoma model, that the Wnt and mitogen-activated protien (MAP) kinase pathways act synergistically to induce melanoma without the need for $p16^{Ink4a}$ mutations, a mechanism likely to occur in some human melanomas. These findings reveal a previously unrecognized function for β-catenin in overcoming the senescence barrier and suggest that fine-tuning of β-catenin levels is essential for controlling various cellular mechanisms during the establishment of this cell lineage and the maintenance of the nontransformed state.

Results

Production of transgenic mice expressing an activated form of β-catenin in cells of the melanocyte lineage

To determine how an activated form of β-catenin exerts its effects on melanocyte transformation in vivo, we generated transgenic mice expressing specifically in melanocytes a stabilized β-catenin that contains four ST–A substitutions. A similar quadruple mutant has been shown previously to be effective in producing aggressive fibromatosis and gastrointestinal tumors (Cheon et al. 2002). We decided to use this ST–A form and not the exon 3 deletion for two main reasons: to minimize the disturbance of the β-catenin structure and to allow the interaction of β-catenin with its natural partners. Moreover, it has been reported that 30% of melanoma biopsies, not mutated for β-catenin, possessed β-catenin in their nuclei, suggesting that the Wnt/β-catenin pathway is activated (Rimm et al. 1999). To eliminate any potential problems arising from cytoplasmic retention of the protein, we added a nuclear localization signal (nls) to the $bcat^{sta}$ transgene construct (Fig. 1A). In melanocytes and melanoma cells, endogenous β-catenin was present at cell–cell contacts on the cell surface whereas $bcat^{sta}$ was detected primarily in the nucleus (Fig. 1B; data not shown). We assessed the transcriptional activity of $bcat^{sta}$ using the "TOP and FOP" flash assay involving luciferase reporter genes under the control of artificial promoters that respond to the β-catenin/Lef transcription complex (see Materials and Methods). The TOP flash construct reports the activity of the TCF/β-catenin complex and the FOP flash construct was used as a negative control. We compared the activity of $bcat^{sta}$ with those of other β-catenin forms that lacked the enhanced green fluorescent protein (EGFP) or the nls in transient cotransfection assays (Fig. 1C). Expression of β-catenin and β-catenin-EGFP resulted in moderate induction (four- to fivefold) of the TOP flash reporter, whereas induction was much stronger (16- to 18-fold) with the $bcat^{sta}$ (=Tyr::β-cat-mut-nls-egfp) and Tyr::β-cat-mut-nls constructs. As expected, similar results were obtained with the Mitf-M promoter, a natural target of β-catenin (data not shown). Thus, the $bcat^{sta}$ expression vector produced a constitutively activated nuclear form of the protein that was comparable in its activity to the mutated form of β-catenin found in melanomas and other cancers. We therefore proceeded to generate transgenic mice with the $bcat^{sta}$ construct.

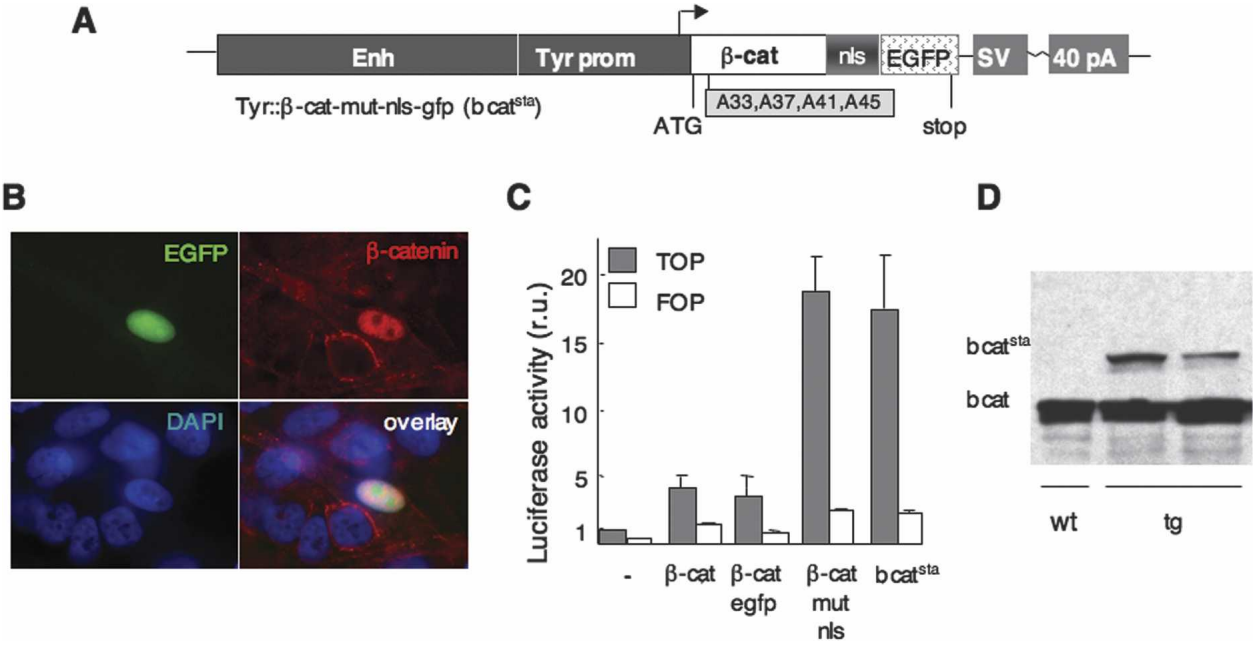

Figure 1. bcat^{sta} construct and characteristics. (*A*) Map of the Tyr::β-cat-mut-nls-egfp (bcat^{sta}) transgene (see Materials and Methods for details). (*B*) Localization of bcat^{sta} in FO-1 melanoma cells. In cells transfected with the bcat^{sta} construct (bcat^{sta}), bcat^{sta} protein was detected by its autofluorescence (EGFP). Note that the cells were transfected when they were at high confluency. Endogenous and exogenous β-catenin was detected with an antibody specific for the protein. Nuclear DNA was detected by DAPI staining. (*C*) Transient transfection of FO-1 melanoma cells with TOP–FOP flash luciferase reporters and various expression vectors. Note that β-cat-mut-nls is similar to bcat^{sta} without egfp. (*D*) bcat^{sta} expression in melanocyte cultures from two independent transgenic bcat^{sta} lines.

bcat^{sta} mice cooperate with NRAS to produce melanoma

Mice of two independent transgenic lines (lines 1 and 2) producing significant amounts of bcat^{sta} were maintained for up to 2 yr. The amount of bcat^{sta} in these transgenic melanocytes was ~10% and 5% of that of the endogenous β-catenin in lines 1 and 2, respectively (Fig. 1D). No melanoma appeared in either mouse line, suggesting that either the amount of the transgene protein was not sufficient or this activated form of β-catenin by itself cannot induce melanoma in mice. Oncogenesis depends on the cooperation of multiple signaling pathways, and in human melanoma the MAP kinase pathway is frequently consititutively activated. We therefore examined the status of N-RAS/B-RAF and β-catenin in 31 human cutaneous melanoma samples. We genotyped these tumors for their N-RAS and B-RAF status and evaluated the amount of β-catenin (Table 1). A significant number of tumors contained activating mutations in N-RAS and produced a relatively large amount of β-catenin, raising the possibility that these two proteins may cooperate during melanomagenesis. To test this possibility directly, we crossed bcat^{sta} mice (line 1) with mice producing an oncogenic form of N-Ras in melanocytes (=Tyr::N-Ras^{Q61K}/°) (Fig. 2). These Tyr::N-Ras^{Q61K}/° mice develop melanoma (Ackermann et al. 2005) after a long latency (54 ± 21 wk) and with <30% incidence (see Fig. 2B). In contrast, the double transgenic animals carrying both the N-Ras mutation and the activated β-catenin (Tyr::N-Ras^{Q61K}/°; bcat^{sta}/°) had a high incidence of melanoma (85%) with a markedly shorter average latency period (27.6 ± 6.7 wk) than single transgenic mice carrying only the N-Ras mutation (Tyr::N-Ras^{Q61K}/°; °/°) (Fig. 2A,B). Mice developed either one or two primary tumors (n = 9) or multiple (three to nine) tumors (n = 8). The development of neoplastic lesions in Tyr::N-Ras^{Q61K}/°; bcat^{sta} mice is therefore a frequent event.

In mice, melanocytes are mainly located in the dermis of the pinna, in the epidermis of the limbs and tail, and in the hair follicles of the hairy parts of the skin. In our study, melanomas developed on the hairy parts of the body (neck, back, and belly) of Tyr::N-Ras^{Q61K}/°; bcat^{sta}/° mice (Fig. 2A) but were not observed on less hairy parts (limb and tail), nor on the pinna. This suggests that the melanomas most probably arose from melanocytes residing within the epidermis and/or the hair follicles. Histological sections of 3-mo-old Tyr::N-Ras^{Q61K}/°; bcat^{sta}/° skins revealed early melanocytic neoplasia emanating from the hair follicles (Fig. 2C). At later stages of melanoma progression, tumors were mainly found in the dermis, always sparing the epidermis (Fig. 2D). Melanoma cells produced tyrosinase, Tyrp-1, and S100 as revealed by immunohistochemistry (Fig. 2E,F; data not shown), though more advanced tumors tended to be less pigmented. The mitotic indices were estimated at 5% in Tyr::N-Ras^{Q61K}/°; bcat^{sta}/° amelanotic melanoma cells and 2% in Tyr::N-Ras^{Q61K}/° amelanotic melanoma cells. In summary, these findings

Table 1. *Melanoma mutated for N-Ras are frequently activated for the Wnt/β-catenin pathway*

	β-Catenin level	
	High	Low
N-Ras mut	7[a] (78%)	2 (22%)
B-Raf mut	4 (36%)	7 (64%)
Wild type	3 (27%)	8 (73%)

Thirty-one tumors were genotyped for N-RAS and B-RAF and the amount of β-catenin was estimated in various melanoma as high or low. The first number corresponds to the quantity of samples. The second number corresponds to the percentage of samples of a certain genotype producing such an amount of β-catenin.

[a]Please note that six out of seven N-Ras mutated melanoma expressing a high level of β-catenin produced a low level of p16. This indicates that in human melanoma, β-catenin could be involved in the repression of p16. None of these tumors was mutated for β-catenin. Standard protocols were used for immunohistochemistry with 3-amino-9-ethylcarbazole used as a chromagen according to the manufacturer's specifications on tissue microarrays as described previously (Curtin et al. 2005). β-Catenin monoclonal antibody (catalog #610153, BD Biosciences; dilution 1:100) was used. Staining intensity levels were scored. We performed sequence analysis of *BRAF* codon 600 and codons 12, 13, and 61 of *NRAS* by direct sequencing of PCR-amplified products generated with specific primers designed to include the codon of interest. PCR products were purified using ExoSAP-IT (USB Corporation) and sequenced directly using an ABI PRISM 3700 DNA Analyzer (Applied Biosystems).

suggest that most melanomas arose from melanocytes located in the hair follicles. The tumor-initiating cells were most likely the melanocytes in the bulb of the hair follicle or the melanocyte stem cells in the bulge region. In conclusion, β-catenin is involved in murine melanoma progression when associated with a mutated form of N-Ras.

bcat^{sta} reduces the number of melanocytes in vivo

Activated β-catenin and Nras can clearly cooperate in generating melanoma in mice. Since proliferation and immortalization are the two main early events during melanoma progression, so we assessed the role of β-catenin in both.

The numbers of melanocytes in the truncal skin region of wild type and bcat^{sta} were evaluated after birth (Fig. 3A) and revealed that there were about three to four times fewer bcat^{sta} melanocytes than wild-type melanocytes. The numbers of wild-type and bcat^{sta} melanoblasts were also determined during embryonic development. Since the Tyrosinase promoter used to drive activated β-catenin in these experiments starts to be active around embryonic day 10 (E10) (data not shown), we evaluated the effect of the production of bcat^{sta} in the increment of melanoblasts between E10.5 and E15.5 in wild-type and bcat^{sta} embryos (Fig. 3B) by crossing Dct::LacZ/°; °/° mice (=Dct::LacZ mice) with °/°; bcat^{sta}/° mice. The melanoblasts of Dct::LacZ mice expressed β-galactosidase, allowing us to detect individual melanoblasts in embryos after staining with X-Gal and genotyping. All the LacZ-positive cells between the front and hind limbs (corresponding approximately to somites 13–25) were counted on each side from E10.5 onward (Fig. 3B; Supplementary Fig. S1). On E10.5, when the transgene starts to be expressed, the number of melanoblasts was similar in wild-type and bcat^{sta} embryos. However, on E11.5, there were fewer melanoblasts in bcat^{sta} than in wild-type embryos, and this difference increased with time.

The decrease in numbers of melanoblasts could result either from decreased proliferation, increased apoptosis, or abnormal changes in cell fate. We evaluated the number of Ki-67-positive cells, the number of apoptotic cells by TUNEL assay, and the number of abnormal melanoblast-committed cells (transdifferentiation or loss of commitment). No difference was observed between wild-type and transgenic embryos, indicating that transgenic and wild-type melanoblasts were cycling, and that cell death, transdifferentiation, and loss of differentiation were not more frequent in the transgenic than wild-type animals (Supplementary Fig. S2; data not shown). Even though it is not formally proven, it is more likely that the in vivo proliferation of melanoblasts was impaired by the production of bcat^{sta} during melanocyte development. We showed previously that Mitf-M represses proliferation in melanoma cells (Carreira et al. 2005). Mitf-M was induced in E13.5 transgenic melanoblasts and was thus more abundant than in wild-type melanoblasts (Supplementary Fig. S3); this may account for the reduction of bcat^{sta} melanoblast number. However, despite the presence of nuclear β-catenin in bcat^{sta} melanocytes (Supplementary Fig. S4), the growth rates of wild-type and bcat^{sta} immortalized melanocyte cell lines in culture, in the presence of the phorbol ester tetradecanoyl phorbol acetate (TPA), were similar (Fig. 3C). The situation in vitro is different from that in vivo because the cells are growing in the presence of TPA, in the absence of heterotypic cells (keratinocytes or other cell types), and are immortalized. In conclusion and contrary to expectations, analysis of melanocyte number during development and in adult mice did not reveal any hyperproliferation of the melanoblasts or melanocytes expressing bcat^{sta}, but rather may suggest that the expression of activated β-catenin led to diminished proliferation.

bcat^{sta} induces immortalization in culture

Since bcat^{sta} did not induce cell proliferation, it presumably acts on at least one other cellular mechanism involved in the early stages of melanocyte transformation. We therefore tested the effect of bcat^{sta} on cell immortalization.

The frequency of activating mutations in the β-catenin gene is higher in melanoma cell lines than in tumors (Rubinfeld et al. 1997; Rimm et al. 1999; Demunter et al. 2002; Pollock and Hayward 2002; Reifenberger et al. 2002). We performed similar experiments on independent melanomas and melanoma cell lines (data

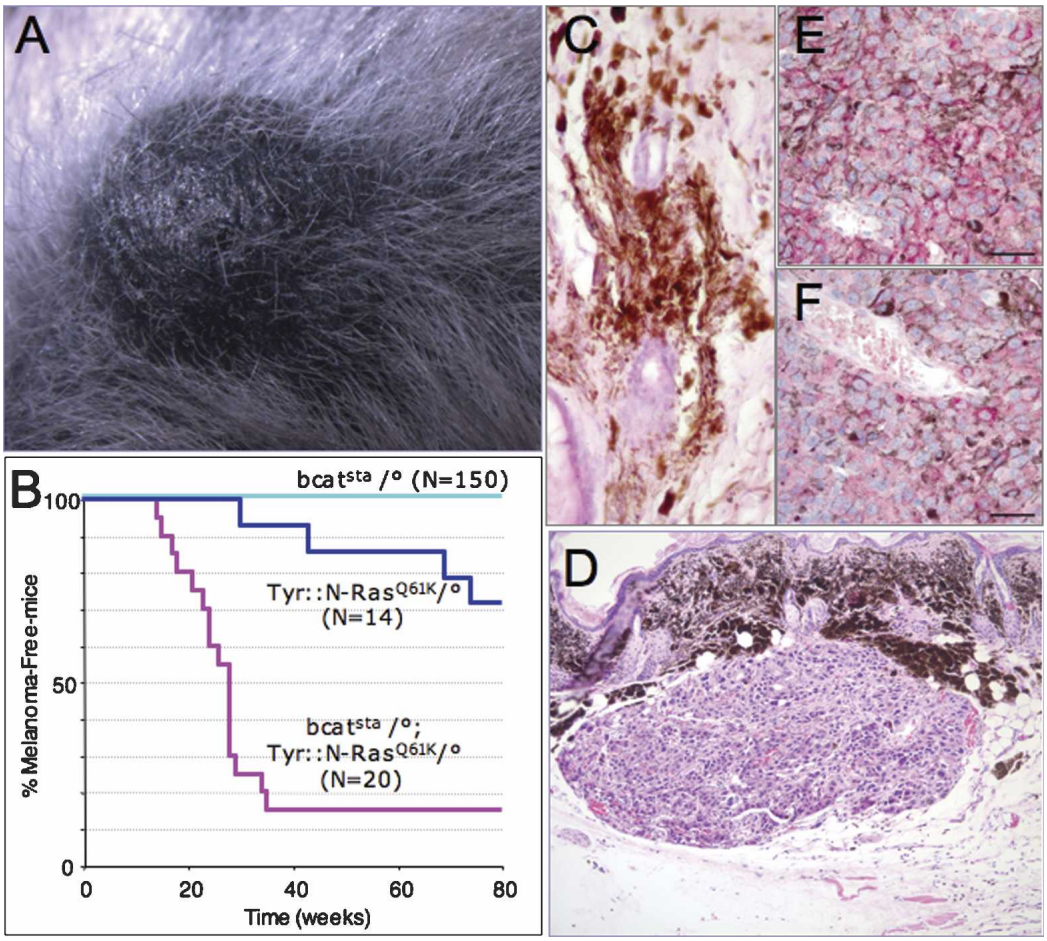

Figure 2. Activated β-catenin cooperates with N-Ras to produce melanoma. (*A*) Photomicrography of a melanoma on the hairy part of the back of a bcat^{sta}/°; Tyr::N-Ras^{Q61K}/° mouse. (*B*) Kaplan-Meier graph of melanoma incidence in various mouse genotypes, as indicated. The age of mice was scored according to the appearance of a cutaneous melanoma and additional signs of morbidity (Serrano et al. 1996; Ackermann et al. 2005). (*C*) Histological section of a hair follicle of a 3-mo-old Tyr::N-Ras^{Q61K}/°; bcat^{sta}/° mouse. (*D*) Histological section of a cutaneous melanoma showing intradermal invasion. Immunostaining for tyrosinase (*E*) and Tyrp-1 (*F*) in an amelanotic melanoma was revealed by the brown color.

not shown). Combining published data with our findings, we estimated that the frequency of activating mutations in the β-catenin gene is 8.5% in melanoma cell lines and 3.3% in melanomas. This discrepancy could be due to two nonexclusive possibilities: Either the β-catenin gene mutated spontaneously in culture, or tumor cells bearing activating β-catenin mutations have a selective advantage when they are transferred into culture during the creation of melanoma cell lines. To test whether the activated β-catenin immortalized cells efficiently in vitro, we established cultures of melanocytes from bcat^{sta} transgenic and wild-type mice. Cultures of bcat^{sta} melanocytes divided continuously (Fig. 4A,B) and rapidly became immortalized. In contrast, wild-type melanocytes stopped expanding in culture after a maximum of 5 wk post-explantation, adopting a flattened morphology and accumulating melanin (Fig. 4A,B). As an indicator of cellular senescence, acidic senescence-associated β-galactosidase was mesured in wild-type and bcat^{sta} melanocytes (Sviderskaya et al. 2002). The number of senescence-associated β-galactosidase is clearly lower among bcat^{sta} melanocytes (Fig. 4C). We noted that around the 16th week of culture, some wild-type melanocytes at the edge of certain colonies became spindle-shaped (see circle in Fig. 4A) and started to divide again. Of these, some proliferated for a limited number of cycles and some immortalized. Melanocyte lines could be established from 95% (19 out of 20) of bcat^{sta} newborn pup skins, but only from 31% (six out of 19) of their wild-type littermates, indicating that bcat^{sta} expression in melanocytes increased the efficiency of immortalization. Neither wild-type nor bcat^{sta} melanocyte cell lines grew in the absence of the phorbol ester TPA or in low serum conditions, and none produced tumors in nude mice (data not shown), indicating that these cells were not fully transformed, but only immortalized. The effect of bcat^{sta} on melanocyte immortalization was not a result of overexpression of the bcat^{sta} protein, as the level of bcat^{sta} transgene expression in melanocytes cultured from newborn skin was ~10% of that of the endogenous

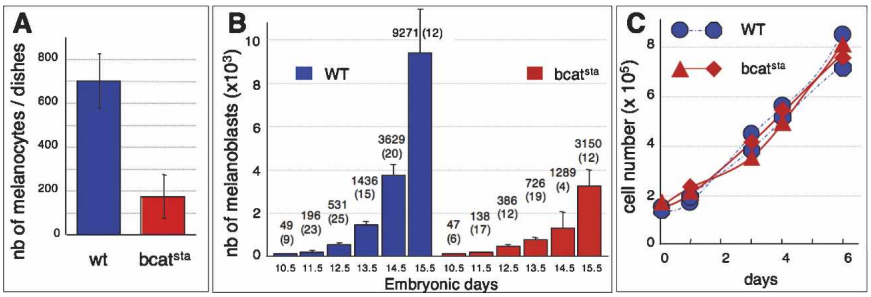

Figure 3. Activated β-catenin does not induce melanocyte proliferation. (*A*) Number of wild-type and bcat^{sta} melanocytes 10 d after the explantation of newborn skin. One-million cells isolated from the skin of wild-type and bcat^{sta} 3-d-old pups were grown in culture for 10 d. The number of melanocytes in each culture was estimated for five independent cultures of wild-type and bcat^{sta} pups. (*B*) The number of melanoblasts in wild-type and bcat^{sta} embryos, from E10.5 to E15.5. The number of melanoblasts, identified as X-Gal-positive cells, was determined by eye and with the help of Adobe Photoshop. The cell number indicated on the figure corresponds to the melanoblasts on one side of the embryo in the trunk region between the front and back limbs, from somites 13–25. The mean number of melanoblasts is indicated, and the standard deviation is shown for each day of development. The number of embryo sides studied is indicated in brackets. Note that the number of melanoblasts is identical in wild-type, Ink4a–Arf$^{+/-}$, and Ink4a$^{-/-}$ embryos (see Supplementary Fig. S9). (*C*) Growth curves of two independent wild-type (blue) and two independent bcat^{sta} (red) melanocyte cell lines. Note that these growth curves were established in the presence of TPA with cells that were immortalized for at least 6 mo. Each point is derived from the mean hemocytometer count of cells from three replicate dishes from two independent experiments. On day 0, 150,000 wild-type and bcat^{sta} cells were seeded. Cell numbers were determined 1, 3, 4, and 6 d after plating.

β-catenin (Fig. 1D). We thus conclude that bcat^{sta} promotes melanocyte immortalization.

bcat^{sta} rapidly represses p16^{INK4a} expression in culture

As inactivation of the *p16^{INK4a}* gene is the most frequent cause of melanocyte immortalization (Sviderskaya et al. 2002), we used immunofluorescence microscopy to study the effect of bcat^{sta} on p16^{INK4a} protein abundance, as well as on the melanocyte differentiation markers melanin and Mitf, in primary melanocyte cultures 2, 6, and 20 wk after skin explantation (Fig. 5A,B). p16^{INK4a} was expressed in all primary wild-type melanocytes 2 and 6 wk after explantation, whereas none of the bcat^{sta} melanocytes expressed p16^{INK4a} (Fig. 5A, arrows). The repression of p16^{INK4a} was melanocyte specific, because contaminating stromal cells (recognized by the absence of Mitf expression and melanin) did express p16^{INK4a} (Fig. 5A, asterisks). Twenty weeks after skin explantation, p16^{INK4a} was no longer detectable in wild-type melanocytes. This suggests that the repression of *p16^{INK4a}* by bcat^{sta} was an early event, leading to the rapid immortalization of these cells. In contrast, loss of p16^{INK4a} expression was a relatively late event during the immortalization of wild-type melanocytes. To confirm that p16^{INK4a} repression is an important determinant of immortalization by bcat^{sta}, we re-expressed p16^{INK4a} using a heterologous promoter in bcat^{sta} melanocytes. This resulted in inhibition of proliferation and the expression of senescence-associated acidic β-galactosidase activity in these cells (data not shown). We next investigated the repression of *p16^{INK4a}* expression in two wild-type (L9 and L14) and three bcat^{sta} (L10, L13, and L17) melanocyte cell lines by Western blotting and by RT–PCR. None of these lines produced significant amounts of p16^{INK4a} protein or mRNA (Fig. 5C), although all expressed *p19^{ARF}* mRNA, which is encoded by the same locus. Thus, melanocyte immortalization is accompanied by specific loss of *p16^{INK4a}* expression. Moreover, in the absence of TPA bcat^{sta}, melanocytes re-expressed p16^{INK4a}, but wild-type melanocytes did not (Fig. 5D). This suggests that the capacity of the *p16^{INK4a}* locus to be expressed remains intact but is suppressed in bcat^{sta} melanocytes and can be reactivated under some circumstances.

bcat^{sta} directly represses p16^{INK4a} *transcription*

The absence of *p16^{INK4a}* expression in wild-type melanocyte/melanoma cell lines is generally the result of one of two independent events: deletion in the *p16^{INK4a}* locus or promoter silencing of *p16^{INK4a}* by methylation. The rapid suppression of p16^{INK4a} and the concomitant maintenance of p19^{ARF} expression in bcat^{sta} melanocytes and its capacity to be re-expressed in the absence of TPA suggested that *p16^{INK4a}* transcription was directly repressed by bcat^{sta}. Examination of the *p16^{INK4a}* promoter revealed potential binding sites for β-catenin, and Lef/Tcf transcription factors have been conserved through evolution in the mouse and human *p16^{INK4a}* promoters (Fig. 6A). Using sequences spanning the putative mouse β-catenin/Lef-binding site as probes in electrophoretic mobility shift assays (EMSAs), we showed that Lef1 binding to both the mouse and human *p16^{INK4a}* promoter elements was almost as efficient as its binding to the control consensus binding site (Fig. 6B). Lef1 did not bind when the core sequence was mutated to GTC tAtGGG (mut1 p16), and binding was reduced if the core sequence was mutated to GTtcAAGGG (mut2 p16). Using a chromatin immunoprecipitation (ChIP) assay to confirm that β-catenin binds to the *p16^{INK4a}* promoter in human melanoma cells, the anti-β-catenin antibody yielded a strong band corresponding to the *p16^{INK4a}* promoter (Fig. 6C) and a weaker band corresponding to the *Brn2* promoter, a known target of β-catenin/Lef in melanomas (Goodall et al. 2004). No PCR product was detected if the ChIP assay was carried out with nonspecific antibody or with primers specific for the *HSP70* gene

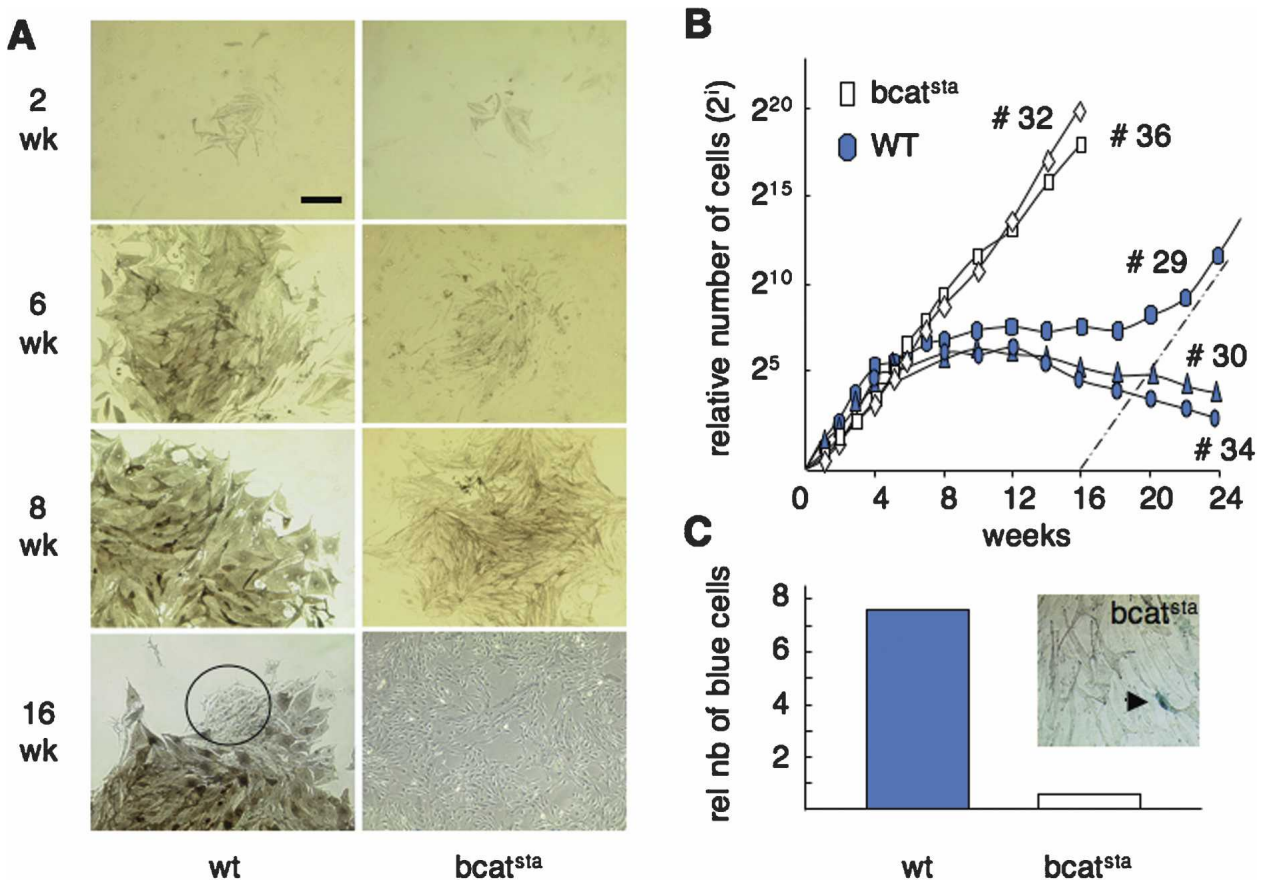

Figure 4. bcat^{sta} promotes melanocyte immortalization in vitro. (*A*) Direct (2, 6, and 8 wk) and phase-contrast (16 wk) photomicrographs of wild-type and bcat^{sta} melanocyte colonies after explantation. The circle indicates a growing melanocyte colony surrounded by senescent cells. Bar, 100 µm. (*B*) Melanocyte growth during the first 24 wk in culture. (*C*) Acidic β-galactosidase is produced in a minority of bcat^{sta} melanocytes (arrow), 8 wk after seeding. The relative number (rel nb) of blue wild-type cells may be underestimated due to the high melanin content of some melanocytes.

(which is not regulated by β-catenin). β-Catenin was detected on *p16^{INK4a}* promoter but not on a *CDH1* region used as a negative control (Fig. 6C). The results using quantitative PCR (qPCR) therefore confirm that β-catenin is present at the *p16^{INK4a}* promoter.

To investigate whether the *p16^{INK4a}* promoter is directly regulated by β-catenin and Lef/Tcf, we used a murine *p16^{INK4a}* promoter–luciferase reporter together with vectors encoding activated β-catenin in transient cotransfection assays in FO-1 cells. The wild-type *p16^{INK4a}* promoter was efficiently repressed both by β-catenin and TCF4 (Fig. 6D; Supplementary Fig. S5) whereas the ability of β-catenin to repress the *p16^{INK4a}* promoter was greatly reduced if the β-catenin/Lef/Tcf-binding site was mutated (GTtcAAGGG). As expected, wild-type β-catenin also repressed expression of *p16^{INK4a}*, but did so about threefold less efficiently than bcat^{sta} (Supplementary Fig. S5). This confirms the crucial role of this element in the response of the *p16^{INK4a}* promoter to β-catenin signaling. Together with the ChIP analysis, these observations strongly indicate that the repression by β-catenin is direct.

Although it is widely believed that p16^{INK4a} is not expressed in melanoma, four of 16 human melanoma cell lines we tested produced *p16^{INK4a}* mRNA (Fig. 6E,F; data not shown). To verify that β-catenin influences *p16^{INK4a}* expression in these human melanoma cells, we used small inhibitory RNA (siRNAs) to suppress β-catenin expression in A375P human melanomas. In this assay, *p16^{INK4a}* levels increased significantly upon knockdown of endogenous β-catenin (Fig. 6E; data not shown). Reciprocally, *p16^{INK4a}* mRNA abundance was reduced in three human melanoma cell lines after transient transfection with bcat^{sta}, and the effect was dose dependent (Fig. 6F). These data show that the repression of *p16^{INK4a}* by β-catenin occurs both in mice and humans, demonstrating that it is a conserved mechanism.

Our data indicate that activated β-catenin directly represses the expression of *p16^{Ink4a}* by binding to its promoter and thereby contributes to the immortalization of melanocytes. Concordantly, bcat^{sta} by itself was insufficient to induce melanoma in mice; this is similar to *p16^{INK4a}* (Serrano et al. 1996; Krimpenfort et al. 2001; Sharpless et al. 2001), the deletion of which does not lead to melanoma formation if not assisted by additional oncogenes. The bcat^{sta}/°; Ink4a–Arf$^{-/-}$ mice that we bred

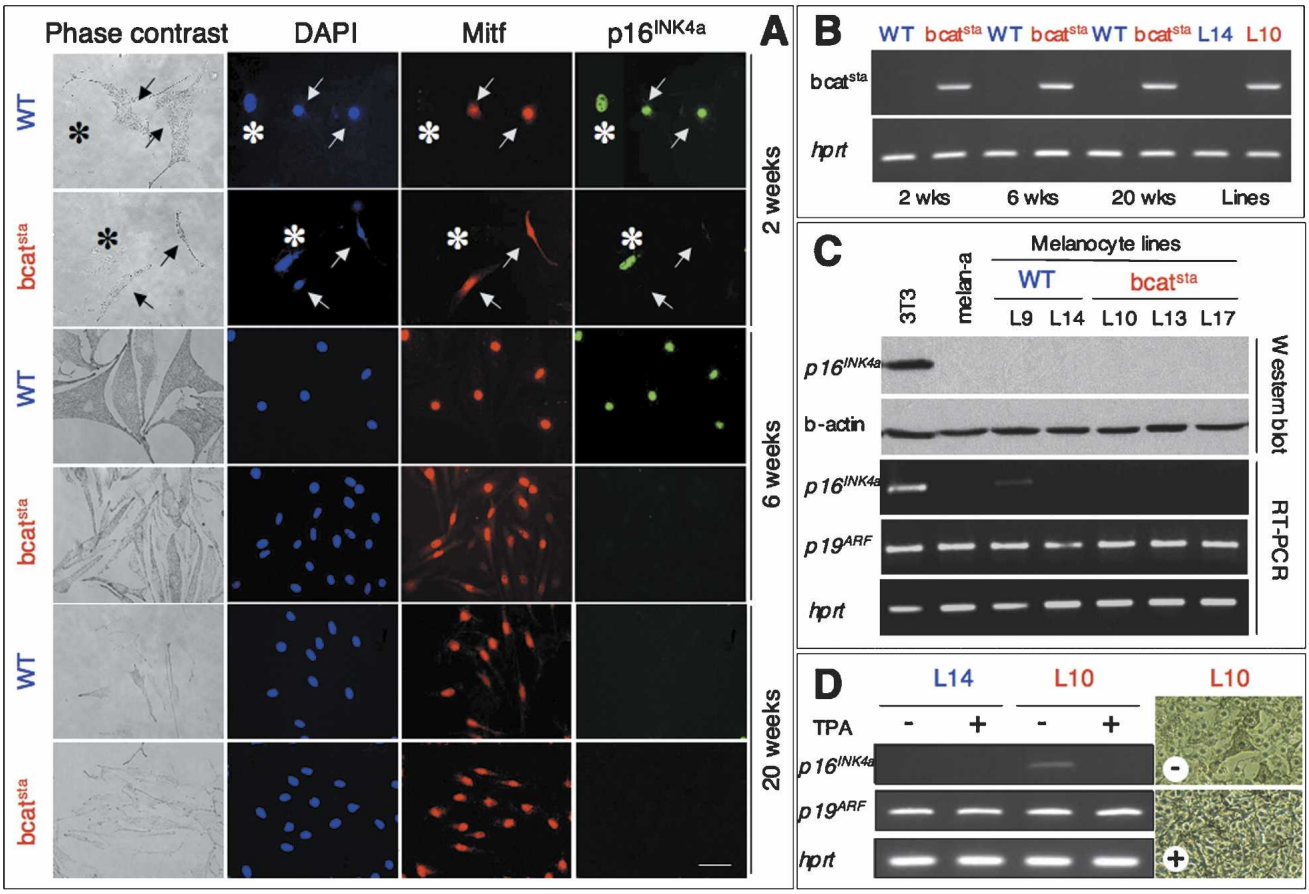

Figure 5. bcat^{sta} melanocyte lines do not express p16^{INK4a}. (*A*) Primary wild-type and bcat^{sta} melanocytes were processed for immunofluorescence microscopy using anti-Mitf and anti-p16^{INK4a} antibodies and DAPI staining 2, 6, and 20 wk after explantation. Arrows indicate Mitf-positive melanocytes and asterisks indicate nonmelanocytic Mitf-negative cells, which express p16^{INK4a}. Bar, 50 µm. Note that the cells were not passaged during the experiments. Morevover, wild-type melanocytes that were not dividing (big, flat, and hyperpigmented) remained p16^{INK4a} positive (not shown). (*B*) Expression of bcat^{sta} in culture 2, 6, and 20 wk after explantation. DNase-treated total RNA was subjected to RT–PCR analysis using oligonucleotides hybridizing to sequences around the intron sequence in the construct. Wild-type L14 and bcat^{sta} L10 melanocyte lines were used as controls. (*C*) Expression of *p16^{INK4a}* and *p19^{ARF}* in two wild-type (L9 and L14) and three independent bcat^{sta} (L10, L13, and L17) immortal melanocyte lines. Experiments were performed 6 mo after immortalization. Immunoblot analyses (*top* panel) and RT–PCR (*bottom* panel) were performed on identical amounts of protein and RNA from wild-type and bcat^{sta} cells. Murine 3T3 fibroblasts and melan-a melanocytes were used as controls. (*D*) RT–PCR analysis of *p16^{INK4a}*, *p19^{ARF}*, and hprt in wild-type (L14) and bcat^{sta} (L10) melanocytes treated with (+) or without (–) TPA for 3 d. The morphology of bcat^{sta} melanocytes (and of wild-type melanocytes, not shown) is greatly affected in the absence (–) of TPA, with cells becoming larger and flatter. The repression of *p16^{INK4a}* is reversible in bcat^{sta} melanocyte. Note that when TPA is removed from untransformed wild-type and bcat^{sta} melanocyte cultures, cells slowly stop growing.

did not develop melanomas, further indicating redundancy between the expression of bcat^{sta} and the absence of *p16^{INK4a}* (Supplementary Fig. S6). The incidence and latency period of melanoma production in Tyr::N-Ras^{Q61K}/°; bcat^{sta}/° mice were similar to those in Tyr::N-Ras^{Q61K}/°; Ink4a–Arf$^{-/-}$ mice. Similar results were obtained when Tyr::N-Ras^{Q61K}/°; bcat^{sta}/° mice carried mutations at p16 (Ink4a–Arf$^{+/-}$ and Ink4a–Arf$^{-/-}$). Finally, in the Tyr::N-Ras^{Q61K}/°; bcat^{sta}/° tumors, p16^{INK4a} production was present at low level and no mutation in the coding region or major alteration was detected in the Ink4a locus (Supplementary Fig. S7). We conclude that the expression of bcat^{sta} bypasses the requirement for genetic targeting of the *p16^{INK4a}* gene in N-Ras-driven melanomas. Hence, β-catenin is genetically upstream of *p16^{INK4a}*, as indicated by our observations that bcat^{sta} can directly repress *p16^{INK4a}* expression.

Discussion

Studies from several cell types have shown that β-catenin is important in promoting cell division and can induce the transcription of genes involved in proliferation, including c-Myc and cyclin D1. By contrast, we show here that β-catenin can reduce the number of melanoblasts in vivo and that it also efficiently immortalizes melanocytes in culture by turning down the

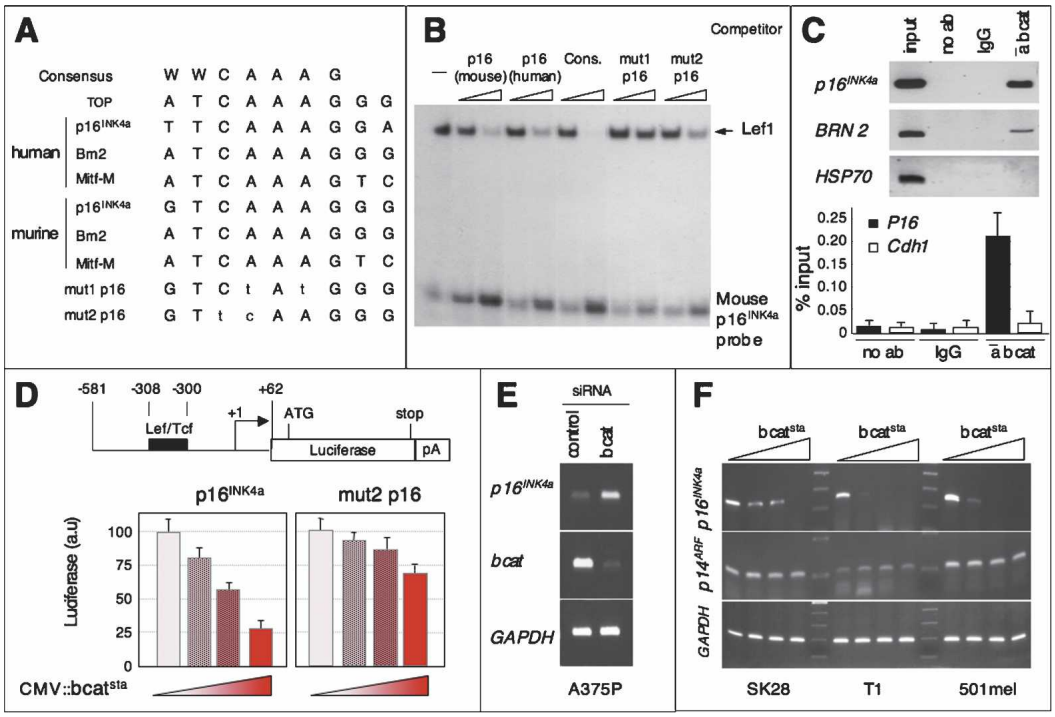

Figure 6. β-Catenin inhibits *p16^INK4a^* transcription. (*A*) β-catenin/Lef-binding sites in mouse and human *p16^INK4a^* promoters aligned with similar sites in mouse and human *Mitf-M* and *Brn-2* genes. The Lef-binding site present in the *p16^INK4a^* human promoter is the same as that published (Saegusa et al. 2006). It is in the same orientation as the Lef-binding sites of MITF-M but opposite to Brn-2 (Saito et al. 2002; Goodall et al. 2004). Mutated β-catenin/Lef sites (mut1 and mut2) of the *p16^INK4a^* promoter used in *B* and *D* are indicated. (*B*) EMSA using *p16^INK4a^* probe with LEF-1 protein produced in bacteria. The arrow indicates the specific *p16^INK4a^*–Lef1 complex. Competitor oligonucleotides were used at 10 and 50 ng. (*C*) ChIP assays of β-catenin binding to the *p16^INK4a^* promoter in 501Mel melanoma cells. (*Top* panel) ChIP assays are performed using antibody against β-catenin and analyzed after 25-cycle PCR (exponential phase). Brn2 promoter is used as a positive control and HSP70 as a negative control. (Lane *1*) Input represents 0.4% of the input used for the ChIP. For each promoter, two negative controls (no Ab and IgG) are included. (*Bottom* panel) qPCR analysis of ChIP on *p16^INK4a^* promoter using specific primers encompassing the Lef site and an antibody against β-catenin were performed in triplicate. No Ab, IgG, and the E-cadherin promoters are used as negative controls. All the data shown are representative of a minimum of two independent assays. (*D*, *top*) Diagram of the mouse *p16^INK4a^* promoter coupled to the luciferase reporter gene. (*Bottom*) Activities of wild-type and mut2 p16^INK4a^–luciferase reporters with various amounts (0, 0.2, 0.4, and 0.8 μg) of bcat^sta^ coexpressed in FO-1 cells, showing means of three independent experiments performed in duplicate; errors bars represent standard deviations. (*E*) siRNA-mediated down-regulation of β-catenin results in increased *p16^INK4a^* expression. (*F*) Increasing amounts of bcat^sta^ inhibit endogenous *p16^INK4a^* mRNA expression in human melanoma cell lines. Note that the cells were transfected when they were at low confluency.

*p16^INK4a^* promoter. Consistent with activated β-catenin silencing *p16^INK4a^* expression, we also show that it cooperates with activated NRas to promote the formation of melanoma in mice. Thus although proliferation is commonly associated with immortalization, these two cellular mechanisms can be uncoupled: N-Ras^Q61K^ induces proliferation but does not induce immortalization (data not shown) while in melanocytes activated β-catenin induces immortalization but does not induce proliferation.

Although β-catenin is likely to regulate a wide range of genes in the melanocyte lineage, including Brn-2 (Larue et al. 2003; Goodall et al. 2004), the ability of β-catenin to regulate melanocyte number is most likely to be related to its capacity to activate Mitf-M expression and activity (Widlund et al. 2002; Larue and Delmas 2006; Schepsky et al. 2006). Recent evidence has revealed that low levels of Mitf activity can promote proliferation (Carreira et al. 2006) while high levels inhibit cell division (Carreira et al. 2005; Loercher et al. 2005; Wellbrock and Marais 2005). Since β-catenin can directly activate the Mitf-M promoter via a LEF/Tcf-binding site (Takeda et al. 2000), any activation of β-catenin would either promote or inhibit proliferation depending on the basal level of Mitf activity in the cell. Thus, activated β-catenin can potentially act both positively and negatively on melanocyte proliferation, though clearly in vivo the net result of the expression of activated β-catenin was the reduction of melanoblast number.

The capacity of activated β-catenin to promote immortalization of primary melanocytes was surprising. Immortalization of melanocytes almost always involves inactivation of the *p16^INK4a^* gene, and, consistent with this, our results suggest that activated β-catenin directly represses the expression of *p16^INK4a^* through an evolutionarily conserved LEF/Tcf site in its promoter. While β-catenin is more usually an activator of transcription, as it is on the Mitf-M promoter, increasing evidence has

emerged to support its role as a transcriptional repressor (Kahler and Westendorf 2003; Kim et al. 2005; Spencer et al. 2006). However, it is yet unclear what signals or cofactors mediate the switch from activator to repressor and whether the effects seen are promoter specific. Although we cannot rule out the possibility that other genes regulated by β-catenin may be involved in immortalization of melanocytes, the absence of genetic alterations of *p16^{INK4a}* that are otherwise very common strongly implicates *p16^{INK4a}* in melanocyte immortalization and in melanomagenesis in cooperation with activated NRas.

It was previously showed that β-catenin may up-regulate p16^{INK4a} in endometrial carcinoma cells in an indirect manner and in a TCF4-independent manner. Note that this activation is cell context dependent (Saegusa et al. 2006). Moreover, Mitf can activate p16^{INK4a} expression in mouse fibroblasts and in uveal primary melanocytes (Loercher et al. 2005). The cellular systems used in these previous studies did not involve skin melanocytes. The results appear to conflict with ours, but this may be explained by the cellular context. Here, we showed that β-catenin down-regulates p16^{INK4a} in melanoma cells in a direct manner and in a TCF4-dependent manner (Supplementary Fig. S5). To test the activity of Mitf in melanocytic cells, we evaluated the activity of Mitf on p16^{INK4a} expression in the FO-1 melanoma cell line (Supplementary Fig. S8): Mitf-M repressed p16^{INK4a} activity in melanoma cells but activated p16 activity in HEK 293 cells. These various findings reveal an important aspect of the modulation of immortalization during melanomagenesis. Indeed, p16^{INK4a} expression is modulated and immortalization of melanocytes may or may not proceed according to the combined amount/activity of bcat, Lef/Tcf, and/or Mitf-M.

The expression of B-Raf^{V600E} in human leads to senescence in vivo (Michaloglou et al. 2005). In nevi, where the N-Ras/B-Raf pathway is commonly activated, induction of the Wnt/β-catenin pathway and the associated repression of *p16^{INK4a}* is likely to facilitate cell immortalization and therefore tumor progression. Senescence bypass may occur in melanocytic nevus cells either transiently, to overcome senescence giving rise to melanoma in situ, or continuously, leading to uncontrolled proliferation associated with invasion of the dermis and metastasis.

Melanoma comprises multiple subtypes, each with specific characteristics. Thus, human melanomas can be classified according to their initial localization on the body, their clinical characteristics, their histopathology, their molecular signature, or various other features. Despite the heterogeneity in the human disease and the limitations of any mouse model for human cancer, the novel mouse NRas/β-catenin melanoma model presented in this study appears to reflect several features associated with human melanoma: (1) β-Catenin is found mutated (activated) in a low percentage of human melanomas, but the Wnt/β-catenin pathway is activated in ~30% of melanomas; (2) NRas mutations are relatively common in melanoma, and some human melanomas with activated N-Ras also overexpress β-catenin (Table 1); (3) β-catenin down-regulates *p16^{INK4a}* both in mice and humans; and (4) melanoma metastases were observed in this model consistent with what is found in humans (data not shown). We are currently analyzing these metastases at the histological and molecular levels. Furthermore, the NRas/β-catenin mouse melanoma model has a precisely defined genetic component on a defined genetic background, with >10 backcrosses to C57BL/6 performed and does not involve chemical or UV induction. As an interesting consequence, NRas/β-catenin primary melanoma and metastasis can be readily transplanted into syngenic or nude mice (data not shown).

The NRas/β-catenin melanomas develop from epidermal melanocytes located in the hair bulge and rapidly invade the dermis. In humans, the exact origin of melanoma cells is unknown and may depend on the type of melanoma: Human melanomas arise either from interfollicular melanocytes, from the bulge of small hairs, or from melanocyte stem cells, and most human melanomas expand in the epidermis. Thus, in the murine melanoma model described here, the disease does not develop in exactly the same way as most human melanomas. However, in our case the origin of the melanoma is the epidermis and the described model presents sufficient features of the human disease to make a valuable resource for understanding the molecular mechanisms underpinning melanoma progression.

The Tyr::N-Ras^{Q61K}/°; Ink4a–Arf$^{-/-}$ and Tyr::N-Ras^{Q61K}/°; bcat^{sta} mice produce melanoma with the same latency and frequency, suggesting that the first cellular events in epidermal melanocytes (proliferation and immortalization) are similar. Intriguingly, the subsequent steps of melanomagenesis appear to be different: Tyr::N-Ras^{Q61K}/°; Ink4a–Arf$^{-/-}$ melanomas develop mainly in the epidermis whereas Tyr::N-Ras^{Q61K}/°; bcat^{sta} develop mainly in the dermis. Since these melanoma models are based on mice with defined genetic backgrounds, these differences can be explained at the molecular level by differences in the p19^{ARF}, p16^{INK4a}, and β-catenin targets, including Mitf-M. p19^{ARF} is still produced and not affected in Tyr::N-Ras^{Q61K}/°; bcat^{sta}, but obviously not produced in Tyr::N-Ras^{Q61K}/°; Ink4a–Arf$^{-/-}$ mice since the full *Cdnk2a* locus is inactivated. p16^{INK4a} is produced at a low level in Tyr::N-Ras^{Q61K}/°; bcat^{sta} melanomas. The expression of β-catenin targets can be induced/repressed in Tyr::N-Ras^{Q61K}/°; bcat^{sta} melanoma, and the production of Mitf-M is clearly induced in this melanoma and may contribute to the invasiveness as concerns the dermis. These two melanoma models (NRas/p16^{INK4a} and NRas/bcat^{sta}) together should provide insight into late events during melanomagenesis. It is also possible that the Braf-activated form (V600E) expressed in melanocytes also cooperates with bcat^{sta}/° or with Ink4a–Arf$^{-/-}$ mice. Experiments addressing these possibilities will be performed in the near future.

Apart from melanoma, the accumulation of nuclear β-catenin is associated with a number of cancers in ad-

dition to melanoma, most notably colon cancer (Giles et al. 2003). Given the potential of β-catenin to suppress $p16^{INK4a}$ expression identified here, it will be interesting to test whether β-catenin also plays an anti-senescence function in colon and other cancers.

Materials and methods

Constructs and transgenic mice

The mouse *Tyrosinase* gene enhancer (Enh) was fused to the promoter region (Tyr prom) to produce a 6.1-kb regulatory element driving the expression of a cDNA encoding a mutated form of β-catenin (b-cat) in which Ser33, Ser37, Ser45, and Thr41 were replaced by alanines (A33, A37, A41, A45) (Aberle et al. 1997). An nls and EGFP sequences were fused in frame to the 3′ end of the mutated β-catenin cDNA. An SV40 small T-antigen splice site and polyadenylation sequence were added to the 3′ end of the construct to produce Tyr::β-cat-mut-nls-gfp ($bcat^{sta}$) (see Fig. 1). The Tyr::β-cat, Tyr::β-cat-egfp, and Tyr::β-cat-mut-nls constructs are similar to the Tyr:: $bcat^{sta}$ construct but lack the EGFP, nls, and/or the β-catenin mutations (mut). Unfortunately, the transgene production could not be followed by direct GFP fluorescence or with antibodies directed against GFP (Roche Molecular).

Transgenic mice were generated with this construct, as described previously (Delmas et al. 2003). The transgene was detected in several founder mice by RT–PCR with RNA isolated from skin biopsy samples. The tyrosinase promoter is mainly specific to the melanocyte lineage. To verify that the expression of $bcat^{sta}$ was specific to the melanocytes of the skin, mouse line 1 was backcrossed to mi^{vga9} mice (Hodgkinson et al. 1993). No transgene expression was detected in such mice. This result indicates that the transgene is specifically expressed in cells of the melanocyte lineage. Transgenic mice were backcrossed >10 times toward C57BL/6, and both lines 1 and 2 presented a hypopigmented coat color phenotype. Mice producing the same type of transgene without NLS were produced. As hemizygotes, transgenic mouse lines did not present any coat color phenotype. As homozygotes, one transgenic mouse line presented a similar coat color phenotype. This transgenic mouse line was not used for further experiments. The transgenic Tyr::N-Ras^{Q61K}/° and Ink4a–Arf knockout mice were described previously (Serrano et al. 1996; Ackermann et al. 2005).

Mice were crossed with Dct::LacZ mice (Mackenzie et al. 1997), and the resulting embryos were collected at various time during pregnancy. Embryos were stained with X-gal, as described previously (Delmas et al. 2003). The number of LacZ-positive cells (melanoblasts) was determined on each embryo side from somites 13–25 (Yajima et al. 2006). We excluded from this count the X-gal staining associated with the nerves. Variations in the number of melanoblasts were found on both sides of embryos.

RT–PCR

Total RNA was prepared for RT–PCR analysis as described previously (Delmas et al. 2003). The primers used were LL636 (5′-ATCTGGAGCAGCATGGAGTC-3′) and LL528 (5′-ACCAGC GTGTCCAGGAAG-3′) for mouse $p16^{INK4a}$; LL922 (5′-CAAC GCACCGAATAGTTACG-3′) and LL923 (5′-CTCCTCAGCC AGGTCCAC-3′) for human $p16^{INK4a}$; LL655 (5′-GTCGCAG GTTCTTGGTCACT-3′) and LL528 for $p19^{ARF}$; LL924 (5′-GGTTTTCGTGGTTCACATCC-3′) and LL926 (5′-CTAGAC GCTGGCTCCTCAGTA-3′) for $p14^{ARF}$; LL17 (5′-CACAGGA CTAGAACACCTGC-3′) and LL18 (5′-GCTGGTGAAAAGGA CCTCT-3′) for *Hprt*; and LL82 (5′-GCTGAGTATGTCGTGG AGTC-3′) and LL83 (5′-TTGGTGGTGCAGGATGCATT-3′) for *Gapdh*.

Western blot analysis and immunofluorescence microscopy

Western blots were performed as described previously (Sviderskaya et al. 2002). The primary antibodies used were rabbit polyclonal anti-$p16^{INK4a}$ antibody from Santa Cruz Biotechnology (SC-1207), mouse monoclonal anti-β-catenin antibody from Transduction Laboratories (#610154), and mouse monoclonal anti-actin antibody from Euromedex (MAB1501). Cells were cultured on glass coverslips and immunostaining was performed as described previously (Morali et al. 2001). For immunofluorescence microscopy, we used mouse monoclonal anti-$p16^{INK4a}$ antibody from Santa Cruz Biotechnology (SC-1661) and rabbit polyclonal anti-MITF antibody prepared in the laboratory of Dr. H. Yamamoto. Histological and immunohistochemical analyses of tumors from Tyr::N-Ras^{Q61K}/°; $bcat^{sta}$/° mice were performed as described previously (Ackermann et al. 2005).

Cell culture and luciferase assays

Primary melanocytes were cultured as described previously (Larue et al. 1992). The number of melanocytes growing in cultured explants from five independent pup skins (#29, 30, 32, 34, and 36) was estimated weekly under the microscope. The mouse melanocyte cell line, melan-a, was kindly provided by Professor D.C. Bennett. These cells were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum and 200 nM TPA (Sigma). FO-1 and 501 mel cells, kindly provided by Drs. R. Baserga and R. Halaban, were cultured in RPMI-1640 medium containing 10% serum. FO-1 cells were transiently transfected in six-well plates, using 6 µL of FuGene (Roche) and 2 µg of total plasmid DNA. TOP and FOP constructs were used. The $p16^{INK4a}$ promoter was cloned and inserted into the pGL3 basic vector (Promega). Cells were cotransfected with the PGK::β-galactosidase construct as a control. The amount of DNA was equalized with pBluescript. We determined luciferase activity and β-galactosidase activity 48 h after transfection. The transfection efficiency of melanoma cells depended on the confluency, from an estimated 10% at high confluency to 80% at low confluency. Luciferase activity was normalized against β-galactosidase activity.

EMSAs and ChIP

EMSAs for Lef1 were performed as described previously (Goodall et al. 2004). LEF1 was produced as a GST fusion protein, purified, and then cleaved with thrombin to release the GST. ChIP assays were performed using goat polyclonal anti-β-catenin antibody from Santa Cruz Biotechnology (SC-1496) or 6 µL of nonspecific anti-IgG antibody (Bio-Rad). Samples were subjected to immunoprecipitation and analyzed by 25-cycle qPCR, ensuring that the reaction was in the exponential phase. The primers used for PCR were as follows: 5′-TCAGAGTCT GCTCTTATACC-3′ and 5′-GAGAAATCGAAATCACCTGT 3′ for the $p16^{INK4a}$ promoter; 5′-GAGGAGGGCTAGGAG GACTCC-3′ and 5′-CGCGTAACTGTCAATGAAAAA-3′ for the *Brn2* promoter; 5′-CCTCCAGTGAATCCCAGAAGACT CT-3′ and 5′-TGGGACAACGGGAGTCACTCTC-3′ for the *HSP70* promoter. The primers used for qPCR were as follows: 5′-AACCCTTGCCCCAGACAG-3′ and 5′-GAGAGCCCCAC CGAGAATC-3′ for the $p16^{INK4a}$ promoter; 5′-TGGCTCAC

ACCTGAAATCCT-3′ and 5′-CGCTGTGTCTCCCTGATATG-3′ for the *Cdh1* promoter.

Acknowledgments

We dedicate this manuscript to the memories of Christine Jeanney and Christian Bonnerot. We are grateful to R. Baserga, C. Gaggioli, R. Halaban, I. Jackson, R. Kemler, S. Saule, M. Serrano, and H. Yamamoto for valuable reagents. We thank H. Arnheiter, D. Bennett, and N. Modjtahedi for helpful discussions, and Delphine Champeval, Aurélie Herbette, and Christophe Alberti for their excellent technical assistance. S.M., M.K. and L.D. are recipients of grants from the ARC, the Ligue Nationale Contre le Cancer, and the LNC Comité de l'Oise, respectively. This work was supported by the Ligue Nationale Contre le Cancer (Equipe labellisée), INCa, cancéropole IdF and Institut Curie, the Swiss Cancer League, the foundation Emma Muschamps, the Swiss National Science Foundation, the NCCR Molecular Oncology, and the Scientific and Technical Research Council of Turkey, as well as Marie Curie Cancer Care and the Association of International Cancer Research.

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Genes Dev. 2007, **21:**
Access the most recent version at doi:10.1101/gad.450107