Stat3 co-operates with mutant $Ezh2^{Y641F}$ to regulate gene expression and limit the anti-tumor immune response in melanoma

Sarah Zimmerman$^{1,2}$, Samantha J. Nixon$^{1,2}$, Leela Raj$^{1,2}$, Pei Yu Chen, Sofia Smith, George P. Souroullas$^{1,2,3, #}$

$^1$ Department of Medicine,
$^2$ Division of Oncology, Molecular Oncology Section
$^3$ Siteman Comprehensive Cancer Center
Washington University School of Medicine in St. Louis, St. Louis, Missouri 63110, USA

$^#$Address correspondence to G.P.S

George P. Souroullas, PhD
Washington University School of Medicine
Department of Medicine, Division of Oncology
Phone: (314) 362-8967
Email: george.souroullas@wustl.edu
ABSTRACT
One of the most frequently genetically altered chromatin modifiers in melanoma is the Enhancer of Zeste Homolog 2 (EZH2), the catalytic component of the Polycomb Repressive Complex 2 (PRC2), which methylates lysine 27 on histone 3 (H3K27me3), a chromatin mark associated with transcriptional repression. Genetic alterations in EZH2 in melanoma include amplifications and activating point mutations at tyrosine 641 (Y641). The oncogenic role of EZH2 in melanoma has previously been determined; however, its downstream oncogenic mechanisms remain underexplored. Here, we found that in genetically engineered mouse models, expression of Ezh2Y641F causes upregulation of a subset of interferon-regulated genes in melanoma cells, suggesting a potential role of the immune system in the pathogenesis of these mutations. Expression of these interferon genes was not a result of changes in H3K27me3, but through a direct and non-canonical interaction between Ezh2 and Signal Transducer And Activator of Transcription 3 (Stat3). We found that Ezh2 directly binds Stat3, and that in the presence of Ezh2Y641F mutant, Stat3 protein is hypermethylated. Expression of Stat3 was required to maintain an anti-tumor immune response and its depletion resulted in faster melanoma progression and disease recurrence. Molecularly, Stat3 and Ezh2 bind together at many genomic loci, and, in association with the rest of the PRC2 complex, repress gene expression. These results suggest that one of the oncogenic mechanisms of Ezh2-mediated melanomagenesis is through evasion of the anti-tumor immune response, and that the immunomodulatory properties of Stat3 are context dependent.
INTRODUCTION

Epigenetic modifiers are frequently mutated in many types of cancers, including melanoma, and are also associated with poor survival, response to treatment or relapse (1, 2). They are thus a relatively new, but very promising class of cancer therapeutic targets. Unlike the genetic code, which is static, the epigenome is reversible, creating numerous opportunities for manipulation using small molecule inhibitors to restore a normal epigenetic state, or to take advantage of genetic or synthetic lethal interactions (3). The availability of small molecules targeting epigenetic/chromatin modifying enzymes has created an enormous interest in their therapeutic properties, with currently hundreds of ongoing clinical trials testing their efficacy (4, 5). Despite clear successes, progress in some areas has been slow, suggesting that we still do not fully understand the downstream mechanisms of these epigenetic modifiers or the interactions between different epigenetic and chromatin modifications. Understanding these mechanisms is, however, an essential element to developing effective therapies.

Epigenetic modifiers regulate fundamental cellular processes such as DNA replication and DNA repair (6), along with many cell-intrinsic hallmarks of cancer, such as the cell cycle, cell growth and proliferation. It is thus not surprising that they also play a significant role in the development of cancer as well. Epigenetic modifiers also contribute towards cell-extrinsic properties relevant to cancer development or progression, such as regulation of various aspects of the immune system (7–9).

One of the most frequently genetically altered chromatin modifiers in melanoma is EZH2, the catalytic component of the Polycomb Repressive Complex 2 (PRC2), which methylates lysine 27 on histone 3 (H3K27me3), a chromatin mark associated with transcriptional repression. Specifically, about 20% of melanoma patients in TCGA datasets exhibit either overexpression of EZH2 or express the activating and neomorphic point mutation \( \text{EZH2}^{\text{Y641}} \) (Supp. Fig 1a, b). The frequency of the \( \text{EZH2}^{\text{Y641}} \) mutations ranges from 5-10% of melanoma patients from numerous studies (10–12). Patients with genetic alterations in PRC2 components and other chromatin modifiers that are highly connected with PRC2, exhibit lower survival rates (Supp. Fig. 1c), as do patients with high protein expression of EZH2 itself (Supp. Fig. 1d).

Previously, we generated a mouse model permitting conditional expression of \( \text{Ezh2}^{\text{Y641F}} \) and showed that \( \text{Ezh2}^{\text{Y641F}} \) expression in murine melanocytes caused a global increase and
redistribution in H3K27me3 across the genome, while accelerating the onset of melanoma (13). 

Ezh2\textsuperscript{Y641F} mutations in mouse models strongly co-operated with mutant \textit{Braf\textsuperscript{V600E}} but failed to co-operate with mutant \textit{Nras\textsuperscript{Q61R}}, two of the main oncogenic drivers in human melanoma (10). This result was consistent with the fact that in human patients, \textit{EZH2\textsuperscript{Y641F}} mutations tend to co-occur with \textit{BRAF\textsuperscript{V600E}} but not \textit{NRAS\textsuperscript{Q61R}} mutations. Additionally, both \textit{EZH2} and \textit{BRAF} are located within 100kb on the same chromosomal arm, and large chromosomal amplifications tend to include both genes. Despite our understanding of the consequences of the \textit{Ezh2\textsuperscript{Y641F}} mutations on chromatin and distribution of H3K27me3, we still do not know which downstream molecular mechanisms mediated by Ezh2 contribute towards melanoma initiation and progression.

In addition to \textit{Ezh2\textsuperscript{Y641F}} mutations, Ezh2 overexpression is also implicated in melanoma development and metastasis (14), suggesting that in general, increased Ezh2 activity in melanocytes may promote transformation through silencing of distinct tumor suppressors (14) or promoting disease progression by controlling mechanisms of adaptive resistance to tumor immunotherapy (15). Melanoma patients have benefited greatly from immunotherapy; however, melanoma remains the deadliest form of skin cancer once it has metastasized, with a significant considerable number of patients relapsing after targeted treatment, not responding, or becoming resistant to checkpoint inhibitors, or experiencing significant toxicity. Therapeutic options at this point become scarce, highlighting our limited understanding of the context and mechanisms of an effective anti-tumor immune response. The epigenome could be an important variable in determining how various immunomodulatory factors affect the anti-tumor immune response.

To understand the oncogenic mechanisms of Ezh2 in melanoma, we analyzed and compared gene expression profiles of \textit{Ezh2\textsuperscript{WT}} vs \textit{Ezh2\textsuperscript{Y641F}} mutant mouse melanoma tumors and identified a unique interferon-related gene signature. Despite the dramatic re-organization of H3K27me3 across the genome mediated by mutant Ezh2\textsuperscript{Y641F}, expression of these genes was not dependent on H3K27me3 at their promoters but was still dependent on Ezh2 methyltransferase activity. We thus hypothesized that expression of these genes is driven via non-canonical interactions of Ezh2 with non-histone proteins. One candidate protein was Stat3 which had previously been shown to be methylated by Ezh2 (16, 17).

Stat3 is a member of the STAT family and is activated in response to extracellular signaling proteins, including growth factors and cytokines such as IL-6 (18, 19). Upon activation, Stat3 is phosphorylated, which promotes its dimerization, transport into the nucleus where it binds a
consensus sequence on its target genes, regulating gene expression (20), often in association with other transcription factors and co-activators (21–23). In addition to being a transcriptional activator, Stat3 has also been shown to promote repression of its direct target genes (24, 25), however, the mechanism of how Stat3 represses gene expression is not clear. Stat3 has many physiological roles in a wide range of biological processes including cell-intrinsic hallmarks of cancer such as cell growth, proliferation, differentiation, apoptosis, and cell-extrinsic properties such as inflammation and immune cell regulation (26). Stat3 is therefore implicated in numerous oncogenic mechanisms and is an attractive therapeutic strategy in some types of cancer (26).

In this study, we investigated the oncogenic mechanisms of Ezh2 in melanoma, and show that activated Ezh2 in melanoma cells causes distinct changes to the anti-tumor immune response, which are dependent on Stat3 expression. We therefore present significant insight into how activated Ezh2 in melanoma promote melanoma progression, a possible mechanism that explains those oncogenic activities, and a possible explanation for how Stat3 can suppress gene expression, via interaction with the PRC2 complex.
MATERIALS & METHODS

Animals
Animals were housed in an AAALAC International-accredited facility and treated in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) for animal research at Washington University School of Medicine in St. Louis.

In vivo tumor models
C57Bl/6 female mice were obtained from Taconic Biosciences. Tumor cells were injected subcutaneously in the flank at 0.5 × 10^6 cells per injection, two injections per mouse. Tumor size was within the size permitted by the Washington University IACUC committee. Tumor volume was measured using calipers to measure length and width; volume was calculated using the formula: length × width^2. Anti-CD8 (InVivoMAb anti-mouse CD8β Cat# BE0223 Lot# 73321901) was administered to the mice intraperitoneally at 10 mg/kg (2.5 mg/ml, 100 µl injection volume). For flow cytometry analysis of infiltrating immune cells, tumors were harvested at Day 9 or, for mice treated with anti-CD8, 15 days after last injection. Tumors were finely chopped, dispersed by passing several times through a syringe with 18G needle, and filtered through a 0.40 µm filter.

Flow Cytometric analysis
Single cell suspension from dissociated tumors were washed with PBS contained 1% FBS and stained with antibody cocktails for detection of tumor-infiltrating lymphocytes. The lymphoid antibody cocktail contained: anti-CD45-PerCP/Cy5.5 (BioLegend 103132), anti-NK1.1-FITC (BioLegend 108706), anti-CD3-PB (BioLegend 100214), anti-CD4-APC (BioLegend 100412), anti-CD8-AF700 (BioLegend 100730), and anti-PD-1(CD279)-PE/Cy7 (BioLegend 135216). The myeloid antibody cocktail contained: anti-CD45-PerCP/Cy5.5 (BioLegend 103132), anti-CD19-FITC (BioLegend 115506), anti-B220-FITC (BioLegend 103206), anti-CD3-FITC (BioLegend 100204), anti-CD11b(Mac1)-PB (BioLegend 101224), anti-CD11c-PE/Cy7 (BioLegend 117318), and anti-Ly-6G(Gr1)-AF700 (BioLegend 127622). Cells were stained with propidium iodide to detect dead cells. Samples were run on an Attune Nxt Flow Cytometer (ThermoFisher Scientific) at the Siteman Flow Cytometry Core Facility. Analysis was done in FlowJo V10 and statistically significant differences were identified using One-way ANOVA and Tukey’s post-hoc test performed in R.
Analysis of RNA-seq data

Gene Set Enrichment Analysis (GSEA) was performed as described here (34). Enrichment of differentially expressed genes was carried out using a pre-ranked gene list using log$_2$ ratio of expression of Ezh2$^{WT}$ vs Ezh2$^{Y641F}$. Independent analysis of the RNA-seq data was performed with the Database for Annotation, Visualization, Integrated Discovery (DAVID) functional annotation tools (27). The functional annotation clustering tool was used to analyze gene ontology (GO) terms associated with the differentially expressed genes in Ezh2$^{Y641F}$ cells vs Ezh2$^{WT}$.

Cell culture, shRNA, cell growth assay

Mouse melanoma cell lines 234 (Tyr-Cre$^{ERT2}$ Braf$^{V600E}$ Pten$^{+/+}$ Ezh2$^{+/+}$), 234D (Tyr-Cre$^{ERT2}$ Braf$^{V600E}$ Pten$^{+/+}$ Ezh2$^{Y641F+/+}$), 234 (Tyr-Cre$^{ERT2}$ Braf$^{V600E}$ Pten$^{+/+}$ Ezh2$^{Y641F+/+}$), 855 (Tyr-Cre$^{ERT2}$ Braf$^{V600E}$ Pten$^{+/+}$ Ezh2$^{+/+}$), 855D (Tyr-Cre$^{ERT2}$ Braf$^{V600E}$ Pten$^{+/+}$ Ezh2$^{Y641F+/+}$), 480 (Tyr-Cre$^{ERT2}$ Braf$^{V600E}$ Pten$^{+/+}$ Ezh2$^{+/+}$) and 480D (Tyr-Cre$^{ERT2}$ Braf$^{V600E}$ Pten$^{+/+}$ Ezh2$^{Y641F+/+}$) were cultured in DMEM high glucose (Sigma) with 10% fetal bovine serum (Corning Cat# MT35010CV Lot# 14020001) and 1% penicillin/streptomycin. Stat3 knockdown cell lines were generated by transducing cells with lentiviral shRNA (TRCN0000071456, TRCN0000071454, TRCN0000071453, Sigma). Lentiviruses were generated using 293T cells via transfection with PEI and appropriate vectors, including a VSV-G envelop vector. Cell lines were transduced at an MOI of 1 and puromycin selection (3 µg/ml) was initiated 48 hours after transfection. Knockdown was confirmed by qPCR and immunoblotting. For the growth assay, cells were plated in triplicate in 96-well plates at 500 cells/well and growth was determined by Alamar Blue staining and detection. Cell were incubated in Alamar blue for 1 hour prior to calorimetric measurement. The average number of cells present at each timepoint was compared between cell lines, and statistically significant differences were detected using Student’s t-test.

Chromatin Immunoprecipitation (ChIP) sequencing and analysis

Ten million cells were used for each ChIP pull down. Cells were prepared using the protocol from Lee et al. (35). Briefly, cells were lysed and sheared using a probe Sonicator to achieve a size of 200-300 bp, and DNA was isolated using phenol: chloroform for library preparation. Immunoprecipitations were performed overnight at 4°C using anti-Stat3 (Cell Signaling #9139) and anti-Ezh2 (Cell Signaling #5246) antibodies at 1:100 dilution. Libraries were prepared using TruSeq ChIP kit (Illumina) and multiplexed for sequencing using 50-bp single reads. Libraries were sequenced on a Hi-Seq 2000 at the Genome Access Center of Washington University in St. Louis. Processing of ChIP-seq data was carried out using Encode standards. Binding sites were
identified using MACS2. Functional significance of Ezh2 and Stat3 binding sites/peaks was carried out using the Genomic Regions Enrichment of Annotations Tool (GREAT) (32). Data was visualized using the Integrated Genomics viewer (36). De novo motif analysis was carried out using HOMER (37) to find 8-20 bp motifs in the mm10 genome. The motif analysis was done on differential Ezh2 and Stat3 peaks that were higher in the Ezh2 wildtype cells, as well as the peaks that were higher in the Ezh2 mutant cells to compare the differences. The Ezh2 and Stat3 differentially bound peaks were annotated with the R package ChipSeeker to look at the differences in feature distributions and visualize the genomic annotation of these peaks between Ezh2 wildtype and mutant cell lines. The python program deetools was used to generate heatmaps and line plots associated with Ezh2 and Stat3 peaks (38). The plotProfile tool was used to create a profile plot for scores over the genomic regions around the TSS for all the Ezh2 and Stat3 marks.

**Protein Immunoprecipitation (IP)**

Cells were lysed using lysis buffer containing protease and phosphatase inhibitors on ice and sonicated briefly to complete lysis. Samples were centrifuged for 10 minutes at 16,000 × g at 4°C, and the supernatant was used for IPs. Lysates were incubated with Stat3 antibody (1:100, Cell Signaling #9139) for 24 hours, then incubated with protein A/G magnetic beads (Bio-Rad SureBeads Cat# 1614023) for 1 hour. Samples were eluted by incubating in 1X Laemmli buffer (Bio-Rad Cat# 161-0747) with beta-mercaptoethanol at 70°C for 10 minutes.

**Immunoblotting**

Samples were prepared by adding 4X Laemmli buffer with beta-mercaptoethanol (except for IP eluants) and boiled for 5 minutes. Samples were cooled briefly and loaded onto 4-20% pre-cast gels (Bio-Rad Mini-PROTEAN TGX Gels Cat# 4561095) and run at 100-120V using the Bio-Rad Mini-PROTEAN Tetra System. The separated proteins were transferred onto nitrocellulose membranes using the Bio-Rad Trans-Blot Turbo Transfer System at 1.0 Amp for 30 minutes. Membranes were blocked for 1 hour in 5% milk in TBS-T (TBS + 0.1% Tween-20), then incubated with primary antibodies overnight at 4°C. Primary antibodies were: anti-Ezh2 Cell Signaling #5246 at 1:1000, anti-Suz12 Cell Signaling #3737 at 1:1000, anti-Eed Cell Signaling #85322 at 1:1000, anti-beta actin Abcam ab213262 at 1:2000, anti-Stat3 Cell Signaling #9139 at 1:500, and anti-phospho-Stat3(Tyr705) Cell Signaling #9145 at 1:2000. The membranes were washed with TBS-T before incubating with the secondary antibodies at room temperature for 1 hour in the dark. Secondary antibodies were: anti-rabbit IgG (H+L) (DyLight 800 4X PEG Conjugate) Cell Signaling
#5151 at 1:50,000 and anti-mouse IgG (H+L) (DyLight 680 Conjugate) Cell Signaling #5470 at 1:50,000. Membranes were imaged using a Licor Odyssey Infrared Imager. Licor Image Studio software was used for densitometry.

**Quantitative PCR**

RNA was isolated from melanoma cells using the Aurum Total RNA Mini Kit (Bio-Rad Cat# 7326820) according to the manufacturer's instructions. The igScript First Strand cDNA Synthesis Kit (Intact Genomics Cat# 4314) was used to synthesize the cDNA with the following modifications: 1.0 µl of random hexamers and 1.0 µl of oligo-dT were used as the primers for the reverse transcriptase reaction. The cDNA was used undiluted for qPCR with the iTaq Universal SYBR Green Supermix (Bio-Rad Cat# 1725120). Primers used for qPCR are in the table below.

| Primer Name | Primer Sequence                  |
|-------------|----------------------------------|
| Ifi44-F     | AACTGACTGCTCGCAATAATGT          |
| Ifi44-R     | GTAACACAGCAATGCCTCTTGT          |
| Stat1-F     | ACCTGGAGGTCTTTGTTCCCT           |
| Stat1-R     | GAGATTCCTGGGCTCTGTCAC           |
| Stat2-F     | CTGAAGGACGAAACAGATGTC           |
| Stat2-R     | CAGGGTGTTAATCGGCCAA             |
| Oas2-F      | CCCTTTCTTCCAAGTGAGGC            |
| Oas2-R      | TGAACACTCCACCATGTCA             |
| Irf7-F      | TCCAGTTGATCCGCATAAGGT           |
| Irf7-R      | CTTCCCTATTTCCGTGGCTG            |
| Ifit3-F     | TCAGGCTTACGTGACAAGGT            |
| Ifit3-R     | CACACTTTAGGGCTGTCATC            |
| Ezh2-F      | GCATCTTTGCTAAGAGGGCT            |
| Ezh2-R      | TCGTTGCAGTGCCACATACT            |
| Stat3-F     | CAACCTTACACCGGCCACA             |
| Stat3-R     | ACGATAGGGTGCCTTGTGTA            |
| Rpl30-F     | CTTGCCAACAACCTGTCCAGC           |
| Rpl30-R     | ACGCTGTGCCAGTTCAATA             |
| Hjurp-ex5/6-F | CATCTTGCAGCTCAAGGATGAG      |
| Hjurp-ex5/6-R | CAGTTTCCAGGTTGTTCCGGG        |
| Hjurp-ex3/7-F | AGAGTTGGGGAGAAAGCTGA       |
| Hjurp-ex3/7-R | CAGAGGCTGTGACAGATGA     |
RESULTS

Expression of Ezh2<sup>Y641F</sup> in melanoma induces an interferon-related gene expression signature

In previous studies we generated a mouse allele modeling the Ezh2<sup>Y641F</sup> mutation in melanoma. We found that this allele was a potent driver of melanoma, especially in combination with the Braf<sup>V600E</sup> mutation. Expression of Ezh2<sup>Y641F</sup> globally increased abundance of H3K27me3, however, it also caused a widespread redistribution of this mark across the genome (13), the mechanisms of which remain unclear. To better understand the underlying oncogenic mechanisms of these mutations, we used isogenic cell lines derived from tumors of Tyr-Cre<sup>ERT2</sup> Braf<sup>V600E</sup> Pten<sup>F/+</sup> mice that differ only in expression of the Ezh2 mutation (WT vs Y641F). Functional annotation (27, 28) of differentially expressed genes identified enrichment for several Gene Ontology terms, including proteolysis, cell-cell adhesion, protein post-translational modifications, transcriptional regulation, and immune response (Fig. 1a). Gene set enrichment analysis additionally identified Interferon signaling, IFNB1 targets and cytokine signaling as also enriched in Ezh2<sup>Y641F</sup> vs Ezh2<sup>WT</sup> tumors (Fig. 1b, c). A subset of these differentially expressed, IFN-related genes was validated on separate biological samples by qPCR (Fig. 1d).

Expression of IFN-related genes in Ezh2<sup>Y641F</sup> melanomas is dependent on Ezh2 activity but not H3K27me3

Next, we wanted to determine if expression of these genes was dependent on Ezh2 activity; therefore, we analyzed expression of these genes after treatment with an Ezh2 inhibitor, JQEZ5. We found that treatment with the Ezh2 inhibitor reversed the overexpression of these genes in the presence of the Ezh2<sup>Y641F</sup>, suggesting that their expression is dependent on Ezh2 activity (Fig. 1e). To determine whether expression of these genes was also dependent on the H3K27me3 mark that is mediated by enzymatically active Ezh2, we analyzed H3K27me3 distribution at the promoter regions of these differentially expressed genes. We expected that genes whose expression is increased in the Ezh2<sup>Y641F</sup> mutants would exhibit a loss of the suppressive H3K27me3 mark along their promoters or gene bodies. Surprisingly, we did not find a decrease in H3K27me3, or any other correlation between expression of these genes and deposition of H3K27me3 at their promoters (Fig. 1h). These data suggest that even though expression of these IFN-related genes is dependent on Ezh2 activity, they are not dependent on its canonical function of methylating histone tails at H3K27, suggesting that expression of these genes is mediated through an indirect way, or a non-canonical function of Ezh2 activity.
Ezh2 directly interacts with Stat3 in melanoma cells

To understand how these IFN targets are upregulated in the presence of Ezh2$^{Y641F}$, we considered whether Ezh2 may interact with, and methylate known IFN regulatory factors, such as the STAT proteins. Previous studies have shown that Ezh2 directly interacts with and methylates Stat3 in several solid tumors (16, 17, 29). Several differences exist between these studies, such as which lysine is methylated and the effect of methylation on Stat3 activity. Yang et al. suggested that Stat3 methylation at lysine 140 (K140) inhibits Stat3 activity (29) whereas Dasgupta et al., reported that Ezh2-mediated methylation at lysine 49 (K49) was activating in colon carcinoma cell lines (16). In glioblastoma, Kim et al reported that methylation at lysine 180 (K180) enhanced Stat3 activation (17). These differences could be explained by tissue-specific functionality of these proteins. First, we determined whether Stat3 was expressed in our mouse model and melanoma cell lines and, indeed, Stat3 is present in both the cytoplasm and the nucleus (Fig. 2a).

Interestingly, there was more Stat3 protein present in the nucleus of Ezh2$^{Y641F}$ cells compared to Ezh2$^{WT}$ cells, however, there was no difference in the amount of activated Stat3 as assayed by how much protein was phosphorylated (Fig. 2a). To test whether Ezh2 interacts with Stat3 in melanoma cells, we performed co-affinity immunoprecipitation in Ezh2$^{WT}$ vs Ezh2$^{Y641F}$ using both a Stat3 antibody and an Ezh2 antibody. We found that we can pull down Ezh2 and Stat3 with the reciprocal antibody in all cell lines tested (Fig. 2b, showing two cell lines, 855 and 480). Interestingly, when probing with a tri-methyl-recognizing antibody, we found that Stat3 was hypermethylated in the presence of Ezh2$^{Y641F}$ mutant protein. A much weaker interaction was also observed in the presence of WT Ezh2 protein as well. These data suggest that Ezh2 directly interacts with and promotes the methylation of Stat3, which could be contributing towards the abnormal expression of IFN-related genes in Ezh2$^{Y641F}$ melanomas shown in Figure 1.

Stat3 knockdown in Ezh2$^{Y641F}$ melanoma cells restores expression of IFN-regulated genes

To investigate the potential role of Stat3 in the differential gene expression patterns we observed in the Ezh2$^{Y641F}$ melanoma tumors, we performed lentiviral, short hairpin RNA (shRNA)-mediated knockdown of Stat3 in the same melanoma cell lines use above expressing either WT or Y641F mutant Ezh2 protein. Stat3 knockdown efficiency was around 90% (Fig. 3a) and was able to decrease expression of many of the upregulated IFN-regulated genes in Ezh2$^{Y641F}$ melanoma cells (Fig. 3b), suggesting that the changes in IFN-related gene expression in Ezh2$^{Y641F}$ mutants is at least partially mediated by Stat3.
Stat3 knockdown has no effect on Ezh2Y641F melanoma cell intrinsic tumor growth

Stat3 can regulate a plethora of biological functions, including many cell-intrinsic hallmarks of cancer, such as cell growth, proliferation, apoptosis, and migration. To determine whether Stat3 regulates cell-intrinsic cellular functions in melanoma, we performed growth assays in Ezh2Y641F melanoma cell lines, with and without shRNA Stat3 knockdown. Growth assays showed that inhibiting Stat3 expression had no significant effect on cell growth of these melanoma cell lines (Fig. 3c), suggesting that if Stat3 plays a role in the pathogenesis of Ezh2Y641F melanoma, it is not through regulation of cell-intrinsic cell growth properties.

Stat3 knockdown accelerates in vivo growth of Ezh2Y641F melanoma tumors.

An important aspect of tumor growth and progression is the interplay between the cell-intrinsic properties of the cancerous cells and the tumor microenvironment. In addition to its role in cell-intrinsic hallmarks of cancer, Stat3 is also implicated in several cell-extrinsic processes related to cancer, such as inflammation and wound healing, persistent Stat3 activation is therefore a recurring feature in many epithelial tumors (26). As a result of its role in wound healing and the consequences of numerous cascades of chemokine and cytokine signaling, Stat3 expression and activation can directly affect the anti-tumor immune response when a cancer develops in a tissue. In most cancers, Stat3 activation and its downstream signaling has strong immunosuppressive properties (30). Stat3 has therefore been an attractive therapeutic strategy to augment the anti-tumor immune response.

To investigate the role of Stat3 in melanoma tumor growth in vivo, we infected Ezh2Y641F and Ezh2WT melanoma cells with anti-Stat3 shRNA or a scrambled control shRNA. These cell lines were transplanted orthotopically into recipient C57Bl6 mice and tumor growth was monitored over time. Knockdown of Stat3 in Ezh2WT cell lines did not affect tumor growth; however, in the epigenetically different background mediated by Ezh2Y641F, Stat3 knockdown, led to increased tumor growth (Fig. 4b). Notably, this result in the Ezh2Y641F mutant cell lines is the opposite of what one would expect based on the previously well-established immunosuppressive properties of activated Stat3 in solid tumors (26).

Expression of Ezh2Y641F in melanoma cells alters tumor-infiltrating immune populations which are dependent on continued Stat3 expression

To understand why Ezh2Y641F mutant melanoma tumors were growing faster despite knocking down an immunosuppressive factor, we carefully analyzed the tumor microenvironment for the
We dissected tumors from mice at day nine after injection, physically dissociated the tumors to isolate tumor-infiltrated hematopoietic cells for analysis via flow cytometry. Analysis for tumor-infiltrating lymphocytes revealed no differences in CD4+ cells, but we identified increased infiltration of cytotoxic CD8+ T cells in Ezh2\textsuperscript{Y641F} compared to Ezh2\textsuperscript{WT} tumors. Stat3 knockdown on Ezh2\textsuperscript{WT} tumors had no effect in the number of infiltrated CD8+ T cells, however, Stat3 knockdown on Ezh2\textsuperscript{Y641F} tumors dramatically decreased the number of CD8+ cells in the tumor microenvironment (Fig. 4c), suggesting that expression of Stat3 becomes critical only when Ezh2\textsuperscript{Y641F} mutant protein is expressed. The increased number of cytotoxic CD8+ cells in the tumor microenvironment in these tumors was not consistent with the increased tumor growth. A possible explanation for this apparent discrepancy is the possibility that these tumor-infiltrating T cells are not functional or are exhausted. We thus looked at expression of the PD-1 receptor on the surface of these cells and found increased expression, which was dependent on continued Stat3 expression (Fig. 4c).

In addition to lymphocyte populations, we also analyzed the tumor microenvironment for the presence of myeloid cells. There was an increase in CD11c+ dendritic cells and Mac1+ cells in Ezh2\textsuperscript{Y641F} cells after Stat3 knockdown, but not in Ezh2\textsuperscript{WT} cells. A different trend was observed with granulocytes (Mac1+ Gr1+ cells), where Stat3 knockdown caused a non-significant increase (compared to Ezh2WT without knockdown) in the Ezh2\textsuperscript{WT} tumors (Fig. 4d). Taken together, these data suggest that expression of Ezh2\textsuperscript{Y641F} in these tumors has created a unique dependency on Stat3 expression, with significant consequences on the tumor microenvironment.

**Stat3 expression limits the anti-tumor immune response in Ezh2\textsuperscript{Y641F} melanomas**

The changes in the composition of the tumor microenvironment described above suggest that it plays a direct role in the growth and progression of these melanoma tumors in vivo. To understand the role of the tumor microenvironment and infiltration of immune cells on tumor growth, we utilized the same in vivo tumor model described above and observed tumor growth over a longer period. Typically, these tumors grow rapidly for about ten days, but then most are spontaneously rejected by the host (Fig. 5a) and shrink to undetectable size by palpation after another ten days. However, some tumors recur, at which point the host can no longer inhibit their growth and the animals must be euthanized (Fig 5a). Interestingly, cell lines with Stat3 knockdown exhibit a much higher incidence of persistent or recurrent tumor growth (Fig 5b and c), regardless of Ezh2 genotype. To determine if the tumor rejection was due to the presence of cytotoxic T cells in the tumor microenvironment, we depleted the CD8+ cells in tumor-bearing animals by administering...
an anti-CD8 antibody. Depletion of CD8+ cells prevented rejection of the tumors, which continued to grow rapidly compared to vehicle-treated controls (Fig. 5d, e). Additionally, we injected the same cell lines into immunodeficient NSG mice and monitored growth over time. In this setting, there was no rejection and tumors continued to grow rapidly (Fig. 5f), clearly suggesting that the effects on tumor growth dynamics that we observe are mediated by the immune system.

**Ezh2<sup>Y641F</sup> changes global Ezh2 and Stat3 association with chromatin and DNA**

To understand the molecular effects and the nature of the interaction between Ezh2 and Stat3 in melanoma, we performed chromatin immunoprecipitation followed by sequencing (ChIP-seq) for both Ezh2 and Stat3 in Ezh2<sup>WT</sup> vs Ezh2<sup>Y641F</sup> melanoma cells. Three separate syngeneic cell lines were used for these experiments as biological controls. Comparative analysis between genotypes revealed several global differences mediated by expression of mutant Ezh2<sup>Y641F</sup>. For example, in Ezh2<sup>Y641F</sup> melanoma cells, Ezh2 protein was bound less frequently on promoter regions but more within the first intron (Supp. Fig. 2a, c). These data are consistent with our previous observations that H3K27me3 in Ezh2<sup>Y641F</sup> is redistributed away from promoters and towards the gene bodies (13). Stat3 binding across the genome was also altered in Ezh2<sup>Y641F</sup> with less binding in promoter regions compared to Ezh2<sup>WT</sup> (Supp. Fig. 2b, d). Systematic analysis of all Stat3 peaks showed binding/association of Stat3 with new genomic loci in Ezh2<sup>Y641F</sup> cells (Supp. Fig. 2e). Analysis of called peaks within proximal genes in Ezh2 and Stat3 ChIP identified numerous already known loci that are bound by either protein. For example, Ezh2 was associated with known PRC2-regulated regions, such as Hoxa and Hoxc clusters, and Stat3 was associated with known Stat3 targets, such as Hif1a, Pim2, Foxo1, Il10 (31) (Supp. Fig. 3). We did not observe a significant change in Stat3 binding at differentially expressed genes in Ezh2<sup>WT</sup> vs Ezh2<sup>Y641F</sup> cells, suggesting that Stat3 does not dramatically alter global gene expression in melanoma cells (Supp. Fig. 4).

Next, we investigated the functional significance of the binding sites that were identified by ChIP-seq. Very often associating only proximal binding events (2-5 kb from the transcriptional start site), results in discarding a significant fraction of the binding events (32), and associating each binding site with the nearest gene also introduces biases and many false positives. Therefore, we used the Genomic Regions Enrichment of Annotations Tool (GREAT) to analyze the functional significance of cis-regulatory regions with Ezh2 and Stat3 binding by modeling the vertebrate genome regulatory landscape from multiple sources (32). Using the GREAT algorithm, we obtained a list of genes previously shown to be regulated by regions bound by Ezh2 and Stat3. Functional annotation of the genes closest to the ChIP-seq peaks identified multiple pathways
implicated in cancer cell-intrinsic and cell-extrinsic mechanisms, such as endothelial cell migration, leukocyte differentiation, regulation of IL-6 signaling, along with other pathways not directly implicated in oncogenic mechanisms (Supp. Fig. 5).

**Ezh2 and Stat3 bind to common regions in the genome**

To understand if Ezh2 and Stat3 co-operate in regulating gene expression, we investigated whether Ezh2 and Stat3 bind to some of the same genomic regions. Comparison of ChIP peaks for Ezh2 and Stat3 in Ezh2<sup>Y641F</sup> cells identified unique loci co-occupied by Ezh2 and Stat3 (Fig. 6a). Binding to these loci was not detected in Ezh2<sup>WT</sup> cells. Motif analysis of these binding sites revealed links to melanoma initiation pathways, such as Mitf binding motifs, in addition to immune regulatory elements, such as Stat4 and Irf6 (Fig. 6b), consistent with a potential role for Ezh2 and Stat3 in regulating the tumor immune response in these cells.

**Ezh2 expression becomes dependent on Stat3 in Ezh2<sup>Y641F</sup> melanoma cells**

Interestingly, one Ezh2 and Stat3 co-occupied locus was on chromosome 6, 70 kb upstream of Ezh2, and was predicted to regulate expression of Ezh2 itself (32). Even though Ezh2 protein binds to that region, no significant H3K27me3 mark was associated with it (Fig 6c), suggesting that expression of Ezh2 in Ezh2<sup>Y641F</sup> cells might be dependent on the interaction between Ezh2 and Stat3. To determine whether Ezh2 expression was dependent on the presence of Stat3, we knocked down Stat3 in multiple melanoma cell lines and assayed Ezh2 expression. Consistent with the ChIP-seq data and the long-range interaction predicted by the GREAT algorithm, Stat3 knockdown caused a ten-fold decrease in Ezh2 expression in Ezh2<sup>Y641F</sup> cells, while only mildly decreasing expression in Ezh2<sup>WT</sup> cells (Fig. 6d), further highlighting this unique interaction between Ezh2 and Stat3 in Ezh2<sup>Y641F</sup> melanoma cells.

**Ezh2 and Stat3 bind at common genomic loci and repress gene expression**

Binding of both Ezh2 and Stat3 at the co-occupied loci proximal to gene start sites and within canonical promoter regions, might result in direct regulation of transcription. We thus directly investigated several of these loci with ChIP-qPCR. An example is shown in Figure 6d and includes the genes Hjurp and Trpm8. To verify that Ezh2 and Stat3 bind to that region, we performed ChIP followed by qPCR using primers that corresponded to the coordinates of specific peaks (Fig. 6f). ChIP-qPCR verified the presence of both Ezh2 and Stat3 at the promoter of Hjurp. To determine if Ezh2 and Stat3 bind together that those regions, we performed sequential ChIP
(ChIP-reChIP), further verifying that Ezh2 and Stat3 co-localize at these genomic loci (data not shown).

To determine the effect of Ezh2 and Stat3 binding on the expression of these genes we performed qPCR in $Ezh2^{WT}$ vs $Ezh2^{Y641F}$ cells with and without shRNA-mediated Stat3 knockdown. We found that $Trpm8$ was not expressed in either genotype, and its expression did not change after Stat3 knockdown. $Hjurp$ expression, however, was readily detectable. $Hjurp$ expression was slightly decreased in $Ezh2^{Y641F}$ cells, and upon Stat3 knockdown, expression of $Hjurp$ significantly increased in both $Ezh2^{WT}$ and $Ezh2^{Y641F}$ cells, but more significantly in $Ezh2^{Y641F}$ cells (Fig. 6g). These data suggest that the presence of both Ezh2 and Stat3 at these loci suppresses $Hjurp$ expression, likely by recruiting the entire PCR2 complex as this region is heavily marked by H3K27me3 (Fig. 6e).

**Stat3 interacts with Ezh2 within the context of the rest of the PRC2 complex**

Previous studies have demonstrated that Stat3 binding to its target genes is not always activating in nature but also repressive (24). This behavior which might depend on Stat3 association with other proteins, co-factors or complexes. The above result suggests that Stat3 is repressive at the $Hjurp$ locus, and the presence of abundant H3K27me3 indicates that $Hjurp$ suppression might be mediated by association with the repressive chromatin modifying complex, PRC2. To determine whether the Stat3 interaction with Ezh2 takes place within the context of the entire PRC2 complex, we performed co-affinity immunoprecipitation assays using a Stat3 antibody and assayed for interactions with Suz12 and Eed, the other two core components of the PRC2 complex. We found that Stat3 interacts with both Suz12 and Eed in both $Ezh2^{WT}$ and $Ezh2^{Y641F}$ cells, but the interaction with the mutant $Ezh2^{Y641F}$ was stronger (Fig. 7a, b), suggesting a possible of mechanisms of how Stat3 may repress gene expression.
**FIGURES AND LEGENDS**

(a) Functional annotation of gene expression profile analysis of Ezh2<sup>WT</sup> vs Ezh2<sup>Y641F</sup> mutant melanoma cells. Upregulated genes in Ezh2<sup>Y641F</sup> cells were analyzed using the DAVID functional annotation clustering tool to identify enriched GO terms.

(b) GSEA analysis of upregulated genes in Ezh2<sup>Y641F</sup> melanoma cell lines showing enrichment for Interferon signaling categories.

(c) Representative GSEA plot of the Hallmark: Interferon gamma response category.

(d) Validation of differentially expressed interferon-related genes by qPCR

(e) Log<sub>2</sub> fold change of upregulated interferon-related genes in Ezh2<sup>Y641F</sup> melanoma cells lines before and after treatment with Ezh2 inhibitor JQEZ5.

(f) Analysis of H3K27me3 signals 5kb upstream and downstream of transcriptional start sites of the genes from (e)

**Figure 1. Ezh2<sup>Y641F</sup> melanoma cells exhibit an interferon-related gene expression profile.**

(a) Functional annotation of gene expression profile analysis of Ezh2<sup>WT</sup> vs Ezh2<sup>Y641F</sup> mutant melanoma cells. Upregulated genes in Ezh2<sup>Y641F</sup> cells were analyzed using the DAVID functional annotation clustering tool to identify enriched GO terms.

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(f) Analysis of H3K27me3 signals 5kb upstream and downstream of transcriptional start sites of the genes from (e)
Figure 2. Stat3 physically interacts with Ezh2 in Ezh2<sup>Y641F</sup> mutant cells, where it is hypermethylated.

(a) Immunoblotting analysis for Stat3 and actin in cytoplasm, and Stat3 and phospho-Stat3 in nucleus of melanoma cell lines 480 and 855 (Δ = Y641F mutant).

(b) Immunoprecipitation for Stat3 and Ezh2, immunoblotted for Stat3, Ezh2, and tri-methyl lysine motif.
**Figure 3.** Stat3 knockdown in melanoma cell lines restores IFN gene expression without affecting intrinsic cell growth.

(a) Lentiviral Stat3 knockdown (KD) in melanoma cell lines confirmed by Western blot and qPCR.

(b) Expression of interferon-related genes is reduced after Stat3 KD.

(c) Stat3 knockdown in Ezh2^Y641F^ melanoma cell lines had no effect on cell growth.
Figure 4. Analysis of tumor-infiltrating immune cells in $Ezh2^{WT}$ vs $Ezh2^{Y641F}$ melanoma cells after Stat3 knockdown.

(a) Schematic of experimental design. Melanoma cells were injected subcutaneously into the flanks of C57Bl/6 mice. The tumors were measured 7 days later. $N = 8-10$/group.

(b) In vivo tumor volume/growth over time of $Ezh2^{WT}$ vs $Ezh2^{Y641F}$ melanoma cell lines, with and without Stat3 knockdown.

(c) Analysis of tumor-infiltrated lymphocytes by flow cytometry, focusing on CD4+, CD8+ and CD8/PD-1+ cells.

(d) Analysis of tumor-infiltrated myeloid cells by flow cytometry.
Figure 5. Analysis of tumor infiltrated immune cells in $Ezh2^{WT}$ vs $Ezh2^{Y641F}$ melanoma cells after Stat3 knockdown
(a) Tumor volume over time for the indicated genotypes. Mice are on a $Braf^{V600E}Pten^{F/+}$ background. Each line represents an individual tumor. N = 10/group. These graphs are from one of two replicate experiments.
(b) Tumor volume over time for $Ezh2^{Y641F}$ cell lines after Stat3 knockdown. N = 10/group. These graphs are from one of two replicate experiments.
(c) Tumor outcome average of two replicate experiments, stratified into 3 groups: cleared (rejected tumors), persistent (tumors that grew consistently), and recurring (tumors that shrunk but then rebounded). N = 10-14/group.
(d) Tumor volume over time with anti-CD8 treatment. N = 8/group. Arrows indicate the time of anti-CD8 treatments.
(e) Representative plots of CD4 and CD8 expression in tumor-infiltrating immune cells from tumors treated with or without anti-CD8 treatment.
(f) Tumor volume from an $Ezh2^{Y641F}$ melanoma cell line from (a), injected in NSG mice. N = 5.
Figure 6. (Legend in next page)
Figure 6. Changes in global DNA and chromatin binding by Ezh2 and Stat3 in Ezh2^{Y641F} mutant melanoma cells.
(a) Distinct and overlapping ChIP-seq peaks for Ezh2 (left) and Stat3 (right) in Ezh2^{Y641F} melanoma cells.
(b) Motifs identified in the common peaks between Ezh2 and Stat3 in Ezh2^{Y641F} melanoma cells from (a).
(c) Ezh2, Stat3 and H3K27me3 ChIP-seq peaks at a regulatory region on chromosome 6, 72kb upstream of Ezh2 that regulate its expression.
(d) Expression of Ezh2 in Ezh2^{WT} vs Ezh2^{Y641F} melanoma cells after Stat3 knockdown.
(e) Unique and representative ChIP-seq peaks of Ezh2 and Stat3 in Ezh2^{Y641F} cells at chromosome 1, proximal to genes Hjurp and Trpm8. H3K27me3 ChIP-seq tracks also visualized for comparison.
(f) Amplified region of the Hjurp promoter highlighting new Ezh2 and Stat3 binding sites at the Hjurp promoter in Ezh2^{Y641F} melanoma cells.
(g) Hjurp gene expression in Ezh2^{WT} and Ezh2^{Y641F} melanoma cells after Stat3 knockdown (KD). Expression of knockdown groups is shown relative to the controls.

Figure 7. Stat3 protein interactions with core components of the PRC2 complex
(a) Immunoblotting analysis of Ezh2, Stat3, Suz12 and Eed after immunoprecipitation with anti-Stat3 antibody in Ezh2^{WT} vs Ezh2^{Y641F} melanoma cell lines.
(b) Quantitative analysis of the blots in (a).
DISCUSSION

To understand the oncogenic mechanisms of Ezh2 in melanoma, we investigated a potential role of hyperactive Ezh2 mutations in the anti-tumor immune response, along with possible non-canonical interactions. Here we report that Ezh2<sup>Y641F</sup> mutations promote a non-canonical interaction between Ezh2 and Stat3, with significant consequences on the anti-tumor immune response in melanoma. Ezh2<sup>Y641F</sup> also affects binding of Ezh2 and Stat3 across the genome. Additionally, Stat3 is responsible for Ezh2<sup>Y641F</sup>-mediated infiltration of lymphocytes in the tumor microenvironment and consequently, for maintaining an anti-tumor immune response. Molecularly, we show that Ezh2 and Stat3 bind to both common and distinct loci across the genome with complicated effects on gene expression. Together, these data suggest that the tumor microenvironment is a significant factor in the oncogenic properties of Ezh2 in melanoma.

Even though numerous sequencing studies have identified frequent mutations in epigenetic and chromatin-modifying factors in many cancers, their downstream oncogenic mechanisms remain underexplored. Understanding these mechanisms is critical in identifying which patients might respond to treatment, which drug combinations would be beneficial, and whether any synthetic lethal interactions might exist that could be manipulated therapeutically. Many small molecule inhibitors targeting epigenetic modifiers are currently approved for use in patients, many of which have significant benefits. Many patients, however, do not respond to treatment. The same is true for immunotherapy. Even though immunotherapy approaches have had great success in many melanoma patients, a significant portion of patients do not respond to treatment or exhibit significant side effects that prevent further use of these compounds. Understanding these mechanisms is therefore very important in developing therapeutic strategies for these patients.

Prior studies have implicated the role of Ezh2 in the anti-tumor immune response (15). Zingg et. al. demonstrated a high correlation between tumor T cell infiltration and high EZH2-PRC2 complex activity in human skin cutaneous melanoma. They further proposed that Ezh2 silenced the tumors’ own immunogenicity and antigen presentation, contributing towards melanoma progression. In this study, we show that Ezh2<sup>Y641F</sup> mutant melanomas also exhibit increased infiltration of cytotoxic T cells, but in this case the T cells do not appear to drive expression of Ezh2, the presence of CD8+ T cells in the tumor microenvironment is in fact dependent on the expression of Ezh2<sup>Y641F</sup> mutant and continued expression of Stat3. These results are not necessarily inconsistent as the genetic background for the melanoma cell lines used in the two
studies were different. In our studies, we have combined \(\text{Ezh2}^{Y641F}\) mutations with the activated \(\text{Braf}^{V600E}\) allele, whereas the Zingg. et. al. study is using Nras-driven melanomas. We have previously shown that \(\text{Ezh2}^{Y641F}\) mutations co-operate with \(\text{Braf}\) mutations but not \(\text{Nras}\) mutations, hence our decision in using the specific additional oncogenic driver. These data is also supported by human melanoma patient data were \(\text{Ezh2}^{Y641F}\) mutations co-occur with \(\text{Braf}\) mutations. The results from the two studies could therefore be mechanistically completely independent. The fact remains, however, that Ezh2 is directly implicated in the anti-tumor immune response in melanoma, under different oncogenic drivers, and potentially through more than one mechanism.

The Ezh2-Stat3 interaction has been observed before in other solid tumor models, however, the functional significance of that interaction was not uncovered in those studies (16, 17, 29). Additionally, those same studies did not agree on whether Stat3 methylation promotes or inhibits phosphorylation and activation of Stat3. In melanoma, using our models, Stat3 methylation does not appear to influence Stat3 phosphorylation, and consequently its activation. This is supported by the fact that RNA-seq analysis of gene expression did not identify a specific Stat3 signature, suggesting that Ezh2-mediated methylation of Stat3 does not have a significant effect on canonical Stat3 activity. Our data suggest that Stat3 interacts with Ezh2 and other PRC2 core components and is associated at common and distinct loci. Furthermore, we show that some loci where Ezh2 and Stat3 interact there are also marked with significant H3K27me3, the repressive chromatin mark mediated by PRC2. At the same time, there are loci where Ezh2-Stat3 binding is not associated with deposition of H3K27me3, such as a distant regulatory region that controls expression of \(\text{Ezh2}\) itself. It is possible, therefore, that the Ezh2-Stat3 interaction is dual in nature. Within the context of the entire PRC2 complex, Stat3 is associated to regions that will be modified with H3K27me3 and will thus be repressed. This behavior would be consistent with the fact that Stat3 can also repress many of its target genes (24). On the other hand, Ezh2 and Stat3 could form a dimer without association with the PRC2 complex and activate transcription. This is also supported by data from other studies. For example, the oncogenic function of Ezh2 in prostate cancer is independent of the PRC2 complex and it involves the ability of Ezh2 to act as a coactivator with an intact methyltransferase domain to regulate expression of the androgen receptor (33).

In summary, we show that expression of activated Ezh2 in melanoma cells has a direct effect on the melanoma tumor immune response, mediated by expression of Stat3. This study also highlights the non-canonical functions of Ezh2, which are enhanced with the mutant and
Several questions remain unanswered. For example, we still do not understand the biochemical details of how Ezh2 may be methylating Stat3, which lysine is methylated, whether it is mono-, di- or tri-methylated, whether methylation of Stat3 requires all members of the PRC2 complex. We also do know if methylation of Stat3 is in fact consequential, and not a mere transient interaction that is enhanced in the presence of a hyperactive Ezh2 protein. Despite these unanswered questions, it is clear that the Ezh2-Stat3 interaction will have clinical importance. Stat3, which typically suppresses the anti-tumor immune response in other solid tumors, has the opposite effect in Ezh2^{Y641F} Braf^{V600E} melanomas. Cellular, genetic and epigenetic context must therefore be taken into consideration when assessing the possible effect of manipulating these pathways for therapeutic purposes. More studies are needed to thoroughly investigate the extent to which the immunomodulatory properties of Stat3 may depend on other genetic or epigenetic pathways in a cell. This work, therefore, opens new avenues of investigation in understanding the biochemical, molecular and clinical implications of these interactions and will have a significant impact on patient stratification and in designing future therapeutic strategies.
ACKNOWLEDGEMENTS
We thank the Siteman Flow Cytometry facility, McDonnel Genome Institute/Genome Access Center for technical assistance, and the Department of Comparative Medicine for animal expertise. We also thank all members of the Souroullas lab for critical input on the manuscript. This work was supported by the US National Cancer Institute K22-CA229612-01 (GPS), T32 CA113275-10 (SZ) and the Cancer Research Foundation, Chicago IL, (GPS).

AUTHOR CONTRIBUTIONS
GPS and SZ designed experiments and wrote the manuscript. GPS, SN and SZ performed experiments, analyzed, and interpreted the data. LR, PYC and SM performed experiments. GPS conceived of and supervised the study.

CONFLICT OF INTEREST
The authors declare no relevant competing financial interests
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Supp. Fig 1. EZH2 expression and overall survival in human melanoma
(a) Frequency of EZH2 overexpression (exp>2) and activating Ezh2\textsuperscript{Y641F} mutations in human melanoma (data from TCGA)
(b) Distribution of point mutations in EZH2 from TCGA data.
(c) Overall survival of melanoma patients with PRC2 mutations/overexpression and other closely related complexes (SWI/SNF, NSD, SETD2)
(d) Overall survival in human melanoma patients with higher-than-average EZH2 protein expression (data from Protein Atlas).
Supp. Fig 2. Global patterns of peak distribution in Ezh2 and Stat3 ChIP-seq data

Distribution of ChIP-seq peaks with the indicated genotypes: (a) Ezh2 peaks in Ezh2^{WT} cells, (b) Stat3 peaks in WT cells, (c) Ezh2 peaks in Ezh2^{Y641F} cells, (d) Stat3 peaks in Ezh2^{Y641F} cells (e) Stat3 ChIP-seq peaks in Ezh2^{Y641F} (top-green) vs Ezh2^{WT} (bottom-blue)
Supp. Fig 3. Representative loci of Ezh2 and Stat3 binding from ChIP-seq data
(a) Ezh2 ChIP-seq data showing binding at Hoxa and Hoxc clusters
(b) Stat3 ChIP-seq data showing Stat3 peaks/binding at known Stat3 target genes: Il10, Pim1, Foxo1, Hif1a.
Supp. Fig 4. Stat3 binding at the transcriptional start site +/- 5kb of all differentially expressed genes in Ezh2<sup>WT</sup> vs Ezh2<sup>Y641F</sup> melanoma cell lines.
Supp. Fig 5. Functional annotation of differential ChIP-seq peaks in Ezh2\textsuperscript{WT} vs Ezh2\textsuperscript{Y641F} for Ezh2 and Stat3 proteins.

(a) Gene Ontology Biological Process annotation for genes in proximity to Ezh2 ChIP-seq peaks unique to Ezh2\textsuperscript{Y641F} cells

(b) Gene Ontology Molecular Function annotation for genes in proximity to Ezh2 ChIP-seq peaks unique to Ezh2\textsuperscript{Y641F} cells

(c) Gene Ontology Biological Process annotation for genes in proximity to Stat3 ChIP-seq peaks unique to Ezh2\textsuperscript{Y641F} cells