Targeting Extracellular Matrix Remodeling Restores BRAF Inhibitor Sensitivity in BRAFi-resistant Melanoma

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ABSTRACT

Purpose: The extracellular matrix (ECM) is an intriguing, yet understudied component of therapy resistance. Here, we investigated the role of ECM remodeling by the collagenase, MT1-MMP, in conferring resistance of v-Raf murine sarcoma viral oncogene homolog B1 (BRAF)-mutant melanoma to BRAF inhibitor (BRAFi) therapy.

Experimental Design: Publicly available RNA-sequencing data and reverse phase protein array were used to determine the relevance of MT1-MMP upregulation in BRAFi-resistant melanoma in patients, patient-derived xenografts, and cell line–derived tumors. Short hairpin RNA (shRNA)-mediated knockdown of MT1-MMP, inhibition via the selective MT1-MMP/MMP2 inhibitor, ND322, or overexpression of MT1-MMP was used to assess the role of MT1-MMP in mediating resistance to BRAFi.

Results: MT1-MMP was consistently upregulated in posttreatment tumor samples derived from patients upon disease progression and in melanoma xenografts and cell lines that acquired resistance to BRAFi. shRNA- or ND322-mediated inhibition of MT1-MMP synergized with BRAFi leading to resensitization of resistant cells and tumors to BRAFi. The resistant phenotype depends on the ability of cells to cleave the ECM. Resistant cells seeded in MT1-MMP uncleavable matrixes were resensitized to BRAFi similarly to MT1-MMP inhibition. This is due to the inability of cells to activate integrinβ1 (ITGB1)/FAK signaling, as restoration of ITGB1 activity is sufficient to maintain resistance to BRAFi in the context of MT1-MMP inhibition. Finally, the increase in MT1-MMP in BRAFi-resistant cells is TGFβ dependent, as inhibition of TGFβ receptors I/II dampens MT1-MMP overexpression and restores sensitivity to BRAF inhibition.

Conclusions: BRAF inhibition results in a selective pressure toward higher expression of MT1-MMP. MT1-MMP is pivotal to an ECM-based signaling pathway that confers resistance to BRAFi therapy.

Introduction

Melanoma is the deadliest form of skin cancer, with an estimated death toll of 6,850 patients in 2020 in the United States and costing approximately $3 billion in treatment. Metastatic melanoma is one of the most aggressive forms of cancer, with a 5-year survival of 16%–20% for distant metastasis. Despite recent advances in melanoma treatment, its incidence is increasing annually. These economic and demographic trends underscore the necessity for developing new therapies to complement current treatment methods, particularly those that target metastasis (1).

In approximately 50% of all melanomas, a mutation in the v-Raf murine sarcoma viral oncogene homolog B1 (BRAF) has been found (2). This results in the constitutive activation of BRAF, resulting in the overactivation of the MAPK growth pathway and melanoma proliferation. Inhibitors specific to mutant BRAF have impressive response rates of 80% initially; however, after 6 months, most patients relapse with BRAF inhibitor(BRAFi)-resistant melanoma, even in combination with an MEK inhibitor (3–6).

Several mechanisms of resistance have been identified mostly consisting of the rewiring of several survival pathways independent of BRAF in tumor cells (4, 7–10). A potential role of the tumor microenvironment, specifically of the extracellular matrix (ECM), in the resistant phenotype has also been suggested. In the work by Fedorenko and colleagues (11), fibronectin was found increased in BRAF-resistant cells and partly responsible of BRAFi resistance through the activation of integrin α5β1/AKT signaling. Also, paradoxical activation of melanoma-associated fibroblasts by PLX4720 has been linked to the promotion of matrix production and induction of integrinβ1 (ITGB1)/FAK/Src signaling in melanoma cells, providing a mechanism of resistance (12).

While activation of integrins may provide survival cues that can promote resistance, the mechanisms linking ECM production and integrin activation to BRAFi resistance remain undescribed. Here, we provide evidence that BRAFi-resistant cells and tumors selectively upregulate the metalloproteinase, MT1-MMP. MT1-MMP is a major collagenase essential for the cleavage and activation of collagen I, II,

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Translational Relevance

While v-Raf murine sarcoma viral oncogene homolog B1 (BRAF) inhibitor (BRAFi) therapies have dramatically changed the outlook for many patients with BRAF-mutant metastatic melanoma, the vast majority of patients inevitably develop resistance. We have previously shown that MT1-MMP inhibition significantly reduces the ability of melanomas to metastasize. Here, we highlight a novel mechanism of resistance to BRAFi involving MT1-MMP. By using BRAFi-resistant xenografts, we demonstrate that targeting of MT1-MMP by a selective MT1-MMP catalytic inhibitor (ND322) with high selectivity toward the target, and low toxicity, can effectively resensitize tumors to BRAFi treatment. MT1-MMP inhibition disrupts the activation of integrinβ1 (ITGB1)/FAK signaling by blunting the cleavage of collagen, a major ligand of ITGB1. This result suggests that targeted inhibition of MT1-MMP by ND322 may be used in the clinic in combination with BRAFi, to prevent both metastasis and treatment resistance.

and III, as well as other substrates such as EGF, CD44, Notch1, and fibronectin, and the invasion promoting, MMP2 (13–20). MT1-MMP has been shown to lead to the activation of ITGB1 via collagen processing (21). We have previously shown that MT1-MMP is highly expressed in melanoma, where it drives invasion and metastases (22, 23); and that inhibition of MT1-MMP by either RNAi or the selective catalytic inhibitor, ND322, significantly impairs metastatic dissemination in a melanoma orthotopic model (22, 24). Here, we demonstrate that BRAFi-resistant cells selectively upregulate ECM components such as collagen and fibronectin, as well as MT1-MMP, their major processing enzyme, via upregulation of TGFβ signaling. Inhibition of MT1-MMP via RNAi or ND322 restores sensitivity to BRAFi in previously resistant cells and tumors. MT1-MMP–dependent resistance to BRAFi is mediated by its ability to remodel the ECM and activate ITGB1 signaling. Thus, severing the interaction of melanoma cells with the supporting ECM by inhibiting MT1-MMP function is an effective means to simultaneously inhibit melanoma growth, metastasis, and treatment resistance.

Materials and Methods

Chemicals

PLX4720 (Vemurafenib) was provided by LC Laboratories and dissolved in DMSO or incorporated into animal chow at a concentration of 200 ppm (OpenSource Diets; ref. 25). ND322 was synthesized as described previously (26). ND322 was solubilized in DMSO in 10 mmol/L stocks and used at 0.32 μmol/L for *in vitro* work. For *in vivo* work, ND322 was solubilized with 25% DMSO, 45% propylene glycol, and 30% H_2O for mouse subcutaneous injections at a dose of 25 mg/Kg once daily. The TGF-receptor type I/II dual inhibitors, LY21109761 and LY364947, were purchased from Selleck Chemicals, dissolved in DMSO, and used at 10 μmol/L concentration in cell cultures.

Viral plasmids and transductions

Short hairpin RNAs (shRNA) against MT1-MMP (TRCN-0000050855 and TRCN0000050856), MMP2 (TRCN00000051526 and TRCN00000051527), and MMP9 (TRCN0000373008) were purchased from Sigma. shMT1-MMP and shMMP2 were described previously (24). MT1-MMP overexpression plasmids were described previously (22). ITGB1 overexpression plasmids were a generous gift from Dr. Valerie Weaver (University of California, San Francisco, CA) and were described previously (21). Plasmids were transfected in HEK-293T (from the ATCC) using XtremeGene-9 (Sigma) to produce viral particles. Supernatant was collected and viral transduction onto primary melanoma cells using 8 μg/mL polybrene was done. Cells were selected using 2 μg/mL puromycin.

Cell lines

The human melanoma cell lines A375, K457, V2387, WM266-4, WM115, 1205Lu, and WM9 acquired resistance to PLX4720 after the chronic treatment with PLX4720 at 5 μmol/L for 1–2 months until no cell death was observed, as described in (27). WM793 and WM164 pairs of parental/resistant cells were a gift from Dr. Keiran Smalley (Moffitt Cancer Institute, Tampa, FL). Resistant cells were designated A375R, WM793R, K457R, V237R, 1205luR, WM9R, WM164R, and WM164RR [dual resistance to BRAFi/MEK inhibitor (MEKi)]. Cells were routinely tested for *Mycoplasma* once a month. Cells were used within a month from thawing.

Reverse phase protein array

Biopsies were collected from xenograft melanoma tumors derived from A375 and 1205Lu cells and from patient-derived xenograft (PDX) lines, WM4007 and WM3929, before and after *in vivo* treatment with PLX4720 or PLX4720 + PD0325901. Samples were prepared as described in (25) and submitted to the University of Texas MD Anderson Center Reverse Phase Protein Array (RPPA) core facility (Houston, Tx) as described in (28), and data were reported as normalized $\log_2$. MT1-MMP expression levels as well as levels of phospho-tyrosine-397-FAK, phospho-serine-473-AKT, and p42/44-MAPK were analyzed.

Western blotting

Cell seeding, collection of protein, and Western blot analysis methods were described previously (22, 23). Membranes were probed with the following antibodies: anti-MT1-MMP (Millipore, MAB3328), anti-GAPDH (Santa Cruz Biotechnology, C65), anti-FAK-(pY397) (BD Biosciences, 611722), anti-FAK (Cell Signaling Technology, D2R2E), anti-ITGB1 (Cell Signaling Technology, D2E5), anti-Cleaved-PARP (Cell Signaling Technology, D64E10), anti-SMAD3-(pS423/S425), and anti-SMAD3 (EP568Y) (Abcam).

Real-Time PCR

cDNA synthesis and PCR amplification were done as described in (24). Primers were selected from the Harvard Primer Bank as described in (29).

Cell viability

Promega's CellTiter-Glo Viability Assay was used to determine relative [ATP]. Cells were seeded at 5×10^3 density in 96-well plates in triplicate in 100 μL. One day after seeding, 100 μL of lysis reagent was added to the time 0 (T_0) plate and baseline luminescence was detected. Media were changed in other plates and drug was added. Three days after treatment, cells were again lysed and luminescence was detected on the basis of total [ATP]. Timepoints were normalized to the T_0 reading.

Cooperativity index

Cooperativity index (CI) was calculated on the basis of viability assay values using CompuSyn as described previously (30). Three doses of PLX4720 were used in combination with shMT1-MMP (for WM266-4) or full-length and ΔCAT (for WM115) MT1-MMP

constructs, in triplicate. Three days after treatment, viability was measured via Promega CellTiter-Glo.

Survival assay

A total of 2×10^4 cells were seeded in 24-well plates. One day after seeding, cells were treated with drug and a time zero was collected. Detached cells present in the media were combined with trypsinized cells, spun down, and suspended in 100 μL of media:trypan blue at a 1:1 ratio. Counting was performed via the T20 Bio-Rad Cell Counter.

Apoptosis assay

Promega's RealTime-Glo Annexin V Apoptosis Assay was used to determine relative Annexin V levels. A total of 5×10^3 cells were seeded in 96-well plates in triplicate. One day after seeding, cells were treated with PLX4720 at 5 μmol/L in DMSO. Three days after treatment, the kit reagents were added and luminescence was detected. Relative Annexin V was normalized to DMSO shGFP controls.

qGEL 3D matrix assay

Cell suspension of 10^6 cells per 100 μL was combined with 400 μL of HEPES with qGEL Lyophilized Powder (formulation IDs: NSC4QA432R and NSC4EN562R, qGel Bio; ref. 31). The mixture was incubated at 37°C, 5% CO_2 for 30 minutes until a solid matrix was formed with cells embedded inside. Media (2 mL) were then added and survival assay was performed as above.

In vivo tumor growth

Female nude mice were provided by the Charles River Laboratories and cared for by the Division of Animal Resources at the University of Miami (Miami, FL). All experimental models were Institutional Animal Care and Use Committee approved. A total of 2×10^6 cells were injected in the dorsal flanks of each mouse, totaling 5 mice and 10 tumors per group. When tumor volumes reached 100 mm^3, tumor volume and body weight were measured every other day. Tumor volume was calculated by using the formula: $[(W^2 \times L) \times 0.5]$. Mice were treated with chow containing 200 ppm PLX4720 and/or daily 25 mg/Kg of ND322s.c. Once tumors reached approximately 1,000 mm^3, mice were euthanized and tumors were collected.

IHC

Formalin-fixed, paraffin-embedded tumor sections derived from 1205Lu (both parental and resistant to BRAFi) were rehydrated and antigen retrieval was performed using a citric acid–based Antigen Unmasking Solution (Vector Laboratories) as per the manufacturer's instructions. Primary anti-MT1-MMP (clone EP1264Y, Abcam) was used at 1:250 dilution overnight at 4°C. Horseradish peroxidase–conjugated secondary antibody was incubated at room temperature as per the manufacturer's instructions (ImmPRESS anti-rabbit, Vector Laboratories) and followed by ImmPACT DAB Substrate (Vector Laboratories).

Statistical analysis

Statistical significance was determined using the Student *t* test with a significant difference being $P < 0.05$. Significance of correlation was detected using the Pearson correlation, calculated via GraphPad prism with a correlation significant when P was at least <0.05. All experiments were repeated at least three times.

Results

MT1-MMP increases after BRAFi treatment

We have previously shown that MT1-MMP expression correlates with reduced outcome for patients with melanoma (22, 23). Furthermore, we have shown that MT1-MMP plays a key role in melanoma invasion and metastasis, in part, through the activation of pro-MMP2 (22, 24). It has been shown that BRAFi-resistant melanoma becomes more aggressive and metastatic. Because MT1-MMP is a key player in cell invasion and migration, we sought to investigate whether MT1-MMP may play a role in resistance to BRAFi (32).

By analyzing two datasets with RNA-sequencing (RNA-seq) data available for patients' pre- and posttreatment tumor specimens, we found that MT1-MMP was significantly increased at the mRNA level in posttreatment tumor biopsies that progressed on BRAFi treatment compared with pretreatment biopsies (**Fig. 1A**; refs. 33, 34). To further support these findings, we created BRAFi-resistant cell lines by chronically treating them with PLX4720 at 5 μmol/L (35). The expression of MT1-MMP at both mRNA (**Fig. 1B**) and protein (**Fig. 1C** and **D**) levels was consistently increased in all seven cell lines that acquired resistance to PLX4720 compared with their treatment-naïve parental counterparts. Interestingly, the analysis of RNA-seq data derived from 4 patients' longitudinal tumor specimens, who were treated at Massachusetts General Hospital (Boston, MA) with sequential targeted therapies and cancer immunotherapies, and then progressed on both, demonstrated an increase in MT1-MMP mRNA (Supplementary Fig. S1).

To further support patients' clinical data, 1205Lu xenografted tumors that progressed on PLX4720 were immunostained with an anti-MT1-MMP antibody. All resistant tumors showed increased expression of MT1-MMP at the protein level compared with parental tumors (**Fig. 1E**). Similarly, the analysis of RPPA data of pre- and posttreatment A375-derived tumors, as well as two PDXs (WM4007 and WM3929; ref. 35), showed increased MT1-MMP protein upon acquiring resistance to PLX4720 (Supplementary Fig. S2). Even short-term treatment with combination BRAFi (PLX4720) and MEKi (PD0325901) demonstrated higher MT1-MMP expression after treatment.

We also found other pathways that were consistently upregulated in resistant tumors, including AKT and ERK signaling, as well as an increase in phosphorylation of FAK at tyrosine Y397, a marker of integrin activation (Supplementary Fig. S3). Together, these data indicate MT1-MMP expression increases in BRAFi-resistant tumors and cell lines, making it a potential novel target to reverse resistance.

MT1-MMP correlates to vemurafenib response in *BRAF^{V600E}*-mutant melanoma cell lines

To better assess the relationship of MT1-MMP with the sensitivity of BRAF-mutant melanoma cells to PLX4720, five melanoma cell lines expressing various relative levels of MT1-MMP (**Fig. 2A**) were treated with increasing doses of PLX4720 and cell viability was determined (**Fig. 2B**). The IC_{50} for each line was calculated by the curve fit method (GraphPad). By comparing each IC_{50} with the relative amount of MT1-MMP expressed by each line, we found that the higher the amount of MT1-MMP, the higher the IC_{50} (**Fig. 2C**), suggesting MT1-MMP is inversely associated to the response of melanoma cells to the BRAFi.

To determine whether MT1-MMP is actively playing a role in the resistance to BRAFi, we next knocked down (**Fig. 2D** and **E**) or overexpressed (**Fig. 2F** and **G**) MT1-MMP in a pair of syngeneic lines that have either high or low relative MT1-MMP expression, respectively (22). Knockdown of MT1-MMP in WM266-4, which has high endogenous MT1-MMP levels, resulted in further decrease in cell growth when coupled with PLX4720 compared with control cells expressing shGFP. On the other hand, overexpression of full-length MT1-MMP in WM115, which has a lower endogenous MT1-

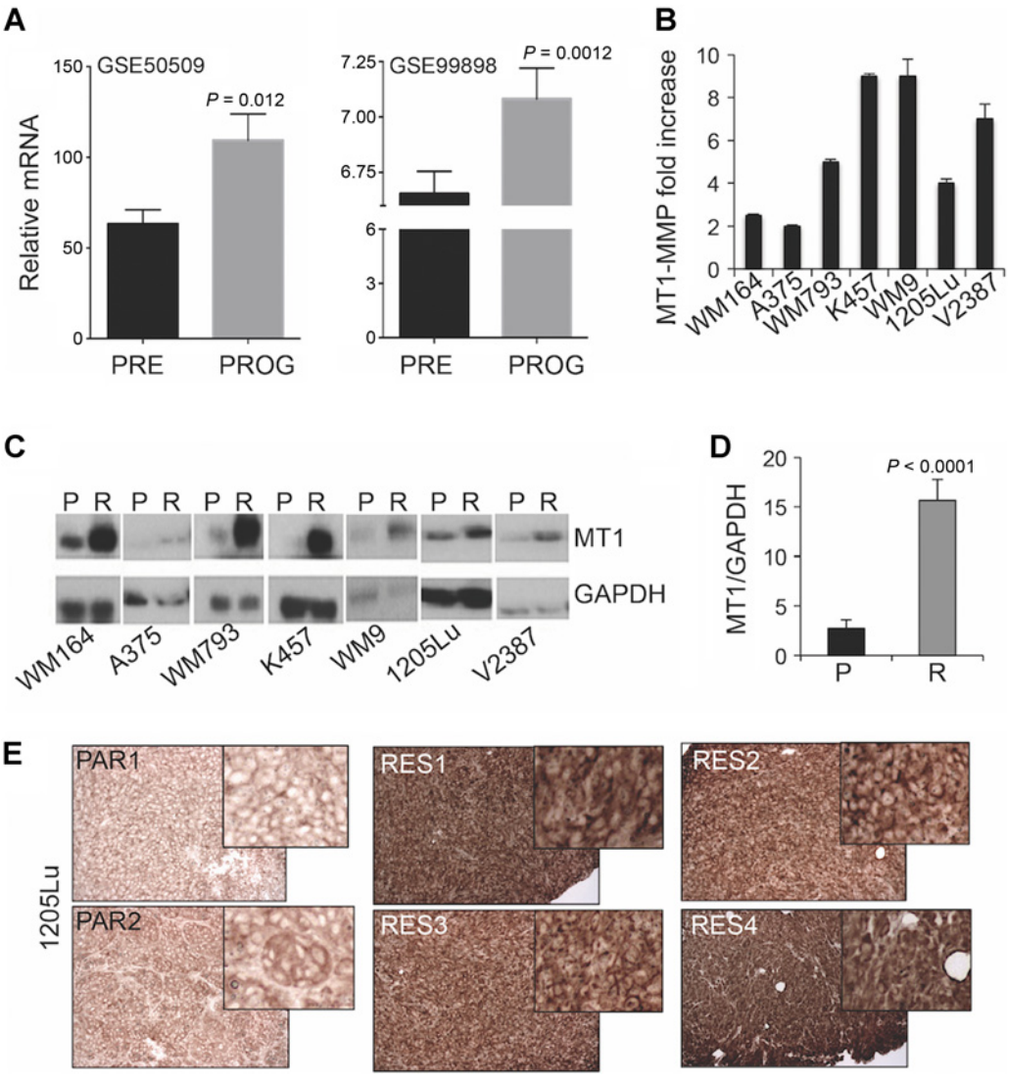

Figure 1.

MT1-MMP is associated with a worse prognosis and increase after BRAFi treatment. **A,** Analysis of RNA-seq geo datasets GSE50509 (left) and GSE99898 (right) for MT1-MMP mRNA expression. Tumor biopsies were separated into two groups: pretreatment biopsy and progressed on treatment with BRAFis (dabrafenib or vemurafenib). **B,** MT1-MMP mRNA levels in resistant cells normalized to each parental counterpart. MT1-MMP was significantly upregulated in all cell lines with respect to parental controls ($P < 0.001$). **C,** MT1-MMP protein levels in parental (P) or PLX4720-resistant (R) cell line pairs. GAPDH was used as loading control. **D,** Densitometry for MT1-MMP parental versus resistant cells obtained with ImageJ. Values were normalized to GAPDH for each sample. **E,** MT1-MMP protein expression in 1205Lu-derived tumors before BRAFi treatment (PAR) and after resistance to BRAFi treatment occurred (RES).

MMP expression, increased growth. A catalytically inactive mutant MT1-MMP (ΔCAT; ref. 22), however, failed to promote cell growth, suggesting MT1-MMP may protect cells from BRAFi via its proteolytic activity. By using CompuSyn to determine the cooperativity of PLX4720 and MT1-MMP expression, we found that PLX4720 synergizes with MT1-MMP inhibition at all doses tested (**Fig. 2H**, top) in WM266-4 cells, whereas in WM115, the overexpression of full length MT1-MMP exerted antagonistic effects (**Fig. 2H**, bottom; refs. 30, 36). Synergy between PLX4720 and shMT1-MMP combination was observed for additional cell lines (Supplementary Fig. S4). These data support a role of MT1-MMP in modulating susceptibility to BRAFi treatment, highlighting MT1-MMP as a potential target for combination therapy and further study.

MT1-MMP knockdown sensitizes cells and tumors to vemurafenib (PLX4720)

Given the link between the expression levels of MT1-MMP and sensitivity of BRAF-mutant melanoma cells to BRAFi, we next sought to determine whether cells that acquired resistance could be resensitized to PLX4720 via the knockdown of MT1-MMP. We first examined cell survival of BRAFi-sensitive and -resistant cells treated with PLX4720 and expressing either a control shRNA (shGFP) or shMT1-MMP. In both PLX4720-sensitive K457 (**Fig. 3A**) and WM793 (**Fig. 3B**) cells, we observed PLX4720 alone significantly decreased cell survival, but did not significantly affect resistant cells. However, the combination of MT1-MMP knockdown with PLX4720 restored sensitivity of resistant cells to the levels of parental cells treated with

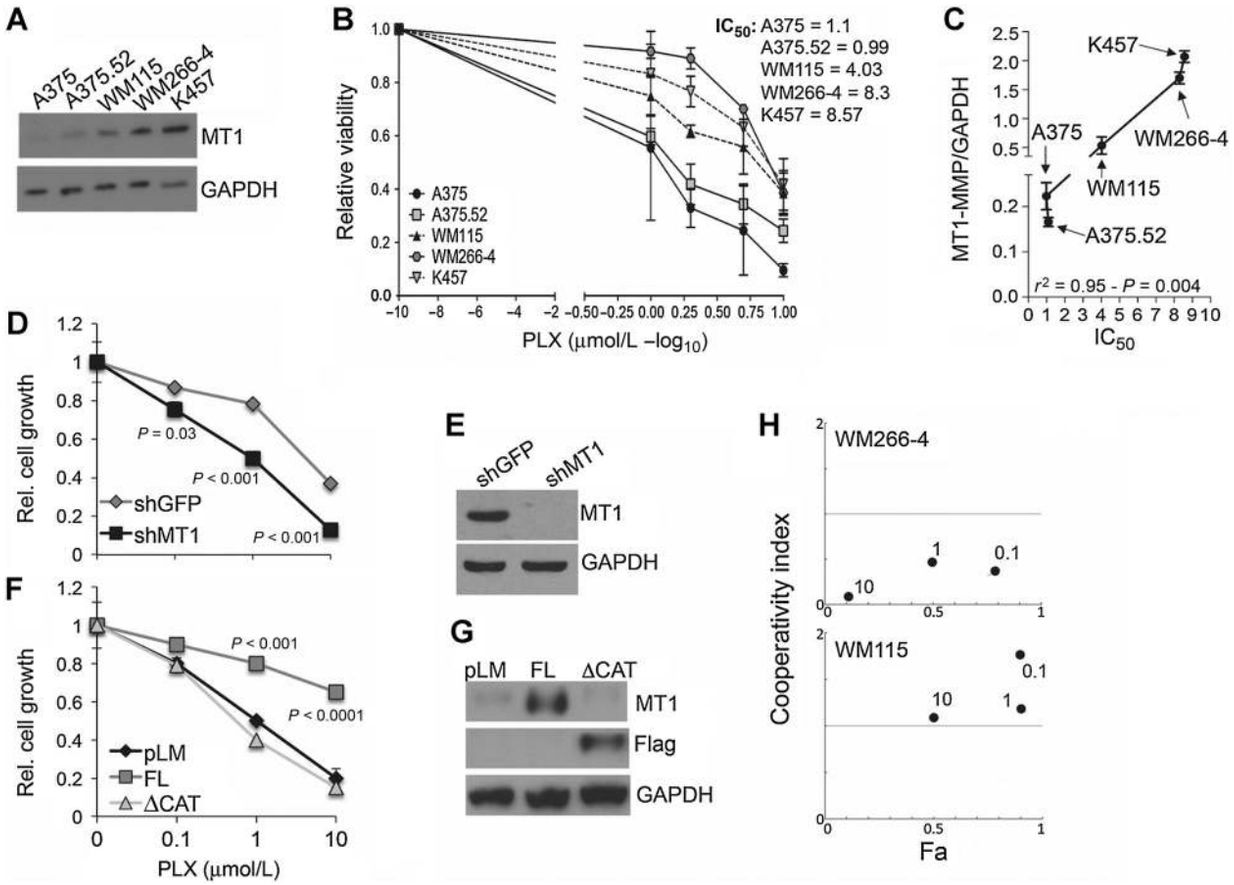

Figure 2.

MT1-MMP correlates to vemurafenib response in $BRAF^{V600E}$-mutant melanoma cell lines. **A,** MT1-MMP expression levels in several melanoma cell lines. **B,** Viability of the cells in **A** after 72 hours of treatment with different doses of PLX4720. IC_{50} values were determine by the curve fit method. Values were normalized to DMSO-treated control. **C,** Correlation between IC_{50} values of the cells in **A** and **B**, and the relative MT1-MMP expression levels determined by densitometry after normalization to GAPDH. **D** and **E,** WM266-4 cells transfected with lentivirus containing shRNA targeting MT1-MMP (shMT1) or GFP (shGFP) and treated with PLX4720 at three different doses. Cell growth was measured 72 hours after treatment and normalized to DMSO control. **F** and **G,** WM115 cells transfected with either PLM (pLM, empty vector), full-length MT1-MMP (FL), or dead mutant MT1-MMP (ΔCAT) and treated with PLX4720 as in **D** and **E**. Viability was measured 72 hours after treatment. **H,** CIs of the cells in **D** and **F**.

PLX4720 alone or even lower. Similar results were observed on an additional pair of parental and resistant cell lines (Supplementary Fig. S5A). Inhibition of MT1-MMP also reduced cell growth in several melanoma cell lines, particularly when combined with PLX4720. Importantly, in resistant cells, in which the BRAFi did not significantly affect cell growth, depletion of MT1-MMP restored the drug inhibitory effects (Supplementary Fig. S5B).

Finally, to address the potential off-target effects, a second shRNA against MT1-MMP, which we have employed previously (22–24), was used. Inhibition of MT1-MMP by this shRNA also led to reduced cell growth and increased cell death when combined with PLX4720, restoring sensitivity to the drug in resistant cells (Supplementary Fig. S6).

To further define the mechanism of action of BRAFi resistance mediated by MT1-MMP, apoptosis was assessed. The combination of MT1-MMP knockdown and PLX4720 increased apoptosis, as measured by the percentage of Annexin V–positive cells (**Fig. 3C** and **D**; ref. 37); and led to higher levels of cleaved PARP (**Fig. 3E** and **F**). Taken together, our results indicate the depletion of MT1-MMP in combination with PLX4720 increases apoptosis and cell death. Likewise, cells resistant to both BRAFi and MEKi were resensitized to the inhibitors by the depletion of MT1-MMP (Supplementary Fig. S7B and S7C).

Having established a strong link between the knockdown of MT1-MMP and the sensitivity to PLX4720, we next sought to verify the effect of the knockdown of MT1-MMP in a mouse xenograft model to investigate its potential as a therapeutic target. K457 parental and resistant cells were transduced with either shMT1-MMP or shGFP and grafted onto mice. Mice were then treated with PLX4720 *ad libitum* and tumor growth was measured over time (**Fig. 3G**). Tumors derived from parental cells, in which MT1-MMP was depleted, displayed the least tumor growth when treated with the BRAFi. The treatment of tumors derived from resistant cells expressing shGFP had little effect on growth as expected; however, tumors derived from resistant cells expressing shMT1-MMP showed a significant reduction in tumor growth. These results indicate that MT1-MMP confers resistance to BRAFi and the depletion of MT1-MMP can overcome resistance.

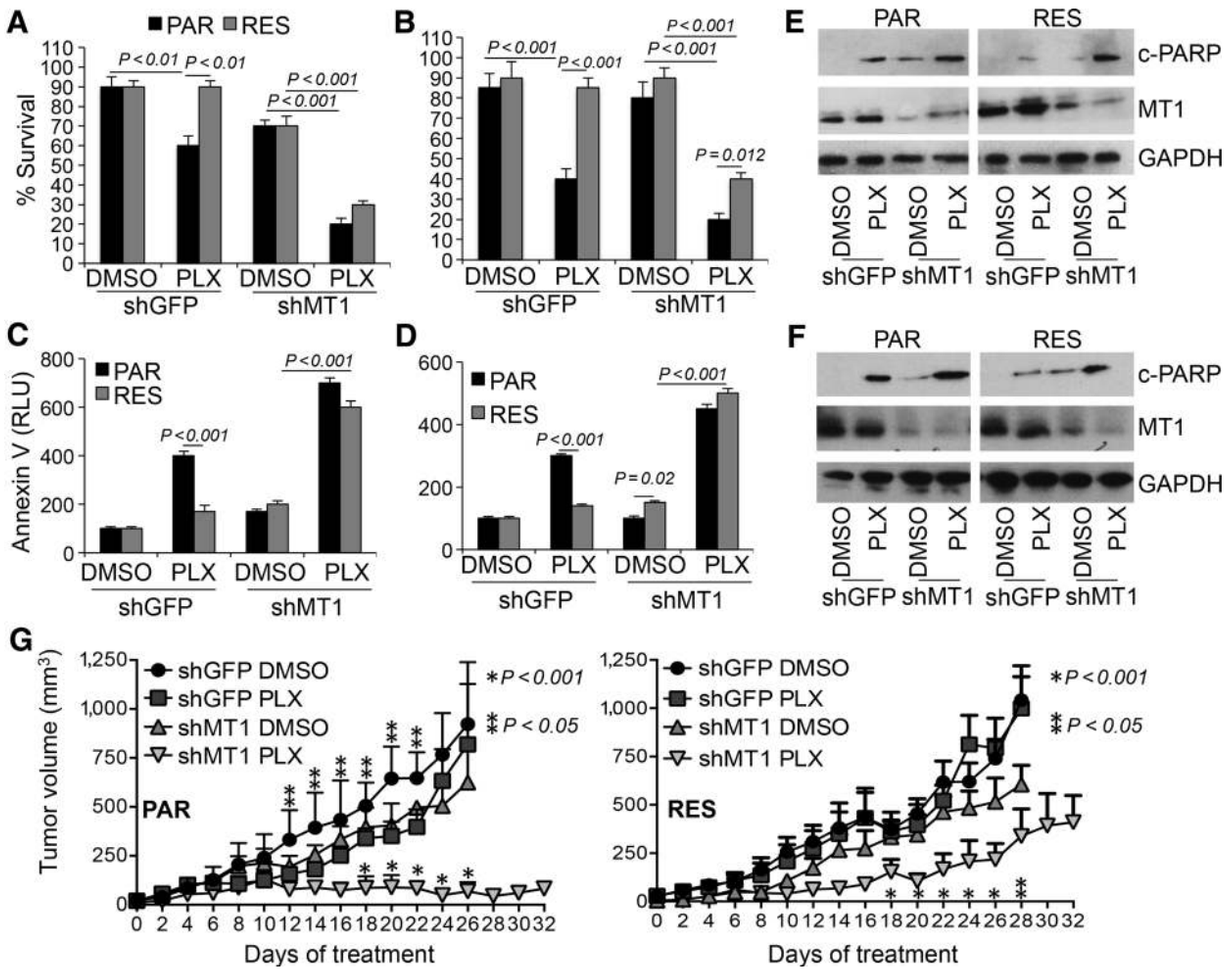

Figure 3.

MT1-MMP knockdown sensitizes cells and tumors to vemurafenib. Survival of K457 (**A**) and WM793 (**B**) parental and PLX4720-resistant cells treated with the BRAFi at 5 μmol/L for 3 day and expressing either shGFP or shMT1-MMP. Cell survival was measured by trypan blue exclusion assay. **C** and **D,** Levels of Annexin V of the cells in **A** and **B**, respectively. **E** and **F,** Western blot analysis showing cleaved PARP (c-PARP) and MT1-MMP levels of the cells in **A** and **B**. **G,** Growth in nude mice of K457 parental (PAR) and resistant (RES) cells expressing either shGFP or shMT1-MMP and fed with control or 200 ppm PLX4720 (PLX) chow. Treatment with the BRAFi started when tumors in all groups reached an average 100 mm^3 in volume. Tumor volumes were measured every other day.

MT1-MMP mediates resistance to vemurafenib via processing of the ECM

MT1-MMP is one of the most important invasion promoting, protumorigenic MMPs that controls progression of cancer cells (35). Active MT1-MMP is able to process a wide variety of ECM proteins, adhesion and signaling receptors, cytokines, and growth factors including EGF, CD44, and Notch1 (13–20). MT1-MMP also activates promigratory/invasive MMPs, such as MMP2 and MMP13, promoting tumorigenesis (38). Although MT1-MMP is known to signal independently of its catalytic function (39–43), data in **Fig. 2F** suggest the response of BRAF-mutant cells to BRAFi is dependent on the catalytic activity of MT1-MMP, as a catalytically inactive MT1-MMP construct (ΔCAT) did not promote cell growth upon BRAFi treatment. To further confirm these data, WM115 cells transduced with either the full-length catalytically-proficient or the ΔCAT MT1-MMP constructs (Supplementary Fig. S8A) were treated with 5 μmol/L PLX4720 and survival was assessed. Only the catalytically-proficient MT1-MMP provided a survival benefit to cells treated with PLX4720 (Supplementary Fig. S8B), indicating MT1-MMP acts through the enzymatic processing of a substrate(s) to mediate resistance to BRAFi.

MMP2 is a main accessory soluble metalloproteinase acting downstream of MT1-MMP in several types of cancers. MMP2 is directly activated by MT1-MMP (44, 45), and we have previously shown MMP2 mediates the migration and invasion of melanoma downstream of MT1-MMP (22). We, therefore, sought to determine whether MMP2 mediates MT1-MMP–dependent BRAFi resistance. MMP2 was depleted by two specific shRNAs (shMMP2-1 and shMMP2-2) in both parental and resistant WM793 cells (Supplementary Fig. S9A). The survival was then measured after 3 days of treatment with PLX4720 at 5 μmol/L (Supplementary Fig. S9B). We found that the depletion of MMP2 did not sensitize cells to BRAFi, supporting a specific role of MT1-MMP in this phenomenon.

We, therefore, asked whether MT1-MMP confers resistance through its ability of directly processing the ECM (46). To answer this question, parental and resistant K457 cells were embedded in a synthetic (ethylene glycol) hydrogel that incorporated MT1-MMP

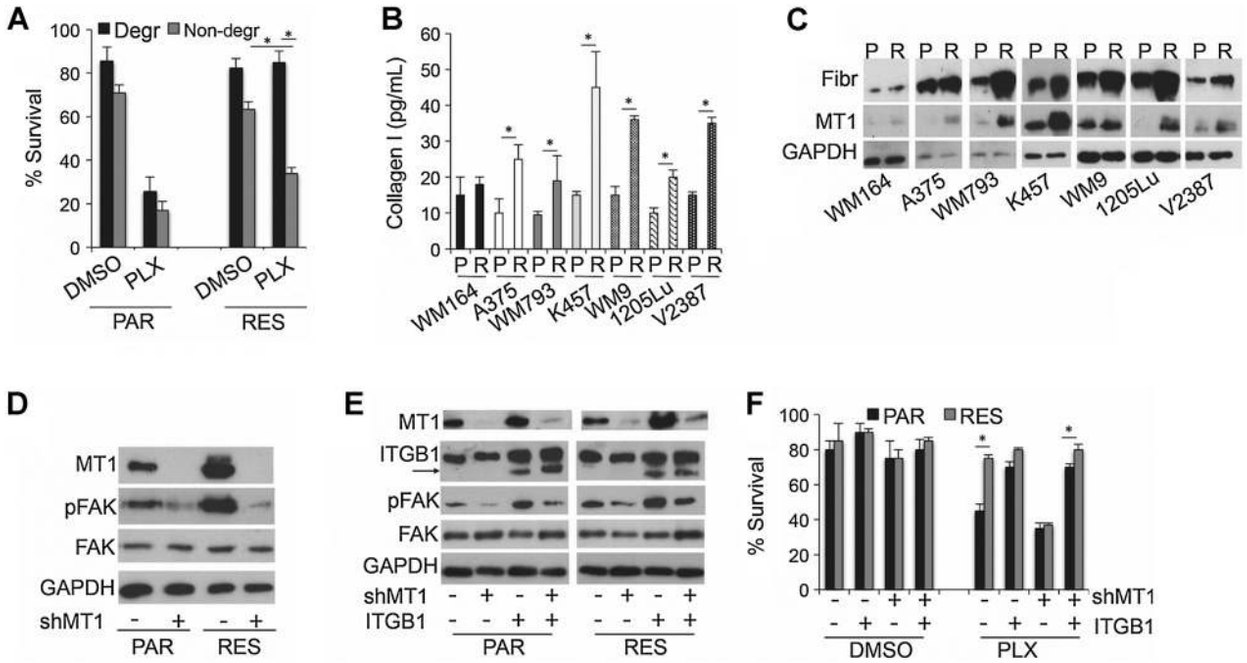

Figure 4.

MT1-MMP mediates resistance to vemurafenib via processing of the ECM. **A,** Survival of parental and resistant K457 cells encapsulated in a hydrogel containing either MT1-MMP degradable (Degr) or not degradable (Non-degr) collagen sites and treated with PLX4720 (5 μmol/L) for 72 hours. Survival was measured via trypan blue exclusion assay. *, $P < 0.01$. **B,** Collagen I secretion in parental (P and PAR) and resistant (R and RES) melanoma pairs. ELISA for collagen I was conducted from serum-deprived conditioned media from 10^6 cells. *, $P < 0.05$. **C,** Fibronectin expression in conditioned media and MT1-MMP from cell lysates of the cells in **B**. **D,** MT1-MMP, FAK, and Phospho-FAK^{Y397} in K457 cells transfected with shGFP or shMT1-MMP. GAPDH was used as loading control. **E,** ITGB1, MT1-MMP, FAK, and phospho-FAK^{Y397} expression levels in K457 cells transfected with a combination of shMT1-MMP or shGFP and a constitutively active ITGB1 overexpression vector (pLV-VN-ITGB1). **F,** Survival of the cells in **E**, treated with 5 μmol/L PLX4720 (PLX) for 72 hours (*, $P < 0.01$).

cleavable or noncleavable collagen sequences (21, 31). Cells were then treated with PLX4720 and the survival was measured by trypan blue staining (**Fig. 4A**). The survival of resistant cells encapsulated in a non-MT1-MMP cleavable gel was significantly decreased compared with cleavable matrix when treated with PLX4720 (Supplementary Fig. S10). This demonstrates that the role of MT1-MMP in ECM cleavage is important for resistance to BRAFi and that inhibiting the catalytic function of MT1-MMP is beneficial to restoring sensitivity to BRAFi.

MT1-MMP mediates BRAFi resistance by engaging ITGB1/FAK signaling

Given the requirement of ECM cleavage in MT1-MMP–dependent BRAFi resistance, we next asked whether ECM components cleaved by MT1-MMP were also upregulated in resistant cells, as a potential mechanism of protection. Indeed, an increase in collagen I secretion as well as fibronectin, was found in resistant cells (**Fig. 4B** and **C**).

These data suggest resistant cells selectively acquire a mesenchymal-like phenotype by upregulating a repertoire of ECM factors as well as MT1-MMP, a major processing enzyme of both collagen and fibronectin.

ITGB1 is a main cell surface receptor of ECM collagen and fibronectin (47, 48). Binding of collagen promotes integrin clustering and activation, which are key steps in allowing ECM-integrin–mediated outside-in signaling (49). The intracellular domain of ITGB1 can bind several effectors. A main signaling factor activated by active ITGB1 is FAK, which autophosphorylates at tyrosine 397 (50). MT1-MMP has been shown to activate ITGB1 and drive osteogenic versus adipogenic differentiation through processing of ECM components and activation of FAK at tyrosine 397 (21). Importantly, ITGB1 has been suggested to play a role in the resistance to BRAFi (11, 12). In the work by Hirata and colleagues (12), treatment with PLX4720 has been shown to activate stromal fibroblasts, which in turn, secrete ECM components leading to ITGB1/Src activation in melanoma cells. Thus, we sought to determine whether in our system, in which resistant melanoma cells themselves secrete more ECM factors and at the same time increase MT1-MMP expression, MT1-MMP might confer resistance to BRAFi via ECM processing and activation of ITGB1. FAK phosphorylation of tyrosine 397 was used as a read out of ITGB1 activity because, as mentioned above, this tyrosine is specifically phosphorylated upon ECM-mediated integrin activation (21). Inhibition of MT1-MMP, in both parental and resistant K457 cells, resulted in a reduction in FAK phosphorylation, indicating reduced ITGB1 activation (**Fig. 4D**). Thus, to test whether ITGB1 indeed mediates resistance to BRAFi downstream of MT1-MMP, parental and resistant cells expressing shMT1-MMP were cotransduced with a construct expressing a self-clustering ITGB1 mutant (ITGB1-VN; ref. 21). This construct causes high ITGB1 clustering at the focal adhesions, which results in its constitutive downstream signaling such as higher FAK phosphorylation (**Fig. 4E**; Supplementary Figs. S7A and S11A). We found that resistant cells that were resensitized to PLX4720 by the knockdown of MT1-MMP regained their resistance when activated ITGB1 was introduced (**Fig. 4F**; Supplementary Fig. S11B). This suggests MT1-MMP acts as a

mediator between the ECM and ITGB1 signaling to promote resistance to BRAFi. Of note, data in Supplementary Fig. S3 show phospho-FAKY397 is upregulated in tumors that progressed on PLX4720, further supporting a link between MT1-MMP, ECM remodeling, and the activation of integrin/FAK signaling.

Inhibition of MT1-MMP activity by ND322 restores sensitivity to vemurafenib *in vivo*

Our data show that MT1-MMP confers resistance to BRAFi through the processing of the ECM and consequent activation of ITGB1 signaling. Thus, we reasoned that specifically targeting the catalytic activity of MT1-MMP would restore responses of resistant cells to BRAFi. To test this, we made use of ND322. ND322 is a selective, slow-binding inhibitor with inhibition constants of 0.02, 0.24, and 0.87 μmol/L, for MMP2, MT1-MMP, and MMP9, respectively (51). ND322, however, is a very poor inhibitor, with short residence time and low affinity, of several other MMPs, as we have shown previously (24, 26, 51). In an earlier study, we have shown that ND322 counteracts melanoma growth and delays metastases in a melanoma orthotopic mouse model while displaying no side-effects *in vivo* (24). At a concentration of 0.32 μmol/L, 2.7 times below the K_i for MMP9, ND322 was able to restore sensitivity of resistant cells to PLX4720 as shown by a reduction in survival compared with cells treated with either agent alone (**Fig. 5A** and **B**).

We excluded that the effects of ND322 on resistance to BRAFi were dependent on MMP2 inhibition because we have shown that MMP2 knockdown does not sensitize resistant cells to PLX4720 (Supplementary Fig. S9). However, because ND322 is also an MMP9 inhibitor, albeit at higher concentrations required to inhibit MT1-MMP, we determined whether MMP9 could play a role in resistance to BRAFi. Knockdown of MMP9 in parental and resistant cells (Supplementary Fig. S12) did not have an impact on cell survival when cells were treated with PLX4720, indicating MMP9 does not play an important role in conferring resistance to BRAFi and that ND322 sensitizes cells to BRAFi mainly through MT1-MMP inhibition.

Next, ND322 was tested *in vivo* to determine whether it could restore responses to PLX4720. BRAF-mutant K457 parental and resistant cells were inoculated subcutaneously in nude mice. Mice were then fed PLX4720 or control chow as described previously (**Fig. 3**; ref. 25). Treatment with PLX4720 and/or ND322 at 25 mg/Kg s.c. daily was started when tumors in all groups reached an average volume of 100 mm^3. The combination of ND322 and PLX4720 demonstrated a significant reduction in tumor growth compared with either agent alone in mice inoculated with BRAFi-sensitive cells, while stable disease was observed in the combination setting in mice inoculated with resistant cells (**Fig. 5C** and **D**). Overall, these data indicate the specific targeting of the catalytic activity of MT1-MMP can restore sensitivity of resistant tumors to BRAFi.

MT1-MMP upregulation in resistant cells is mediated by TGFβ signaling

It has been previously shown that resistance to BRAFi causes an increase in TGFβ release, which in turn, induces the secretion of ECM components, such as fibronectin, from the surrounding fibroblasts (52). In our experimental system, we found that melanoma cells themselves secreted ECM components and even more so when they acquired resistance to BRAFi. Also, MT1-MMP has been shown

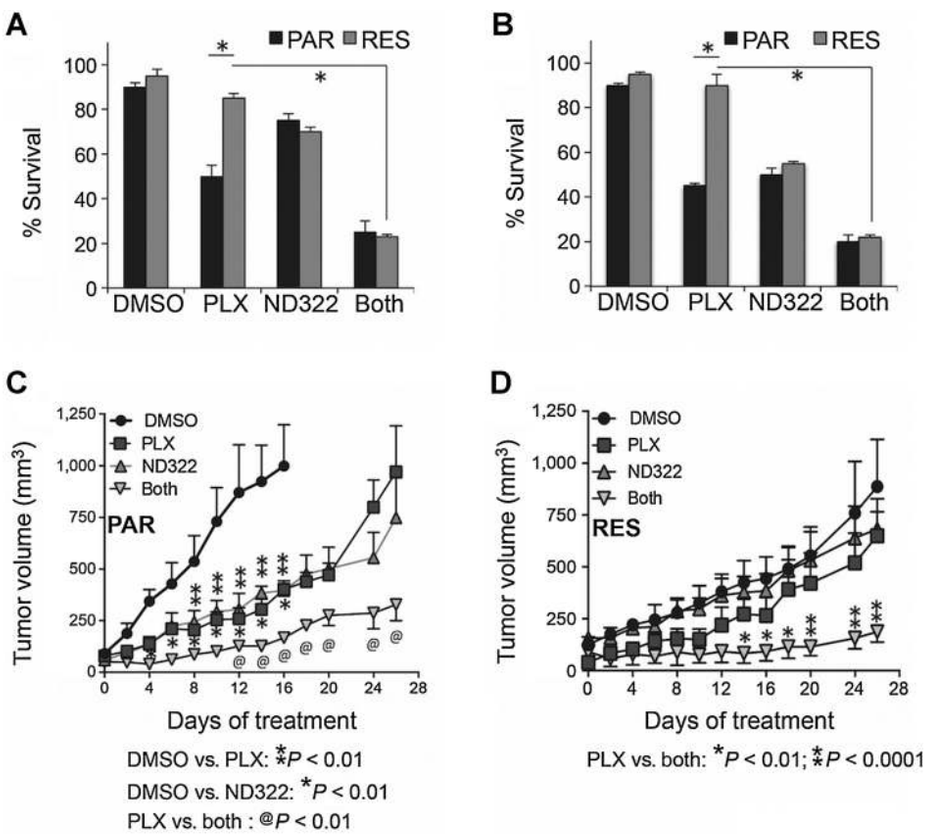

Figure 5.
Inhibition of MT1-MMP activity by ND322 restores sensitivity to vemurafenib. Survival of parental (PAR) and resistant (RES) K457 (**A**) and WM793 (**B**) cells treated with 5 μmol/L PLX4720 (PLX) and 0.32 μmol/L ND322 either alone or in combination for 72 hours. *, $P < 0.01$. **C** and **D,** Growth of K457 parental and resistant cells in mice treated with daily doses of 25 mg/Kg ND322 (s.c.) and fed with 200 ppm PLX4720 in chow. Treatment started when tumors in all groups reached an average of 100 mm^3 in volume. Tumor volumes were measured every other day. Statistically significant difference between the combination and the individual treatment groups is shown.

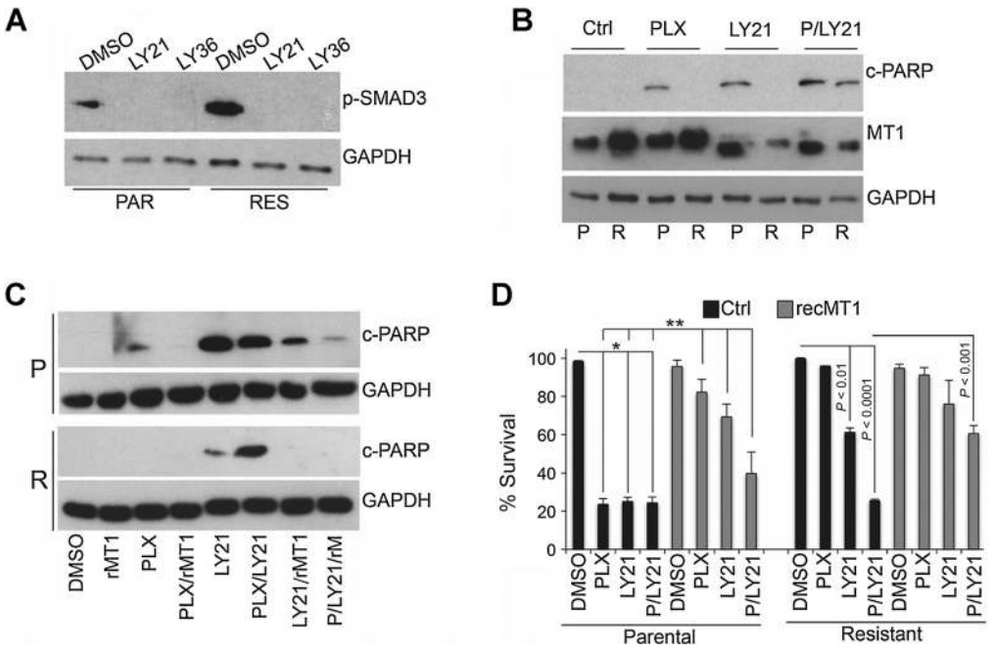

Figure 6.

MT1-MMP upregulation is dependent on TGFβ signaling. **A,** levels of phohpo-SMAD3$^{S423/S425}$ in parental (PAR) and resistant (RES) K457 cells treated with 10 μmol/L of the TGF receptor I/II dual inhibitors, LY21109761 (LY21) and LY364947 (LY36), overnight. **B,** Cleaved PARP and MT1-MMP expression levels in parental (P) and resistant (R) K457 cells treated with 5 μmol/L PLX4720 and 10 μmol/L LY21109761 for 72 hours. **C,** Cleaved PARP in parental and resistant K457 treated as in **B**, but with the addition of 20 ng/mL active recombinant MT1-MMP (rMT1) where indicated. **D,** Survival of the cells in **C** (*, $P < 0.001$). Ctrl, control; PLX, PLX4720.

to be controlled by TGFβ signaling (53, 54). Hence, we posited that increased MT1-MMP in BRAFi-resistant cells could be a function of TGFβ signaling. To test this possibility, we used the TGF receptor I/II dual inhibitors, LY2109761 and LY364947. Both inhibitors effectively blunt the signaling downstream of TGFβ as indicated by a reduction of SMAD3 phosphorylation (**Fig. 6A**). Treatment of both parental and resistant K457 cells with LY2109761 led to a decrease in MT1-MMP expression in resistant cells, likely because the upregulation of MT1-MMP in these cells was TGFβ dependent, and was accompanied by an increase in cleaved PARP, suggesting inhibition of MT1-MMP by blockade of TGFβ signaling may lead to cell death. To definitively assess this possibility, cells were treated with PLX4720 and LY2109761 either alone or in combination, and then they were stimulated by active recombinant MT1-MMP, to determine whether the latter could rescue cell survival. Indeed, the addition of active MT1-MMP reduced the amount of cleaved PARP in both parental and resistant cells, and importantly, rescued cell survival (**Fig. 6C** and **D**). Similar results were obtained with the inhibitor LY364947 (Supplementary Fig. S13). Hence, these data indicate TGFβ signaling in BRAFi-resistant cells may lead to an increase in MT1-MMP resulting in cell protection.

Discussion

In this study, we show that MT1-MMP–dependent remodeling of the ECM is a novel mechanism of resistance to BRAFis. A selective advantage exists in BRAFi-resistant melanomas to overexpress MT1-MMP, as well as components of the ECM (i.e., collagen and fibronectin), likely as a mechanism of protection against BRAFi-mediated cytotoxicity. Indeed, we show that MT1-MMP activates an outside-in survival signaling pathway through the activation of ITGB1, and that genetic or pharmacologic inhibition of MT1-MMP synergizes with BRAF inhibition restoring sensitivity to BRAFis both *in vitro* and *in vivo*. This indicates MT1-MMP is playing a role beyond its canonical migratory and invasive functions and that MT1-MMP inhibition can not only counteract tumor progression by inhibiting metastases, as we have shown previously (22–24), but, importantly, can be combined with BRAFis to increase their efficacy and prevent or revert resistance.

It has been previously shown that matrix deposition by tumor-associated fibroblasts following BRAFi treatment can induce an ITGB1-dependent survival pathway in melanoma cells (11, 12). Here, we show that melanoma cells under the selective pressure of BRAF inhibition consistently activate an autogenous mechanism of protection by producing their own ECM, such as collagen I and fibronectin, as well as MT1-MMP, the rate-limiting enzyme in ECM remodeling and of paramount importance in the activation of ITGB1 and its downstream survival effects.

Inhibition of integrins has been investigated as an anticancer target, however, targeting them specifically has proved difficult because of high homology between the different integrins and the important role of integrins in general cell homeostasis (55). Instead, targeting of MT1-MMP is a feasible alternative in view of its role in linking the ECM to ITGB1 signaling and the availability of novel selective MMP inhibitors.

Indeed, earlier trials with pan-MMP inhibitors failed mostly due to severe side-effects such as musculoskeletal pain and inflammation, accompanied by negligible anticancer effects (56). This is because several MMPs play important roles in inflammation and immune responses and some, such as MMP8, possess anticancer and proimmune surveillance properties, as well as the fact that these inhibitors

were tested on patients with late-stage melanoma, when pan-MMP inhibition might be more useful as an adjuvant (56).

However, research into the individual roles of the MMPs in cancer has revealed MT1-MMP as a major driver of melanomagenesis. MT1-MMP increases in melanoma as it progresses, it inversely correlates to BRAF treatment responses as demonstrated here, it is a poor prognosis marker, and it is critical for metastasis (22–24, 38). Selective targeting of MT1-MMP would, therefore, provide effective antitumor responses while reducing deleterious side-effects. In fact, while daily treatment with the selective MT1-MMP/MMP2 inhibitor, ND322, either alone or in combination with PLX4720 revealed significant anticancer activity, and it did not show any evident morbidity such as changes in body weight, hunched posture, and reduced motility, highlighting its potential safety and efficacy even in a combination setting. Similar safety profiles were previously observed in animal models of brain ischemia and wound healing (57–59).

That said, it is worth mentioning that MT1-MMP–knockout mice are the only MMP-knockout mice that cannot fully develop, with systemic growth defects across the body that eventually lead to mortality (60). These defects are likely due to the inability to properly deposit collagen early on in development. This may, therefore, potentially limit the use of MT1-MMP inhibitors to nonpediatric cancers (61). Still, MT1-MMPs' established roles in metastasis combined with our data demonstrating its importance in cell survival and BRAFi resistance makes it an attractive target for a wide range of aggressive cancers, in addition to melanoma.

In summary, our findings highlight a previously unidentified role of MT1-MMP in mediating BRAFi resistance and demonstrate that MT1-MMP inhibition via the selective inhibitor, ND322, when combined with BRAFi in $BRAF^{V600E}$-mutant melanoma, has a profound synergistic inhibitory response, reverting resistant tumors to responsive ones. Overall, blockade of MT1-MMP provides an effective means to simultaneously inhibit melanoma growth, metastasis, and treatment resistance by severing the interaction of melanoma cells with the supporting ECM.

Disclosure of Potential Conflicts of Interest

Y. Li reports grants from NIH during the conduct of the study and outside the submitted work. M. Chang is listed as a coinventor on a nonprovisional patent application on ND-322 that is owned by the University of Notre Dame. G.B. Mills reports honoraria from Astra-Zeneca, PDX Pharma, Signalchem Lifesciences, Symphogen, Tarveda, and Zentalis; stock options from Catena, Signalchem Lifesciences, Tarveda; personal fees and non-financial support from Astra-Zeneca, Chyrsalis, ImmunoMET, Ionis, Lilly, Turbine, Genentech, and GSK; and royalties from Myriad Genetics during the conduct of the study. K.T. Flaherty reports serving/having served on the board of directors of Loxo Oncology, Clovis Oncology, Strata Oncology, Vivid Biosciences, and Checkmate Pharmaceuticals; on scientific advisory boards of Array Biopharma, Monopteros, X4 Pharmaceuticals, PIC Therapeutics, Fount Therapeutics, Shattuck Labs, Apricity, Oncoceutics, Fog Pharma, Tvardi, Sanofi, Amgen, Asana Biosciences, Aeglea, Tolero, Neon Therapeutics, and Cell Signaling Technology; and as consultant to Novartis, Genentech, and Pierre Fabre, Bristol Myers Squibb, Merck, Takeda, Verastem, Boston Biomedical, Cell Medica, and Debiopharm. R.J. Sullivan reports grants and personal fees from Merck, grants from Amgen, and personal fees from Bristol Myers Squibb, Novartis, Array Biopharma, Asana Biosciences, Replimune, Syndax, Iovance, and Compugen outside the submitted work. G.M. Boland reports other from Nektar Therapeutics (SAB) and Novartis (SAB), grants from Palleon Pharmaceuticals (SRA), Olink Proteomics (SRA) outside the submitted work. No potential conflicts of interest were disclosed by the other authors.

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Authors' Contributions

C. Marusak: Investigation, writing-original draft. **V. Thakur:** Investigation. **Y. Li:** Investigation. **J.T. Freitas:** Investigation. **P.M. Zmina:** Investigation. **V.S. Thakur:** Methodology. **M. Chang:** Resources. **M. Gao:** Resources. **J. Tan:** Data curation. **M. Xiao:** Resources. **Y. Lu:** Investigation. **G. Mills:** Resources. **K.T. Flaherty:** Resources. **D.T. Frederick:** Resources. **B. Miao:** Resources. **R.J. Sullivan:** Resources. **T. Moll:** Resources. **G. Boland:** Resources. **M. Herlyn:** Resources. **G. Zhang:** Resources. **B. Bedogni:** Supervision, funding acquisition, investigation, writing-original draft.

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