LETTER

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DHODH modulates transcriptional elongation in the neural crest and melanoma

Richard Mark White[1,2], Jennifer Cech[1], Sutheera Ratanasirintrawoot[1], Charles Y. Lin[3,4], Peter B. Rahl[3], Christopher J. Burke[1], Erin Langdon[1], Matthew L. Tomlinson[5], Jack Mosher[6], Charles Kaufman[1,2], Frank Chen[7], Hannah K. Long[8], Martin Kramer[9], Sumon Datta[1], Donna Neuberg[10], Scott Granter[11], Richard A. Young[3,4], Sean Morrison[6], Grant N. Wheeler[5] & Leonard I. Zon[1]

Melanoma is a tumour of transformed melanocytes, which are originally derived from the embryonic neural crest. It is unknown to what extent the programs that regulate neural crest development interact with mutations in the *BRAF* oncogene, which is the most commonly mutated gene in human melanoma[1]. We have used zebrafish embryos to identify the initiating transcriptional events that occur on activation of human *BRAF(V600E)* (which encodes an amino acid substitution mutant of BRAF) in the neural crest lineage. Zebrafish embryos that are transgenic for *mitfa:BRAF(V600E)* and lack *p53* (also known as *tp53*) have a gene signature that is enriched for markers of multipotent neural crest cells, and neural crest progenitors from these embryos fail to terminally differentiate. To determine whether these early transcriptional events are important for melanoma pathogenesis, we performed a chemical genetic screen to identify small-molecule suppressors of the neural crest lineage, which were then tested for their effects on melanoma. One class of compound, inhibitors of dihydroorotate dehydrogenase (DHODH), for example leflunomide, led to an almost complete abrogation of neural crest development in zebrafish and to a reduction in the self-renewal of mammalian neural crest stem cells. Leflunomide exerts these effects by inhibiting the transcriptional elongation of genes that are required for neural crest development and melanoma growth. When used alone or in combination with a specific inhibitor of the *BRAF(V600E)* oncogene, DHODH inhibition led to a marked decrease in melanoma growth both *in vitro* and in mouse xenograft studies. Taken together, these studies highlight developmental pathways in neural crest cells that have a direct bearing on melanoma formation.

In melanoma, it is unknown to what extent *BRAF(V600E)* mutations depend on transcriptional programs that are present in the developmental lineage of tumour initiation. These programs may be therapeutic targets when combined with BRAF(V600E) inhibition. We have used zebrafish embryos to identify small-molecule suppressors of neural crest progenitors that give rise to melanoma. Transgenic zebrafish expressing human *BRAF(V600E)* under the melanocyte-specific *mitfa* promoter, *Tg(mitfa:BRAF(V600E))*, develop melanoma at 4–12 months of age when crossed with $p53^{-/-}$ mutant zebrafish, *Tg(mitfa:BRAF(V600E))*; $p53^{-/-}$ (Fig. 1a). Because the *mitfa* promoter drives the expression of *BRAF(V600E)* from 16 h after fertilization (a time point that overlaps with the expression of embryonic neural crest markers such as *sox10*), events that occur early in embryogenesis are analogous to those that occur at tumour initiation. To gain insight into these initiating events, we compared the gene expression profiles of *Tg(mitfa:BRAF(V600E))*; $p53^{-/-}$ embryos with those of *Tg(mitfa:BRAF(V600E))*; $p53^{-/-}$ melanomas by using gene set enrichment analysis (Fig. 1b). This approach uncovered a signature of 123 overlapping genes, which is enriched for markers of embryonic neural crest progenitors (*crestin*, *sox10* and *ednrb* (also known as *ednrb1*)) and melanocytes (*tyr* and *dct*) (see Supplementary Table 1 for full gene sets). The overlapping gene signature is similar to the signature of a multipotent neural crest progenitor, suggesting that the melanomas have adopted this cell fate.

We analysed alterations in embryonic neural crest development by using *in situ* hybridization. At 24 h post fertilization, *Tg(mitfa:BRAF(V600E))*; $p53^{-/-}$ embryos show an abnormal expansion in the number of $crestin^{+}$ progenitors, together with an increase in other markers from the 123 gene signature such as *spry4* and *rab3il1* (Supplementary Fig. 1). By 72 h post fertilization, *crestin* persists aberrantly in the head, tail and dorsal epidermis only in *Tg(mitfa:BRAF(V600E))*; $p53^{-/-}$ embryos but not in embryos with either single mutation (Supplementary Fig. 2a). The gene encoding Crestin is zebrafish specific[2] and is normally downregulated after the terminal differentiation of neural crest progenitors[3]. Our finding therefore suggests that activated BRAF(V600E) promotes the maintenance of multipotency in neural crest progenitors, which become expanded during tumorigenesis. In adult *Tg(mitfa:BRAF(V600E))*; $p53^{-/-}$ melanomas, almost all tumour cells, but no normal cells, were positive for *crestin* (Fig. 1c). Only 10–15% of the melanoma cells were pigmented (Supplementary Fig. 2b), which is consistent with the concept that adult zebrafish melanomas retain a progenitor-like state. A human melanoma tissue array yielded similar results: 52 out of 70 of the melanomas on the array (74.3%) were positive for the neural crest progenitor gene *ednrb*, but only 9 of 70 (12.9%) were positive for the melanocyte lineage marker *dct* (Supplementary Fig. 3), in agreement with the finding that most human melanomas express the neural crest marker *sox10* (ref. 4). These data indicate that the majority of human melanomas reflect events that lead to the maintenance of a neural crest progenitor phenotype[5].

We proposed that chemical suppressors of neural crest progenitors would be useful for treating melanoma. We screened 2,000 chemicals to identify compounds that inhibit the $crestin^{+}$ lineage during embryogenesis. Most chemicals (90%) had a minimal effect or were toxic (Supplementary Fig. 4). NSC210627, a molecule of unknown function, strongly abrogated the expression of *crestin* (Fig. 2a, centre and left). The chemoinformatic algorithm DiscoveryGate[6] revealed similarity between NSC210627 and brequinar (Supplementary Fig. 5), an inhibitor of DHODH[7]. NSC210627 inhibited DHODH activity *in vitro* (Supplementary Fig. 6). Leflunomide, a DHODH inhibitor that is structurally distinct from NSC210627 (ref. 8), phenocopied NSC210627 (Fig. 2a, right) and was used for further studies because of its availability.

We examined neural crest derivatives affected by leflunomide. Treated zebrafish embryos were devoid of pigmented melanocytes at 36–48 h post fertilization (Fig. 2b) and iridophores at 72 h post fertilization

[1]Stem Cell Program and Hematology/Oncology, Children's Hospital Boston, Howard Hughes Medical Institute, Harvard Stem Cell Institute, Harvard Medical School, Boston, Massachusetts 02115, USA. [2]Department of Medical Oncology, Dana Farber Cancer Institute, Boston, Massachusetts 02115, USA. [3]Whitehead Institute for Biomedical Research, Cambridge, Massachusetts 02142, USA. [4]Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02142, USA. [5]School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, UK. [6]Center for Stem Cell Biology, University of Michigan, Howard Hughes Medical Institute, Ann Arbor, Michigan 48109, USA. [7]Harvard University, Cambridge, Massachusetts 02138, USA. [8]Cambridge University, Cambridge CB2 1TN, UK. [9]Genzyme Corporation, Cambridge, Massachusetts 02142, USA. [10]Department of Biostatistics and Computational Biology, Dana Farber Cancer Institute, Boston, Massachusetts 02115, USA. [11]Department of Pathology, Brigham and Women's Hospital, Boston, Massachusetts 02115, USA.

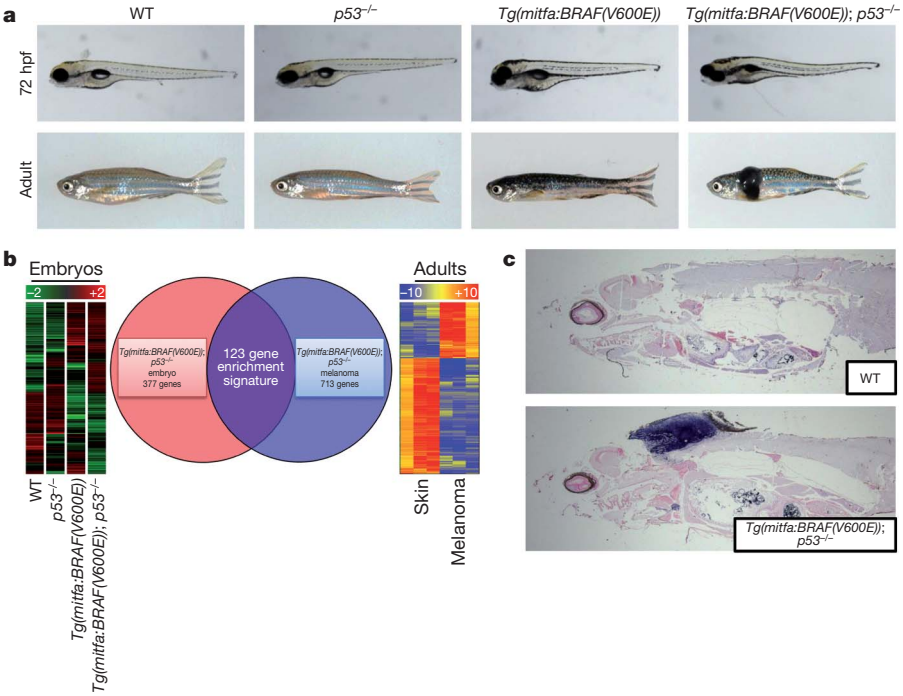

Figure 1 | Transgenic zebrafish melanoma and neural crest gene expression. **a**, Transgenic zebrafish expressing BRAF(V600E) under the control of the promoter of the melanocyte-specific gene *mitfa*, *Tg(mitfa:BRAF(V600E))*, develop pigmentation abnormalities and melanoma when crossed with $p53^{-/-}$ fish. Their gross embryonic development is largely normal. hpf, hours post fertilization; WT, wild type. **b**, Gene expression analysis revealed a unique gene signature at 72 h post fertilization in the *Tg(mitfa:BRAF(V600E)); $p53^{-/-}$* strain (left). Gene set enrichment analysis uncovered an enrichment between the embryonic gene signature and the adult melanomas that form 4–12 months later (centre and right; see the Supplementary Information for full protocol details). Embryo heat-map columns represent an average of three clutches ($\log_2$ scale, range −2-fold to +2-fold increase); adult heat-map columns represent individual fish ($\log_2$ scale, range −10-fold to +10-fold increase). **c**, *In situ* hybridization of sagittal sections of WT and *Tg(mitfa:BRAF(V600E)); $p53^{-/-}$* adults reveal homogeneous *crestin* expression (blue) only within the dorsal melanoma; it is absent from normal adult tissues.

(Supplementary Fig. 7a). dhodh inhibition led to a loss of ventral melanocytes in stage 38 *Xenopus* embryos (Supplementary Fig. 7b). Leflunomide treatment led to an almost complete loss of melanocyte progenitors at 24 h post fertilization (Fig. 2c), a reduction in the number of glial cells at 72 h post fertilization (Fig. 2d) and disruption of jaw cartilage at 72 h post fertilization (data not shown). Leflunomide reduced the expression of *sox10* and *dct*, which are expressed by neural crest progenitors and melanocytes, respectively, while leaving other lineages such as blood and notochord less affected (Supplementary Fig. 8). Microarray analysis of leflunomide-treated embryos showed downregulation of 49% of the genes that were upregulated in the 123-gene melanoma signature, and more than half of these are neural crest related (see Supplementary Table 2 for the complete list).

The loss of several types of neural crest derivative suggested that leflunomide acts on neural crest stem cells. We tested leflunomide, and its derivative A771726, on neural crest stem cells isolated from the fetal (embryonic day 14.5) rat gut[9,10]. Both chemicals reduced the number of self-renewing neural crest stem cells in primary stem cell colonies, to 27±5.35% (leflunomide) and 35±6.16% (A771726) of control numbers ($P < 0.0003$ and $P < 0.00007$, respectively, Student's *t*-test; Fig. 2e and Supplementary Fig. 9a). Colony size was also reduced compared with controls (by 18% and 24%, respectively; $P < 0.02$, Student's *t*-test), but there was no effect on the differentiation or survival of specific progeny (Supplementary Fig. 9b, c). These results demonstrate that DHODH inhibitors negatively regulate the self-renewal of neural crest stem cells and have an affect on these cells in multiple species.

DHODH catalyses the fourth step in the synthesis of pyrimidine nucleotides (NTPs)[11]. We noted striking morphological similarity between leflunomide-treated embryos and *spt5/spt6* mutants[12], suggesting that leflunomide acts to suppress transcriptional elongation. In the $spt5^{sk8}$ null mutant, we found a lack of both *crestin* expression and pigmented melanocytes (similar to leflunomide-treated embryos) (Supplementary Fig. 10a). At 24 h post fertilization, the gene expression profiles of $spt5^{sk8}$ mutants and leflunomide-treated embryos[13] were nearly identical; of 223 genes downregulated after leflunomide treatment, 183 were similarly downregulated in $spt5^{sk8}$ mutants (Supplementary Table 3 and Supplementary Fig. 10b). These downregulated genes include neural crest genes (*crestin, sox10* and *mitfa*) and members of the *notch* pathway (*her2* and *dlb*). We examined the interaction of Dhodh with *spt5* by incubating the hypomorphic $spt5^{m806}$ mutant (which has only mild melanocyte defects)[14] in low concentrations of leflunomide (3–5 μM) and then analysing the number of pigmented melanocytes. Enhanced sensitivity to leflunomide was shown by $spt5^{m806}$ embryos (Fig. 3a and Supplementary Fig. 11); at 3 μM leflunomide, 99% of mutant embryos had few or no melanocytes, compared with 0% of wild-type embryos ($P = 0.000018$, Kruskal–Wallis test; Supplementary Fig. 11b). These data confirm that DHODH inhibition affects transcriptional elongation, which is consistent with previous data demonstrating that a reduction in nucleotide pools *in vitro* leads to defects in elongation[15].

We assessed whether leflunomide specifically caused defects in the transcriptional elongation of genes required for neural crest development by using reverse transcription followed by quantitative PCR (Supplementary Fig. 10c and Supplementary Table 4). Leflunomide caused either no change or an increase in 5′ transcript abundance but a significant downregulation of 3′ transcripts of *mitfa* (for 5′ transcripts 3.75±1.19-fold increase versus 0.39±0.07-fold increase for 3′ transcripts; $P < 0.05$, Student's *t*-test) and *dlb* (5′ transcripts 1.13±0.14-fold increase versus 3′ transcripts 0.74±0.07-fold increase; $P < 0.05$). Leflunomide did not have a similar effect on control genes such as the gene encoding β-actin (5′ transcripts 1.03±0.07-fold increase versus 3′ transcripts 0.99±0.06-fold increase; *P* is not significant, Student's *t*-test). In the presence of leflunomide, transcription is initiated normally, but the transcripts accumulate and do not undergo productive elongation.

To confirm that this mechanism is conserved in human melanoma, we performed chromatin immunoprecipitation using an antibody

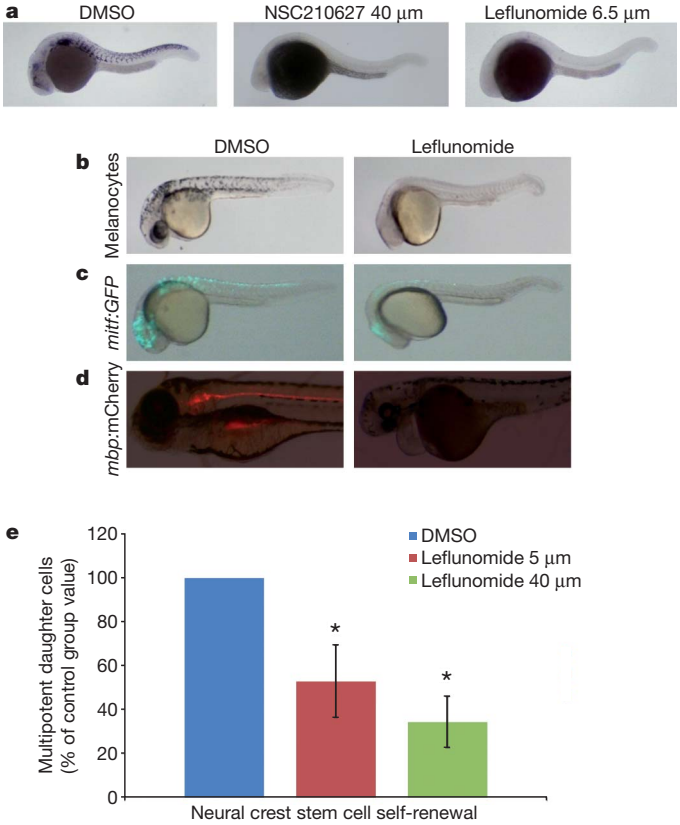

Figure 2 | A chemical genetic screen to identify suppressors of neural crest development. **a**, A search for chemical suppressors of the *crestin*$^{+}$ lineage during embryogenesis identified NSC210627, a compound that completely abrogates the expression of *crestin* (which is normally present in the head, along the dorsum and in ventrally migrating neural crest cells), as shown by *in situ* hybridization (**a**, left and centre). DMSO is used as a control. The DiscoveryGate chemoinformatic algorithm revealed structural similarity between NSC210627 and brequinar (Supplementary Fig. 5), an inhibitor of DHODH. Leflunomide, a structurally distinct DHODH inhibitor, phenocopies the *crestin* phenotype induced by treatment with NSC210627 (**a**, right). **b**–**d**, Treatment with leflunomide caused an absence of multiple neural crest derivatives, including pigmented melanocytes (**b**); melanocyte progenitors, which were visualized by expressing green fluorescent protein (GFP) under the control of the *mitfa* promoter (**c**); and glial cells, which were visualized by expressing the fluorescent protein mCherry under the control of the myelin basic protein (*mbp*) promoter (**d**). **e**, Treatment with leflunomide, or A771726 (Supplementary Fig. 9a), significantly reduced the number of multipotent daughter cells that could be subcloned from individual primary neural crest stem cell colonies. Values shown are mean ± s.d. of three replicates; *, $P < 0.05$ compared with control, Student's *t*-test.

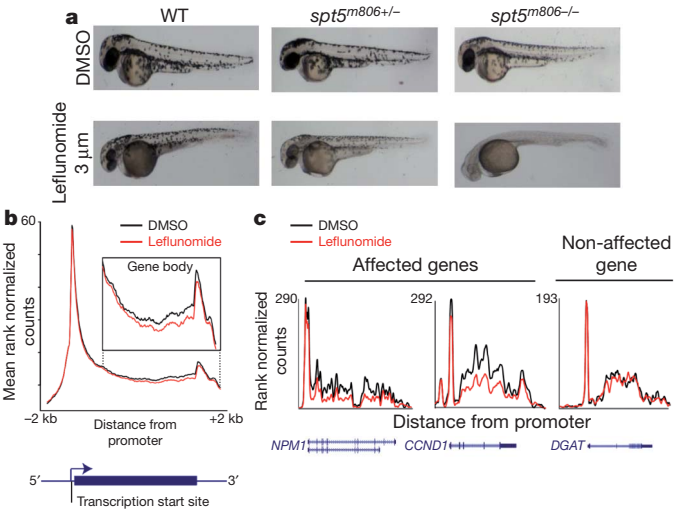

Figure 3 | DHODH inhibition modulates transcriptional elongation. **a**, The hypomorphic *spt5*^{m806} homozygous mutant (top right) has only a mild pigmentation defect compared with WT (top left) or heterozygous (top centre) animals. Treatment with a low dose of leflunomide (3 μM) leads to an almost complete absence of neural-crest-derived melanocytes in the mutant line. See Supplementary Fig. 11 for dose–response quantification of this effect. **b**, Metagene analysis of RNA Pol II occupancy in A375 human melanoma cells after treatment with leflunomide. The metagene plot allows visualization of all of the genes that are occupied by RNA Pol II, corrected for individual gene lengths. Genome-wide RNA Pol II occupancy at the promoter region is unaffected but is diminished at the 3′ end of the genes. The inset shows a higher magnification of the 3′ region of the genes. **c**, Representative examples of Myc target genes (*NPM1* and *CCND1*), which demonstrate defects in transcriptional elongation after treatment with leflunomide. A gene that is minimally affected (*DGAT*) is also depicted. For *NPM1*, the TR is 5.04 after DMSO treatment and 8.10 after leflunomide treatment. For *CCND1*, the TR is 3.47 after DMSO treatment and 4.67 after leflunomide treatment. For *DGAT*, the TR is 5.19 after DMSO treatment and 5.34 after leflunomide treatment.

specific for RNA polymerase II (RNA Pol II), followed by sequencing (ChIP-seq). Transcriptional elongation was measured using the travelling ratio (TR)[16], in which the ratio of RNA Pol II density in the promoter-proximal region is compared with that in the gene body. In the human melanoma cell lines A375 and Malme-3M, leflunomide treatment caused a significant inhibition of transcriptional elongation (measured as an increase in the TR), particularly for genes with a TR that was initially low (<7.5). For example, in A375 cells, the TR increased by >1.3 fold at 21.3% of loci; in Malme-3M cells, this occurred for 36.3% of loci (Supplementary Table 5). Examination of RNA Pol II occupancy using metagene analysis at a variety of fold-change cutoffs (Fig. 3b (A375), Supplementary Fig. 12 (Malme-3M) and Supplementary Table 5) revealed no defect in transcription initiation but a decrease in elongation that was pronounced at the 3′ end of genes such as *NPM1* and *CCND1* (Fig. 3c). Ingenuity Pathway Analysis on the loci affected in both cell lines revealed a strong enrichment for Myc targets and pathway members[17] (Supplementary Fig. 13a, b). Myc, in addition to its requirement for neural crest development[18], was recently shown to be a potent regulator of transcriptional pause release in embryonic stem cells[16]. Our data suggest that the mechanism by which Myc target genes are regulated at the transcriptional elongation level operates in neural-crest-derived melanoma as well. Taken together, the genetic and biochemical data demonstrate that leflunomide acts to modulate transcriptional elongation in both neural crest development and human melanoma.

Given the effect of DHODH inhibition on neural crest development, we tested its effects on melanoma growth. A771726 caused a dose-dependent decrease in the proliferation of human melanoma cell lines (A375, Hs 294T and RPMI-7951; Fig. 4a). Similarly, a short hairpin RNA directed against DHODH led to a 57.7% decrease in the proliferation of A375 cells, as well as a decrease in elongation as measured by ChIP-PCR (Supplementary Fig. 14). Microarray analysis of the A375 cell line treated with leflunomide revealed downregulation of genes that are required for neural crest development (such as *SNAI2*) and members of the *NOTCH* pathway (for example, *HES6* and *JAG1*), which is consistent with the effects of leflunomide on zebrafish embryos (Supplementary Table 6).

Pyrimidine NTP production is regulated at the level of carbamoyl-phosphate synthetase (CAD)[19], the enzyme that is directly upstream of DHODH. CAD is phosphorylated by the mitogen-activated protein kinase ERK2[20], a protein that would be activated in melanoma as a result of the BRAF(V600E) mutation. We reasoned that combined blockade of BRAF(V600E) and DHODH would suppress melanoma growth. We measured melanoma cell proliferation after treatment with the BRAF(V600E) inhibitor PLX4720 (ref. 21) together with A771726 (Fig. 4b, c and Supplementary Fig. 15a, b), and we found that the combination led to a cooperative suppression of melanoma growth.

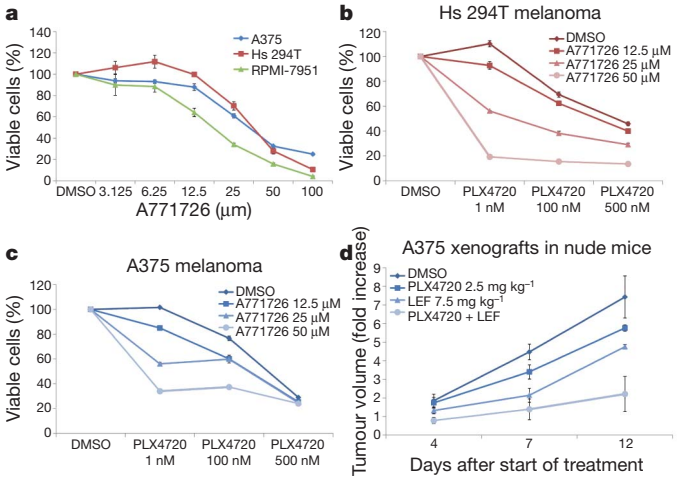

Figure 4 | Melanoma growth is suppressed by DHODH blockade in concert with BRAF(V600E) inhibition. **a**, A771726 causes a dose-dependent decrease in melanoma cell proliferation, as measured by the CellTiter-Glo assay, in three human melanoma cell lines that contain the BRAF(V600E) mutation (A375, Hs 294T and RPMI-7951). **b**, **c**, A771726 cooperates with the BRAF(V600E) inhibitor PLX4720 in inhibiting melanoma cell proliferation in the Hs 294T (**b**) and A375 (**c**) cell lines, as well as other melanoma cell lines (Supplementary Fig. 15). **d**, After subcutaneous transplantation of 3×10^5 A375 cells into nude mice ($n = 4$–5), both leflunomide (LEF) alone and PLX4720 alone impair tumour progression; the combination of these chemicals elicits an almost complete abrogation of tumour growth and results in complete tumour regression in two of five animals (DMSO versus PLX4720, $P = 0.036$; DMSO versus LEF, $P = 0.006$; PLX4720 or LEF versus PLX4720 + LEF, $P = 0.006$; PLX4720 versus LEF, P is not significant; analysis of variance, followed by Tukey's post hoc test). **a**–**d**, Values shown are mean ± s.e.m. of three to five replicates.

PLX4720 had no effect in non-melanoma cell lines (Supplementary Fig. 15c, $BRAF^{WT}$). A771726 demonstrated some antiproliferative activity in non-melanoma cell lines but was less potent in these cells than in melanoma cells (Supplementary Fig. 15d).

We examined the *in vivo* effects of leflunomide and PLX4720 by using xenografts of A375 cells transplanted into nude mice (Fig. 4c and Supplementary Fig. 16). At 12 days post treatment, tumours in mice that had been treated with dimethylsulphoxide (DMSO) as a control had grown 7.4±1.3 fold. By contrast, tumours in PLX4720-treated mice had grown 5.7±0.16-fold, and those in leflunomide-treated mice had grown 4.7±0.12 fold. The combination of PLX4720 and leflunomide led to an enhanced abrogation of tumour growth, with only 2.2±0.9 fold growth. In 40% of animals, this combination led to almost complete tumour regression (PLX4720 and leflunomide versus PLX4720 alone or leflunomide alone, $P < 0.001$, analysis of variance followed by Tukey's post hoc test). Therefore, we have found that an inhibitor of embryonic neural crest development, leflunomide, blocks *in vivo* tumour growth in combination with the BRAF(V600E) inhibitor PLX4720 when used at clinically meaningful doses.

Our data suggest that inhibition of DHODH abrogates the transcriptional elongation of genes that are required for both neural crest development and melanoma growth, including Myc target genes and *mitfa*. Although DHODH inhibition would be expected to lead to ubiquitous defects, human mutations in *DHODH* cause Miller's syndrome[22], a craniofacial disorder that is similar to syndromes with defects in neural crest development. Our data support recent findings that elongation factors are important for both neural crest development[23] and cancer growth[24]. Developmental regulators of transcriptional elongation have recently been identified to be important in haematopoiesis[25], and identification of such factors in the neural crest awaits further study.

Using chemical genetic approaches in zebrafish and *Xenopus* allows the identification of molecules that require *in vivo* contexts for the expression of relevant phenotypes[26]. Inhibition of DHODH may be a unique *in vivo* mechanism for modulating transcriptional elongation. Leflunomide is a well-tolerated anti-arthritis drug in humans[27], and our data suggest that, for the treatment of melanoma, it would be most effective in combination with a BRAF(V600E) inhibitor. This combination therapy may help to overcome resistance to BRAF(V600E) inhibitors[28]. As an increasing number of genomic changes are identified in cancerous cells, the challenge is to target these in concert with lineage-specific factors to achieve therapeutic synergy. Our approach to identifying lineage-specific suppressors in zebrafish embryos can be generalized to other cell types, with direct relevance to human cancer.

METHODS SUMMARY

Microarray analysis was performed on four groups of embryos at 72 h post fertilization: wild type, *Tg(mitfa:BRAF(V600E))*, $p53^{-/-}$, and *Tg(mitfa:BRAF(V600E)); $p53^{-/-}$* double mutants. Array analysis was also performed for adult *Tg(mitfa:BRAF(V600E)); $p53^{-/-}$* melanomas and for adjacent skin. The transcriptional signature of the melanomas was used in gene set enrichment analysis to identify genes that were significantly enriched in the *Tg(mitfa:BRAF(V600E)); $p53^{-/-}$* embryos. The 123 genes that make up this signature, which is enriched for markers of the neural crest, were concordantly upregulated or downregulated in both *BRAF(V600E); $p53^{-/-}$* embryos and tumours. *In situ* hybridization was used to examine the expression of *crestin* (a pan neural crest marker) and that of other neural crest genes in embryos (at 24–72 h post fertilization) and adult tumours. Chemical screening was performed to identify suppressors of the *crestin*$^+$ lineage by treating wild-type embryos from 50% epiboly to 24 h post fertilization with various chemicals, followed by robot-controlled *in situ* hybridization. Two inhibitors of DHODH abrogated *crestin* expression: NSC210627 and leflunomide. The latter was used for further studies owing to its more widespread availability. The effect of leflunomide on embryonic neural crest development in zebrafish was assessed by scoring embryos for melanocytes, iridophores and glial cells. Leflunomide was further assessed for its ability to affect the multipotent self-renewal of purified $p75^+\alpha_4$-integrin$^+$ rat neural crest stem cells (referred to in the main text as neural crest stem cells). The effects of leflunomide on transcriptional elongation in the neural crest were tested using the *$spt5^{m806}$* allele and measuring pigmentation in response to 3–5 μM leflunomide. Elongation in melanoma cells was assayed by ChIP-seq using an antibody specific for RNA Pol II and measuring the TR. Leflunomide was tested for anti-melanoma effects on human melanoma cell lines in the presence or absence of the BRAF(V600E) inhibitor PLX4720. *In vitro* proliferation assays were performed using the CellTiter-Glo system (Promega). *In vivo* effects were tested by treating established A375 xenografts with daily intraperitoneal doses of PLX4720 alone, leflunomide alone or both, and the tumour growth rate was measured on days 4, 7 and 12.

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