DHODH modulates transcriptional elongation in the neural crest and melanoma

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Abstract

Melanoma is a tumor of transformed melanocytes, which are derived from the embryonic neural crest. It is unknown to what extent the programs regulating neural crest development interact with mutations in the BRAF oncogene, the gene most commonly mutated in human melanoma[1]. We have utilized the zebrafish embryo to identify initiating transcriptional events upon BRAF^{V600E} activation in the neural crest lineage. Transgenic *mitf*-BRAF^{V600E};p53$^{-/-}$ zebrafish embryos

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demonstrate a gene signature enriched for markers of multipotent neural crest cells, and exhibit a failure of terminal differentiation of neural crest progenitors. To determine if these early transcriptional events were 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 effects in melanoma. One class of compounds, inhibitors of dihydroorotate dehydrogenase (DHODH) such as leflunomide, led to an almost complete abrogation of neural crest development in the zebrafish and a reduction in self-renewal of mammalian neural crest stem cells. Leflunomide exerts these effects by inhibiting transcriptional elongation of genes 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 direct bearing upon subsequent melanoma formation.

In melanoma, it is unknown to what extent BRAF^{V600E} mutations depend upon transcriptional programs present in the developmental lineage of tumor initiation. These programs may be therapeutic targets when combined with BRAF^{V600E} inhibition. We have utilized zebrafish embryos to identify small molecule suppressors of neural crest progenitors which give rise to melanoma. Transgenic zebrafish expressing human BRAF^{V600E} under the melanocyte-specific *mitf* promoter (*mitf*-BRAF^{V600E}) develop melanoma at 4-12 months of age when crossed with p53$^{-/-}$ mutants (Figure 1a). As the *mitf* promoter drives BRAF^{V600E} starting at 16 hours post fertilization (hpf), overlapping with other markers such as *sox10,* events that occur early in embryogenesis are analogous to those occurring at tumor initiation. To gain insight into initiating events, we compared gene expression profiles of BRAF^{V600E};p53$^{-/-}$ embryos to BRAF^{V600E};p53$^{-/-}$ melanomas using Gene Set Enrichment Analysis (GSEA) (Figure 1b). This revealed a 123 gene overlap signature, notable for markers of embryonic neural crest progenitors (*crestin, sox10, ednrb*) and melanocytes (*tyr, dct*) (See Supplemental Table 1 for full gene sets). This is similar to that of a multipotent neural crest progenitor, and suggested that the melanoma adopted this fate.

We analyzed alterations in embryonic neural crest development using in situ hybridization(ISH). At 24hpf, BRAF^{V600E};p53$^{-/-}$ embryos exhibit an abnormal expansion in the number of *crestin*$^+$ progenitors, along with an increase in other markers from the 123 gene signature such as *spry4* and *rab3il1* (Supplemental Figure 1). By 72hpf, *crestin* aberrantly persists within the head, tail and dorsal epidermis only in BRAF^{V600E};p53$^{-/-}$ embryos (Supplemental Figure 2a). c*restin*, a zebrafish specific gene[2], is normally downregulated after terminal differentiation of neural crest progenitors[3], suggesting that activated BRAF^{V600E} promotes maintenance of multipotency in neural crest progenitors, which become expanded during tumorigenesis. In adult BRAF^{V600E};p53$^{-/-}$ melanomas, virtually all tumor cells, but no normal cells, were positive for *crestin* (Figure 1c). Only 10-15% of the melanoma cells are pigmented (Supplemental Figure 2b), consistent with the concept that adult zebrafish melanomas retain a progenitor-like state. A human melanoma tissue array showed similar findings: 75.0% were positive for the neural crest progenitor gene *ednrb,* but 12.8% for the melanocyte lineage marker *dct* (Supplemental Figure 3), in agreement with findings that most human melanomas express the neural crest marker *sox10*[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 hypothesized that chemical suppressors of neural crest progenitors would have utility in treatment of melanoma. We screened 2,000 chemicals to identify inhibitors of the *crestin*$^+$ lineage during embryogenesis. Most chemicals (90%) had minimal effect or were toxic (Supplemental Figure 4). NSC210627, a molecule of unknown function, strongly abrogated

expression of *crestin* (Figure 2a, left and middle). The chemoinformatic Discoverygate algorithm[6] revealed similarity between NSC210627 and brequinar (Supplemental Figure 5), an inhibitor of dihydroorotate dehydrogenase (DHODH)[7]. NSC210627 inhibited DHODH activity *in vitro* (Supplemental Figure 6). Leflunomide, a structurally distinct DHODH inhibitor[8], phenocopied NSC210627 (Figure 2a, right) and was used for further studies given its availability.

We examined neural crest derivatives affected by leflunomide. Treated zebrafish embryos were devoid of pigmented melanocytes at 36-48hpf (Figure 2b) and iridophores (Supplemental Figure 7a) at 72hpf. DHODH inhibition led to a loss of ventral melanocytes in stage 38 *Xenopus* embryos (Supplemental Figure 7b). Leflunomide led to a nearly complete loss of *mitf*-GFP+ cells at 24hpf (Figure 2c), reduction of myelin basic protein (*mbp*)-mCherry glial cells, and jaw cartilage disruption at 72hpf (Figure 2d and data not shown). Leflunomide reduced expression of *sox10* and *dct* while leaving other lineages such as blood and notochord less affected (Supplemental Figure 8). Microarray analysis of leflunomide treated embryos showed downregulation of 49% of the genes upregulated in the 123-gene melanoma signature, and over half of those are neural crest related (see Supplemental Table 2 for complete list).

The loss of multiple neural crest derivatives suggested that leflunomide acts on neural crest stem cells. We tested leflunomide, and its derivative A771726, on neural crest stem cells (NCSCs) isolated from the fetal(E14.5) rat gut[9, 10]. Both reduced the number of self-renewing NCSCs from primary stem cell colonies to 27+/-5.35% and 35+/-6.16% of controls (p<0.0003 and p<0.00007, t-test, Figure 2e and Supplemental Figure 9a). Colony size was reduced compared to controls (by 18% and 24%, respectively, p<0.02, t-test) but there was no effect on differentiation or survival of specific progeny (Supplemental Figure 9b,c). These results demonstrate that DHODH inhibitors negatively regulate NCSC self-renewal and affect NCSCs from multiple species.

DHODH is the fourth step in the synthesis of pyrimidine nucleotides(NTPs)[11]. We noted striking morphological similarity between leflunomide treated embryos and the *spt5/spt6* mutants[12], suggesting that leflunomide acted to suppress transcriptional elongation. We found a lack of *crestin* expression and pigmented melanocytes (similar to leflunomide) in the *spt5^{sk8}* null mutant (Supplemental Figure 10a). The expression profiles of 24hpf *spt5^{sk8}* mutants and leflunomide treated embryos[13] were nearly identical; of 223 genes downregulated after leflunomide treatment, 183 of these were similarly downregulated in *spt5^{sk8}* (Supplemental Table 3 and Supplemental Figure 10b) including neural crest genes (*crestin, sox10, mitf*) and members of the *notch* pathway (*her2, dlb*). We examined the interaction of DHODH with *spt5* using low-doses of leflunomide (3-5uM) incubated with the hypomorphic *spt5^{m806}* mutant (which has only mild melanocyte defects)[14] and then analyzing the number of pigmented melanocytes. *spt5*^{m806} embryos showed enhanced sensitivity to leflunomide (Figure 3a and Supplemental Figure 11); at 3uM, 99% of mutant embryos had few or no melanocytes compared to 0% of wildtype embryos (Supplemental Figure 11b, Kruskal-Wallis, p=0.000018). These data confirm that DHODH inhibition impacts transcriptional elongation, consistent with previous data demonstrating that 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 using qRT-PCR (Supplemental Figure 10c and Supplemental Table 4). Leflunomide caused no change or an increase of 5′ transcript abundance, but a significant downregulation of 3′ transcripts of *mitf* (5′: 3.75+/-1.19 vs.3′: 0.39+/-0.07 fold, p<0.05), and *dlb* (5′:1.13+/-0.14 vs. 3′:0.74+/-0.07 fold, p<0.05), but not in control genes such as *beta-actin* (5′:1.03+/-0.07 vs. 3′:0.99+/-0.06

fold, p=NS, t-test). In the presence of leflunomide, transcription is initiated normally, but these 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 to RNA polymerase II (Pol II), followed by sequencing (ChIP-seq). Transcriptional elongation was measured using the traveling ratio, TR[16], where the ratio of Pol II density in the promoter-proximal region is compared to the gene body. In both A375 and MAMLE-3M cells, leflunomide caused a significant inhibition of transcriptional elongation (measured as an increase in the TR), particularly for genes with an initially low TR<7.5. For example, in A375, the TR increased by >1.3 fold in 21.3% of loci; in MAMLE-3M, this was 36.3% of loci (Supplemental Table 5). Examination of pol II occupancy using metagene analysis at a variety of fold-change cutoffs (Figure 3b [A375], Supplemental Figure 12[MAMLE-3M], and Supplemental Table 5) revealed no defect in transcription initiation, but a decrease in elongation pronounced at the 3′ end of genes such as Npm1 and Ccnd1 (Figure 3c). Ingenuity Pathway Analysis on the loci affected in both cell lines revealed a strong enrichment for c-Myc targets and pathway members[17] (Supplemental Figure 13a,b). c-Myc, in addition to its requirement in neural crest development,[18] was recently shown to be a potent regulator of transcriptional pause release in ES cells[16]. Our data suggests the regulation of c-Myc target genes at the transcriptional elongation level is an operative mechanism 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 proliferation of human melanoma cell lines (A375, Hs.294t, RPMI7951; Figure 4A). Similarly, an shRNA against DHODH led to a 57.7% decrease in proliferation of A375 cells, as well as a decrease in elongation as measured by ChIP-PCR (Supplemental Figure 14). Microarray analysis of the A375 cell line treated with leflunomide revealed downregulation of genes required for neural crest development (i.e. *snai2*) and members of the *notch* pathway (e.g. *hes6, jag1*), consistent with the effects in embryos (Supplemental Table 6).

NTP production is regulated at the level of carbamoyl-phosphate synthetase (CAD)[19], the enzyme upstream of DHODH. CAD is phosphorylated by MAP kinase[20], which would be activated in melanoma due to the BRAF^{V600E} mutation. We reasoned that combined blockade of BRAF^{V600E} and DHODH would cooperate to suppress melanoma growth. We measured melanoma proliferation utilizing the BRAF^{V600E} inhibitor PLX4720[21] together with A771726 (Figure 4b,c and Supplemental Figure 15a,b), and found that the combination led to a cooperative suppression of melanoma growth. PLX4720 had no effect in non-melanoma cell lines (BRAF^{WT}, Supplemental Figure 15c). A771726 demonstrated mild antiproliferative activity but was less potent in these cells (Supplemental Figure 15d).

We examined the *in vivo* effects of leflunomide and PLX4720 using xenografts of A375 melanoma cells transplanted into nude mice (Figure 4c and Supplemental Figure 16). At 12 days post-treatment, DMSO tumors grew 7.4+/-1.3-fold, compared to 5.7+/-0.16-fold with PLX4720 and 4.7+/-0.12-fold with leflunomide. The combination of PLX4720 and leflunomide led to an enhanced abrogation of tumor growth, with only 2.2+/-0.9-fold growth, and in 40% of animals led to nearly complete tumor regression (p<0.001, PLX/LEF vs. PLX or LEF alone, ANOVA followed by Tukey's LSD). At clinically meaningful doses, we find that an inhibitor of embryonic neural crest development, leflunomide, blocks *in vivo* tumor growth in combination with the oncogenic BRAF^{V600E} inhibitor PLX4720.

Our data suggest that inhibition of DHODH abrogates transcriptional elongation of genes required for both neural crest development and melanoma growth, including *mitf* and *myc* targets. Although DHODH inhibition would be expected to lead to ubiquitous defects, human mutations in DHODH cause Miller's syndrome[22], a craniofacial disorder 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 in hematopoiesis[25], and identification of such factors in the neural crest awaits further study.

Chemical genetic approaches in zebrafish and *Xenopus* allow for identification of molecules that require *in vivo* contexts for the expression of relevant phenotypes[26]. Inhibition of DHODH may provide a unique *in vivo* mechanism for modulating transcriptional elongation. Leflunomide is a well-tolerated arthritis drug in humans[27], and our data suggest it would be most effective in combination with a BRAF^{V600E} inhibitor. This may help to overcome resistance to BRAF^{V600E} inhibitors[28]. As an increasing number of genomic changes are identified in cancer, the challenge is to target these in concert with lineage-specific factors to uncover 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 4 groups of 72hpf embryos: 1) WT, 2) *mitf*-BRAF^{V600E}, 3) p53$^{-/-}$ or 4) *mitf*-BRAF^{V600E};p53$^{-/-}$. Arrays were similarly performed on adult *mitf*-BRAF^{V600E};p53$^{-/-}$ melanomas and adjacent skin. The transcriptional signature of the melanomas was used in GSEA to identify genes significantly enriched in the *mitf*-BRAF^{V600E};p53$^{-/-}$ embryos. This signature (123 genes), enriched for markers of the neural crest, were concordantly up/downregulated in both BRAF^{V600E};p53$^{-/-}$ embryos and tumors. In situ hybridization (ISH) for *crestin* (a pan NC marker) and other NC genes, was examined in embryos (24-72hpf) and adult tumors. Chemical screening was performed to identify suppressors of the *crestin$^+$* lineage by treating wild-type embryos from 50% epiboly to 24hpf, followed by robotic ISH. Two inhibitors of DHODH abrogated *crestin* expression: NSC210627 and leflunomide. The latter was used for further studies due to more widespread availability. The effect of leflunomide on zebrafish embryonic neural crest development was assessed by scoring for embryonic melanocytes, iridophores, and glial cells. Leflunomide was further assessed for its ability to affect multipotent self-renewal of purified p75$^+$α$_4^+$ rat neural crest stem cells. The effects of leflunomide on transcriptional elongation in the neural crest was tested using the spt5^{m806} allele, and measuring pigmentation in response to 3-5uM leflunomide. Elongation in melanoma cells was assayed by ChIP-Seq using an antibody to RNA Polymerase II and measuring the traveling ratio. Leflunomide was tested for anti-melanoma effects in human melanoma cells lines in the presence or absence of the BRAF^{V600E} inhibitor PLX4720. *In vitro* proliferation assays were performed using the CellTiterGlo system (Promega). *In vivo* effects were tested by treatment of established A375 xenografts by daily intraperitoneal dosing of PLX4720 alone, leflunomide alone or the combination, and tumor growth rate measured on day 4, 7 and 12.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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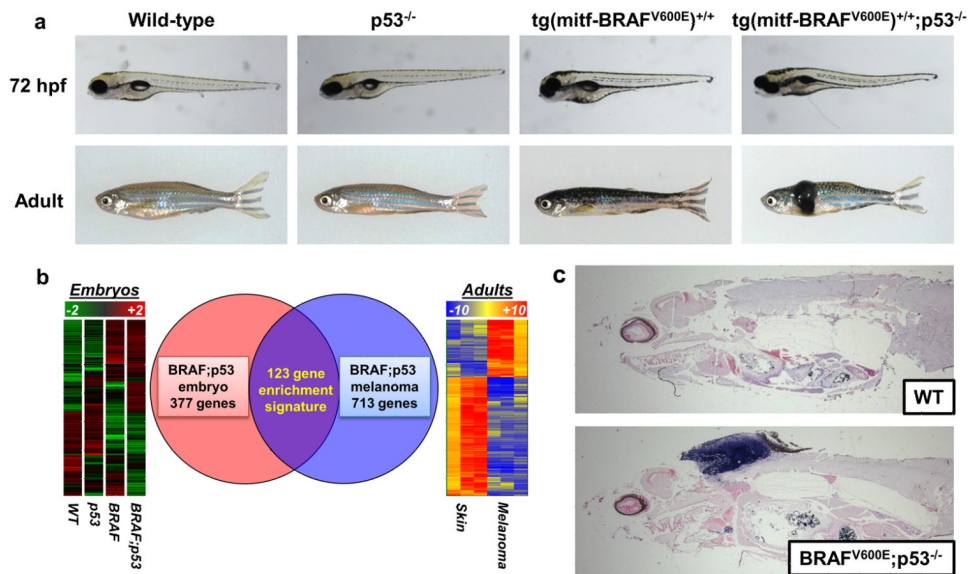
Figure 1. Transgenic zebrafish melanoma and neural crest gene expression
a, Transgenic zebrafish expressing BRAF^{V600E} under the melanocyte specific *mitf* promoter develop pigmentation abnormalities, and melanoma when crossed with p53$^{-/-}$ fish. Gross embryonic development is largely normal. b, Gene expression analysis reveals a unique gene signature at 72hpf in the BRAF^{V600E};p53$^{-/-}$ strain (left). Gene set enrichment analysis (GSEA) reveals an enrichment between the embryonic gene signature and the adult melanomas which form 4-12 months later (middle and right; see Methods for full GSEA methods). Embryo heatmap columns represent average of 3 clutches (log$_2$ scale, range -2 to +2 fold); adult heatmap columns represent individual fish (log$_2$ scale, range -10 to +10 fold). c, Sagittal section of WT and BRAF^{V600E};p53$^{-/-}$ adults reveal homogeneous *crestin* expression (blue staining) only within the dorsal melanoma, whereas it is absent in normal adult tissues.
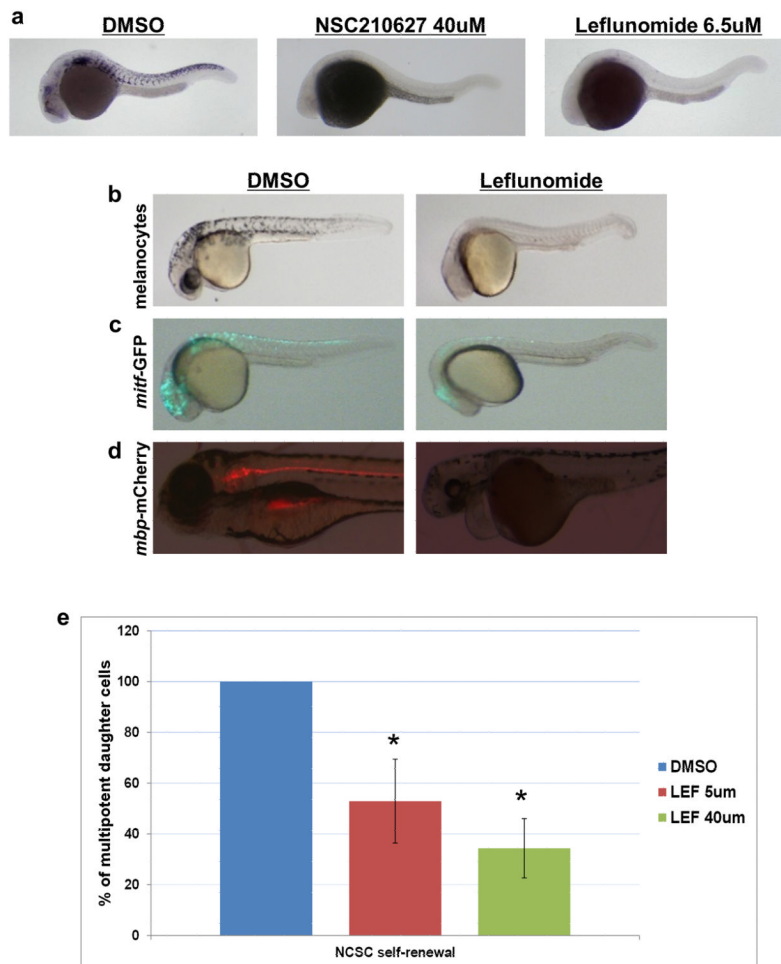
Figure 2. A chemical genetic screen to identify suppressors of neural crest development
a, A chemical genetic screen to identify suppressors of the *crestin*[+] lineage during embryogenesis identified NSC210627, a compound which completely abrogates expression by ISH (a, left and middle). The Discoverygate chemoinformatic algorithm revealed structural similarity between NSC210627 and brequinar (see Supplemental Figure 5), an inhibitor of dihydroorotate dehydrogenase (DHODH). Leflunomide, a structurally distinct DHODH inhibitor, phenocopies the *crestin* phenotype of NSC210627 (a, right). b-d, Leflunomide caused an absence of multiple neural crest derivatives, including pigmented melanocytes (b), *mitf*-GFP[+] melanocyte progenitors (c), and *mbp-m*Cherry^{+} glial cells (d). e, Leflunomide or A771726 (see Supplemental Figure 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 +/-SD of n=3 replicates; *, p<0.05 compared to control, t-test).
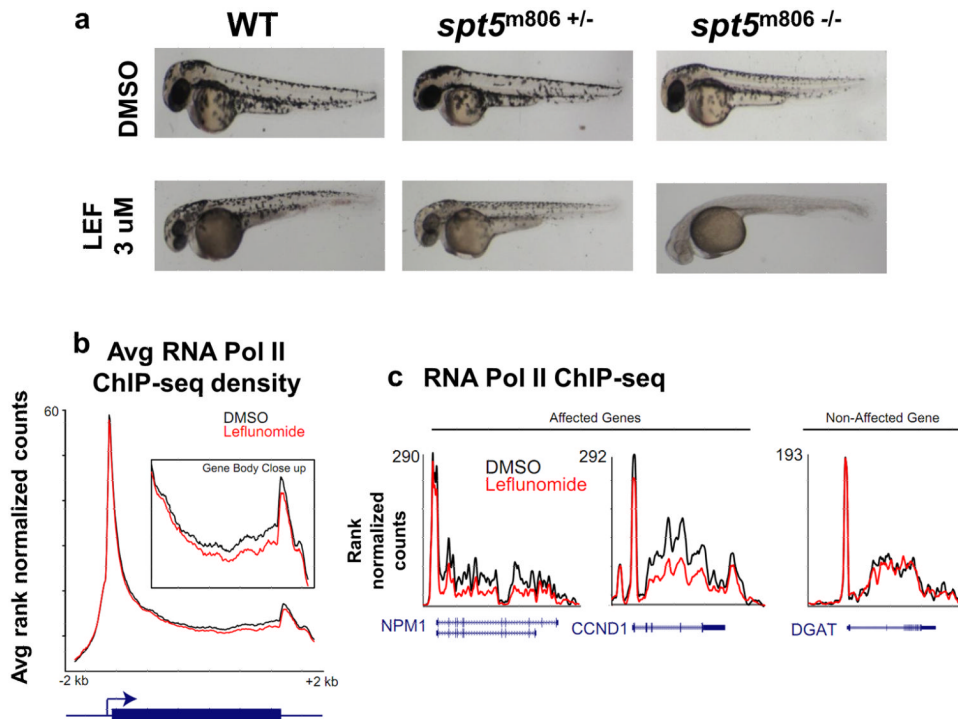
Figure 3. DHODH inhibition modulates transcriptional elongation

a, The hypomorphic *spt5*^m806 mutant has only a mild pigment defect on its own (top). Treatment with low-dose leflunomide (3uM) leads to an almost complete absence of neural crest derived melanocytes in the mutant line. See Supplemental Figure 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. Pol II occupancy at the promoter region is unaffected, but diminished at the 3′ end of the genes. Inset shows a higher magnification of the 3′ region of the genes. c, Representative examples of *myc* target genes which demonstrate defects in transcriptional elongation after leflunomide treatment, along with a non-affected gene. For Npm1, the TR in DMSO=5.04, and in LEF=8.10. For Ccnd1, the TR in DMSO=3.47, and in LEF=4.67.
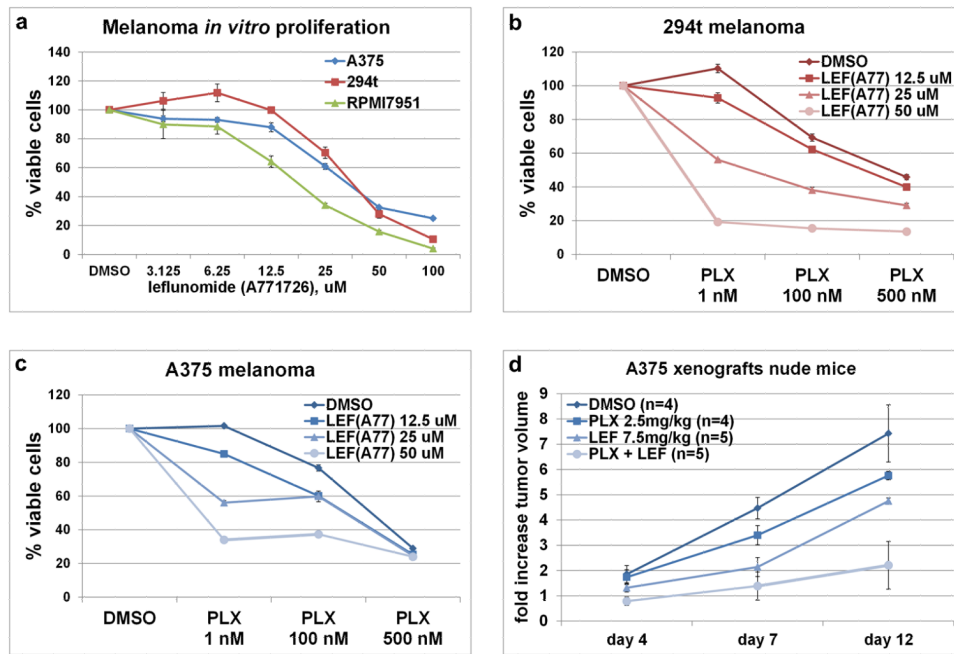
Figure 4. DHODH blockade suppresses melanoma growth in concert with BRAF^{V600E} inhibition

a, Leflunomide causes a dose-dependent decrease in melanoma proliferation as measured by CellTiterGlo assay in 3 BRAF^{V600E} melanoma cells lines tested (A375, RPMI7951, Hs. 294T). b, c Leflunomide cooperates with the BRAF^{V600E} inhibitor PLX4720 in inhibiting melanoma cell proliferation in the A375 (b) and Hs.294T (c) cell lines as well as the other tested lines (See Supplemental Figure 15). d, After subcutaneous transplantation of A375 cells (3×10^5) into nude mice, both leflunomide and PLX4720 impair tumor progression, with the combination showing a nearly complete abrogation of tumor growth and in 2/5 animals complete tumor regression. (*p=0.036 DMSO vs. PLX; **p=0.006 DMSO vs. LEF; ***p=0.006 PLX or LEF vs. PLX/LEF; PLX vs. LEF: p=NS, ANOVA followed by Tukey post-hoc analysis). Values shown are mean +/- SEM of n=3-5 replicates, as shown.