EZH2 Cooperates with DNA Methylation to Downregulate Key Tumor Suppressors and IFN Gene Signatures in Melanoma

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The histone methylase EZH2 is frequently dysregulated in melanoma and is associated with DNA methylation and silencing of genes involved in tumor suppression. In this study, we used chromatin immunoprecipitation and sequencing to identify key suppressor genes that are silenced by histone methylation in constitutively active EZH2(Y641) mutant melanoma and assessed whether these regions were also sites of DNA methylation. The genes identified were validated by their re-expression after treatment with EZH2 and DNA methyltransferase inhibitors. The expression of putative EZH2 target genes was shown to be highly relevant to the survival of patients with melanoma in clinical datasets. To determine correlates of response to EZH2 inhibitors, we screened a panel of 53 melanoma cell lines for drug sensitivity. We compared RNA sequencing profiles of sensitive to resistant melanoma cells and performed pathway analysis. Sensitivity was associated with strong downregulation of IFN-γ and IFN-α gene signatures that were reversed by treatment with EZH2 inhibitors. This is consistent with EZH2-driven dedifferentiated invasive states associated with treatment resistance and defects in antigen presentation. These results suggest that EZH2 inhibitors may be most effectively targeted to immunologically cold melanoma to both induce direct cytotoxicity and increase immune responses in the context of checkpoint inhibitor immunotherapy.

INTRODUCTION

Melanoma is the deadiest type of skin cancer and continues to represent a significant global health burden. Despite recent advances using immunotherapy and targeted therapies, not all patients respond to such treatments, and therapy resistance occurs in most initially responding patients (Eggermont et al., 2018; Long et al., 2018; Wolchok et al., 2017). Epigenetic changes associated with methylation are increasingly appreciated to govern major hallmarks of cancer. Therefore, there remains a need for treatments that target such epigenetic factors.

EZH2 is the catalytic subunit of PRC2, which deposits methyl groups on lysine amino acids of type 3 histones (H3K27me3), associated with gene repression (Margueron and Reinberg, 2011). EZH2 is essential in early embryonic development and adult lymphopoiesis but is often dysregulated in many types of cancer, including melanoma, and is associated with poor survival (Kim and Roberts, 2016; Yamagishi and Uchimaru, 2017). It is postulated that in cancer, aberrant EZH2-driven histone methylation represses the expression of critical tumor suppressors that would normally constrain cell growth. EZH2 was shown to interact with DNA methyltransferases (DNMTs) DNMT1, DNMT3A, and DNMT3B in cancer cells (Viré et al., 2006) and to result in hypermethylation of genes, leading to more permanent silencing of target genes (Tan et al., 2014). However, the exact association of EZH2 with DNMTs remains controversial. In normal cell biology, DNA methylation has been shown to overlap with histone methylation (H3K27me3) at multiple sites throughout the genome; however, the two marks are mutually exclusive at CpG dinucleotides (Bogdanovic et al., 2011; Brinkman et al., 2012; de la Calle Mustienes et al., 2015). A recent study implicated unmethylated CpG sites in directing PRC2 binding to target genes (Li et al., 2017a), and once H3K27me3 is in place, it is thought to limit the spreading of DNA methylation (Das et al., 2015).

The Australian melanoma whole genome sequencing study on 183 patients showed that 27% of melanoma specimens were found to contain EZH2 aberrations, consisting of...
amplifications (21%), single nucleotide variants or insertion-deletions (5%), or structural variants (1%) (Hayward et al., 2017). EZH2 is implicated in melanoma progression in both human and animal studies and is associated with more invasive melanoma (Fane et al., 2017). Conditional knockout of EzH2 in the melanocytes of transgenic mice virtually abolished melanoma growth and metastases in amurine model of melanoma (Zingg et al., 2015).

We and others (Tiffen et al., 2015; Zingg et al., 2015) have reported that a small molecule inhibitor of EZH2 could induce growth inhibition and cell death in melanoma in vitro. Several small molecule EZH2 inhibitors have entered phase 1/2 clinical trials (Fioravanti et al., 2018; Yamagishi and Uchimaru, 2017), particularly in lymphomas that harbor the same activating mutation in the catalytic SET domain of EZH2(Y641) as identified in 1% of melanomas (Hayward et al., 2017; Tiffen et al., 2016). As the EZH2(Y641) activating mutation is known to increase global H3K27me3 levels (McCabe et al., 2012), we questioned whether this might also be associated with increased DNA methylation and whether combinations of EZH2 and DNMT inhibitors might be more effective in melanoma treatment. We also questioned whether particular EZH2 target genes might be identified in melanoma that might help in the selection of patients that would respond to these treatments.

To answer these questions, we examined EZH2 target genes in EZH2(Y641) activated melanoma using chromatin immunoprecipitation followed by sequencing (ChIP-seq) and transcriptional profiling. In addition, DNA methylation sequencing by reduced representation bisulfite sequencing (RRBS) was performed in the same cell line to examine the relationship between H3K27me3 peaks and DNA methylated regions throughout the genome. The relevance of these findings was shown by reference to survival of patients with melanoma in clinical datasets. In addition, to identify potential biomarkers of responses to EZH2 inhibitors, we screened a panel of 53 melanoma cell lines for their sensitivity to EZH2 and compared RNA sequencing (RNAseq) profiles of sensitive to resistant melanoma cells using gene set enrichment analysis (GSEA). The results suggested that IFN signaling may be a major target of silencing by EZH2 in melanoma in addition to downregulation of tumor suppressor genes.

**RESULTS**

**EZH2 silences important tumor suppressors by histone methylation in EZH2 mutant melanoma**

To identify EZH2 target genes in melanoma, ChIP-seq was performed in MM386 cells that harbor the EZH2(Y641) activating mutation. Immunoprecipitation was performed using an H3K27me3 antibody in cells that were either untreated or treated with the EZH2 inhibitor GSK126 for 48 hours to identify specific H3K27me3 peaks that were depleted following GSK126 treatment (Figure 1a). Sequencing revealed a total of 27,193 H3K27me3 peaks that were unique to the vehicle control, corresponding to 8,272 genes (Table 1). Most peaks were intergenic (16,802) and within intronic regions (9,300), compared with 363 exon peaks and 263 peaks occurring within promoter—transcription start site regions (Table 2).

To narrow the list of putative EZH2 target genes, we used gene expression array data from MM386 and a second EZH2(Y641) mutant cell line, IGR1, treated ± GSK126 for 48 hours. Bona fide EZH2 target genes should be derepressed following GSK126 treatment. We identified 200 genes containing H3K27me3 peaks within a genomic distance of 10 kilobases of their transcription start site, whose expression increased by >0.4 (log2-fold units) following drug treatment. Gene Ontology terms related to cancer were used to further reduce the list to 58 genes, including regulation of cell growth, death, and differentiation and immune system processes (Figure 1b, Supplementary Data File).

We selected 18 putative EZH2 target genes with well-defined tumor suppressive functions in melanoma or other cancers (Table 3) for ChIP-qPCR validation in MM386 (Figure 1c) and IGR1 cells (Figure 1d). For most targets, gene expression was significantly enriched by pull-down with an H3K27me3 antibody compared with IgG controls that could be reduced by treatment with GSK126. A diverse array of tumor suppressor genes were identified, with most playing a role in differentiation pathways (OCAs, MC2R, ARHGAP22, SH2B2, EPAS1, and VAX2) as well as growth suppression (NDRG1, BOK, ABTB1, VAX2, and ARHGAP22) and anti-tumor immunity (ITGB2, RASSF5, TNFRSF25, TNFRSF14, and IL9).

**EZH2 target genes show a dose-dependent upregulation following treatment with an EZH2 inhibitor or RNA inhibition**

To validate a selection of putative EZH2 target genes, MM386 and IGR1 cells were treated over a 6-day period with increasing doses of GSK126. Gene expression measured by RT-qPCR showed a significant dose-dependent upregulation of NDRG1, RASSF5, and ITGB2 in both cell lines, whereas EPAS1 and ABTB1 were significant in one (Figure 2a and b). Consistent with ChIP-seq data, a higher-fold induction was observed in IGR1 cells compared with MM386 cells.

The expression of bona fide EZH2 target genes would be expected to increase following EZH2 knockdown. We interrogated publicly available datasets from the Gene Expression Omnibus that used RNA inhibition to inactivate EZH2 in melanoma (GSE63165) and human dermal fibroblasts (GSE109064). Small interfering RNA directed against EZH2 led to significant increases in the expression of ABTB1, ITGB2, IL9, EPAS1, TNFRSF14, and IL24 in at least one melanoma cell line (Figure 2c). In normal cells (human dermal fibroblasts), many putative targets showed a time-dependent increase in expression following EZH2 short hairpin RNA inhibition (Figure 2d) that was followed by senescence as reported by Ito et al. (2018).

**EZH2 mRNA positively correlates with both maintenance and de novo DNMTs in melanoma**

The association of DNA methylation with histone modification by EZH2 in melanoma is relatively understudied. To explore this relationship, we compared the expression of EZH2 with mediators of DNA methylation in large melanoma datasets. We observed a striking positive correlation between EZH2 levels with maintenance DNMTs (DNMT1) and de novo DNMT (DNMT3B) but not DNMT3A in 51 melanoma cell lines that have undergone RNAseq (Supplementary
Figure 1. Identification of EZH2 target genes with diverse tumor suppressor functions in EZH2 mutant melanoma. (a) MM386 (EZH2Y641H) cells were treated with GSK126 for 48 hours before ChIP-seq using an H3K27me3 antibody. Two biological replicates were performed for each treatment. (b) Criteria for selecting putative EZH2 target genes for validation. (c) qPCR validation of ChIP-seq target genes was performed in MM386 (EZH2Y641H) and (d) IGR1 (EZH2Y641N) melanoma cell lines. Known EZH2 target gene CCND2 was included as a positive control, whereas CDC6 represents a negative control (not identified by ChIP-seq). pos = primers designed proximal to an H3K27me3 peak, neg = primers designed distally to an H3K27me3 peak with diminished enrichment. Data represent two biological replicates performed in duplicate for each cell line. ChIP-seq, chromatin immunoprecipitation and sequencing; H3K27me3, histone methylation; kb, kilobase; TSS, transcription start site.
EZH2 Suppresses IFN Signalling in Melanoma

Figure 2. EZH2 target gene expression is upregulated following treatment with an EZH2 inhibitor or EZH2 knockdown. (a) Dose-dependent upregulation of EZH2 target gene expression following 6 days of treatment with GSK126 in MM386 (EZH2Y641H) or (b) IGR1 (EZH2Y641H) cells measured by RT-qPCR with GAPDH used as a housekeeping gene to adjust for RNA loading. Data is normalized to vehicle control-treated cells and represents two biological replicates performed in triplicate using a two-way ANOVA. (c) Depletion of EZH2 by RNA interference in two melanoma cell lines or (d) dermal fibroblasts upregulates expression of some EZH2 target genes. shControl; control short hairpin RNA; shEZH2, EZH2 short hairpin RNA; siControl, control small interfering RNA; siEZH2, EZH2 small interfering RNA.
EZH2 target genes display a high level of DNA methylation that silences their expression in melanoma

Given the positive association between EZH2 and DNMT expression, we assessed whether EZH2 target genes identified by ChIP-seq also contain high levels of DNA methylation that could silence gene expression. RRBS performed on MM386 cells treated with an EZH2 inhibitor for 48 hours identified that 5% of H3K27me3 ChIP-seq peaks overlap with methylated DNA fragments in the control group. This equates to approximately 9% of unique genes that are potentially regulated by both histone and DNA methylation in MM386 cells (Table 1, Supplementary Data File), including validated EZH2 target genes EPAS1, VAX2, and OCA2. However, cells treated with GSK126 showed no significant difference in DNA methylation at this timepoint (Supplementary Data File). We then explored the TCGA skin cutaneous melanoma DNA methylation dataset (HM450K arrays) to see if DNA hypermethylation represses the expression of EZH2 target genes in multiple melanoma samples. Indeed, significant inverse correlations were identified for ITGB2, NDRG1, ABTB1, EPAS1, and RASSF5 (Supplementary Figure S2a–e). A comparison of EZH2 target gene expression in EZH2-activated (single nucleotide variant, copy number gains, and mRNA overexpression) versus EZH2 wild-type (WT) revealed significant repression in the activated group (Supplementary Figure S2f–j). Collectively, these data suggest that the putative EZH2 target genes identified in our ChIP-seq studies are not unique to the EZH2<sup>Y641</sup> activating mutation in melanoma.

Combined EZH2 and DNMT inhibition derepresses target genes in EZH2 mutant and EZH2 WT melanoma

Given the presence of both H3K27me3 and DNA methylation on some EZH2 target genes, we speculated that combining an EZH2 inhibitor (GSK126) with a DNMT inhibitor (decitabine) would lead to more potent upregulation of target genes compared with treatment with either inhibitor alone. After six days of drug treatment with both GSK126 and decitabine, western blots showed complete elimination of H3K37me3 with reductions in DNMT1A, DNMT3A, and DNMT3B (Figure 3a). As expected, no significant reduction of EZH2 protein was observed because GSK126 affects EZH2 methylation activity, not total EZH2 protein levels (Figure 3a). Consistent with RRBS data, EZH2 inhibition alone showed no reduction in the levels of DNMTs. Significant upregulation of RASSF5, EPAS1, ITGB2, and NDRG1 target gene expression was seen in EZH2 mutant cell lines (Figure 3b). A similar trend was observed in EZH2 WT cells (KMJR138), indicating that the effect is not restricted to EZH2 mutant cells (Figure 3b). A similar yet not statistically significant upregulation was seen in the MelRMU EZH2 WT cell line, which was the least sensitive to EZH2 inhibition or combination treatment (Figure 4a).

Combining an EZH2 inhibitor with a DNMT inhibitor results in synergistic killing of melanoma

As EZH2 and DNMTs tended to be coexpressed and could independently suppress tumor suppressor gene expression, we tested the ability of the drug combination to initiate apoptosis in two EZH2 mutants and two EZH2 WT melanoma cell lines, using Annexin V/propidium iodide staining and flow cytometry. All four cell types showed a significant increase in cell death following treatment with the combination compared with the control (Figure 4a). To test whether the drug combination was truly synergistic and not additive, the combination index was calculated for each cell type using multiple drug concentrations with a CellTiter-Glo assay. A combination index < 1 indicates synergism; that indeed was the case for all cell lines. Consistent with the apoptosis assay, IGR1 EZH2 mutant cells were the most sensitive (combination index = 0.117), whereas MelRMU EZH2 WT were the least sensitive (combination index = 0.666) (Figure 4b).

The combination not only led to reduced numbers of melanoma cells but also induced morphological changes in the cells observed under light microscopy. The drug combination-treated cells appeared more melanocyte-like with pronounced dendrites (Figure 4c), and increased pigmentation, possibly melanin, was observed in cell pellets. This was supported by a significant increase in the expression of pigmentation genes MITF and TYR in drug-treated cells (Figure 4e).

To test the drug combination in vivo, xenograft studies were performed in nude mice with a subclone of the IGR1 EZH2 mutant cell line (IGR1-EV3), as the growth of the parental line was slow. Therefore, we generated a subclone of IGR1 cells called ex vivo 3 from a parental tumor with the fastest growth characteristics. IGR1-EV3 cells were implanted subcutaneously and, upon reaching 125 mm<sup>3</sup>, groups of 8 mice received intraperitoneal injections of either vehicle control (captisol), GSK126, decitabine, or a combination of GSK126 and decitabine. After 25 days of treatment, the combination led to a 36% reduction in tumor growth; however, the effect was not synergistic. Decitabine alone lead to a 15% reduction in tumor growth, and 37% growth inhibition was seen with GSK126, relative to control (Figure 4f). We speculated that the lack of synergistic growth inhibition was due to the absence of adaptive immune cells in nude mice. This was supported by RT-qPCR data showing a dramatic increase in the expression of antigen-presenting genes (HLA-A/B/C), chemokines (CXC19/10), and EZH2 target genes (RASSF5 and ITGB2) by the drug combination in the presence of IFN-γ (Supplementary Figure S4a and b).

Expression of EZH2 target genes correlates with survival outcome in patients with melanoma and tumor-infiltrating lymphocytes

If genes have genuine tumor suppressive functions, it is expected that patients with a low expression of these genes would have a poor survival outcome compared with those with high expression in their melanomas. We interrogated 18 putative EZH2 targets validated by ChIP-qPCR in the skin cutaneous melanoma TCGA and found this to be the case for ITGB2, RASSF5, ABTB1, NDRG1, TNFRSF25, and ARHGAP22 (Supplementary Figure S3a–f). The same observation...
was made for RASSF5 and ITGB2 in an independent dataset of 83 stage III melanoma specimens (Mann et al., 2013) (Supplementary Figure S3g and h).

As several putative EZH2 target genes are implicated in the antitumor immune response, we used the Tumor Immune Estimation Resource webtool (Li et al., 2017b) to see whether the expression of any genes was associated with tumor-infiltrating lymphocytes in the TCGA skin cutaneous melanoma dataset. Both RASSF5 and ITGB2 expression (lymphocyte adhesion) were positively correlated with infiltrating dendritic cells and CD4+ and CD8+ T cells (Supplementary Figure S3i). Collectively, our data suggest that EZH2 represses these genes, thus inhibiting the antitumor immune response that may account for poor survival outcome in patients with melanoma.

Identifying potential biomarkers of EZH2 inhibitor sensitivity and resistance

To identify genes associated with EZH2 inhibitor sensitivity and resistance, we screened a panel of 53 melanoma cell lines with the EZH2 inhibitor GSK126 (Figure 5a, Supplementary Data File). Human dermal fibroblasts and melanocytes (HEMNNMP) were included as controls. As shown in Figure 5a, the screen identified a range of responses to GSK126 including sensitive (40% < 30 μM) and resistant (60% > 30 μM) cells. RNAseq did not show correlations between EZH2 mRNA levels or common melanoma driver mutations and drug sensitivity (Supplementary Figure S4a–n db).

We compared the 15 most GSK126-sensitive with the 15 most GSK126-resistant melanoma cell lines and identified genes that are differentially expressed between the two groups (Supplementary Figure S5). GSEA showed that sensitivity to GSK126 was positively correlated with the MTOR.N4 sensitivity profile induced by rapamycin in lymphoid malignant cells, aminoacyl transfer RNA synthesis, and the unfolded protein response (Figure 5b). In contrast, sensitivity to GSK126 was negatively correlated with IFN-α and IFN-γ responses (Figure 5c). When RNAseq data for the IFN-related genes shown in Figure 5c were assembled as heat maps, there were clear differences between the sensitive and resistant melanoma lines (Figure 5d). In particular, the resistant lines appeared to have two subgroups, with one group having high expression of IFN-α and IFN-γ response genes.
EZH2 represses IFN signaling that can be reversed by EZH2 inhibitors.

IFN responses are critical for antigen presentation and adaptive immune responses (Dunn et al., 2006), so we reasoned that the low expression of IFN gene sets would be consistent with the low antigen presentation mediated by aberrant EZH2 shown in previous studies (Zingg et al., 2017). To see if these changes could be reversed by EZH2 inhibitors,
we analyzed expression array data from four drug-treated cell lines described previously (Tiffen et al., 2015). GSEA showed an increase in the expression of genes related to IFN following EZH2 inhibitor treatment, consistent with EZH2 suppressing IFN signaling (Figure 6a). Myc gene sets were downregulated following EZH2 inhibition (Figure 6b), consistent with previous studies showing interactions between EZH2 and Myc (Perotti et al., 2019; Qu et al., 2017). Heat maps of the individual IFN genes from four individual cell lines are shown in Figure 6c. These indicated that the MelRMU line that was resistant to the GSK126 inhibitor had high pre- and post-treatment levels of IFN-related genes, whereas the sensitive lines had relatively low expression before treatment with the EZH2 inhibitor.

**DISCUSSION**

We have shown that EZH2, alone or in conjunction with DNMTs, is able to reduce the expression of many tumor suppressor genes. This ability provides the incentive for tumor cells to increase EZH2 activity and provides a rationale to target EZH2, either alone or in combination with DNA methylation, in cancer therapy. The genes were validated as EZH2 targets by their loss of H3K27me3 and upregulation of expression following EZH2 inhibition.

The genes suppressed by EZH2 include those involved in antitumor immune responses in melanoma, including antigen processing and presentation, cytokine receptor interactions, and natural killer cell-mediated cytotoxicity. We identified RASSF5 and ITGB2 as two EZH2 target genes associated with leukocyte transendothelial migration. RASSF5 is a tumor suppressor gene frequently inactivated by DNA hypermethylation in multiple cancers. It is thought to suppress cell growth in response to activated RAS (Li et al., 2018), NRAS being the second most common activating gene mutation found in melanoma. RASSF5 regulates lymphocyte adhesion (Katagiri et al., 2004) and has recently been shown to maintain oncogene-induced senescence (Barnoud et al., 2017). ITGB2 forms a receptor for cell adhesion molecules to assist transmigration of leukocytes, including T cells and neutrophils (van de Vijver et al., 2012). It is thought to contribute to natural killer cell development and function (Crozet et al., 2011). Data from the Tumor Immune Estimation Resource webtool showed a strong positive correlation between tumor RASSF5 and ITGB2 expression and infiltrating lymphocytes, and low expression was linked with poor survival outcomes in patients with melanoma. Our data suggest that EZH2-driven hypermethylation silences these genes in melanoma.

There is much evidence to suggest a linkage between histone methylation and DNA methylation (Cedar and Bergman, 2009), either by direct interactions between DNMTs and EZH2 protein domains or by directing the PRC2 complex containing EZH2 to unmethylated CpG islands (Moran et al., 2018). Supporting this association, we found a strong positive correlation between EZH2 expression and expression of maintenance (DNMT1) and de novo (DNMT3B) methyltransferases in two independent melanoma datasets. Additionally, there was overlap of H3K27me3 and DNA methylation at 9% of genes in EZH2WT mutant melanoma cells. Extension to the TCGA melanoma cohort data also showed that many EZH2 target genes were heavily methylated and silenced. However, previous studies have shown that the relationship between EZH2 and DNA methylation is not absolute (Kondo et al., 2008), and therefore further investigation is required. Nevertheless, we showed that EZH2 target genes could be upregulated more effectively with combined treatment of EZH2 and DNMT inhibitors compared with single agents, inducing synergistic cell death in vitro. Although this observation did not extend to our own xenograft studies, the lack of inhibition is likely explained by the lack of immune cells present in nude mice to tolerate human xenografts. In light of this, it appears important to conduct future studies in models with intact immune systems to observe the influence of antitumor immune responses.

Further strong evidence for the importance of suppression of immune responses by EZH2 came from GSEA of RNAseq data from sensitive versus resistant cell lines. These revealed, to our knowledge, previously unreported evidence that sensitivity to EZH2 inhibitors was associated with low IFN-α and IFN-γ signaling, and that high IFN was associated with treatment resistance. Such states have been described in innate resistance to immunotherapy (Benci et al., 2016; Hugo et al., 2016; Mehta et al., 2018) and were associated with a dedifferentiated invasive phenotype. EZH2 expression was one of the epigenetic regulators of this resistance (Zingg et al., 2017) and in one study was associated with NFAT upregulation of EZH2 (Perotti et al., 2019). These results were supported by GSEA of expression array data from four melanoma lines treated with an EZH2 inhibitor, which showed increases in gene sets for IFN signaling. There was also a strong correlation between resistance to EZH2 inhibitors and high levels of IFN genes. The signaling events downstream of EZH2 causing downregulation of IFN gene sets remains to be investigated.

Given that dedifferentiated invasive phenotypes are associated with innate resistance to treatment of both targeted and checkpoint blockade immunotherapy, EZH2 inhibition may be most suitable to this resistant subset as a single agent or in combination treatment.

**MATERIALS AND METHODS**

ChiP-seq and RRBS was performed on MM386 (EZH2Y641H) melanoma cells, and qPCR validation included an additional mutant cell line (IGR1 EZH2Y641H), treated ± the EZH2 inhibitor GSK126 for 48 hours. An additional 53 melanoma cell lines were screened by CellTiter-Glo after 72 hours.

**Cell lines and drug treatment**

IGR1 (EZH2Y641N), MM386 (EZH2Y641H), KMJR138 (EZH2WT), and MelRMU (EZH2WT) cells have been described previously (Tiffen et al., 2015), and all contain the BRAFV600E activating mutation. Cells were cultured in DMEM supplemented with 10% fetal calf serum (AusGeneX, Brisbane, Queensland, Australia) and penicillin/streptomycin (Sigma, St. Louis, MO). The collection of 55 early passage cell lines used for RNAseq were derived from stage III lymph node and stage IV metastatic melanomas established at the QIMR Berghofer Medical Research Institute (Brisbane, Australia). These cell lines were first described by Payve et al. (2004) and Dutton-Regester et al. (2012), and clinical information is available in the Supplementary Data File. All cell lines were cultured in RPMI-1640
Figure 5. EZH2 inhibitor screening reveals sensitivity is associated with low IFN signaling. (a) Heatmap of IC50s for 53 melanoma cell lines plus normal melanocytes (HEMNP) and HDFs treated with GSK126 for 72 hours. Cell viability was calculated from dose-response curves using CellTiter-Glo. (b, c) GSEA using RNAseq data from untreated cell lines comparing GSK126-sensitive melanoma with GSK126-resistant melanoma cell lines. (d) Top ranked gene sets
supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100μg/ml streptomycin and incubated at 37 °C (5% CO₂). Authenticity was confirmed using an AmpFISTR Profiler Plus PCR amplification kit (Life Technologies, Carlsbad, CA) after DNA extraction with QIAamp Blood Maxi Kits (Qiagen, Hilden, Germany). All cell lines were mycoplasma-negative as determined using a PCR-based assay or MycoAlert Mycoplasma Detection Kit (Lonza, Basel, Switzerland).

The EZH2 inhibitor GSK126 (PubChem CID:68210102) and the DNMT inhibitor decitabine were purchased from MedChemExpress (Princeton, NJ) and dissolved in DMSO that was used as the vehicle control in all in vitro experiments.

**Cell line screen**

Cells were seeded into white 96-well plates at 4,000 cells per well and treated the next day with seven different concentrations of each drug, ranging from 20 nM to 20 mM, or carrier control. Biological duplicates were performed. After 72 hours, cell viability was measured using CellTiter-Glo (Promega, Madison, WI) reagent. Luminescence was detected using POLARstar Omega microplate reader (BMG Labtech, Ortenberg, Germany). Luminescence values were normalized to a percentage of control cells. Half maximal inhibitory concentration was calculated using djvMixedIC50 in R Studio.

**ChiP-seq and qPCR**

MM386 cells were fixed with 1% formaldehyde for 10 minutes, glycine was added to neutralize the formaldehyde, and nuclei were prepared from the cells. Immunoprecipitation was performed overnight with antibodies against H3K27me3 (Active Motif clone MAB0323) or IgG control (Dako, Carpinteria, CA) coupled to magna-ChIP beads (Millipore, Burlington, MA). Immunoprecipitates were washed five times in LiCl buffer and reverse cross-linked overnight at 65 °C before being column purified (Qiagen). Real-time PCR was performed using primers (Supplementary Data File) on an ABI H77900 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA), and values were normalized to the amount of input chromatin.

**Expression arrays and RNAseq**

Expression arrays for IGR1, MM386, KMJR138, and MelRMU cells ± GSK126 treatment are previously described (Tiffen et al., 2015). Pellets from the 55-cell line collection were harvested at low passage number (under 15 passages) during logarithmic growth phase at approximately 70% confluence. Total RNA was extracted using RNeasy Mini Plus Kits (Qiagen) with cells being lysed directly in culture flasks. Lysates were stored at −80 °C until sufficient samples could be batch processed for RNA extraction. Samples were then sent to Macrogen (Seoul, South Korea) on dry ice for RNAseq using 100 base pair paired-end sequencing on a HiSeq 2000 (Illumina, San Diego, CA). Fastq files were checks for quality control using FASTQC. Reads were aligned to GRCh38 release 93 using STAR with default values and gene counts generated with featurecounts from the Subread package and alignment quality checked with MultiQTC 1.5, which showed over 88% of reads aligned in all samples. Differential expression was performed using DeSeq2 in R studio running R version 3.5.3, and normalized RNAseq counts per cell lines were also calculated using DeSeq2.

**RT-qPCR**

RNA was extracted from cells using the RNeasyPlus mini prep kit (Qiagen), quantified using a Nanodrop (Thermo Fisher Scientific, Waltham, MA), and 1 μg was reverse transcribed into cDNA using SuperScriptIII (Life Technologies).

**RRBS library preparation and sequencing**

RRBS was used to map DNA methylation in MM386 cells treated with 7.5 μM GSK126 for 48 hours. Genomic DNA was digested with MspI enzyme followed by end-repair and ligation of sequencing adaptors. The fragments were size-selected and bisulfite-converted before a PCR amplification step. The quality and size distribution of the library was determined using a bioanalyzer and sequenced on an Illumina HiSeq2500 machine (100 base pair reads, single-ended).

**DNA methylation data analysis**

The quality check and processing of the sequenced RRBS reads was performed using in-house bioinformatics tools as previously described (Chatterjee et al., 2012; Stockwell et al., 2014). Bismark tool (Krueger and Andrews, 2011) was used to align the processed sequence reads to the reference human genome (GRCh37). Stringent mapping criteria was applied by allowing only one mismatch (default = 2) in the seed (i.e., in the first 28 base pairs of the sequenced reads).

Differential methylation analysis was performed with in-house Differential Methylation Analysis Pipeline, which contains two main programs (diffmeth and idenentgenloc) (Stockwell et al., 2014). Briefly, we applied an F statistic (ANOVA test) on fragments that had high-quality methylation information (at least two CpG sites covered by 10 or more sequenced reads, F2 t10 switch in the diffmeth program of the Differential Methylation Analysis Pipeline tool) and identified regions showing the largest methylation difference and significant P-value. We applied a false discovery rate of 5% on the analyzed fragments (at an alpha level = 0.05) to filter for significant fragments. We further filtered this list and obtained fragments with 0.25 (i.e., 25%) or higher methylation difference (mean methylation on fragments) between the GSK126 treated and untreated group.

**Annexin V/propidium iodide staining**

Cells were seeded and treated after 24 hours with control, GSK126, decitabine, or a combination, with drugs refreshed after 3 days, retaining supernatants. After 6 days, cells were harvested, washed, and stained with Annexin V/propidium iodide staining according to the manufacturer's instructions (BD Biosciences, San Jose, CA) and analyzed by flow cytometry with Flowjo software.

**Animal studies**

All animal experiments were performed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Approval was obtained from the Peter MacCallum Cancer Centre Animal Experimentation Ethics Committee. Female 8-week-old Balb/c nu/nu mice were injected subcutaneously into the flank with 4 × 10⁶ IGR1-EV3 cells. Once tumors had grown to approximately 125 mm³, mice were randomized into four groups of eight mice per group and each group administered GSK126 (daily, 150 mg/kg, intraperitoneal), decitabine showing higher expression and (c) top ranked gene sets showing lower expression in GSK126-sensitive cells. (d) Heatmaps showing expression of individual genes in the IFN-α gene set and the IFN-γ gene set in GSK126-resistant versus GSK126-sensitive cell lines. FDR, false discovery rate; GSEA, gene set enrichment analysis; HDF, human dermal fibroblast; IC50, half maximal inhibitory concentration; NES, normalized enrichment score; RNAseq, RNA sequencing.
(3 times per week, 0.5 mg/kg, intraperitoneal), both drugs, or the control solvent carrier. GSK126 was dissolved in 20% captisol, and decitabine was dissolved in saline. The size of palpable tumors was measured by Vernier calipers twice per week. Treatments were continued for 31 days or when maximal growth was reached.

![Enrichment plots for GSEA pathways]

**Figure 6. IFN signaling increases following EZH2 inhibitor treatment, but killing is less effective in cells with high levels of endogenous IFN-related genes.** (a) GSEA from expression arrays of pathways significantly increased or (b) decreased following 48-hour treatment with GSK126 in four melanoma cell lines. (c) Heatmaps showing expression of individual genes in the IFN-α gene set and the IFN-γ gene set in three GSK126-sensitive cell lines versus one GSK126-resistant cell line. FDR, false discovery rate; GSEA, gene set enrichment analysis; NES, normalized enrichment score.
Western blots
Cell pellets were harvested, washed in ice cold PBS, and lysed using RIPA buffer containing a protease inhibitor cocktail on ice for 30 minutes. Cells were centrifuged at 13,000 rpm for 30 minutes at 4 °C, and supernatants were collected. For detection of histone methylation, acid extraction was used to purify histones as described previously (Shechter et al., 2007). Total protein was quantified using the Biorad DC protein assay. Next, 20 μg of total protein was run in Tris-buffered saline/Tween 20 and incubated overnight in the following primary antibodies: H3K27me3 (#61017, Active Motif, Carlsbad, CA), total histone 3 (#ab1791, Abcam, Cambridge, United Kingdom), EZH2 (#5246, Cell Signaling Technology, Danvers, MA), DNMT1 (#5119, Cell Signaling Technology), DNMT3A (#3598, Cell Signaling Technology), DNMT3B (#67259, Cell Signaling Technology), and β-actin (#A2228, Sigma). Following washing in Tris-buffered saline/Tween 20, membranes were blocked using 5% BSA or 5% skim milk powder in Tris-buffered saline/Tween 20 and incubated overnight in the following primary antibodies: H3K27me3 (#61017, Active Motif, Carlsbad, CA), total histone 3 (#ab1791, Abcam, Cambridge, United Kingdom), EZH2 (#5246, Cell Signaling Technology, Danvers, MA), DNMT1 (#5119, Cell Signaling Technology), DNMT3A (#3598, Cell Signaling Technology), DNMT3B (#67259, Cell Signaling Technology), and β-actin (#A2228, Sigma). Following washing in Tris-buffered saline/Tween 20, membranes were incubated in appropriate secondary antibodies conjugated to horseradish peroxidase and chemiluminescent imaging performed using Clarity Western ECL substrate (Biorad, Hercules, CA) and Chemidoc imaging system.

Statistical analysis
Statistical significance was assessed using nonparametric analyses (Mann-Whitney U-test) using GraphPad Prism 5.01 unless otherwise indicated in the figure legend. All error bars represent the standard error from three independent experiments (n = 3), unless otherwise stated. ****P < 0.0001, ***P < 0.001, **P < 0.01, or *P ≤ 0.05 was considered statistically significant.

Data availability statement

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CONFLICT OF INTEREST
The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS
Conceptualization: PH, JT; Funding Acquisition: PH, JT; Investigation: JT, SIG, FF, AAE, AC, EJR; Methodology: JT, DG, CC; Project Administration: JT, PH; Resources: KD, LA, NH; Supervision: PH, SIG; Validation: JT, DG; Writing - Original Draft Preparation: JT, SIG, PH; Writing - Review and Editing: JT, SIG, FF, AAE, NH, AC, EJR, MRE, PH

SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2020.02.042.

REFERENCES


SUPPLEMENTARY MATERIAL
The Supplementary Data File contains target gene selection, cell line information, primers, and overlapping chromatin immunoprecipitation and sequencing, reduced representation bisulfite sequencing, and regulatory element coordinates.

Supplementary Figure S1. EZH2 mRNA expression positively correlates with DNMT1 and DNMT3B expression. (a–c) RNA-seq expression data from 51 melanoma cell lines from the Hayward sample collection and (d–f) 472 specimens from the SKCM TCGA showing Pearson’s correlation. RNA-seq, RNA sequencing; SKCM, skin cutaneous melanoma; TCGA, The Cancer Genome Atlas.
Supplementary Figure S2. DNA hypermethylation of EZH2 target genes and aberrant EZH2 activity correlates with low mRNA expression. (a–e) DNA methylation scores (HM450 from cBioPortal) versus mRNA expression of EZH2 target genes from the SKCM TCGA (n = 472) showing Pearson’s correlation. (f–j) mRNA expression of EZH2 target genes in activated EZH2 (SNV, copy number gains, and mRNA overexpression) versus EZH2 wild-type from the SKCM TCGA. SKCM, skin cutaneous melanoma; SNV, single nucleotide variant; TCGA, The Cancer Genome Atlas.
Supplementary Figure S3. Low expression of EZH2 target genes are associated with poor survival and low levels of TILs in patients with melanoma. (a–f) Kaplan-Meier plots comparing survival outcomes of patients with melanoma from the TCGA (n = 302) with expression of EZH2 target genes (low = bottom third, high = top third). Log-rank (Mantel-Cox) test was used to compare groups. (g) Kaplan-Meier plots comparing survival outcomes of Australian patients with melanoma. **P < 0.0001, ****P < 0.0001, **P < 0.001, *P < 0.05.
stage III melanoma (n = 79) with expression of EZH2 target gene transcripts ITGB2 and RASSF5. Low = bottom half, high = top half. (i) TIMER webtool showing tumor infiltration versus expression of ITGB2 and RASSF5 for SKCM TCGA (n = 425). Purity-corrected partial Spearman’s correlation is indicated. GAPDH is included as a gene unrelated to the level of TILs. SKCM, skin cutaneous melanoma; TCGA, The Cancer Genome Atlas; TIL, tumor-infiltrating lymphocyte; TIMER, Tumor Immune Estimation Resource.
Supplementary Figure S5. Genes differentially expressed between GSK126-sensitive versus GSK126-resistant melanoma cell lines. Heatmap indicating top ranked genes that were differentially expressed between the 15 most GSK126-sensitive versus the 15 most GSK126-resistant melanoma cell lines. Driver mutation status and log_{10} GSK126 IC50 is stated. IC50, half maximal inhibitory concentration.