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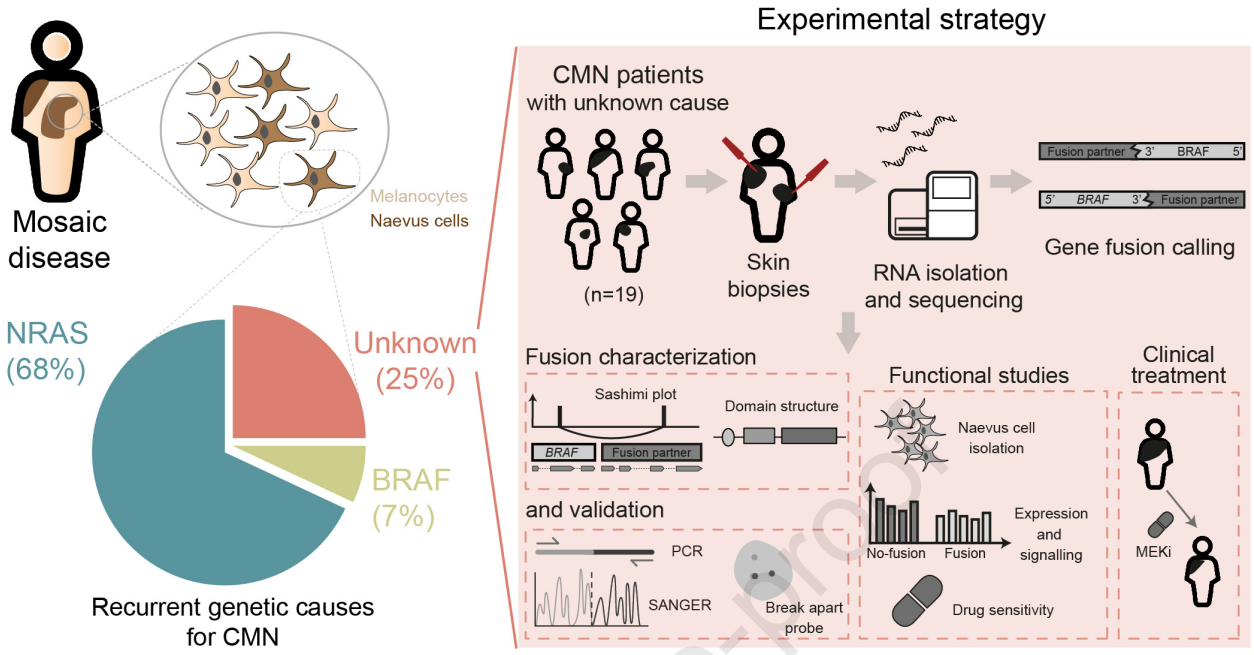
Congenital Melanocytic Naevi (CMN)



TITLE

Mosaic *BRAF* fusions are a recurrent cause of congenital melanocytic naevi targetable by MEK inhibition

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ABSTRACT

Among children with multiple congenital melanocytic naevi (CMN), 25% have no established genetic cause, of which many develop a hyperproliferative and severely pruritic phenotype resistant to treatment. Gene fusions have been reported in individual cases of CMN. Here, we study 169 CMN patients, 38 of whom were double wild-type for *NRAS/BRAF* mutations. Nineteen of these 38 patients had sufficient tissue to undergo RNAseq, which revealed mosaic *BRAF* fusions in 11/19 patients and mosaic *RAF1* fusions in 1/19. Recurrently, fusions involved the loss of the 5' regulatory domain of *BRAF* or *RAF1* but preserved the kinase domain. We validated all cases and detected the fusions in two separate naevi in 5/12 patients,

confirming clonality. The absence of the fusion in blood in 8/12 patients indicated mosaicism. Primary culture of *BRAF*-fusion naevus cells from 3/12 patients demonstrated highly increased MAPK activation, despite only mildly increased *BRAF* expression, suggesting additional mechanisms of kinase activation. Trametinib quenched MAPK hyperactivation *in vitro* and treatment of two patients caused rapid improvement in bulk tissue, improving bodily movement, and reducing inflammation and severe pruritus. These findings offer a genetic diagnosis to an additional group of patients and trametinib as a treatment option for the severe associated phenotypes.

INTRODUCTION

Congenital melanocytic naevi (CMN) are moles present from birth, termed CMN syndrome when associated with other features. Known recurrent causes of CMN are mosaic heterozygous missense mutations in *NRAS* (Kinsler et al., 2013) or *BRAF* (Etchevers et al., 2018), at 68% and 7% frequencies respectively in the largest prospective study (Polubothu et al., 2019), with the remaining 25% unknown. The condition is thus monogenic but mosaic, with the causative mutation occurring to a single cell during embryonic or fetal development**.** The highly variable severity of the phenotype is likely related to the timing of the mutation and the multipotency of the mutated cell amongst many other potential factors (reviewed in (Kinsler et al., 2019)), with earlier mutations in general thought to lead to more severe disease affecting more tissue types. Thus far the causative clonal mosaic genotype has not been linked to disease severity, in so much that there has been no differences between *NRAS* and *BRAF* missense mutations or the unknown group in incidence of associated neurological abnormalities, or incidence of melanoma in childhood although numbers for melanoma are small (Polubothu et al., 2019). There have been however early indications that the genotype may be related to the behavioural phenotype of the skin lesions, with *BRAF*^{V600E}-CMN more likely than *NRAS*-CMN to present

with multiple benign nodules (Polubothu et al., 2019, Salgado et al., 2015). In addition, the unknown genotype (WT) group appeared to us to contain some of the most proliferative and symptomatic cutaneous phenotypes. We have termed this phenotype "hyperproliferative", as defined as recurrently developing distinct nodular or widespread proliferative areas within the CMN in the post-natal period. In addition, these areas are typically clinically inflamed (erythematous, warm), often hairless, and usually highly pruritic.

Gene fusions have previously been reported in a small number of cases of CMN, and in two cases have been demonstrated in more than one naevus from the same patient. This demonstration of clonality within a patient helps to define likely causality in the context of the multiple non-causative somatic mutations that can be detected in skin naevi. The first description was of two patients with translocations involving *BRAF* in a single sample each (Dessars et al., 2007), followed by single cases of likely causative *RAF1* and *ALK* fusions in two samples from each patient (Martins da Silva et al., 2018), and single cases with single samples of *RAF1, BRAF* (two cases) and *RASGRF2* fusions (Baltres et al., 2019, Houlier et al., 2021, Mir et al., 2019, Molho-Pessach et al., 2022). As to the pre-causal somatic mutational origins of CMN, recent data suggest a contribution from mismatch repair in some patients (Boxuan et al., 2023). Over the last 15 years we have collected a cohort of patients with CMN for in-depth phenotypic and genotypic studies and undertook whole transcriptome RNAseq on 19/169 who were wildtype for *NRAS* and *BRAF* missense mutations and for whom we still had sufficient tissue. We were particularly interested in learning more about this group of patients as the common occurrence of post-natal proliferation and intractable pruritus is classically resistant to treatments.

RESULTS

Mosaic *BRAF* fusions are a recurrent cause of multiple CMN

A total of 15 different mosaic gene fusions were identified in CMN tissue samples from 12/19 patients (7% of the total 169 patient cohort): 13 fusions involving *BRAF* in 11 patients and two involving *RAF1* in one patient (**Fig.1a,b; Table S1**). The *BRAF* fusions identified consisted of both inter- (11/13) and intra-chromosomal rearrangements (2/13), while both *RAF1* fusions were intra-chromosomal (2/2) (**Fig.1c**). All patients but one (10/11) presented at least one *BRAF* fusion consisting of the 5' regulatory region of the partner gene fused to the 3' portion of *BRAF*, which encodes for the tyrosine kinase domain (5'partner-3*'BRAF*). Within those ten patients, two (patient 3 and 10) had an additional *BRAF* fusion in the opposite direction (5'BRAF-3' partner) involving the same (patient 10) or a different partner gene (patient 3). In the one remaining patient (patient 11) the only identifiable fusion was 5' *BRAF* fused to the 3' partner gene (5'*BRAF*-3'partner) (**Fig.1a**). For the single *RAF1* patient (patient 12), we identified two fusions involving the same partner gene, one in each orientation (**Fig.1b**). Examples of sashimi plots showing the spanning and junction reads supporting the rearrangements are shown in **Fig.1d,e** and **Fig. S1.**

Mosaic *BRAF* fusions have varied but some recurrent breakpoints

The location of the breakpoints within *BRAF* varied between fusions, although a breakpoint at the start of exon 9 was recurrent and the most common (8/13 fusions) (**Fig.1a, Table S1)**. For *RAF1*, two different breakpoints were found in the two fusions (**Fig.1b, Table S1**). Assessment of break points in the fusion genes did not implicate segmental duplications or SINE/LINE involvement in most cases as assessed by RepeatMasker (Kent et al., 2002) (**Fig.S2**).

Mosaic *BRAF* fusions have multiple partner genes which contain predicted dimerisation domains

Ten different partner genes were identified (*AGAP3, AKAP9,* EEA1, GOLGA4, *LCA5, MIER3, PHIP, QKI, SEC31A, STRN3).* Of those only *EEA1* and *GOLGA4* were recurrent partners (**Fig.1a,b**). The functional domains contributed by each partner include the promoter regions, which would be predicted to drive expression of the *BRAF/RAF1* kinases. A diverse mix of other domains are predicted *in silico* in partner genes, in particular dimerisation domains in 10/15 fusions (**Fig.1a, b**).

Mosaic *BRAF* fusions are associated with the hyperproliferative CMN phenotype

Phenotypic description of the 19 patients included in this study is detailed in **Table S1**. The presence of a *BRAF/RAF1* fusion is significantly associated with a hyperproliferative phenotype ($p<0.001$)(**Fig.2a,b,c**) observed in 8/12 patients (66%) compared with 6/119 patients (5%) from the *NRAS* mutant cohort. Other factors to note in *BRAF/RAF1* fusion patients are the chronic intractable pruritus interfering with everyday life, in 8/12, and the frequent requirement for surgical intervention for debulking of the tissue overgrowth and its associated pruritus in 6/12.

***BRAF*-fusion CMN exhibit similar histological features to *BRAF*-fusion acquired naevi**

Tissues sections were available for review for 8/12 fusion patients. Multiple blocks were reviewed from the same patient when available (4/8). In total, 25 different blocks were reviewed from 8 patients (**Table S2**). Key defining features identified in this cohort were desmoplasia and fibrosis in 6 patients (cords in whorled fibrosis in 6/6 cases and buckshot fibrosis and cords in whorled fibrosis in 1/6 (**Fig.2 d,e**)). This has been previously reported in acquired *BRAF*-fusion melanoctyic tumors (Perron et al., 2018). Some cases exhibited small melanocytes (n=4/8) whilst some cases also exhibited a more spitzoid cytomorphology (n=3/8) (**Fig.2 f**). One patient showed evidence of pagetoid melanocytes.

Mosaic gene fusions validate by alternative methods and clonality is confirmed within patients

Fusions were validated by PCR and Sanger sequencing of patient CMN tissue cDNA using fusion specific primers (**Fig.3a,b and Table S1**). All RNAseq-detected fusions were confirmed (**Fig.S3**). In all patients where samples were available from more than one physically distinct naevi (5/12) the same fusion was in addition validated in each sample from the same patient (**Fig.3a**), demonstrating clonality and likely disease causality. Blood samples were available for eight patients, in which absence of the fusion was demonstrated by an absence of amplification by PCR (**Fig.3a**), as is the pattern for mosaicism in CMN of other genotypes.

As a further validation method, we stained patient derived naevus cells (from patients 1,2 and 3) with a *BRAF* break-apart probe (**Fig.3c**). The absence of colocalization of the two probes surrounding the genomic region of *BRAF* demonstrates the presence of a rearrangement involving *BRAF* in the three cell lines (arrowheads in **Fig.3c**). No rearrangement is present in the melanocyte control cell line (Hermes-1) as seen by colocalization of the probes.

***BRAF* fusions are associated with increased *BRAF* expression and hyperactivation of the MAP-kinase pathway**

Considering that most of the *BRAF* fusions identified involved loss of the autoinhibitory domain of *BRAF*, likely leaving the control of its expression to the partner gene (**Fig.1a, b**), we sought to investigate whether the baseline levels of expression of *BRAF* were altered by the fusion events. Assessing expression levels from the RNAseq was not thought to be accurate. The reasons for this are twofold: firstly, the gene fusions are mosaic, only present in naevus cells and not in other cell types in affected skin biopsy, whereas the bulk RNAseq data was

from whole skin biopsies. Differences in expression due to the fusion may therefore be lost within the bulk tissue. Secondly, only the spanning reads on RNAseq capture the fusion transcript, whereas junctional reads end at breakpoints, and cannot be definitely attributed to the fusion. Expression analyses were therefore performed in the three primary cell lines derived from patients 1,2,3 (sample details listed in **Table S1**). *BRAF* expression was significantly increased in fusion patient cell lines compared to a control melanocyte cell line (Hermes-1) (**Fig.4a**) but to a similar degree as cell lines derived from patients harbouring the *NRAS* p.(Q61K) mutation. In contrast, all three *BRAF*-fusion cell lines showed markedly increased levels of MAP-kinase signalling activation compared to controls and the same *NRAS*-missense cell lines (**Fig.4b**).

BRAF fusion cell lines are highly sensitive to trametinib treatment

Taking advantage of the three patient cell lines isolated in this study we were able to assess their sensitivity to a MEK inhibitor (Trametinib) treatment *in vitro* before translating its use to the clinic. Patient cell line proliferation was significantly sensitive to trametinib treatment, in a similar way as the control and the *NRAS* p.(Q61K) cell lines (**Fig.5a, b**). Most importantly, the decreased proliferation was accompanied by a significant reduction in MAPK signalling activation as measured by phosphorylation of ERK (**Fig.5c**).

CMN patient hyperproliferative phenotype responds rapidly to oral MEKi treatment

On the basis of preliminary data, Great Ormond St Hospital Drug and Therapeutics Committee approval was granted to trial trametinib in two patients with severe mosaic *BRAF*-fusion CMN. The first patient (who was not part of the original study), a three-year old boy, was referred to our department with a known *EVI5-BRAF* fusion. This patient exemplified the hyperproliferative and severely pruritic phenotype with a very bulky main CMN in a bathing

trunk distribution, including affecting the genital area. The weight of the main CMN was considered to be impairing his gross motor development, including his ability to stand up from a sitting position. Sleep was being impaired by severe pruritis. Recurrent cutaneous infections within the main CMN were arising due to the chronic inflammatory and hairless desmoplastic appearance of the surface of the lesion coupled with excoriations. Neurodevelopment was otherwise normal. The patient was started on trametinib 0.025mg/kg/day given as 0.5mg every other day. Within four weeks there had been a visible reduction in CMN bulk, a reduction in erythema, and a reduction in pruritus. Within twelve weeks there had been further visible and continued symptomatic improvement (**Fig.5d**), a reduction in overall body weight of 1kg (equivalent to 6.6%) **(Fig.S4)**, and clear improvement in gross motor ability. The only adverse effect seen during this time was a rise in creatine kinase (CK), higher than baseline but only just out of the normal range and stable between weeks four and eight, and resolving by week 12. This rise in CK is recognised as a side effect of trametinib and we have previously reported similar in the context of this drug in CMN syndrome where melanoma has arisen (Kinsler et al., 2017). Patient 2, a five year old girl with *QKI-BRAF* fusion had a bulky, nodular CMN in the bathing trunk area with severe pruritis refractory to treatment with anti-histamines and topical corticosteroids, but no obvious effects on motor development. She was commenced on trametinib at a dose 0.025mg/kg/day equating to 0.5mg on alternate days. Within one week her pruritis was reported to have completely resolved and within four weeks she had a visible reduction in tissue CMN bulk and underlying erythema (**Fig.5d**). Again, there was a reduction in body weight noted at one month of treatment with an increase in height over the same period of 2.5cm **(Fig.S4)**. The only adverse effect was a slight increase in liver transaminases at four weeks which is under review.

DISCUSSION

The finding of mosaic gene fusion events as a recurrent cause of the CMN phenotype described here may suggest that mosaic gene fusions could be considered as a mechanism of disease in other congenital mosaic disorders which are yet unexplained. We have provided a genotype to a further 7% (12/169) of patients with CMN in our cohort, and the functional exploration of the ensuing pathobiology has offered the rationale for targeted therapeutic intervention. Gene discovery in the field of mosaics therefore continues to break ground in disease biology and to drive treatment for these severe conditions.

Detection using whole genome RNAseq was relatively challenging at bioinformatics level due to the mosaic nature of the disease together with a poor concordance between callers, a situation we are familiar with from detection of mosaic missense mutations by DNA NGS. Where naevus cell culture is possible, we would recommend the use of diagnostic break-apart probes as a relatively rapid method for detection, although this method is agnostic for the partner gene and does not give detailed information on breakpoints.

BRAF fusions are a well-described although relatively rare driver in different solid tumours, most commonly melanoma at approximately 3% (Botton et al., 2013, Forbes et al., 2015, Hutchinson et al., 2013, Ross et al., 2016). The fusions found here follow the same pattern as previously described, particularly as regards to the multiplicity of partner genes, and the presence of dimerisation domains within those partner genes (Botton et al., 2013). *BRAF* fusions in melanoma are seen twice as commonly in females than in males, and this too has been mirrored in this small cohort of 11 patients (8 females). Given the parallel in a congenital disease, this sex difference is likely to reflect something fundamental about the mechanisms underlying fusion generation rather than an environmental influence.

One patient had the same *RAF1* fusions in two CMN samples demonstrating clonality, with two others cases previously described in the literature, one clonal (Martins da Silva et al., 2018) and one from more than one area of the same CMN which had developed a rhabdomyosarcoma

(Baltres et al., 2019). Taken together these data likely support *RAF1* fusions as a recurrent cause of CMN. The patient with the *RAF1* fusion in this study does not have a hyperproliferative phenotype.

Given the recurrence of *BRAF* and *RAF1* in the gene fusions, these kinases are clearly key to the development of the naevus phenotype in these cases. However, a role or roles for the partner genes is also at least potentially contributory, particularly perhaps for the post-natal behaviour where a few remain stable but most become highly proliferative and pruritic. Expression levels of *BRAF* in *BRAF*-fusion naevus cells in culture were not substantially higher than in those with *NRAS* mutations. Simply increased levels of expression driven by a more highly expressed partner gene is therefore not the whole story. Other than dimerisation driving kinase activation, there could be other mechanisms by which the partner genes are involved in pathology, such as the spatiotemporal expression of the fusion proteins.

We have shown a statistically significant association between *BRAF* fusion patients and a hyperproliferative phenotype however it is important to note the small total number in the cohort, so this remains to be confirmed in larger cohorts.

The pruritus in these cases is unresponsive to all non-targeted topical and oral medications we have tried so far. Alternative treatment for those patients is therefore highly desirable. Previous *in vitro* data from six melanoma cell lines harbouring *BRAF* fusions demonstrated responsiveness to MEK inhibition (Botton et al., 2019), and two cases of *BRAF*-fusion in single samples of CMN treated with oral trametinib demonstrated reduction in the bulk and pruritus of the main lesion (Mir et al., 2019, Molho-Pessach et al., 2022). Our findings *in vitro* demonstrate high sensitivity of *BRAF*-fusion patient naevus cells to trametinib, over and above that of *NRAS*-missense cells, and that this sensitivity is due to quenching of MAPK hyperactivation. Our subsequent clinical data from two patients described here demonstrates substantial and rapid clinical benefit from the first four-twelve weeks of oral trametinib,

without clinically-relevant side effects. Importantly however, this only treats the post-natal hyperproliferation, and not the underlying congenital naevus, a similar situation to the tumour-specific effects of MEK inhibition seen in the treatment of melanoma in patients with CMN.

In conclusion mosaic gene fusions are an important disease mechanism and mosaic *BRAF* fusions and *RAF1* fusions are a recurrent cause of CMN. Exploration of the biological effects of these fusions has demonstrated hyperactivation of the MAPK pathway over and above that of *NRAS*-missense CMN, by as yet unknown mechanisms which could include dimerisation of partner gene products. This translates clinically into a hyperproliferative and highly pruritic phenotype in most cases, which has been rapidly sensitive to oral trametinib administration in our trial patients. These studies have given a further 7% of patients a causative genotype and helped open the door to targeted therapies in this particularly severe phenotype.

MATERIALS AND METHODS

Patient recruitment and sample collection

All children with CMN seen in the paediatric dermatology department of a tertiary referral centre between January 2015 and October 2020 were offered participation in a genotyping study, and written informed consent was obtained from their parents/guardians under the local NHS Research Ethics Committee (London Bloomsbury). No specific selection was done based on the phenotypic characteristics (cohort details are provided in Supplementary Materials and Methods). CMN tissue was obtained either during routine surgery or by a single 4-mm punch skin biopsy for genotyping for *NRAS* and *BRAF* mutational 'hotspots', and/or genotyping from archival formalin-fixed paraffin-embedded (FFPE) tissue as previously described (Polubothu et al., 2019). Parents/guardians consented to the publication of patient images.

RNA sequencing

Total RNA was extracted from CMN tissue of the 19 patients (sample details are listed in **Table S1**), using the RNeasy Fibrous Tissue Mini Kit (Qiagen 74704) according to the manufacturer's protocol. RNA integrity was assessed using a Bioanalyser (Agilent). Library preparation using KAPA mRNA HyperPrep Kit with RiboErase (Roche) using 80ng of total RNA and sequenced using a HiSeq (Illumina, San Diego, US), with a 150-bp paired-end run at ~40 million reads per lane giving a total of ~120 million (pairs of) reads per sample. Details of the alignment and bioinformatic analysis are available in Supplementary Materials and Methods.

Histology

Haematoxylin and eosin stained FFPE tissue sections from all available samples from each *BRAF* fusion patient were reviewed by an independent expert histopathologist. Findings were reviewed in the context of recently published features of *BRAF*-fusion acquired melanocytic naevi, and in the context of the well-known histological features of *NRAS*-mosaic CMN (Yeh et al., 2023).

Naevus cell isolation and culture

Skin biopsies were collected from patients, as described in the sample collection section (sample details are listed in **Table S1),** and transported fresh in a saline soaked gauze to the laboratory within two hours. Detailed culturing and media preparation protocol is provided in Supplementary Materials and Methods.

Break-apart probe staining

BRAF break-apart probe was purchased from Empire Genomics (BRAFBA-20-ORGR). Patient cell lines (from patient 1, 2 and 3) were seeded, as detailed in Supplementary Materials

and methods, and staining was performed following the probe manufacturer's protocol. Representative images form n=30 cells were taken with Zeiss Axio Imager M1.

Gene expression and Western analyses

Patient-derived naevus cells (from patients 1, 2 and 3) were seeded in 6-well plates at $0.5x10^6$. 24h later, RNA and protein was extracted from cell lysates to perform gene expression and pathway activation (Western) analyses respectively. A full detailed protocol is available in Supplementary Materials and Methods.

In vitro drug treatment

For proliferation studies patient-derived naevus cells (from patients 1,2 and 3) were seeded and treated 24 hours later with increasing concentrations of trametinib (12.5, 25, 50, 100nM), while only one concentration (12.5nM) was used for pathway activation analyses (Western). A full detailed protocol is available in Supplementary Materials and Methods.

Patient Treatment

Two patients with *BRAF*-fusion CMN were recruited for treatment with trametinib following approval from Great Ormond Street Hospital Drugs & Therapeutics Committee. Treatment dosing and monitoring schedule was as previously described (Kinsler et al., 2017).

DATA AVAILABILITY STATEMENT

Raw RNAseq data supporting the findings presented in this manuscript is available in ArrayExpress (https://www.ebi.ac.uk/biostudies/arrayexpress) under the accession number E-MTAB-13182

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS STATEMENT

Conceptualization: VK; Data curation: SB, SP; Formal analysis: SB, AB, AP, GK, SH; Funding acquisition: VK; Investigation: SB, AB, SP, IY; Methodology: SB, DB, AS; Project administration: DZ, MR, FM; Resources: NMN, PLB, AS, NK and NB; Supervision: VK; Validation: SB, MP; Visualization: SB, MP, AB; Writing-original manuscript: SB, SP, AB and VK.

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FIGURE LEGENDS

Figure 1 – ***BRAF/RAF1* fusions identified in CMN patients**. Schematic illustration of the identified *BRAF* **(a)** and *RAF1* **(b)** fusions showing the wide range of fusion partners detected. Most fusions consist of the loss of the regulatory domain but retention of the *BRAF/RAF1* kinase domain. Recurrent *BRAF* breakpoints were identified in exon 9 (dotted red line) and relevant protein domains were identified using InterProScan. Asterisks highlight patients with more than one fusion. **c)** Circos plot representation of *BRAF* and *RAF1* fusions identified by RNAseq. Sashimi plots showing **d)** the single inter-chromosomal rearrangement of *BRAF* with the partner gene *QKI* found in patient 2 and **e)** the complex complementary intra-chromosomal rearrangement of *RAF1* with *GOLGA4* found in patient 12.

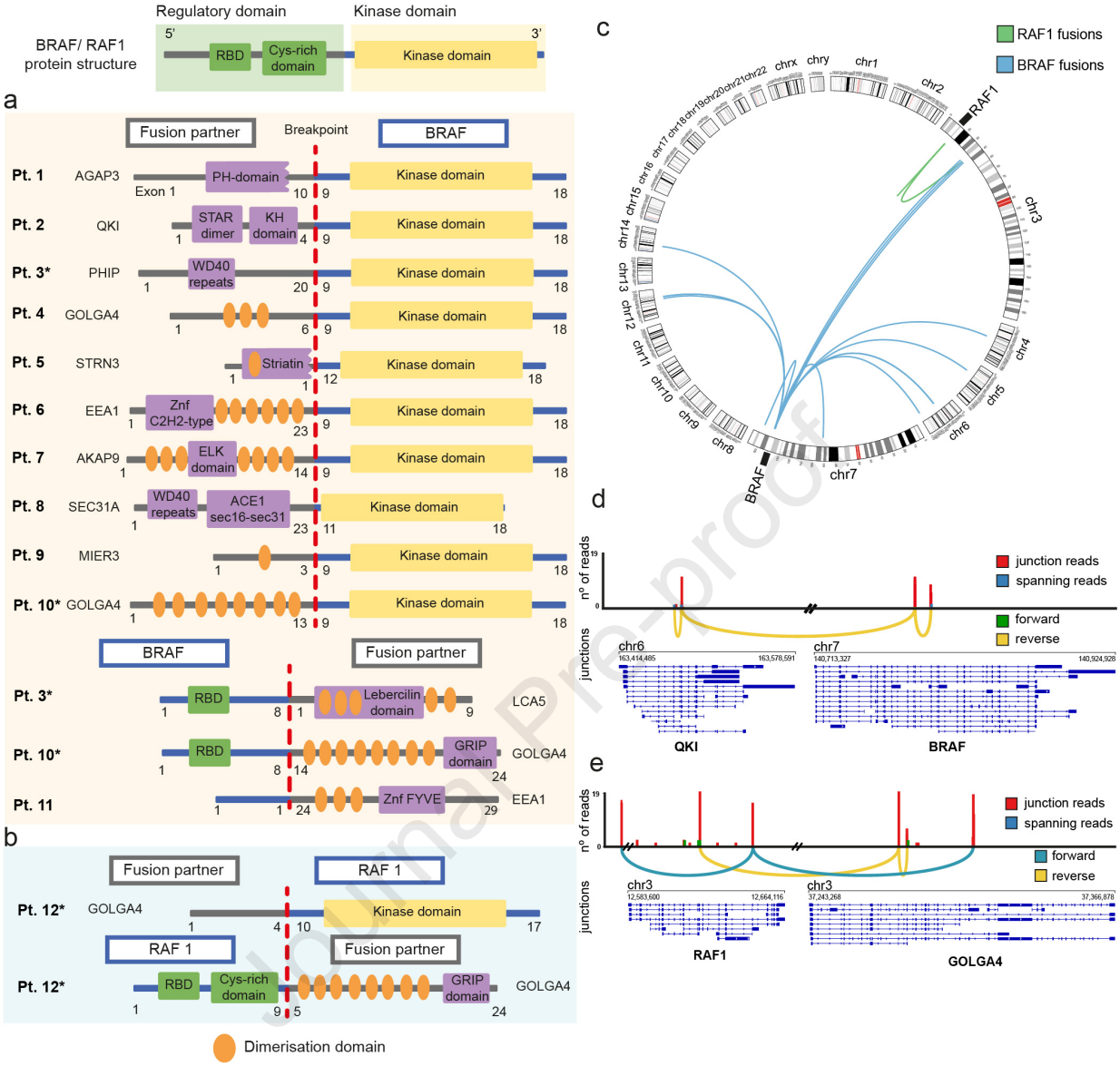
Figure 2 – **Clinical and histological features of CMN patients harbouring *BRAF* fusions**. **a)** Patient with hyperproliferative and multinodular phenotype, with excoriations demonstrating evidence of the chronic pruritus. **b)** Patient with more diffusely bulky and progressive hyperproliferation, also chronically pruritic. **c)** Patient with hyperproliferative and multinodular phenotype on the scalp, also chronically pruritic. **d)** Nevus with adjacent proliferative nodule area with slightly epithelioid melanocytes. **e)** and **f)** CMN demonstrating storiform fibrosis with high degree of cellularity. Parents/guardians consented to the publication of patient images. Scale bar= 500 μm in **d/e** and 100 μm in **f**.

Figure 3 –All ***BRAF/RAF1* fusions were validated by additional methods**. **a)** Image of an agarose gel showing the PCR amplification of *QKI-BRAF* fusion transcript and the control Tubulin in cDNA from patient 2 blood, two different CMN lesions (main CMN and nodular area) and primary nevus cells. The fusion transcript was detected in the two lesions plus nevus cells but absent in blood. **b)** Sanger sequencing showing the breakpoint junction between *QKI* and *BRAF* (lower case and uppercase nucleotides distinguish between *QKI* and *BRAF* fragment respectively) . **c)** Fluorescence in situ hybridization using a *BRAF* break-apart probe demonstrating the presence of the *BRAF* rearrangement in three fusion patient cell lines (arrowheads) compared to the control cell line. Scale bar = 10 μm.

Figure 4 – **Increased *BRAF* expression and MAPK pathway activation in cell lines derived from *BRAF* fusion patients a)** Graph representing the significant increase in *BRAF* expression detected in fusion patient cell lines compared to control cell lines. **b)** A significantly higher basal activation of the MAPK pathway was observed in fusion cell lines, detected by Western blot, compared to control cell lines. Only a representative blot, of the six

independent ones performed to assess statistical differences, is shown. All statistical comparisons were performed by two-tailed unpaired t-test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$)

Figure 5 – **In vitro and in vivo response to Trametinib (MEK inhibitor). a)** Control, *NRAS* mutant and *BRAF* fusion cells lines were treated with increasing concentrations of Trametinib (12.5, 25, 50,100nM) and proliferation rates were assessed by EdU staining. **b)** A significant decrease in proliferation was observed in all cell lines starting with the lowest trametinib concentration (12.5 nM) onwards. **c)** Significant reduction on MAPK activation levels after trametinib treatment (12.5 nM) in the three *BRAF* fusions cells lines. **d)** Clinical images of two patients before and after treatment with trametinib (0.025mg/kg/day given as 0.5mg every other day) reveal an improvement with visible reduction in CMN bulk, a reduction in erythema, and a reduction in pruritus. All graphs represent an average of three independent experiments and statistical comparisons performed by two-tailed unpaired t-test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$). Scale bar = 100 μm.


BRAF/ RAF1
protein structure
Regulatory domain
Kinase domain
5'
3'
RBD
Cys-rich domain
Kinase domain
a
Fusion partner
Breakpoint
BRAF
Pt. 1 AGAP3 Exon 1 PH-domain
Pt. 2 QKI STAR dimer KH domain
Pt. 3* PHIP WD40 repeats
Pt. 4 GOLGA4
Pt. 5 STRN3 Striatin
Pt. 6 EEA1 Znf C2H2-type
Pt. 7 AKAP9 ELK domain
Pt. 8 SEC31A WD40 repeats ACE1 sec16-sec31
Pt. 9 MIER3
Pt. 10* GOLGA4
Kinase domain
BRAF
Fusion partner
Pt. 3* RBD Lebercilin domain LCA5
Pt. 10* RBD GRIP domain GOLGA4
Pt. 11 Znf FYVE EEA1
b
Fusion partner
RAF 1
Pt. 12* GOLGA4
Kinase domain
RAF 1
Fusion partner
Pt. 12* RBD Cys-rich domain GRIP domain GOLGA4
Dimerisation domain
c
RAF1 fusions
BRAF fusions
d
junction reads
spanning reads
forward
reverse
nº of reads
junctions
chr6
chr7
QKI
BRAF
e
junction reads
spanning reads
forward
reverse
nº of reads
junctions
chr3
chr3
RAF1
GOLGA4