Clinical Cancer Research

MAPK Pathway Inhibitors Sensitize BRAF-Mutant Melanoma to an Antibody-Drug Conjugate Targeting GPNMB

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Abstract

Purpose: To determine if BRAF and/or MEK inhibitor–induced GPNMB expression renders melanomas sensitive to CDX-011, an antibody-drug conjugate targeting GPNMB.

Experimental Design: The Cancer Genome Atlas melanoma dataset was interrogated for a panel of MITF-regulated melanosomal differentiation antigens, including GPNMB. BRAF-mutant melanoma cell lines treated with BRAF or MEK inhibitors were assessed for GPNMB expression by RT-qPCR, immunoblot, and FACS analyses. Transient siRNA-mediated knockdown approaches were used to determine if MITF is requirement for treatment-induced GPNMB upregulation. GPNMB expression was analyzed in serial biopsies and serum samples from patients with melanoma taken before, during, and after disease progression on MAPK inhibitor treatment. Subcutaneous injections were performed to test the efficacy of MAPK inhibitors alone, CDX-011 alone, or their combination in suppressing melanoma growth.

Results: A MITF-dependent melanosomal differentiation signature is associated with poor prognosis in patients with this disease. MITF is increased following BRAF and MEK inhibitor treatment and induces the expression of melanosomal differentiation genes, including GPNMB. GPNMB is expressed at the cell surface in MAPK inhibitor–treated melanoma cells and is also elevated in on-treatment versus pretreatment biopsies from melanoma patients receiving MAPK pathway inhibitors. Combining BRAF and/or MEK inhibitors with CDX-011, an antibody-drug conjugate targeting GPNMB, is effective in causing melanoma regression in preclinical animal models and delays the recurrent melanoma growth observed with MEK or BRAF/MEK inhibitor treatment alone.

Conclusions: The combination of MAPK pathway inhibitors with an antibody-drug conjugate targeting GPNMB is an effective therapeutic option for patients with melanoma. *Clin Cancer Res; 22(24); 6088–98. ©2016 AACR.*

Introduction

Melanoma can be divided into four groups based on the prevalence of specific genetic mutations, including *BRAF, RAS, NF1,* and triple wild-type (1, 2). The mutant BRAF subtype is most common, comprising approximately 50% of all melanomas. Introduction of BRAF (vemurafenib, dabrafenib) and MEK (trametinib, cobimetinib) inhibitors has significantly affected the treatment of BRAF-mutant melanoma (3). These inhibitors elicit therapeutic responses in the majority of BRAF-mutant patients with melanoma; however, they are rarely durable. As a result, there is considerable interest in defining the molecular mechanisms that drive intrinsic or acquired resistance to BRAF and MEK inhibitors in order to devise alternate therapeutic strategies.

Overexpression of Microphthalmia-Associated Transcription Factor (MITF) promotes acquired resistance to BRAF/MEK inhibitors (4, 5). Although MITF is a master regulator of melanosomal pigmentation/function (6), it also promotes melanoma cell proliferation and is required for the maintenance of BRAF-mutant melanoma (7, 8). Patients with pigment-producing metastatic melanomas have shorter disease-specific survival compared with those with nonpigmented melanoma (9, 10). Notably, treatment with BRAF and MEK inhibitors induces melanosomal antigen expression in patients with BRAF-mutant melanoma (11). These data suggest that MITF-regulated pigmentation promotes melanoma progression in patients receiving MAPK pathway inhibitors (MAPKi). However, the role of MITF in the development of therapeutic resistance to MAPKi is complex. Recent work defined a subset of melanomas expressing very low levels of MITF, which are intrinsically resistant to MAPKi (12). Given its conflicting roles in melanoma progression, MITF remains problematic as a potential therapeutic target.

We sought to identify MITF-regulated genes that are amenable to targeted intervention. We demonstrate that Glycoprotein

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Translational Relevance

BRAF and MEK inhibitors have dramatically improved the clinical management of BRAF-mutant melanoma; however, patients receiving these treatments often develop resistance. MITF is a transcription factor required for melanoma formation and functions as a modifier of therapeutic response in patients with melanoma. Our results demonstrate that clinically approved MAPK inhibitors, which are currently used to treat patients with melanoma, cause the MITF-dependent upregulation of GPNMB. Importantly, GPNMB is the target of an antibody-drug conjugate (CDX-011) that is currently in clinical trials for the treatment of multiple cancers, including melanoma. We demonstrate that combining BRAF and/or MEK inhibitors with CDX-011 enhanced melanoma tumor regression and delayed emergence of acquired resistance when compared with treatment with MAPK pathway inhibitors alone. These findings support the initiation of clinical trials to assess the efficacy MAPK pathway inhibitors combined with CDX-011 for the treatment of melanoma.

(transmembrane) Nmb (GPNMB), a MITF target gene, is upregulated in response to MAPKi. GPNMB is a heavily glycosylated transmembrane protein that localizes to late-stage melanosomes, lysosomes, and the cell surface. Moreover, GPNMB is required for melanin production in melanocytes (13, 14). In breast cancer, GPNMB promotes breast tumor migration, invasion, and metastasis (15–17). It forms a heterodimer with EGFR to facilitate HB-EGF–mediated signals that stabilize HIF1α expression, under normoxic conditions, to promote glycolytic reprogramming and tumorigenesis (18). In melanoma, GPNMB is also required for tumor growth and metastasis, and drives tumor progression via its immunomodulatory and immune suppressive effects (19). We show that GPNMB is elevated in melanoma biopsies from patients receiving BRAF and/or MEK inhibitors and demonstrate that combining BRAF and/or MEK inhibitors with Glembatumumab vedotin (CDX-011), an antibody-drug conjugate that targets GPNMB, impairs MAPKi-mediated pigmentation, and effectively controls the growth of melanoma.

Materials and Methods

Melanoma gene expression and survival analysis

Illumina HiSeq gene expression data [The Cancer Genome Atlas (TCGA) Skin Cutaneous Melanoma dataset] were downloaded via the UCSC Cancer Genomics Browser. A MITF-dependent melanosomal signature was designed by incorporating the following genes (*MITF+GPNMB+RAB38+MCOLN3+TRPM1+SNCA+TYR+DCT+TYRP1+MLANA+RAB27A+MC1R+OCA2+SLC45A2+GPR143*). Survival data (TCGA dataset) were modified using the methods described in ref. 1. MITF-signature data were linked to survival data via patient number, and survival analysis was restricted to patients with metastatic disease at the time of TCGA tissue collection. Data were reordered on the melanosomal signature, and samples were segregated into three groups possessing "high," "medium," and "low" expression of the MITF/melanosomal signature. Associated survival data for each tumor were used to generate Kaplan–Meier survival curves. Statistical analyses were performed with MedCalc (v9) software (MedCalc Software).

Immunofluorescence

Cells on coverslips were washed with 1X PBS, fixed with 4% paraformaldehyde for 15 minutes, and permeabilized with 0.2% Triton X-100 in PBS for 10 minutes at room temperature. Cells were then incubated with a mouse anti-MITF antibody (1:200 dilution; Cat. #MS-771-P0; NeoMarkers) for 1 hour in 1X PBS containing 10% FBS and 0.1% (wt/v) saponin (Cat. #47036; Sigma). After three washes in 1X PBS, cells were incubated with Alexa 555–conjugated anti-mouse secondary antibody (1:500 dilution; Cat. #A21424; Invitrogen) and 1 μg/mL DAPI for 1 hour at room temperature. Coverslips were then washed and mounted with fluorescence mounting medium (Cat. #9990402; Thermo Scientific). Images were taken on ZEISS LSM 800 at 60X magnification. The percentage of nuclei positive for MITF staining and MITF nuclear intensity were quantified using MetaMorph Software.

Fluorescence-activated cell sorting analysis

For flow cytometric analysis, cells were stained for cell surface GPNMB expression as previously described (16). Data analysis was performed using FlowJo software (v7.5; Tree Star, Inc.).

Patient tumor specimens and serum samples

Melanoma biopsy specimens from patients were obtained (i) before treatment with BRAFi alone (vemurafenib) or a combination of BRAFi + MEKi, (ii) while patients were receiving MAPK pathway inhibitors, and (iii) after progression as indicated in Supplementary Table S1. Acquisition of tissue, and companion serum samples, was conducted under a protocol approved by the Dana-Farber/Harvard Cancer Center (legacy #11-181) in accordance with the Declaration of Helsinki.

Immunohistochemistry

Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded tumor biopsy specimens, according to standard procedures (16). A polyclonal goat anti-GPNMB antibody (1:500 dilution; Cat. #AF2550; R&D Systems) and a biotin-conjugated donkey anti-goat secondary antibody (1:500 dilution; Cat. #705-065-147; Jackson ImmunoResearch Laboratories) were employed for the staining. Sections were developed with 3-3-diaminobenzidine-tetrahydrochloride and counterstained with hematoxylin.

Quantification of kinase inhibitor–induced melanoma pigmentation

Subcutaneous WM2664 melanoma tumors were excised and fixed overnight in paraformaldehyde, prior to paraffin embedding. Sections were stained for melanin pigment using a Fontana-Masson stain (20). Stained sections were scanned, and the percentage of positive pixel intensity (2+/3+ positive pixels/total pixels) was quantified using Aperio ImageScope software (Leica Biosystems).

Results

A MITF-driven melanosomal signature correlates with poor prognosis in melanoma

Melanosomal differentiation is a multi-step process. Stage I melanosomes represent endosomal precursors, stage II–III

melanosomes are non- or partially pigmented organelles characterized by longitudinal stria, and stage IV melanosomes lack recognizable internal structures and possess an electron dense melanized lumen (ref. 21; Fig. 1A). MITF is a MAPK pathway–regulated transcription factor that controls the expression of genes involved in multiple stages of melanosomal differentiation (5, 6). We asked whether BRAF (vemurafenib) or MEK (trametinib) inhibitors could regulate the expression of MITF, and MITF-

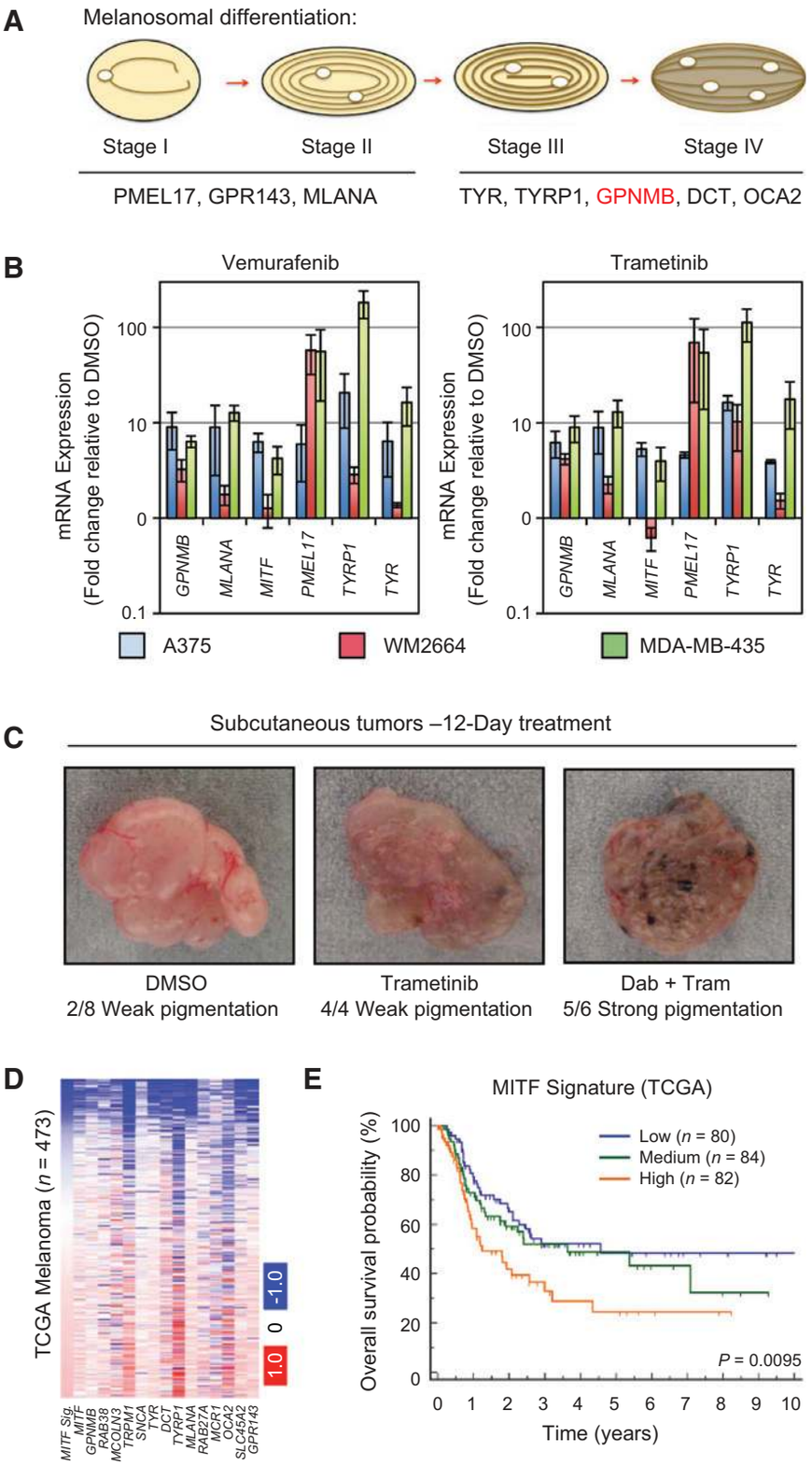

Figure 1.
A MITF transcriptional signature composed of melanosomal differentiation antigens correlates with poor outcome in patients with melanoma. **A,** Structural/enzymatic proteins and the stages during melanosomal differentiation in which they function: PMEL: premelanosome protein 17; GPR143: G protein-coupled receptor 143; MART1: melanoma antigen recognized by T cells 1; TYR: tyrosinase; TYRP1, tyrosinase-related protein 1; GPNMB: glycoprotein (transmembrane) Nmb; DCT: dopachrome tautomerase; OCA2: oculocutaneous albinism II. **B,** The mRNA expression levels of selected melanosomal differentiation antigens following 48-hour treatment of melanoma cells (A375, WM2664, MDA-MB-435) with a BRAF (Vemurafenib) or MEK (trametinib) inhibitor. Error bars represent SEM from three independent experiments. **C,** Gross images of subcutaneous WM2664 melanomas from mice treated for 12 days with vehicle (DMSO), trametinib (2 mg/kg/day), or dabrafenib (10 mg/kg/day) plus trametinib (2 mg/kg/day). **D,** Heat map depicting mRNA expression of individual melanosomal differentiation antigens within the MITF-melanosomal differentiation signature (MITF Sig.) in the TCGA dataset. **E,** Kaplan–Meier analysis of patients with melanoma separated into high, medium, or low expression of a MITF-melanosomal differentiation signature.

dependent melanosomal target genes, in a panel of BRAF-mutant melanoma cell lines. MITF and a subset of its target genes (*GPNMB*, *MART1*, *PMEL17*, *TYR*, *TYRP1*) were transcriptionally upregulated by vemurafenib and trametinib treatment (Fig 1B). Upregulation of melanosomal differentiation genes was also associated with increased pigmentation of WM2664 melanoma cells grown as subcutaneous xenografts in mice treated with BRAF/MEK inhibitors (Fig. 1C). We next analyzed a set of MITF targets, previously characterized as bona fide pigmentation genes (22), in the TCGA melanoma dataset and found that many are strongly correlated with *MITF* (Fig. 1D; Supplementary Table S2). Similar results were observed in an independent dataset comprised primarily of short-term cultures from human melanoma tumors (Supplementary Fig. S1; Supplementary Table S2). We designed a MITF-melanosomal differentiation signature encompassing each of these genes and show that an elevated signature associates with worse disease-specific survival in patients with metastatic melanoma compared with those with low [HR, 1.98; 95% confidence interval (CI), 1.22–3.21] or medium (HR, 1.60; 95% CI, 0.97–2.63) expression levels (Fig. 1E). These data suggest that MITF-mediated hyperpigmentation is upregulated in a subset of patients treated with MAPKi; and this MITF-mediated melanosomal signature correlates with aggressive disease in patients with metastatic melanoma.

GPNMB is a MITF-dependent transcriptional target that is induced by MAPK pathway inhibition

We sought to identify MITF-dependent genes within this melanosomal signature that were induced by MAPKi treatment and amenable to therapeutic intervention. Glycoprotein (transmembrane) Nmb (GPNMB) is the target of CDX-011 (Glembatumumab vedotin), an antibody-drug conjugate whose efficacy is correlated with increased cell-surface GPNMB expression. CDX-011 is in development for the treatment of melanoma and has shown clinical activity as a single agent (23). Interestingly, treatment-induced GPNMB expression occurs in multiple BRAF-mutant melanoma cells (Fig. 2A; Supplementary Fig. S2). To confirm that GPNMB is a transcriptional target of MITF in response to MAPK pathway inhibition, we performed siRNA-mediated knockdown of MITF in A375 cells treated with either vemurafenib or trametinib. *GPNMB* transcripts were induced 17.0- (vemurafenib) and 22.5-fold (trametinib) following treatment, whereas this induction was reduced to 10.6- and 10.9-fold, respectively, by diminishing *MITF* levels (Supplementary Fig. S3). These data suggest that MITF is required, in part, for MAPKi-mediated upregulation of *GPNMB* expression. Consistent with these results, treatment of WM2664 melanoma cells with either dabrafenib or trametinib resulted in a significantly increased percentage of MITF nuclear positivity and also its nuclear fluorescence intensity (Fig. 2B; Supplementary Fig. S4). MAPKi-mediated induction of GPNMB protein expression is partly MITF-dependent in BRAF-mutant A375 and WM2664 melanoma cells (Fig. 2C). We further show that MAPKi significantly increases cell surface expression of GPNMB in melanoma cells (Fig. 2D), suggesting that MAPKi-treated cells might be more sensitive to CDX-011.

GPNMB is upregulated by MAPK-inhibitor therapy in melanoma biopsies

We analyzed serial biopsies obtained from patients prior to initiation of MAPKi therapy (pretreatment), approximately 2 weeks after treatment was initiated (on-treatment) and from patients who progressed while on therapy (progression; Supplementary Table S1). We show that both *MITF* and *GPNMB* transcripts were elevated in 69% (11/16) of the on-therapy biopsies relative to the pretreatment biopsies and were coordinately regulated in 62.5% (10/16) of the samples examined (Fig. 3A). Indeed, expressions of these two genes were positively correlated ($R = 0.5271$; Supplementary Fig. S5). To further assess whether *MITF* levels were correlated with *GPNMB* and other MITF-regulated melanosomal genes, we analyzed RNA-seq data generated from a subset of these biopsy samples (24). In on-treatment ($n = 11$) and progression ($n = 6$) samples, *GPNMB* and *MITF* were again strongly correlated ($R = 0.61$), second only to *DCT* ($R = 0.80$; Supplementary Fig. S6). In comparison, *PMEL* and *RAB27A* expression levels were moderately correlated with *MITF* transcript ($R = 0.37$ and 0.41, respectively), whereas no correlation between *MLANA* or *TYR* transcripts levels and MITF expression was evident ($R = 0.09$ and 0.02, respectively; Supplementary Fig. S6). Finally, we validated that GPNMB protein levels were increased in on-treatment patient biopsies relative to pretreatment expression levels (Fig. 3B). Next, we asked whether elevated GPNMB levels were maintained in patients who had progressed on MAPKi therapy. *GPNMB* transcript levels were increased an average of 4.27-fold (Fig. 3C), and *MITF* mRNA expression was elevated 2.95-fold (Fig. 3D) in on-treatment melanoma biopsy specimens. However, following disease progression, both *MITF* (0.63-fold) and *GPNMB* (0.57-fold) transcript levels were significantly reduced relative to pretreatment levels (Fig. 3C and D). Together, these data suggest that MAPKi-induced, MITF-mediated upregulation of GPNMB expression is transient and occurs only when patients are receiving the inhibitor.

Circulating levels of the shed extracellular domain of GPNMB are elevated following MAPK pathway inhibitor treatment

The extracellular domain (ECD) of GPNMB is shed from the surface of cancer cells and can be detected in the serum of cancer patients (25, 26). We first measured shed levels of the GPNMB ECD in melanoma cell lines treated with MAPKi by ELISA. GPNMB ECD levels are enhanced 1.8 to 3.1 fold in conditioned media from melanoma cells (A375 and WM2664) treated with either trametinib or vemurafenib (Supplementary Fig. S7A). We also detected elevated levels (2.79-fold) of the shed GPNMB ECD in the serum of mice bearing subcutaneous WM2664 xenografts that were treated with trametinib when compared with vehicle-treated (DMSO) animals (Supplementary Fig. S7B). In agreement with the changes in *GPNMB* mRNA levels within patient-derived biopsies, shed GPNMB levels in the serum of patients with melanoma on-treatment with MAPK pathway inhibitors were increased relative to pretreatment levels (1.68-fold; Fig. 3E). Similarly, soluble GPNMB levels decreased as patients progressed on these therapies (Fig. 3E). These data argue that serial analysis of soluble GPNMB levels in patient sera may provide clinical utility as a noninvasive, blood biomarker to predict response to, and impending progression on, MAPKi therapy in patients with melanoma.

Intermittent MAPK pathway inhibition coupled with a GPNMB-targeted therapy effectively suppresses melanoma growth

Emerging data suggest that intermittent dosing of MAPKi forestalls the development of acquired resistance and minimizes MAPKi-related toxicity (27, 28). Given that treatment-induced

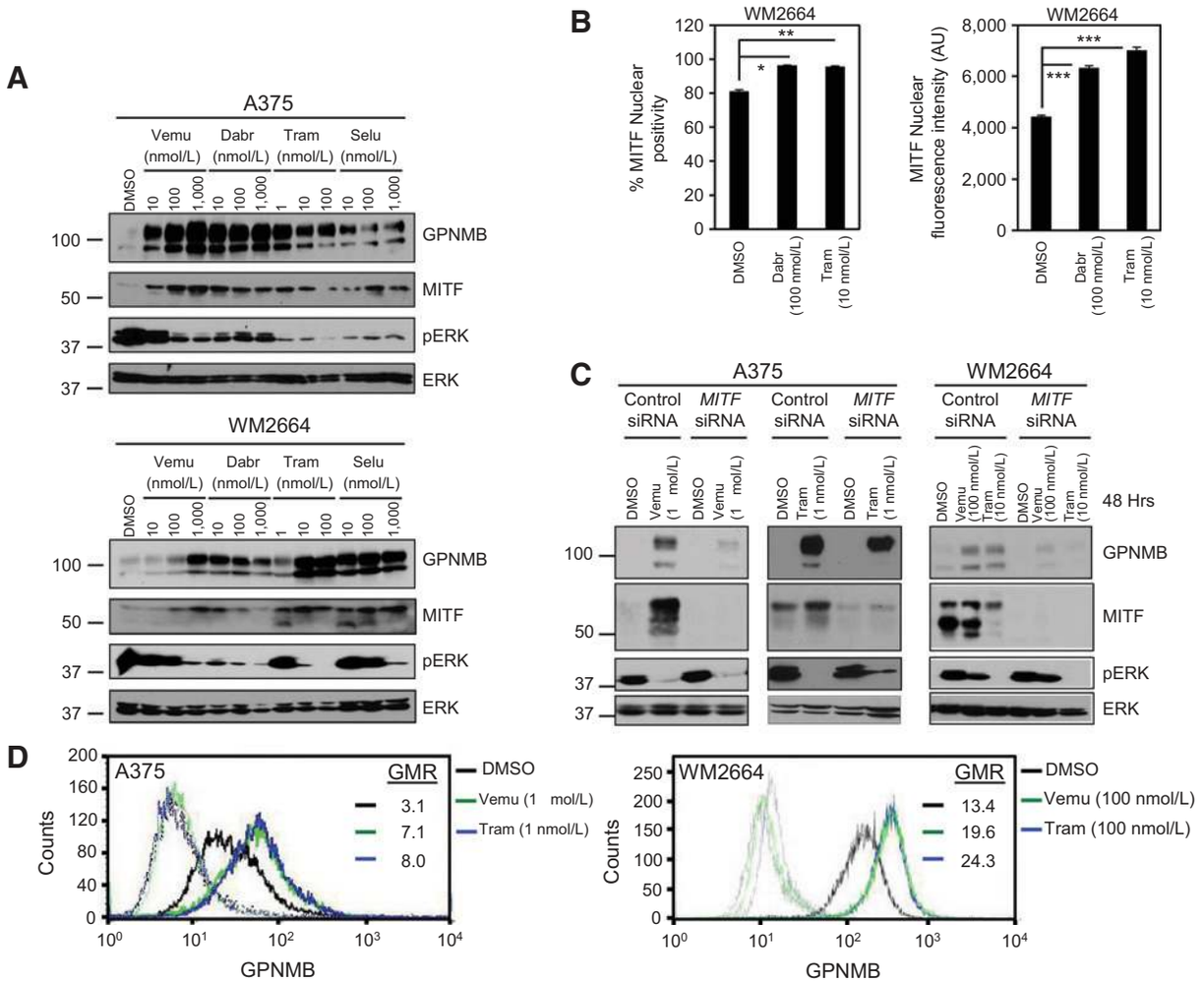

Figure 2.

GPNMB expression is upregulated in melanoma cells following MAPK pathway inhibition in a MITF-dependent manner. **A,** Immunoblot analysis of GPNMB and MITF expression in melanoma cells (A375, WM2664) following 48-hour treatment with vehicle (DMSO) or increasing concentrations of BRAF (Vemu, Dabr) or MEK (Tram, Selu) inhibitors. Inhibitor efficacy was assessed by pERK/ERK immunoblot analysis. Vemu: vemurafenib; Dabr: dabrafenib; Tram: trametinib; Selu: selumetinib. **B,** Quantification of the percentage MITF nuclear positivity and immunofluorescence intensity of nuclear localized MITF following treatment with vehicle (DMSO), Dabrafenib (Dabr; 100 nmol/L), or Trametinib (Tram; 10 nmol/L). The number of cells analyzed from three independent experiments for each condition: $n = 645$ for DMSO; $n = 351$ for Dabrafenib; $n = 361$ for Trametinib. Error bars represent SEM. *, $P < 0.001$; **, $P < 0.01$; ***, $P < 0.0001$. **C,** Immunoblot analysis of GPNMB expression in response to vehicle (DMSO), vemurafenib (Vemu), or trametinib (Tram) treatment in melanoma cells with normal (Control siRNA) or reduced (MITF siRNA) MITF levels. Inhibitor efficacy was assessed by pERK/ERK immunoblot analysis. **D,** Fluorescence-activated cell sorting analysis of cell surface GPNMB expression in DMSO (*black lines*), vemurafenib (*green lines*), or trametinib (*blue lines*) treated melanoma cells. The geometric mean ratio (GMR) indicates the fluorescence intensity (*solid lines*)/fluorescence intensity measured with the secondary antibodies alone (*dotted lines*).

GPNMB upregulation only occurs while tumors are responding to therapy, we reasoned that discontinuous MAPKi treatment in combination with CDX-011 might achieve maximal clinical benefit. Using two independent model systems (A375, WM2664), mice bearing subcutaneous xenografts were exposed to intermittent dosing of trametinib alone, CDX-011 alone, or combination of both agents. Immunoblot analysis of A375 or WM2664 tumor lysates following 7 days of trametinib treatment reveals reduced ERK phosphorylation, which correlates with increased GPNMB protein levels (Fig. 4A and B). Discontinuous trametinib treatment impaired both A375 and WM2664 tumor growth while mice received the inhibitor; however, tumor growth resumed when trametinib treatment was withheld (Fig. 4C and D). In contrast, continuous daily trametinib initially resulted in a marked impairment of tumor growth; however, tumors acquired resistance to MAPKi 4 weeks after treatment and started growing progressively, albeit slower than their vehicle-treated counterparts (Fig. 4D). A375-derived tumors were modestly growth-inhibited by CDX-011 alone (Fig. 4C), whereas, the addition of CDX-011, on day 3 of each intermittent trametinib cycle, significantly impaired WM2664 tumor growth even during the "off-treatment" week (Fig. 4D). Mice bearing A375 tumors treated with a combination of trametinib/CDX-011 experienced significant tumor regression relative to either agent alone ($P < 0.0001$ vs. trametinib;

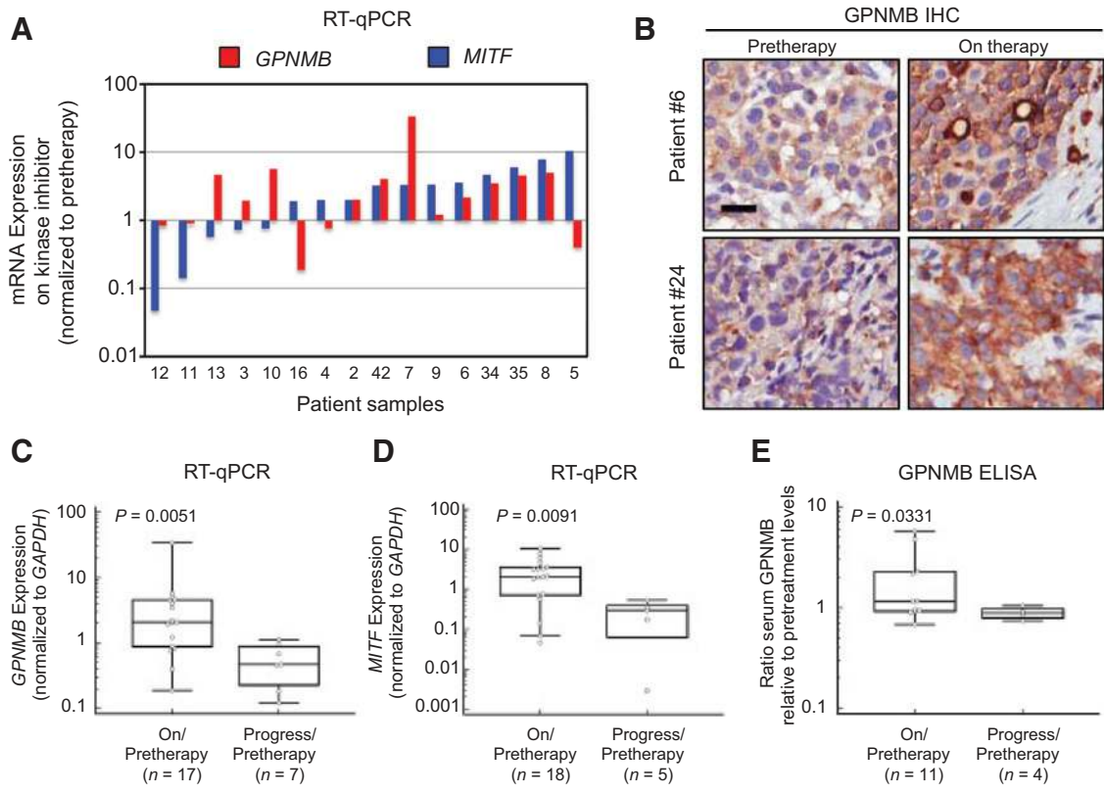

Figure 3.

GPNMB mRNA and protein expression is increased in patients with melanoma undergoing treatment with MAPK pathway inhibitors. **A,** RT-qPCR of *MITF* and *GPNMB* expression performed on melanoma biopsy specimens taken from patients prior to treatment (pretherapy) and while receiving therapy (on-treatment). The data are expressed as the on-treatment/pretreatment ratio for *MITF* (blue) and *GPNMB* (red). **B,** Immunohistochemical (IHC) staining for GPNMB in select patients prior to initiation of therapy and while receiving MAPK inhibitors. Scale bar represents 20 μm and applies to all plots. **C** and **D,** RT-qPCR analysis of *GPNMB*(**C**) and *MITF* (**D**) Expression performed on RNA extracted from pretreatment, on-treatment, and progression melanoma biopsy specimens from patients with melanoma undergoing MAPKi therapy. The data are presented as on-therapy/pretherapy or progression/pretreatment ratios. **E,** ELISA analysis of soluble GPNMB ECD in pretreatment, on-treatment, and progression serum samples from patients with melanoma undergoing MAPK pathway inhibitor therapy. The data are presented as on-therapy/pretherapy or progression/pretreatment ratios.

$P < 0.0001$ vs. CDX-011; Fig. 4C), and 33% of these mice achieved complete response (CR), compared to 0% CR in the other groups (Supplementary Table S3). More striking results were observed in the WM2664 cohort. Tumors treated with a combination of intermittent trametinib and CDX-011 continuously regressed with each treatment cycle and were significantly smaller than mice treated with either intermittent ($P < 0.0001$) or continuous trametinib ($P = 0.0061$) alone (Fig. 4D). Indeed, 50% of melanomas (WM2664) treated with the combination therapy achieved CR, which was not observed in any of the other treatment groups (Supplementary Table S3). Taken together, these data show that CDX-011 enhances melanoma tumor regression when used in combination with MAPK pathway inhibitors.

Addition of CDX-011 to continuous dabrafenib and trametinib treatment inhibits MAPKi-mediated pigmentation and impairs the development of acquired resistance

Most patients with advanced BRAF-mutant melanoma currently will receive a combination of BRAF and MEK inhibitors in a continuous fashion until their tumors show signs of progression. Therefore, we examined whether addition of CDX-011 to this standard regimen would provide additional clinical benefit. WM2664 cells grew aggressively in vehicle-treated mice, whereas tumor growth was delayed in the cohort of mice receiving CDX-011 injections (Fig. 5A). In this case, WM2664 tumors treated with CDX-011 alone showed progressive tumor growth at a slower rate relative to vehicle-treated tumors. Combined dabrafenib and trametinib treatment suppressed melanoma growth for 6 weeks, at which time the tumors reinitiated growth, reaching 150% of the initial tumor volume, suggesting that they acquired resistance to dabrafenib/trametinib combination therapy (Fig. 5A). A combination of dabrafenib, trametinib, and CDX-011 resulted in the progressive shrinkage of melanomas (Fig. 5A). These tumors were significantly smaller than tumors treated with CDX-011 ($P < 0.0001$) or dab/tram alone ($P < 0.0001$). We confirmed that combined dabrafenib and trametinib treatment effectively reduced pERK levels in tumors following 2 weeks of continuous inhibitor treatment, which again coincided with increased GPNMB expression (Fig. 5B). At experimental endpoint (9 weeks), WM2664-derived tumors treated continuously with dab/tram had regained ERK phosphorylation, which was coincident with progressive growth of these tumors, suggesting the emergence of acquired resistance. However, the tumors treated with dabrafenib,

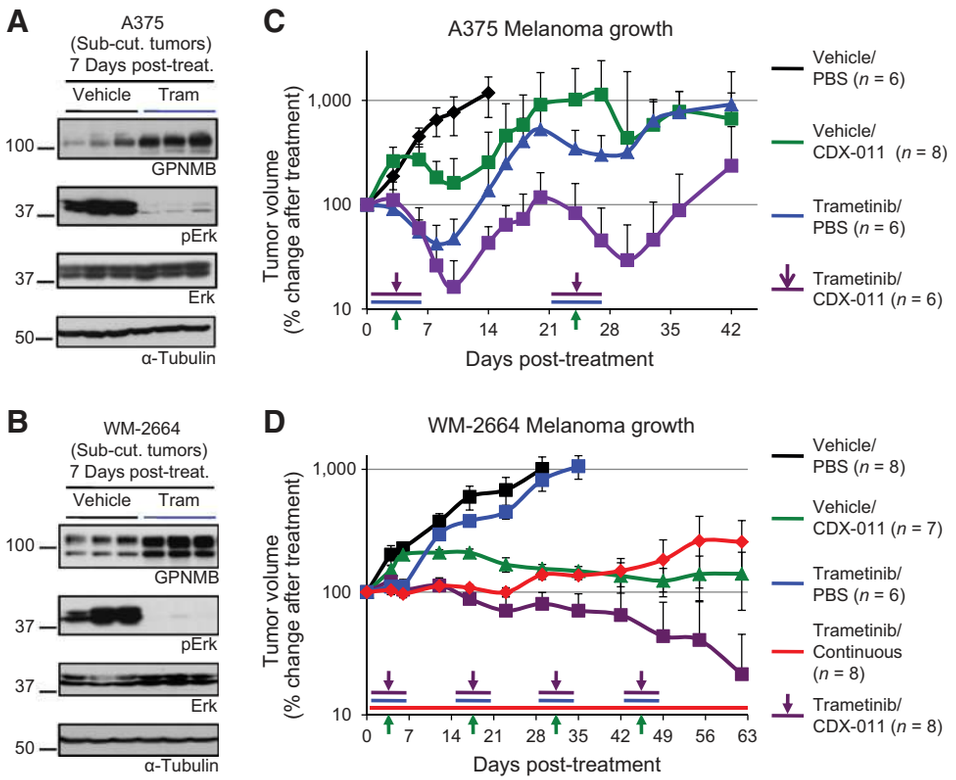

Figure 4.

Combination of intermittent MAPK pathway inhibition and CDX-011 effectively impairs melanoma growth. **A** and **B,** Tumor lysates derived from the subcutaneous injection of A375 and WM2664 melanoma cells were subjected to immunoblot analysis for GPNMB, pERK, and ERK. Tumor lysates were prepared following 7 days of treatment with trametinib or vehicle alone. Immunoblots for α-tubulin served as a loading control. **C** and **D,** Subcutaneous tumor growth of A375 and WM2664 melanoma cells in mice treated with vehicle (*black line*), CDX-011 alone (*green line*), discontinuous trametinib alone (*blue line*), continuous daily trametinib alone (*red line*, **D**) and a combination of discontinuous trametinib plus CDX-011 (*purple line*). All treatments were started when subcutaneous tumors reached 250 mm^3 and were administered as follows: CDX-011 (*green arrows below x-axis*) was administered as a single agent once every 3 weeks (**C**) or once every 2 weeks (**D**); Continuous daily trametinib treatment (**D,** *red line*); discontinuous trametinib treatment (*blue line*) was administered daily for 1 week on and 2 weeks off inhibitor (**C**) or 1 week on and 1 week off inhibitor (**D**); Discontinuous trametinib treatment with CDX-011 administered during the mid-point of the on-treatment cycle (*purple line*). In all cases, tumor growth is plotted as the percentage change in tumor volume relative to the size of the tumors when treatment was initiated.

trametinib, and CDX011 showed relatively impaired ERK reactivation (Fig. 5B), which suggests that addition of CDX-011 to dab/tram inhibits the development of acquired resistance to dual MAPKi therapy. Finally, to determine whether targeting GPNMB affected MAPKi-mediated pigmentation, we assessed melanin production by Fontana-Masson staining of WM2664 melanoma tumors at experimental endpoint. Mice bearing WM2664 tumors treated with either vehicle or CDX-011 alone stained very weakly for melanin, whereas tumors treated with dabrafenib/trametinib showed intense melanin staining (6-fold increase in dabrafenib/trametinib-treated vs. vehicle-treated mice). Notably, tumors treated with CDX011 in addition to dabrafenib/trametinib were markedly impaired in their ability to produce melanin (3-fold increase in dabrafenib/trametinib/CDX-011 vs. vehicle-treated mice) relative to dabrafenib/trametinib-treated tumors (Fig. 5C).

Discussion

Previous data indicate that patients with pigmented melanomas experience worse outcomes compared with those with amelanotic melanomas (9, 10). MAPK pathway inhibitors enhance melanosomal gene expression (11), and our data confirm that melanoma cells respond to MAPK pathway inhibitors by exhibiting increased pigmentation and upregulating several melanosomal differentiation antigens. We show that patients with metastatic melanomas whose tumors display strong expression of a MITF-driven melanosomal gene signature experience worse overall survival. Indeed, MITF expression is reversibly enhanced by MAPK inhibitors in melanoma and is associated with a drug-tolerant phenotype that allows melanomas to acquire resistance to these inhibitors (29).

Glycoprotein (transmembrane) Nmb (GPNMB) is a normal constituent of melanosomes and is required for UVB or endothelin-1–stimulated melanogenesis (13, 14). Our data demonstrate that GPNMB levels are induced in BRAF-mutant melanoma cells in response to clinically indicated BRAF and MEK inhibitors; this is in agreement with previous studies that employed experimental MAPK pathway inhibitors (30). We observe a coincident increase in MITF expression, which is required for elevated GPNMB levels. MITF stability and localization is tightly controlled. Sustained MAPK signaling can lead to a reduction in MITF levels via

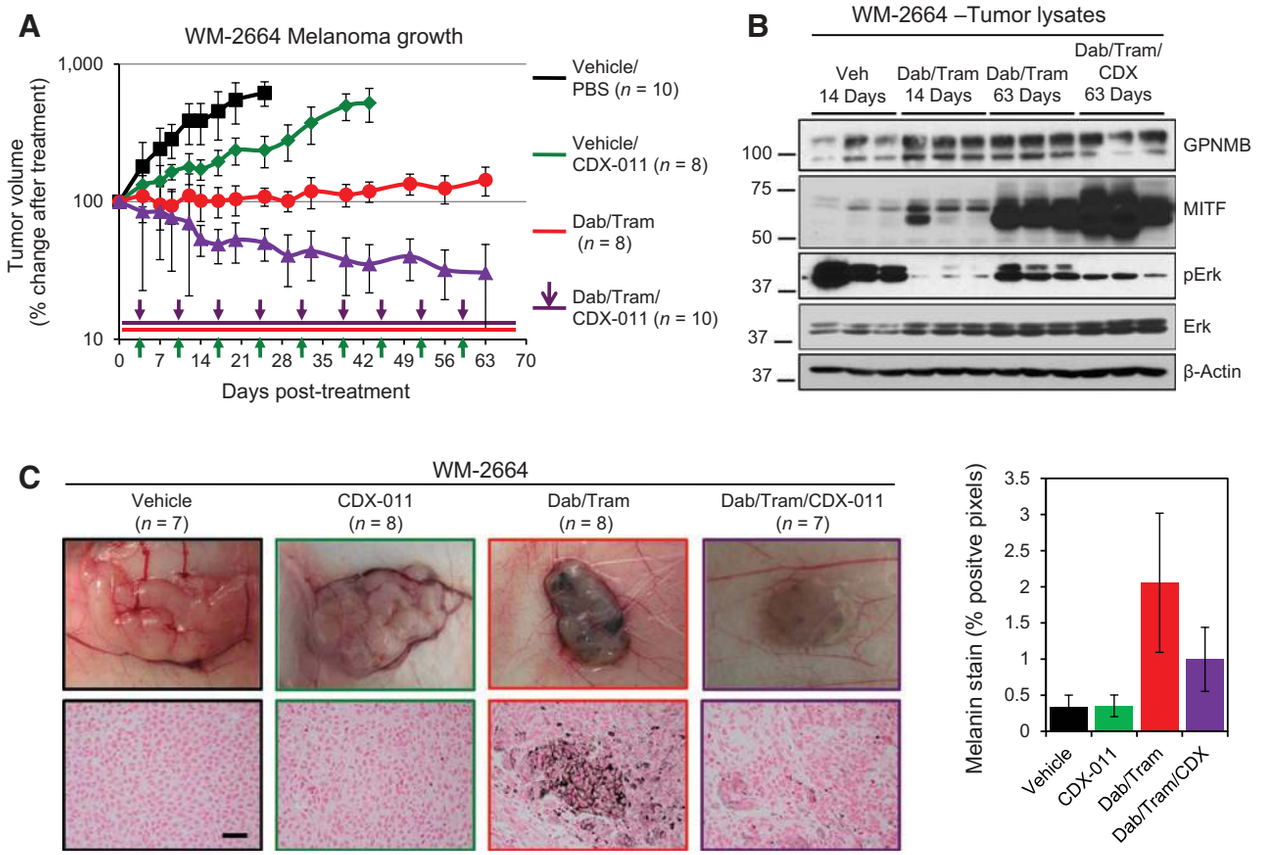

Figure 5.

Continuous treatment with BRAF/MEK inhibitors and CDX-011 significantly impairs melanoma growth. **A,** Subcutaneous tumor growth of WM2664 melanoma cells in mice treated with vehicle (*black line*), CDX-011 alone (*green line*), continuous dabrafenib + trametinib (*red line*), and a combination of continuous dabrafenib/trametinib plus CDX-011 (*purple line*). Treatments were started when subcutaneous tumors reached 400 mm^3 and were administered as follows: CDX-011 was administered once weekly as a single agent (*green arrows*); daily dabrafenib/trametinib treatment (*red line*); daily dabrafenib/trametinib treatment with CDX-011 administered once weekly (*purple line*). In all cases, tumor growth is plotted as the percentage change in tumor volume relative to the size of the tumors when treatment was initiated. **B,** Tumor lysates from WM2664 melanomas were subjected to immunoblot analysis for GPNMB, MITF, pERK and ERK, and β-actin following 2 weeks of treatment with vehicle alone, dabrafenib, and trametinib or at experimental endpoint (9 weeks) from mice treated with dabrafenib and trametinib +/- CDX-011. **C,** Gross images of subcutaneous WM2664 melanomas from mice treated with vehicle, CDX-011, dabrafenib/trametinib (Dab/Tram), or dabrafenib/trametinib/CDX-011 (Dab/Tram/CDX-011). Fontana-Masson stained sections are shown and quantification of the degree of pigmentation is indicated below each image. Scale bar, 100 μm and applies to all sections.

proteosomal degradation (5). Thus, reduced MAPK pathway activation following treatment with BRAF or MEK inhibitors may stabilize MITF and subsequent induction of melanosomal differentiation genes, including GPNMB (Fig. 6). Our data reveal that treatment-induced GPNMB expression is reversible, and lasts only during MAPK-inhibitor treatment. This is consistent with previous observations showing that other melanosomal genes, including *MLANA*, *TYRP1*, and *TYRP2*, are transiently upregulated by MAPKi early during treatment, and return to or fall below pretreatment levels at progression (11). As melanomas develop resistance to MAPKi therapy, these treatments are suspended and melanosomal gene expression, including GPNMB levels diminish. As such, any melanosomal-targeted therapies would be more beneficial if implemented early during MAPKi therapy instead of after MAPKi resistance is established. Of note, we show that elevated serum levels of GPNMB are detectable in melanoma-bearing mice and patients with melanoma that are undergoing MAPKi therapy, which coincides with increased GPNMB expression in the tumors. Thus, serum GPNMB may serve as a biomarker to predict benefit of adding CDX-011 to MAPKi therapy, and warrants further investigation in the clinic.

GPNMB is the target of an antibody-drug conjugate (CDX-011, Glembatumumab vedotin) that is currently in clinical trials for the treatment of metastatic melanoma (NCT02302339; ref. 23). Our observations reveal intermittent dosing of MAPK pathway inhibitors, when combined with CDX-011, provide superior control of melanoma growth compared with continuous or intermittent treatment of MAPK inhibitors alone. One of the issues facing continuous administration of BRAF and/or MEK inhibitors is the cumulative toxicities associated with this treatment approach. Dose reduction or intermittent dosing has been implemented to manage these toxicities (31, 32). Moreover, results using preclinical models suggest that intermittent dosing may represent a strategy to forestall the onset of therapeutic resistance to BRAF inhibitors (27). Our data suggest that the addition of CDX-011 to an intermittent MAPKi dosing regimen

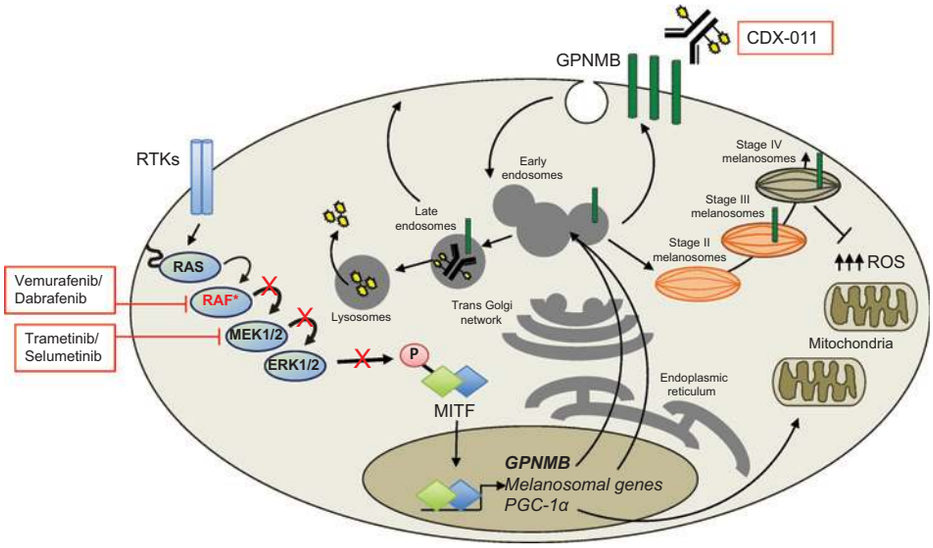

Figure 6.

Combination therapy targeting the MAPK pathway and GPNMB in BRAF-mutant melanoma. BRAF mutations (*) are present in approximately 50% of melanomas, which constitutively activate the MAPK pathway. Downstream ERK activation leads to MITF phosphorylation, resulting in decreased MITF protein stability and cytoplasmic retention. Clinically approved MAPK pathway inhibitors targeting BRAF (vemurafenib, dabrafenib) or MEK (trametinib, selumetinib, cobimetinib) stabilize and increase MITF nuclear localization. Nuclear MITF transcriptionally regulates many melanosomal genes, including GPNMB, which causes an increase in melanosome biogenesis and hyperpigmentation. MITF also induces PGC-1α expression, a master regulator of mitochondrial biogenesis. Thus, MAPKi-treated melanoma cells undergo a metabolic shift from anerobic glycolytic metabolism to aerobic oxidative metabolism, leading to increased oxygen consumption and ROS production. Elevated melanin associated with increased melanosome biogenesis can serve as a scavenger for excess ROS produced during a MAPKi-induced switch to oxidative phosphorylation. GPNMB is a transmembrane glycoprotein present in endosomes, late-stage melanosomes, and at the plasma membrane. Cell surface GPNMB is the target of the antibody-drug conjugate, CDX-011 (Glembatumumab vedotin). The GPNMB:CDX-011 complex is internalized and traffics to lysosomes, at which point the linked cytotoxin, monomethyl auristatin E (MMAE), is liberated from the antibody by proteolytic cleavage of an amino-acid linker. MMAE is a potent inhibitor of tubulin dynamics and causes apoptotic cell death.

could forestall resistance while limiting melanoma growth during off-treatment periods.

Addition of CDX-011 to dabrafenib/trametinib leads to impaired MAPKi-mediated pigmentation and robust tumor regression. These data suggest that melanosomes and/or melanin may serve a functional role in facilitating tumor growth in the setting of MAPK pathway inhibition, and targeting the molecular mediators of this process is sufficient to impair the development of intrinsic or acquired resistance to MAPKi. It has been reported that melanosomes can sequester cytotoxic drugs, thereby limiting the efficacy of these agents against malignant melanoma (33). Antagonizing melanosome formation promotes chemotherapeutic sensitivity, whereas stimulating melanosome formation enhances resistance to chemotherapy in melanoma cells (34, 35).

An additional mechanism by which melanin synthesis may promote melanoma growth in response to treatment is by modulating the oxidative state within melanoma cells. Treatment of BRAF-mutant melanoma with MAPK inhibitors supresses glycolysis and induces oxidative phosphorylation, the latter resulting from increased mitochondrial biogenesis (36). PGC-1α, which is a master regulator of mitochondrial biogenesis, is upregulated in response to MAPKi, in a MITF-dependent fashion, and promotes melanoma resistance to MAPKi (36, 37). Thus, MITF controls both the expression of a melanosomal signature and the switch from glycolytic to oxidative metabolism in response to MAPKi (38). Moreover, in a similar fashion to our melanosomal signature, melanoma patients with high expression of a mitochondrial biogenesis signature also experience poor survival (39).

Elevated production of reactive oxygen species (ROS) accompanies increased engagement of oxidative phosphorylation. However, the relationship between melanin and the oxidative state within cells is complex as melanin exhibits both pro-oxidant and anti-oxidant properties. Melanin synthesis involves oxidation steps and ROS generation; however, much of the ROS that is generated remains confined to the melanosomes (40). Conversely, enzymes involved in melanin synthesis, such as DCT, are capable of inducing anti-oxidants, such as glutathione (41). Furthermore, melanin itself can directly act as a scavenger for excess ROS (42, 43). In agreement with a treatment-induced metabolic shift toward oxidative phosphorylation, short-term MAPKi treatment of BRAF-mutant melanoma initially down regulates HIF1α and its glycolytic target genes (44). However, at tumor progression, glycolysis-promoting HIF1α target genes were again upregulated, indicating that glucose metabolism is restored in melanoma cells that develop resistance to MAPK pathway inhibitors (44). Interestingly, stimulation of melanogenesis promotes HIF1α stabilization and upregulation of target genes, such as *GLUT-1*, *PDK-1*, and *HK2* that promote glucose metabolism (45). Together, these observations demonstrate that metabolic programming is dynamic in response to targeted inhibitors and that pigmented melanomas may be better equipped to deal with MAPKi-induced ROS production. Furthermore, pigmentation may contribute to the re-emergence of protumorigenic glycolytic metabolism that accompanies MAPKi resistance.

Emerging literature has implicated melanin production, or more specifically an increased pheomelanin:eumelanin ratio, with melanomagenesis (46, 47). Animal studies have shown that spontaneous melanoma arises only in pigment producing, but not albino, HGF transgenic mice (46). Thus, in the context of excessive ROS produced by a MAPKi-mediated shift to oxidative phosphorylation, it is possible that the protumorigenic properties of melanin outweigh the potentially deleterious prooxidants associated with melanin synthesis (Fig. 6). This hypothesis is supported by the observation that patients with pigmented melanoma experience shorter survival times than those with amelanotic melanoma following exposure to oxidizing radiotherapy (48).

Our data contribute to this growing field by showing that MAPKi therapy induces melanosomal gene expression and hyperpigmentation in a subset of BRAF-mutant melanomas, and this melanosomal signature correlates with poorer survival among patients with metastatic melanoma. We confirm that MAPKi treatment induces hyperpigmentation *in vivo*, which can be disrupted with an antibody-drug conjugate (CDX-011) targeting GPNMB. We argue that such a strategy can impair MAPKi-mediated pigmentation and effectively induce tumor regression, while delaying the emergence of resistant melanoma. Strategies involving intermittent or continuous dosing of MAPKi in conjunction with CDX-011 may improve efficacy while affording flexibility to reduce cumulative toxicities associated with MAPKi currently used in the treatment of melanoma.

Disclosure of Potential Conflicts of Interest

T. Keler holds ownership interest (including patents) in Celldex Therapeutics, Inc. No potential conflicts of interest were disclosed by the other authors.

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