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β-Catenin Signaling Controls Metastasis in Braf-Activated Pten-Deficient Melanomas

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SUMMARY

Malignant melanoma is characterized by frequent metastasis, however, specific changes that regulate this process have not been clearly delineated. Although it is well known that Wnt signaling is frequently dysregulated in melanoma, the functional implications of this observation are unclear. By modulating β-catenin levels in a mouse model of melanoma that is based on melanocyte-specific Pten loss and $Braf^{V600E}$ mutation, we demonstrate that β-catenin is a central mediator of melanoma metastasis to the lymph nodes and lungs. In addition to altering metastasis, β-catenin levels control tumor differentiation and regulate both MAPK/Erk and PI3K/Akt signaling. Highly metastatic tumors with β-catenin stabilization are very similar to a subset of human melanomas. Together these findings establish Wnt signaling as a metastasis regulator in melanoma.

INTRODUCTION

Malignant melanoma is the most deadly form of skin cancer, and its incidence is increasing both in the United States and worldwide (Purdue et al., 2008). Nearly all melanoma deaths are a result of metastasis, which can occur early in disease progression, even from thin primary tumors (Bedrosian et al., 2000). Recent advances in melanoma therapeutics have led to improved survival in patients with metastatic melanoma, however, prognosis is still poor for most patients (Hodi et al., 2010; Chapman et al., 2011). Despite an improving understanding of the genetic alterations present in human melanomas, how these changes relate to the metastatic process is not well understood. Metastasis is difficult to study in vitro, making the use of mouse models central to the functional evaluation of the roles of genetic changes observed in human melanomas in the metastatic process.

β-catenin associates with E-cadherin as part of the adherens junction and can also interact with the T cell factor/lymphoid enhancer factor (TCF/LEF) to activate the transcription of target genes as part of the canonical Wnt signaling pathway. β-catenin mutations were originally found to be present in 23% of melanoma cell lines (Rubinfeld et al., 1997) and have since been consistently described in about 5% of primary, uncultured melanomas (Omholt et al., 2001; Demunter et al., 2002; Reifenberger et al., 2002; Forbes et al., 2010). Nuclear β-catenin, which is

Significance

Melanoma has a predilection for early and extensive metastasis. Despite an improved understanding of genetic changes found in human melanoma, how these changes relate to the metastatic process is unclear. Here, using an inducible genetically engineered mouse model of melanoma that is driven by some of the most common alterations found in human melanoma, we identify β-catenin as a central mediator of the metastatic process in vivo. In this study, the relation between β-catenin, MITF, tumor differentiation, metastasis, and signaling through core pathways that regulate proliferation and survival are evaluated. This study also introduces a mouse model characterized by inducible and rapid primary tumor growth, high melanocytic antigen expression, and numerous metastases.

thought to reflect canonical Wnt pathway activation, is present in approximately one-third of human melanoma specimens (Rimm et al., 1999). The mechanisms of β-catenin dysregulation are likely diverse but are at least in part due to mutations both in β-catenin as well as other Wnt pathway components (reviewed by Larue and Delmas, 2006). Subsequent analyses have also reported abnormal cellular localization of β-catenin in the cytoplasm and nucleus of melanomas (Silye et al., 1998; Sanders et al., 1999; Omholt et al., 2001; Kageshita et al., 2001; Demunter et al., 2002; Kielhorn et al., 2003; Tucci et al., 2007; Chien et al., 2009). The consequences of this dysregulation, as well as the functional implications of β-catenin activating mutations in vivo, remain somewhat unclear. For example, whereas Wnt signaling activation has been shown to contribute to melanoma formation in mice (Delmas et al., 2007), other studies have reported that increased β-catenin staining is associated with improved survival in melanoma patients (Chien et al., 2009). These findings suggest that the results of Wnt/β-catenin signaling activation are complex and likely context-dependent.

β-catenin is important for normal melanocyte biology and is required for survival of melanocytes from neural crest precursors (Hari et al., 2002). Part of this requirement relates to the direct transcriptional activation of MITF by β-catenin when Wnt signaling is activated. MITF is a transcription factor that regulates melanocyte function and survival. Similarly to β-catenin, MITF is dysregulated in melanoma, where it is has been shown to be amplified in 10% of primary and 21% of metastatic melanomas (Garraway et al., 2005). The functional impact of MITF amplification and overexpression in human melanoma are also somewhat unclear (Garraway et al., 2005; Ugurel et al., 2007; Nazarian et al., 2010). The implications of the activation of both Wnt signaling and melanocytic differentiation in human melanomas remain unresolved and highlight the need for systematic in vivo evaluation of the relation between Wnt/MITF alterations and disease progression in the context of the genetic alterations that are commonly observed in human melanomas.

In this study, we utilized a conditional mouse model of melanoma that is based on melanocyte-specific Pten loss and the $Braf^{V600E}$ activating mutation in order to help clarify the role of Wnt/β-catenin signaling and downstream factors, such as MITF in melanoma formation and progression. By either inactivating or stabilizing β-catenin in these melanomas, the functional role of β-catenin was specifically evaluated. The mouse models of melanoma generated in this study were closely characterized and compared to human melanomas with analogous genetic changes.

RESULTS

β-Catenin Loss Inhibits Melanoma Formation in *Pten/Braf*-Driven Melanomas

In order to evaluate the role of canonical Wnt signaling and β-catenin in melanoma formation and progression, we used a previously characterized conditional mouse model of melanoma that is based on melanocyte-specific Pten-inactivation and the $Braf^{V600E}$ activating mutation (Dankort et al., 2009). In this model (henceforth referred to as the "*Pten/Braf*" model), genetic recombination of floxed alleles and melanoma formation occurs after topical application of 4-hydroxtamoxifen (4-HT). Furthermore, mice develop melanoma from endogenous melanocytes, closely recapitulating the complex nature of tumor initiation and formation in a physiologically accurate microenvironment. In the *Pten/Braf* model, just these two genetic changes are sufficient to cause melanoma in 100% of mice within one month of generalized tumor induction by topical application of 4-HT (Figure 1A; Dankort et al., 2009).

To study the role of endogenous β-catenin in melanoma formation and progression, β-catenin was inactivated in the *Pten/Braf* model by using a previously characterized conditional knockout *Ctnnb1* allele (Brault et al., 2001; referred to as *Bcat-KO*). In these mice, wild-type β-catenin is expressed in melanocytes until Cre-mediated recombination results in β-catenin inactivation at the time of tumor induction. *Pten/Braf/*$Bcat^{+/-}$ or *Pten/Braf/*$Bcat^{-/-}$ (KO) mice were perinatally treated with topical 4-HT in order to induce generalized recombination in melanocytes. In mice in which both copies of β-catenin were inactivated, a significant increase in median survival was observed relative to the *Pten/Braf* mice (Figure 1A). Although pigmented lesions did eventually develop in *Pten/Braf/Bcat-KO* mice, melanoma formation was significantly delayed, especially on footpads and peri-oral skin and mucosa (Figure 1B). Histologically, *Pten/Braf/Bcat-KO* melanomas invade deep into the dermis and subcutis. In contrast with *Pten/Braf* melanomas, which are pigmented, these tumors show only rare pigmented tumor cells and exhibit focal nerve sheath-like histopathological features (Figure 1C).

Pten/Braf melanomas metastasize to draining lymph nodes in 100% of mice that were perinatally treated with 4-HT (Dankort et al., 2009). However, when β-catenin is inactivated, metastatic tumor cells in draining lymph nodes become undetectable, even at up to 80 days of age (Figures 1B and 1D). These findings suggest that endogenous β-catenin is important both in melanoma formation, as well as in the subsequent spread of tumor cells to draining lymph nodes. Melanomas did not form when β-catenin was inactivated in the context of either $Braf^{V600E}$ mutation or Pten loss alone, demonstrating that β-catenin inactivation does not promote tumorigenesis in the context of these individual oncogenic changes (Figure S1A available online).

β-Catenin Stabilization Accelerates *Pten/Braf*-Driven Melanomagenesis

Activation of the canonical Wnt signaling pathway results in stabilization, accumulation, and nuclear translocation of β-catenin. In the nucleus, β-catenin interacts with the TCF/LEF family of transcription factors and leads to transcriptional activation of target genes, such as *MYC*, *CCND1* (Cyclin D1), and *AXIN2*, as well as melanocyte-specific transcripts, such as *MITF* (Larue and Delmas, 2006; MacDonald et al., 2009). Mutations to exon 3 phosphorylation sites, which regulate β-catenin stability, provide an alternate means for increasing β-catenin activity, even in the absence of Wnt ligands (Omholt et al., 2001; Demunter et al., 2002; Reifenberger et al., 2002). In light of the inhibitory effect of β-catenin loss in *Pten/Braf* melanomas and the prevalence of β-catenin stabilizing mutations in human melanomas, we tested the effects of β-catenin stabilization in the *Pten/Braf* tumor model. In order to do this, *Pten/Braf* mice were crossed with mice bearing the previously characterized $Ctnnb1^{loxex3}$ allele (Harada et al., 1999). Prior to recombination, wild-type β-catenin is expressed in melanocytes, but after

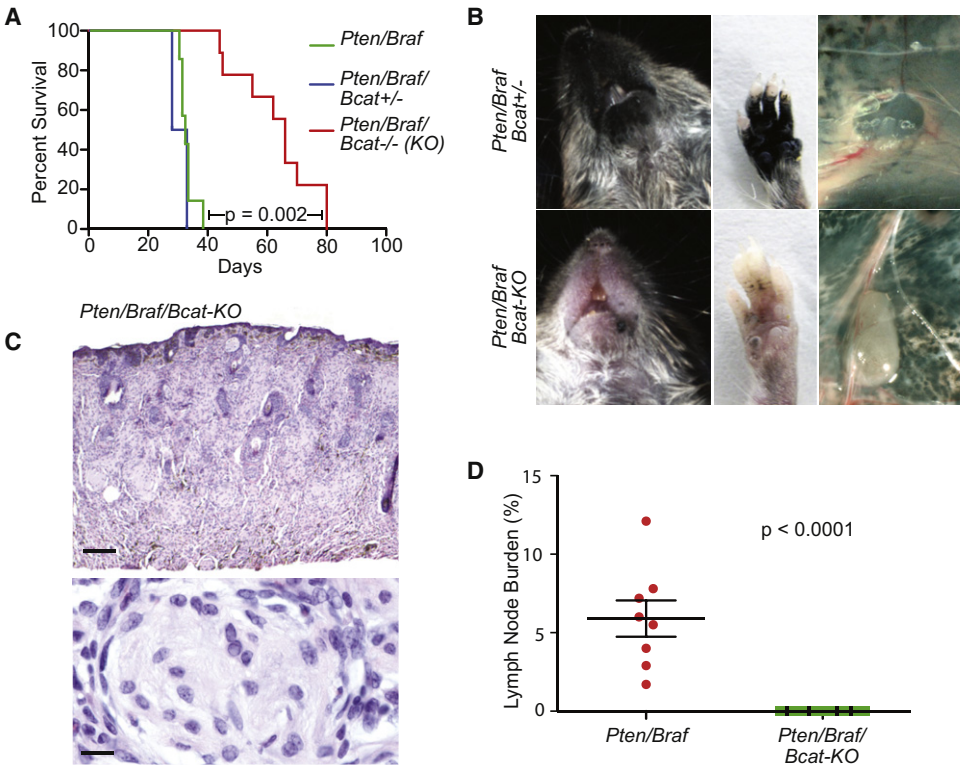

Figure 1. β-Catenin Loss Inhibits Melanoma Formation in *Pten/Braf*-driven Melanomas

(A) Survival analysis of *Pten/Braf* cohorts after perinatal/generalized tumor induction.

(B) Comparison of *Pten/Braf/Bcat+/−* (top) and *Pten/Braf/Bcat-KO* (bottom) tumor burden at 28 days of age. Metastasis to inguinal lymph node is also shown (right panels).

(C) H&E stained *Pten/Braf/Bcat-KO* flank tumor, scale 300 μM (top) and 10 μM (bottom).

(D) Quantification of metastasis to the inguinal lymph nodes in *Pten/Braf/Bcat-KO* mice (n = 13) compared to *Pten/Braf* mice (n = 8). Error bars represent SEM. See also Figure S1.

Cre-mediated excision of exon 3, a stabilized (degradation-resistant) version of β-catenin is expressed (*Bcat-STA* allele). Mutations in human melanomas almost always affect exon 3 phosphorylation sites (Forbes et al., 2010) and result in analogous stabilization of β-catenin.

After generalized induction of recombination, *Pten/Braf/Bcat-STA* mice exhibit reduced survival relative to *Pten/Braf* mice (Figures 2A and 2B). By the time of weaning, *Pten/Braf/Bcat-STA* mice are moribund with heavily pigmented tumors (Figures 2C and 2D). Necropsy revealed enlarged, pigmented lymph nodes in all mice, which were confirmed to contain metastatic melanoma (Figures 2C and 2E). Upon histological examination, tumors were markedly pigmented throughout their entire thickness and tumor cells could be found surrounding and directly abutting the microvasculature.

Melanomas also formed in mice with Braf activation and β-catenin stabilization in the presence of wild-type Pten after generalized induction of recombination. These tumors, in contrast with the *Pten/Braf/Bcat-STA* tumors, had a very long latency, with a median survival of 250 days (Figure 2A). *Braf/Bcat-STA* tumors generally grew very slowly and were heavily pigmented (Figure 2F). Approximately 10% of tumors exhibited more rapid growth, which was always accompanied by relative loss of melanocytic differentiation antigen expression (Table S1). In contrast with the *Pten/Braf/Bcat-STA* model, neither manifestation of the *Braf/Bcat-STA* model exhibited distant metastasis.

β-Catenin Signaling Controls the Ability of Melanomas to Metastasize to Lung, Bowel, and Spleen

As most melanoma deaths are due to metastasis to distant organs, we characterized the pattern of visceral metastasis in *Pten/Braf* melanomas with altered β-catenin status. In *Pten/Braf/Bcat-STA* mice, lung metastases were present at nearly a 100-fold greater frequency than in *Pten/Braf/Bcat-KO* mice (Figures 3A and 3B and S2A–C). The metastases were irregular in shape and ranged in size from <0.1mm to >0.5 mm (Figure 3C and S2A–C). These pigmented metastases stained strongly for the melanocytic marker Tyrp1 (Figure S2D–E). β-catenin stabilization in *Pten/Braf* melanomas also resulted in metastasis to other visceral sites, such as bowel and spleen (Figure 3D–3G).

Locally Induced Melanomas are Highly Metastatic and Can Form Lethal Metastases

The conditional and inducible nature of this mouse model system allows for the generation of small, anatomically restricted melanomas by focal application of a small volume of 4-HT to adult mice (Dankort et al., 2009). Using this approach, rather than

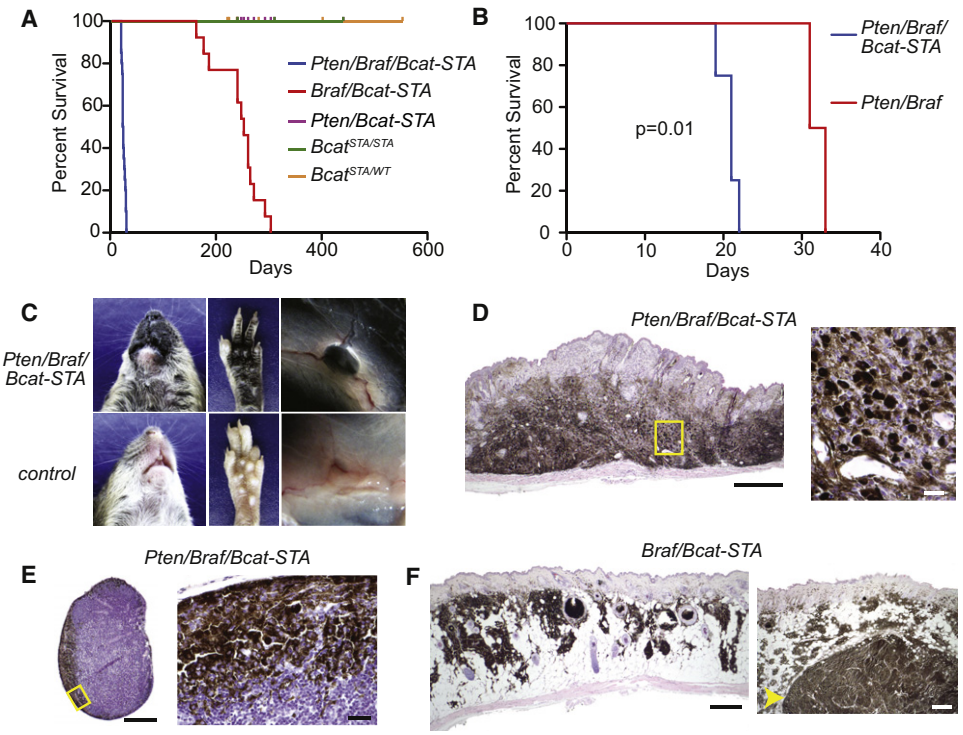

Figure 2. β-Catenin Stabilization Accelerates *Pten/Braf*-Driven Melanomagenesis

(A) Survival analysis of cohorts with β-catenin stabilization after perinatal tumor induction.

(B) Survival analysis of *Pten/Braf* littermates, either with or without β-catenin stabilization, after perinatal tumor induction.

(C) Tumor phenotype in *Pten/Braf/Bcat-STA* mice (top panels) compared to non-tumor prone mice (bottom panels) at 21 days of age.

(D) H&E stained *Pten/Braf/Bcat-STA* flank tumor, scale 500 μM. Right panel: high power of outlined field, scale 20 μM.

(E) H&E stained tumor-draining lymph node from a *Pten/Braf/Bcat-STA* mouse, scale 400 μM. High power of outlined field, scale 20 μM, right panel.

(F) H&E stained nevi (left panel) and tumor (right panel, arrow) from a *Braf/Bcat-STA* mouse, scale 500 μM (left) and 500 μM (right).

See also Table S1.

the generalized, perinatal inductions used until this point, melanomas were induced on the flank skin of *Pten/Braf* mice with wild-type, inactivated, or stabilized β-catenin. Melanocytic proliferations generally became grossly apparent approximately two weeks after induction of recombination, growing to 1 cm tumors by four to eight weeks, at which point mice were euthanized for analysis (Figure 4A). When β-catenin was stabilized, tumors grew more rapidly to 1 cm (Figure S3A). Melanomas still formed in mice with inactivation of both copies of β-catenin (Figure 4A). PCR combined with western blotting and immunohistochemistry confirmed recombination of floxed *Ctnnb1* alleles as well as the predicted changes to β-catenin at the protein level (Figure S3B–D). When β-catenin was stabilized, it was frequently found in the nucleus of tumor cells, and the stabilized protein (lacking exon 3) could be visualized by western blot. In *Pten/Braf* melanomas, wild-type β-catenin was generally found within the cytoplasm but could also be visualized in the nucleus. β-catenin staining was absent in tumor cells from *Pten/Braf/Bcat-KO* mice.

In addition to changes in β-catenin, these tumors displayed vastly different histology (Figure 4B). *Pten/Braf/Bcat-STA* tumors were characterized by large pigmented cells that were present throughout the thickness of the tumor. *Pten/Braf* tumors were characterized by increased edema in deeper parts of the tumor, in which tumor cells were often relatively smaller in size. Similarly to the generalized tumor inductions, *Pten/Braf/Bcat-KO* melanomas had relatively little pigmentation, bland spindled cytology, and often exhibited areas of nerve sheath-like differentiation. Metastases were infrequent in mice with locally induced *Pten/Braf* melanomas with either wild-type or inactivated β-catenin. However, when β-catenin was stabilized, lung metastases and expansile lymph node metastases were prevalent (Figure 4C).

In the various iterations of the *Pten/Braf* model, flank melanomas grow rapidly, leaving little time for metastasis to occur. To circumvent this issue and develop models in which morbidity and mortality were directly related to metastases, rather than the primary tumor, two approaches were taken. First, tumors were induced on the distal tail of *Pten/Braf/Bcat-STA* mice (Figure S3F). Second, localized tumors were induced on the flank in *Braf/Bcat-STA* mice that had only one copy of Pten inactivated (Figures 4A and 4B). Both approaches resulted in slower primary tumor growth, allowing mice to be followed for metastasis for up to one year. Clinically symptomatic metastases developed in two-ninths of tail melanoma-bearing mice and in one-fifth of flank melanoma-bearing mice (Figures S3E and S3G; Table S2). Similar approaches in *Pten/Braf* mice, without β-catenin stabilization, resulted in primary tumor formation, but not distant metastasis (Table S2).

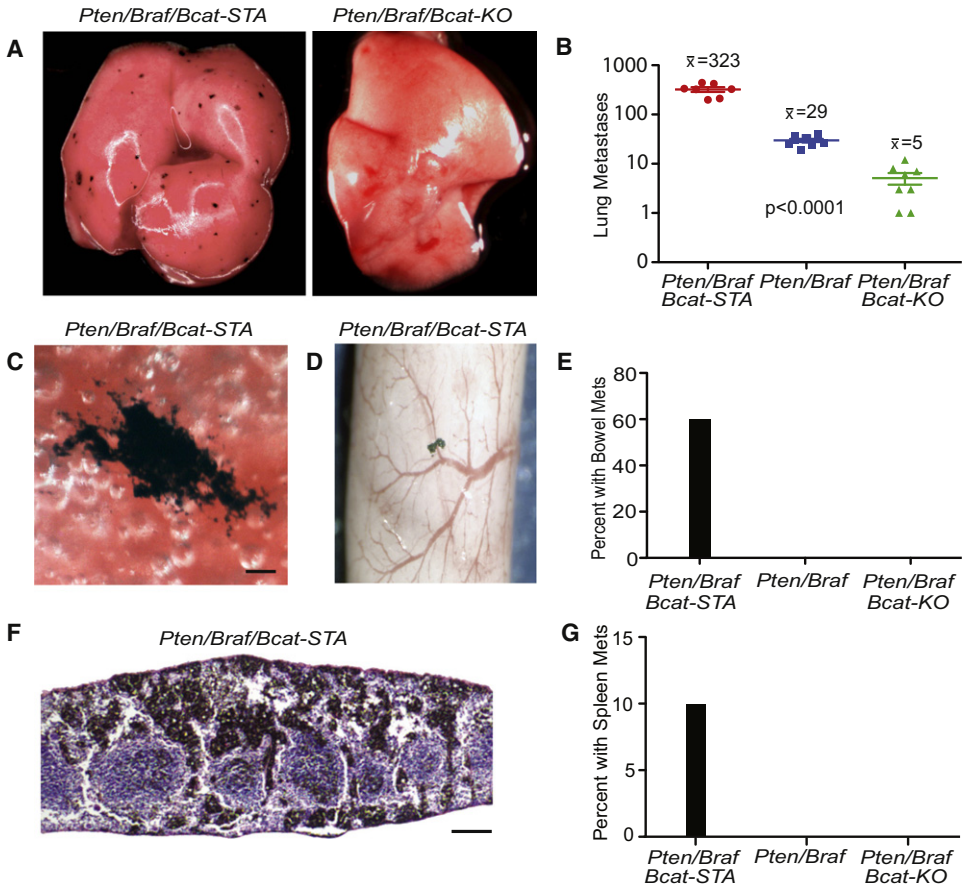

Figure 3. β-Catenin Signaling Controls the Ability of Melanomas to Metastasize to Lung, Bowel, and Spleen

(A) Lungs from a perinatally treated *Pten/Braf/Bcat-STA* mouse at 27 days of age (left panel) and a perinatally treated *Pten/Braf/Bcat-KO* mouse at 56 days of age (right panel).

(B) Comparison of number of lung metastases visible on the surface of the lung in perinatally treated litters. Error bars represent SEM.

(C) Lung metastasis from a perinatally treated *Pten/Braf/Bcat-STA* mouse, scale 100 μM.

(D) Bowel metastasis from a perinatally treated *Pten/Braf/Bcat-STA* mouse.

(E) Comparison of number of bowel metastases in perinatally treated litters.

(F) H&E stained spleen metastasis from a perinatally treated *Pten/Braf/Bcat-STA* mouse, scale 200 μM.

(G) Comparison of number of spleen metastases in perinatally treated litters.

See also Figure S2.

Enhanced Metastasis in *Pten/Braf/Bcat-STA* Melanomas Is Accompanied by an Increase in Melanocytic Differentiation Markers

Pten/Braf/Bcat-STA tumors were much more pigmented than were *Pten/Braf* tumors (Figure 5A–5B). *Pten/Braf* tumors showed pigmented melanoma cells at the superficial aspect of the tumor, whereas deeper parts of the tumor were edematous and amelanotic. In contrast, *Pten/Braf/Bcat-STA* melanomas were heavily pigmented and remained so, even in deeper portions of the tumor. As the expression of many melanocytic pigmentation genes is controlled by MITF-M, a melanocyte-specific isoform of MITF that is a transcriptional target of β-catenin/LEF1 (Widlund et al., 2002; Cheli et al., 2010), we investigated alterations to both Wnt and Mitf target genes in these tumors. qRT-PCR using RNA prepared directly from uncultured tumors showed transcriptional upregulation of both canonical Wnt and Mitf target genes in *Pten/Braf/Bcat-STA* tumors (Table S3). Conversely, many of these transcripts were downregulated in *Pten/Braf/Bcat-KO* tumors. Increased or decreased Mitf-M levels were confirmed by qRT-PCR and western blot analysis (Figure 5C and 5D). Global expression profiling was also performed on a subset of uncultured melanomas, which more broadly supported these trends in both Wnt and Mitf target gene expression. Together, these models encompass a continuum of β-catenin/Mitf transcriptional activity in the context of Pten loss and Braf activation. *Pten/Braf/Bcat-STA* melanomas show the highest, *Pten/Braf/Bcat-KO*, the lowest, and *Pten/Braf* an intermediate level of β-catenin/Mitf transcriptional activity (Table S3). Activated transcriptional activity of endogenous β-catenin in *Pten/Braf* models is also supported by the presence of S552 phosphorylation of β-catenin in this model (Figure S3D), a posttranslational modification associated with nuclear translocation and transcriptional activation (Zhang et al., 2010).

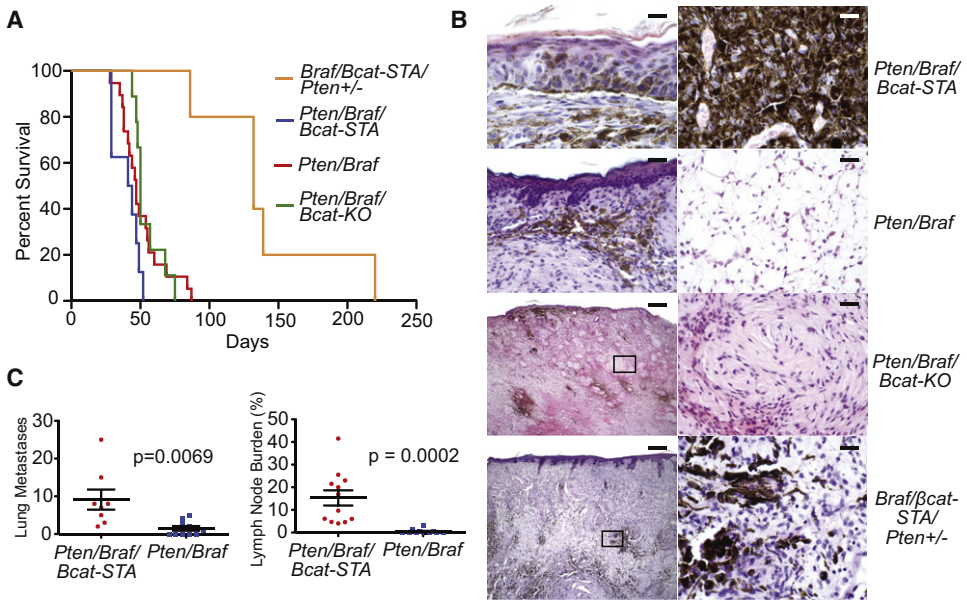

Figure 4. Locally Induced Melanomas are Highly Metastatic and Can Form Lethal Metastases

(A) Survival analysis of cohorts after localized tumor induction. Mice were euthanized when tumors reached 1 cm.

(B) H&E stained sections of locally induced tumors from a *Pten/Braf/Bcat-STA* mouse (top panels, scale 20 μM left, 20 μM right), a *Pten/Braf* mouse (upper middle panels, scale 100 μM left, 50 μM right), a *Pten/Braf/Bcat-KO* mouse (lower middle panels, scale 400 μM left, 50 μM right), and a *Braf/Bcat-STA/Pten+/−* mouse (bottom panels, scale 400 μM left, 50 μM right).

(C) Quantification of lung (left panel) and lymph node (right panel) metastasis in mice with focally induced melanomas. Error bars represent SEM.

See also Figure S3 and Table S2.

Increased levels of melanocyte differentiation markers in *Pten/Braf/Bcat-STA* tumors are also present at the protein level. *Pten/Braf/Bcat-STA* tumors have elevated Tyrp1 staining relative to *Pten/Braf* tumors (Figure 5E), and they also stain strongly for S100 (Figure 5F). Other melanocytic differentiation markers, such as Kit, Mitf, Met, and E-cadherin, are also increased at the protein level (Figure 5D and S4A–C). N-cadherin levels were also highest in *Pten/Braf/Bcat-STA* melanomas (Figure 5D). Initially, the finding of increased melanocytic differentiation seemed somewhat counterintuitive as tumor metastasis is often thought to be associated with relative loss of differentiation markers. Along these lines, we decided to determine the effects of conditional inactivation of E-cadherin in the *Pten/Braf/Bcat-STA* model. E-cadherin loss is thought to result in increased metastasis in some cancers (Kalluri and Weinberg, 2009). Along these lines, E-cadherin loss in *Pten/Braf/Bcat-STA* melanomas might be predicted to further facilitate metastasis if differentiation in these tumors was functionally restricting metastasis. However, when E-cadherin was inactivated in these tumor cells, neither primary tumor growth nor metastasis was altered (Figure 5G–5H). These data support the notion that enhanced differentiation in melanocytic tumors does not functionally restrict metastasis and can even be associated with increased metastasis.

β-Catenin Stabilization Is Associated with Hyperactivation of PI3K/Akt and MAPK/Erk Signaling

As reduced levels of differentiation did not seem to promote or even correlate with enhanced metastasis, we next compared activation of core pathways mediating growth and proliferation: MAPK/Erk and PI3K/Akt. Protein lysates were prepared from uncultured melanomas with either wild-type, stabilized, or inactivated β-catenin. *Pten/Braf/Bcat-STA* tumors tended to exhibit a relative decrease in phosphorylated Erk and p90Rsk compared with *Pten/Braf* and *Pten/Braf/Bcat-KO* tumors (Figure 6A). Previously, it has been proposed that very high levels of Erk phosphorylation may actually impede proliferation (Cheung et al., 2008). Furthermore, it has been recently suggested that an 18-gene transcriptional signature may be the best indicator of MAPK pathway activity in tumor cells (Packer et al., 2009; Dry et al., 2010). In order to complement the phospho-protein analysis and gain a more comprehensive understanding of MAPK pathway activation in these tumors, we also investigated the expression of this gene set. In fact, most of these 18 genes were upregulated in *Pten/Braf/Bcat-STA* tumors relative to *Pten/Braf* tumors, which suggests that the transcriptional output of the MAPK pathway may actually be elevated, despite slightly attenuated phosphorylated Erk levels (Figure S5A). Increased MAPK/Erk signaling in *Pten/Braf/Bcat-STA* melanomas was accompanied by an increased Ki67 index and mitotic figure count relative to *Pten/Braf* tumors (Figure S5B). As MITF has been shown to be required for proliferation induced by $Braf^{V600E}$ (Wellbrock et al., 2008), it could be reasonably hypothesized that increased Mitf in *Pten/Braf/Bcat-STA* tumors may be related to MAPK pathway hyperactivation.

Next, we checked the phosphorylation status of Akt at S473 and T308, two phosphorylation events required for full activation of Akt (Guertin and Sabatini, 2007). *Pten/Braf/Bcat-STA* tumors showed an increase in both phosphorylation events, in addition to increases in total Akt protein levels (Figure 6B). *Pten/Braf/Bcat-KO*

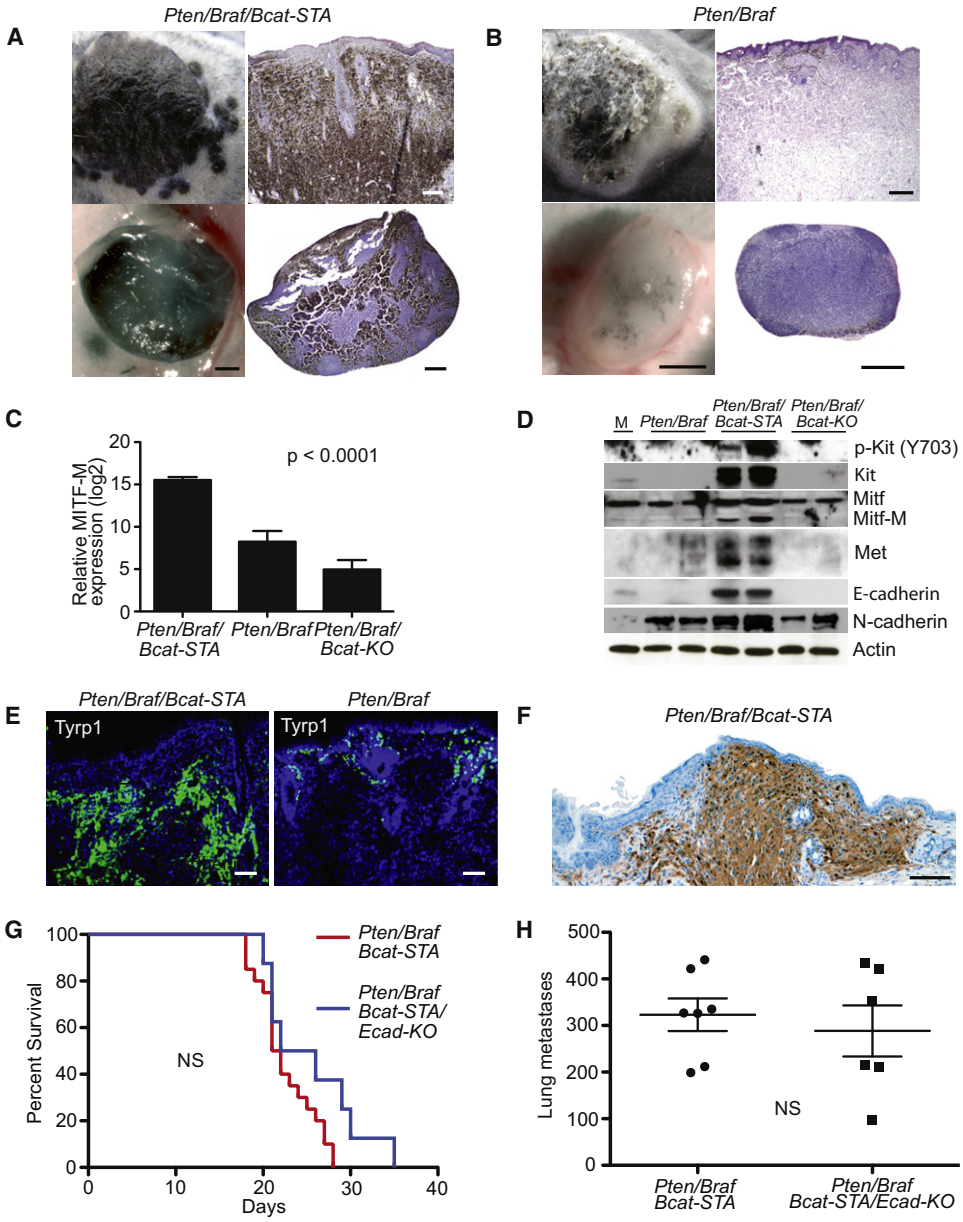

Figure 5. Enhanced Metastasis in *Pten/Braf/Bcat-STA* Melanomas Is Accompanied by an Increase In Melanocytic Differentiation Markers

(A) Locally induced flank melanoma (upper left panel) with H&E (upper right panel, scale 400 μM) and draining lymph node (lower left panel, scale 500 μM) with H&E (lower right panel, scale 500 μM) in a *Pten/Braf/Bcat-STA* mouse.

(B) Locally induced flank melanoma (upper left panel) with H&E (upper right panel, scale 400 μM) and draining lymph node (lower left panel, scale 500 μM) with H&E (lower right panel, scale 500 μM) in a *Pten/Braf* mouse.

(C) Mean expression of Mitf-M determined by qRT-PCR from *Pten/Braf/Bcat-STA*, *Pten/Braf*, and *Pten/Braf/Bcat-KO* melanomas (n = 4 per genotype). Expression levels normalized to GAPDH control. Error bars represent SEM.

(D) Western blots using protein lysates prepared directly from uncultured flank melanomas. M, normal cultured melanocytes.

(E) Tyrp1 immunofluorescence of locally induced *Pten/Braf/Bcat-STA* (left panel) and *Pten/Braf* (right panel) flank melanomas, scale 200 μM.

(F) S100 immunohistochemistry of a locally induced *Pten/Braf/Bcat-STA* melanoma from an albino mouse, scale 100 μM.

(G) Survival analysis of *Pten/Braf/Bcat-STA* mice with or without E-cadherin inactivation after perinatal tumor induction. NS, not statistically significantly different.

(H) Quantification of lung metastases in perinatally induced *Pten/Braf/Bcat-STA* mice with or without E-cadherin inactivation. Error bars represent SEM.

See also Figure S4 and Table S3.

tumors showed decreased Akt activation and protein levels. The phosphorylation status of Akt substrates (Pras40 and Gsk3β) also supported this trend (Figure 6B). The activation status of mTORC1, which lies downstream of Akt, also seemed highest in *Pten/Braf/Bcat-STA* melanomas, which had relatively elevated phosphorylation of S6, an indicator of mTORC1 activity (Figure 6B).

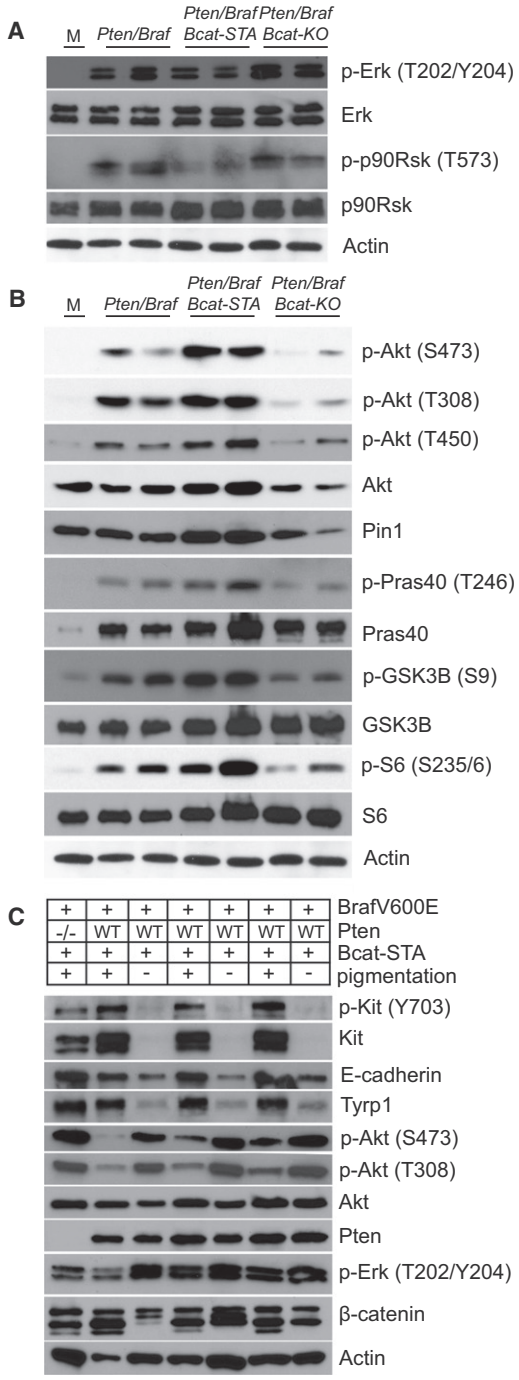

Figure 6. β-Catenin Stabilization Is Associated with Hyperactivation of PI3K/Akt and MAPK/Erk Signaling

(A–C) Western blots using protein lysates prepared from uncultured flank melanomas. M, Normal cultured melanocytes.

See also Figure S5.

At the transcriptional level, *Akt* gene expression was similar among the models, suggesting that differences in posttranscriptional regulation of Akt might underlie differences in Akt protein levels (Table S3). Pin1, a protein isomerase known to regulate Akt stability and phosphorylation (Liao et al., 2009; Nakatsu et al., 2011), was increased in *Pten/Braf/Bcat-STA* tumors and decreased in *Pten/Braf/Bcat-KO* tumors (Figure 6B). Pin1 is transcriptionally upregulated in *Pten/Braf/Bcat-STA* melanomas (Table S3), and by increasing Akt stability, Pin1 may enable increased levels of activated Akt protein. Furthermore, T450 turn motif phosphorylation of Akt by mTORC2 also promotes Akt stability (Oh et al., 2010) and was highest (independent of increases in total protein level) in *Pten/Braf/Bcat-STA* tumors (Figures 6B and S5C). Taken together, these observations suggest that Wnt signaling, by promoting Akt stability through Pin1, can enhance PI3K/Akt pathway activty.

Increases in Akt levels and phosphorylation status in the context of Braf activation and β-catenin stabilization seemed to correlate most closely with a metastatic phenotype. To address this further, we reconsidered the *Braf/Bcat-STA* cohort, which has wild-type Pten and thus reduced Akt activation. These mice form heavily differentiated melanomas that grow slowly and do not metastasize. When Akt is activated via Pten deletion, as in the *Pten/Braf/Bcat-STA* model, enhanced metastasis is observed in the context of increased melanocytic differentiation, providing strong evidence that activation of Akt through loss of Pten drives metastasis of pigmented melanomas. Interestingly, full Akt activation in *Braf/Bcat-STA* melanomas was only observed in a small subset of tumors and always in the context of relative decreases in melanocyte differentiation antigen expression (Figure 6C). As neither manifestation of the *Braf/Bcat-STA* melanomas (low Akt activation with high differentiation or high Akt activation with low differentiation) metastasize, a complex relation is implied in which both melanocytic differentiation and Akt activation are required for metastasis in the context of *Braf/Bcat-STA* melanomas.

An alternative explanation to increases in Akt activation status and metastasis in the *Pten/Braf/Bcat-STA* melanomas could involve increased receptor tyrosine kinase (RTK) expression and/or activation, which are known to signal through this pathway. As the RTKs Kit and Met are both upregulated in *Pten/Braf/Bcat-STA* melanomas, these receptors emerged as possible mediators of this effect. In vivo functional evaluation of the potential role of each of these receptors in mediating the metastatic phenotype of β-catenin stabilization was evaluated. Overexpression of Met in *Pten/Braf* melanomas by using a doxycycline-inducible mouse strain did not recapitulate the metastatic phenotype induced by β-catenin stabilization (Figure S5D, data not shown). This was not entirely unexpected; although Met was overexpressed in *Pten/Braf/Bcat-STA* melanomas, it did not appear to be activated (Figure S5E). Similarly, inhibition of Kit receptor signaling by treating with imatinib in *Pten/Braf/Bcat-STA* melanomas significantly slowed growth of the primary tumor but did not alter the frequency of metastasis (Figure S5F and 5G). These data suggest that increased expression of Met and Kit observed in *Pten/Braf/Bcat-STA* melanomas is not sufficient to explain the metastatic phenotype.

Pten/Braf/Bcat-STA Murine Melanomas Faithfully Recapitulate Human Melanomas

In order to understand the relevance of this model to human melanomas, several approaches were taken. First, human melanoma cell lines with Braf activating mutation and Pten loss/Akt

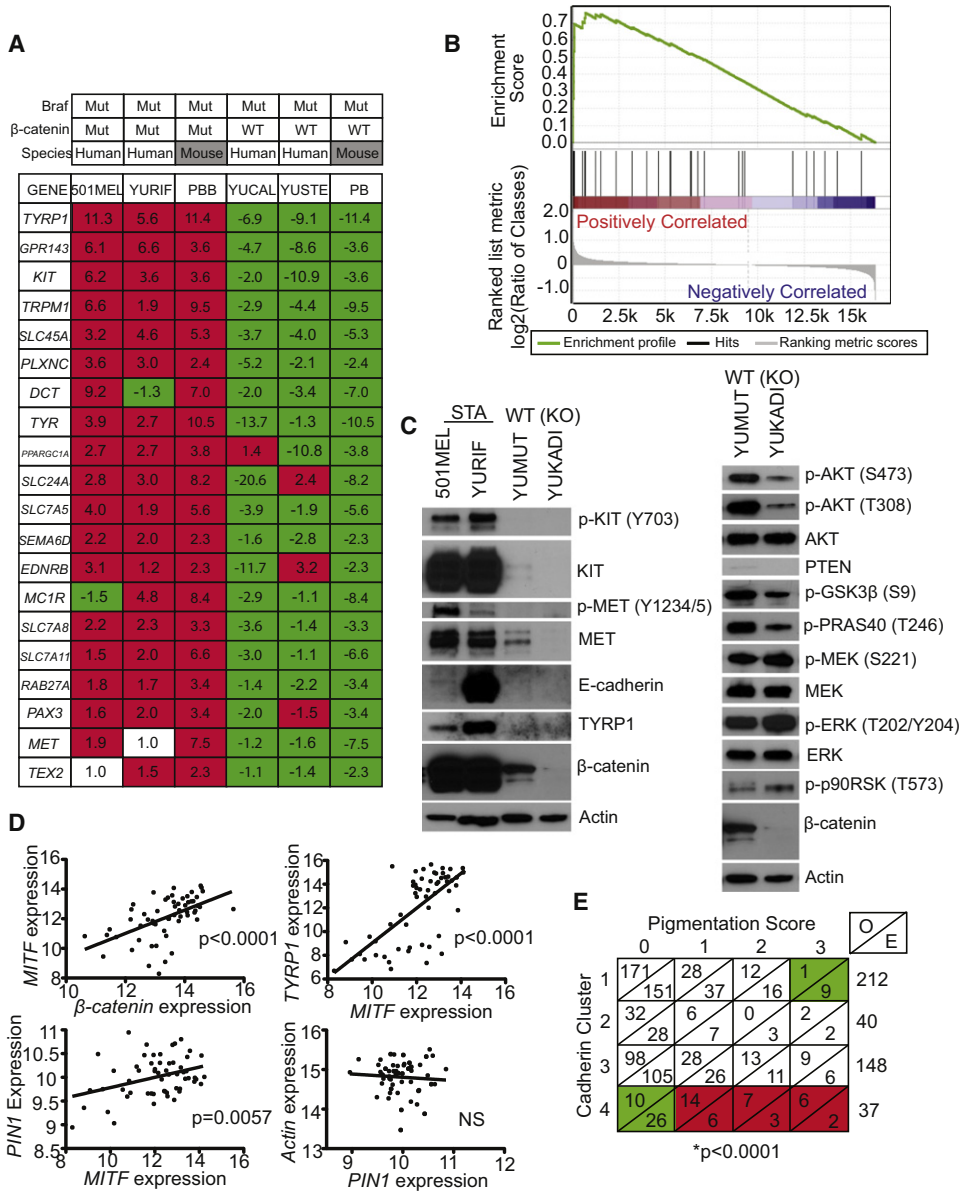

Figure 7. *Pten/Braf/Bcat-STA* Murine Melanomas Faithfully Recapitulate Human Melanomas

(A) Fold-change in expression of individual transcripts relative to mean for each transcript compared between human and murine melanomas. PBB, *Pten/Braf/Bcat-STA*; PB, *Pten/Braf*.

(B) GSEA was performed on human melanomas with β-catenin stabilizing mutation using a subset of differentially regulated transcripts identified using murine melanomas, $p < 0.001$.

(C) Western blot analysis using protein lysates prepared from human melanoma cell lines. β-catenin status indicated for each sample.

(D) Regression analysis comparing *MITF*, *TYRP1*, and *PIN1* expression in a large human melanoma expression data set.

(E) Contingency table of pigmentation status in human melanomas based on cadherin clusters (Kreizenbeck et al., 2008). O, observed; E, expected. Green, less than expected by chance; red, greater than expected by chance.

See also Figure S6 and Table S4.

activation were identified. Within this group, we characterized two tumors with β-catenin stabilizing mutations, one with wild-type β-catenin and one without detectable β-catenin protein levels. These cell lines were characterized at both the mRNA and protein levels. The most differentially expressed transcripts between *Pten/Braf/Bcat-STA* and *Pten/Braf* murine melanomas were identified (Figure 7A; Table S4) and used to perform gene set enrichment analysis (GSEA) on the human melanoma expression data (Subramanian et al., 2005). These analyses showed a strong concordance in the expression of this gene subset in human melanomas with β-catenin stabilization (Figure 7B; $p < 0.001$). A similar methodology was also used to identify differentially expressed genes in the human melanoma cell lines with and without β-catenin stabilization and used for

GSEA with mouse melanoma expression data (Figure S6A; Table S4). Melanomas with analogous changes showed significant enrichment of these transcripts, whereas those without the same changes exhibited an inverse relation for this gene subset (Figure S6B, $p < 0.001$). Together, these analyses suggest that the mouse and human melanomas with the same genetic changes were more similar to each other than were melanomas with different genetic changes but from the same species.

Western blotting, using protein lysates from these human melanoma cell lines, showed similar changes to mouse melanomas with analogous genetic changes. Melanomas with β-catenin stabilization showed much higher expression of melanocytic differentiation markers relative to tumors with wild-type or no β-catenin (Figure 7C). Also, human melanomas with wild-type β-catenin compared with those without β-catenin staining, mirrored changes to MAPK and PI3K/Akt signaling identified by comparing *Pten/Braf* and *Pten/Braf/Bcat-KO* mouse melanomas (Figures 7C and S6C). Lastly, analysis of a large expression dataset from a genetically diverse collection of cultured human melanomas supported the finding that increased β-catenin expression is associated with increased *MITF*, which was, in turn, associated with increased *TYRP1* and *PIN1* expression (Figure 7D). Analysis of the human *PIN1* promoter revealed a CATNNG motif E box (−457CACATG-452), a promoter element to which MITF is specifically known to bind (Khaled et al., 2010). Together, these data support the hypothesis that alterations in β-catenin levels in human melanomas result in similar phenotypic changes to those observed in the murine melanoma models.

In order to assess how frequently human melanomas exhibit characteristics as seen in mouse models with Braf, Akt, and Wnt signaling activation, we employed a previously published data set (Kreizenbeck et al., 2008). Here, primary and metastatic melanoma biopsies were stained in order to assess cadherin and catenin expression patterns, which formed the basis for clustering into four groups. One of these four clusters (cluster 4) was characterized by relatively high levels of N-cadherin, E-cadherin, with several cases also showing relatively elevated β-catenin. This phenotype is very similar to the *Pten/Braf/Bcat-STA* mouse melanomas, which exhibit increased E-cadherin, β-catenin, and high N-cadherin levels, in addition to being heavily pigmented. To follow up on these observations, we conducted additional analyses of this human melanoma data set, which revealed that melanomas in cluster 4 were significantly more pigmented than would be expected by chance, with the largest proportion of heavily pigmented melanomas compared to other clusters ($p < 0.0001$; Figure 7E). Other clinical features of these melanomas are described in Table S4. These data support the notion that the *Pten/Braf/Bcat-STA* murine melanomas closely recapitulate an entire class of human melanomas, not just those with Braf activation, Pten loss, and β-catenin stabilization.

DISCUSSION

The MAPK and Akt pathways are nearly universally dysregulated in human melanomas, with specific occurrence of Pten loss together with Braf activation in at least 20% of tumors (Tsao et al., 2004; Curtin et al., 2005). The precise role of Wnt/β-catenin signaling in human melanoma has remained elusive, despite extensive in vitro study, mouse modeling efforts, and immunohistochemical analyses of human melanoma specimens. Here, by characterizing mouse models that are based on genetic alterations commonly observed in human melanoma, a definitive role for β-catenin as a mediator of tumor progression and metastasis in Pten inactivated, Braf activated melanomas has been established. In the *Pten/Braf* mouse models, metastasis can be either enhanced or repressed by either increasing or decreasing β-catenin levels, respectively.

In recent analytical studies of human melanoma specimens, it has been proposed that reduced β-catenin levels are associated with a relatively worse prognosis (Chien et al., 2009). However, when β-catenin is inactivated in *Pten/Braf* melanomas, tumor formation is delayed, survival is extended, and metastasis is nearly eliminated. These data demonstrate the requirement for endogenous β-catenin in melanoma formation and progression. The phenotypic alterations in *Pten/Braf/Bcat-KO* tumors seem to be related to decreased Wnt transcriptional output, as well as decreased Mitf levels and Mitf target gene expression. In a similar vein, when β-catenin is stabilized in the *Pten/Braf* melanomas, survival is reduced and metastasis to lymph node, lung, bowel, and spleen is enhanced, some of the most common sites of metastasis in human melanoma patients. In addition to being more metastatic, *Pten/Braf/Bcat-STA* melanomas are also characterized by increased Wnt-related transcription, primarily as an increase in Mitf and Mitf target gene expression. Thus, in the *Pten/Braf* model, more metastatic tumors are characterized by enhanced melanocyte differentiation, whereas less metastatic tumors exhibit reduced melanocytic differentiation.

The relation between melanoma differentiation/pigmentation and metastasis has long been of research interest. Twenty-five years ago it was observed that more pigmented mouse melanoma cell lines had an enhanced ability to metastasize (Bennett et al., 1986). Since this time, other work has suggested that the melanocytic differentiation program may intrinsically predispose melanomas to metastasis after oncogenic transformation (Gupta et al., 2005). The finding of Mitf amplification in >20% of metastatic melanomas, also suggests a powerful oncogenic potential for melanocyte differentiation programs (Garraway et al., 2005). These observations are also supported clinically, as melanomas are known to frequently metastasize early in disease progression, even from relatively thin primary tumors (Bedrosian et al., 2000). These observations are contrasted with observations suggesting that in general melanomas with increased MITF staining (and β-catenin staining) are associated with relatively improved survival in melanoma (Chien et al., 2009; Nazarian et al., 2010).

An improved understanding of human melanoma genetics has begun to allow for classification of melanomas based on fundamental driving mutations, such as mutant NRAS or BRAF. Here, specific evaluation of the consequences of β-catenin stabilization or inactivation in the context of precisely defined genetic changes was possible. When β-catenin is stabilized in the context of Pten loss and Braf activation, melanomas are very metastatic, grow rapidly, and are highly differentiated. However, in the presence of wild-type Pten, Braf activation and β-catenin stabilization have very different effects. Although highly differentiated tumors form, they do not metastasize. Further, *Braf/Bcat-STA* melanomas grow very slowly and it is only in association with relative loss of melanocytic differentiation, that tumors exhibit

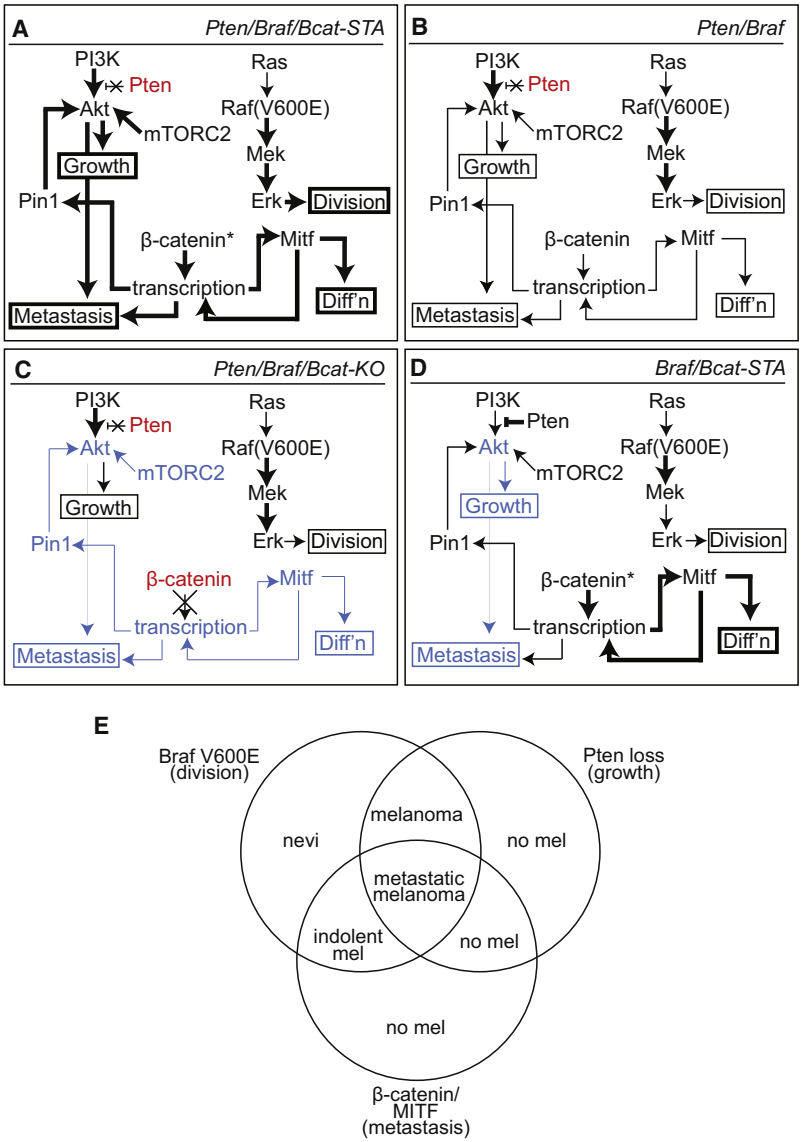

Figure 8. Signaling and Phenotypic Overview of Murine Melanoma Models

(A) *Pten/Braf/Bcat-STA* melanomas are characterized by rapid growth, high degree of melanocytic differentiation, and extensive metastasis. These phenotypes are associated with increased MAPK/Erk and PI3K/Akt signaling, as well as elevated Mitf and Pin1 levels. Bold text, relatively increased levels and/or activity compared to other models; blue shading, relatively decreased compared to other models; red shading, genetically inactivated; *, stabilized.

(B) *Pten/Braf* melanomas exhibit rapid tumor growth but only intermediate melanocytic differentiation and metastasis. Signaling pathway activation, Mitf, and Pin1 are intermediate in this model.

(C) *Pten/Braf/Bcat-KO* melanomas exhibit tumor growth but significantly reduced melanocytic differentiation and almost no metastasis. Inactivation of β-catenin in this model is associated with reduced PI3K/Akt signaling and Mitf levels.

(D) *Braf/Bcat-STA* melanomas are characterized by long latency and slow tumor growth, as well as lack of metastases. These tumors are differentiated and heavily pigmented because of high Mitf levels but show reduced PI3K/Akt pathway activation.

(E) A three-pathway synergy is observed when MAPK/Erk, PI3K/Akt, and β-catenin/Mitf signaling pathways are simultaneously activated in melanoma. If any of these three changes are not present, the metastatic melanoma phenotype is abrogated.

more rapid growth. Comparison of *Pten/Braf/Bcat-STA* melanomas to *Braf/Bcat-STA* melanomas revealed that activation of Akt in the context of Braf activation and β-catenin stabilization is strongly associated with metastasis in the highly differentiated melanomas. Furthermore, when one considers the amelanotic *Braf/Bcat-STA* melanoma variant, in which accelerated tumor growth occurred only in the context of relative loss of differentiation markers, it becomes clear that in some contexts melanocytic differentiation may actually restrict exuberant melanoma growth. It is notable that in this model loss of Pten has the potential to switch the melanocyte differentiation program from a transforming, but relatively growth restrictive pathway to a pathway that promotes growth and metastasis. It is also notable that increased differentiation, which includes increased E-cadherin expression, rather than preventing metastasis, is associated with an increase in this process.

These findings raise the possibility that factors promoting metastasis in one melanoma may not promote metastasis in other genetically distinct melanomas. These considerations suggest that broadly defining the biological effects of pathways, such as Wnt signaling and MITF, in melanoma may not be possible out of context of other mutational changes and help to explain the relative inconsistency of previous studies in this regard. Such context-specific roles of mutated proteins in cancer are likely to emerge as a paradigm as we learn better ways to classify melanomas into biologically meaningful groups.

In the *Pten/Braf* melanomas, simultaneous activation of core signaling pathways mediating growth and survival correlate very closely with the ability to metastasize. Concurrent activation of MAPK, PI3K/Akt, and Wnt/Mitf pathways are all required for the full metastatic melanoma phenotype, as loss of any component abrogates the effect (summarized in Figure 8). Furthermore, changes to Wnt signaling not only alter Wnt output and the melanoma phenotype but also impact signaling through other core pathways regulating cellular growth and survival. *Pten/Braf/Bcat-STA* melanomas show enhanced MAPK and PI3K/Akt activation, whereas *Pten/Braf/Bcat-KO* melanomas show decreased PI3K/Akt signaling. It is likely that MAPK pathway activation in *Pten/Braf/Bcat-STA* tumors is at least in part related to increased Kit and/or Mitf levels, especially given the growth inhibitory effect of imatinib treatment, however, other possible mediators of this effect are not excluded. Additionally, increased Mitf may also play a role in the elevated proliferation rates observed in *Pten/Braf/Bcat-STA* melanomas, as Mitf has been shown to be an important integrator of upstream signaling pathways in regulating

proliferation and differentiation in the melanocytic lineage. Increased Akt activity, however, is likely due to transcriptional upregulation of Pin1, which enhances Akt stability, leading to an increase in Akt protein levels, and ultimately activated Akt. This link between Wnt signaling and Akt in melanoma is likely also important in human melanomas, where increased PIN1 transcription is associated with increased Wnt/MITF. As PIN1 has also been shown to potentiate the effects of additional oncogenes, such as Jun (Han et al., 2011), mutant p53 (Girardini et al., 2011), and Cyclin D1 (Li et al., 2006), PIN1-dependent effects are likely to play a broad role in cancer.

Components of the MAPK and also the PI3K/Akt pathways are central targets for new cancer therapeutics. For example, vemurafenib (PLX4032), a mutant Braf inhibitor has been shown to improve survival in late-stage melanoma patients with Braf mutations (Chapman et al., 2011). Along these lines, understanding how simultaneous activation of additional signaling pathways in a given tumor can impact inhibitor-targeted pathways is essential and may assist in understanding why some patients with a given driver mutation respond differentially to treatment with the same inhibitor. In this context, it is particularly noteworthy that changes to β-catenin can have such profound effects to both the MAPK and PI3K/Akt signaling pathways.

Here we have described several mouse models of melanoma that have provided fundamental insight into mechanisms by which β-catenin/Wnt signaling alterations can impact melanoma formation and progression in vivo. Extensive analyses were conducted in order to ensure that these models closely resemble human melanomas with analogous genetic changes and cellular phenotypes. Faithful recapitulation of human melanoma, combined with features, such as rapid tumor growth and reproducible metastasis to lymph nodes and lung, make this model attractive for future studies in melanoma.

EXPERIMENTAL PROCEDURES

Mouse Strains and In Vivo Experiments

The *Ctnnb1*^{lox}, *E-cadherin*^{lox}, and *TRE::Met* strains were obtained from Jackson Labs. These mice were genotyped and assayed for recombination using PCR as described previously (Brault et al., 2001; Wang et al., 2001; Boussadia et al., 2002). The *Tyr-CreER*, *Pten*^{lox}, *Braf*^{CA}, and *Ctnnb1*^{loxex3} alleles have also been described previously and were genotyped and assayed for recombination using PCR (Harada et al., 1999; Dankort et al., 2009). The *Tyr::rtTA* strain was obtained from MMHCC and genotyped as described previously (Chin et al., 1999). All strains were maintained on a mixed C57BL/6, FVB, 129 background. For imatinib experiments, *Pten/Braf/Bcat-STA* mice were treated daily with 200mg/kg imatinib (LC Laboratories) in water by oral gavage. All experiments involving animals were reviewed and approved by the Yale Institutional Animal Care and Use Committee.

Immunohistochemistry, Immunofluoresence, and Western Blotting

Immunofluoresence and immunohistochemistry were performed on formalin-fixed, paraffin-embedded, and frozen tumor sections. Western blotting was performed using standard methods on uncultured, macrodissected tumor protein lysates or lysates from cultured human melanoma cell lines.

RNA Purification and qRT-PCR

Total RNA was isolated from homogenous portions of macrodissected, uncultured tumors. cDNA was prepared for qRT-PCR analysis using SYBR green detection methods and ΔΔCt quantification relative to an endogenous GAPDH control.

Statistical Analyses

Kaplan-Meyer survival curves were compiled using Prism statistical analysis software. Significance was assessed using the Log-rank (Mantel-Cox) test. For comparison of pooled data between two different groups, unpaired t tests were used to determine significance. For comparison of data among three groups, one-way ANOVA was used to determine significance. R was used for hierarchical clustering, and Fisher's Exact test was used to determine significance for contingency table analysis.

ACCESSION NUMBERS

Microarray datasets were deposited in the National Center for Biotechnology Information's Gene Expression Omnibus database with the accession number GSE32907.

SUPPLEMENTAL INFORMATION

Supplemental information includes six figures, four tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.ccr.2011.10.030.

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