Title
Recurrent Tumor Cell-Intrinsic and -Extrinsic Alterations during MAPKi-Induced Melanoma Regression and Early Adaptation.

Permalink
https://escholarship.org/uc/item/9626v45g

Journal
Cancer discovery, 7(11)

ISSN
2159-8274

Authors
Song, Chunying
Piva, Marco
Sun, Lu
et al.

Publication Date
2017-11-01

DOI
10.1158/2159-8290.cd-17-0401

Peer reviewed
Recurrent Tumor Cell–Intrinsic and –Extrinsic Alterations during MAPKi-Induced Melanoma Regression and Early Adaptation

Chunying Song1,2, Marco Piva1,2, Lu Sun1,2, Aayoung Hong1,2,3, Gatien Moriceau1,2, Xiangju Kong1,2, Hong Zhang1,2, Shirley Lomeli1,2, Jin Qian1,2, Clarissa C. Yu1,2, Robert Damaoiseaux2,3,4, Mark C. Kelley5, Kimberley B. Dahlman6, Philip O. Scumpia1, Jeffrey A. Sosman7, Douglas B. Johnson7, Antoni Ribas2,3,4,8,9, Willy Hugo1,2, and Roger S. Lo1,2,3,4

ABSTRACT

Treatment of advanced BRAFV600-mutant melanoma using a BRAF inhibitor or its combination with a MEK inhibitor typically elicits partial responses. We compared the transcriptomes of patient-derived tumors regressing on MAPK inhibitor (MAPKi) therapy against MAPKi-induced temporal transcriptomic states in human melanoma cell lines or murine melanoma in immune-competent mice. Despite heterogeneous dynamics of clinical tumor regression, residual tumors displayed highly recurrent transcriptomic alterations and enriched processes, which were also observed in MAPKi-selected cell lines (implying tumor cell–intrinsic reprogramming) or in bulk mouse tumors (and the CD45-negative or CD45-positive fractions, implying tumor cell–intrinsic or stromal/immune alterations, respectively). Tumor cell–intrinsic reprogramming attenuated MAPK dependency, while enhancing mesenchymal, angiogenic, and IFN-inflammatory features and growth/survival dependence on multi-RTKs and PD-L2. In the immune compartment, PD-L2 upregulation in CD11c+ immunocytes drove the loss of T-cell inflammation and promoted BRAFi resistance. Thus, residual melanoma early on MAPKi therapy already displays potentially exploitable adaptive transcriptomic, epigenomic, immune-regulomic alterations.

SIGNIFICANCE: Incomplete MAPKi-induced melanoma regression results in transcriptome/methylome-wide reprogramming and MAPK-redundant escape. Although regressing/residual melanoma is highly T cell–inflamed, stromal adaptations, many of which are tumor cell–driven, could suppress/eliminate intratumoral T cells, reversing tumor regression. This catalog of recurrent alterations helps identify adaptations such as PD-L2 operative tumor cell intrinsically and/or extrinsically early on therapy. Cancer Discov; 7(11); 1248–65. ©2017 AACR.

Cancer Discov; 7(11); 1248–65. ©2017 AACR.
See related commentary by Haq, p. 1216.

Note: Supplementary data for this article are available at Cancer Discovery Online (http://cancerdiscovery.aacrjournals.org/).

C. Song, M. Piva, and L. Sun contributed equally to this article.

Corresponding Author: Roger S. Lo, University of California, Los Angeles, 10833 Le Conte Avenue, 52-121 CHS, Los Angeles, CA 90095-1750; Phone: 310-794-6608; Fax: 310-206-9878; E-mail: rlo@mednet.ucla.edu. doi: 10.1158/2159-8290.CD-17-0401
©2017 American Association for Cancer Research.
INTRODUCTION

The only clinically validated approach (i.e., BRAF inhibitor + MEK inhibitor) to suppress acquired BRAF inhibitor (BRAFi) resistance in melanoma addresses only MAPK-reactivating mechanisms (1–7). Recent appreciation of the pervasiveness of nongenomic and immune evolution in melanoma with acquired or late MAPK inhibitor (MAPKi) resistance (2) suggests dynamic and continuous alterations early on therapy. This is consistent with the clinical experience that the majority of initial tumor responses to MAPKi are partial, with many acquired resistant tumors later growing in situ from these residual tumors. The equilibrium between the tumor cell and stromal/immune compartments is expected to be highly dynamic immediately after initiating therapy. Thus, there is a need to develop models that would recapitulate temporally either tumor cell–intrinsic or tumor cell–extrinsic adaptations in regressing/persisting melanoma early during MAPKi therapy.

It is known that melanoma tumors responding to MAPKi therapy display robust T-cell infiltration (8). However, the extent and nature of stromal/immune compartment remodeling and potential adaptations, including adaptive immune resistance, has not been systematically characterized in regressing tumors responding to targeted therapy. Compensatory survival signaling, through therapy-induced relief of feedback inhibition on the MAPK pathway (9) and rebound upregulation of the PI3K–AKT pathway (10, 11), has been shown to participate as adaptive mechanisms. However, it is not clear whether early adaptation of melanoma to MAPKi is limited to compensatory signaling or involves transcriptomic or epigenomic reprogramming. Recently, tumor cell mesenchymal transition has been linked to an immune-suppressive microenvironment (12, 13), wound-healing signatures, and innate anti–PD-1 resistance (14). Thus, in theory, large-scale phenotypic changes of tumor cells in response to targeted therapy can have profound effects on the tumor microenvironment and antitumor immunity. We postulated that macroscopic or clinically evident tumor shrinkage occurs in parallel with the development of therapy tolerance followed by microscopic foci of resistance. In this case, understanding subclinical resistance evolution may offer insights into combinatorial therapeutic targets to suppress such early resistance.
Despite the heterogeneous kinetics of melanoma regression and timing of tumor biopsies on MAPKi therapy, we sought insights into the early adaptive landscape by cataloging recurrent gene and gene set expression alterations across patient-matched tumors on treatment (On-Tx) versus pretreatment (Fig. 1A). Across distinct tumors or patients, transcriptome matched tumors on treatment (On-Tx) versus pretreatment (patient-matched baseline tumors) versus gene expression alterations at multiple time points (after initiating MAPKi treatment) in human melanoma cell lines or immune-competent murine melanoma tumors after increasing durations of continuous MAPKi exposure (Fig. 1B). To nominate tumor cell–intrinsic and tumor cell–extrinsic (stromal/immune) transcriptomic alterations, we compared regressing clinical tumors against in vitro–treated human melanoma cell lines and in vitro–treated murine melanoma tumors.

RESULTS
Detection of Transcriptomic Reprogramming Associated with MAPK-Redundant Resistance in Patient-Derived Melanoma Regressing on MAPKi

To analyze stromal/immune transcriptomic alterations in murine melanoma, we subtracted transcriptomic alterations in the CD45-negative subpopulation from those in the bulk regressing tumors. We also provided proof-of-concept functional studies to support adaptive, tumor cell–intrinsic and/or extrinsic roles of RTK or PD-L2 (encoded by PDCD1LG2) upregulation.

**Figure 1.** Nongenomic evolution within regressing/residual clinical tumors on MAPKi therapy. A. Clinical photos of MAPKi therapy (patient #4) showing a partial response and serial tumor biopsies. B. Overall study design of comparative transcriptomic analysis of gene expression alterations in On-Tx tumors (relative to patient-matched baseline tumors) versus gene expression alterations at multiple time points (after initiating MAPKi treatment) in human melanoma cell lines or murine melanoma tumors. C. Heat map showing unsupervised hierarchical clustering based on top variant gene expression levels of parental lines (P-lines) and isogenic single drug-resistant (SDR) or double drug-resistant (DDR) lines. Bottom, GSVA enrichments of a BRAF\textsuperscript{V600E} signature. D, PCA of BRAFi-induced temporal transcriptomic states in BR\textsuperscript{A} melanoma cell lines (P-lines at origin; Ra, resistance with MAPKi addiction; Rr, resistance with MAPK-redundancy; Rr or Rr lines include SDR and DDR lines). E. Projection of transcriptomes from pretreatment tumors (origin) versus patient-matched On-Tx or disease progression (DP) tumors onto the PC1/2 space in D. F. Numbers of up-expressed genes (fold change ≥2) in ≥50% of drug-tolerant persisters (DTP), drug-tolerant proliferating persister (DTPP), or Rr lines and their pattern of overlap. G, GO (biological processes) enrichments of temporal stage-specific or overlapping up-expressed and down-expressed (FC ≥2) genes in DTP, DTPP, and Rr lines.
on day 22 or earlier) after objective clinical responses (Fig. 1A; Supplementary Table S1A; ref. 15). Residual melanoma cells in “responding” (vs. baseline) tumors showed marked decreases in Ki-67 staining or proliferation (Supplementary Fig. S1A). In vitro, BRAFi-sensitive BRAFV600E melanoma cell lines on continuous BRAFi treatment reached a population nadir, consisting of slow-cycling, drug-tolerant persisters (DTP), prior to resuming growth as drug-tolerant proliferating persisters (DTPP; Fig. 1B; refs. 11, 16). In most cell lines examined, DTPPs acquired a flattened morphology and displayed senescence-associated β-galactosidase (SA-βGal) activity, which was later lost in fibroblastoid DTPP cells (Supplementary Fig. S1B–S1D; refs. 3, 11).

To delineate dynamic transcriptomic states, we profiled parental (P) cell lines treated with DMSO/vehicle, temporal subpopulations (2 d, DTP, DTPP; days to weeks on BRAFi), and long-term sublines (months to years on BRAFi or BRAFi + MEKi, resulting in single drug-resistant (SDR) or double drug-resistant (DPR) lines (R-line); respectively; Fig. 1B; Supplementary Fig. S1E and S1F). We then performed unsupervised clustering based on the top 3,000 most variant genes expressed by 18 isogenic P-lines versus R-lines (SDR or DDR; Fig. 1C). Two major clusters emerged: one group of R-lines clustered with all the P-lines; another group of R-lines clustered away from their isogenic P-lines. The group of R-lines clustering with all the P-lines harbored specific drivers of MAPK-reactivation/addiction (i.e., BRAFV600E ultra-amplification, M249 DDR4; BRAFV600E amplification + MEK1/2 amplification; M249 DDR5; BRAFV600E splicing, M397 SDR and M395 SDR1; BRAFV600E amplification, M395 SDR2; NRAS MUT, M249 SDR; refs. 3, 5, 6, 17). The second group of R-lines displayed transcriptomic reprogramming, relatively weak BRAFV600E signature enrichment, and attenuated MAPK dependency (Supplementary Fig. S1G and S1H). Thus, there are two distinct functional and transcriptomic classes of MAPKi resistance: Resistance with MAPK addiction (Ra) versus Resistance with MAPK redundancy (Rr). Accordingly, we observed from principal component analysis (PCA) two temporal transcriptomic trajectories: (i) for Rr, successive transcriptomic reprogramming away from MAPKi-sensitive isogenic P-lines (positioned at the origin), and (ii) for Ra, transient transcriptomic reprogramming followed by restablishment of a transcriptomic state closely matching the isogenic P-lines (Fig. 1D).

We then assessed how transcriptomic changes within On-Tx or disease-progressive (DP) tumors (vs. patient-matched baseline tumors) would compare relative to the PC trajectory observed for Rr lines (Fig. 1E). Notably, in contrast to the heterogeneous patterns of DP tumors, nearly all On-Tx tumors shifted with the directionality of the DTP → DTPP → Rr trajectory, suggesting converging phenotypes. To understand the biological processes of Rr transcriptomic reprogramming, we performed gene ontology (GO) enrichment analysis of upregulated or downregulated genes unique to each stage, overlapping between two consecutive stages or among all three stages (Fig. 1F and G). For example, DTP was characterized by enhanced neural differentiation, DTPP by enhanced interferon signaling, and Rr by reduced melanocyte differentiation. Also, upregulated genes shared by DTPP and Rr or all three stages enriched for cytokine signaling/migration or extracellular matrix (ECM) remodeling/angiogenesis. In contrast, Ra cells displayed few unique or overlapping differentially expressed genes (DEG; Supplementary Fig. S1I). Thus, BRAFi can induce temporally distinct transcriptomic and phenotypic states leading to MAPK-redundant resistance.

**Recurrent Tumor Cell–Intrinsic Alterations, Including an Epigenetically Driven Mesenchymal–Angiogenic Switch, in Melanoma Regressing on MAPKi**

Using gene signatures unique to or shared by DTP, DTPP, and Ra, we detected recurrent and often concurrent positive enrichments in the On-Tx tumors (Fig. 2A). We assessed the significance of transcriptomic similarity between the On-Tx tumors and Rr lines and found 533 upregulated and 457 downregulated genes being shared between On-Tx tumors (≥50% of biopsies) and DTP, DTPP, Rr cells (>50% of cell lines in any samples of the On-Tx group; Fig. 2B; Supplementary Tables S2A and S2B). This overlap of DEGs was significant across all pair-wise comparisons (Supplementary Table S2A). The 533 upregulated genes shared across tumor/cell line samples were enriched for the GO terms “extracellular matrix organization,” “response to IFNA,” “neuron projection,” and “mesenchyme development” (Fig. 2C). Although On-Tx (vs. baseline) tumors harbored higher immune/stromal contents (Supplementary Fig. S2A), the significant overlap of their DEGs with those in MAPKi-treated cell lines suggests that the mesenchymal phenotype could arise, in part, tumor cell autonomously.

We listed the gene sets (C2/6, Molecular Signature Database and Supplementary Table S2C) that were enriched in On-Tx tumors (≥50% of biopsies) and any of the in vitro temporal stages (Fig. 2D). We observed no gene set recurrently enriched (positive or negative) among the Ra transcriptional classes. The top gene sets positively enriched were related to cancer-associated fibroblasts (CAF)/mesenchymal transition, ECM, IFN/IRF3, and JNK/TNF signaling. Consistently, network analysis of lineage-specific transcription factors (TF) and target genes (CellNET; ref. 18) showed that Ra lines expressed more fibroblast-specific TFs and target genes (Supplementary Fig. S2B). For recurrently (>50%) differentially expressed mesenchymal, neural crest, and melanocytic genes found during in vitro evolution (Supplementary Table S2C), we observed corresponding DEG patterns in On-Tx tumors (Supplementary Fig. S2C–S2E). The heterogeneity of DEG patterns in neural crest–melanocytic differentiation observed in On-Tx tumors may be related to heterogeneous intratumoral abundance of MITF-high versus MITF-low, AXIN/NGFR-high melanoma cells in treatment-naïve tumors (19). We validated BRAFi-induced altered differentiation states based on protein levels of TPAP2A, MITF, FOXD3, CD271/NGFR, ZEB2, SNAI2, and TAGLN/SM22 (Supplementary Fig. S2F–S2I). Consistent with therapy-induced changes in chromatin regulators of melanoma cell lines (10, 16, 20, 21), we observed highly dynamic temporal expression patterns of chromatin regulator genes, including changing ratios of the atypical histone splice variants mH2A1.1 and mH2A1.2, during BRAFi resistance development (Supplementary Fig. S2J–S2N). Mutually exclusive splicing of mH2A1.1 versus mH2A1.2 is known to regulate cancer cell senescence (e.g., heterochromatin, secretory phenotype) and developmental genes (22, 23).

We then sought an epigenomic perspective of the invasive/mesenchymal switch in Rr lines versus On-Tx tumors. We first analyzed published RNA-sequencing (RNA-seq) and H3K27ac sequencing data derived from proliferative (n = 9) and invasive (n = 2) short-term melanoma cultures (Supplementary Table S2F–S2I).
Figure 2. MAPKi-induced transcriptomic and methylomic alterations shared by patient-derived regressing tumors and cell lines with temporal transcriptomic reprogramming. A, Recurrent enrichment of cell line–derived, stage-specific, and stage-overlapping signatures (see Fig. 1F) across On-Tx tumors from patients. Orange, positive enrichment; blue, negative enrichment. B, Numbers of genes recurrently up-expressed (>50% of cell line samples; 2-fold) in DTP, DTPP, or Rr lines versus On-Tx tumors (≥50% of tissue samples; 1.5-fold) and their overlaps. C, GO enrichments of overlapping up-expressed genes in B (red numbers). D, Enrichment pattern across On-Tx tumors of gene sets recurrently (>50%) enriched within any of the DTP, DTPP, Rr, or Ra set of samples. E and F, Changes in CpG methylation levels in On-Tx versus baseline tumors in E or SDR or DDR Rr lines versus isogenic P-lines in F at chromosome locations of differential H3K27ac peaks between invasive and proliferative melanoma cultures. R, Pearson correlation between differential CpG methylation and log2 fold change (FC) of H3K27ac levels. P, t test. G, Number of genes proximal to H3K27ac peaks with indicated differential mRNA (FC ≥ 1.5) and CpG methylation (LAPP ≥ 10%) for FDR adjusted p ≤ 0.05 patterns in Rr lines in F. Bottom, GO enrichments of genes within blue and orange circles.
Transcriptomic Alterations of Regressing Melanoma on MAPKi

To identify gene-level consequences of MAPKi-induced tumor cell–intrinsic transcriptomic reprogramming, we ranked-ordered the recurrence of mRNA up-expression and down-expression in On-Tx tumors based on those genes displaying DEG patterns in >50% of any in vitro temporal stage (Fig. 3A; Supplementary Table S2B). DEGs were highly concordant among the DTP–DTPP–Rr stages but discordant between Rr and Ra groups. Importantly, recurrence of DEGs in On-Tx tumors was highly consistent with those observed during in vitro transcriptional evolution of the Rr phenotype. Among the top 50 recurrently up-expressed genes were those involved in immunity, inflammation, wound healing, and cell motility, as well as growth and differentiation. Many up-expressed genes (e.g., GAS6, LGALS9, IL34, IL32, IL15) constituted a shared secretome response (Supplementary Fig. S3A and Table S3A). PDGFRB up-expression has been shown to be highly recurrent during disease progression (2) and was also highly recurrent early On-Tx, suggesting an adaptive role. Including only RTK genes that met minimal expression cutoff off (FPKM ≥ 1) and dynamic range [interquartile range (IQR) ≥ 2-fold], we found that Rr lines formed a distinct cluster at CpG sites or both hypermethylated and hypomethylated at distinct CpG sites within the same genes (e.g., LOXL2, WNT5A, VEGFC). This, Rr lines and On-Tx residual melanoma tumors underwent an epigenome-based mesenchymal–invasive–angiogenic switch.

MAPK-Redundant Resistance Displays Up-Expression of Functionally Redundant RTKs and PD-L2 in Melanoma Cells

To identify gene-level consequences of MAPKi-induced tumor cell–intrinsic transcriptomic reprogramming, we ranked-ordered the recurrence of mRNA up-expression and down-expression in On-Tx tumors based on those genes displaying DEG patterns in >50% of any in vitro temporal stage (Fig. 3A; Supplementary Table S2B). DEGs were highly concordant among the DTP–DTPP–Rr stages but discordant between Rr and Ra groups. Importantly, recurrence of DEGs in On-Tx tumors was highly consistent with those observed during in vitro transcriptional evolution of the Rr phenotype. Among the top 50 recurrently up-expressed genes were those involved in immunity, inflammation, wound healing, and cell motility, as well as growth and differentiation. Many up-expressed genes (e.g., GAS6, LGALS9, IL34, IL32, IL15) constituted a shared secretome response (Supplementary Fig. S3A and Table S3A). PDGFRB up-expression has been shown to be highly recurrent during disease progression (2) and was also highly recurrent early On-Tx, suggesting an adaptive role. Including only RTK genes that met minimal expression cutoff off (FPKM ≥ 1) and dynamic range [interquartile range (IQR) ≥ 2-fold], we found that Rr lines formed a distinct cluster at CpG sites or both hypermethylated and hypomethylated at distinct CpG sites within the same genes (e.g., LOXL2, WNT5A, VEGFC). Thus, Rr lines and On-Tx tumors were supraphysiologic (i.e., above pretreatment levels) in range (Fig. 3B; Supplementary Fig. S3D and S3E). Among RTK genes recurrently up-expressed during DTP–DTPP–Rr evolution, PDGFRB (3) and other RTK genes (AXL, c-MET, EGF) have been linked to MAPKi resistance (2, 11, 25, 26). Using validated antibodies (Supplementary Fig. S3F), we detected PDGFRB up-expression as tumor cell–intrinsic and concurrent with EGF or c-MET up-expression (Fig. 3C).

As in On-Tx tumors, PDGFRB, EGF, c-MET, and AXL in cell lines displayed supraphysiologic and concurrent up-expression in Rr (but not Ra) lines, along with higher pAKT but lower pERK levels (Supplementary Fig. S4A). We then tested whether concurrent PDGFRB and EGF or supraphysiologic up-expression could result in PDGF-BB–dependent EGFRI transactivation. After serum starvation, PDGFB–EGFR treatment strongly induced pPDGFRB, pEGFR, pAKT, and pERK levels in M229 and M238 SDR lines (versus P lines; Supplementary Fig. S4B). This PDGFB–EGFR signaling axis did not depend on the kinase activity of PDGFRB, as sunitinib (PDGFRBi) treatment inhibited PDGFB–BB–induced pPDGFRB levels but not pEGFR accumulation. Interestingly, downstream pAKT and pERK induction was attenuated but not abolished (Supplementary Fig. S4C). However, cotreatment of the Rr lines with gefitinib (EGFRI) on top of PDGFB–BB and sunitinib strongly reduced PDGFB–BB–induced pAKT and pERK levels and their clonogenic growth (Supplementary Fig. S4D and S4E).

Among immune-regulomic genes (Supplementary Table S3A) with DEGs coherent between On-Tx tumors and DTP–DTPP–Rr temporal evolution were immune checkpoint genes such as PDCD1LG2/PD-L2, CD274/PD-L1, and LAG3. IFN signatures were enriched positively starting at DTPP in vitro, but, unlike in On-Tx tumors, there was no upregulation of IFNA/B/G (Supplementary Fig. S5A). This finding suggested that tumor cell–intrinsic IFN pathway activation in response to MAPKi could remodel the tumor immune microenvironment independently of an adaptive response to antitumor T cells. In On-Tx tumors, T-cell infiltration occurred along with a whole immune compartment expansion (Supplementary Fig. SSB and Table S3B), consistent with a tissue injury or wound-healing response, and with a lower ratio of T-cell exhaustion or checkpoint inhibitory genes to CD8A (Supplementary Fig. S5C). Because eventual disease progression is commonly associated with loss of CD8 T-cell inflammation (2), the MAPKi–responsive “phase” may be a window of opportunity to augment antitumor immunity and suppress resistance development. Among the top recurrently up-expressed immune genes, PD-L2 was expressed at a supraphysiologic range (Fig. 3E and F). Methylation levels (FC or absolute levels) at a specific CpG site in PD-L2 were significantly anticoordinated with mRNA levels in this melanoma tissue and cell line cohort and The Cancer Genome Atlas (TCGA) melanoma (Fig. 3G). The identified PD-L2 CpG site lost methylation with increasing duration of BRAF selection and PD-L2 mRNA expression (Fig. 3H; Supplementary Fig. SSD and SSE) and located within H3K27ac peaks of short-term invasive melanoma cultures (Fig. 3I). Moreover, PD-L2 (vs. PD-L1) protein up-expression in Rr lines (versus P lines) was more robust; neither PD-L1 nor PD-L2 was up-expressed in Ra lines (Fig. 3J; Supplementary
Figure 3. Recurrent tumor cell-intrinsic transcriptomic, RTKomic, and immune regulomic alterations in regressing melanoma on MAPKi therapy.

A, B, Pattern of differential expression of genes in the On-Tx tumor samples with recurrent (>50%) up-expression in any of the DTP, DTPP, Rr, or Ra set of samples (A, top 50 genes; B, RTK genes only; red and green, up-expression and down-expression).

C, Immunofluorescence of PDGFRβ, EGFR, and c-MET using formalin-fixed, paraffin-embedded (FFPE) tumor tissues designated by letters (ruler, 100 μm); negative staining by gray circles.

D, As in A and B except showing only top recurrently up-expressed immune regulomic genes.

E and F, Expression ranges of immune genes in D among baseline versus On-Tx tumors (E) or Rr versus Rr lines (F). G, Correlation between FCs in PD-L2 mRNA expression and % methylation changes at the expression anticorrelated CpG site of PD-L2 across comparisons of DP versus baseline tumors and Rr versus P lines. Middle, correlation across all samples (from the left panel) and (right) all TCGA melanoma samples. R, Pearson correlation coefficient; P, Student t test. (continued on following page)
**Figure 3.** (Continued) **H** Methylations changes at all profiled CpG sites (green bubble, expression-correlated site; left) or absolute methylation levels at the expression-correlated CpG site (right) versus PD-L2 mRNA expression FC (left) or absolute levels (right). All changes relative to vehicle-treated M229. **I,** H3K27ac chromatin immunoprecipitation sequencing (ChIP-seq) peaks covering the PD-L2 expression-correlated CpG site in invasive versus proliferative melanoma cultures. PD-L2 mRNA levels also shown. **J,** Median fluorescence intensities (MFI) of PD-L1 and PD-L2 staining in P versus Rr or Ra lines. **K,** PD-L2 immunofluorescence using frozen tissues from patient #3 (100× scale bar; 50 μm; 400× scale bar; 20 μm). Several views shown for On-Tx tumor.

In paired frozen tumors, we observed strong PD-L2 protein up-expression in residual tumor cells on MAPKi (Fig. S5F–S5H). Given a reported tumor cell–intrinsic, antiapoptotic role of PD-L1 (27), we first assessed a potential tumor cell–intrinsic, survival of MAPK-redundant resistance tumor cell–intrinsically and in a manner augmented by interaction with PD-1.

**Tumor Cell–Intrinsic PD-L2 Upregulation Promotes Survival of In Vitro Models of MAPK-Redundant Resistance**

Given a reported tumor cell–intrinsic, antiapoptotic role of PD-L1 (27), we first assessed a potential tumor cell–intrinsic, adaptive function of PD-L2’s supraphysiologic expression by testing whether PD-L2 knockdown by shRNAs in Rr lines would suppress clonogenic growth and induce apoptosis (Fig. 4A and B; Supplementary Fig. S6A–S6C). Indeed, partial knockdown of PD-L2 levels in Rr lines by five independent shRNAs consistently and strongly reduced clonogenic growth and induced apoptosis. Interestingly, coculture with HEK293T cells expressing PD-1 attenuated or rescued basal or PD-L2 knockdown–induced apoptosis in Rr lines (Supplementary Fig. S6D; Fig. 4C and D), which was reversed by coexpression of soluble PD-L2 in HEK293T cells (Supplementary Fig. S6E and S6F). Additionally, we induced apoptosis in Rr lines with staurosporine (STP) prior to cocultures (with STP washed away before coculture) and observed that PD-1 expression by neighboring cells protected Rr lines from basal and STP-induced apoptosis (Fig. 4E). This PD-1–mediated protection of apoptosis was not observed in the P-lines or Ra lines, which did not up-express PD-L2 (Supplementary Fig. S6G and S6H). Thus, PD-L2 up-expression promotes survival of MAPK-redundant resistance tumor cell–intrinsically and in a manner augmented by interaction with PD-1.

To gain insights into tumor cell–intrinsic antiapoptotic processes mediated by PD-L2–PD-1 interaction, we sorted two Rr lines (based on negative GFP expression) in the baseline growth condition or after brief STP treatments from cocultures with HEK293T cells, which did or did not overexpress PD-1. PCA of RNA-seq data generated from nonapoptotic, GFP-negative Rr cells showed that STP treatment was associated with a negative shift along the PC2 axis (Fig. 4F), where PC2 positive-driving genes were enriched for cell-cycle progression and PC2 negative-driving genes were enriched for cell death genes (Supplementary Fig. S6I). Although this reflected the effects of STP pretreatment on gene expression, we also observed that, regardless of STP pretreatment, coculture with PD-1–expressing cells invariably resulted in a positive shift along PC3 (Fig. 4F), where PC3 positive-driving genes were enriched for angiogenic processes and PC3 negative-driving genes were enriched for inflammation.
Figure 4. PD-L2 upregulation promotes tumor cell–intrinsic MAPKi resistance. A, PD-L2 surface detection in P and Rr lines with stable expression of empty vector (shVec) or shPD-L2. Error bars, SEM. B, Clonogenic growth of indicated Rr lines, with shVec or indicated shPD-L2, for 9 days. Quantifications relative to each cell line culture with shVec. C, Apoptosis (%) in Rr lines stably expressing shVec or shPD-L2#9 + #24 on indicated days after lentiviral infection. GFP– (VEC) or PD-1–GFP-expressing HEK293T cells were added to melanoma cell cultures on day 2 of infection at a ratio of 1 to 2. D, Apoptosis on indicated days in Rr lines cocultured from the outset with GFP– (VEC) or PD-1–GFP-expressing HEK293T cells at a ratio of 2 to 1. (continued on following page)
Transcriptomic Alterations of Regressing Melanoma on MAPKi

YUMM1.7 tumors regressed soon after daily gavage with BRAFi and further by in vivo experiments (see below).

MAPKi Remodels the Stromal/Immune Compartment with PD-L2 Uregulation in CD11c-Positive Immunocytes

We also assessed gene- and gene signature-level consequences of MAPKi-induced tumor stromal/immune compartment remodeling by comparing clinical On-Tx tumors to immune-competent murine melanoma temporally transcriptome profiled after BRAFi treatment. A syngeneic, transplantable murine melanoma model (YUMM1.7 Braf/Pten/Cdkn2a-mutant cells subcutaneously transplanted into C57BL/6 mice) was used for this comparative transcriptomic analysis, but the clinical relevance of this murine melanoma model was first assessed. YUMM1.7 tumors regressed soon after daily gavage with BRAFi (vemurafenib), and residual tumors persisted before resuming growth (Fig. 5A). Transcriptomes derived from vehicle-treated tumors [day 3 (d 3)] and BRAFi-treated tumors at d 6 (regressing), d 15 (residual), and d 30 (regrowing) were analyzed for expression levels of CD8A/B and CD8 T-cell activation/cytolytic genes (Fig. 5B). This analysis showed heterogeneous but progressive induction of CD8 T-cell activation/cytolytic genes early On-Tx, which peaked in the residual, maximally regressed tumors but ceased when the tumors regrew. Thus, YUMM1.7 tumors in response to BRAFi therapy recapitulated the transcriptomic evolution of clinical melanoma.

We first determined in patient-derived On-Tx tumors the differential expression patterns of immune genes recurrently and...
coherently up-expressed or down-expressed in any stage (regressing, residual, or progressing) of YUMM1.7 tumors on BRAFi (Fig. 5C; Supplementary Table S2B). By rank-ordering up-expressed immune genes in clinical On-Tx tumors based on the frequency of recurrence (Fig. 5C), we could appreciate the immune-regulomically similar behavior between On-Tx and regressing/residual YUMM1.7 tumors. To define tumor cell-intrinsic versus tumor cell-extrinsic immune-regulomically alterations, we annotated the recurrence profile (Fig. 5C) with DEG patterns in the CD45-negative fraction of residual YUMM1.7 tumors and in human melanoma cell samples (i.e., DTP, DP, Rr or Ra). We first determined that DEG (in particular gene up-expression) overlapped significantly between MAPKi-selected human cell lines in vitro and the CD45-negative fraction of mouse tumors in vitro (Supplementary Fig. S7). These overlapping up-expressed genes enriched for GO terms such as ECM organization and cell migration. Additionally, a gene set enrichment analysis that incorporated recurrent alterations in the CD45-negative fraction of residual YUMM1.7 tumors (Supplementary Fig. S7B) revealed concordant and recurrent enrichment of CAF, mesenchymal transition and ECM-remodeling signatures in On-Tx tumors, DTP–DP–Rr temporal populations, and CD45-negative residual YUMM1.7 tumors. By excluding the characteristic MAPKi-induced (tumor cell-intrinsic) tumor transcriptomic alterations observed in human melanoma cell lines in vitro and in the CD45-negative fraction of residual YUMM1.7 tumors, we nominated the majority of recurrent gene up-expression in On-Tx tumors (Fig. 5C) as tumor cell-extrinsic alterations. Consistently, at the gene signature enrichment level (Fig. 5D), the top recurrently enriched gene signatures in the On-Tx tumors, with support in any of the temporally sampled YUMM1.7 tumors, were all related to immune cell function, indicating wholesale immune cell infiltration (including CD8+ T cells) as during wound healing. Interestingly, the loss of CD8+ T cells noted earlier (Fig. 5B) in progressing YUMM1.7 tumors was accompanied by loss of pan-immune subsets (Fig. 5D). Although the top recurrently up-expressed immune genes in On-Tx tumors were not commonly attributable to the tumor cell compartment, the top recurrently up-expressed genes in general (including the secretome; Supplementary Fig. S3A) were frequently attributable...
Transcriptomic Alterations of Regressing Melanoma on MAPKi

RESEARCH ARTICLE

Figure 5. (Continued) D, Pattern of enrichment in On-Tx tumors (rank ordered by recurrence frequency of positive enrichment) of gene sets with support from YUMM1.7 tumors as in C. E, Representative scanned and quantified images of Ki-67/CD8 immunofluorescence of tumor sections from A (DAPI, nuclear counterstain). F, Ki-67 or CD8 quantifications from E (n = 5 tumors per time point; 2–3 regions per tumor; error bars, SEM; Student t test, P values: *, ≤0.05; **, ≤0.01; n.s., not significant; reference, DMSO 3 d). G, Immunofluorescent costaining of CD11c and PD-L2 of tumor sections from A (scale bar, 20 μm). Representative low and high magnification images shown.
to the CD45-negative residual YUMM1.7 tumor cells or DTP-DTPP-R subpopulations in vitro (Supplementary Fig. S7C).

By comparing results in Figs. 3D versus 5C, we noted a few exceptions where recurrent immune gene (e.g., CD36, CXCL12, and CX274/PD-L1) up-expression in On-Tx tumors may be attributable, at least partially, to the tumor cell compartment. We also noticed that, although PDCD1LIG2/PD-L2 was up-expressed robustly during the DTP–DTPP-R evolution in human melanoma cell lines (Fig. 3), it was not in the CD45-negative fraction of residual YUMM1.7 tumors (Fig. 5C). In fact, the full-length PD-L2 transcript was detected by RNA-seq only with sufficient intratumoral inflammation (e.g., when YUMM1.7 tumors were treated with BRAFi; Supplementary Fig. S7D). As a cell line in response to BRAFi treatment, YUMM1.7 expressed only the 3′ short transcript, which is missing the 5′ exons required to translate the extra-cellular domain (Supplementary Fig. S7E and S7F). Thus, this model presented an opportunity to assess the impact of PD-L2 induction specifically in the immune compartment. Furthermore, in YUMM1.7 tumors, we confirmed by Ki-67 and CD8 quantitative immunofluorescence highly dynamic alterations in the proliferative status and intratumoral CD8+ T-cell abundance (Fig. 5E and F). We also confirmed PD-L2 accumulation and observed its frequent colocalization with CD11c (a myeloid dendritic marker) in the immune compartment of regressing/residual YUMM1.7 tumors (Fig. 5G).

**PD-L2 Blockade in the Immune Compartment Prolongs BRAFi Response and CD8+ T-Cell Accumulation**

Importantly, cotreatment of BRAFi with a PD-L2-blocking antibody (aPD-L2) consistently delayed growth of resistant tumors, whereas aPD-L2 monotherapy did not lead to tumor growth inhibition or shrinkage (Fig. 6A and B; Supplementary Fig. S7G–S7I). We hypothesized that aPD-L2 acts by delaying the loss of CD8+ T-cell inflammation that occurs during the evolution of BRAFi resistance. Consistently, at d23, BRAFi + aPD-L2–responsive tumors, compared with tumors that had escaped either BRAFi alone or BRAFi + aPD-L2, displayed the highest number of CD8+ T cells (Fig. 6C). We also profiled via RNA-seq YUMM1.7 tumors that had escaped BRAFi (n = 3) or BRAFi + aPD-L2 (n = 2) or remained responsive to BRAFi + aPD-L2 (n = 2) at d 33 (Fig. 6D) and compared their PC positions relative to those from the BRAFi treatment time course (Fig. 5A). From this transcriptomic perspective, we found that d 33 YUMM1.7 tumors that remained responsive to BRAFi + aPD-L2 segregated with d 15 residual tumors on BRAFi alone, whereas d 33 tumors that escaped BRAFi or BRAFi + aPD-L2 shifted positively along the PC1 axis toward d 30 BRAFi-treated tumors (Fig. 6D). To understand this pattern further, we analyzed the GO term enrichments of PC1− and PC1+ tumors (Fig. 6D). From this transcriptomic perspective, we found that delayed aPD-L2 treatment acts by delaying the loss of CD8+ T-cell inflammation, we found that, in the residual tumors, aPD-L2 cotreatment already led to a significantly higher number of CD8+ T cells (Fig. 6F). Also, as
Transcriptomic Alterations of Regressing Melanoma on MAPKi

DISCUSSION

The scale and nature of tumor cell–intrinsic and –extrinsic alterations, during the early course of MAPKi therapy for advanced melanoma, have not been defined systematically. This characterization is essential to start to address the potential adaptive nature of these changes and define therapeutic opportunities. We integrated transcriptomic analysis of patient-matched pretreatment and regressing/residual melanoma with analysis of MAPKi-induced temporal transcriptomic alterations in human melanoma cell lines in vitro and murine melanoma tumors in an immune-competent context. By focusing on recurrent transcriptomic alterations during each stage of response/adaptation in the experimental microenvironment, potentially through upregulating hypoxia-driven oncogenic processes and downregulating inflammatory signaling.

Figure 6. PD-L2 blockade delays BRAFi resistance in murine melanoma by enforcing a CD8+ T cell–inflamed residual tumor state. A, YUMM1.7 tumor volumes with indicated treatments (vehicle, 0.1% methylcellulose and 10% DMSO; BRAFi, vemurafenib 100 mg/kg/d gavage; isotype (iso) or aPD-L2 antibody at 300 μg/mouse twice per week i.p.; n = 8 per group; error bars, SEM). Data representative of three independent experiments. B, Progression-free survival (cutoff tumor size at ≥150% of size at treatment initiation) of mice in A. P values, log-rank test. C, Tumors from experiment in A harvested on day 33 available as FFPE tissue for CD8 and Ki-67 quantifications (n = 6, 2, and 4 tumors, BRAFi + isotype, BRAFi + aPD-L2 responsive, BRAFi + aPD-L2 unresponsive, respectively; 3 regions per tumor; error bars, SEM; Student t test, P values: *, ≤0.05; n.s., not significant; reference, BRAFi + isotype). D, Tumors from experiment in A with available RNA-seq profiles analyzed by PCA along with RNA-seq profiles derived from tumors in Fig. 5A. E, GO enrichment analysis of PC1− and PC1+ driving genes from D. F, YUMM1.7 tumors treated with BRAFi + isotype (n = 6) or BRAFi + aPD-L2 (n = 8), harvested on day 8, and analyzed by CD8 and Ki-67 immunofluorescence. Quantification. 3 regions per tumor; error bars, SEM; Student t test, P values: *, ≤0.05; reference, BRAFi + isotype.
models, we uncovered similar highly recurrent gene and gene program expression alterations in patient-derived On-Tx tumors. These recurrent transcriptomic alterations originated in the tumor cell and/or immune compartment, as inferred by concordant changes in (i) On-Tx clinical tumors versus human cell lines (i.e., putative tumor cell–intrinsic alterations) or (ii) On-Tx versus mouse tumors (but absent in human cell lines (i.e., putative tumor cell–intrinsic alterations). This approach incorporating temporal dynamics in experimental systems enhanced our view of heterogeneous On-Tx clinical tumors, which were biopsied at varied times and responded to therapy with distinct kinetics. Importantly, this integrative analysis of patient-derived tumors and experimental systems enabled functional interrogation of clinically relevant and highly ranked alterations in patient tumors as potentially adaptive and targetable responses.

This study identified regressing/residual melanoma “responder” to MAPKi therapy as a nidus of dynamic transcriptomic–CpG methylation reprogramming and of tumor cell persistence and resistance through attenuated MAPK dependency. MAPK-redundant tumor growth control by multi-RTK upregulation associated with a mesenchymal/invasive/angiogenic switch, highlighting therapeutic challenges. Recent pan-cancer studies (12–14) have linked mesenchymal, angiogenic, and immune-suppressive transcriptomic features, which together are associated with clinical innate (14) and adaptive (28) anti-PD-1 resistance in melanoma. Thus, MAPKi induction of
Transcriptomic Alterations of Regressing Melanoma on MAPKi

such microenvironmental alterations, including CD8+ T-cell infiltration, reflects an immune phenotypic switch that presages loss of T-cell inflammation and likely loss of anti-PD-1 responsiveness. In the immune-competent context, MAPKi should induce release by tumor cells of danger-associated molecular patterns (DAMP), which would promote noninfectious inflammation. Surviving malignant cells appear to undergo mesenchymal/angiogenic/fibroblastic transition and, through an expanded secretome and cell-surface expression of immunomodulatory molecules, actively participate in chronic wound healing or inflammation, which is immune suppressive. Hence, it may be critical to disrupt this immune-phenotypic transition early during “successful” MAPKi-targeted therapy in order to prevent the eventual loss of T-cell inflammation.

As a proof of concept, we showed that, by inducing infiltration of PD-L2–expressing immune cells and tumor cell–intrinsic PD-L2 upregulation, BRAFi unmasks the protumorigenetic and resistance functions of PD-L2. These functions of PD-L2 expressed by nontumor cells in the immune microenvironment are at least partially dependent on the ability of PD-L2 to downregulate intratumoral CD8+ T-cell function and/or number. Tumor cell–intrinsic upregulation of PD-L2 and its interaction with PD-1 increased fitness of melanoma cells that have epigenomically adapted to MAPKi. Recent work showed that PD-L2 expression, independently of PD-L1 expression, predicted clinical response to pembrolizumab in head and neck squamous cell carcinoma, suggesting that clinical responses to PD-1 blockade may be related partly to blockade of PD-1/PD-L2 interactions (29). It also remains to be determined how much of PD-L2’s tumor cell–intrinsic function is relevant clinically in the context of MAPKi resistance. Further mechanistic understanding and biomarker discovery of PD-L2’s tumor cell–intrinsic function would facilitate this evaluation.

The recognition of tumor cell–intrinsic transcriptomic reprogramming and its potential immune-suppressive, immune-editing or prometastatic impacts early during therapy calls for preemptive, up-front combinatorial therapies. Although the intrinsic requirement of tumor cell mesenchymal transition for metastatic capability has been called into question (30–32), its importance in early adaptive resistance to targeted therapy is strongly suggested by our study. We also observed, in On-Tx clinical tumors, MAPKi inducing tumor cell–intrinsic (in human melanoma cell lines and the CD45-negative fraction of YUMM1.7 tumors) upregulation of multiple factors that could promote metastasis. One example is the fatty-acid receptor CD36, which has been nominated recently (33) as a critical driver of metastasis-initiating cells. Tumor neoantigens can result from somatic mutations or reexpression of cancer testis antigens. MAPKi-induced epigenomic reprogramming raises the possibility of cancer testis antigens as endogenous immune or exogenous immunotherapeutic targets. Furthermore, immune-suppressive candidates include tumor cell–intrinsic elaboration of TGFβ2, VEGFA/C, and IFN upregulation. Interestingly, chronic IFN stimulation of tumor cells has been shown to induce T-cell inhibitory receptors and reduce sensitivity to aPD-1 blockade (34). Also, EGFR, VEGF, and other triggers of STAT3 signaling could promote immunosuppression (35). Loss of PTEN or induction of AKT has been associated with decreased T-cell infiltration into tumors and T cell–mediated tumor killing through expression of immunosuppressive cytokines (36). Importantly, MAPK-redundant resistance that results from transcriptomic reprogramming invariably upregulates AKT signaling. Thus, further analysis of this data set and exploitation of these experimental models should generate additional combinatorial targets with the potential to tighten the bottleneck of melanoma evolution in response to MAPKi therapy.

METHODS

Analysis of Tumor Specimens

All melanoma tumors (pretreatment, on-treatment) were obtained with the approval of institutional review boards (IRB) and patients’ consent. mRNAs from 24 snap-frozen specimens were extracted by the mirVana RNA Isolation Kit (Life Technologies) and paired-end sequenced with read length of 2 × 100 bps (Illumina HiSeq2000, unstranded TrueSeq RNA Sample Preparation Kit v2), except for patient #9 (2 × 150 bps, Illumina HiSeq3000, Kapa Stranded mRNA-seq kit) and patient #5 (for which microarray was used). Also, RNA-seq data of 22 independent specimens (15) were analyzed in parallel. Selected tumor samples were also subjected to whole-exome sequencing (n = 8 pairs) to verify tumor content and to methylene profiling (n = 4 pairs) to compare with methylation profiles of cell lines.

Cell Culture, Subline Derivation, Infection, and Treatments

All cell lines were routinely tested for Mycoplasma, and cell line and subline identities have been ensured by DNA-seq and the GenePrint 10 system (Promega) at routine intervals during the course of this study for banking and experimental studies. All M series cell lines were established from patient-derived tumors at the University of California, Los Angeles. SKMEL28 was obtained from Dr. Alan Houghton (between 2008 and 2010), YUMM1.7 was obtained from Dr. Marcus Bosenberg (between 2014 and 2016). All cell lines were maintained in DMEM high glucose with 10% heat-inactivated FBS (Omega Scientific) and 2mmol/L glutamine in a humidified, 5% CO2 incubator. To derive DTPP clones, parental melanoma cells seeded at low density were treated with drugs as indicated every 2 to 3 days for 3 to 6 weeks, and highly proliferative colonies were ring-isolated and expanded. SDR and DDR sublines were derived previously as stated in Supplementary Methods. ShPD-L2 and vector control (Thermo Fisher) were packaged into lentiviral particles for infection. To derive DTPP clones, parental melanoma cells seeded at low density were treated with drugs as indicated every 2 to 3 days for 3 to 6 weeks, and highly proliferative colonies were ring-isolated and expanded. SDR and DDR sublines were derived previously as stated in Supplementary Methods. ShPD-L2 and vector control (Thermo Fisher) were packaged into lentiviral particles for infection. PD-1–GFP and its empty lentiviral vector were purchased from Origene. See Supplementary Methods for details on all antibody, small-molecule inhibitor, and growth factor treatments.

Protein Detection

Cells were lysed in RIPA buffer (Sigma) with protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktails (Santa Cruz Biotechnology) for Western blotting. Paraffin-embedded formalin-fixed sections were subjected to heat-induced antigen retrieval. The biotin–streptavidin system (Vector Laboratories) and DAB were used to visualize human Ki-67, and Alexa Fluor conjugates (Life Technologies) and the TSA plus fluorescein system (Perkin Elmer) were used to visualize human PDGFRβ, EGFR, and c-MET, and mouse Ki-67, PD-L2, CD8, and CD11c. For immunocytochemistry, cells were fixed with 4% paraformaldehyde and stained with Alexa Fluor 555 (for Ki-67) and Alexa Fluor 488 (for CD271) conjugates (Life Technologies). Nuclei were counterstained by hematoxylin or DAPI. Fluorescent images were digitized using the Applied Imaging Axio Imager SL–50 scanner at 20× magnification. Quantification of CD8 and Ki-67 signals was performed using the Definiens Tissue Studio 4.3 (Definiens, Inc.) according to the manufacturer’s instructions. Please refer to Supplementary Methods for primary antibody information.
Cell-surface PD-L1 and PD-L2 were detected by flow cytometry using APC-conjugated anti-PD-L1 and PE-conjugated anti-PD-L2 antibodies (BioLegend).

**Cell Line-Based Assays**

Senescence was assessed by senescent-β-galactosidase staining kit (Cell Signaling Technologies). CSFE (Molecular Probes) dilution was monitored by flow cytometry to follow cell division. Clonogenic and cell-cycle assays are described in Supplementary Methods. For vital imaging, cells were plated onto gridded-dishes (Sigma) and imaged every 2 days at five predesignated areas.

**Treatment and Analysis of YUMM1.7 Tumors**

YUMM1.7 cells (1 million per flank, two flanks per mouse) were subcutaneously implanted into C57BL/6 mice. Tumors were treated with BRAFi when the volumes reached to 150–200 mm³. aPD-L2 antibodies and isotype control were administered intraperitoneally. Tumors were dissociated into single cells and stained with a CD45 antibody. The CD45-negative population was sorted for RNA extraction. See Supplementary Methods for additional details.

**RNA-seq Analysis of Melanoma Cell Lines and Tumors**

FASTQ reads were mapped to the UCSC hg19 or mm9 (for the YUMM1.7 mouse melanoma study) reference genome using TopHat2. Normalized expression levels of genes (and transcripts in the case of mH2A1.1 and mH2A1.2) were expressed in FPKM values as generated by cuffquant and cuffnorm. Because we observed overexpression of the last two exons in the Pkdhl2 gene without the accompanying expression of the other exons in the YUMM1.7 tumors treated with BRAFi for 30 days, we excluded the reads mapping to the last two exons when we quantified full-length Pkdhl2 mRNA expression in the YUMM1.7 RNA-seq dataset. A gene was defined as differentially expressed when its expression increased or decreased by at least 2-fold for cell lines or 1.5-fold (|log₂ FC| ≥ 0.585) for tumors (including YUMM1.7 tumors). To overcome noise in differential expression values caused by extremely low FPKM levels, we added a pseudo-FPKM value of 0.1 to all expression values. Recurrence analysis of up-expression or down-expression of genes was performed for cell lines/YUMM1.7 tumors/patient tumors with respect to their parental/vehicle-treated/pretreatment lines or tumors, respectively. We used the ESTIMATE algorithm (37) to calculate the extent of immune infiltration of the baseline and On-Tx tumors based on gene expression. Tumor purities were called using WES analysis by Sequenza (38). The purities of samples without WES data were estimated by the fraction of Sanger-derived MUT peaks compared with the wild-type peaks, assuming heterozygous BRAF mutation. Paired gene set enrichment was performed as described (2); single-sample gene set enrichment was quantified using GSEA (39). GO term enrichment was computed based on the online functional annotation tool ENRICHR (40). RNA-seq data have been made available through the Gene Expression Omnibus (GEO) at the accession number GSE75313.

**Analysis of Methylome and H3K27ac ChIP-seq**

Differentially methylated CpG sites in tumor/cell line samples (profiled by Illumina 450K Methylation arrays) were computed as described (2). We intersected the proliferative and invasive H3K27ac peak regions (24) with the Illumina 450K Methylation probes. For each peak region with more than one CpG probe, we calculated the median of the methylation changes across the CpG probes. Using the independently derived H3K27ac signals and methylation changes from our studies, we computed the Pearson correlation between the fold differences of H3K27ac signals (invasive vs. proliferative cultures) and % methylation changes (in Rr lines or On-Tx tumors vs. their respective P lines or pretreatment tumors) of the CpG sites within the H3K27ac peaks. To analyze differential mRNA expression (between Rr and P lines) within regions of differential H3K27ac and methylation, we selected those H3K27ac peaks overlapping with one or more differentially methylated CpG sites (p-value ≤ 0.05, |ΔB| ≥ 10%) and whose nearest genes were differentially expressed (≥1.5-fold up-expression or down-expression) in more than half of the Rr lines. These genes were grouped into four different categories (hypomethylation or hypermethylation with mRNA up-expression or down-expression).

**Disclosure of Potential Conflicts of Interest**

P.O. Scumpia is a consultant/advisory board member for EMD Serono and Pfizer. D.B. Johnson is a consultant/advisory board member for BMS, Merck, Incyte, and Genoptix. R.S. Lo served on the advisory boards of Shire, Novartis, and Amgen. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**

Conception and design: C. Song, A. Hong, W. Hugo, R.S. Lo Development of methodology: C. Song, M. Piva, L. Sun, A. Hong, S. Lomeli, J. Qian, C.C. Yu, W. Hugo, R.S. Lo Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Song, M. Piva, L. Sun, G. Moriceau, S. Lomeli, J. Qian, C.C. Yu, R. Darnoiseaux, M.C. Kelley, K.B. Dahlman, J.A. Sosman, D.B. Johnson, A. Ribas, W. Hugo, R.S. Lo Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Song, M. Piva, L. Sun, A. Hong, X. Kong, S. Lomeli, J. Qian, C.C. Yu, P.O. Scumpia, A. Ribas, W. Hugo, R.S. Lo Writing, review, and/or revision of the manuscript: C. Song, M. Piva, L. Sun, J.A. Sosman, D.B. Johnson, W. Hugo, R.S. Lo Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Sun, H. Zhang, R. Darnoiseaux, M.C. Kelley, W. Hugo, R.S. Lo Study supervision: W. Hugo, R.S. Lo

**Acknowledgments**

We thank G. Bollag (Plexxikon Inc.) for providing PLX4032/4720, M.W. Rosenberg for YUMM1.7, and H. Shi for technical assistance. We thank all patients who donated tissues for this research. Patient-informed consent was obtained for the research performed in this study.

**Grant Support**

This work has been funded by the Burroughs Wellcome Fund (to R.S. Lo), the NIH (1R01CA176111 to R.S. Lo; P01CA168585 to A. Ribas and R.S. Lo; K12CA0906525 to D.B. Johnson), the Ressler Family Foundation (to R.S. Lo and A. Ribas), the Melanoma Research Alliance (to R.S. Lo), D.B. Johnson, and W. Hugo), the Ian Copeland Melanoma Fund (to R.S. Lo), the SWOG/Hope Foundation (to R.S. Lo and A. Ribas), the Steven C. Gordon Family Foundation (to R.S. Lo), the American Skin Association (W. Hugo), the American Association for Cancer Research-Amgen, Inc. Fellowship in Clinical/Translational Cancer Research (16-40-11-HUGO to W. Hugo), the Department of Defense Horizon Award (to A. Hong), the Dermatology Foundation (to G. Montecu), a National Cancer Center Aggressive Cancer Research Postdoctoral Fellowship (to J. Qian), the ASCO Conquer Cancer Career Development Award (to D.B. Johnson), and the American Cancer Society Research Professorship (to J.A. Sosman).

Received April 14, 2017; revised August 22, 2017; accepted August 28, 2017; published OnlineFirst September 1, 2017.
Transcriptomic Alterations of Regressing Melanoma on MAPKi

REFERENCES


ity to anti-PD-1 therapy in metastatic melanoma. Cell 2016;165:35–44.


Recurrent Tumor Cell–Intrinsic and –Extrinsic Alterations during MAPKi-Induced Melanoma Regression and Early Adaptation

Chunying Song, Marco Piva, Lu Sun, et al.

Cancer Discov 2017;7:1248-1265. Published OnlineFirst September 1, 2017.

Updated version
Access the most recent version of this article at:
doi:10.1158/2159-8290.CD-17-0401

Supplementary Material
Access the most recent supplemental material at:
http://cancerdiscovery.aacrjournals.org/content/suppl/2017/08/31/2159-8290.CD-17-0401.DC1

Cited articles
This article cites 38 articles, 13 of which you can access for free at:
http://cancerdiscovery.aacrjournals.org/content/7/11/1248.full#ref-list-1

Citing articles
This article has been cited by 2 HighWire-hosted articles. Access the articles at:
http://cancerdiscovery.aacrjournals.org/content/7/11/1248.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://cancerdiscovery.aacrjournals.org/content/7/11/1248.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.