NRAS mutations are common in human melanoma. To produce a mouse model of NRAS-driven melanoma, we expressed oncogenic NRAS (NRAS\textsuperscript{G12D}) in mouse melanocytes. When NRAS\textsuperscript{G12D} was expressed in the melanocytes of developing embryos, it induced melanocyte proliferation and congenital melanocytic lesions reminiscent of human blue nevi but did not induce cutaneous melanoma. Unexpectedly, however, it did induce early-onset primary melanoma of the central nervous system (CNS). The tumors were rapidly proliferating and caused neurologic symptoms, rapid health deterioration, and death. NRAS is not a common driver oncogene of primary melanoma of the CNS in adults, but we report two cases of primary melanoma of the CNS in children, both of which carried oncogenic mutations in NRAS. We conclude that acquisition of somatic mutations in NRAS in CNS melanocytes is a predisposing risk factor for primary melanoma of the CNS in children, and we present a mouse model of this disease.

**SIGNIFICANCE:** We show that the acquisition of NRAS mutations in melanocytes during embryogenesis is a risk factor for early-onset melanoma of the CNS. We have developed a powerful mouse model to study this rare but devastating childhood disease, and to develop therapeutic approaches for its treatment. Cancer Discov; 3(4): 458–69. © 2013 AACR.

See related commentary by Ciarlo and Zon, p. 382.
INTRODUCTION

Malignant melanoma is a potentially fatal form of cancer that develops from specialized pigment cells called melanocytes. In humans, melanocytes are common in the epidermis (cutaneous melanocytes), but they also inhabit the dermis, eyes, ears, heart, central nervous system (CNS), and mucosal surfaces of the mouth and genital areas (1, 2). Thus, although the most common form of melanoma (~90% of cases) occurs on hair-bearing skin (cutaneous melanoma), primary melanomas also develop in other sites of the body. Melanoma of the non–hair-bearing skin (acral and mucosal melanomas) accounts for approximately 5% (3), and 1% (4) of cases, respectively, whereas uveal melanoma accounts for approximately 3% (5). In general, the rare forms have poorer prognosis, probably because they are diagnosed at a late stage.

Genetic analyses suggest that melanomas from different anatomic sites represent genetically distinct diseases. BRAF and NRAS are mutated in approximately 45% and 20%, respectively, of cutaneous melanomas. In contrast, in acral melanoma, BRAF mutations occur in only approximately 16% of cases, NRAS mutations are absent or very rare, and KIT mutations occur in approximately 20% of patients (6, 7). Furthermore, in uveal melanomas BRAF, NRAS, and KIT mutations seem to be extremely rare, and this disease instead is driven by mutations in GNAQ, GNA11, and BAP1 (8–10). Primary melanoma of the CNS is another rare melanoma and is thought to arise from the melanocytes of the leptomeninges. Melanocytic lesions of the CNS range from benign (leptomeningeal melanocytosis and melanocytoma) to malignant (leptomeningeal melanomatosis and melanoma) tumors (11–13). In children, these neoplasms often (but not always) occur in the context of neurocutaneous melanosis, a rare nonhereditary neurocutaneous syndrome presenting with giant (>20 cm) and/or multiple congenital melanocytic nevi (CMN) in association with leptomeningeal melanocytic lesions (14–16). Until recently, little was known about the genetic drivers of CNS melanoma, particularly in children. However, it was recently reported that the adult disease is associated with mutations in GNAQ and GNA11 (17–19) and, in one case, NRAS (19). Notably, approximately 80% of human CMN harbor somatic mutations in NRAS (20, 21), and giant CMN are associated with increased risk of cutaneous and leptomeningeal melanoma (22).

To study melanoma biology, mouse models of melanoma driven by oncogenic BRAF or RAS have been developed (23–29), and we have used conditionally inducible alleles based on Cre-recombinase/loxP technology to express oncogenes in mouse melanocytes (24, 25, 30, 31). In adult mice, BRAF\textsuperscript{V600E} induced skin darkening at 2 months, blue nevus–like lesions at 4 months, and melanoma in approximately 80% of the animals within 2 years (24). In contrast, when BRAF\textsuperscript{V600E} was expressed in the melanocytes of developing embryos (congenital expression), it induced developmental abnormalities and embryonic lethality (31).

Here, we investigated whether oncogenic NRAS could induce melanoma when it was expressed at physiologic levels using the endogenous Nras gene. We found that expression of NRAS\textsuperscript{G12D} in the melanocytes of adult mice induced skin darkening and blue nevus–like lesions, but not cutaneous melanoma. Expression of NRAS\textsuperscript{G12D} in the melanocytes of embryonic mice also induced skin darkening and congenital blue nevus–like lesions, but again, NRAS\textsuperscript{G12D} did not induce cutaneous melanoma.
However, when it was expressed in congenital nevi, NRAS\textsuperscript{G12D} induced melanoma of the CNS, and critically, the course of the disease in these mice closely resembled that in 2 children who developed melanoma of the CNS driven by oncogenic NRAS.

We conclude that acquired somatic mutation in NRAS drives melanoma of the CNS, and we have developed a mouse model of this disease.

RESULTS

To develop NRAS-driven melanoma models, we expressed NRAS\textsuperscript{G12D} at physiologic levels in mouse melanocytes. To achieve this, we used mice in which NRAS\textsuperscript{G12D} is expressed from the endogenous \textit{Nras} gene under the control of a \textit{LoxP-STOP-LoxP} (\textit{LSL}) cassette (\textit{Nras\textsuperscript{LSL-G12D}}, ref. 32), the removal of which by Cre-recombinase released NRAS\textsuperscript{G12D} expression in a conditional-inducible manner (Supplementary Fig. S1A). We crossed the \textit{Nras\textsuperscript{LSL-G12D}} mice onto mice in which tamoxifen-activated Cre-recombinase (CreERT2) was expressed in melanocytes using a tyrosinase enhancer/promoter fragment (\textit{Tyr::CreERT2} mice; see Supplementary Fig. S1A; refs. 32, 33). Although CreERT2 was expressed in the melanocytes of these mice from approximately embryonic day 10.5 (E10.5), it was only activated when the mice were treated with tamoxifen.

We painted tamoxifen onto the shaven skin on the backs of the mice at approximately 2 months of age to induce NRAS\textsuperscript{G12D} expression. Within 4 to 8 months, we observed visible darkening of the skin (Supplementary Fig. S1B) in the homozygous (\textit{Nras\textsuperscript{LSL-G12D};Tyr::CreERT2}) mice and weak darkening of the skin in 50% of the heterozygous (\textit{Nras\textsuperscript{LSL-G12D};Tyr::CreERT2}) mice. The darkening was more apparent in the tamoxifen-treated areas, although systemic effects also occurred, with darkening of the tails of the homozygous mice (Supplementary Fig. S1B). Microscopic examination of the skin revealed small paucicellular nevi in the deep dermal and periadnexal regions of the skin that followed the gyri and sulci and enveloped the ventricles (melanocytosis) (Fig. 2C). The darkly pigmented areas were composed of large melanocytic lesions comprising pleomorphic cells that invaded the brain parenchyma (Fig. 2D). The tumor cells were rapidly dividing (mitotic index >1/mm\textsuperscript{2}) and stained positive for Ki67 (Fig. 2E), phosphorylated extracellular signal–regulated kinase (ppERK), and cyclin D1 (Supplementary Fig. S3A and S3B). They were melanin-laden (Fig. 2F) and stained positive for HMB45/MelanA (Fig. 2G and Supplementary Fig. S3A and S3B) and S100 (Supplementary Fig. S3C), but negative for the glial cell marker glial fibrillary acidic protein (GFAP) (Supplementary Fig. S3D).

To provide evidence that oncogenic NRAS was expressed, we reverse transcribed RNA from the tumors and PCR amplified an \textit{Nras} fragment across the exon 2/exon 3 boundary. Sequencing of this fragment revealed that \textit{Nras\textsuperscript{G12D}} was expressed in the tumors, but not the normal brains of the \textit{Tyr::CreA/°} littermate controls (Supplementary Fig. S4). Macroscopic and microscopic examination of the skin did not present evidence of cutaneous melanoma (Fig. 1B–D), and we did not observe primary tumors in the uvea, hearts (Supplementary Fig. S2), or oral and genital mucosa (data not shown), the other tissues in which melanocytes reside. We therefore diagnosed primary melanoma of the CNS, and cells derived from these tumors displayed constitutive ERK activity that was sensitive to the MAP-ERK kinase (MEK) inhibitors PD184352, U0126, and A2700 (Fig. 2H). Importantly, PD184352 also delayed the growth of tumor allografts formed by these cells in syngeneic immunocompetent mice (Fig. 2I).

We observed hyperpigmentation of the leptomeninges in the brains of asymptomatic mice (melanocytosis). The pigmented cells followed the gyri and sulci and covered the parietal lobe in an arboriform pattern (Fig. 3A). Even in the immediate postnatal period (1-day-old mice), the leptomeninges were thickened and hyperpigmented and presented HMB45/MelanA-positive cells (Fig. 3B and C). Thus, although NRAS\textsuperscript{G12D} induced hyperproliferation of melanocytes in both the skin and the CNS, those in the skin developed into congenital cutaneous nevi,
**Figure 1.** NRAS<sup>G12D</sup> induces skin pigmentation and congenital nevi. A, schematic representation of the conditional-inducible approach used to express NRAS<sup>G12D</sup> in embryonic mouse melanocytes. A tyrosinase gene enhancer/promoter construct (Tyr enh/prom) was used to express Cre-recombinase (Cre) in melanocytes from approximately E 10.5 (38). NRAS<sup>G12D</sup> was expressed from the endogenous mouse Nras gene using a conditional-inducible targeted allele in which exon 2 is mutated to introduce the G12D mutation (32). The LSL cassette blocks NRAS<sup>G12D</sup> expression, but its removal by Cre-recombinase releases the block on expression. B, photographs showing skin pigmentation in control, Nras<sup>+/+</sup>;Tyr::Cre<sup>+/−</sup> (<+/G12D>) and Nras<sup>LSL-G12D/LSL-G12D</sup>;Tyr::Cre<sup>+/−</sup> (G12D/G12D) mice at 1 day, 3 weeks, and in adulthood. C, top, photomicrographs of hematoxylin and eosin (H&E)–stained skin in 1-day-old, 3-week-old, and adult control mouse skin. Scale bar, 200 μm. Bottom, low-power photomicrographs of H&E–stained skin in 1-day-old, 3-week-old, and adult Nras<sup>+/−</sup>;G12D/G12D;Tyr::Cre<sup>+/−</sup> mice. Hyperpigmented dendritic melanocytes are visible at low magnification in the 3-week-old and adult mice. Scale bar, 200 μm (n = 6 mice per experimental group). D, high-power photomicrographs of H&E–stained skin from boxed areas in the bottom panel from C in 1-day-old, 3-week-old, and adult Nras<sup>+/−</sup>;G12D/G12D;Tyr::Cre<sup>+/−</sup> mice. Hyperpigmented dendritic melanocytes in the papillary and reticular dermis, and along the hair follicles and adnexal glands, are indicated (black arrows) and are sparse in the skin of 1-day-old mice, but prominent in 3-week-old and adult mice. Scale bar, 20 μm. E, photomicrographs of HMB45/MelanA–stained skin from a 1-day-old Nras<sup>+/−</sup>;G12D/G12D;Tyr::Cre<sup>+/−</sup> mouse, showing the presence of melanocytes (black arrows). The area boxed in the left photomicrograph is enlarged in the right. Scale bars, 200 μm (left) and 20 μm (right).
Figure 2. Nras<sup>G12D</sup> induces CNS tumors in mice. A, Kaplan-Meier plot showing survival in months (mo) of study mice. The experimental groups consisted of Nras<sup>G12D/GL20; Tyr::CreA/+</sup> (n = 33) and Nras<sup>G12D/GL20; Tyr::CreA/> (G12D/G12D; n = 23) mice. The control groups consisted of Tyr::CreA/+ (n = 22), Nras<sup>G12D/GL20; Tyr::CreA/+</sup> (n = 13), and Nras<sup>G12D/GL20; Tyr::CreA/+</sup> (n = 15) mice. B, photographs showing representative whole-brain and sagittal sections of the brains of control, Nras<sup>G12D/GL20; Tyr::CreA/+</sup>, and Nras<sup>G12D/GL20; Tyr::CreA/+</sup> mice. C, photomicrograph of an H&E-stained brain section from an Nras<sup>G12D/GL20; Tyr::CreA/+</sup> mouse (boxed area in the bottom image of B), displaying proliferation of pigmented melanocytes along the external surface of the brain parenchyma (black arrow). Scale bar, 100 μm. D, photomicrograph of an H&E-stained frontal lobe melanoma from an Nras<sup>G12D/GL20; Tyr::CreA/+</sup> mouse, showing leptomeningeal spread of melanoma cells (black arrows). Scale bar, 1 mm. E, photomicrograph showing nuclear Ki67 staining (white arrows) of a representative melanoma from an Nras<sup>G12D/GL20; Tyr::CreA/+</sup> mouse. Note that some cells are melanin laden (black arrowheads). Scale bar, 20 μm. F, photomicrograph showing HMB45/Melan-A staining in a representative melanoma from an Nras<sup>G12D/GL20; Tyr::CreA/+</sup> mouse. Note the predominant membranous staining and presence of intracytoplasmic deposits of melanin in the melanoma cells (black arrowheads). Scale bar, 50 μm. G, Western blot analysis of ppERK and total ERK levels of Nras-mutant melanoma cells following MEK inhibition for 3 hours using PD184352, U0126, and AZD6244. H, in vivo allograft experiment, using Nras-mutant cells from a mouse brain melanoma showing the effect of the MEK inhibitor PD184352 on intradermal tumor growth in C57Bl/6 mice (n = 6 PD184352-treated mice and 9 vehicle-treated mice).
Figure 3. NRAS<sup>G12D</sup> induces melanocytosis in embryonic mice. A, photograph showing a representative whole brain from an Nras<sup>LSL-G12D/LSL-G12D;Tyr::CreA/°</sup> (G12D/G12D) and control mouse at 3 weeks of age. Note the hyperpigmentation of leptomeninges following the gyri (black arrow) and sulci (black arrowhead) and the arboriform pattern over the parietal lobe (white arrow). B, top, photomicrographs of H&E-stained mouse brains of control mice at 1 day and 3 weeks of age showing a single array of nonpigmented leptomeninges lining the cerebral parenchyma (black arrows). Scale bar, 50 μm. Bottom, photomicrographs of H&E-stained mouse brains of Nras<sup>LSL-G12D/LSL-G12D;Tyr::CreA/°</sup> (G12D/G12D) mice at 1 day and 3 weeks of age, showing hyperpigmentation and thickening of the leptomeninges (black arrows). Scale bar, 50 μm (n = 6 mice per experimental group). C, photomicrographs of an HMB45/MelanA-stained mouse brain (arrows indicate individual cells) from an Nras<sup>LSL-G12D/LSL-G12D;Tyr::CreA/°</sup> (G12D/G12D) mouse at 1 day of age. Scale bar, 50 μm. The boxed area in the left photomicrograph is shown at higher magnification in the right. Scale bar, 20 μm.
whereas those in the leptomeninges progressed to become aggressive and invasive primary CNS melanoma.

We were struck by the similarities between the disease in our mice and the disease we observed in 2 cases of primary melanoma of the CNS that occurred in young children. The first case involved a 4-year-old Caucasian boy who presented with somnolence and motor dysfunction. Initial clonic seizures of the right leg led to secondary generalized tonic-clonic seizures followed by paralysis of the right arm and leg that slowly recovered over 24 hours. Magnetic resonance imaging (MRI) revealed a hypertensive, contrast-enhancing lesion in the left parieto-occipital region, following the gyri and sulci. Cerebrospinal fluid (CSF) analysis with cytologic examination and computerized tomography (CT) scans of the thorax and abdomen yielded normal findings, and no pigmented lesions were found on the skin.

Despite treatment with valproic acid, carbamazepine, and phenytoin, the seizures recurred and subsequent MRI revealed extension of the lesion to the right parietal cortex (Fig. 4A). After 6 months, the child developed permanent right hemiparesis and aphasia. The lesion was resected, and despite radiotherapy, the child passed away. Macroscopic examination of the cerebral tissue showed marked thickening and brownish discoloration of the leptomeninges and a zone of intense black discoloration of the underlying cerebral cortex (Fig. 4B). Microscopically, the lesions revealed a proliferation of atypical cells in the leptomeninges and Virchow–Robin spaces, with invasion of the adjacent cerebral cortex (Fig. 4C). The tumor cells were highly mitotic (>10/mm²) and adopted an epithelioid morphology with nuclear pleomorphism (Fig. 4D and E). Tumor cells stained positive for MelanA (Fig. 4F), HMB45, and S100 (Supplementary Fig. SSA and SSB), and the malignant cells in the Virchow–Robin spaces and cerebral cortex contained copious amounts of melanin, whereas cells in the leptomeninges were sparsely pigmented (Fig. 4C and D). These features are consistent with a diagnosis of leptomeningeal melanomatosis, and DNA sequencing did not uncover mutations in NRAS, GNAQ, GNA11, CDKN2A, or TPS3 (data not shown), but did reveal a c.181C>G, p.(Q61R) mutation in NRAS (Fig. 4G).

The second case involved a boy with a giant cutaneous congenital melanocytic nevus over the lumbarosacral region, with multiple satellite congenital nevi over the trunk, face, arms, and upper legs. Biopsies at 2 and 5 years revealed a congenital melanocytic nevus with diffuse infiltration of nevomelanocytes into the dermis and subcutaneous tissue (Fig. 5A–C). Single cells in the dermis splayed the collagen bundles and extended around and within the peridural structures (Fig. 5A, 5C). At age 7, the boy presented with motor dysfunction and paresthesia of the left hand, progressively weakened leg, and accompanying headache and vomiting. MRI revealed a large, heterogeneous, hyperintense tumor in the right frontotemporal area, causing midline shift and compression of the ventricles and mesencephalon (Fig. 5D). The patient quickly deteriorated and passed away despite tumor debulking and supportive treatment with mannitol and dexamethasone. The tumor was composed of highly mitotic (>10/mm²) epithelioid cells with irregular nuclei, prominent nucleoli (Fig. 5E and F), and positive staining for MelanA (Fig. 5G), HMB45, and S100 (Supplementary Fig. S5C and D). Dermatologic examination did not reveal any clinical changes to suggest primary cutaneous melanoma arising over CMN. In the absence of cutaneous melanoma, we diagnosed primary melanoma of the CNS arising in the context of neurocutaneous melanosis. DNA sequencing did not reveal mutations in BRAF, HRAS, GNAQ, GNA11, CDKN2A, or TPS3 (data not shown), but did reveal a c.181C>G, p.(Q61R) mutation in NRAS in the tumor (Fig. 5H) and the congenital nevus (Supplementary Fig. S5E).

**DISCUSSION**

Here we show that oncogenic NRAS induced dose-dependent hyperpigmentation of the skin when expressed in the mature melanocytes of adult mice or the developing melanocytes of embryonic mice. This finding is consistent with previous data showing that NRASQ61K also increased skin pigmentation when expressed in embryonic mouse melanocytes using the tyrosinase promoter, and that oncogenic KRAS induced skin hyperpigmentation when expressed in mature melanocytes from the endogenous Kras gene (30) or from an Actb (β-Actin) promoter fragment (25). We also show that like KRASG12V, when NRASG12D was expressed in mature melanocytes, it induced paucicellular nevi in the deep dermal layers of the skin that resembled human blue nevi. We also show that when NRASG12D was expressed in developing melanocytes, it induced congenital blue nevus–like lesions, complementing a recent report showing that NRASQ61K also induced congenital nevi when expressed using a tyrosinase promoter fragment (34).

We previously reported that when BRAFV600E was expressed in embryonic melanocytes, it disrupted heart and eye development and caused embryonic lethality (31), but here we show that NRASG12D did not induce these effects. The basis of this difference is unclear, but a possible explanation is that BRAFV600E transforms developing melanocytes more readily than does NRASG12D, causing them to disrupt the development of the organs they colonize. Alternatively, perhaps oncogenic NRAS induces melanocyte senescence or apoptosis, so that unlike the BRAFV600E melanocytes, the NRASG12D melanocytes are unable to disrupt the development of their host organs.

Of note, although none of the mice developed cutaneous melanoma when NRASG12D was expressed in the melanocytes of embryonic or mature mice, when NRASG12D was expressed in the melanocytes of the embryos, the mice developed leptomeningeal melanoma that presented as neuronal symptoms at a median of 4 months for homozygous animals and 12.5 months for heterozygous animals. The tumors generally affected the frontoparietal region of the brain and presented as darkly pigmented lesions that followed the sulci and fissures and invaded the normal CNS parenchyma. The tumors were aggressive and had a high proliferative index, and showed evidence of RAS pathway activation, as well as expression of melanocytic, but not neuronal, cell markers.

Cerebral metastases of cutaneous melanomas are usually multifocal, homogeneous, and well-circumscribed nodules, whereas the tumors in our mice arose from areas connected to leptomeningeal melanocytic hyperproliferation. They were firmly adhered to the surface of the dura and diploe, and invaded the CNS in a highly destructive infiltrative pattern.
Figure 4. Diagnosis of leptomeningeal melanomatosis carrying an oncogenic mutation in NRAS in patient 1. A, axial T1-weighted MRI revealing a hyperintense, contrast-enhancing lesion in the left parieto-occipital region following the gyri and sulci. B, photograph showing macroscopic appearance of the cerebral tissue from patient 1. Brownish discoloration of the thickened leptomeninges (L) and black discoloration of the underlying cerebral cortex (C) are evident. C, photomicrograph showing H&E staining of the melanoma revealing the proliferation of hypopigmented cells in the leptomeninges (L) and invasion of hyperpigmented cells (black arrowheads) into the CNS parenchyma (P). Scale bar, 100 μm. D, photomicrograph showing H&E staining of the melanoma. Note the epithelioid morphology of the nonpigmented atypical cells in the leptomeningeal compartment (L) and pigmented tumor cells (white arrows) invading the CNS parenchyma (P). Scale bar, 25 μm. E, photomicrograph showing details of epithelioid morphology of pleomorphic melanoma cells in the leptomeningeal compartment. Scale bar, 50 μm. F, photomicrograph showing MelanA staining of the pigmented melanoma cells (black arrowheads) in the CNS parenchyma (black stars). Scale bar, 50 μm. G, forward sequence of DNA from the primary CNS melanoma from patient 1 showing the presence of an NRAS c.182A>G, p.(Q61R) mutation (arrow).
NRAS-induced prenatal hyperproliferation of the melanocytes were more susceptible than his cutaneous melanocytes to transformation by oncogenic NRAS. Mouse leptomeningeal melanocytes also seem to be more susceptible than cutaneous melanocytes to transformation by oncogenic NRAS. Thus, the evidence that NRASG12D-induced proliferation of both cutaneous and leptomeningeal melanocytes, none of the animals developed cutaneous melanoma, whereas 75% of the heterozygous mice and all of the homozygous mice developed melanoma of the CNS in both cases. Sequencing revealed the presence of an NRAS mutation. Furthermore, one of the children presented with a giant congenital melanocytic nevus that shared the same NRAS mutation as his CNS melanoma. Because this child did not present cutaneous melanoma arising in CMN, it seems that his leptomeningeal melanocytes were more susceptible than his cutaneous melanocytes to transformation by oncogenic NRAS. Mouse leptomeningeal melanocytes also seem to be more susceptible than cutaneous melanocytes to transformation by oncogenic NRAS. Thus, despite the evidence that NRASG12D-induced proliferation of both cutaneous and leptomeningeal melanocytes, none of the animals developed cutaneous melanoma, whereas 75% of the heterozygous mice and all of the homozygous mice developed melanoma of the CNS.

It is unclear why developing leptomeningeal melanocytes are more susceptible than cutaneous melanocytes to transformation by oncogenic NRAS, but it seems unlikely that this is due to differences in the numbers of melanocytes in these 2 sites. More plausibly, it seems likely that although oncogenic NRAS induced prenatal hyperproliferation of the melanocytes arise in the absence of any other primary tumors, and we diagnosed primary melanoma of the CNS.

We were intrigued that although we observed melanoma of the CNS when we expressed NRASG12D off the endogenous Nras gene, when NRASQ61K was expressed using the tyrosinase promoter, it did not induce leptomeningeal melanoma (26). However, as discussed below, the disease in our mice, which was driven by NRASG12D, mimicked the cardinal features of the disease in the children, which was driven by NRASQ61K. We therefore posit that the differences between our mouse model and the mice that were previously reported lies in differences in the pattern, levels, or timing of oncogenic NRAS expression, rather than differences in the biology of NRASG12D or NRASQ61K.

This melanoma model is the first driven by oncogenic RAS expressed using the endogenous gene that did not require additional genetic engineering of the mice, or exposure to carcinogens or tumor promoters. In most previously described models of RAS-driven melanoma, tumor induction was inefficient unless the mice carried a second genetic lesion or were exposed to an environmental stress. For example, HRASG12V-induced melanoma was inefficient unless the mice were exposed to ultraviolet light or the tumor promoter TPA (23, 35, 36). Similarly, deletion of p16INK4A cooperated with HRASG12V and NRASQ61K to induce melanoma (26, 28), and KRAS-driven melanomagenesis was inefficient unless BRAFV600E was also expressed (30) or KRASG12V was strongly overexpressed using the Actb promoter (25). Note, however, that we do not interpret this finding to mean that NRASG12D alone was sufficient to induce leptomeningeal melanomagenesis, and we are currently working to identify the cooperating events, using insertional mutagenesis and genomics.

Importantly, our mice presented the same cardinal clinical features of the disease as those manifested in the 2 children. As in the mice, the children presented with neurologic symptoms, then experienced rapid health deterioration and death, and the cells in their tumors were highly proliferative, invaded the CNS parenchyma, and stained positive for melanoma markers. We did not observe primary melanoma of the skin in either child and therefore diagnosed primary melanomatosis and melanoma or primary malignant melanocytic tumors of the CNS.

No macroscopic or histologic changes were observed in the skin of our mice, and at time of sacrifice, the nevi in the skin retained their paucicellularity, benign architecture, and cytomorphology. We also ruled out primary tumors in the other organs colonized by melanocytes. Thus, the brain melanomas
in both the skin and brains, intrinsic (cell autonomous) or extrinsic (microenvironment) differences between the melanocytes in these 2 sites mean that those in the leptomeninges are more susceptible to transformation. This idea is supported by the observation that one of the children’s tumors had the same NRAS mutation as his giant congenital melanocytic nevus, suggesting a common ancestry, but nevertheless a more susceptible population in the leptomeninges. We anticipate that identifying the events that cooperate with NRAS to transform melanocytes will go some way toward explaining the underlying biology.

Although we have reported 2 cases of childhood melanoma of the CNS that carry NRAS mutations, we note that in adults, melanoma of the CNS is associated with mutations in GNAQ and GNA11, whereas NRAS mutations are rare (17–19). Previous studies have shown that truly congenital cutaneous melanocytic nevi harbor NRAS mutations and patients with these lesions may also have congenital deposits of leptomeningeal melanocytes (20, 21). Furthermore, in humans, and in children in particular, CNS melanoma often arises in patients with giant cutaneous congenital melanocytic nevi (12–16), approximately 80% of which carry somatic mutations in NRAS, but not in BRAF (20, 21). Our data show that leptomeningeal melanocytes are more susceptible to transformation by oncogenic NRAS than are cutaneous melanocytes. We posit that a link exists between prenatal proliferation of melanocytes and early-onset melanoma of the CNS and that acquisition of somatic oncogenic mutations in NRAS in the melanocytes of the CNS is a predisposing risk factor for melanoma of the CNS. We present a mouse model that can be used to study this rare and devastating disease and note that the tumor cells from our mice are susceptible to MEK inhibitors, suggesting a potential therapeutic approach for these patients.

**METHODS**

**Animal Procedures**

All procedures involving animals were approved by the Animal Ethics Committees of the Institute of Cancer Research and the Cancer Research–UK London Research Institute in accordance with National Home Office regulations under the Animals (Scientific Procedures) Act 1986 and according to the guidelines of the Committee of the National Cancer Research Institute (37). Tamoxifen (Sigma-Aldrich T5648) was freshly prepared in 100% ethanol. For allograft experiments, 0.5 × 10⁶ Nras-mutant cells in 0.1 mL PBS were inoculated intradermally into the flanks of female C57Bl/6 mice (Charles River). Mice were treated daily by oral gavage with vehicle (n = 9 mice) or PD184352 (25 mg/kg; n = 6 mice). Tumor volumes were determined using volume = length × width × depth (mm) × 0.5236.

**RNA Extraction and Sequence Analysis of Mouse Tissue**

RNA was extracted from frozen tissues using an RNAsesy Kit (QIA-GEN), and first-strand cDNA was synthesized and DNase treated as previously described (24). Subsequently, cDNA was amplified by PCR and the products were sequenced using dye-terminator chemistry with Nras primers: 5′-ATGACTGAGTACAAACTGGTGGTGG-3′ and 5′-CCATCAATCACCACTTGGCTTTCGTAAG-3′. Sequences were visualized using Sequencer software.

**Tumor DNA Extraction and Sequence Analysis of Human Cases**

Three manually dissected sections of 10 μm formalin-fixed and paraffin-embedded tissue with an estimated tumor cell percentage of at least 80% were used for DNA extraction. DNA extraction and sequence analysis of BRAF, NRAS, Hras, GNAQ, CDKN2A, and TP53 were conducted as previously described (17). The GNAQ gene sequence amplified was gene-specific, as GNAQ pseudogene–specific nucleotides were not detected. Sequence analysis for GNA11 was conducted using M13-tailed GNA11-specific primers: 5′-TGTAAACCGACGGCAGTGGTGGGAGCGTCTGGAGATGC-3′ and 5′-CAGGAAACACACTGACCTCCTGTTGAGACGC-3′. All PCR reactions were conducted in duplicate using 2 independent PCR products for sequence analysis.

**Histology and Immunohistochemistry**

Mouse tumors were formalin fixed and analyzed as previously described (24), and subsequently stained with H&E, Ki-67 (Dako M7249), and HMB45/MelanA (Abcam ab732). All procedures involving the use of human tissue were in accordance with valid standards for this type of investigation in the Netherlands (39). For tumor staining, the following antibodies were used: S100 (Dako 20311), HMB45 (Novocastra NCL-HMB45), and MelanA (Marti clone M2-7C10, Neomarkers M5-716). Sample preparation for S100 staining was conducted without retrieval, whereas sample preparation for HMB45 and MelanA staining was conducted with retrieval (NAcitrate, pH 6.0 and pH 9.0, respectively).

**Immunofluorescence Labeling of FFPE Samples**

Three-millimeter sections of formalin-fixed paraffin-embedded (FFPE) material were used. Slides were dewaxed, and antigen retrieval was conducted using citrate buffer pH 6.0 followed by blocking in PBS-Tween 0.1% + 1% BSA for 15 minutes and overnight incubation with primary antibody (1:100 in PBS + 1% BSA). The following primary antibodies were used: phospho-ERK1/2 (Cell Signaling Technology), GFAP (Dako), HMB45/MelanA and cyclin D1 (Abcam), and S100 (MenaPath). Antibody detection was conducted using Alexa Fluor–conjugated secondary antibodies (Invitrogen). Slides were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Samples were analyzed, and pictures were taken using a Leica SP2 confocal scanning microscope (Leica Microsystems).

**Cell Culture and Western Blotting**

The Nras-mutant tumor cell line was established by collecting murine brain melanoma in sterile PBS on ice and mechanically dissociating tumors in Dulbecco’s modified Eagle medium (DMEM). The cells were continuously cultured in DMEM supplemented with 10% PBS and 10 U/mL penicillin and 100 mg/mL streptomycin. Cells were exposed to the MEK inhibitor PD184352, and cell lysates were prepared as previously described (40). The following primary antibodies were used: anti-phospho-p42/p44 MAPK, and total ERK1/2 (Cell Signaling Technology).

**Disclosure of Potential Conflicts of Interest**

C.J. Springer has ownership interest (including patents) in Rewards to Inventors Scheme (The Institute of Cancer Research). R. Marais has honoraria from speakers bureau from Roche and is a consultant/advisory board member of Servier, Novartis, and GSK. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**

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