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ErbB3 targeting enhances the effects of MEK inhibitor in wild-type BRAF/NRAS melanoma

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Abstract

MEK-ERK1/2 signaling is elevated in melanomas that are wild-type for both BRAF and NRAS (WT/WT), but patients are insensitive to MEK inhibitors. Stromal-derived growth factors may mediate resistance to targeted inhibitors, and optimizing the use of targeted inhibitors for WT/WT melanoma patients is a clinical unmet need. Here, we studied adaptive responses to MEK inhibition in WT/WT cutaneous melanoma. TCGA dataset and tumor microarray studies of WT/WT melanomas showed that high levels of neuregulin-1 (NRG1) were associated with stromal content and ErbB3 signaling. Of growth factors implicated in resistance to targeted inhibitors, NRG1 was effective at mediating resistance to MEK inhibitors in patient-derived WT/WT melanoma cells. Furthermore, ErbB3-ErbB2 signaling was adaptively upregulated following MEK inhibition. Patient-derived cancer-associated fibroblast studies demonstrated that stromal-derived

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NRG1 activated ErbB3/ErbB2 signaling and enhanced resistance to MEK inhibitor. ErbB3- and ErbB2-neutralizing antibodies blocked the protective effects of NRG1 in vitro and cooperated with MEK inhibitor to delay tumor growth in both cell line and patient-derived xenograft models. These results highlight tumor microenvironment regulation of targeted inhibitor resistance in WT/WT melanoma and provide a rationale for combining MEK inhibitors with anti-ErbB3/ErbB2 antibodies in WT/WT cutaneous melanoma patients for whom there are no effective targeted therapies options.

Keywords

NRG1; MEK; ErbB3; wild-type BRAF/NRAS melanoma; cancer-associated fibroblasts

Introduction

Effective targeted therapy for wild-type BRAF/wild-type NRAS (WT/WT) cutaneous melanoma remains an unmet clinical need (1). WT/WT tumors account for approximately 30% of cutaneous melanoma (2, 3). Approximately one-third of WT/WT melanoma harbor NF1 alterations; cKIT, cyclin D1 and CDK4 amplifications are also detected (2). Despite elevated MEK-ERK1/2 signaling in most WT/WT melanoma, the response of these patients to MEK inhibitors is poor (4). This contrasts with the efficacy of MEK inhibitors in V600E/K BRAF-harboring melanoma, for which trametinib is FDA-approved alone (5) and in combination with BRAF inhibitor (6). Additionally, the MEK inhibitor, binimetinib, showed improved response rates and progression-free survival compared to dacarbazine in mutant NRAS melanoma patients (7). The standard of care for WT/WT melanoma patients is immune checkpoint inhibitor therapy with anti-CTLA-4 (ipilimumab) and/or anti-PD-1 (pembrolizumab or nivolumab) (8). Since many WT/WT patients do not respond to immune checkpoint inhibitors and immune-related adverse effects are common, there is a critical clinical need for additional therapeutic options.

Tumors that have primary/intrinsic resistance to ERK1/2 pathway inhibitors frequently show adaptive up-regulation of expression of receptor tyrosine kinases (RTK) including IGF1R, EGFR, PDGFR β , AXL, MET and ErbB3 and consequential activation of ERK1/2-independent survival pathways (9–12). In mutant BRAF melanoma and thyroid carcinoma, ErbB3/HER3 (v-erb-b2 erythroblastic leukemia viral oncogene homolog 3/human epidermal receptor 3) up-regulation is a cellular adaptation to BRAF/MEK inhibition (9, 13). ErbB3 has weak kinase activity compared to other EGFR family members (14) and is activated via heterodimerization with other family members. ErbB2 is the most potent signaling partner for ErbB3 (15–17). The stroma has been strongly implicated melanoma progression (18). Growth factors that activate RTKs can be derived from the tumor microenvironment, suggesting a potential role for cells such as cancer-associated fibroblasts (CAFs) in mediating resistance to targeted therapies (9, 19–23). While up-regulated expression of ErbB3 and other RTKs are linked to resistance to BRAF and MEK inhibitors in BRAF mutant melanoma, the mechanisms underlying resistance in WT/WT melanoma remain unclear.

Here, we studied adaptive responses to MEK inhibitor in WT/WT cutaneous melanoma. Our results demonstrate that fibroblast-derived NRG1 promotes resistance to MEK inhibitor therapy in these tumors. Resistance is not dependent upon ErbB3 up-regulation, but rather is mediated via enhanced phosphorylation of ErbB2 and ErbB3 and increased ErbB2 cell surface levels. Targeting adaptive ErbB3/ErbB2 signaling with neutralizing antibodies to either ErbB2 or ErbB3 enhances the effects of MEK inhibitors in WT/WT melanoma *in vitro* and *in vivo*. These findings provide pre-clinical data to support the use of anti-ErbB2 and/or anti-ErbB3 antibodies to maximize the effects of MEK inhibitors in WT/WT cutaneous melanoma patients.

Materials and Methods

Cell culture

Details of the cell lines utilized in this study are provided in Supplementary Materials.

Isolation of CAFs

Human melanoma biopsies (TJUMEL40 and TJUMEL45) were obtained from Thomas Jefferson University Hospital under IRB-approved protocol (#10D.341) that include written informed consent and was in accordance with recognized ethical guidelines. Post-surgery, excess adipose tissue was removed. Tumors were cut into small pieces and digested with collagenase (Sigma-Aldrich Co) in complete medium at 37°C for 2–4 hours. Samples were washed with PBS and resuspended in DMEM supplemented with 10% FBS containing 5 µg/ml insulin. CAFs cultures were maintained for 5 passages. CAFs were authenticated by morphology and by the expression of α -smooth muscle actin (α -SMA), fibroblast activation protein (FAP) and PDGFR α and by the absence of CD45.

Short term culture

Patient derived xenografts (PDX) were generated in NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice. Two human melanoma biopsies (TJUMEL40 and WM4279) were processed, as described above, except samples were incubated with collagenase in complete medium at 37°C for 30 min. For TJUMEL40, the sample was divided and implanted into the back of two NSG mice with Matrigel (Corning). Tumors were harvested when they reached 1000 mm³ in size (passage 1) and re-implanted in mice (passage 2). The WM4279 PDX was obtained from the Wistar Institute as passage 3 (24, 25). Tumor pieces from either passage 1 or 2, for TJUMEL40, and passage 4 for WM4279 were collected, cut into small pieces and digested with collagenase in complete medium at 37°C for 2–4 hours. Cells were then centrifuged at 4000 rpm for 4 min; the pellet was washed with PBS and centrifuged again. The pellet was resuspended and cells were cultured in DMEM supplemented with 10% FBS for a single passage (TJUMEL40) or used directly for experiments (WM4279).

Growth factors, antibodies and inhibitors

Recombinant human NRG1 and insulin-like growth factor 1 (IGF1) were purchased from Cell Signaling Technology. Recombinant human epidermal growth factor (EGF), platelet-derived growth factor beta (PDGFbb), and hepatocyte growth factor (HGF) were purchased from Lonza, R&D Systems and Proteintech, respectively. LJM716 was produced and

purified by Novartis (Cambridge, MA). Trametinib and PD0325901 were purchased from Selleck Chemicals (Houston, TX).

Western blot analysis

Proteins were extracted with Laemmli sample buffer, resolved by SDS-PAGE and transferred to PVDF membranes. Immunoreactivity was detected using HRP-conjugated secondary antibodies (CalBioTech, Spring Valley, CA) and chemiluminescence HRP-recognizing substrates (ThermoScientific, Waltham, MA) on a VersaDoc Multi-Imager. Primary antibodies used are listed in Supplementary Material and Methods.

Crystal violet assays

Crystal violet assays were performed and analyzed as described (20). Plates were scanned and cell density was quantified using ImageJ software. The intensity mean was calculated for each well. Pictures were taken with a Nikon Eclipse Ti inverted microscope with NIS-Elements AR 3.00 software (Nikon, Melville, NY).

Reverse phase protein array (RPPA) analysis

WT/WT cells (B6, FEMX, MeWo, YUROL and WM3928) were treated, as indicated, and proteins analyzed, as previously described (26). Samples were normalized as described at <https://www.mdanderson.org/research/research-resources/core-facilities/functional-proteomics-rppa-core/faq.html>.

Conditioned medium preparation

For Western blot analysis, conditioned medium was prepared, as previously described (20). For growth assays, conditioned medium was prepared as previously described (20) except that cells were cultured in 18 ml of serum-free medium (SFM). For normalization, fibroblasts were counted afterwards. Cell counts per plate were: $3.34 \pm 0.55 \times 10^6$ HFF cells, $3.37 \pm 0.7 \times 10^6$ for HTERT cells and $1.44 \pm 0.24 \times 10^6$ CAFs.

Flow cytometry

Cells were treated, detached using 2 mM EDTA and washed twice with PBS. Samples were stained with Zombie NIR (Biolegend), for 10 min at room temperature in the dark. Following two washes with FACS buffer (PBS with 1% FBS and 0.5% sodium azide), cells were stained with either ErbB3-PE or ErbB2-PE (Biolegend) for 30 min at room temperature. Cells were fixed with cytofix/cytosperm and analyzed on an LSR II (BD Biosciences) using FlowJo software (TreeStar, Ashland, OR).

Xenograft experiments

Animal experiments were performed at a Thomas Jefferson University facility that is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. The Institutional Animal Care and Use Committee approved these studies. FEMX (1×10^6) or MeWo (2×10^6) cells were injected intradermally into the backs of athymic mice (NU/J, homozygous, Jackson, 6–8 weeks, 20–25 g). When tumors were palpable, mice were randomly sorted into four cohorts. For FEMX xenografts, mice were treated with trametinib

diet (1 mg/kg) and/or injected intraperitoneally with LJM716 (400 µg/mouse). AIN-76A diet and PBS injection (100 µl) were used as vehicle. Treatments were discontinued from day 9–12 and diet gel 76A (Scanbur) given to minimize weight loss. Drug treatments were resumed on day 12 and were continued to the end of the experiment. In the MeWo experiment PD0325901 diet (7 mg/kg), instead of trametinib, was utilized and LJM716 injected (400 µg/mouse to day 9, 200 µg/mouse after day 9). For TJUMEL40 PDX experiments, tumors were processed as described in short term culture except that when tumors were palpable, mice were randomly sorted into three cohorts. Five mice (4 female, F and 1 male, M) were treated with PD0325901 diet (7 mg/kg; Research Diets, Inc., New Brunswick, NJ), five mice (3F and 2M) were treated with PD0325901 diet (7 mg/kg) and injected intraperitoneally with LJM716 (200 µg/mouse for F and 300 µg/mouse for M). AIN-76A diet and PBS injection (500 µl) were used as vehicle in three mice (2F and 1M). Digital caliper measurements of the tumors were taken every 3 days and tumor volumes were calculated using the formula: $\text{volume} = (\text{length} \times \text{width}^2) \times 0.52$.

Immunohistochemistry

Tissue microarrays were generated at The University of Texas MD Anderson Cancer Center containing 345 tumor samples from 164 WT/WT melanoma patients. Of these, 110 samples were from 43 Stage III patients and 235 samples were from 121 Stage IV WT/WT patients. Expression of phospho-ErbB2 in the tissue microarray was run on the Ventana Benchmark Discovery staining platform. Discovery CC1 epitope retrieval was followed by antibody (pErbB2 Y1221/Y1222, 6B12, Cell Signaling Technology, at a 1:200 dilution) incubation for 44 min at ambient temperature. Phospho-ErbB2 was visualized with the Ventana ultra View Universal Alkaline Phosphatase Red Detection Kit and lastly counterstained with hematoxylin for 8 min. The intensity of staining and the percentage of positive cells were evaluated with the VisioPharm system. Samples were first separated by Stage. Samples with no detectable level were classified as negative and a mean intensity cutoff value of 50 was used to separate the remaining samples into high and low expression groups. The highest scoring call was kept for patients with multiple samples.

Tumor purity analysis and CAF gene correlation

The Cancer Genome Atlas (TCGA) cutaneous melanoma RNA sequencing (RNA-seq) V2 normalized gene expression and mutation call data were retrieved from the latest Broad GDAC Firehose data run (stddata__2016_01_28). Similarly, the TCGA cutaneous melanoma replicates-based normalized RPPA data (v 4.0) were collected from The Cancer Proteome Atlas (TCPA) (27). GSVA (28) was used to calculate a CAF signature score for each sample from the list of 88 CAF signature genes in Tirosh, I., et al. (29). ESTIMATE, LUMP, hematoxylin and eosin (H&E) staining and CPE tumor purity data were collected from Aran, D., et al. (30). ESTIMATE, LUMP and H&E staining purity values are calculated using RNA-seq V2, methylation, H&E staining data, respectively. CPE purity values are calculated from the combination of ESTIMATE, LUMP and H&E staining values. ABSOLUTE tumor purity data were gathered from the Cancer Genome Atlas Network (2). ABSOLUTE tumor purity values are calculated using copy number segment data. Eight of the CAF signature genes were removed from the list due to their use in calculating ESTIMATE purity values. Metastatic samples were retained when there were multiple

samples from the same patient. In total, 74 melanoma patient samples had an estimated purity level, RNA-seq data and a WT/WT mutation profile, with 54 also having RPPA data. Gene expression levels were log2-transformed then median-centered and clustered. Samples were then sorted based on CPE value. Colorblind-safe color schemes were derived from ColorBrewer (v2.0) (31).

Statistical analysis

Crystal violet quantification was performed using Student's two sample *t*-tests, assuming unequal variance. For RPPA analysis the two sample *t*-test with 1,000 permutations and assumed unequal variance was used to compare protein expression levels between NRG1 and $-/+$ trametinib groups at different time intervals for each cell line. Additional details are described in Supplementary Data.

To evaluate the percentage of WT/WT melanoma patient samples that co-express pErbB2 and pErbB3, the TCGA Skin Cutaneous Melanoma (SKCM) replicates-based normalized (v 4.0) RPPA dataset was collected from TCPA (27). Correlation analysis was performed between NRG1 (heregulin), pErbB2 and pErbB3 data for WT/WT samples ($n = 54$). Z-score values were calculated for pErbB2 and pErbB3 expression levels using all samples ($n = 354$). Samples with both pErbB2 and pErbB3 z-scores >1 and >-1 were classified as high and mid, respectively. The remaining samples were classified as low.

Associations between NRG1, pErbB2 and pErbB3 protein expression levels were determined by correlation analysis of RPPA data. For the association between NRG1 mRNA expression and CAF levels, NRG1 RNA-seq and CAF GSVA scores were used for correlation analysis. Tumor purity and NRG1 expression level associations were determined by correlation analysis of NRG1 RNA-seq and ABSOLUTE, ESTIMATE, LUMP, H&E staining and CPE tumor purity estimates plus NRG1 RPPA and CPE tumor purity estimates data. For FEMX and MeWo *in vivo* studies, the repeated over time log-transformed tumor volumes were modeled as a low order polynomial function of day using a linear mixed effects (LME) model adjusting for the random effects of animal and allowing for animal-specific growth trajectories. Additional details are described in Supplementary Material. For MeWo xenograft and TJUMEL40 PDX tumors, volume day-to-day comparisons were performed using Student's two sample *t*-tests, assuming unequal variance.

Results

High levels of ErbB3 and ErbB2 phosphorylation in WT/WT melanoma patient samples

It is important to provide the rationale for targeted therapy strategies in WT/WT melanoma. TCGA SKMC dataset analysis performed according to Liu et al. guidelines (32), showed that ErbB3 expression level is inversely correlated with Stage III WT/WT melanoma patient survival before treatment (Supplemental Fig. S1A). Furthermore, expression of NRG1, the ligand for ErbB3, positively correlated with the phosphorylation state of both ErbB3 and its co-receptor ErbB2 in WT/WT melanoma samples within the SKMC dataset (Fig. 1A). Analysis of the SKMC replicates-based normalization RPPA dataset using the z-score classification methods, showed that 78% (42 out of 54) of WT/WT melanoma patients co-

expressed medium-high levels of both phosphorylated ErbB3 and ErbB2 (Fig. 1B). A comparable co-expression was observed in BRAF mutant melanoma samples and similar results were also observed in NRAS mutant melanoma samples (Supplemental Fig. S1B). Similar findings were evident when the z-score was calculated based on the Pan-Cancer 19 RPPA data collected from The Cancer Proteome Atlas (TCPA) with nearly 60% of patients co-expressing phospho-ErbB3 and phospho-ErbB2 (Supplemental Fig. S1C). Based on these findings, we stained a tissue microarray (TMA) of WT/WT melanoma patient samples containing 43 Stage III tumors and 121 Stage IV tumors. Due to the suitability of antibodies for immunohistochemistry, phospho-ErbB2 was analyzed. 74% of Stage III and Stage IV WT/WT melanoma patient samples expressed phosphorylated ErbB2. 30% and 43% of 43 Stage III and 121 Stage IV WT/WT melanoma had high levels of phosphorylated ErbB2, respectively (Fig. 1C). These data indicate that ErbB3 and ErbB2 are activated in a high proportion of WT/WT melanoma patient tumors.

NRG1 expression is associated with the tumor stroma

While autocrine production of NRG1 mediates phosphorylation of ErbB3/ErbB2 in a small cohort WT/WT melanoma (33), ErbB3 may be a relevant target across WT/WT melanoma, possibly in combinatorial approaches. To assess tumor versus stromal source of NRG1, we analyzed the correlation between tumor purity estimates and NRG1 mRNA across 74 WT/WT patient samples from the SKMC TCGA dataset (2). Tumor purity estimates (ESTIMATE, LUMP, H&E, ABSOLUTE and CPE) derived from RNA-seq, H&E staining, methylation (30) and somatic copy number data (2) all showed statistically significant inverse correlations with NRG1 RNA level (Fig. 2A). CPE, which incorporates multiple tumor purity estimates, showed the strongest negative correlation with NRG1 RNA (Fig. 2A lower panel) and similar results with NRG1 protein levels within the TCGA dataset (Fig. 2B). Comparing RNA levels, NRG1 showed a statistically significant positive correlation with CAF signature genes (29) (Fig. 2C and 2D). The low level of endogenous NRG1 expression was confirmed in multiple WT/WT melanoma cell lines in both basal conditions and following MEK inhibition (Supplemental Fig. S2A and S2B). These data show that NRG1 RNA levels in human melanoma patient samples are associated with a high CAF content.

NRG1 partially reverses trametinib-mediated inhibition of cell growth

Since the MEK-ERK1/2 pathway is activated in WT/WT melanoma but MEK inhibitors are ineffective in patients, we analyzed the ability of NRG1 to mediate resistance to the MEK inhibitor, trametinib. We also tested the effect of four additional growth factors (EGF, HGF, IGF, and PDGFbb), which have been implicated in resistance to targeted therapies in mutant BRAF melanoma (9–12, 19). FEMX and MeWo cell lines were utilized, which are wild-type for BRAF and NRAS but differ in their NF1 status. FEMX retain NF1 expression but MeWo are negative for NF1 (Supplemental Fig. S3A and S3B) (34). In the absence of trametinib, all growth factors elicited no to modest increases in colony formation (Fig. 3A and Supplemental Fig. S3C). In trametinib-treated cells, NRG1 strongly rescued growth (Fig. 3B, Supplemental Fig. S3D and S3E). Contrary to the effects observed in BRAF mutant melanoma, EGF, HGF, IGF, and PDGFbb had little to no protective effect in MEK-inhibited cells (Fig. 3B and Supplemental Fig. S3D and S3E). NRG1-mediated protection was

generalizable as we observed similar effects in B6 and YUROL cells (Fig. 3C and Supplemental Fig. S3F). More modest effects were observed in WM3928. NRG1 effects were independent of NF1 expression. Notably, NRG1 stimulated high level phosphorylation of ErbB3 and ErbB2 in trametinib-treated cells compared to untreated cells (Fig. 3D). MET and IGFR were phosphorylated following treatment with HGF and IGF, respectively; however, phosphorylation of EGFR and PDGFR β was weak following growth factor stimulation, likely due to low receptor expression (Supplemental Fig. S3G and S3H). These data show that NRG1 modulates the response to MEK inhibitors in WT/WT melanoma cells.

NRG1 effects on cell growth in MEK-inhibited WT/WT melanoma cells are associated with ErbB3/ErbB2 adaptive activation

To investigate NRG1-mediated signaling, we performed Reverse Phase Protein Array (RPPA) in five WT/WT melanoma cell lines treated with/without MEK inhibitor (Supplemental Fig. S4A). NRG1-mediated phosphorylation of ErbB2 and ErbB3 was enhanced in trametinib-treated cells (Fig. 4A, 4B and Supplemental Fig. S4B), although enhanced ErbB3 phosphorylation did not meet statistical significance in all cell lines. Analysis of individual time points showed that ErbB2 phosphorylation was transiently enhanced in MEK-inhibited, NRG1-stimulated cells and was associated with prolonged AKT activation (quantitated in Fig. 4B lower graph and Supplemental Fig. S4B). In contrast to some findings on ERK1/2 pathway re-activation in mutant BRAF melanoma (21), NRG1 elicited minimal effects on ERK1/2 phosphorylation in MEK-inhibited WT/WT melanoma cells (Fig. 4C and Supplemental Fig. S4C). Western blot analysis corroborated the RPPA data showing enhanced NRG1-stimulated ErbB3/ErbB2 and AKT phosphorylation in MEK-inhibited cells (Fig. 4C; Supplemental Fig. S4D and S4E). The level of NRG1-mediated ErbB2 phosphorylation correlated with the ability of NRG1 to rescue MEK inhibitor effects on cell growth and to induce a higher and prolonged AKT activation (compare Fig. 4B with Fig. 3B and 3C). NRG1-stimulated AKT phosphorylation was lower and transient in WM3928 compared to the other cell lines, consistent with the lower activation of ErbB2 (Fig. 4C). This result correlated with lower level of protection afforded by NRG1 on cell growth following trametinib treatment (Fig. 3C). ErbB3 partners with other ErbB family members, such as EGFR and ErbB4, as well as MET (15–17, 35, 36). EGFR and ErbB4 were lowly expressed/undetectable and MET phosphorylation was unchanged following NRG1 treatment (Supplemental Fig. S3H, S3G and S4B). These data indicate that adaptive activation of ErbB3/ErbB2 and AKT signaling is associated with protection against MEK inhibition in WT/WT melanoma. Since NRAS mutant melanoma patients showed similar co-expressed medium-high levels of both phosphorylated ErbB3 and ErbB2 compared to WT/WT (Supplemental Fig. S1B), we next analyzed the effect of NRG1 in MEK-inhibited NRAS mutant cell lines. Out of 4 cell lines just one expressed ErbB3 and ErbB2 and showed adaptive activation of ErbB3/ErbB2 pathways in the presence of trametinib and NRG, confirming that NRG1 effects rely on the expression of ErbB3 and its co-receptor, ErbB2 (Supplemental Fig. S4F).

Enhanced ErbB2 phosphorylation and cell surface levels are associated with NRG1-mediated protection

Next, we tested for changes in the expression of ErbB3 and ErbB2 that would underlie enhanced signaling in MEK-inhibited cells. Increased NRG1-initiated ErbB3 and ErbB2 phosphorylation occurred within 6 hours of MEK inhibition in WT/WT melanoma cells suggesting a non-transcription-based mechanism (Fig. 5A). Total cellular levels of ErbB3 were either unaltered or only slightly up-regulated following MEK inhibition (Supplemental Fig. S4B and S4E). Both total and cell surface levels of ErbB2 expression also remained unchanged (Fig. 5A and Supplemental Fig. S5A). These data suggest that short-term MEK inhibition renders ErbB3/ErbB2 complexes more responsive to NRG1.

We also considered effects at later time points. Cell surface levels of ErbB3 after NRG1 stimulation were comparable in the absence and presence of MEK inhibitor (Supplemental Fig. S5B). By contrast, cell surface ErbB2 levels were consistently up-regulated by 24 hours of MEK inhibition but were subsequently down-regulated by NRG1 stimulation (Fig. 5B and Fig. 5C; Supplemental Fig. S5C and S5D). Importantly in NRG1-stimulated conditions, cell surface ErbB2 was increased in trametinib-treated cells versus control cells between 6 and 24 hours (Fig. 5C and Supplemental Fig. S5C). ErbB3 cell surface expression levels were also reduced following NRG1 stimulation (Fig. 5D and Supplemental Fig. S5E), consistent with previous studies (37, 38). The level of reduction was equivalent at most time points in MEK-inhibited versus vehicle cells and ErbB3 levels were restored by 24 hours of stimulation, making ErbB3 available for dimerization. Together, these data suggest the presence of a second mechanism that prolongs cell surface levels of ErbB2 following MEK inhibition to maintain durable activation of downstream signaling. These findings contrast with the mechanism identified in BRAF mutant cell lines in which NRG1 effects are mediated by the up-regulation of FOXD3 and its ability to induce ErbB3 transcript levels (9, 13).

Targeting ErbB3/ErbB2 complexes reverses NRG1-mediated effects on cell growth in MEK-inhibited WT/WT melanoma cells

To address the translational potential of our studies, we tested the ability of ErbB3 and ErbB2 neutralizing antibodies to block the protective effects of NRG1 in MEK-inhibited cells. We utilized LJM716, an ErbB3-neutralizing antibody that selectively binds an epitope created by domains II and IV of the ErbB3 extracellular domain and pertuzumab, a clinical-grade antibody that binds to ErbB2 subdomain II (39–41). LJM716 and pertuzumab individually reduced NRG1-induced phosphorylation of ErbB3, ErbB2 and AKT (Fig. 6A and Supplemental Fig. S6A). In colony growth assays, neither LJM716 nor pertuzumab affected cell growth in normal conditions (Supplemental Fig. S6B) but both antibodies partially reversed the protective effect of NRG1 in MEK-inhibited cell lines (Fig. 6B and Supplemental Fig. 6SC). Similarly, the β -sparing phosphoinositide 3-kinase (PI3K) inhibitor, GDC0032 (42), reduced NRG1-induced phosphorylation of ErbB3 and AKT and reversed NRG1 effect on cell growth in MEK-inhibited cells (Supplemental Fig. S6D and S6E). These data corroborate the notion that ErbB3 effects, in response to MEK inhibitor, are mediated through the activation of PI3K-AKT pathway.

Given the poor response to MEK inhibitors in WT/WT melanoma patients (43), there are few on-treatment WT/WT melanoma patient samples available for analysis. As an alternative, we generated short term cultures from WT/WT PDXs. Melanoma cultures from TJUMEL40 and WM4279 were validated by SOX10 expression and the absence of the fibroblast marker, PDGFR α (Supplemental Fig. S6F). Consistent with the established melanoma cell lines, TJUMEL40 cultures did not show activation of ErbB3 in basal conditions (Fig. 6C) and LJM716 did not affect their proliferation in the presence of NRG1 (Supplemental Fig. S6G). Furthermore, MEK inhibition increased phospho-ErbB3 and phospho-ErbB2 levels following NRG1 treatment; effects that were reversed by LJM76 and pertuzumab antibodies (Fig. 6C). LJM716 and pertuzumab partially reversed the protective effect of NRG1 on cell growth in MEK-inhibited TJUMEL40 cultures (Fig. 6D and Supplemental Fig. S6H). Thus, increased ErbB3/ErbB2 phosphorylation following MEK inhibition is targetable by ErbB3/ErbB2 neutralizing agents.

ErbB3/ErbB2 targeting blocks the protective effects of fibroblast-derived NRG1 and enhances the effects of MEK inhibitors *in vivo*

To test the role of the stromal TME, we evaluated the effects of fibroblast-derived conditioned medium. MEK-inhibited MeWo and FEMX cells showed robust phosphorylation of ErbB3, ErbB2 and AKT after treatment with conditioned medium from two different fibroblast cell lines (HFF and HTERT) (Fig. 7A and Supplemental Fig. S7A). Additionally, we detected NRG1 in isolated CAF cultures, which were verified by expression of α SMA, FAP and PDGFR α , from two WT/WT patient-derived melanoma (Supplemental Fig. S7B, S7C and S7D). Conditioned media derived from CAF40 and CAF45 stimulated phosphorylation of ErbB3, ErbB2 and AKT in MEK-inhibited WT/WT melanoma cells (Fig. 7B and Supplemental Fig. S7E). ErbB3, ErbB2 and AKT phosphorylation induced by fibroblast and CAF conditioned media was reversed by LJM716 or pertuzumab treatment (Fig. 7A, 7B and Supplemental Fig. S7A and S7E). Notably, CAF40 conditioned medium was able to induce ErbB3 and ErbB2 phosphorylation in the matched melanoma cells extracted from the same patient (TJUMEL40) (Fig. 7C). Conditioned media from fibroblasts and CAFs rescued the inhibitory effects of trametinib on cell growth (Fig. 7D, 7E and Supplemental Fig. S7F, S7G). Consistently, these effects were partially reversed by LJM716 and pertuzumab (Fig. 7D and 7E; Supplemental Fig. S7F and S7G). These data support the notion that paracrine NRG1 production promotes resistance to trametinib in WT/WT melanoma cells.

Our results provide the rationale to test the combination of MEK inhibitors with anti-ErbB3 antibodies in WT/WT melanoma *in vivo*. To assess the effects of MEK inhibitor and the anti-ErbB3 antibody, LJM716, in a fibroblast-rich microenvironment, we generated FEMX and MeWo intradermal xenografts. Treatment of xenografts with LJM716 alone did not alter tumor growth, supporting the notion that ErbB3 activation is an adaptive response to MEK inhibition (Fig. 7F and 7G). MEK inhibitor reduced tumor growth in both models, which was significantly further delayed when LJM716 was combined with MEK inhibitor treatment (Fig. 7F and 7G; Supplemental Fig. S7H). Furthermore, similar results were observed utilizing *in vivo* PDXs derived from TJUMEL40 (Fig. 7H). Together, these data

suggest that the ErbB3 blocking agents significantly enhance the growth reduction effect of MEK inhibitors of WT/WT melanoma.

Discussion

Our findings demonstrate that the ErbB3/ErbB2 pathway is adaptively activated in MEK inhibited WT/WT melanoma by stromal NRG1 and that targeting this compensatory pathway with clinical grade antibodies increases the efficiency of MEK inhibitors. Our findings underscore the influence of the tumor microenvironment in mediating resistance to targeted agents and support testing of MEK inhibitors and in combination with ErbB3/ErbB2 targeting antibodies in WT/WT cutaneous melanoma.

Our studies address an important clinical need. Major advances have been made for the treatment of V600-mutant BRAF melanoma. By contrast, targeted inhibitor trials in non-mutant

BRAF melanoma have elicited poor response rates. In a study from Falchook and colleagues, a 20% response rate to the MEK inhibitor, trametinib, was observed in WT/WT (although 2 of these samples harbored atypical BRAF mutations) (43). Thus, new strategies are needed for the treatment of this subgroup of melanoma. Our findings may extend to mutant NRAS melanoma. While bioinformatic analysis showed strong basal pErbB3 and pErbB2 levels in mutant NRAS melanoma, *cell-based* studies showed various levels of ErbB3 adaptive responses. These data reflect the high level of heterogeneity present in NRAS mutant melanoma and need further investigation to clarify the role of NRG1 in driving resistance to MEK inhibitor in this subgroup.

In the mutant BRAF setting, multiple growth factors and their cognate receptors have been shown to mediate resistance to BRAF inhibitors (9, 12, 19–22, 44). WT/WT melanoma are frequently sensitive to MEK inhibitors in monocultures (data within and (34, 45)). We show that NRG1 protects against MEK inhibitors in this subset of melanoma. By contrast, other growth factors linked to resistance to BRAF inhibitors in mutant BRAF melanoma, elicit little to no reversal of growth inhibition. Nevertheless, we do not rule out the possible involvement of other growth factors in protecting WT/WT melanoma from growth blockade mediated by MEK inhibitors. Indeed, LJM716 and pertuzumab partially, but not completely, reversed the effects of CAF conditioned medium on cell growth in MEK-inhibited cells. PI3K and AKT inhibitors may broadly block signaling downstream of multiple RTKs; however, the combination of MEK inhibitors and either PI3K or AKT inhibitors has been challenging with high toxicity and poor response rate issues (46). The use of bi- and multi-valent antibodies may represent a more efficient alternative to block the compensative activation of other RTKs (47). Clinical grade anti-ErbB3 targeting agents are being developed and tested in clinical trials for many solid malignancies (NCT02387216, NCT02167854, NCT01602406, NCT02980341) (48–50). Considering the high percentage of tumors co-expressing pErbB3 and pErbB2, drug-conjugated ErbB3/ErbB2 antibodies may increase the cytotoxic effect and the efficacy of treatment. Previous studies have shown that ErbB2 antibody drug conjugates have a favorable safety profile compared with other treatments and a notable survival benefit in heavily pretreated patients, including patients

treated with pertuzumab or lapatinib, with less severe toxic effects than treatment of physician's choice (51).

We show up-regulation ErbB3/ErbB2 phosphorylation in MEK-inhibited WT/WT melanoma. In contrast to previous studies published in mutant BRAF melanoma and mutant KRAS lung and colon cancer (9, 13, 20–22, 52, 53), NRG1 effects in MEK-inhibited WT/WT cells are likely driven by an increase in ErbB2 phosphorylation that occurs within hours of stimulation, suggestive of a non-transcriptional mechanism, and by the retention of ErbB2 protein at the cell surface. Recent studies in breast cancer cells demonstrate ErbB2 is inactivated by ERK1/2-dependent phosphorylation of threonine 677 in the juxtamembrane region. By contrast, in MEK-inhibited cells, dephosphorylation of threonine 677 enables ErbB2 to be a more effective co-receptor for ErbB3 (53, 54). In our studies, phosphorylation of ErbB2 at T677 was not detected in basal conditions (Supplemental Fig S7I).

We previously showed that a small subset of WT/WT melanoma express NRG1 which acts in an autocrine manner and these melanomas are highly sensitive to ErbB3 mono-therapy (33) and show less sensitivity to MEK inhibition, *in vitro*, compared to the subgroup tested in this study. Through analysis of patient database samples and use of conditioned medium derived from CAFs isolated from tumors, we show that ErbB3/ErbB2 activation is mediated via a paracrine NRG1 mechanism in the majority of WT/WT melanoma, including our PDX-derived cells, TJUMEL40 and WM4279. In our studies, these tumors do not respond to ErbB3 monotherapy. The use of PDX models as well as patient-matched CAFs and tumor cell cultures strengthens the notion that stromal NRG1 drives adaptive resistance in WT/WT melanoma. The mechanism(s) regulating NRG1 expression in CAFs remains to be determined. We show that phosphorylated ErbB3 and ErbB2 are co-expressed in nearly 80% of WT/WT melanoma, suggesting that a high percentage of patients could potentially benefit of the combination of MEK inhibitor plus ErbB3/ErbB2 antibodies. The ability of NRG1 to rescue MEK inhibitors effect on cell growth is linked to its ability to phosphorylate ErbB2, identifying phospho-ErbB2 as a possible biomarker for testing the combinatory effects of MEK inhibitors and ErbB3/ErbB2 targeting antibodies in WT/WT cutaneous melanoma.

Heterogeneity in melanoma is an important issue and moving forward it will be critical to identify biomarkers for patients who are likely to respond to MEK inhibitor/ErbB3 targeting treatment combinations. Given the intra-tumor heterogeneity, this should ideally be performed at the single cell level. The transcription factor SOX10 has been described as a direct regulator of ErbB3 in neural crest-derived cells (55); however, SOX10 in mutant BRAF melanoma cells is associated with repression of EGFR and PDGFR (11). Additionally, depletion of SOX10 is associated with a slow-growing phenotype that may contribute to resistance to targeted inhibitors (11). A future direction will be to determine the effect of MEK inhibitors on intra-tumor heterogeneity in WT/WT melanoma.

Significance

This work suggests a mechanism by which NRG1 regulates the sensitivity of WT NRAS/BRAF melanomas to MEK inhibitors and provides rationale for combining MEK inhibitors with anti-ErbB2/ErbB3 antibodies in these tumors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Merlino G, Herlyn M, Fisher DE, Bastian BC, Flaherty KT, Davies MA, et al. The state of melanoma: challenges and opportunities. *Pigment Cell Melanoma Res.* 2016; 29:404–16. [PubMed: 27087480]
- Cancer Genome Atlas N. Genomic Classification of Cutaneous Melanoma. *Cell.* 2015; 161:1681–96. [PubMed: 26091043]
- Hodis E, Watson IR, Kryukov GV, Arold ST, Imielinski M, Theurillat JP, et al. A landscape of driver mutations in melanoma. *Cell.* 2012; 150:251–63. [PubMed: 22817889]
- Falchook GS, Lewis KD, Infante JR, Gordon MS, Vogelzang NJ, DeMarini DJ, et al. Activity of the oral MEK inhibitor trametinib in patients with advanced melanoma: a phase 1 dose-escalation trial. *Lancet Oncol.* 13:782–9.
- Flaherty KT, Robert C, Hersey P, Nathan P, Garbe C, Milhem M, et al. Improved survival with MEK inhibition in BRAF-mutated melanoma. *N Engl J Med.* 2012; 367:107–14. [PubMed: 22663011]
- Flaherty KT, Infante JR, Daud A, Gonzalez R, Kefford RF, Sosman J, et al. Combined BRAF and MEK inhibition in melanoma with BRAF V600 mutations. *N Engl J Med.* 2012; 367:1694–703. [PubMed: 23020132]
- Dummer R, Schadendorf D, Ascierto PA, Arance A, Dutriaux C, Di Giacomo AM, et al. Binimetinib versus dacarbazine in patients with advanced NRAS-mutant melanoma (NEMO): a multicentre, open-label, randomised, phase 3 trial. *Lancet Oncol.* 2017; 18:435–45. [PubMed: 28284557]
- Larkin J, Chiarion-Sileni V, Gonzalez R, Grob JJ, Cowey CL, Lao CD, et al. Combined nivolumab and ipilimumab or monotherapy in untreated melanoma. *N Engl J Med.* 2015; 373:23–34. [PubMed: 26027431]
- Abel EV, Basile KJ, Kugel CH 3rd, Witkiewicz AK, Le K, Amaravadi RK, et al. Melanoma adapts to RAF/MEK inhibitors through FOXD3-mediated upregulation of ERBB3. *J Clin Invest.* 2013; 123:2155–68. [PubMed: 23543055]
- Kugel CH 3rd, Aplin AE. Adaptive resistance to RAF inhibitors in melanoma. *Pigment Cell Melanoma Res.* 2014; 27:1032–8. [PubMed: 24828387]
- Sun C, Wang L, Huang S, Heynen GJ, Prahallad A, Robert C, et al. Reversible and adaptive resistance to BRAF(V600E) inhibition in melanoma. *Nature.* 2014; 508:118–22. [PubMed: 24670642]
- Villanueva J, Vultur A, Lee JT, Somasundaram R, Fukunaga-Kalabis M, Cipolla AK, et al. Acquired resistance to BRAF inhibitors mediated by a RAF kinase switch in melanoma can be overcome by cotargeting MEK and IGF-1R/PI3K. *Cancer Cell.* 2010; 18:683–95. [PubMed: 21156289]

13. Montero-Conde C, Ruiz-Llorente S, Dominguez JM, Knauf JA, Viale A, Sherman EJ, et al. Relief of feedback inhibition of HER3 transcription by RAF and MEK inhibitors attenuates their antitumor effects in BRAF-mutant thyroid carcinomas. *Cancer Discov.* 2013; 3:520–33. [PubMed: 23365119]
14. Shi F, Telesco SE, Liu Y, Radhakrishnan R, Lemmon MA. ErbB3/HER3 intracellular domain is competent to bind ATP and catalyze autophosphorylation. *Proc Natl Acad Sci U S A.* 2010; 107:7692–7. [PubMed: 20351256]
15. Tzahar E, Waterman H, Chen X, Levkowitz G, Karunakaran D, Lavi S, et al. A hierarchical network of interreceptor interactions determines signal transduction by Neu differentiation factor/neuregulin and epidermal growth factor. *Mol Cell Biol.* 1996; 16:5276–87. [PubMed: 8816440]
16. Pinkas-Kramarski R, Soussan L, Waterman H, Levkowitz G, Alroy I, Klapper L, et al. Diversification of Neu differentiation factor and epidermal growth factor signaling by combinatorial receptor interactions. *EMBO J.* 1996; 15:2452–67. [PubMed: 8665853]
17. Wallasch C, Weiss FU, Niederfellner G, Jallat B, Issing W, Ullrich A. Heregulin-dependent regulation of HER2/neu oncogenic signaling by heterodimerization with HER3. *EMBO J.* 1995; 14:4267–75. [PubMed: 7556068]
18. Villanueva J, Herlyn M. Melanoma and the tumor microenvironment. *Curr Oncol Rep.* 2008; 10:439–46. [PubMed: 18706274]
19. Straussman R, Morikawa T, Shee K, Barzily-Rokni M, Qian ZR, Du J, et al. Tumour micro-environment elicits innate resistance to RAF inhibitors through HGF secretion. *Nature.* 2012; 487:500–4. [PubMed: 22763439]
20. Capparelli C, Rosenbaum S, Berger AC, Aplin AE. Fibroblast-derived neuregulin 1 promotes compensatory ErbB3 receptor signaling in mutant BRAF melanoma. *J Biol Chem.* 2015; 290:24267–77. [PubMed: 26269601]
21. Lito P, Pratilas CA, Joseph EW, Tadi M, Halilovic E, Zubrowski M, et al. Relief of profound feedback inhibition of mitogenic signaling by RAF inhibitors attenuates their activity in BRAFV600E melanomas. *Cancer Cell.* 2012; 22:668–82. [PubMed: 23153539]
22. Kugel CH 3rd, Hartsough EJ, Davies MA, Setiady YY, Aplin AE. Function-blocking ERBB3 antibody inhibits the adaptive response to RAF inhibitor. *Cancer Res.* 2014; 74:4122–32. [PubMed: 25035390]
23. Wilson TR, Fridlyand J, Yan Y, Penuel E, Burton L, Chan E, et al. Widespread potential for growth-factor-driven resistance to anticancer kinase inhibitors. *Nature.* 2012; 487:505–9. [PubMed: 22763448]
24. Krepler C, Sproesser K, Brafford P, Beqiri M, Garman B, Xiao M, et al. A Comprehensive Patient-Derived Xenograft Collection Representing the Heterogeneity of Melanoma. *Cell Rep.* 2017; 21:1953–67. [PubMed: 29141225]
25. Garman B, Anastopoulos IN, Krepler C, Brafford P, Sproesser K, Jiang Y, et al. Genetic and Genomic Characterization of 462 Melanoma Patient-Derived Xenografts, Tumor Biopsies, and Cell Lines. *Cell Rep.* 2017; 21:1936–52. [PubMed: 29141224]
26. Tibes R, Qiu Y, Lu Y, Hennessy B, Andreeff M, Mills GB, et al. Reverse phase protein array: validation of a novel proteomic technology and utility for analysis of primary leukemia specimens and hematopoietic stem cells. *Mol Cancer Ther.* 2006; 5:2512–21. [PubMed: 17041095]
27. Li J, Lu Y, Akbani R, Ju Z, Roebuck PL, Liu W, et al. TCPA: a resource for cancer functional proteomics data. *Nat Methods.* 2013; 10:1046–7.
28. Hanzelmann S, Castelo R, Guinney J. GSEA: gene set variation analysis for microarray and RNA-seq data. *BMC Bioinformatics.* 2013; 14. [PubMed: 23323936]
29. Tirosh I, Izar B, Prakadan SM, Wadsworth MH 2nd, Treacy D, Trombetta JJ, et al. Dissecting the multicellular ecosystem of metastatic melanoma by single-cell RNA-seq. *Science.* 2016; 352:189–96. [PubMed: 27124452]
30. Aran D, Sirota M, Butte AJ. Systematic pan-cancer analysis of tumour purity. *Nat Commun.* 2015; 6:8971. [PubMed: 26634437]
31. Brewer CA. Basic mapping principles for visualizing cancer data using Geographic Information Systems (GIS). *Am J Prev Med.* 2006; 30:S25–36. [PubMed: 16458787]

32. Liu J, Lichtenberg T, Hoadley KA, Poisson LM, Lazar AJ, Cherniack AD, et al. An Integrated TCGA Pan-Cancer Clinical Data Resource to Drive High-Quality Survival Outcome Analytics. *Cell*. 2018; 173:400–16e11. [PubMed: 29625055]
33. Capparelli C, Rosenbaum S, Berman-Booty LD, Salhi A, Gaborit N, Zhan T, et al. ErbB3-ErbB2 Complexes as a Therapeutic Target in a Subset of Wild-type BRAF/NRAS Cutaneous Melanomas. *Cancer Res*. 75:3554–67. [PubMed: 26206558]
34. Nissan MH, Pratilas CA, Jones AM, Ramirez R, Won H, Liu C, et al. Loss of NF1 in cutaneous melanoma is associated with RAS activation and MEK dependence. *Cancer Res*. 2014; 74:2340–50. [PubMed: 24576830]
35. Engelman JA, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, Park JO, et al. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. *Science*. 2007; 316:1039–43. [PubMed: 17463250]
36. Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol*. 2001; 2:127–37. [PubMed: 11252954]
37. Jeong J, VanHouten JN, Dann P, Kim W, Sullivan C, Yu H, et al. PMCA2 regulates HER2 protein kinase localization and signaling and promotes HER2-mediated breast cancer. *Proc Natl Acad Sci U S A*. 2016; 113:E282–90. [PubMed: 26729871]
38. Sorkin A, Goh LK. Endocytosis and intracellular trafficking of ErbBs. *Exp Cell Res*. 2009; 315:683–96. [PubMed: 19278030]
39. Garner AP, Bialucha CU, Sprague ER, Garrett JT, Sheng Q, Li S, et al. An antibody that locks HER3 in the inactive conformation inhibits tumor growth driven by HER2 or neuregulin. *Cancer Res*. 2013; 73:6024–35. [PubMed: 23928993]
40. Garrett JT, Sutton CR, Kurupi R, Bialucha CU, Ettenberg SA, Collins SD, et al. Combination of antibody that inhibits ligand-independent HER3 dimerization and a p110alpha inhibitor potently blocks PI3K signaling and growth of HER2+ breast cancers. *Cancer Res*. 73:6013–23.
41. Franklin MC, Carey KD, Vajdos FF, Leahy DJ, de Vos AM, Sliwkowski MX. Insights into ErbB signaling from the structure of the ErbB2-pertuzumab complex. *Cancer Cell*. 2004; 5:317–28. [PubMed: 15093539]
42. Deuker MM, Marsh Durban V, Phillips WA, McMahon M. PI3'-kinase inhibition forestalls the onset of MEK1/2 inhibitor resistance in BRAF-mutated melanoma. *Cancer Discov*. 2015; 5:143–53. [PubMed: 25472943]
43. Falchook GS, Lewis KD, Infante JR, Gordon MS, Vogelzang NJ, DeMarini DJ, et al. Activity of the oral MEK inhibitor trametinib in patients with advanced melanoma: a phase 1 dose-escalation trial. *Lancet Oncol*. 2012; 13:782–9. [PubMed: 22805292]
44. Nazarian R, Shi H, Wang Q, Kong X, Koya RC, Lee H, et al. Melanomas acquire resistance to B-Raf(V600E) inhibition by RTK or N-Ras upregulation. *Nature*. 2010; 468:973–7. [PubMed: 21107323]
45. Ranzani M, Alifrangis C, Perna D, Dutton-Regester K, Pritchard A, Wong K, et al. BRAF/NRAS wild-type melanoma, NF1 status and sensitivity to trametinib. *Pigment Cell Melanoma Res*. 2015; 28:117–9. [PubMed: 25243813]
46. Jokinen E, Koivunen JP. MEK and PI3K inhibition in solid tumors: rationale and evidence to date. *Ther Adv Med Oncol*. 2015; 7:170–80. [PubMed: 26673580]
47. Fitzgerald JB, Johnson BW, Baum J, Adams S, Iadevaia S, Tang J, et al. MM-141, an IGF-IR- and ErbB3-directed bispecific antibody, overcomes network adaptations that limit activity of IGF-IR inhibitors. *Mol Cancer Ther*. 2014; 13:410–25. [PubMed: 24282274]
48. Abramson VG, Supko JG, Ballinger T, Cleary JM, Hilton JF, Tolaney S, et al. Phase Ib study of safety and pharmacokinetics of the PI3K inhibitor SAR245408 with the HER3 neutralizing human antibody SAR256212 in patients with solid tumors. *Clin Cancer Res*. 2016; 23:3520–8. [PubMed: 28031425]
49. Liu JF, Ray-Coquard I, Selle F, Poveda AM, Cibula D, Hirte H, et al. Randomized phase II trial of seribantumab in combination with paclitaxel in patients with advanced platinum-resistant or -refractory ovarian cancer. *J Clin Oncol*. 2016; 34:4345–53. [PubMed: 27998236]

50. Takahashi S, Kobayashi T, Tomomatsu J, Ito Y, Oda H, Kajitani T, et al. LJM716 in Japanese patients with head and neck squamous cell carcinoma or HER2-overexpressing breast or gastric cancer. *Cancer Chemother Pharmacol.* 2017; 79:131–8. [PubMed: 27942917]
51. Krop IE, Kim SB, Martin AG, LoRusso PM, Ferrero JM, Badovinac-Crnjevic T, et al. Trastuzumab emtansine versus treatment of physician's choice in patients with previously treated HER2-positive metastatic breast cancer (TH3RESA): final overall survival results from a randomised open-label phase 3 trial. *Lancet Oncol.* 2017; 18:743–54. [PubMed: 28526538]
52. Sun C, Hobor S, Bertotti A, Zecchin D, Huang S, Galimi F, et al. Intrinsic resistance to MEK inhibition in KRAS mutant lung and colon cancer through transcriptional induction of ERBB3. *Cell Rep.* 2014; 7:86–93. [PubMed: 24685132]
53. Turke AB, Song Y, Costa C, Cook R, Arteaga CL, Asara JM, et al. MEK inhibition leads to PI3K/AKT activation by relieving a negative feedback on ERBB receptors. *Cancer Res.* 2012; 72:3228–37. [PubMed: 22552284]
54. Kawasaki Y, Sakimura A, Park CM, Tomaru R, Tanaka T, Ozawa T, et al. Feedback control of ErbB2 via ERK-mediated phosphorylation of a conserved threonine in the juxtamembrane domain. *Sci Rep.* 2016;6. [PubMed: 28442741]
55. Prasad MK, Reed X, Gorkin DU, Cronin JC, McAdow AR, Chain K, et al. SOX10 directly modulates ERBB3 transcription via an intronic neural crest enhancer. *BMC Dev Biol.* 2011;11. [PubMed: 21352525]

Figure 1. The majority of WT/WT melanoma patient samples co-express pErbB3 and pErbB2

A. Scatter plot of neuregulin vs pErbB2 (blue) and pErbB3 (red) normalized protein values from WT/WT (n=54) in the TCGA cutaneous replicates-based normalized (v 4.0) RPPA melanoma dataset. **B.** Scatter plot of pErbB2 and pErbB3 z-score values from WT/WT TCGA cutaneous melanoma samples (n=54). Samples were classified as having high, mid or low pErbB2 and pErbB3 co-expression based on z-score cutoffs of 1 and -1 (gray dotted lines). Z-scores were calculated using all TCGA cutaneous melanoma samples (n=354). **C.** TMA samples were stained for pErbB2. The graph represents the proportion of WT/WT samples from Stage III (n=43) and Stage IV (n=121) patients. Samples with no detectable level were classified as negative and a mean intensity cutoff value of 50 was used to separate the remaining samples into high and low expression groups (left panel). The representative images show samples that scored negative, low and high for pErbB2. Pictures were taken at 200x magnification. Scale bars, 100 μ m.

Figure 2. High NRG1 is associated with the tumor stroma
A. Table with Spearman's rank-order correlation results comparing NRG1 mRNA expression levels to all tumor purity estimation metrics (upper panel). Moving average plot of NRG1 mRNA expression versus CPE tumor purity (lower panel). **B.** Moving average plot of NRG1 protein expression versus CPE tumor purity. **C.** Heat map of median-centered, log2-transformed mRNA expression values for NRG1 and 80 CAF genes with samples sorted by CPE values. **D.** Moving average plot of NRG1 mRNA expression versus CAF GSVA scores. Moving average plots display GSVA or tumor purity values (black), individual sample NRG1 levels (gray) and average NRG1 levels over a 15-sample window (red) for RNA-seq (n=74) or RPPA (n=54) data from TCGA WT/WT cutaneous melanoma patients.

Figure 3. NRG1 recovery of cell growth in MEK-inhibited cells is associated with activation of the ErbB3/ErbB2 pathway

A. FEMX and MeWo cells were treated with either EGF, HGF, IGF, NRG1 or PDGFbb (50 ng/ml) twice per week. After 6 days, cells were fixed and stained with crystal violet. Quantification was performed using ImageJ software. Graphed is the mean intensity $\pm$ SD from three independent experiments. * $p < 0.05$.

B. FEMX and MeWo were treated as in A. except that cells were pretreated overnight with trametinib (50 nM). Medium and treatment were replaced twice per week. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. **C.** B6, YUOL and WM3928 cells were pre-treated overnight with trametinib and on the next day NRG1 (10 ng/ml) added. Medium and treatments were replaced twice per week. After 6 days, cells were fixed and stained with crystal violet. Quantification was performed using ImageJ software. Graphed is the mean intensity $\pm$ SD from three independent experiments. * $p < 0.05$; ** $p < 0.01$; ns = not significant.

D. FEMX and MeWo cells were treated overnight with trametinib and on the next day stimulated with either EGF, HGF, IGF, NRG1 or PDGFbb (50 ng/ml) for 10 min. Cell lysates were analyzed by Western blot, as indicated.

Figure 4. NRG1 induces adaptive activation of ErbB2 and prolonged AKT phosphorylation in MEK-inhibited cells

A. B6, FEMX, MeWo (2×10^5), WM3928 (2.5×10^5) and YUROL (3×10^5) cells were seeded in single wells of a 6-well plate. Cells were treated for a total of 48 hours $\pm$ trametinib (50 nM) and for the last 0, 1, 24 hours with NRG1 (10 ng/ml). Cells were lysed at the same time and processed for RPPA. Shown is a heat map from three independent experiments with median-polished, log2-transformed group linear average values for antibodies passing significance cutoffs. **B.** Quantitation of pErbB2 Y1248 and pAKT S473 expression levels for cell lines treated as in A. (n=3; errors bars, S.E.M.; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, NS = not significant). **C.** The same number of cells were plated as in A. Cells were treated for a total of 48 hours $\pm$ trametinib (50 nM) and for the last 0, 1, 24 hours with NRG1 (10 ng/ml). Cells were lysed at the same time and analyzed by Western blotting with the antibodies indicated.

Figure 5. Cell surface ErbB2 levels are increased following MEK inhibition and reduced by NRG1 stimulation

A. FEMX and MeWo cells were treated with trametinib for 0, 0.5, 1, 3 and 6 hours before adding NRG1 for 10 min. Cells were lysed and lysates analyzed by Western blotting, as indicated. **B.** Cells were treated with trametinib (50 nM) for 24 hours before stimulation with NRG1 (10 ng/ml) for a further 24 hours. Cells were collected and analyzed by flow cytometry for ErbB2 surface expression, as described in Materials and Methods. Graphed is the mean intensity $\pm$ SD from three independent experiments. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, ns = not significant. **C.** Cells were treated for 24 hours with trametinib before stimulation with NRG1 for 0, 1, 3, 6, and 24 hours. Cells were collected and analyzed by flow cytometry for ErbB2 surface levels. Graphed is the mean intensity $\pm$ SD from three independent experiments. ns = not significant. **D.** As for C, except that ErbB3 was analyzed. * = $p < 0.05$, ns = not significant comparing control versus trametinib at the same time point.

Figure 6. ErbB3/ErbB2 neutralizing antibodies reverse NRG1 protection in MEK-inhibited WT/WT melanoma cells

A. B6, FEMX, MeWo (2×10^5), WM3928 (2.5×10^5) and YUROL (3×10^5) cells were seeded in single wells of a 6-well plate. Cells were treated $\pm$ trametinib (overnight, 50 nM) and LJM716 (25 μ g/ml) or pertuzumab (10 μ g/ml) added for 45 min before stimulation with NRG1 (10 ng/ml) for 1 hour. Cells were lysed and analyzed by Western blot, as indicated. The black horizontal lines indicate the separation of blots from independent experiments. **B.** The same number of cells was plated as in A. Cells were treated with trametinib and then with LJM716 or pertuzumab for 45 min after which NRG1 was added. Medium and treatment were renewed twice per week. After 6 days, cells were fixed and stained with crystal violet. Scale bars, 50 μ m. Quantification was performed using ImageJ software. Graphed is the mean intensity $\pm$ SD from three independent experiments. * $p < 0.05$; ** $p < 0.01$. **C.** TJUMEL40 and WM4279 were generated from WT/WT PDX, as described in Materials and Methods. Cells were treated $\pm$ trametinib and LJM716 or pertuzumab added for 45 min before stimulation with NRG1 for 1 hour. Cell lysates were analyzed by Western blot. **D.** TJUMEL40 cells were treated with trametinib and then with LJM716 or pertuzumab for 45 min after which NRG1 was added. Medium and treatment were renewed twice per

week. After 6 days, cells were fixed and stained with crystal violet. Scale bars, 50 μm . Quantification was performed using ImageJ software. Graphed is the mean intensity $\pm$ SD from three independent experiments. * $p < 0.05$; ** $p < 0.01$.

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Figure 7. ErbB3 and ErbB2-neutralizing antibodies enhance effects of MEK inhibitor

A. MeWo cells (2×10^5) were seeded in single wells of a 6-well plate. Cells were treated overnight with trametinib (50 nM), and then LJM716 (25 μ g/ml) or pertuzumab (10 μ g/ml) added for 45 min. Finally, fibroblast cond. med. was added for 1 hour. Concentrated DMEM alone was used as control. Cells lysates were analyzed by Western blot. **B.** Western blot analysis as in A, except cond. med. from CAFs was utilized. **C.** Western blots of TJUMEL40 cells treated as described in B with CAF40 conditioned medium. **D.** The same number of cells as in A. was plated. Cells were treated twice per week as in A for 6 days and crystal violet analysis performed. Mean intensity $\pm$ SD from three independent experiments. *p<0.05; **p<0.01. **E.** The same number of cells was plated as in A. Cells were treated as in D., cond. medium from CAF40 was utilized. **F.** Average tumor volume $\pm$ SEM in FEMX xenografts. Mice were treated with: CTL; MEK inhibitor (1 mg/kg trametinib); anti-ErbB3 (400 μ g LJM716); or the combination. p=0.026 and p=0.001 for overall comparison of time trends between combo treatment and MEK inhibitor or LJM716, respectively. **G.** MeWo xenografts were treated with vehicle, MEK inhibitor (MEKi: 7 mg/kg PD0325901), anti-ErbB3 (LJM716: 400 μ g/mouse to day 9 and 200 μ g/mouse after day 9) or the combination. Average tumor volume $\pm$ SEM. *p<0.05; **p<0.01; ***p<0.001 for day-to-day comparison.

p=0.001 and p<0.001 for overall comparison of time trends between combo treatment and MEK inhibitor or LJM716, respectively. **H.** TJUMEL40 PDXs were treated with: vehicle (CTL), MEK inhibitor (MEKi: 7 mg/kg PD0325901), MEKi in combination with anti-ErbB3 (LJM716: 200 µg/mouse for females and 300 µg/mouse for males). Average tumor volume ± SEM. *p<0.05; **p<0.01 for day-to-day comparison.

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