Combined activation of MAP kinase pathway and β-catenin signaling cause deep penetrating nevi

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Deep penetrating nevus (DPN) is characterized by enlarged, pigmented melanocytes that extend through the dermis. DPN can be difficult to distinguish from melanoma but rarely displays aggressive biological behavior. Here, we identify a combination of mutations of the β-catenin and mitogen-activated protein kinase pathways as characteristic of DPN. Mutations of the β-catenin pathway change the phenotype of a common nevus with BRAF mutation into that of DPN, with increased pigmentation, cell volume and nuclear cyclin D1 levels. Our results suggest that constitutive β-catenin pathway activation promotes tumorigenesis by overriding dependencies on the microenvironment that constrain proliferation of common nevi. In melanoma that arose from DPN we find additional oncogenic alterations. We identify DPN as an intermediate stage in the step-wise progression from nevus to melanoma. In summary, we delineate specific genetic alterations and their sequential order, information that can assist in the diagnostic classification and grading of these distinctive neoplasms.
Common nevi are benign melanocytic neoplasms located superficially in the skin. Nevus cells become smaller and less pigmented as their distance from the surface epithelium increases. This characteristic is termed ‘maturation’ and is an important diagnostic feature to distinguish benign nevi from melanoma.1, 2. In 1989 Helwig and colleagues described a neoplasm – deep penetrating nevus (DPN) – as an exception to this rule in which the constituent melanocytes maintain their cell size and pigmentation throughout the dermis.3 Before their initial description, these microscopic characteristics resulted in the misdiagnosis of almost one-third of DPN as melanoma. While the establishment of DPN as a diagnostic entity has reduced the number of cases falsely classified as melanoma, tumors with the characteristic phenotypic features can present diagnostic challenges, and occasional tumors classified as DPN metastasize with fatal outcome.4

While the majority of common nevi are clonal proliferations of BRAF V600E mutant melanocytes5, 6, the genetic drivers of DPN are largely unknown. DPN have overlapping phenotypic features with blue nevi in that they are mainly dermal based and contain numerous melanophages. However, they lack the GNAQ and GNA11 mutations that are found in the majority of blue nevi.8. DPN also have overlapping phenotypic features with Spitz nevi in that they have large melanocytes with abundant cytoplasm and have previously been shown to harbor HRAS mutations in 6% of cases.8 To investigate oncogenic alterations in DPN, we sequenced
DNA from 18 DPN and 10 common nevi, using a platform that includes exons of several hundred cancer genes and introns involved in oncogenic rearrangements. We sequenced additional nevi with overlapping features of DPN and blue nevi. The resulting sequence data was analyzed for single-nucleotide variants, insertion/deletions (indels), structural variants and copy number changes. We identify MAPK activating mutations in combination with β-catenin activating mutations as a characteristic feature of DPN. We demonstrate that CTNNB1 mutations arise after BRAF mutation in DPN arising from common acquired nevi and confer the characteristic phenotypic features of DPN. In DPN-like melanomas, we identify the presence of additional genetic alterations indicating that DPN are genetically intermediate between benign nevi and DPN-like melanomas.

Results

β-catenin and MAPK pathway mutations define DPN. Cases with typical histological features of DPN were genetically distinct from common nevi and nevi whose features overlapped features of DPN and blue nevi. 17 out of 18 (94%) DPN had activating mutations of the β-catenin pathway, mostly affecting CTNNB1 (16 cases) with rare APC mutations (Fig. 1a and Supplementary Table 1), whereas no common nevi harbored β-catenin activating mutations (P = 0.007214, binomial test). By contrast, nevi with overlapping features of DPN and blue nevi were devoid of CTNNB1 and APC mutations (P = 0.03, binomial test), instead harboring GNAQ activating mutations in 5 out of 7 (71%) cases (P = 0.02, binomial test). Thus, the latter are better classified as blue nevi based on their genetic profile. Only one DPN harbored GNAQQ209I without mutation in CTNNB1 or APC. This tumor also had an IDH1R132C mutation that was not present in the other GNAQ mutant tumors in our series. Perhaps this mutation led to epigenetic changes that altered histopathological features contributing to the apparent morphological misclassification of this lesion that genetically represents a blue nevus.

Most β-catenin pathway activating mutations were missense mutations in exon 3 of CTNNB1 that disrupt phosphorylation of β-catenin and its ubiquitin-mediated degradation, leading to increased β-catenin levels (Fig. 1b)9–11. In one DPN, two inactivating mutations of APC were present, representing an alternative mechanism of β-catenin activation.

16 out of 18 DPN had mutations of the mitogen-activated protein kinase (MAPK) pathway in addition to the activating mutations of the β-catenin pathway, affecting BRAF, MAP2K1 and NRAS in a mutually exclusive pattern. 9 out of 18 DPN (50%) had BRAF mutations: BRAFV600E in six cases, BRAF(599_B599insI, A598S, T959I) in one case each. One DPN harbored an HRASQ61R mutation. MAP2K1 alterations were present in 6 of 18 DPN (33%) consisting of small indels near codons 102 to 107, within helix C of MEK1, which is displaced by the activation loop in the active conformation12, 13. In one case, a small deletion affected the negative regulatory region (Fig. 1c)13. Similar MAP2K1 mutations occur in Langerhans cell histiocytosis and hairy cell leukemia, also in a mutually exclusive pattern with BRAFV600E14, 15.

Three of the five MAP2K1 indels have been previously observed in melanoma or other cancers, and two have been experimentally demonstrated to constitutively activate MAP2K114, 16, 17. We expressed 4 of the observed MAP2K1 mutations in 293FT cells and found that they activate MAP kinase signaling, which could be inhibited by the MEK inhibitor trametinib (Fig. 1d). Notably, the MAP2K1 mutations present in DPN are distinct from those at positions P124 and E203, which often occur in combination with BRAF or NRAS mutations in melanoma18, 19.

By contrast, common nevi harbored BRAFV600E in 7/10, NRASQ61R in 3/10, with no additional identified mutations. Thus DPN are genetically defined by the combination of MAPK activating and β-catenin activating mutations and are distinct from common and blue nevi.

To further investigate the role of CTNNB1 mutation in DPN, we used immunohistochemistry to evaluate β-catenin expression level and localization in DPN and common nevus controls. In common nevi, nuclear β-catenin was expressed in superficial melanocytes situated near the epidermis, whereas the smaller melanocytes in the deeper portions of the nevus had considerably lower expression levels, consistent with prior reports (Fig. 2)20.
Cyclin D1 is a direct transcriptional target of β-catenin and is frequently amplified in melanoma. Melanocytes in DPN demonstrated strong and uniform nuclear expression of cyclin D1 by immunohistochemistry. In contrast, immunoreactivity for cyclin D1 in common nevi was limited to melanocytes near epithelia, mimicking the expression pattern of CTNNB1 (Fig. 2, Supplementary Figs. 1, 2).

**CTNNB1 mutations and confer phenotypic characteristics of DPN.** Some DPN arise juxtaposed with a common nevus. We hypothesized genetic progression of a BRAFV600E melanocyte within a common nevus by acquisition of a β-catenin activating mutation results in an adjacent DPN. In 66 out of 68 (97%) bi-phenotypic cases the DPN displayed increased nuclear β-catenin and cyclin D1 compared to the adjacent common nevus. β-catenin and cyclin D1 levels in the DPN remained uniform, whereas they decreased in the adjacent common nevus with distance from the epithelium (Fig. 3). We genotyped the adjacent common nevus component and found identical BRAFV600E mutations as in the adjacent DPN but no CTNNB1 mutations. These results indicate that common nevi can evolve to DPN by acquiring β-catenin activating mutations.

We transduced immortalized mouse melanocytes (melan-a) with BRAFV600E either alone or in combination with CTNNB1I337F. Melanocytes expressing BRAFV600E and CTNNB1I337F were larger, more pigmented, and expressed higher levels of cyclin D1 than melanocytes expressing BRAFV600E alone (Fig. 4), recapitulating the characteristic features of DPN. These experimental findings indicate that CTNNB1 mutations are sufficient to induce the phenotypic changes characteristic of DPN in BRAF mutant melanocytes.

**DPN-like melanomas harbor additional oncogenic mutations.** Rare DPN metastasize with lethal outcomes. It is not known whether DPN that metastasize are genetically different from those that do not. We genotyped two metastases that originated from DPN within a common nevus by acquisition of a β-catenin activating mutation (Fig. 5, Supplementary Table 3). In 2 cases we genotyped the adjacent common nevus component and found identical BRAFV600E mutations as in the adjacent DPN. In 66 out of 68 (97%) bi-phenotypic cases the DPN displayed increased nuclear β-catenin and cyclin D1 compared to the adjacent common nevus. In contrast, immunoreactivity for cyclin D1 in common nevi was limited to melanocytes near epithelia, mimicking the expression pattern of CTNNB1 (Fig. 2, Supplementary Figs. 1, 2).

**Discussion**

In summary, our study shows that DPN result from the combined mutational activation of the MAP kinase and β-catenin pathways. Some DPN evolve from pre-existing nevi by acquiring a β-catenin pathway mutation secondary to the initiating MAP kinase pathway mutation. Prior work demonstrates that β-catenin signaling...
in melanocytes results in reduced T-cell response\textsuperscript{30}, which could contribute to the deeply invasive phenotype of DPN. The differences in β-catenin pathway activation between common nevi and DPN reveal another possible mechanism by which β-catenin pathway activation contributes to neoplastic progression. In DPN, melanocytes remain large and pigmented throughout the entire neoplasm, whereas in common nevi without mutational activation of the β-catenin pathway these cytomorphic features are constrained to areas in proximity to epithelia, and melanocytes become smaller and less pigmented with increasing distance from epithelia. Epithelia secrete WNTs\textsuperscript{31}, which may explain the decrease in WNT signaling with distance from epithelia. A dependence on WNT signaling may prevent common nevi from invading the deeper dermis. Constitutive β-catenin pathway activation in DPN may override this limitation, allowing these tumors to ‘deeply penetrate’.

Our data further indicates that β-catenin and MAPK pathway mutations alone are insufficient to fully transform melanocytes, and that additional mutations such as immortalizing mutations of TERT and loss of CDKN2A are required for DPN to progress to melanoma, (Fig. 6) consistent with prior work in mouse models\textsuperscript{32, 33}. Our results identify DPN as an intermediate melanocytic neoplasm, with a progression stage positioned between benign nevus and DPN-like melanoma.

**DNA extraction and targeted next-generation sequencing.** Areas of tumor were microdissected from 20 µm sections of formalin-fixed, paraffin-embedded (FFPE) tumor. After deparaffinization by washing with SafeKlear and ethanol DNA was extracted by phenol chloroform extraction. Multiplex library preparation was performed using the Ovation Ultralow Library System (NuGEN, San Carlos, CA, p/n No. 5140-53) or Kapa Hyper Prep Kit (Kapa Biosystems, Wilmington, MA, p/n 07962363001) according to the manufacturer’s specifications with up to 200 ng of sample DNA. Hybridization-capture of pooled libraries was performed using custom-designed bait libraries (Nimblegen SeqCap EZ Choice, p/n 065887001) including the exons of genes targeted to comprise common cancer genes with particular relevance to melanoma. The target intervals cover mostly exonic but also some intronic and untranslated regions of 293 (version 1), 538 (version 2), and 480 (version 3) target genes. The target intervals cover mostly exonic but also some intronic and untranslated regions of 293 (version 1), 538 (version 2), and 480 (version 3) target genes. The target genes were curated to comprise common cancer genes with particular relevance to melanoma.

Captured libraries were sequenced as paired-end 100 bp reads on a HiSeq 2000 or HiSeq 2500 instrument (Illumina). Sequence reads were mapped to the reference human genome (hg19) using the Burrows-Wheeler aligner (BWA)\textsuperscript{34}. Recalibration of reads was performed using the Genome Analysis Toolkit (GATK)\textsuperscript{35}. Mutation calling was performed with FreeBayes\textsuperscript{36}. Coverage and sequencing statistics were determined using Picard CalculateHsMetrics and Picard CollectInsertSizeMetrics (Supplementary Table 5). Variant annotation was performed with Annovar\textsuperscript{37} and variants with frequency in the 1000 genomes or exome sequencing project datasets of 0.001 were excluded from further analysis. For fusion detection, read pairs with one or more reads unaligned, insert sizes greater than 1000 bp, or with soft clipping of at least one read were extracted and re-aligned using BWA-SW\textsuperscript{38} and used as input to CREST\textsuperscript{40}. Structural variants predicted by CREST were reviewed by visual inspection in the Integrative Genomics Viewer\textsuperscript{41}. We predicted the resulting fusion transcript by joining the exon directly upstream from the genomic
Fig. 5 Additional genetic alterations in DPN-like melanoma. Distant metastases of DPN harbored a combination of MAPK and β-catenin pathway activating mutations in addition to oncogenic point mutations and copy number alterations, left. Half of primary melanomas with features of DPN harbored a combination of MAPK and β-catenin pathway activating mutations, also in combination with oncogenic point mutations and copy number alterations, right.

breakpoint with the exon directly downstream. Predicted protein sequences were then determined from the predicted transcripts. Copy number analysis was performed using CNVkit42.

Statistical analysis. We used the binomial test with the null hypothesis that β-catenin activating mutations are equally distributed between DPN and common nevi, and DPN and nevi with features of DPN and blue nevus. We also tested the null hypothesis that GNAQ mutations distinguish nevi with features of DPN and blue nevus from DPN and nevi lacking such characteristics.

Plasmid construction. Total RNA from 293FT cells (purchased from Life Technologies) was reverse transcribed with random primers, MAP2K1 cDNA was amplified with primer pairs 5′-caccatgaagggagagcttggg-3′ and 5′-tgagggattgttcagaattgct-3′, cloned into pENTR™D-TOPO vector (ThermoFisher, p/n K240020). BRAFV600E and CTNNB1 cDNAs were cloned into pENTR™/D-TOPO using primer pairs 5′-caccatggagccacggag-3′ and 5′-tcgagaatgctccacccctg-3′, or 5′-caccatggagccacggag-3′ and 5′-tcgagaatgctccacccctg-3′, respectively, and subsequently cloned into pLenti6.3/V5-DEST (i.e., BRAF, MAP2K1, and GFP constructs) or pLenti CMV/TO Puro DEST (670-1), a gift from Eric Campeau (Addgene plasmid #17293), (CTNNB1 construct)43, CTNNB1V537F and MAP2K1 mutations were introduced by site-directed mutagenesis using the following primer pairs: 5′-cttgctccattacgcttgattcagtgctt-3′ and 5′-tcgtggtctttctcagcttctgc-3′.

Immunohistochemistry. Immunohistochemical analysis was performed on archival FFPE tumor specimens using the following antibodies: CTNNB1 clone B-catenin 1 (Dako catalog# M5559 or IR702), CCND1 clone EP-12 (Dako, catalog #IR083) or clone SP4 (Thermo Scientific, catalog #RM-9104-R7).

RNA in situ hybridization. Formalin-fixed, paraffin-embedded tissue sections were pretreated with 13 min of boiling followed by 12 min of protease digestion (RNAscope Protease Plus). Hybridization for hs-Axin2 (catalog #400249) was performed in parallel with DapB (catalog # 310043) and PPIB (catalog # 313901) as negative and positive controls respectively. Scoring for each case was performed in melanocytes near the epidermis and at a distance from epithelium (epidermis and adnexal epithelium). A semi-quantitative scoring method was used for each area (Supplementary Table 6)44.
**Sanger sequencing.** Sanger sequencing was performed for CTNNB1 exon 3, BRAF exon 15, MAP2K1 exons 2 and 3. After selection of the relevant paraffin blocks or hematoxylin-eosin stained sections, genomic DNA was extracted from macrodissected FFPE material (using the QiAamp DNA FFPE extraction kit, Qiagen, Courtaboeuf, France) according to the manufacturer’s guidelines. Sanger sequencing analysis of BRAF exon 15, CTNNB1 exon 3, and MAP2K1 exons 2 and 3 were performed.

BRAF: Forward 5′-TCTAATAGTTCTTCAGATGAGA-3′; Reverse 5′-TCTAATGCAGAGGAATCTC-3′; CTNNB1: Forward 5′-TAAAGTTACATTCTTTAAC-3′; Reverse 5′-TCTTTGAGTAAAGGACTGAGA-3′ MAP2K1, exon 2: Forward 5′-ACCTGAGGCTCTTCTTCTCATG-3′; Reverse 5′-AATGAGCTCTTCTTCTATTACCCG-3′; MAP2K1, exon 3: Forward 5′-GCTCTACATTTAAAGGACTTACAA-3′; Reverse 5′-TACCTCCAGACAAAGGATTA-3′.

**Generation of stably transduced cell lines.** Melan-a cells were generously provided by Dr Dorothy C. Bennett (St George’s Hospital, University of London, London, UK) and maintained in glutamine-containing RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum, 200 mM of 12-0-tetradecanoylphorbol-13-acetate (TPA), penicillin (100 units/ml) and streptomycin (50 mg/ml). 293FT cells were transfected with 1 μg of plasmid (a gift from Dr. Peter Openshaw, University of London, UK) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Supernatants were harvested 48 h after transfection and filtered on 0.45-μm polyvinylidene difluoride (PVDF) membranes and used to transduce melan-a cells in the presence of 10 μg/ml polybrene (Santa Cruz Biotechnology). Cells were selected using either 5 μg/ml blasticidin or 1 μg/ml puromycin for 1–3 weeks. Mycoplasma contamination was tested using MycoSensor PCR Assay Kit (Agilent Technologies).

**Western blotting.** Cells were lysed in RIPA buffer supplemented with Halt protease and phosphatase inhibitor cocktail (Thermo Scientif.

**TGFβ assay.** Stably transduced melan-a cell lines cultured in 12-well plates were transfected with 1 μg of β-catenin reporter construct (MSO Super 8x TOP-Flash, a gift from Randall Moon, Aldgen pharmaceuticals, #12456) and renilla control reporter (pRL-CMV Vector, Promega, E2231). At 24 h after transfection, luciferase activity was assayed in 10 μl of lysate using the Dual-Luciferase reporter assay system (Promega, E1910) and a Glomax-Multi luminometer (Promega).

**Melanin quantitation.** Cells were trypsinized and centrifuged and the resulting cell pellet was dissolved in 1N NaOH 10%-DMSO, and incubated at 74 °C for 2 h. The absorbance was measured at 420 nm. Melanin content was determined using a standard curve of melanin from Sepia officinalis (Sigma, #M6249).

**HoloMonitor imager measurements.** Stably transduced cell lines were plated in 6-well plates (Griener-EC 27160) with 20,000 cells/well in serum free conditions. Digital holograms of the cells were generated 24–48 h after plating when they were at approximately 30% cell density using a HoloMonitor M4 Digital Holography Cytometer (Phase Holographic Imaging) PHA AB, Lund, Sweden). Cell volume was calculated using Hstudo M4 software (Phase Holographic Imaging) PHA, Lund, Sweden).

**Data availability.** Sequence data that support the findings of this study have been deposited in the Sequence Read Archive (SRA), with the accession code BioProject ID: PRJNA384770 (http://www.ncbi.nlm.nih.gov/bioproject/384770). All other remaining data are available within the Article and Supplementary Files, or available from the authors upon request.
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Author contributions
I.Y., B.C.B. and A.d.l.F. designed the study, critically reviewed the data and wrote the manuscript. I.Y., A.H.S., V.H., D.P. and A.d.l.F. performed DNA sequence analysis. U.E.L., M.K.T., A.J., X.C. and R.I.J. performed in vitro studies. I.Y., B.C.B., A.d.l.F., E.D., L.C., P.E.L. and T.H.M. identified and reviewed the histopathological features of the study cohort. I.Y., A.d.l.F. and E.D. performed the data collection and analysis of immunohistochemical and in situ hybridization results.

Additional information
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