Primary melanoma of the CNS in children is driven by congenital expression of oncogenic NRAS in melanocytes.

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ABSTRACT

NRAS mutations are common in human melanoma. To produce a mouse model of NRAS-driven melanoma, we expressed oncogenic NRAS (NRAS<sup>G12D</sup>) in mouse melanocytes. When NRAS<sup>G12D</sup> 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 neurological 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 to primary melanoma of the CNS in children and 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.
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) occur 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 ~5% (3), and 1% (4) of cases respectively, while uveal melanoma accounts for ~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 anatomical sites represent genetically distinct diseases. BRAF and NRAS are mutated in ~45% and ~20% respectively of cutaneous melanomas. By contrast, in acral melanoma, BRAF mutations occur in only ~16% of cases, NRAS mutations are absent or very rare, and KIT mutations occur in ~20% of patients (6, 7). Furthermore, in uveal melanomas BRAF, NRAS and KIT mutations appear to be extremely rare and instead this disease is driven by mutations in GNAQ, GNA11 and BAP1 (8-10). Primary melanoma of the central nervous system (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 (NCM), a rare non-hereditary neurocutaneous syndrome presenting with giant...
(>20cm) and/or multiple congenital melanocytic nevi (CMN) (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 \textit{GNAQ} and \textit{GNA11} (17-19), and in one case, \textit{NRAS} (19). Notably, ~80% of human CMN harbor somatic mutations in \textit{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/\textit{LoxP} technology to express oncogenes in mouse melanocytes (24, 25, 30, 31). In adult mice, \textit{BRAF}^{V600E} induced skin darkening at 2 months, blue nevus-like lesions at 4 months, and melanoma in ~80% of the animals within 2 years (24). In contrast, when \textit{BRAF}^{V600E} was expressed in the melanocytes of developing embryos (congenital expression), it induced developmental abnormalities and embryonic lethality (31).

Here we investigated if oncogenic NRAS could induce melanoma when it was expressed at physiological levels using the endogenous \textit{Nras} gene. We found that expression of \textit{NRAS}^{G12D} in the melanocytes of adult mice induced skin darkening and blue nevus-like lesions, but not cutaneous melanoma. Expression of \textit{NRAS}^{G12D} in the melanocytes of embryonic mice also induced skin darkening and congenital blue nevus-like lesions, but again, it did not induce cutaneous melanoma. However, when it was expressed in congenital nevi, \textit{NRAS}^{G12D} induced melanoma of the CNS and critically, the course of the disease in these mice closely resembled the course of disease in two children who developed melanoma of the CNS driven by oncogenic NRAS. We conclude that acquired somatic mutation in NRAS in the melanocytes of
the leptomeninges is a predisposing risk factor to childhood 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 physiological 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} (LSL) cassette (\textit{Nras\textsuperscript{LSL-G12D}})(32), the removal of which by Cre-recombinase released NRAS\textsuperscript{G12D} expression in a conditional-inducible manner (Supplementary Fig. 1A). We crossed the \textit{Nras\textsuperscript{LSL-G12D}} mice onto mice in which tamoxifen-activated Cre-recombinase (CreERT\textsubscript{2}) was expressed in melanocytes using a tyrosinase enhancer/promoter fragment (\textit{Tyr::CreERT\textsubscript{2}} mice; see Supplementary Fig. 1A)(32, 33). Although CreERT\textsubscript{2} was expressed in the melanocytes of these mice from approximately embryonic day 10.5 (E10.5), it was only activated when the mice are treated with tamoxifen.

We painted tamoxifen onto the shaven skin on the backs of the mice at approximately two months of age to induce NRAS\textsuperscript{G12D} expression. Within 4-8 months, we observed visible darkening of the skin (Supplementary Fig. 1B), in the homozygous (\textit{Nras\textsuperscript{LSL-G12D/LSL-G12D};Tyr::CreERT\textsubscript{2}}) mice and weak darkening of the skin in 50\% of the heterozygous (\textit{Nras\textsuperscript{+/LSL-G12D};Tyr::CreERT\textsubscript{2}}) 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. 1B). Microscopic examination of the skin revealed small paucicellular nevi in the deep dermal and perianexial regions of the skin of the heterozygous mice and larger multicellular nevi in the homozygous mice (Supplementary Fig. 1C). However, despite this clear evidence that NRAS\textsuperscript{G12D} drives melanocyte proliferation, none of the mice developed tumors even after 24-months of expression (Supplementary Fig. 1D).
Next we crossed the $N\text{ras}^{\text{LSL-G12D}}$ mice onto mice expressing unmodified Cre-recombinase from the tyrosinase promoter/enhancer ($\text{Tyr::CreA}$; see Fig. 1A) to induce NRAS$^{G12D}$ expression in the developing melanocytes of embryonic mice. We previously reported that expression of BRAF$^{V600E}$ in embryonic melanocytes caused hydrocephaly, disruption to the development of the eyes and hearts, and embryonic lethality (31). We were therefore intrigued that live-born $N\text{ras}^{+/\text{LSL-G12D}};\text{Tyr::CreA}^{+/+}$ and $N\text{ras}^{\text{LSL-G12D}/\text{LSL-G12D}};\text{Tyr::CreA}^{+/+}$ offspring were obtained at the expected ratio and did not present any signs of hydrocephaly (Fig 1B), or developmental abnormalities of the eyes or hearts (Supplementary Fig. 2). Notably, all mice presented with darkening of the skin, tails, paws and snouts that was evident from day 1, persisted throughout life and was more pronounced in the homozygous than heterozygous mice (Fig. 1B).

These mice also developed benign paucicellular dermal melanocytic lesions reminiscent of human blue nevi (Fig. 1C). Hyperpigmented dendritic melanocytes were visible in 3 week old and adult mice, particularly between the collagen bundles of the reticular dermis and along the adnexae of the deep dermal layers (Fig. 1C). Notably, these hyperpigmented dendritic melanocytic lesions stained positive for HMB45/MelanA and were present in 1 day old mice (Fig. 1D, 1E) confirming that expression of NRAS$^{G12D}$ in embryonic melanocytes induced congenital nevi.

Despite clearly driving melanocyte proliferation in the congenital setting, NRAS$^{G12D}$ did not induce cutaneous melanoma. However, at a median of 4 months for the homozygous animals, and 12.5 months for the heterozygous animals (Fig. 2A), the mice developed neurological symptoms that presented as hyperreactivity to normal stimuli and motor dysfunction, including incoordination and tremor with impaired gait. Most animals developed increasing cranial perimeter or cranial...
deformity and the symptoms progressed rapidly to akathisia and marked general malaise that necessitated sacrifice.

The brains of the affected animals revealed marked darkening of the leptomeninges that followed the sulci and fissures, and was more prominent in the frontoparietal region (Fig. 2B). We observed an increased number of pigmented melanocytes that followed the gyri and sulci and enveloped the ventricles (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 >10 per mm²) and stained positive for Ki67 (Fig. 2E), ppERK and Cyclin D1 (Supplementary Fig. 3A, B). They were melanin-laden (Fig. 2F) and stained positive for HMB45/MelanA (Fig. 2G, Supplementary Fig. 3A, B) and S100 (Supplementary Fig. 3C), but negative for the glial cell marker GFAP (Supplementary Fig. 3D).

To provide evidence that oncogenic NRAS was expressed, we reverse transcribed RNA from the tumors and PCR amplified an Nras fragment across the exon 2/exon 3 boundary. Sequencing of this fragment revealed that NrasG12D was expressed in the tumors, but not the normal brains of the Tyr::CreA/+ littermate controls (Supplementary Fig. 4). 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. 2) 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 MEK inhibitors PD184352, U0126 and AZD6244 (Fig. 2H). Importantly, PD184352 also delayed the
growth of tumor allografts formed by these cells in syngeneic immuno-competent mice (Fig. 2I).

We observed hyperpigmentation of the leptomeninges in the brains of asymptomatic mice. The pigmented cells followed the gyri and sulci and covered the parietal lobe in an arboriform pattern (Fig. 3A). Even in the immediate post-natal period (1 day old mice), the leptomeninges were thickened and hyperpigmented and presented HMB45/MelanA positive cells (Fig. 3B, 3C). Thus, although NRASG12D induced hyperproliferation of melanocytes in both the skin and the CNS, those in the skin developed into congenital cutaneous nevi, whereas those in the leptomeninges progressed to become aggressive and invasive primary CNS melanoma.

We were struck by the similarities in the disease in our mice and those we observed in two 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 that were followed by paralysis of the right arm and leg that slowly recovered over 24 hours. Magnetic resonance imaging (MRI) revealed a hyperintense, contrast-enhancing lesion in the left parieto-occipital region, following the gyri and sulci. Cerebral spinal fluid (CSF) analysis with cytology and CT scans of the thorax and abdomen were normal 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 six months, the child developed permanent right hemiparalysis 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 underlying the 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 per mm²) and adopted an epithelioid morphology with nuclear pleomorphism (Fig. 4D, 4E). Tumor cells stained positive for MelanA (Fig. 4F), HMB45 and S100 (Supplementary Fig. 5A, 5B) 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, 4D). These features are consistent with a diagnosis of leptomeningeal melanomatosis and DNA sequencing did not uncover mutations in \textit{BRAF}, \textit{HRAS}, \textit{GNAQ}, \textit{GNA11}, \textit{CDKN2A} or \textit{TP53} (data not shown), but did reveal a c.182A>G, p.(Q61R) mutation in \textit{NRAS} (Fig. 4G).

The second case involved a boy with a giant cutaneous congenital melanocytic nevus over the lumbrosacral 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 periannexal structures (Fig. 5A, 5C). At age 7 the boy presented with motor dysfunction and paraesthesia of the left hand, progressive weakness of the left 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 per mm²) epithelioid cells with irregular nuclei, prominent nucleoli (Fig. 5E, 5F) and positive staining for MelanA (Fig. 5G), HMB45 and S100 (Supplementary Fig. 5C, 5D). Dermatological 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. DNA sequencing did not reveal mutations in \textit{BRAF}, \textit{HRAS}, \textit{GNAQ}, \textit{GNA11}, \textit{CDKN2A} or \textit{TP53} (data now shown), but did reveal a c.181C>A, p.(Q61K) mutation in \textit{NRAS} in the tumor (Fig. 5H) and the congenital nevus (Supplementary Fig. 5E).
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 is consistent with previous data showing that NRAS\textsuperscript{Q61K} 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 \textit{Kras} gene (30) or from an \textit{Actb} (\textit{\beta-Actin}) promoter fragment (25). We also show that like KRAS\textsuperscript{G12V}, when NRAS\textsuperscript{G12D} 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 NRAS\textsuperscript{G12D} was expressed in developing melanocytes it induced congenital blue nevus-like lesions, complementing a recent report showing that NRAS\textsuperscript{Q61K} also induced congenital nevi when expressed using a tyrosinase promoter fragment (34).

We previously reported that when BRAF\textsuperscript{V600E} was expressed in embryonic melanocytes, it disrupted heart and eye development, and caused embryonic lethality (31), but show here that NRAS\textsuperscript{G12D} did not induce these effects. The basis of this difference is unclear, but a possible explanation is that BRAF\textsuperscript{V600E} transforms developing melanocytes more readily than NRAS\textsuperscript{G12D}, causing them to disrupt the development of the organs that they colonize. Alternatively, perhaps oncogenic NRAS induces melanocyte senescence or apoptosis, so that unlike the BRAF\textsuperscript{V600E} melanocytes, the NRAS\textsuperscript{G12D} melanocytes are unable to disrupt the development of their host organs.
Critically, although none of the mice developed cutaneous melanoma when NRAS\textsuperscript{G12D} was expressed in the melanocytes of embryonic or mature mice, when NRAS\textsuperscript{G12D} 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 the homozygous animals and 12.5 months for the 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, showed evidence of RAS pathway activation, and expression of melanocytic, but not neuronal cell markers.

Cerebral metastases of cutaneous melanomas are usually multifocal, homogenous 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. There were no macroscopic or histological changes 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 arose 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 NRAS\textsuperscript{G12D} off the endogenous \textit{Nras} gene, when NRAS\textsuperscript{G61K} 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 NRAS\textsuperscript{G12D},
mimicked the cardinal features of the disease in the children, which was driven by
NRAS<sub>Q61K/R</sub>. We therefore posit that the differences between our mouse 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 NRAS<sup>G12D</sup> or
NRAS<sup>Q61K</sup>.

This is the first melanoma model 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,
HRAS<sup>G12V</sup>-induced melanoma was inefficient unless the mice were exposed to
ultraviolet (UV) light or the tumor promoter TPA (23, 35, 36). Similarly, deletion of
p16<sup>INK4A</sup> cooperated with HRAS<sup>G12V</sup> and NRAS<sup>Q61K</sup> to induce melanoma (26, 28), and
KRAS driven melanomagenesis was inefficient unless BRAF<sup>D594A</sup> was also expressed
(30), or KRAS<sup>G12V</sup> was strongly over-expressed using the Actb promoter (25). Note
however that we do not interpret this to mean that NRAS<sup>G12D</sup> 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 cardinal clinical features of the disease
presented by two children. As in the mice, the children presented with neurological
symptoms, rapid health deterioration and death, and the cells in their tumors were
highly proliferative, they invaded the CNS parenchyma and they stained positive for
melanoma markers. We did not observe primary melanoma of the skin in either child
and therefore diagnosed primary melanoma of the CNS. Sequencing revealed the
presence of a NRAS mutation. Furthermore, one of the children presented with a giant congenital melanocytic nevus that shared the same NRAS mutation as his CNS melanoma. Since this child did not present cutaneous melanoma arising over CMN, it appears that his leptomeningeal melanocytes were more susceptible than his cutaneous melanocytes to transformation by oncogenic NRAS. Mouse leptomeningeal melanocytes also appear to be more susceptible than cutaneous melanocytes to transformation by oncogenic NRAS. Thus, despite the evidence that NRAS\textsuperscript{G12D} 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 more susceptible than cutaneous melanocytes to transformation by oncogenic NRAS, but it seems unlikely that this is due to differences in the numbers of melanocyte in these two sites. More plausibly, it seems likely that although oncogenic NRAS induced prenatal hyperproliferation of the melanocytes in both the skin and brains, intrinsic (cell autonomous) or extrinsic (microenvironment) differences between the melanocytes in these two sites mean that those in the leptomeninges were more susceptible to transformation. This is supported by the observation that one of the children’s tumors shared 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 the identification of the events that cooperate with NRAS to transform melanocytes will go some way explaining the underlying biology.
Although we have reported two 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 present 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 cutaneous melanocytes. We posit that there is a link 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 to 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 CR-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 genotyping, genomic DNA was prepared from tail biopsies and PCR was performed using the primers previously described (32, 38). 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; UK). 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 RNAeasy kit (QIAGEN) and first-strand cDNA synthesized and DNase treated as previously described (24). Subsequently, cDNA was amplified by PCR and the products were sequenced using dye-terminator chemistry using NRAS primers: 5’-ATGACTGAGTACAAACTGGTGGTGG-3’ and 5’-CCATCAATCACCACTTGCTTTCGGTAAG-3’. Sequences were visualized using Sequencher 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 was performed 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 performed using M13-tailed GNA11 specific primers 5’-TGAAACGACGCCAGTGGGTGGAGCCGCTCTGGATTC-3’ and 5’-CAGGAAACAGCTATGACCCACCTTCGCTGGTCCGAC-3’. All PCR reactions were performed in duplicate using two independent PCR products for sequence analysis.
Histology and immunohistochemistry (IHC). Mouse tumors were formalin-fixed and analyzed as previously described (24), and subsequently stained with hematoxylin and eosin, Ki-67 (Dako M7249) and HMB45/MelanA (Abcam ab732).

All procedures concerning 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 Z0311), HMB45 (Novocastra NCL-HMB45) and MelanA (Mart clone M2-7C10, Neomarkers MS-716). Sample preparation for S100 staining was performed without retrieval while sample preparation for HMB45 and MelanA staining was performed with retrieval (NACitrate pH6.0 and pH9.0 respectively).

Immunofluorescence (IF) labeling of FFPE samples. Three mm sections of formalin-fixed paraffin embedded (FFPE) material were used. Slides were dewaxed and antigen retrieval was performed using citrate buffer pH6.0 followed by blocking in PBS-Tween 0.1% + 1% BSA for 15 minutes and over night incubation with primary antibody (1:100 in PBS+1%BSA). The following primary antibodies were used: a-phospho-ERK1/2 (Cell Signalling), Glial Fibrillary Acidic Protein, GFAP, (Dako), HMB45/MelanA and Cyclin D1 (Abcam), and S100 (Menapath). Antibody detection was performed using AlexaFluor-conjugated secondary antibodies (Invitrogen). Slides were counterstained with DAPI. Samples were analysed and pictures were taken using a Leica SP2 confocal scanning microscope (Leica Microsystems, Milton Keynes, Bucks, UK).

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’s Medium (DMEM). The cells were continuously cultured in DMEM supplemented with 10% fetal bovine serum and 10units/mL penicillin and 100mg/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, β-tubulin (Sigma-Aldrich) and total ERK1/2 (Cell Signalling).
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REFERENCES.

FIGURE LEGENDS.

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 embryonic day ~10.5 (38). NRAS<sup>G12D</sup> was expressed from the endogenous mouse <i>Nras</i> gene using a conditional-inducible targeted allele in which exon 2 is mutated to introduce the G12D mutation (32). The loxP-STOP-loxP 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, <i>Nras</i><sup>+/LSL-G12D;Tyr::CreA/°</sup>(+/G12D), and <i>Nras</i><sup>LSL-G12D/LSL-G12D;Tyr::CreA/°</sup>(G12D/G12D) mice, at 1 day, 3 weeks, and in adulthood.

(C) Upper panels: photomicrographs of H&E stained skin in 1 day old, 3 week old and adult mouse skin. Scalebar = 200 µm. Lower panels: low power photomicrographs of H&E stained skin in 1 day old, 3 week old and adult <i>Nras</i><sup>LSL-G12D/LSL-G12D;Tyr::CreA/°</sup>(G12D/G12D) mice. Hyperpigmented dendritic melanocytes are visible at low magnification in the 3 week old and adult mice. Scalebar = 200 µm. n=6 mice / experimental group.

(D) High power photomicrographs of H&E stained skin from boxed areas in the lower panel from (C) in 1 day old, 3 week old and adult <i>Nras</i><sup>LSL-G12D/LSL-G12D;Tyr::CreA/°</sup>(G12D/G12D) 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 day 1 old mice, but are prominent in 3 week old and adult mice. Scalebar = 20 µm.

(E) Photomicrographs of HMB45/MelanA stained skin from a 1 day old <i>Nras</i><sup>LSL-G12D/LSL-G12D;Tyr::CreA/°</sup>(G12D/G12D) mouse, demonstrating the presence of melanocytes (black arrows). The area boxed in the left hand panel is enlarged in the right hand panel. Scale bars = 200 µm (left panel) and 20 µm (right panel).

Figure 2. Nras<sup>G12D</sup> induces CNS tumors in mice.

(A) Kaplan-Meier plot showing survival in months (m) of study mice. The experimental groups consisted of <i>Nras</i><sup>+/LSL-G12D;Tyr::CreA/°</sup>(+/G12D; n=33) and <i>Nras</i><sup>LSL-G12D/LSL-G12D;Tyr::CreA/°</sup>(G12D/G12D; n=23) mice. The control groups consisted of <i>Tyr::CreA/°</i> (n=22), <i>Nras</i><sup>+/LSL-G12D</sup>(n=13) and <i>Nras</i><sup>LSL-G12D/LSL-G12D</sup>(n=15) mice.
(B) Photographs showing representative whole brain and sagital sections of the brains of control, Nras<sup>LSL-G12D;Tyr::CreA*/+</sup> (+/G12D) and Nras<sup>LSL-G12D;Tyr::CreA*/G12D/G12D</sup> mice.

(C) Photomicrograph of an H&E stained brain section from a Nras<sup>LSL-G12D;Tyr::CreA*/</sup> mouse (boxed area in the left image), displaying proliferation of pigmented melanocytes in the leptomeninges along the external surface of the brain parenchyma (black arrow). Scalebar = 100μm.

(D) Photomicrograph of an H&E stained frontal lobe melanoma from an Nras<sup>LSL-G12D;Tyr::CreA*/</sup> mouse, showing leptomeningeal spread of melanoma cells (black arrows). Scalebar = 1mm.

(E) Photomicrograph showing nuclear Ki67 staining (white arrows) of a representative melanoma from a Nras<sup>LSL-G12D/LSL-G12D;Tyr::CreA*/</sup> mouse (boxed area in upper left image). Note that some cells are melanin-laden (black arrowheads). Scalebar = 20μm.

(F) High power photomicrograph of boxed area in upper left image of an H&E stained melanoma with atypical, epithelioid cells. Tumor cells frequently presented intracytoplasmic melanin (black arrowheads). Scalebar = 25μm.

(G) Photomicrograph showing HMB45/MelanA staining in a representative melanoma from a Nras<sup>LSL-G12D/LSL-G12D;Tyr::CreA*/</sup> mouse. Note the predominant membranous staining and presence of intracytoplasmic deposits of melanin in the melanoma cells (black arrowheads). Scalebar = 50μm.

(H) Western blot analysis of ppERK and total ERK levels of Nras mutant melanoma cells following MEK inhibition for 3h using PD184352, U0126 and AZD6244.

(I) 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) Upper panels: photomicrographs of H&E stained mouse brains of control mice at 1 day and 3 weeks of age showing a single array of non-pigmented leptomeninges lining the cerebral parenchyma (black arrows). Scalebar = 50μm. Lower panel: 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). Scalebar = 50μm. n = 6 mice / experimental group.

(C) Photomicrographs of an HMB45/MelanA stained mouse brain (arrows indicate individual cells) from an NrasLSL-G12D/LSL-G12D;Tyr::CreA/+ (G12D/G12D) mouse at 1 day of age. Scalebar = 50μm. The boxed area in the left panel is shown in higher magnification in the right panel.

Figure 4. Diagnosis of leptomeningal 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). Scalebar = 100μm.

(D) Photomicrograph showing H&E staining of the melanoma. Note the epithelioid morphology of the non-pigmented atypical cells in the leptomeningeal compartment (L) and pigmented tumor cells (white arrows) invading the CNS parenchyma (P). Scalebar = 25μm.

(E) Photomicrograph showing detail of epithelioid morphology of pleomorphic melanoma cells in the leptomeningeal compartment. Scalebar = 50μm.

(F) Photomicrograph showing MelanA staining of the pigmented melanoma cells (black arrowheads) in the CNS parenchyma (black stars). Scalebar = 50μm.

(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).

Figure 5. Diagnosis of NRAS mutated leptomeningal melanomatosis in patient 2.

(A) Photomicrograph showing H&E staining of full-thickness skin from the congenital melanocytic nevus (CMN). Scalebar = 0.5mm.

(B) Photomicrograph of the CMN showing detailed H&E staining of melanocytic nests (arrows) in the papillary dermis and single melanocytes in the reticular dermis (arrowhead). Scalebar = 300μm.
(C) Photomicrograph of the CMN showing detailed H&E staining of the deep dermal melanocytes in the collagen and along the hair follicle (arrows). Scalebar = 300μm.

(D) T1-weighted magnetic resonance image (MRI) with contrast, revealing a large tumor in the right frontotemporal region with meningeal attachment.

(E) H&E staining of the CNS melanoma showing epithelioid tumor cells with low pigment content. Scalebar = 50μm.

(F) H&E staining of the CNS melanoma. Note the atypical tumor cells with irregular nuclei and frequent nucleoli. Scalebar = 30μm.

(G) Photomicrograph showing cytoplasmic brown chromogen MelanA staining in melanoma cells (black arrowhead) invading brain parenchyma (black star) of patient 2. Scalebar = 50μm.

(H) Forward sequence of DNA from the primary CNS melanoma from patient 2 showing the presence of an NRAS c.181C>A, p.(Q61K) mutation (arrow).
FIGURE 1

A

Congenital NRAS<sup>G12D</sup> expression in melanocytes

B

C

D

E

H&E

H&E

H&E

H&E

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FIGURE 2

A

![Graph showing survival over time for different genotypes: Controls, +/G12D, and G12D/G12D.]

B

Control

+/G12D

G12D/G12D

C

![Image of H&E staining showing tissue sections.]

D

![Image of Ki-67 staining highlighting proliferating cells.]

E

![Image of HMB45/MelanA staining indicating melanoma cells.]

F

![Image of H&E staining showing tissue morphology.]

G

![Image of HMB45/MelanA staining indicating melanoma cells.]

H

![Image of ppERK and ERK1/2 expression levels across different treatments.]

I

![Graph showing tumor volume over days with Vehicle and PD164352 treatments.]

**Analysis**

- **P-value**: p=0.00006***

**Research.**

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Figure 4
FIGURE 5

A

B

C

D

E

F

G

H

T

A

G

Q/K

E

E

Y

A

C

A

G

T

G

A

Y

A

A

G

A

G

A

G

T

A

C

H&E

H&E

H&E

H&E

MelanA
Primary melanoma of the CNS in children is driven by congenital expression of oncogenic NRAS in melanocytes.

Malin Pedersen, Heidi V.N. Küsters-Vandevelde, Amaya Viros, et al.

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