Acute Inhibition of MEK Suppresses Congenital Melanocytic Nevus Syndrome in a Murine Model Driven by Activated NRAS and Wnt Signaling

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Congenital melanocytic nevus (CMN) syndrome is the association of pigmented melanocytic nevi with extra-cutaneous features, classically melanocytic cells within the central nervous system, most frequently caused by a mutation of NRAS codon 61. This condition is currently untreatable and carries a significant risk of melanoma within the skin, brain, or leptomeninges. We have previously proposed a key role for Wnt signaling in the formation of melanocytic nevi, suggesting that activated Wnt signaling may be synergistic with activated NRAS in the pathogenesis of CMN syndrome. Some familial pre-disposition suggests a germ-line contribution to CMN syndrome, as does variability of neurological phenotypes in individuals with similar cutaneous phenotypes. Accordingly, we performed exome sequencing of germ-line DNA from patients with CMN to reveal rare or undescribed Wnt-signaling alterations. A murine model harboring activated NRASQ61K and Wnt signaling in melanocytes exhibited striking features of CMN syndrome, in particular neurological involvement. In the first model of treatment for this condition, these congenital, and previously assumed permanent, features were profoundly suppressed by acute post-natal treatment with a MEK inhibitor. These data suggest that activated NRAS and aberrant Wnt signaling conspire to drive CMN syndrome. Post-natal MEK inhibition is a potential candidate therapy for patients with this debilitating condition.

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INTRODUCTION

Congenital melanocytic nevus (CMN) syndrome (formerly known as neurocutaneous melanosis) is the involvement of typically large pigmented melanocytic nevi with extra-cutaneous features, such as certain facial characteristics and melanotic cells within the central nervous system (CNS; Barkovich et al., 1994; Kinsler et al., 2008; Kinsler et al., 2012). Pigmented hairy nevi can cover up to 80% of the body surface area, and neurological abnormalities can lead to intractable seizures and neurodevelopmental delay. There is also a significant risk of primary melanoma within the skin, brain, or leptomeninges (Krengel et al., 2006; Kinsler et al., 2009). Previous work has shown that CMN syndrome is caused, in roughly 80% of cases, by a single post-zygotic mutation of NRAS codon 61 in a neuro-ectodermal precursor (Kinsler et al., 2013b). However, some familial pre-disposition also suggests a germ-line contribution to the development of CMN (Danarti et al., 2003; Kinsler et al., 2009; de Wijn et al., 2010). There is currently no treatment for this condition, and where melanoma develops the outcome is almost universally fatal. Despite recent advances in treatment of some types of melanoma with targeted therapies, a cure for melanoma harboring NRAS mutation remains particularly elusive (Shtivelman et al., 2014). Therefore, a potential treatment for the cutaneous and extra-cutaneous features of CMN syndrome is most likely prior to its acquisition of additional genetic alterations en route to melanoma.

In both CMN and acquired melanocytic nevi, nevus cells are thought to be maintained in a proliferation arrested state
by cellular senescence (Michaloglou et al., 2005; Gray-Schopfer et al., 2006; Suram et al., 2012), a tumor-suppressive, stable proliferation arrest triggered by activated oncoproteins and other molecular stresses (Salama et al., 2014). A complex network of effectors enacts the senescence response, including DNA damage signaling, activation of the pRb and p53 pathways and, notably, repression of Wnt signaling (Ye et al., 2007; Pawlikowski et al., 2013; Juan et al., 2014; Salama et al., 2014). Paradoxically, however, we and others have shown that nevus melanocytes, including in CMN, often exhibit markers of activated Wnt signaling (Bergman et al., 1997; King et al., 2001; Ramirez et al., 2005; Kinsler et al., 2013a; Pawlikowski et al., 2013), and activated Wnt signaling is able to bypass or delay senescence in mouse models (Delmas et al., 2007; Pawlikowski et al., 2013; Juan et al., 2014). We therefore reasoned that activated Wnt signaling, in addition to the NRAS mutation, might influence the varied phenotype of CMN syndrome.

RESULTS

Altered Wnt signaling in human CMN syndrome

To more directly assess the status of Wnt signaling in human CMN, we stained such nevi for markers of activated Wnt signaling. As shown previously (Kinsler et al., 2013a), CMN expressed nuclear and cytoplasmic localized β-catenin. In addition, these nevi consistently expressed cyclin D1 and c-MYC (Figure 1) indicative of activated Wnt signaling and in contrast to normal epidermal melanocytes that do not express nuclear cyclin D1 and c-myc (Pawlikowski et al., 2013). We conclude that human CMN are associated with activated Wnt signaling.

A higher prevalence of CMN within some families suggests a germ-line predisposition to CMN (Danarti et al., 2003; Kinsler et al., 2009; de Wijn et al., 2010), and neurological phenotypic variability between patients with similar cutaneous phenotypes also supports a role for a modifying germline genotype. Accordingly, we performed whole-exome next-generation sequencing on DNA from peripheral white blood cells of 32 children with extensive CMN (phenotypic variability). One hundred and nine variants in 26 genes were included in the analyses. Genetic variation in FZD1 was seen in six CMN patients and therefore considered a possible undescribed SNP (pending confirmation of its prevalence by direct screening of a large control population). These 19 variants seen in 14 CMN patients are listed in Table 1. This list includes variants in the APC, APC2, FZD6, TCF3, and WNT16 genes that are absent from control population databases. Although functional characterization of each individual variant is beyond the scope of this study, this analysis is supportive for a role for germ-line alterations of Wnt signaling, together with somatic activation of NRAS, in the development of extensive CMN.

Activation of Wnt signaling and NRAS recapitulates CMN syndrome in a mouse model

On the basis of this finding, we asked whether combined activation of NRAS and Wnt signaling in the melanocytic lineage could drive formation of CMN syndrome in an animal model. We therefore generated mice with either activated NRAS (Tyr-NRasQ61K (Ackermann et al., 2005) or activated Wnt signaling alone (Tyr-Cre Apcfl/fl (Shibata et al., 1997)) or both activated NRAS and Wnt signaling (Tyr-Cre Apcfl/fl/Tyr-NRasQ61K (Pawlikowski et al., 2013)), all under the control of the mouse Tyr promoter to restrict altered signaling largely to the melanocytic lineage (Delmas et al., 2003). These mice were then examined for features characteristic of CMN syndrome in humans, including skin hyperpigmentation and excessive infiltration of pigmented melanocytes into the leptomeninges and brain (Supplementary Figure S1 online). As reported previously, Tyr-NRasQ61K mice

Figure 1. Human congenital melanocytic nevi harbor activated Wnt signaling. Serial sections of a CMN stained for melan A, β-catenin, cyclin D1, and c-myc. Images are representative of at least four congenital human nevi (Scale bar = 25 μm).
### Table 1. Details of 19 rare germline variants found in 14 CMN patients on whole-exome sequencing

| Gene symbol | Position (Hg19) | Reference allele/Sample allele | Protein variants | Translation impact | Predicted functional impact: SIFT function prediction | Conservation phyloP p-value | dbSNP ID | Frequency in control population: 1,000 Genomes/CG public genomes/NHLBI ESP |
|-------------|----------------|-------------------------------|-----------------|-------------------|------------------------------------------------------|-----------------------------|----------|--------------------------------------------------|
| APC         | 5:112176056    | C/T                           | p.R1571C; p.R1589C | Missense          | Damaging (0.05, possibly damaging)                   | 0.005152                    | —        | —/0.01                                           |
| APC2        | 19:1453017     | C/T                           | p.A6V           | Missense          | Damaging (0.05), probably damaging                   | 1.83E-05                    | rs200897976 | 0.19/—/—/0.02                                   |
| APC2        | 19:1465820     | 5′-GGCCAA GGCCAAAGGCC AAG-3′   | p.K846_L847insAK | In-frame          | —                                                    | —                          | —        | —/0.01                                           |
| EP300       | 22:41569633    | A/G                           | p.K1542E        | Missense          | —, Possibly damaging                                 | 3.52E-04                    | rs374162524 | —/—/0.01                                        |
| FZD1        | 7:90894459     | ACC/ACCACC                    | p.P989_P90insP  | In-frame          | —                                                    | —                          | —        | —/0.01                                           |
| FZD6        | 8:104341919    | AT/---                        | p.C527*; p.C495* | Frameshift        | —                                                    | —                          | —        | —/0.01                                           |
| HNF1A       | 12:121426790   | G/A                           | p.A161T         | Missense          | Damaging (0.03), probably damaging                   | 1.32E-04                    | rs201095611 | —/—/0.01                                        |
| LRP1        | 12:57577915    | C/T                           | p.R1993W        | Missense          | Damaging (0), probably damaging                      | rs141826184                 | 0.05/—/0.22 | —/0.11/0.11                                     |
| LRP1        | 12:57602881    | A/T                           | p.Y4054F        | Missense          | Damaging (0.02), probably damaging                   | rs79435985                  | 0.96/—/0  | —/0.11/0.11                                     |
| MAP4K1      | 19:39108034    | G/A                           | p.R70W          | Missense          | Damaging (0), probably damaging                      | rs35079766                  | 0.11/—/0.11 | —/0.11/0.11                                     |
| PPP2R1B     | 11:111608216   | T/A                           | p.N623Y; p.N559Y | Missense          | Damaging (0.01), possibly damaging                   | rs61756429                  | 0.35/0.92/0.76 | —/—/0.06                                       |
| PPP2R1B     | 11:111631738   | AC/---                        | p.V115fs*3; p.V51fs*3 | Frameshift        | —                                                    | rs20159112                  | 0.01/—/0  | —/0.05/0.04                                     |
| PPP2R3B     | X:308383       | C/T                           | p.A186T         | Missense          | Damaging (0.04), possibly damaging                   | rs201733691                 | 0.01/—/0  | —/0.04/0                                         |
| RARB        | 3:25636151     | C/T                           | p.R266C; p.R378C | Missense          | Damaging (0), probably damaging                      | rs201359112                 | 0.01/—/0  | —/0.04/0                                         |
| SOX8        | 16:1034833     | A/T                           | p.N2631         | Missense          | Damaging (0.01), possibly damaging                   | rs20159112                  | 0.01/—/0  | —/0.04/0                                         |
| TCF3        | 19:1621908     | GAGGAG/GAG                     | p.S295del       | In-frame          | —                                                    | rs201232229                 | 0.06/—/0  | —/0.06/0                                         |
| TCF7        | 5:133480486    | A/G                           |                 |                   | —                                                    | rs201733691                 | —/—/0.04 | —/0.04/0                                         |
| WNT16       | 7:120965470    | CCA                           | p.M16fs*141     | Frameshift        | —                                                    | —                          | —        | —/0.01                                           |
| WNT9B       | 17:44950095    | G/A                           | p.R97H          | Missense          | Damaging (0), probably damaging                      | rs201232229                 | 0.06/—/0  | —/0.06/0                                         |

Abbreviation: APC, adenomatous polyposis coli.

Whole-exome sequencing was performed on peripheral blood leukocyte DNA from children with CMN. Data were analyzed to identify rare pathogenic variants (<1% of the population in published control data sets) in genes in the APC/Wnt-signaling pathway.
exhibited an excess of melanocytes in the dermis and became hyperpigmented with melanin within a few days of birth, compared with either wild-type mice or Tyr-Cre Apc<sup>fl/fl</sup> mice (Ackermann et al., 2005; Pawlikowski et al., 2013). Strikingly, activation of Wnt-signaling in Tyr-Cre Apc<sup>fl/fl</sup>/Tyr-NRas<sup>Q61K</sup> mice greatly exacerbated the NRas<sup>Q61K</sup>-induced proliferative expansion of melanocytes and skin melanization ((Supplementary Figure S2 online) and previously quantitated in (Pawlikowski et al., 2013)), comparable to the cutaneous features of extensive CMN (Supplementary Figure S1A online). In addition, on post mortem, Tyr-Cre Apc<sup>fl/fl</sup>/Tyr-NRas<sup>Q61K</sup> mice, but neither Tyr-Cre Apc<sup>B/B</sup> nor Tyr-NRas<sup>Q61K</sup> mice, were found to have neurological features characteristic of human CMN syndrome, including hyperpigmented spines (Figure 2a and Supplementary Figure S1B online) and the pathognomonic leptomeningeal melanosis around the spinal cord (Figure 2b and c). In the head, the mice showed melanosis and thickening of the leptomeninges, as well as melanosis in olfactory bulbs and nasal turbinates (Figure 2d). Invasion of melanin-producing cells around the spinal cord and into the brain was also observed by MRI (Figure 2e and f and Supplementary Figure S1C, S1D and Supplementary Video S1 online), highly reminiscent of the malignant progression seen in patients with CMN complicated by melanoma (Rhodes et al., 1991; Kinsler et al., 2008). The abnormal infiltrating pigment-producing cells in the mice were confirmed as melanocytes based on morphology, as well as expression of the neural crest cell marker S100 (Supplementary Figure S3 online).

**Features of CMN syndrome are blocked through post-natal MEK inhibition**

As proliferative expansion of the melanocyte population in the Tyr-Cre Apc<sup>B/B</sup>/Tyr-NRas<sup>Q61K</sup> mice was dependent on...
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Dual activation of NRAS and Wnt signaling, we reasoned that inhibition of NRAS signaling alone should be sufficient to inhibit proliferation of these cells. To test this in vitro, we prepared cutaneous melanocytes from Tyr-Cre Apcfl/fl/Tyr-NRasQ61K mice. These genetically altered cells were highly pigmented and showed a dendritic morphology, characteristic of melanocytes (Figure 3a). To test the influence of reducing activity of the MAPK/ERK pathway, we treated Tyr-Cre Apcfl/fl/Tyr-NRasQ61K mouse melanocytes in vitro with the allosteric MEK inhibitor, AZD6244 (selumetinib, ARRY-142886; Yeh et al., 2007). Upon MEK inhibition, proliferation was significantly reduced, as measured by a decrease in Edu incorporation and suppression of cyclin A expression (Figure 3b-d).

A massive expansion of melanocytes in the skin of Tyr-Cre Apcfl/fl/Tyr-NRasQ61K mice occurs predominantly within the first 2 weeks of life (Pawlikowski et al., 2013). Therefore, we reasoned that treatment of such neonatal mice with a MEK inhibitor over the first 2 weeks of life could potentially decrease melanocytic proliferation and suppress features of CMN syndrome. Mice were treated three times weekly by intraperitoneal injection with AZD6244 or DMSO vehicle for 2 weeks after birth (Figure 4a). PhosphoERK staining in the liver was markedly decreased, confirming that this regimen effectively inhibited MEK–ERK signaling in the mice (Supplementary Figure S4 online). Treatment with AZD6244 greatly reduced the population of melanocytes in the skin dermis (Figure 4b) compared to vehicle and, crucially, significantly decreased numbers of melanocytes in the CNS (Figure 4c and d). Indicative of at least partial sustained benefit from drug treatment, acute post-natal treatment with AZD6244 over 2 weeks, followed by withdrawal from therapy, resulted in a significant decrease in pigmentation within the CNS 4 weeks after the end of drug treatment (Figure 5).

DISCUSSION

Here we present several lines of evidence to indicate that, in at least a subset of cases of human CMN syndrome, the phenotype is likely to be driven by concerted aberration of NRAS and Wnt signaling. First, by whole-exome sequencing we identified a number of sequence variants in genes of the Wnt signaling pathway in DNA of peripheral white blood cells. Several of these variants are to our knowledge previously unreported, and those described are detected only at extremely low frequency in published databases. All are predicted to impair the function of the encoded protein, where in silico prediction is possible. Given the complexity of the Wnt signaling pathway, comprising positive and negative regulators and antagonistic interactions between canonical Wnt/β-catenin signaling and the non-canonical Wnt pathway (Niehrs, 2012), it is difficult to confidently ascertain whether these variants activate or inhibit Wnt/β-catenin signaling, without extensive additional functional studies. At this time, these sequence variants are good candidates for heritable, germ-line variants that alter activity of the Wnt signaling pathway, which we postulate may have a synergistic effect in the presence of a somatic NRAS mutation. Second, we observed that human CMN exhibit markers of activated Wnt signaling, suggesting that the effect of activated Wnt is not only important in the congenital development of the disease but may be involved in the post-natal persistence of the nevus. Third, in a mouse model that to our knowledge is previously unreported, we showed that melanocyte-specific activated NRASQ61K and activated Wnt signaling, through inactivation of APC, cooperate to generate features of CMN syndrome, most notably a massive excess of melanocytes in the skin and infiltration of melanocytes into the leptomeninges and CNS. Finally, post-natal systemic treatment with a MEK inhibitor, an inhibitor of NRAS signaling, reversed both the cutaneous and neurological features of this mouse model.

Previous reports have described other genetically modified mice exhibiting phenotypes that partially resemble CMN syndrome. Merlino and coworkers showed that constitutive expression of Scatter factor/hepatocyte growth factor under control of the mouse metallothionein 1 promoter induced melanosis in the CNS and hyperpigmentation in the skin (Takayama et al., 1996). Although we cannot exclude a role for Scatter factor/hepatocyte growth factor signaling in CMN syndrome, this model does not recapitulate the most common hallmark of human CMN syndrome, NRAS codon 61 mutation (Kinsler et al., 2013b). Several groups...
have previously reported hyperpigmentation in the skin of mice expressing NRasQ61K in the melanocyte lineage (Ackermann et al., 2005; Delmas et al., 2007; Shakhova et al., 2012). However, consistent with our observations, none of these groups reported any neurological involvement. One of these studies found that a stabilized β-catenin allele cooperated with NRasQ61K to promote melanoma (Delmas et al., 2007). However, these melanomas were proposed to arise from the hair follicle bulb or the bulge region in the skin. As discussed previously (Pawlikowski et al., 2013), we have not observed any frank melanoma in Tyr-Cre Apcfl/fl/Tyr-NRasQ61K mice, likely because the behavioral and neurological abnormalities associated with CNS infiltration, themselves characteristic of CMN syndrome (Barkovich et al., 1994; Kinsler et al., 2009; Kinsler et al., 2008), preclude keeping any live mice beyond 4 months for ethical reasons.

Marais and coworkers reported in a murine model that expression of NRASG12D in the melanocyte lineage induced melanocyte proliferation and congenital melanocytic skin lesions and also primary melanoma of the CNS of variable latency but high penetrance (Pedersen et al., 2013). Human CMN syndrome, however, is typically characterized by mutation of NRAS at codon 61, not codon 12 (Kinsler et al., 2008), although an uncommon variant of CMN is rarely associated with codon 13 mutations (Kinsler et al., 2014), with a single case report of brain involvement in a patient with p.G13R (Shih et al., 2014). In addition, the most common congenital CNS finding is melanosis in the parenchyma or the leptomeninges, with intracerebral primary...
melanoma being a more rare and acquired phenomenon. Thus, the Tyr-Cre Apcfl/fl/Tyr-NRasQ61K mice reported here appear, to date, to be the best combined genetic and phenotypic recapitulation of human CMN syndrome.

The idea that CMN syndrome results from activated NRAS and Wnt signaling extends the model previously proposed for human acquired melanocytic nevi. Specifically, their formation is thought to depend on activated NRAS or BRAF signaling, due to oncogenic mutation of NRAS or BRAF (Omholt et al., 2002; Pollock et al., 2003), and also depends on input from the Wnt signaling pathway (Pawlowski et al., 2013). Normal human melanocytes are exposed to activated Wnt signaling as they migrate from the neural crest to the dermis (Ikeya et al., 1997; Dorsky et al., 1998; Dunn et al., 2000), and this might be the source of Wnt signaling for benign nevogenesis (Pawlowski et al., 2013), either congenital in utero or acquired, which typically occurs in young children (Bataille et al., 2007; Zalaudek et al., 2011). In some cases of CMN syndrome, we propose that somatic NRAS mutation cooperates with a higher level of sustained Wnt signaling resulting from germ-line genetic variants or other activators of the Wnt pathway to drive a much more severe melanocytic expansion. This germ-line modifier could provide a possible explanation for the unexplained observation of the presence or absence of neurological involvement in unrelated CMN syndrome individuals with the same cutaneous phenotype—a clinical problem that leads to all children with multiple CMN having MRI screening of the CNS in the first few months of life (Waelchli et al. 2015).

Alterations of Wnt signaling in pediatric pathologies are not exclusive to CMN syndrome. For instance, more than half of all pediatric Wilms’ tumor (the fourth most common childhood malignancy) have been reported to show Wnt activation (Su et al., 2008). In the mouse, activation of KRAS and stabilization of β-catenin, specifically in the developing kidney, cause metastatic renal epithelial tumors to develop that mimic the epithelial component of Wilms’ tumor (Clark et al., 2011). As in CMN syndrome, the molecular basis of Wnt signaling activation driving this disease is often unclear (Su et al., 2008). With a greater understanding of the cross-talk between RAS and Wnt signaling in benign and advanced disease, we may better decipher the inner workings of multiple childhood malignancies.

An understanding of the genetic basis of CMN syndrome is a likely first step to therapeutic interventions. On the basis of our previous finding that CMN syndrome is caused by somatic NRAS mutation (Kinsler et al., 2013b), a recent report administered an oral MEK inhibitor to a 13-year-old boy with CMN syndrome in the very advanced stages of leptomeningeal melanoma (Kusters-Vandevelde et al., 2014). Despite biomarker responses to the drug, the child passed away within days, as would be expected from the natural history of this malignancy at this stage. As onset of melanoma in CMN is known to require further key mutations affecting proliferative pathways (Kinsler et al., 2013b), intervention before progression to malignancy is likely to be the key to success. In support of further testing of this approach, here we report that acute post-natal inhibition of MEK–ERK signaling downstream of NRASQ61K does substantially rescue the key features of CMN syndrome in this mouse model, specifically the massive expansion of melanocytes in the skin, brain, and leptomeninges. These results suggest that MEK inhibitors, currently Food and Drug Administration–approved or being trialed for use in melanoma (Sullivan and Flaherty, 2013), might also benefit patients with CMN syndrome. Importantly, although the cure of advanced melanoma through application of targeted therapies, such as MEK and BRAFV600E inhibitors (Shitvelman et al., 2014), has ultimately been frustrated often by acquisition of drug resistance, conceivably this might not be a confounding factor in CMN syndrome with its many fewer genetic alterations.

**MATERIALS AND METHODS**

**Human tissues**

Congenital melanocytic nevi were obtained under an Institutional Review Board approved protocol by a board-certified dermatopathologist (HWM) from Fox Chase Cancer Center, Philadelphia. These tissues were fixed in 10% (vol/vol) buffered formalin for 1–3 days and embedded in paraffin, following routine histology procedure.

For whole-exome sequencing, peripheral blood leukocyte DNA was extracted from samples from 32 children with CMN attending a specialist clinic at Great Ormond St Hospital, London. Written consent was obtained in all cases, and the study was approved by the Great Ormond Street Hospital/UCL Institute of Child Health Research Ethics Committee. Detailed phenotyping of the cutaneous and neurological signs of the participants is shown in Supplementary Table S1 online, demonstrating that this group is at the severe end of the phenotypic spectrum of individuals with CMN.

**Immunoblotting**

Cells were lysed in 1× Laemmeli sample buffer, and 30–50 μg of protein was resolved by SDS-polyacrylamide gel electrophoresis followed by transfer onto PVDF membrane and probing with antibodies. Antibodies used include the following: anti-cyclin A (SC-754, Santa Cruz Biotechnology, Dallas, TX), anti-β-actin (A1978, Sigma, St. Louis, MO), p44/42 MAPK (Erk1/2, 4695, Cell Signaling, Danvers, MA) and phospho-p44/42 MAPK (Erk1/2, Thr202/Tyr204, 9101, Cell Signaling), anti-rabbit IgG, horseradish peroxidase-linked (NA934, GE Healthcare, Fairfield, CT), and anti-mouse IgG, horse-radish peroxidase-linked (P0447, Dako, Carpinteria, CA).

**Cell proliferation assays, immunofluorescence**

The Click-iT EdU Alexa Fluor 594 Imaging Kit (C10339, Life Technologies, Carlsbad, CA) was used according to the manufacturer’s instructions to monitor cellular proliferation.

**Immunohistochemistry**

Briefly, formalin fixed, paraffin embedded sections were deparaffinised, rehydrated, blocked for endogenous peroxidases, and underwent antigen retrieval according to antibody specifications. Tissues

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were incubated overnight with the following primary antibodies: Anti-human melan-a clone A103 (Dako, M7196), S100 (Dako, Z0311), β-catenin (610154, BD Transduction Laboratories, San Jose, CA), CyclinD1 clone EP12 (Dako, M3642), c-myc clone 9E10, and phospho-p44/42 MAPK (Erk1/2; Cell Signaling, 4695). Samples were then counterstained with haematoxylin, dehydrated, and coverslipped.

**Genetically modified mice**

Tyr-NRasC61K mice and Apcfl mice were mated to Tyr-Cre mice (Shibata et al., 1997; Delmas et al., 2003; Ackermann et al., 2005). Progenies from these crosses were then interbred to obtain Tyr-Cre Apcfl/Tyr-NRasC61K and were maintained on a C57BL6 background. Mice were kept in conventional animal facilities, monitored frequently, and experiments were carried out in compliance with UK Home Office guidelines at the Beatson Institute for Cancer Research mouse facility (Home Office PCD 60/2.607) under project license 60/4,079. Mice were genotyped by Transnetyx. Mice were first treated at their day of birth or at 1 day of age with AZD6244 (10 mg kg$^{-1}$) or DMSO vehicle via intraperitoneal injection and then three times per week for 2 weeks. Mice were euthanized following a Schedule 1 method, and tissues were collected for histological analyses.

**Whole-exome sequencing**

Whole-exome sequencing was performed in two batches of 23 and 10 samples. For the first batch, a total of 3 μg of DNA was used to prepare a DNA library using Covaris DNA sonication in the skin melanocyte lineage. *Genesis* (2003) 36:73–80

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**CONFLICT OF INTEREST**

The authors state no conflict of interest.

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**Author Contributions**

JSP, majority of experiments and manuscript preparation. S-CC, statistical analysis. VAK, patient phenotyping and recruitment, sample collection, and exome sequencing analysis. LA-O, Sanger DNA sequencing. SP, immunohistochemistry on brain sections. EC, library prep for exome sequencing. CB, CN, FM, WL, WMH, and JMM, technical assistance. AK and KB, mouse pathology and advice. HW, human tissue and human dermatopathology, PDA, VK, and AR, supervision and manuscript preparation.

**SUPPLEMENTARY MATERIAL**

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid
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