

Braf^{V600E} cooperates with *Pten* loss to induce metastatic melanoma

David Dankort^{1,5,6}, David P Curley^{2,6}, Robert A Cartledge¹, Betsy Nelson², Anthony N Karnezis³, William E Damsky, Jr², Mingjian J You^{4,5}, Ronald A DePinho⁴, Martin McMahon¹ & Marcus Bosenberg^{2,5}

Mutational activation of *BRAF* is the earliest and most common genetic alteration in human melanoma. To build a model of human melanoma, we generated mice with conditional melanocyte-specific expression of *BRAF*^{V600E}. Upon induction of *BRAF*^{V600E} expression, mice developed benign melanocytic hyperplasias that failed to progress to melanoma over 15–20 months. By contrast, expression of *BRAF*^{V600E} combined with *Pten* tumor suppressor gene silencing elicited development of melanoma with 100% penetrance, short latency and with metastases observed in lymph nodes and lungs. Melanoma was prevented by inhibitors of mTORC1 (rapamycin) or MEK1/2 (PD325901) but, upon cessation of drug administration, mice developed melanoma, indicating the presence of long-lived melanoma-initiating cells in this system. Notably, combined treatment with rapamycin and PD325901 led to shrinkage of established melanomas. These mice, engineered with a common genetic profile to human melanoma, provide a system to study melanoma's cardinal feature of metastasis and for preclinical evaluation of agents designed to prevent or treat metastatic disease.

Malignant melanoma is noted for its aggressive clinical behavior, propensity for lethal metastasis and therapeutic resistance. This clinical picture, coupled with an increase in incidence, has motivated efforts to understand the genetic underpinnings of melanoma initiation and progression and translate such insights into effective preventive and therapeutic strategies^{1,2}.

Several key genetic lesions governing melanoma initiation and progression have been identified, the earliest and most common being a point mutation (T1799A) in the *BRAF* proto-oncogene, which is detected in ~65% of affected individuals^{1,3–7}. *BRAF*^{T1799A} encodes *BRAF*^{V600E}, a constitutively active protein serine kinase that elicits sustained activation of the *BRAF*→*MEK1/2*→*ERK1/2* MAP kinase pathway. Although it contributes to the aberrant pathophysiological characteristics of the melanoma cell^{8,9}, oncogenic activation of *BRAF* in melanocytes is insufficient for full malignant conversion owing to activation of senescence^{10,11}. Hence, progression to malignant melanoma is invariably accompanied by silencing of one or more tumor suppressor genes, most commonly *PTEN* or *CDKN2A*^{5,12–14}. The combination of mutated *BRAF* and silencing of *PTEN* expression is common in human melanoma (~20%), although genetic evidence for *BRAF* and *PTEN* cooperation in this disease has not been obtained to date^{14–16}.

To understand and validate the role of such genetic events and their interactions in melanoma initiation, progression and response to

therapy, we have generated a conditional mouse model of *BRAF*^{V600E}-induced, *Pten*-deficient metastatic melanoma. This genetic model recapitulates key pathophysiological aspects of the human disease and offers control over the timing and location of melanoma initiation. Moreover, we demonstrate the utility of this model in the evaluation of pharmacologic agents that target key signaling pathways linked to these signature events in the melanoma cell, supporting the utility of this disease model in preclinical experimental therapeutics.

RESULTS

BRAF^{V600E} promotes benign melanocytic hyperplasia *in vivo*

BRAF^{CA} mice harbor a germline conditional *BRAF*^{V600E} allele, the expression of which is initiated at physiological levels under the control of the gene's chromosomal promoter by the action of Cre recombinase (Fig. 1a)¹⁷. When the *BRAF*^{CA} allele was combined with a constitutively active Cre transgene under the control of the *Tyr* promoter (*Tyr::Cre*, *Tec1*) no viable offspring were obtained (Supplementary Table 1 online)¹⁸. Similarly, compound *BRAF*^{CA} and *Dct* promoter-directed Cre (*Dct::Cre*) transgenic mice succumbed within 3 months of age¹⁹ (D. Matzen *et al.*, unpublished data). To circumvent lethality associated with embryonic expression of *BRAF*^{V600E}, we used a *Tyr::CreER* transgene, which encodes conditionally active CreER^{T2} specifically in melanocytes (Fig. 1a)²⁰. In the absence of 4-hydroxytamoxifen

¹Cancer Research Institute & Department of Cell and Molecular Pharmacology, Helen Diller Family Comprehensive Cancer Center, University of California, San Francisco, California, USA. ²Department of Pathology, University of Vermont College of Medicine, Burlington, Vermont, USA. ³Cancer Research Institute & Department of Pathology, Helen Diller Family Comprehensive Cancer Center, University of California, San Francisco, California, USA. ⁴Belfer Institute for Applied Cancer Science, Departments of Medical Oncology, Medicine & Genetics, Dana-Farber Cancer Institute and Harvard Medical School, Boston, Massachusetts, USA. ⁵Present addresses: Department of Biology, McGill University, Montreal, Canada, H3A 1B1 (D.D.); Department of Hematopathology, Division of Pathology and Laboratory Medicine, University of Texas, M.D. Anderson Cancer Center, Houston, Texas, USA (M.J.Y.); Department of Dermatology, Yale University School of Medicine, New Haven, Connecticut, USA (M.B.). ⁶These authors contributed equally to this work. Correspondence should be addressed to M.M. (mcmahon@cc.ucsf.edu) or M.B. (marcus.bosenberg@yale.edu).

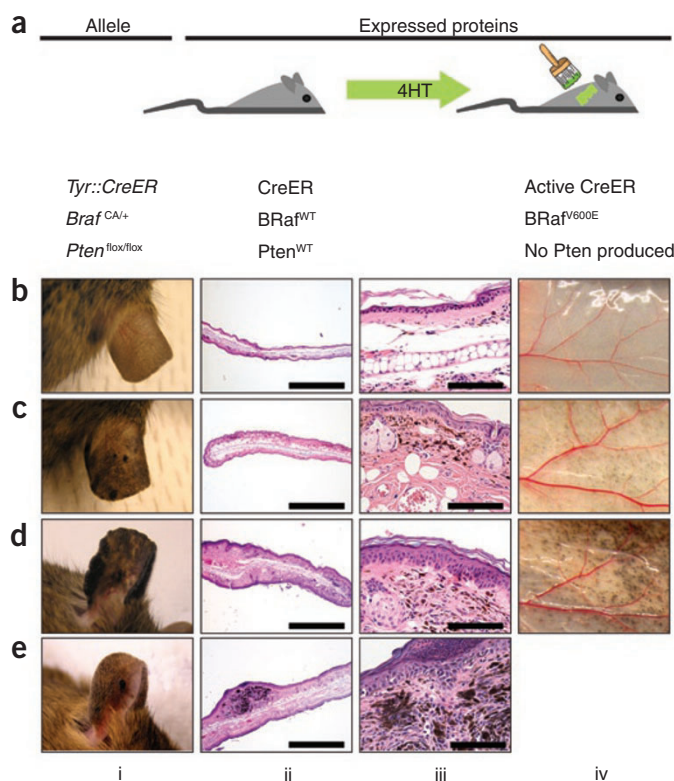


Figure 1 Benign hyperplasias induced by melanocyte-specific expression of BRAF^{V600E}. (a) Mice carrying various conditional alleles of *Braf* (*Braf^{CA}*) and/or *Pten* (*Pten^{lox4-5}* or *Pten^{lox5}*) were crossed to *Tyr::CreER* mice with melanocyte-specific expression of a hormone-dependent form of Cre recombinase (CreERT²)^{17,20}. Activation of CreER by 4-HT leads to melanocyte-specific conversion of *Braf^{CA}* to *Braf^{V600E}* and the conversion of the *Pten^{lox}* alleles to null alleles. (b–d) *Braf^{CA/+}* (b), *Tyr::CreER; Braf^{CA/+}* (c) and *Tyr::CreER; Braf^{CA/CA}* (d) mice were treated topically with 4-HT and monitored for 75 weeks for signs of melanocytic proliferation (i). Mice were euthanized and skin from the ear (ii, iii) and flank (iv) was stained with hematoxylin and eosin and inspected for the presence of pigmented cells. (e) A *Tyr::CreER; Braf^{CA/+}* mouse developed a papular pigmented lesion ~60 weeks after topical administration of 4-HT (i). This mouse was euthanized and skin sections encompassing the lesion were stained with hematoxylin and eosin and inspected for the presence of pigmented cells (ii, iii). Scale bars: ii, 2 mm; iii, 0.1 mm.

(4-HT), *Tyr::CreER; Braf^{CA/+}* mice did not show any discernable phenotype over 18 months of observation ($n \geq 50$, Fig. 1b, i). However, topical administration of 4-HT to either fur bearing or glabrous skin or systemic 4-HT administration led to the appearance of highly pigmented lesions that were detected by 21–28 days after 4-HT administration (Fig. 1c, i). Such lesions were not detected in 4-HT-treated *Tyr::CreER* mice, indicating that they require the conversion of the *Braf^{CA}* allele to *Braf^{V600E}* (Supplementary Fig. 1a online)²⁰.

Braf^{V600E}-induced pigmented lesions were frequently located at the dermal-epidermal junction with extension into the dermis, as is seen in human compound melanocytic nevi (Fig. 1c, iii). Other melanocytic proliferations formed in the dermis with no apparent junctional component. *Braf^{V600E}*-induced pigmented lesions appeared only in regions of the skin to which 4-HT was applied and were not induced by topical administration of solvent as control (Supplementary Fig. 1). Occasionally, when high concentrations of 4-HT were topically administered to mice, we observed periocular lesions on the face and on the glabrous skin of the paws, most likely owing to groom-

ing behavior (Supplementary Fig. 1b,c). However, by reducing the amount of 4-HT administered, we could elicit spatially restricted melanocytic lesions with no evidence of inappropriate spreading to untreated sites.

To determine whether BRAF^{V600E}-induced pigmented lesions would progress spontaneously to malignant melanoma, we monitored 35 4-HT-treated *Tyr::CreER; Braf^{CA}* mice over 15–20 months. Although two mice developed small papular pigmented lesions, these lesions were not considered malignant in nature given their lack of significant invasion or metastasis, the small and self-limited tumor growth, the banal cytologic features of the cells and the absence of aberrant mitotic figures within the lesions (Fig. 1). These data are consistent with the hypothesis that BRAF^{V600E} expression in human melanocytes promotes an initial phase of proliferation that is subsequently restrained from malignant progression by engagement of a cell cycle arrest program with features of senescence, reflecting the clinical observation of growth arrest of pigmented nevi in humans^{10,21}.

In addition, we generated a small number of *Tyr::CreER* mice that were homozygous for *Braf^{CA}* to assess the impact of two copies of the *Braf^{V600E}* allele ($n = 6$). Topical 4-HT administration to the ear, tail or flank of these mice led to the appearance of benign melanocytic hyperplasias that were considerably larger and more highly pigmented than in mice heterozygous for *Braf^{CA}* expressing only one copy of *Braf^{V600E}* (Fig. 1d, i–iv, respectively). Indeed, this phenotypic presentation was so reproducible that mice could be genotyped (*Braf^{CA/+}* versus *Braf^{CA/CA}*) on the basis of the size and pigmentation of the melanocytic lesions that developed. These data indicate that *Braf^{V600E}* gene dosage exerts a physiologically relevant impact on melanocyte proliferation, an experimental observation that may rationalize the observation of increased copy number of oncogenic BRAF in human melanoma and other tumors^{22,23}.

BRAF^{V600E} cooperates with Pten loss to induce metastatic melanoma

Human melanomas expressing BRAF^{V600E} often show activation of the PI3'-kinase→PDK→AKT pathway through silenced expression of the PTEN tumor suppressor gene^{14,15,22}. To model this genetic profile, we generated *Tyr::CreER; Braf^{CA}* mice that were also homozygous for either one of two different conditional *Pten* alleles. One allele (*Pten^{loxP-neo}*) conditionally deletes *Pten* exons 4 and 5 (ref. 24). The second allele (not previously described) permits Cre-mediated deletion of *Pten* exon 5. We refer to these alleles as *Pten^{lox4-5}* and *Pten^{lox5}*, respectively. Notably, the action of Cre recombinase on both alleles leads to deletion of exons that encode amino acids essential for the Pten's PI3'-lipid phosphatase activity²⁴.

In contrast to *Braf^{V600E}*-induced melanocytic neoplasia (Fig. 1), 4-HT treatment of *Tyr::CreER; Pten^{lox4-5/lox4-5}* or *Tyr::CreER; Pten^{lox5/lox5}* mice failed to elicit any melanocytic phenotype over ~18 months ($n = 20$). These data are consistent with observations that *Dct::Cre; Pten^{lox/lox}* mice do not develop melanoma unless treated with carcinogen²⁵. However, 7–10 d following 4-HT administration, all *Tyr::CreER; Braf^{CA}; Pten^{lox/lox}* mice showed a marked expansion of highly pigmented cells regardless of where the 4-HT was administered to the skin and regardless of which *Pten* allele was used (Fig. 2a–c). Indeed, the cooperation between *Braf^{V600E}* and *Pten* silencing was so strong that confluent melanocytic proliferation was observed in 4-HT-treated regions of the skin of *Tyr::CreER; Braf^{CA}; Pten^{lox/lox}* mice. By contrast, treatment with dimethylsulphoxide solvent control was without effect (Fig. 2 and Supplementary Figs. 2 and 3b online). Abrogation of *Pten* expression was confirmed by protein blotting analysis of extracts of primary lesions derived from

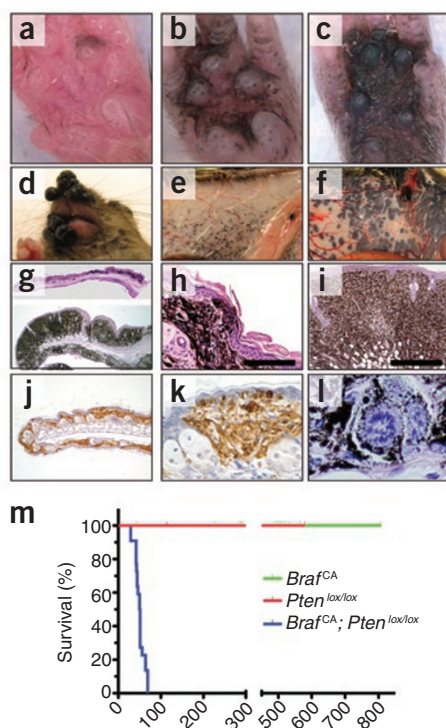


Figure 2 *Braf^{V600E}* cooperates with *Pten* loss in the induction of malignant melanoma. (a–c) *Tyr::CreER; BrafcA/+; Pten^{lox5/lox5}* mice were treated topically on the paw with 4-HT to elicit *Braf^{V600E}* and to silence *Pten* expression. The presence of pigmented lesions was assessed 6 (a), 8 (b) and 10 (c) days following 4-HT administration. (d–l) *Tyr::CreER; BrafcA/+; Pten^{lox4-5/lox4-5}* mice were treated topically with 4-HT on the right ear and flank. Mice were monitored for 7 weeks. Mice were euthanized at ~7 weeks with the presence of malignant melanoma lesions assessed by visual inspection of the underside of the ventral/lateral skin. *Tyr::CreER; BrafcA/+; Pten^{lox5/lox5}* mice were treated topically on the ear, flank and tail with 4-HT. Twenty-five days later, mice were euthanized and skin sections were prepared, stained with hematoxylin and eosin and examined for the presence of pigmented cells. Almost confluent proliferation of densely pigmented cells was observed in the ear and skin (g) that penetrated deep into the dermis and the subcutis (h,i) and showed signs of pagetoid spread into the epidermis (h, k). Cells in these lesions stained positive with antisera to PEP1 that detects expression of Tyrp1 (j,k). (l) Pigmented cells in the lesions displayed histological characteristics of malignant melanoma, including marked cytological atypia, prominent nucleoli and aberrant mitotic figures. (m) Kaplan-Meier survival analysis of 4-HT-treated *Tyr::CreER; BrafcA/+; Pten^{+/+}* (*n* = 22), *Tyr::CreER; BrafcA/+; Pten^{lox4-5/lox4-5}* (*n* = 5) and *Tyr::CreER; BrafcA/+; Pten^{lox4-5/lox4-5}* (*n* = 22) mice. Log rank tests of survival plots of the data indicated a statistically significant difference between the following survival curves: *Tyr::CreER; BrafcA/+; Pten^{+/+}* versus *Tyr::CreER; BrafcA/+; Pten^{lox4-5/lox4-5}* (*P* < 0.0001) and; *Tyr::CreER; BrafcA/+; Pten^{lox4-5/lox4-5}* versus *Tyr::CreER; BrafcA/+; Pten^{lox4-5/lox4-5}* (*P* < 0.0002).

4-HT-treated *Tyr::CreER; BrafcA; Pten^{lox5/lox5}* mice (Supplementary Fig. 4 online). Pigmented melanoma cells were found throughout the dermis and subcutis and showed pagetoid spread into the epidermis (Figs. 2h,i,k). Moreover, there was rapid emergence of highly pigmented malignant lesions elicited by 4-HT in the face, flank or tail skin, the location of which could be highly restricted by topical administration of low-dose 4-HT (Fig. 2d–f and Supplementary Fig. 3a,c). These lesions progressed rapidly to advanced malignancy such that all *Tyr::CreER; BrafcA; Pten^{lox/lox}* mice required euthanasia 25–50 d following 4-HT administration (Fig. 2m).

Histological analysis of primary skin lesions revealed highly pigmented cells with variably shaped enlarged nuclei containing clumped chromatin, prominent nucleoli and atypical mitoses (Fig. 2l). In addition, the melanocytic origin of cells in the lesions was confirmed by the presence of abundant melanin and by immunostaining with antibody to PEP1 to detect expression of tyrosinase-related protein 1 (Fig. 2j,k)²⁶.

To further document the rate-limiting role of *Pten* in constraining malignant progression, we followed a small number of 4-HT-treated

Tyr::CreER; BrafcA mice that were heterozygous for the *Pten^{lox4/5}* allele. These mice initially developed benign melanocytic lesions similar to those observed in *Tyr::CreER; BrafcA* mice. However, they eventually developed focal malignant melanomas that we believe are most likely due to stochastic silencing of the wild-type *Pten* allele, as has been observed with other tumor suppressor genes (Supplementary Fig. 2).

Braf^{V600E}; Pten^{-/-} melanoma cells are invasive and metastatic

At euthanasia *Tyr::CreER; BrafcA; Pten^{lox/lox}* mice were subjected to complete histopathologic survey for evidence of melanoma invasion and metastasis. As described above, all mice analyzed had evidence of melanoma cells throughout the dermis with evidence of invasion into the subcutis (Fig. 2). In addition, spread of melanoma was observed into the regional draining lymph nodes in 100% of mice, with expansile growth noted in most mice (Fig. 3a–c). Metastases were also noted in mice with localized tumors in the distal region of the tail that had clear evidence of pigmented cells in the iliac lymph nodes (Fig. 3d and Supplementary Fig. 3c) and lungs (data not shown). Examination of

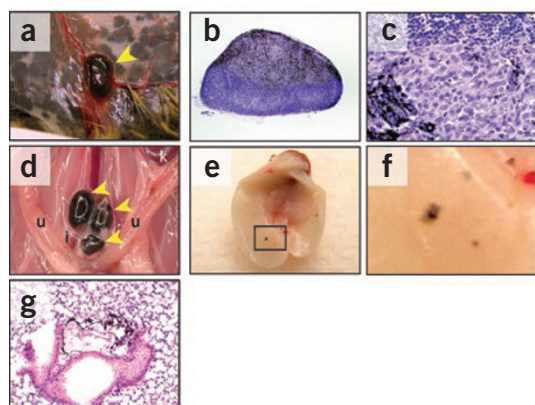


Figure 3 *Braf^{V600E}* cooperates with *Pten* loss in the induction of invasive and metastatic melanoma. (a) *Tyr::CreER; BrafcA/+; Pten^{lox4-5/lox4-5}* mice were treated topically on the ear, flank and tail with 4-HT; six weeks later mice were euthanized and inspected for evidence of pigmented cells in the mammary gland lymph node. (b,c) *Tyr::CreER; BrafcA/+; Pten^{lox5/lox5}* mice were treated similarly to those described in a, euthanized and then inspected by microscopy at low (b) and high (c) power for the presence of pigmented cells in the lymph nodes. (d) A *Tyr::CreER; BrafcA/+; Pten^{lox5/lox5}* mouse was treated topically with 5mM 4-HT in 100% (v/v) ethanol on the distal tail. The mouse had a visible tumor within 4 weeks but did not require euthanasia until 24 weeks later after 4-HT administration. The presence of pigmented cells in the iliac, lumbar, inguinal, axillary and submandibular lymph nodes was assessed by stereomicroscopy. Tumor cells were restricted to the iliac nodes (pictured). u, uterus; i, right common iliac artery. (e) *Tyr::CreER; BrafcA/+; Pten^{lox4-5/lox4-5}* mice were treated topically on the ear, flank and tail with 4-HT. Mice were euthanized 6–7 weeks after 4-HT treatment at which times the lungs were excised, cleared of blood and visually inspected for the presence of pigmented lesions. (f) The insert in e is shown at higher magnification in f. (g) Lung sections were prepared, stained with hematoxylin and eosin and examined for the presence of pigmented cells in the lung parenchyma.

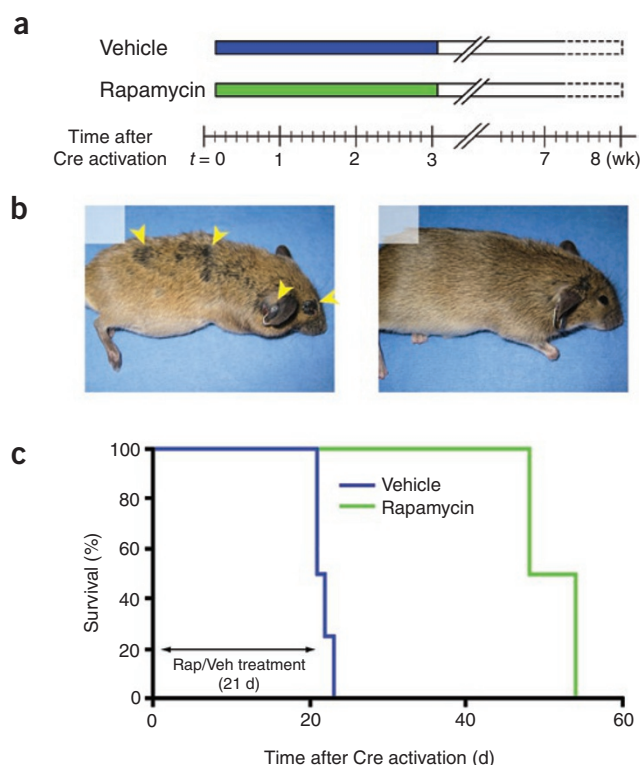


Figure 4 Prevention of BRAF^{V600E}-induced melanomas by rapamycin. (a) Adult *Tyr::CreER; Bra^f^{CA/+}; Pten^{lox5/lox5}* mice were treated topically on the ear, flank and tail with 4-HT. The next day, mice were randomly assigned to be administered rapamycin (7.5 mg/kg, *n* = 5) or solvent control (*n* = 4) for 21 d. Mice were monitored daily for the presence of cutaneous malignant melanoma and euthanized according to a standard body conditioning score. After three weeks of rapamycin treatment, drug administration was ceased and mice were monitored for the presence of cutaneous malignant melanoma as described above. Mouse survival was plotted using a Kaplan-Meier survival curve. (b) Representative images of a vehicle control (left) or a rapamycin (right) treated mouse immediately after the end of the 21-d drug administration are presented. (c) Kaplan-Meier survival curve of vehicle (*n* = 4) or rapamycin (*n* = 5) treated cohorts of mice analyzed in this experiment. All mice were treated for the indicated 21 d as indicated in a.

the lungs revealed visible evidence of at least one and, in many cases, multiple nests of metastatic melanoma cells (Figs. 3e–g). These data indicate that activated BRAF^{V600E} and Pten deficiency cooperate to produce primary cutaneous malignant melanoma with robust capacity to invade into the skin and to metastasize to draining lymph nodes and distal organs that are commonly targeted in the human disease.

Prevention and therapy of BRAF^{V600E}-induced melanomas by pharmacological inhibition of MEK1/2 and mTORC1

Melanoma is noted for its resistance to chemotherapy, although there are anecdotal reports of dramatic responses to therapy targeted against mutated *KIT* (c-KIT) in humans^{13,27}. Consequently, we wished to determine whether targeted inhibition of downstream effectors of BRAF^{V600E} or PI3'-kinase signaling could prevent the development of melanoma in this model system. To do so, we used specific and selective inhibitors of MEK1/2 (PD325901) or mTORC1 (rapamycin), which are downstream of BRAF^{V600E} or PI3'-kinase, respectively^{28,29}. Melanoma was initiated in adult *Tyr::CreER; Bra^f^{CA}; Pten^{lox/lox}* mice

by topical administration with 4-HT. Either 1 d (rapamycin, Fig. 4) or 1 week (PD325901, Fig. 5) later the mice were treated systemically either with the relevant solvent control or with rapamycin (7.5 mg/kg) or PD325901 (15 mg/kg) for 3 or 6 weeks, respectively. Solvent-treated mice rapidly developed melanoma that required euthanasia within 3–6 weeks (Figs. 4b and 5b, panel i). By contrast, treatment with either rapamycin or PD325901 markedly inhibited melanoma formation, such that by the time all of the solvent-treated mice were euthanized, drug-treated mice were alive and showed no signs of melanoma formation (Figs. 4b and 5b, panel ii).

To determine whether treatment with either rapamycin or PD325901 had completely eliminated melanoma cells initiated by 4-HT administration, we ceased drug treatment in both groups of animals and monitored the animals for an additional 3–11 weeks (Figs. 4c and 5c). Regardless of the drug used, all of the animals subsequently developed malignant melanoma requiring their euthanasia within 5–11 weeks following cessation of drug administration (Figs. 4c and 5b (bottom), c). These data indicate that the prevention of melanoma in the drug-treated mice was not a result of a technical flaw in the activation and function of CreER following 4-HT administration. Moreover, these data are consistent with the presence of latent melanoma-initiating cells that arise shortly after the CreER activation and that can survive extended exposure to agents that target signaling pathways essential for the melanoma cell division cycle and survival.

To determine whether melanomas that recur following cessation of MEK1/2 inhibitor treatment are resistant to retreatment of mice with this agent, melanoma was initiated in a cohort of 10 3-week-old *Tyr::Cre; Bra^f^{CA}; Pten^{lox4-5/lox4-5}* mice. Ten days later, all mice were treated systemically with PD325901 for a period of 6 weeks. At this time, consistent with results described above, none of the mice showed any signs of melanoma. Upon cessation of PD325901 administration, the mice were left untreated for a period of 8 weeks. At this time mice were randomized into two groups of five, one of which was administered solvent control (group A) and the other PD325901 (group B) for a further 6 weeks. As described above, group A mice treated only once with PD325901 succumbed to disease 8–12 weeks following cessation of drug administration (Fig. 5d). By contrast, group B mice that received two cycles of PD325901 remained alive at a time when all of the group A mice had been euthanized. However, all group B mice displayed signs of melanoma regrowth which, although not requiring euthanasia, strongly suggested that these mice would eventually succumb to disease. Regardless, these data indicate that mice treated once with PD325901 to prevent melanomagenesis remain sensitive to the antimelanoma action of a second round of PD325901 administration.

The results described above document the potent melanoma-prevention activity of PD325901 and rapamycin. However, to determine whether these agents can elicit regression of preexisting tumors in mice, we initiated melanoma in a group of 20 *Tyr::Cre; Bra^f^{CA}; Pten^{lox5/lox5}* mice by local administration of 4-HT to back skin (Supplementary Fig. 3a). Three weeks later, when the animals had readily measurable melanoma lesions, mice were randomly divided into four groups that were administered either solvent control (Vehicle), PD325901 (PD, 12.5 mg/kg), rapamycin (Rapa, 7.5 mg/kg) or the combination of both rapamycin and PD325901 (PD + Rapa) at the same dose used in the single-agent arms (Fig. 6a). We treated mice with these agents for 3 weeks and measured tumor size every day. Melanoma lesions in the vehicle-treated animals progressed steadily over the time course analyzed and had expanded in size in all animals by ~40% by the end of the experiment (Fig. 6b). By contrast, mice treated with either PD325901 or rapamycin as single

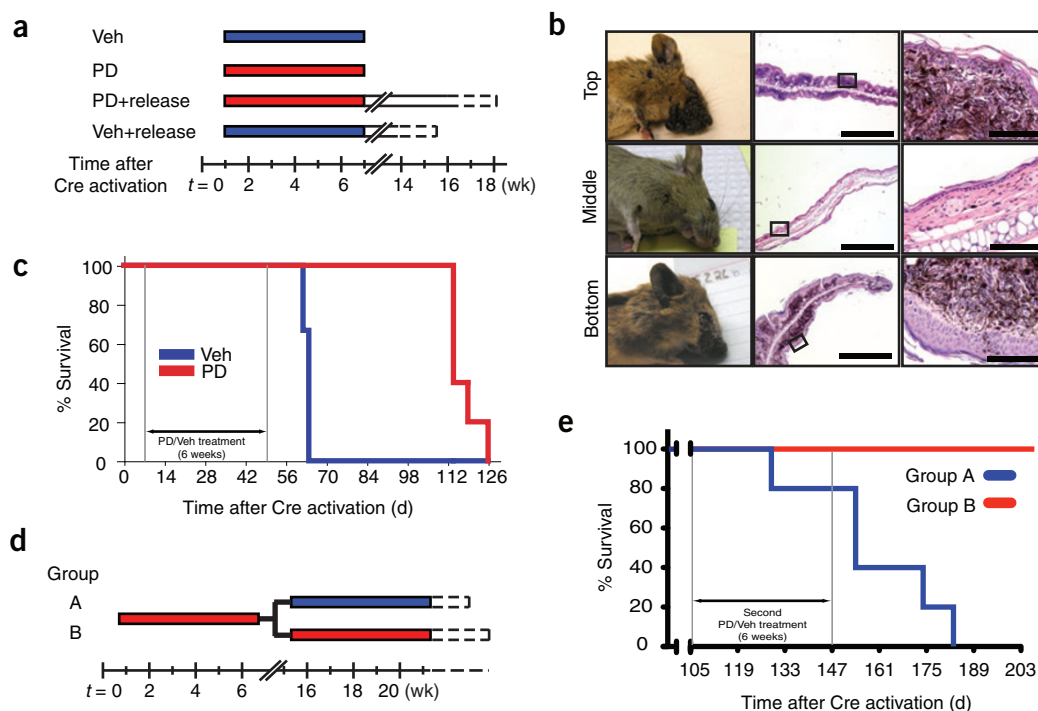


Figure 5 Prevention of BRAF^{V600E}-induced melanomas by PD325901. (a) Adult *Tyr::CreER; Braf^{CA/+}; Pten^{lox4-5/lox4-5}* mice were treated topically on the ear with 4-HT. One week later, mice were randomly assigned to be administered PD325901 (12.5 mg/kg, $n = 12$) or the solvent control ($n = 12$) for 6 weeks. Mice were analyzed daily for the presence of cutaneous malignant melanoma and euthanized according to a standard body conditioning score. After 6 weeks of PD325901 treatment, seven drug-treated and six control mice were euthanized for analysis of skin sections. A further five PD325901-treated and six control mice were monitored over the course of a further 11 weeks without any further drug administration. These mice were analyzed for the presence of cutaneous malignant melanoma as described above. Mouse survival was plotted using a Kaplan-Meier survival curve. (b) Representative anatomical and histological images of skin from 4-HT treated *Tyr::CreER; Braf^{CA/+}; Pten^{lox4-5/lox4-5}* mice analyzed after 6 weeks of treatment with control solvent ($n = 6$, top) or PD325901 ($n = 7$, middle) or from PD325901 treated mice left without further drug administration for an additional 9–11 weeks (bottom). Scale bars: center column, 2 mm; top right, 0.4 mm; center and bottom right, 0.1 mm. (c) Kaplan-Meier survival curve of vehicle ($n = 6$) or PD325901 ($n = 5$) treated cohorts of mice analyzed in this experiment. All mice were treated for the indicated 6 weeks as in b. Log rank tests of survival plots of the data demonstrate a statistically significant difference between vehicle and PD325901 treated animals ($P = 0.0024$). (d) Three week old *Tyr::CreER; Braf^{CA/+}; Pten^{lox4-5/lox4-5}* mice ($n = 10$) were treated topically on the right ear with 4-HT. Ten days later all mice were administered PD325901 (12.5mg/kg) for 6 weeks. Drug administration was then ceased for 8 weeks. Mice were then randomized into two groups: group A (blue) was administered vehicle control and group B (red) was administered PD325901 for a further 6 weeks. At the end of this period mice were monitored prospectively for disease progression as described above. (e) Mouse survival was plotted using a Kaplan-Meier survival curve that demonstrated statistically significant difference ($P = 0.0018$) between the groups by log rank tests.

agents had marked inhibition of tumor growth, but with little or no evidence of tumor regression. Mice treated with a combination of rapamycin and PD325901 showed a ~20% reduction in tumor size that was statistically significant. These data suggest that the combination of MEK1/2 and mTorC1 inhibition can lead to significant tumor shrinkage in the BRAF^{V600E}; *Pten*^{null} melanoma model.

Two hours after the final administration of the various agents in the experiment described above, tumor specimens were prepared and stained with (i) hematoxylin and eosin; (ii) antisera against Ki67 to assess cell proliferation; (iii) antisera against phospho-ERK1/2 (pERK1/2); and (iv) antisera against phospho-4EBP1 (p4EBP1) as indicated (Fig. 6c). The last two stains were used to assess the effects of PD325901 or rapamycin on the relevant signaling pathways. Histological analysis of sections stained with hematoxylin and eosin revealed the presence of viable tumor cells in all treatment groups, but with an increase in the number of cells with pyknotic features of apoptosis in mice treated with PD325901 or PD325901 in combination with rapamycin (Supplementary Fig. 6 online). Administration of PD325901 or rapamycin, either alone or in combination, led to a reduction in cell proliferation as measured by Ki67 staining. As

expected, treatment with PD325901 led to decreased pERK1/2 staining but had little or no effect on p4EBP1. Conversely, treatment with rapamycin led to decreased p4EBP1 staining but had little or no effect on pERK1/2. As expected, the combination of PD325901 and rapamycin led to decreased staining of both pERK1/2 and p4EBP1. These data suggest that, as single agents, PD325901 and rapamycin have predominantly a cytostatic effect on melanoma initiated by expression of BRAF^{V600E} and extinction of *Pten*. By contrast, evidence of melanoma regression in mice treated with the combination of PD325901 and rapamycin suggests that these agents may combine to elicit a cytotoxic effect on preexisting lesions, supported by the decrease in tumor size accompanied by an increase in pyknotic tumor cells. However, it remains possible that both tumor cell autonomous and nonautonomous effects of these agents may contribute to melanoma regression in this experimental system.

Response of BRAF^{V600E}-induced melanoma cell lines to pharmacological inhibition of MEK1/2 and mTorC1

To characterize potential melanoma cell-autonomous effects of rapamycin and PD325901, we established a small number of mel-

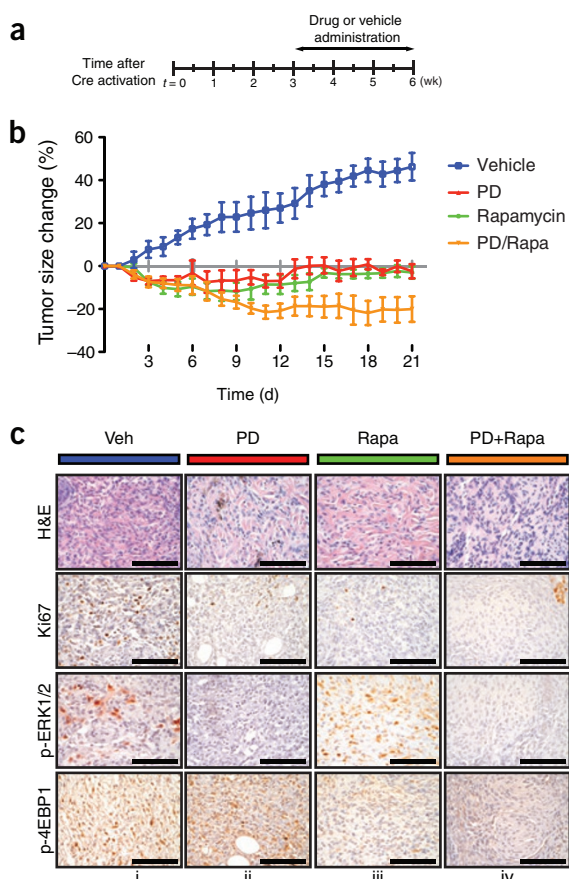
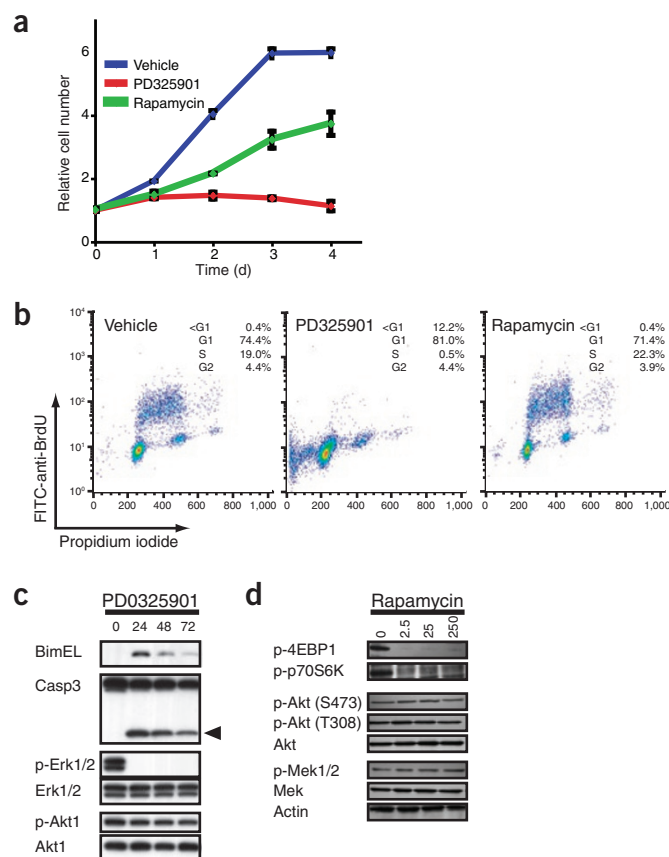


Figure 6 Melanoma regression in response to combination treatment with PD325901 and rapamycin. **(a)** Melanoma was initiated in a group of 20 *Tyr::Cre; Braf^{CA}; Pten^{lox/lox}* mice by local administration of 1–2 μ l of 5 mM 4-HT to back skin. Three weeks later, when the animals had readily measurable melanoma lesions, mice were randomly divided into four groups that were administered either (i) vehicle control (blue); (ii) PD325901 (PD, 12.5 mg/kg, red); (iii) rapamycin (Rapa, 7.5 mg/kg, green); or (iv) the combination of both rapamycin and PD325901 (PD+Rapa, orange) for three weeks as indicated. **(b)** Tumor size in each of the treatment groups described in **a** was measured daily and plotted as percentage change in tumor size compared to the starting size. Two-way ANOVA analysis demonstrates statistical significance: Vehicle versus Rapa or PD or PD+Rapa ($P < 0.0001$); PD versus PD+Rapa ($P = 0.0024$) and Rapa versus PD+Rapa ($P = 0.0120$). **(c)** Two hours following the final administration of the various agents, mice were euthanized and melanoma specimens were prepared for staining with hematoxylin and eosin or antisera against Ki67, pERK1/2 or p4EBP1 as indicated. Scale bars, 0.1 mm.

lowest dose tested (2.5 nM, Fig. 7d). Rapamycin had no effect on the phosphorylation of upstream Akt (T308 or S473) nor on the phosphorylation of MEK1/2. Treatment of 2697T cells with PD325901 (2 μ M) led to complete abrogation of cell proliferation (Fig. 7a), a marked reduction in S-phase cells (Fig. 7b) and a modest reduction in cell number at the latest time point. Consistent with this, MEK1/2 inhibition elicited a substantial reduction of phospho-ERK1/2 with no effect on phospho-Akt (Fig. 7c). Moreover, MEK inhibition was accompanied by induction of the proapoptotic Bcl-2 family protein Bim-EL, cleavage of caspase 3 (Fig. 7c) and an increase in cells with a sub-G1 DNA content (Fig. 7b). These data are similar to those obtained in bona fide human melanoma cell lines where regulation of BIM-EL expression downstream of oncogenic NRAS or BRAF contributes to suppression of melanoma cell apoptosis^{30,31}.

anoma-derived cell lines. One such line that grew well in culture (2697T) was derived from a primary cutaneous lesion arising in a 4-HT-treated *Tyr::CreER; Braf^{CA}; Pten^{lox5/lox5}* white mouse and was confirmed to have recombined the *Braf^{CA}* and the *Pten^{lox}* alleles by PCR (Supplementary Fig. 5 online). These cells showed evidence of phosphorylated MEK1/2, ERK1/2, p70^{S6K} and 4EBP1, consistent with the genetic alterations in *Braf* and *Pten* that promote melanoma initiation and progression in this model (Fig. 7). Treatment of melanoma cells with rapamycin (25 nM) for either 24 or 48 h led to 50% inhibition of cell proliferation after 72 h compared to the control (Fig. 7a). The antiproliferative effects of rapamycin were accompanied by robust inhibition of phospho-p70^{S6K} and 4EBP1, even at the

Figure 7 Inhibition of mouse melanoma cell proliferation by rapamycin or PD325901 *in vitro*. A melanoma cell line (2697T) was derived from a 4-HT-induced lesion that developed on the skin of a white *Tyr::CreER; Braf^{CA}; Pten^{lox5/lox5}* mouse. As indicated, 2697T cells growing asynchronously in full media were treated with PD325901 (2 μ M) or rapamycin (50 nM). **(a)** Cell growth was measured using a standard crystal violet staining assay. **(b)** DNA synthesis and content was assessed at 48 h using standard anti-BrdU/Propidium Iodide FACS analysis following a 2 h BrdU pulse. The percentage of cells in each portion of the cell cycle is indicated. **(c)** 2697T cells, growing asynchronously in full media, were treated with 2 μ M PD325901 for 24–72 h at which time cell extracts were prepared. Expression of Bim-EL, ERK1/2, Akt1, Caspase 3 and its active cleavage product (arrowhead) as well as the phosphorylation of ERK1/2 and Akt1 was assessed by immunoblotting. **(d)** Serum-deprived 2697T cells were pre-treated with different concentrations of rapamycin (2.5–250 nM) for 30 min before stimulation with 10% (v/v) FCS for a further 20 min. Expression of Akt1, MEK1/2 and actin as well as the phosphorylation of 4EBP1, p70^{S6K}, and Akt1 (S473 and T308) was assessed by immunoblotting.



DISCUSSION

Mouse models have provided key insights into cancer initiation, progression and therapy, and the model described here offers additional distinct and important advantages in the study of melanoma and its treatment^{32–34}. Here, we provide evidence of cooperative interactions of two signature mutations found in human melanoma, enabling the generation of a mouse that recapitulates hallmark features of the disease, including metastases to relevant organ sites. Moreover, the use of *Braf*^{CA} mice allows accurate modeling of the earliest effects of physiological levels of expression of *Braf*^{V600E}, the purported initiating mutation that promotes the conversion of normal human melanocytes to benign nevus cells^{4,7}. Because expression of *Braf*^{V600E} promotes the development of benign melanocytic hyperplasias that fail to progress to malignant melanoma, this system may be used to explore mechanisms constraining progression *in vivo*, to discover and validate tumor suppressor genes governing such progression checkpoints and to define the mechanisms driving invasion and metastasis^{10,35,36}. Next, use of the *Tyr::CreER*^{T2} transgene affords the experimental advantage of exquisite spatial and temporal control over the genetic alterations that initiate melanoma formation in mice. Finally, and most notably, we provide evidence that this model is useful to study the signaling axis of these key genetic lesions and to assess the impact of agents targeting these pathways that may facilitate the development of new strategies to target malignant melanoma using conventional or targeted therapies³⁷.

Data presented here are consistent with effects of transgenic overexpression of *BRAF*^{V600E} in zebrafish, which leads to the development of ‘fish nevi’³⁸. Moreover, when combined with loss of *Trp53* expression, *BRAF*^{V600E} elicited malignant fish melanoma. However, the relevance of this combination of genetic events to human melanoma is unclear given the relatively low frequency of *TP53* mutations in the human disease^{1,13}. However, these data stand in contrast to the reported effects of *BRAF*^{V600E} overexpression in human melanocytes engineered to express the catalytic subunit of telomerase (hTERT), dominant-negative TP53^{R248W} and CDK4^{R24C}, the last being resistant to the inhibitory effects of the melanoma suppressor, p16^{INK4A} (ref. 39). In this context, the effects of ectopic *BRAF*^{V600E} expression were benign, inducing only mild junctional melanocyte nesting. By contrast, ectopic expression of oncogenic variants of RAS (*NRAS*^{G12V} or *HRAS*^{G12V}) or the catalytic subunit of PI3'-kinase- α (p110-CAAX) led to marked malignant transformation of such ‘initiated’ human melanocytes. It is unclear why the effect of *BRAF*^{V600E} expression is so benign in this context, especially given the notable effects of *BRAF*^{V600E} in zebrafish and mice.

Although genetic analysis of human melanomas suggests that mutated *BRAF* cooperates with loss of *PTEN* expression in melanomagenesis, it has yet to be unequivocally demonstrated that such is the case^{14,15}. However, data presented here demonstrate the ability of *Braf*^{V600E} expression to cooperate with *Pten* silencing in the genesis of metastatic melanoma. It remains to be seen whether PI3'-lipid phosphatase activity is key to *Pten*'s tumor suppressor activity, especially in view of recent demonstrations of effects of *Pten* on other signaling pathways that are independent of its lipid phosphatase activity⁴⁰.

A key feature of this model is the ability to control melanoma initiation and progression in response to expression of oncogenic *Braf*^{V600E} in combination with alterations in other genes implicated in melanoma initiation, progression or response to therapy, such as *Cdkn2a*, *Ctnnb1*, *Mitf* or various alleles of *Mcl1*^{5,41,42}. Given the high frequency of invasion and metastasis observed in this model, it may be possible to initiate a primary malignant melanoma in a readily accessible site, resect the primary lesion and then monitor the mice prospectively for local

or distant recurrence. This aspect of the model may afford a detailed analysis of mechanisms that promote melanoma cell migration, local invasion and metastasis in a manner that more closely recapitulates the clinical course of melanoma recurrence in humans.

Metastatic melanoma presents one of the most difficult diseases to treat in medical oncology. Indeed, there has not been a major breakthrough in the treatment of this disease in over 25 years. However, considerable excitement has been generated by the identification of genetic and epigenetic alterations that contribute to melanomagenesis, all the more so given the accelerated pace of development of pharmacological agents that target key signaling pathways in the melanoma cell, although it remains unclear how best to use such agents in the clinic. Studies with the broad spectrum Raf inhibitor Sorafenib, either as a single agent or in combination chemotherapy, have proven disappointing in treating metastatic melanoma, but this may be a result of incomplete inhibition of *BRAF*^{V600E} activity^{43,44}. Moreover, individual case reports indicate that even highly aggressive melanomas expressing mutationally activated *KIT* can show a marked response to the potent c-KIT inhibitor, Gleevec^{27,45}. Such observations raise hope that pharmacological targeting of relevant molecular target(s) may lead to clinical responses even in individuals with late-stage melanoma. Given the prevalence of *BRAF* and *PTEN* alterations in melanoma, testing such agents in a validated preclinical mouse model may inform the clinical development of various new and more selective *BRAF*, *MEK*, *PI3'-kinase*, *AKT* and *mTor* inhibitors^{46–48}. Experiments presented here suggest such agents may have value in melanoma prevention and therapy, but the relapse of mice following cessation of drug administration indicates that single-agent therapy with *MEK1/2* or *mTor* inhibitors is unlikely to cure individuals with this disease. However, this model system is readily tractable to test combinations of targeted agents or targeted therapy combined with conventional cytotoxics or other agents such as biochemotherapy or immune-based approaches⁴⁹. Such studies might provide useful guides to the best dosage and scheduling of antimelanoma therapeutics in clinical trials in humans.

METHODS

Mouse breeding, activation of *Tyr::CreER* transgene and treatment of mice with targeted therapeutics. *Braf*^{CA}, *Tyr::CreER* and *Pten*^{lox4-5} mice were genotyped as previously described^{17,20,24}. Cre-mediated conversion of *Braf*^{CA} to *Braf*^{NE} and the deletion of exons 4 and 5 of *Pten* were assessed by PCR as previously described. Topical administration of 4-hydroxytamoxifen (4-HT) was conducted by preparing a 25–50 mg/ml (65–130 mM) solution of 4-HT (70% Z-isomer, Sigma) in dimethylsulphoxide and applying enough solution to wet the right ear, right flank and tail with a small paint brush on postnatal days 2, 3 and 4. For localized melanoma induction on the back skin, adult (6–8 weeks of age) mice were treated topically with 1–2 μ l of 1.9 mg/ml (5 mM) 4-HT at 6–8 weeks of age using a similar protocol. Generalized induction in adult mice was performed by intraperitoneal injection of 1 mg of tamoxifen per 40 g mouse on three consecutive days. In this case tamoxifen was prepared as a 10 mg/ml suspension in peanut oil. PD352901 was dissolved in 0.5% (w/v) hydroxy-propyl-methylcellulose, 0.2% (v/v) Tween 80 (Sigma) and administered to mice daily by oral gavage at a dose of 12.5 mg/kg. Rapamycin (LC Laboratories) was suspended in 0.5% (w/v) methylcellulose and administered to mice daily by oral gavage at a dose of 7.5 mg/kg. The relevant solvent was administered to control animals in the melanoma prevention studies. Tissues were prepared for analysis as previously described^{17,20}.

Generation of mice carrying a Cre-inactivated allele of *Pten*. A targeting vector was generated in pKO SelectDT-915 using a 12-kb fragment of the mouse *Pten* gene by standard techniques. A cassette carrying *Pten* exon 5 flanked by *loxP* sites and *PGK::Neo* flanked by FRT sites was then subcloned between the genomic DNA arms. This construct was linearized, transfected into TC1 ES cells and genomic DNA from G418 and diphtheria toxin-resistant ES cells were screened by Southern blot and PCR for homologous recombination events. Blastocyst injections were carried out with three different targeted clones, and transmit-

ting chimaeric mice were bred from CAGG-*Flpe* transgenic mice to generate the *Pten^{lox5}* allele³⁰. Chimeric mice were mated with FVB/N mice and transmission verified by Southern blotting, and PCR with the primers listed in **Supplementary Table 2** online. All mice were maintained on a mixed C57Bl/6 and FVB/N background by random interbreeding.

Melanoma cell line derivation and culture. Melanoma cell lines were derived by physical resection of primary cutaneous lesions followed by trituration through a 20-gauge syringe needle and filtration through a 100 μ m nylon cell strainer (BD/Falcon) before being placed into media. Cell suspensions were plated in media (a 1:1 mix of DMEM and Hams F12) supplemented with FCS (5% v/v) and penicillin, streptomycin and glutamine. Serum starved cells were treated with different doses of rapamycin (2.5–250 nM as indicated) for 30 min before 10%(v/v) serum stimulation for an additional 20 min. Asynchronously growing cells in full media were treated with PD325901 (2 μ M) for different periods of time (24–96 h as indicated) with cell proliferation quantified by staining of fixed cells with crystal violet. Data presented in **Figure 6a** are results from two separate experiments in which each time point was measured in triplicate. Subconfluent cells grown in full melanocyte media treated with PD325901 (2 μ M) or rapamycin (50nM) were cultured for 46 h when BrdU was added to 20 μ M for 2 h. Single cell suspensions fixed in ethanol were stained with FITC-labeled BrdU (BD Pharmingen 556028), incubated with propidium iodide (10 μ g/ml), and analyzed with a BD-FACS caliber. Cell extracts were prepared by standard techniques and resolved using SDS-polyacrylamide gel electrophoresis. Protein blots prepared by standard techniques, and were probed with commercially available antisera against various proteins as indicated³¹.

Immunocytochemistry and immunoblotting. The expression of tyrosinase (Tyr, Albino), tyrosinase-related protein 1 (Tyrp1, Brown) or tyrosinase-related protein 2 (Tyrp2, Slaty) was detected by staining with antisera against α PEP7, α PEP1 or α PEP8, respectively (gift of V. Hearing, National Cancer Institute)²⁶. Slides were developed using a di-amino-benzidine-peroxidase substrate kit (Vector Labs), according to the manufacturer's instructions. The expression and phosphorylation of proteins was detected by immunoblotting using appropriate commercially available antisera: phospho-4EBP1, phospho-p70^{S6K}, phospho-MEK1/2, total MEK1/2, phospho-ERK1/2; total ERK1/2; phospho S473-AKT; phospho-T308-AKT; total AKT; caspase 3 (all from Cell Signaling Technology), Bim-EL (Calbiochem mAb 14A8); and actin (Sigma).

Note: Supplementary information is available on the Nature Genetics website.

AUTHOR CONTRIBUTIONS

M.M. and M.B. established a collaboration for the exchange of relevant mouse strains to enable the generation and analysis of *Tyr::CreER*, *Braf^{CA}*, *Pten^{lox}* mice and contributed to the manuscript equally as senior authors. D.D. (UCSF) and D.P.C. (University of Vermont) performed all of the mouse experiments in parallel. Representative figures were selected for publication by D.D., D.P.C., M.M. and M.B. and were prepared for publication by D.D. R.A.C. performed immunoblot analysis of 2697T cells treated with PD325901. B.N. and W.E.D. assisted with and optimized mouse tumor induction and performed immunohistochemical analysis. A.N.K. analyzed *Tyr::CreER*, *Braf^{CA}*, *Pten^{lox4-5}* mice treated with multiple cycles of PD325901. M.J.Y. generated and characterized *Pten^{lox5}* mice in the laboratory of R.A.D. M.M. wrote the manuscript and shepherded it through review with contributions from D.D., D.P.C., M.J.Y., R.A.D. and M.B.

ACKNOWLEDGMENTS

We thank the members of the McMahon and Rosenberg laboratories as well as B. Bastian, L. Chin, E. Filenova, B. Hann, M. Herlyn, L. Johnson, G. Merlino, P. P. Pandolfi, V. Hearing, M. Held, G. Kay and D. Matzen for the provision of mouse strains, reagents, advice and support. M.M. thanks A. Ricart and J. Sebolt-Leopold (Pfizer) for provision of PD325901 and acknowledges the support of the University of California San Francisco Helen Diller Family Comprehensive Cancer Center Mouse Pathology and Pre-Clinical Therapeutics cores. R.A.D. is supported as an American Cancer Society Research Professor. This work was supported by grants from the Melanoma Research Foundation, U.C. Discovery Award and from the US National Institutes of Health (CA 108972 to M.M., CA 84313 to R.A.D. and CA 89124 and CA 112054 to M.B., respectively).

Published online at <http://www.nature.com/naturegenetics/>

Reprints and permissions information is available online at <http://npg.nature.com/reprintsandpermissions/>

- Chin, L., Merlino, G. & DePinho, R.A. Malignant melanoma: modern black plague and genetic black box. *Genes Dev.* **12**, 3467–3481 (1998).
- Gray-Schopfer, V.C., da Rocha Dias, S. & Marais, R. The role of B-RAF in melanoma. *Cancer Metastasis Rev.* **24**, 165–183 (2005).
- Chin, L. The genetics of malignant melanoma: lessons from mouse and man. *Nat. Rev. Cancer* **3**, 559–570 (2003).
- Davies, H. *et al.* Mutations of the BRAF gene in human cancer. *Nature* **417**, 949–954 (2002).
- Garraway, L.A. *et al.* Integrative genomic analyses identify MITF as a lineage survival oncogene amplified in malignant melanoma. *Nature* **436**, 117–122 (2005).
- Hayward, N.K. Genetics of melanoma predisposition. *Oncogene* **22**, 3053–3062 (2003).
- Pollock, P.M. *et al.* High frequency of BRAF mutations in nevi. *Nat. Genet.* **33**, 19–20 (2003).
- Wellbrock, C., Karasides, M. & Marais, R. The RAF proteins take centre stage. *Nat. Rev. Mol. Cell Biol.* **5**, 875–885 (2004).
- Mercer, K.E. & Pritchard, C.A. Raf proteins and cancer: B-Raf is identified as a mutational target. *Biochim. Biophys. Acta* **1653**, 25–40 (2003).
- Michaloglou, C. *et al.* BRAF600-associated senescence-like cell cycle arrest of human naevi. *Nature* **436**, 720–724 (2005).
- Sviderskaya, E.V. *et al.* p16(Ink4a) in melanocyte senescence and differentiation. *J. Natl. Cancer Inst.* **94**, 446–454 (2002).
- Wellbrock, C. *et al.* V599EB-RAF is an oncogene in melanocytes. *Cancer Res.* **64**, 2338–2342 (2004).
- Chin, L., Garraway, L.A. & Fisher, D.E. Malignant melanoma: genetics and therapeutics in the genomic era. *Genes Dev.* **20**, 2149–2182 (2006).
- Lin, W.M. *et al.* Modeling genomic diversity and tumor dependency in malignant melanoma. *Cancer Res.* **68**, 664–673 (2008).
- Tsao, H., Goel, V., Wu, H., Yang, G. & Haluska, F.G. Genetic interaction between NRAS and BRAF mutations and PTEN/MMAC1 inactivation in melanoma. *J. Invest. Dermatol.* **122**, 337–341 (2004).
- Tsao, H., Zhang, X., Fowlkes, K. & Haluska, F.G. Relative reciprocity of NRAS and PTEN/MMAC1 alterations in cutaneous melanoma cell lines. *Cancer Res.* **60**, 1800–1804 (2000).
- Dankort, D. *et al.* A new mouse model to explore the initiation, progression, and therapy of BRAFV600E-induced lung tumors. *Genes Dev.* **21**, 379–384 (2007).
- Tonks, I.D. *et al.* Tyrosinase-Cre mice for tissue-specific gene ablation in neural crest and neuroepithelial-derived tissues. *Genesis* **37**, 131–138 (2003).
- Guyonneau, L., Murisier, F., Rossier, A., Moulin, A. & Beermann, F. Melanocytes and pigmentation are affected in dopachrome tautomerase knockout mice. *Mol. Cell. Biol.* **24**, 3396–3403 (2004).
- Bosenberg, M. *et al.* Characterization of melanocyte-specific inducible Cre recombinase transgenic mice. *Genesis* **44**, 262–267 (2006).
- Bennett, D.C. Human melanocyte senescence and melanoma susceptibility genes. *Oncogene* **22**, 3063–3069 (2003).
- Jonsson, G. *et al.* Genomic profiling of malignant melanoma using tiling-resolution arrayCGH. *Oncogene* **26**, 4738–4748 (2007).
- Willmore-Payne, C., Holden, J.A., Hirschowitz, S. & Layfield, L.J. BRAF and c-kit gene copy number in mutation-positive malignant melanoma. *Hum. Pathol.* **37**, 520–527 (2006).
- Trotman, L.C. *et al.* Pten dose dictates cancer progression in the prostate. *PLoS Biol.* **1**, E59 (2003).
- Inoue-Narita, T. *et al.* Pten deficiency in melanocytes results in resistance to hair graying and susceptibility to carcinogen-induced melanomagenesis. *Cancer Res.* **68**, 5760–5768 (2008).
- Jimenez, M., Tsukamoto, K. & Hearing, V.J. Tyrosinases from two different loci are expressed by normal and by transformed melanocytes. *J. Biol. Chem.* **266**, 1147–1156 (1991).
- Hodi, F.S. *et al.* Major response to imatinib mesylate in KIT-mutated melanoma. *J. Clin. Oncol.* **26**, 2046–2051 (2008).
- Ohren, J.F. *et al.* Structures of human MAP kinase kinase 1 (MEK1) and MEK2 describe novel noncompetitive kinase inhibition. *Nat. Struct. Mol. Biol.* **11**, 1192–1197 (2004).
- Sabatini, D.M., Erdjument-Bromage, H., Lui, M., Tempst, P. & Snyder, S.H. RAFT1: a mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs. *Cell* **78**, 35–43 (1994).
- Sheridan, C., Brumatti, G. & Martin, S.J. Oncogenic B-RafV600E inhibits apoptosis and promotes ERK-dependent inactivation of Bad and Bim. *J. Biol. Chem.* **283**, 22128–22135 (2008).
- Cartledge, R.A. *et al.* Oncogenic BRAF(V600E) inhibits BIM expression to promote melanoma cell survival. *Pigment Cell Melanoma Res.* **21**, 534–544 (2008).
- Walker, G.J. & Hayward, N.K. Pathways to melanoma development: lessons from the mouse. *J. Invest. Dermatol.* **119**, 783–792 (2002).
- Bardeesy, N., Wong, K.K., DePinho, R.A. & Chin, L. Animal models of melanoma: recent advances and future prospects. *Adv. Cancer Res.* **79**, 123–156 (2000).
- Tietze, M.K. & Chin, L. Murine models of malignant melanoma. *Mol. Med. Today* **6**, 408–410 (2000).
- Woods, D. *et al.* Raf-induced proliferation or cell cycle arrest is determined by the level of Raf activity with arrest mediated by p21Cip1. *Mol. Cell. Biol.* **17**, 5598–5611 (1997).
- Zhu, J., Woods, D., McMahon, M. & Bishop, J.M. Senescence of human fibroblasts induced by oncogenic Raf. *Genes Dev.* **12**, 2997–3007 (1998).
- Olive, K.P. & Tuveson, D.A. The use of targeted mouse models for preclinical testing of novel cancer therapeutics. *Clin. Cancer Res.* **12**, 5277–5287 (2006).

38. Patton, E.E. *et al.* BRAF mutations are sufficient to promote nevi formation and cooperate with p53 in the genesis of melanoma. *Curr. Biol.* **15**, 249–254 (2005).
39. Chudnovsky, Y., Adams, A.E., Robbins, P.B., Lin, Q. & Khavari, P.A. Use of human tissue to assess the oncogenic activity of melanoma-associated mutations. *Nat. Genet.* **37**, 745–749 (2005).
40. Freeman, D.J. *et al.* PTEN tumor suppressor regulates p53 protein levels and activity through phosphatase-dependent and -independent mechanisms. *Cancer Cell* **3**, 117–130 (2003).
41. Larue, L. & Delmas, V. The WNT/Beta-catenin pathway in melanoma. *Front. Biosci.* **11**, 733–742 (2006).
42. Landi, M.T. *et al.* MC1R germline variants confer risk for BRAF-mutant melanoma. *Science* **313**, 521–522 (2006).
43. Eisen, T. *et al.* Sorafenib in advanced melanoma: a Phase II randomised discontinuation trial analysis. *Br. J. Cancer* **95**, 581–586 (2006).
44. Flaherty, K.T. Chemotherapy and targeted therapy combinations in advanced melanoma. *Clin. Cancer Res.* **12**, 2366s–2370s (2006).
45. Lutzky, J., Bauer, J. & Bastian, B.C. Dose-dependent, complete response to imatinib of a metastatic mucosal melanoma with a K642E KIT mutation. *Pigment Cell Melanoma Res* **21**, 492–493 (2008).
46. Maira, S.M. *et al.* Identification and characterization of NVP-BEZ235, a new orally available dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor with potent in vivo antitumor activity. *Mol. Cancer Ther.* **7**, 1851–1863 (2008).
47. Tsai, J. *et al.* Discovery of a selective inhibitor of oncogenic B-Raf kinase with potent antimelanoma activity. *Proc. Natl. Acad. Sci. USA* **105**, 3041–3046 (2008).
48. Yeh, T.C. *et al.* Biological characterization of ARRY-142886 (AZD6244), a potent, highly selective mitogen-activated protein kinase kinase 1/2 inhibitor. *Clin. Cancer Res.* **13**, 1576–1583 (2007).
49. Sosman, J.A. & Puzanov, I. Molecular targets in melanoma from angiogenesis to apoptosis. *Clin. Cancer Res.* **12**, 2376s–2383s (2006).
50. Rodriguez, C.I. *et al.* High-efficiency deleter mice show that FLPe is an alternative to Cre-loxP. *Nat. Genet.* **25**, 139–140 (2000).