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Somatic Copy Number Amplification and Hyperactivating Somatic Mutations of EZH2 Correlate With DNA Methylation and Drive Epigenetic Silencing of Genes Involved in Tumor Suppression and Immune Responses in Melanoma

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Abstract

The epigenetic modifier EZH2 is in the center of a repressive complex controlling differentiation of normal cells. In cancer EZH2 has been implicated in silencing tumor suppressor genes. Its role in melanoma as well as target genes affected by EZH2 are poorly understood. In view of this we have used an integrated systems biology approach to analyze 471 cases of skin cutaneous melanoma (SKCM) in The Cancer Genome Atlas (TCGA) for mutations and amplifications of EZH2. Identified changes in target genes were validated by interrogation of microarray data from melanoma cells treated with the EZH2 inhibitor GSK126. We found that EZH2 activation by mutations, gene amplification and increased transcription occurred in about 20% of the cohort. These alterations were associated with significant hypermethylation of DNA and significant downregulation of 11% of transcripts in patient RNASeq data. GSK126 treatment of melanoma lines containing EZH2 activation reversed such transcriptional repression in 98 candidate target genes. Gene enrichment analysis revealed genes associated with tumor suppression, cell differentiation, cell cycle inhibition and repression of metastases as well as antigen processing and presentation pathways. The identified changes in EZH2 were associated with an adverse prognosis in the TCGA dataset. These results suggest that inhibiting of EZH2 is a promising therapeutic avenue for a substantial fraction of melanoma patients.

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Introduction

During cancer progression a tissue-specific dedifferentiation towards an immortal state takes place [1], a change that requires concerted alterations at the genomic, epigenomic, and transcriptional level [2]. The polycomb repressive complex (PRC) 2 is instrumental for chromatin remodeling and recruitment of proteins required for epigenetic modifications [1,3]. Crucial to PRC2 activity, the histone methyltransferase enhancer of zeste homolog 2 (EZH2) [GenBank:2146] tri-methylates lysine 27 of histone 3 (H3K27me3), leading to chromatin condensation and transcriptional repression. EZH2 can also direct DNA methylation via recruitment of DNA methyltransferases (DNMTs), thus linking histone methylation to DNA methylation [3]. The cellular networks targeted by EZH2 are essential in early development but downregulated in normal adult tissues.

In many types of cancers including lymphomas and leukemia, EZH2 is postulated to exert its oncogenic effects via aberrant histone and DNA methylation, causing silencing of tumor suppressor genes [4–9]. Recent studies have identified reversible H3K27me3 levels in response to aberrant EZH2 activity in melanoma suggesting suitability for pharmacological targeting [10–14]. In particular our recent studies have shown that small molecule inhibitors of EZH2 could induce cell cycle arrest and apoptosis of melanoma cells harboring somatic mutations of EZH2 [14].

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In this study, we capitalize on the druggability of EZH2 and shed light on its role as an epigenetic regulator. We apply a comprehensive systems biology approach to the skin cutaneous melanoma (SKCM) dataset of 471 patients and in total to 12366 Pan-cancer specimens of 32 tissues of The Cancer Genome Atlas (TCGA). We connect somatic mutations and somatic copy number alterations (SCNAs) of EZH2 to epigenetic and transcriptional control of its target genes. Methylation status and transcriptional activity of target genes is combined with the transcriptional response of cellular melanoma models of activating EZH2 mutations to treatment with an EZH2 inhibitor. The rationale behind combining transcriptional data from inhibitor studies is to reveal or confirm genes repressed by EZH2 activation.

Methods

We utilized files from 471 SNP arrays, 120 whole-genome, 339 whole-exome, and 440 clinical datasets with normal reference samples from 471 TCGA SKCM patients. In addition, we selected 458 patients of the SKCM cohort with complete methylome and transcriptome data. Genomic regions of TCGA SKCM data set aligned to HG19 were determined using the tool genomic identification of significant targets in cancer 2.0.21 at confidence level of 0.99 and cutoff q-value of 0.01. Somatic mutation and somatic copy number alterations were assessed for 32 different cancer tissues covering a total cohort size of 9833 and 6506 TCGA patients for somatic copy number alteration data and whole exome sequencing data, respectively (Supplementary Table 1). The study was carried out as part of IRB of the University of California Merced approved study dbGap ID 5094 "Somatic mutations in melanoma" and conducted in accordance with the Helsinki Declaration of 1975. The results shown are based upon next generation sequencing data generated by the TCGA Research Network http://cancergenome.nih.gov. Restricted access clinical, RNASeq, and whole-exome sequences were obtained from the TCGA genome data access center and the data portal.

Illumina HiSeq 2000 V2 RNA Sequencing by expectation-maximization normalized Log2 data was filtered for differential expression in patients with activating EZH2 mutations in two-tailed Z-tests and p-values below 0.05 in 458 and 12633 patients in TCGA SKCM and Pan-cancer, respectively. Pearson's correlation coefficient was calculated for paired differential methylation and RNASeq data classified according to moderate negative correlation ($-0.2 \geq \rho > -0.4$) or strong negative correlation ($-0.4 \geq \rho$) and associated with methylation dependent transcriptional silencing. Pairwise average-linkage in combination with Pearson's correlation was used as distance measure for both, column (patients) and row (genes or markers) hierarchical clustering. Methylation data was thresholded for differential methylation in patients with activating EZH2 mutations in two-tailed Z-tests and p-values below 0.05. Differentially regulated methylation markers were mapped to HG19 and only kept if a gene association was detected at least twice. Statistical hypotheses testing, in detail Fisher's exact method, was used to determine significant enrichment of somatic mutation in given patient cohorts. Microarray analysis was performed on two melanoma cell lines, IGR1 and MM386, each conducted as duplicates 48 hours after treatment with DMSO as control or 7.5 μM GSK126 (IC 50 value between 5-8 μM) [PubChem CID:68210102]. Transcriptomic data were normalized using the normal-exponential deconvolution method, corrected for multiple samples in two-tailed T-tests, and adjusted p-values below 0.05. Pathway enrichment was determined using the web-based gene set analysis toolkit at p-values below 0.05 and mapped onto the Kyoto Encyclopedia of Genes and Genomes (KEGG).

Somatic mutations of selected PRC genes were called by multi-step filtering after cohort selection, mapping of human genome and patient specific somatic references, assessment of recurrence, evolutionary conversation, basal mutation rate based on frequency of mutations of introns vs exons, and structural analysis [10]. TCGA patients showed recurring mutations TCGA-BF-A1PV-01 EZH2(Y641N), TCGA-D9-A1JW-06 EZH2(Y641F), and TCGA-EE-A3AF-06 EZH2(Y641N). EZH2 Y641 mutant melanoma cell lines C001 and MM386 were a generous gift from Dr. Chris Schmidt and Dr. Nick Hayward, QIMR, Brisbane, Australia. IGR1 cells were gifted from Dr. David Adams of the Welcome Trust Sanger Institute, Cambridge, UK. EZH2 wild type status Y641 of MELJD, ME1007, and KMJR138 cell lines was confirmed by sequenom or sanger sequencing, while IGR1 had in-frame point mutations EZH2(Y641N), MM386(Y641H) and C001(Y641S). All cells lines were authenticated by short tandem repeat validation. Cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (AusGeneX, Brisbane, Queensland, Australia) and Pen/Strep (Sigma, St. Louis, MO, USA). Human dermal fibroblasts (HDF) and human epithelial melanocytes (HEM) were used as untransformed controls (ATCC, Manassas, VA, USA). HEM were cultured in M254 containing HGMS and all cells were maintained at 37°C in 5% CO_2.

Cell pellets were lysed with radioimmunoprecipitation (RIPA) buffer and subjected to western blot analysis. Total protein was determined using a BCA assay (Bio-Rad, Hercules, CA, USA). Labeled bands were detected by Immune-Star horseradish peroxidase chemiluminescence kit (Bio-Rad, Hercules, CA, USA) and images were captured by the Fujifilm LAS-4000 image system. Antibodies used were as follows: EZH2 (5246, Cell Signaling, Danvers, MA, USA), Beta Actin (AC-74, Sigma, St. Louis, MO, USA), p21 (SC-397, Santa Cruz Biotechnology, Dallas, TX, USA). GSK126 [PubChem CID:68210102] was purchased from Medchemexpress (New Jersey, NJ, USA) and dissolved in DMSO that was used as the vehicle control in all experiments. Cells were seeded in 6 well plates and treated the following day with DMSO, 2.5 μM, 5 μM or 7.5 μM GSK126. After 48 hours RNA was extracted from cells using the RNeasyPlus mini prep kit (Qiagen, Hilden, Germany), quantified using a Nanodrop (Thermo Scientific, Waltham, MA, USA) and 1 μg was reverse transcribed using SuperScriptIII (Life Technologies, Carlsbad, CA, USA). cDNA was amplified using the AB7900 real-time quantitative PCR (RT-qPCR) system (Life Technologies, Carlsbad, CA, USA) using Universal PCR Mastermix and Taqman probes (Life Technologies, Carlsbad, CA, USA) specific for CDKN1A [GeneBank:1026] (Hs00355782_m1) and normalized to levels of 18 s (Hs99999901_s1).

Results

Enrichment of Activating Somatic Mutations of EZH2 in Melanoma

EZH2 has somatic mutations in several regions of the molecule. The proportion of mutations that affect the different domains of EZH2 is similar between the melanoma cohort and the Pan-cancer average (DMNT binding domain 42. 9%/39.3%, CDYL binding domain 21.4%/16.7%, CXC domain 14.3%/13.1%, SET domain 21.4%/30.9% in SKCM and TCGA Pan-cancer respectively)

(Figure 1*A*, Supplementary Table 1). Alternative splice events in exons 3, 4, and 8 give rise to at least 5 mRNA isoforms (Figure 1*B*). In total, 100 coding mutations including 22 silent mutations were detected in EZH2: TCGA SKCM showed a mutation frequency of 4.1% in 339 exomes and TCGA Pan-cancer 1.3% in 6506 exomes. However, a somatic mutation in the center of the active site of the SET domain at residue Y641 was recurring. It was detected in 7 cases of all reported whole exome sequencing melanoma studies and 5 times across the TCGA Pan-cancer cohort. There were 2 cases in 278 metastatic tumors of TCGA SKCM; 1 case in 61 primary tumors of TCGA SKCM (Figure 1*C*); 2 cases in 121 metastatic Broad tumors; 2 cases in 91 metastatic Yale tumors [10,13,15]. The replacement of the JAK2 phosphorylation target residue Y641 alters EZH2 conformation, increasing its tri-methylation activity and stabilizing the protein [16,17]. Therefore it is expected that EZH2 Y641 mutants in melanoma cells exhibit increased H3K27me3 levels. High levels of activating mutations affecting the SET domain were also evident in other cancers such as Lymphoid Neoplasm Diffuse Large B-cell Lymphoma (DLBC) including 2 Y641 mutations, uterine corpus endometrial carcinoma (UCEC), lung cancer (LUAD), colorectal cancer (COAD), stomach cancer (STAD), and acute myeloid leukemia (LAML). Isoform-specific RNASeq analysis resolved splice variants of EZH2 (Figure 2*A*).

Major EZH2 Cancer Transcript NM_001203247 Lacks Extension of Exon 8, Hence Down-Stream Somatic Mutation Target Location is at Y641

Next, we investigated the TCGA RNASeq dataset to identify which isoforms of EZH2 were predominantly transcribed. Across the TCGA PAN-cancer panel, the predominantly transcribed isoform is mRNA transcript variant 3 [GeneBank:NM_001203247], 746 amino acids [GeneBank:NP_001190176], corresponding Uniprot entry [Swiss-Prot:Q15910-1], lacking a short segment at the end of exon 8 coding for 5 amino acids (Figure 2*A*). This is in contrast to the longest and first described transcript variant 1 [GeneBank:NM_004456], 751 amino acids [GeneBank:NP_004447], corresponding Uniprot isoform 2 [GeneBank:Q15910-2], [18]. While consistently being the major transcript across TCGA Pan-cancer as well as SKCM, Melanoma patients show 1.46-fold higher expression of the major EZH2 transcript compared to the TCGA Pan-cancer median in Z-tests with p-values below 1.0e-10 (Figure 2*A*). Patients with Y641 mutations share the common transcript [GeneBank:NM_001203247] and no switching of the 5' splice donor site of exon 8 was detected. Other alternative minor transcripts detected in cancer lacked mRNA segments in exon 3, 4, and 14 result in shorted isoforms, [GeneBank:NM_152998], 707 amino acids and [GeneBank:NM_001203248], 737 amino acids,

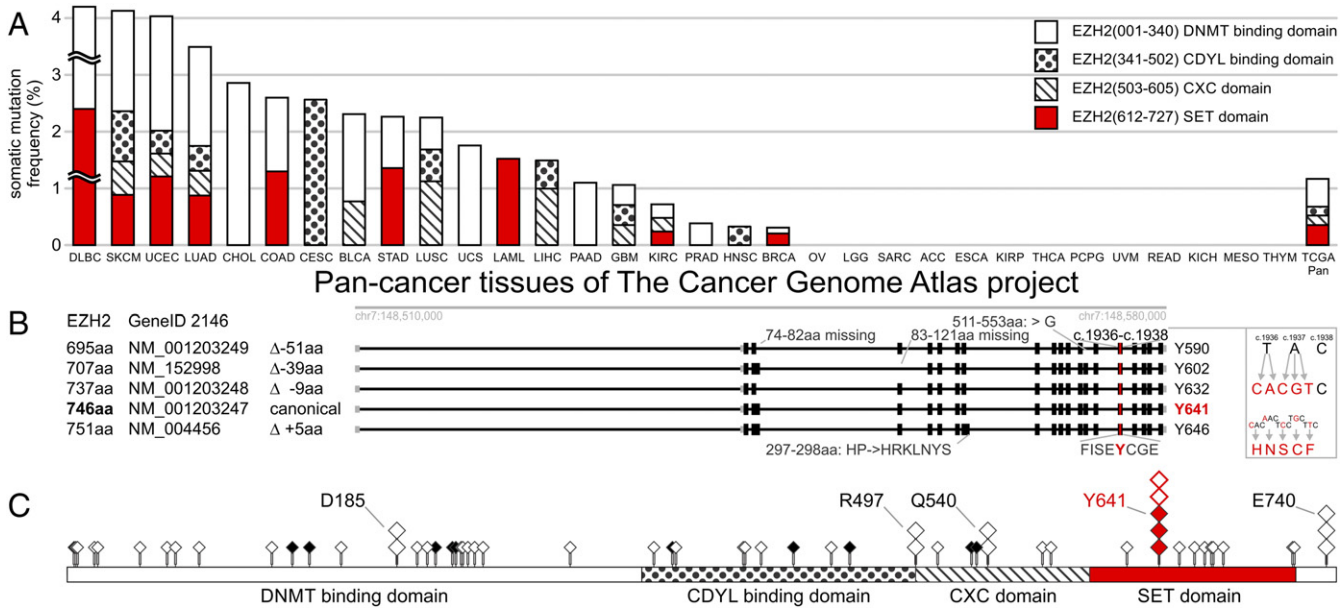

Figure 1. Distribution of somatic mutations in EZH2 in skin cutaneous melanoma (SKCM) and across Pan-cancer tissues of The Cancer Genome Atlas (TCGA) project. (A) EZH2 has a high somatic mutation frequency in melanoma of Pan-cancer tissues within TCGA. The frequency of non-silent somatic mutations (number of observed somatic mutations divided by cohort size) in different human cancer tissues within TCGA is sorted from most to least frequent. The fraction of affected protein domains of EZH2 is shaded white for DNMT binding domain, dotted for CDYL binding domain, striped for CXC domain, and red for SET domain. Lymphoid Neoplasm Diffuse Large B-cell Lymphoma (DLBC) has a mutation frequency of 14.6% exceeding the chosen y-axis range. Tildes indicate truncation of the DLBC data, while maintaining the relative domain distribution of observed DLBC mutations in DNMT and SET domains. TCGA Pan-cancer average across 6506 specimens is displayed at the very right of panel. (B) Isoform-specific transcripts of EZH2 affect numbering of somatic mutations in the C-terminal SET domain. Total amino acid count, NCBI Gene ID, amino acid difference to isoform NM_001203247, exon usage, and numbering of tyrosine in the SET-domain are provided. Somatic mutation observed in cDNA positions c.1936-1938 are boxed. (C) Somatic mutations of EZH2 in TCGA melanoma and Pan-cancer cohorts are plotted onto its amino acid sequence as filled and empty diamonds, respectively. Amino acid residues with recurring mutations across TCGA Pan-cancer tissues are shown as stacks of multiple diamonds: Y641 in red has three recurrences in melanoma, and two in lymphoma; D185H, R497Q, Q540P/*, K660E/R, and E740K/fs, each have recurrences in TCGA Pan-cancer.

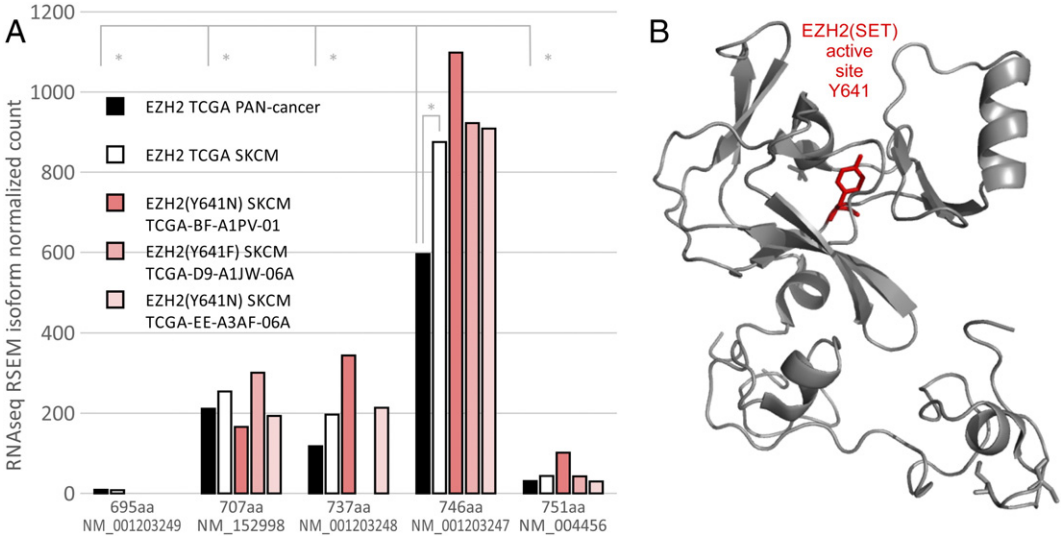

Figure 2. Isoform-specific RNASeq data across PAN-cancer panel assigns recurring somatic mutation of EZH2 in cancer to Y641 in center of SET domain. (A) Distribution of RNASeq data show isoform-specific counts for PAN-cancer and TCGA SKCM patients across 12633 and 458 specimens, respectively (somatic genotype and TCGA patient ID are indicated). Transcript NM_001203247 is the predominant isoform of wildtype and somatically mutated EZH2. We recommend mRNA transcript NM_001203247 and its corresponding 746 amino acid-long protein as canonical sequence framework. Asterisk indicated significant difference by Z-test with p-value below 1.0e-10 between isoforms or between TCGA Pan-cancer and SKCM cohorts. (B) The residue with the most frequent recurring somatic mutations of the polycomb repressive complex in SKCM (as well as in TCGA) is residue Y641 located in the active site of the SET domain of EZH2 plotted on ribbon structure of 4mi0.pdb.

which did not affect the SET-domain. Given the consistent and predominant transcriptional pattern of EZH2, we recommend cataloging of isoform [GeneBank:NM_001203247] as canonical transcript (Figures 1*B* and 2*A*). Numbering according to this transcript identifies the recurring somatic mutation in the SET-domain as Y641 (Figure 2*B*).

Significant Copy Number Gain of EZH2 in Melanoma

In addition to activating somatic mutations, TCGA melanoma patients showed strong somatic copy number alterations of EZH2 (Figure 3*A*). The EZH2 locus is over-expressed at the level of somatic copy number alterations (5.0% significant SCNA gain with a q-value below 0.01), which is higher than the TCGA Pan-cancer average of 1.8% (Figure 3*A*, Supplementary Table 2). The EZH2 locus at band 7q36.1 chr7:148,504,464-148,581,441 is in close proximity (8Mbp) to BRAF [GeneBank:673] and CDK5 [GeneBank:1020]. These and other proto-oncogenes share a hotspot at the end of the q-arm of chromosome 7 which is implicated in a significant amplification event with q-values below 10e-05 at a frequency of more than 50% for WGS (120 patients) and SNP-arrays (471 patients) (Figure 3*B*). Transcriptional levels of EZH2 are also increased in a subset of melanoma patients and 14.2% of patients showed differentially upregulated RNASeq levels. As expected, SCNA amplification of EZH2 correlates with mRNA upregulation and patients with SNCA gains showed increased EZH2 expression at the RNASeq level (Figure 3*C*). In comparison to Pan-cancer levels, the normalized RNASeq reads of EZH2 of SKCM patients are elevated by 46.7% (Figure 2*A*). Further, the pattern of SCNAs in melanoma is tightly linked to its mutational signature and tumor samples of patients with EZH2 and BRAF mutations are significantly enriched in somatic copy number amplifications with p-values below 0.05 and q-values below 10e-05 in comparison to BRAF wild type status (Figure 3*C*). EZH2 activation correlates with decreased patient survival (Figure 4). Kaplan–Meier curves of patients with EZH2 activating mutation, EZH2 amplification, or high normalized RNASeq counts show reduced patient survival of 1.8 years in comparison to curves of patients with EZH2 wild-type status (Figure 4*A*). RNASeq expression levels of EZH2 correlate with reduced overall median survival of melanoma patients of 1.8 years (Figure 4*B*).

Multi-Omics Study of Cancer Patient Data Correlates Epigenomic Silencing, DNA Hypermethylation, and Transcriptional Derepression by EZH2

We sought to identify EZH2 target genes in melanoma by combining three separate data sets. We combined gene transcription and DNA methylation data from the SKCM cohort in the TCGA with our own gene expression data based on response to small molecule inhibition of EZH2 in melanoma cell lines with activating mutations (Figure 5).

In SKCM, about 1/5th of the patient cohort is affected by somatic mutations, somatic copy number amplification, or transcriptional upregulation of EZH2. These tumors were considered likely to have high EZH2 activity and were compared to all other SKCM tumors. Comparing these cohorts, we overlaid gene sets of the TCGA SKCM transcriptome and methylome data looking for genes with both decreased expression and increased DNA methylation (Figure 5, *A–B*). In addition to histone tri-methylation, which compacts chromatin structure and represses transcription, EZH2 recruits DNA methyltransferases to methylate DNA and thereby enhances transcriptional repression. We therefore considered that EZH2 target genes would show decreased transcription and increased DNA methylation levels in the patient tumor data. To further increase confidence that these were EZH2 targets, we combined these results with expression array results from melanoma cells treated with the EZH2 inhibitor GSK126 [PubChem CID:68210102] (Figure 5, *A–C*).

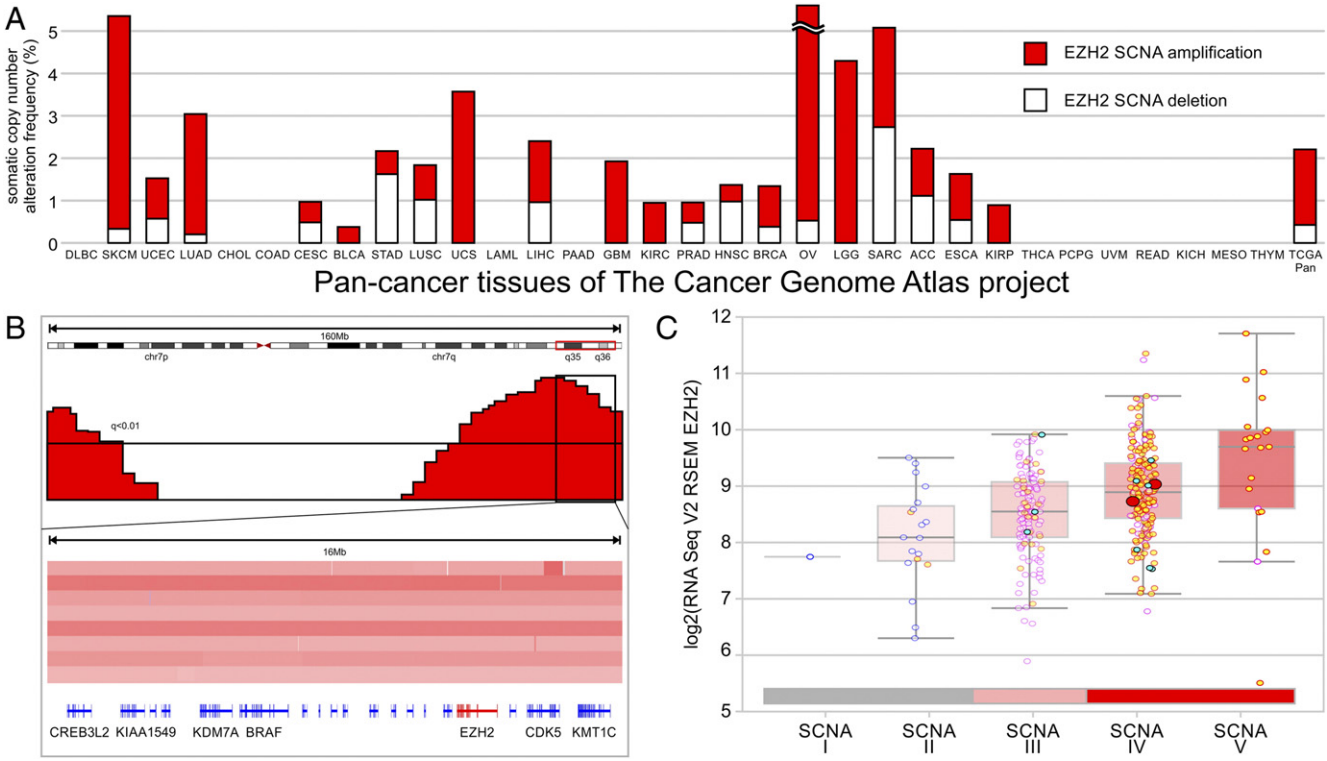

Figure 3. Somatic copy number amplification of EZH2 results in hyperactivation of methyltransfer activity of EZH2. (A) EZH2 is predominantly amplified at the somatic copy number level across a comprehensive panel of TCGA Pan-cancer patients. Frequency of EZH2 somatic copy number alterations (SCNAs) is shown in red for amplifications and white for deletions. Ovarian serous cystadenocarcinoma (OV) has a SCNAs frequency of 11.4% exceeding the chosen y-axis range indicated by tilde. TCGA Pan-cancer average across 9833 specimens is displayed at the very right of panel. (B) Amplification of chromosome band 7q includes EZH2 and other oncogenes. Segmented SNP array data of 8 representative patients is plotted relative to genomic coordinates. Bands 7q35 and 7q36 are framed on chromosome 7 and exonic regions of EZH2 are highlighted in red. Significance threshold of q = 0.01 is indicated as black line. (C) Somatic copy number alterations of EZH2 are plotted against the mRNA expression level of EZH2. Status of somatic copy number alterations are classified as deep deletions (SCNA I), shallow deletions (SCNA II), diploid (SCNA III), gain (SCNA IV), or amplification (SCNA V). Somatic copy number amplification is marked with red bar below data points. Deletions are circled in blue, amplifications in red. Patients with EZH2 mutations are highlighted as filled cyan circles and those with the Y641 mutation are shown as large red circles. Patients with BRAF mutations are marked with filled yellow circles.

As expected, patients with high EZH2 activity had differentially expressed genes that were mostly decreased (55.8% down) (Figures 5*B*, 6). Specifically, there were 2052 of 18088 transcripts differentially down regulated. Heatmaps of RNASeq data of top 50 genes with somatic EZH2 alterations visualize the transcriptional response (Figure 6). Also as expected, these patients showed mostly increased gene hypermethylation (74.7% up)—consisting of 82639 (61703 up, mapped to 12125 genes) of 485577 CG islands (Figure 5*B*).

Following treatment of melanoma cells with EZH2 inhibitor GSK126, H3K27me3 levels are expected to be decreased leading to increased transcriptional levels of EZH2 target genes. We performed protein immunoblotting to analyze the impact of EZH2 on H3K27me3 levels in melanoma cell lines. Common to all tested melanoma cell lines was presence of H3K27me3 that could be reduced by the EZH2 inhibitor GSK126 (Figure 7*A*). GSK126 is a competitive inhibitor of EZH2's methyltransferase activity that does not degrade EZH2 protein. Cell lines with active site mutations in the SET domain at Y641 had elevated H3K27me3 levels compared to wild-type cells. Upon treatment with GSK126, all tested cell lines had strongly reduced H3K27me3 levels (Figure 7*A*). In our experiments 539 transcripts showed significantly deregulated gene expression following GSK126 treatment with log2-fold changes above 2 and p-values below 0.05 (Figure 5*B*). 74.6% (402) of the deregulated genes were increased. Candidate EZH2 target genes were taken to be those both repressed in the SKCM cohort with EZH2 amplification or activation (threshold p-value below 0.05) and increase of transcription in melanoma cells following EZH2 inhibition by GSK126 (p-value below 0.05). The overlap of TCGA and drug response transcriptomes resulted in 98 responsive genes, of which 65 genes also showed DNA hypermethylation in TCGA tumors with high EZH2 activity (Figure 5*B* and *C*, Supplementary Table 3). These 65 candidate EZH2 target genes in melanoma included CDKN1A [GeneBank:1026] (Figures 7, *B–C* and 8), NDRG1 [GeneBank:10397], NFKB2 [GeneBank:4791], NFKBIA [GeneBank:4792], EPAS1 [GeneBank:2034], JUN [GeneBank:3725], JUND [GeneBank:3727], FOS [GeneBank:2353], FOSB [GeneBank:2354], IRF9 [GeneBank:10379], ITGA3 [GeneBank:3675], PLCG2 [GeneBank:5336], BCL6 [GeneBank:604], BOK [GeneBank:666], CD74 [GeneBank:972], HLA-A [GeneBank:3105], HLA-B [GeneBank:3106], HLA-DPA1 [GeneBank:3113], and HLA-F [GeneBank:3134] (Figure 8, Supplementary Table 4).

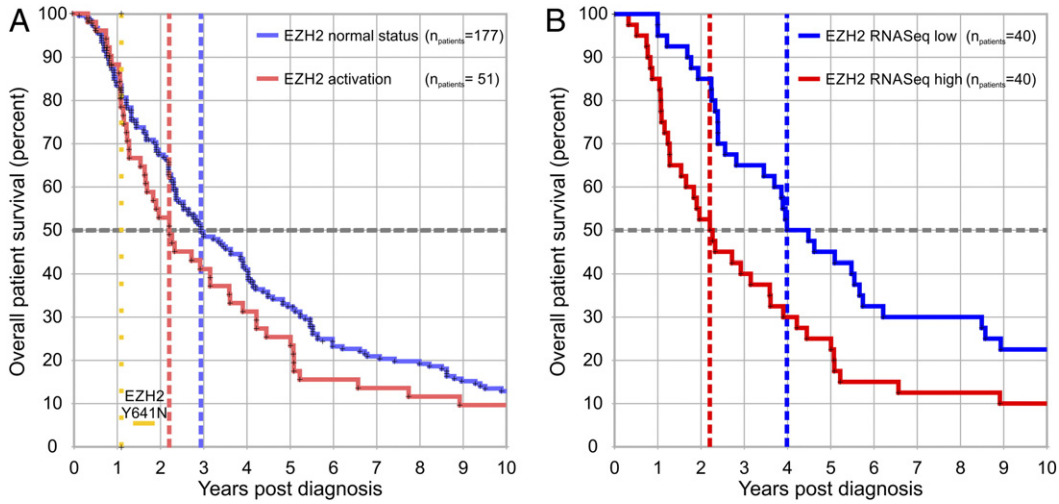

Figure 4. EZH2 is activated in melanoma and correlates with adverse patient survival. (A) Kaplan–Meier curves show shorter survival of TCGA SKCM patients with activated EZH2 (red) compared to patients with EZH2 wild-type status (blue). Median overall survival is indicated as dashed line (2.2 years post diagnosis for EZH2 activation defined as EZH2(Y641) mutation, SCNA amplification, or RNASeq upregulation; 3.0 years post diagnosis for EZH2 wild-type status; log-rank test p-value below 0.05). Survival record of patient with EZH2(Y641N) mutation is highlighted as dotted yellow line. RNASeq expression levels of EZH2 correlate with reduced overall median survival of melanoma patients. Patients with high EZH2 normalized RNASeq counts (red, top 20% of transcripts, median overall survival 2.2 years post diagnosis) show shorter survival in comparison to low EZH2 transcript levels (blue, bottom 20% of transcripts, median overall survival 4.0 years post diagnosis, log-rank test p-value below 0.05).

Multi-Omics Study Shows Enrichment of Transcriptional Silencing of Important Tumor Suppressors, Differentiation Factors, and Immune Response Genes

In order to assess any potential functional impact of high EZH2 activity in melanoma, we conducted a gene enrichment analysis of the 65 candidate EZH2 target genes derived from the above analysis. A KEGG pathway analysis showed that these candidate genes were significantly enriched in pathways deregulated in cancer including CDKN1A, NFKB2, NFKBIA, EPAS1, JUN, FOS, ITGA3, and PLCG2 (KEGG ID:05200; p-value = 6.33e-08; Supplementary Table 5). In addition, genes involved in antigen processing and presentation were significantly enriched (KEGG ID: 04612; p-value = 1.02e-05; Supplementary Table 5). We conducted a motif search of corresponding promotor and transcription start sites to identify transcriptional cooperation of PRC2. The identified target genes share a purine-rich motif of GGA(G/A)(G/A) at their promotors typical for recognition by E2F-related factors. We further assessed the DNA methylation pattern of this test group of silenced target genes. Unsupervised hierarchical clustering of their DNA methylation pattern recapitulated EZH2 status (Figure 9). The effect on DNA methylation was particularly pronounced in patients with activation mutations of EZH2 (Figure 10). Specifically we analyzed all CpG markers with significant elevation of methylation in patients with enhanced somatic EZH2 activity. The relative locations of methylation revealed accumulation of methylation sites at the transcription start site of candidate target genes. Besides chromatin histone tri-methylation by somatically activated EZH2 (Figure 7, Supplementary Table 4), such DNA hypermethylation pattern contributes to dampening the transcriptional response of affected target genes (Figures 6, 9, and 10).

Activating Somatic Mutations of EZH2 Y641 Contribute to Melanomagenesis by Tumor Suppressor Gene Silencing

Of the identified genes, we selected CDKN1A for characterization by RT-qPCR and western blot analysis (Figure 7). EZH2 may exhibit its oncogenic potential through CDKN1A by transcriptionally downregulating its gene product, the tumor suppressor and cell cycle regulator p21 (Figure 7*B*) [17,19]. While EZH2 is absent or lowly expressed in human epithelial melanocytes (HEM) or human dermal fibroblasts (HDF), it is highly expressed in melanoma cells MELJD, ME1007, KMJR138 (Figure 7*B*). We were able to identify three cellular melanoma models, IGR1, C001, and MM386, which recapitulate the Y641 point mutations. All three melanoma cell lines showed evidence of expression of EZH2 at the protein level (Figure 7*A*). The gene product of CDKN1A, p21, is absent in melanocytes but present in melanoma cells. In stark contrast, melanoma cell lines with activating EZH2 mutations show absence of p21 (Figure 7*B*). Using a small molecule EZH2 inhibitor, we monitored the transcriptional response of CDKN1A by RT-qPCR. MM386 and IGR-1 cells showed a dose dependent upregulation of CDKN1A by RT-qPCR (Figure 7*C*), following treatment with GSK126, which is a competitive inhibitor of EZH2s methyltransferase activity [20]. Taken together, the transcriptional reactivation of CDKN1A following EZH2 inhibition by GSK126 shows reversal of the transcriptional silencing observed with activating EZH2 mutations.

Interaction of EZH2 With DNA Remodelers

Lastly, we sought to investigate if EZH2 alterations co-occur with changes to other DNA remodelers. Overexpressed in many types of cancers, EZH2 is postulated to exert its oncogenic effects via aberrant methylation, causing silencing of tumor suppressor genes. It tri-methylates histones, ultimately causing gene repression by chromatin condensation thus blocking access of gene promotors to transcription initiation machinery and facilitates DNA methylation. The group of PRC genes, EZH1 [GeneBank:2145], EZH2 [GeneBank:2146], EED [GeneBank:8726], SUZ12 [GeneBank:23512], RBBP4 [GeneBank:5928], RBBP7 [GeneBank:5931], as well as PRC-associated DNMTs show high enrichment of somatic mutations affecting 10.5% and 15.5%, respectively of 343 patients

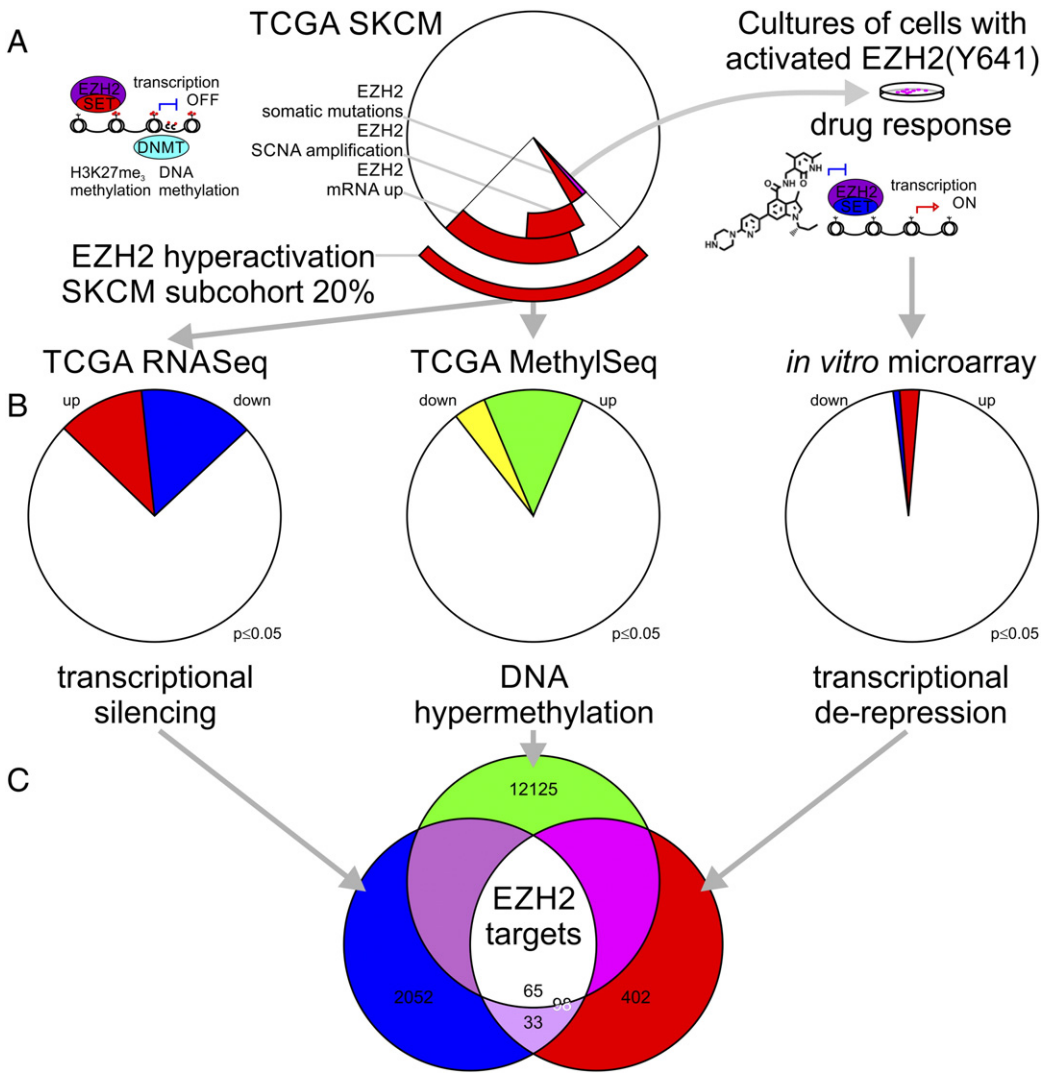

Figure 5. Multiomics analysis of EZH2 activation across different platform combined with responsiveness to drug treatment in melanoma. (A) The histone methyltransferase EZH2 transcriptionally silences target genes by tri-methylation of histone 3 K27. Cooperation with DNA methyltransferases helps manifest the transcriptional inactivation. 88 patients with EZH2 somatic mutations, somatic copy number amplification, and/or somatic mRNA upregulation were combined into a subhort comprising about 20% of all patients. Melanoma cells of TCGA patients with activating somatic mutations of EZH2(Y641) were cultured and subjected to a drug response assay using the EZH2 inhibitor GSK126. (B) Three genomics datasets, TCGA RNASeq, TCGA MethylSeq, and microarray of the drug response of cell lines, were filtered by p-value threshold below 0.05 and sorted by significantly up or down regulation. (C) EZH2 target genes show overlap of transcriptional silencing, DNA methylation, and responsiveness to drug exposure.

with whole exome sequencing data. The mutational signature of EZH2 (5.04% of SKCM patients) (Figure 1*A*) is mutually exclusive to non-synonymous somatic mutations in DNMT3A [GeneBank:1788] (occurring in 2.88% of patients) or DNMT3B [GeneBank:1789] (4.68% of patients) with an odds-ratio of 3.8, but is not strictly mutually exclusive to DNMT1 [GeneBank:1786] mutations (6.47% of patients) (Figure 10). EZH2-D142V in the DNMT binding domain and DNMT1-I531R co-occur as well as EZH2-C535W and DNMT1-R1466C. Further somatic mutations of PRC-associated DNMTs occur in methyltransfer domains, replication foci domains, and bromo-adjacent homology domains displaying an EZH2-distinct route of epigenetic changes in cancer. The mutual exclusivity (reflected in all cancers including blood-derived cancer with high EZH2 alterations LAML, acute myeloid leukemia, and DBLC, Lymphoid Neoplasm Diffuse Large B-cell Lymphoma) points towards a potential mechanism of somatic mutations in PRC2 driving cancer through EZH2 or alternatively DNMT3A/B (Figures 9, 10). Identification of genes whose expression is specifically modulated by EZH2, DNMT1 vs DNMT3A/B, and combination of EZH2 and DNMT inhibitors are the next logical steps in this line of research. Taken together, amplified methylation patterns as observed in EZH2-activated patients are part of the transcriptional response of affected target genes and regulate gene clusters critical for oncogenic signaling.

Discussion

This analysis on the large SKCM dataset from TCGA appears to have revealed several hitherto unappreciated aspects of EZH2 biology in melanoma. Although the analysis verified that activating mutations of EZH2 are relatively uncommon [15] when added to copy number

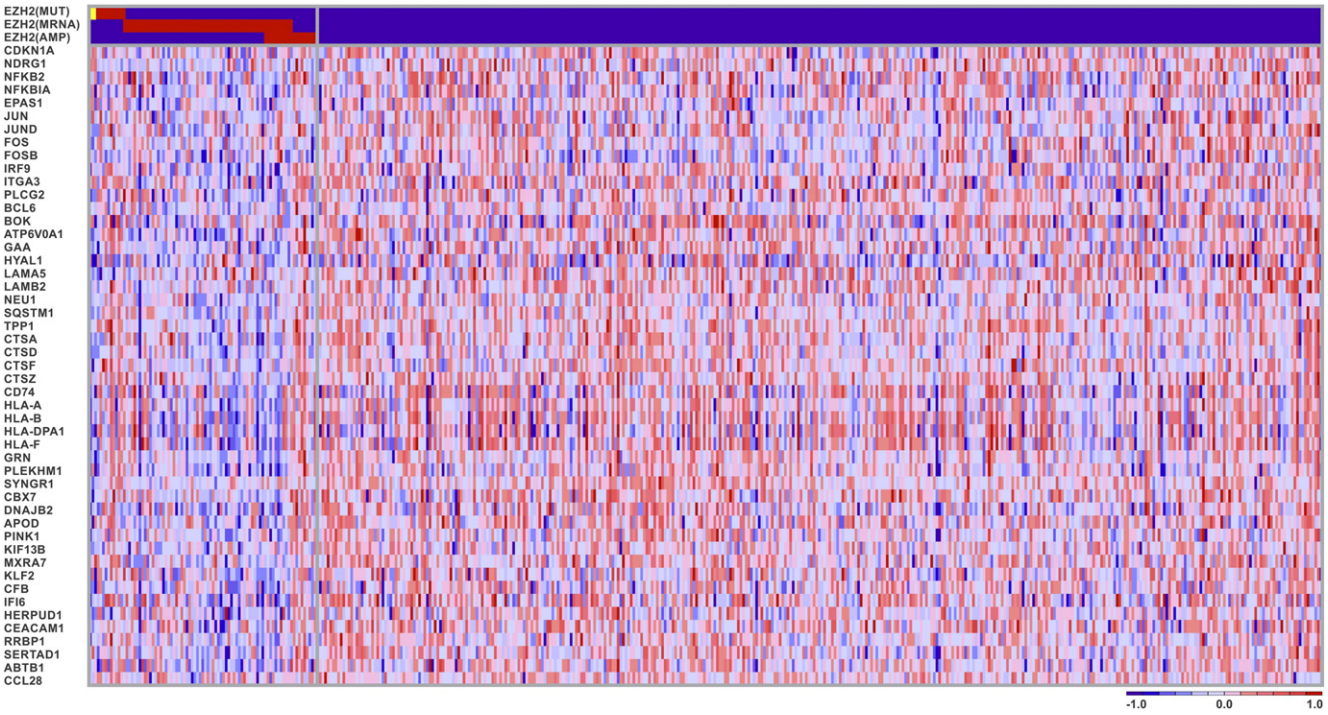

Figure 6. Activation of EZH2 and pattern of transcriptional silencing in melanoma. 88 of 470 cases in the skin cutaneous melanoma dataset show somatic mutations, somatic copy number amplification, or upregulation of transcription of EZH2. Top 50 genes are shown for patients with EZH2 alterations vs patients with EZH2 wild-type status. Transcripts show significant deregulation with p-values below 1.0e-04. RNASeq transcription levels are shown as log2 normalized heat map from low values in blue to high values in red according to color scheme legend. Somatic EZH2 status is indicated in red in the first three rows. Activating somatic mutations of Y641 in EZH2 are highlighted in yellow in the first row.

gains and amplification of gene expression the number of patients with dysregulation of EZH2 approaches 20% of patients (88 of 471 patients). Pathological hyperactivity of EZH2 can arise from somatic amplification persisting at the transcriptional level or from somatic mutations but both events eventually result in a disruption of the epigenetic homeostasis. By looking into isoform-specific RNASeq data of more than 12,000 patients we were able to map the major cancer transcript [GeneBank:NM_001203247] of EZH2, which is an essential step to unambiguously identify non-synonymous somatic mutations. Irrespectively of lengths of N-terminal domains, somatic mutations across different cancers hit a unique phosphorylation site in the center of the SET domain resulting in hyperactivation and increased tri-methylation activity. Somatic Y641 mutations correlate with hypermethylation and lead to prominent gene suppression in the TCGA dataset. Patients with somatic copy number amplifications of EZH2 showed a similar functional impact: gene suppression was most pronounced in cases where SCNA events translated to significant upregulation of EZH2 transcripts. Importantly, the SCNA event amplifying EZH2 impacts a broad genomic environment including CREB3L2 [GeneBank:64764], KIAA1549 [GeneBank:57670], KDM7A [GeneBank:80853], BRAF [GeneBank:673], CDK5 [GeneBank:1020], and KMT2C [GeneBank:58508]. This suggests that a broader genomic context of chromosome 7 is functionally important for the tumor instead of a focal event amplifying an isolated proto-oncogene in melanoma. Our analysis identified a coincidence of BRAF activation with EZH2 amplification providing a mechanistic link for previous studies, which showed an association between BRAF mutations and DNA hypermethylation [21].

Abnormal DNA methylation of CpG markers is a well-known epigenetic feature of cancer [22]. Melanoma exhibits global hypomethylation within the bulk genome and local hypermethylation at specific tumor suppressor genes [23,24]. Gene-specific DNA hypermethylation might serve as classifier for melanoma, as studies indicate that multilocus DNA methylation signature genes may differentiate melanomas from nevi [25]. The characterized and confirmed candidate target genes susceptible to reversible EZH2 histone modification upon drug treatment agreed with the transcriptional signature of TCGA SKCM patients with EZH2 hyperactivity. The majority of the 98 candidate target genes also showed high levels of DNA methylation. This is consistent with suggested concerted action of histone modifiers and DNA methyltransferases at the epigenomic level resulting in transcriptional repression [2]. Methylation data in TCGA showed that DNA methylation appeared to be the common cause of gene suppression associated with EZH2. This may not be that surprising given that DNA methyltransferases DNMT1, DNMT3A, and DNMT3B are closely associated with polycomb repressive complex 2 and are required for epigenetic modifications [1,3]. The mechanism of suppression of genes not associated with DNA methylation may represent transcriptional repression resulting from EZH2 mediated histone methylation but was not further explored in this analysis.

Recent studies in murine melanoma models and human melanoma cultures have supported a role for EZH2 in the proliferation and metastasis of melanoma and have linked these properties to expression of possible suppressor genes identified in TCGA [10–14]. Gene expression studies carried out on melanoma lines with known

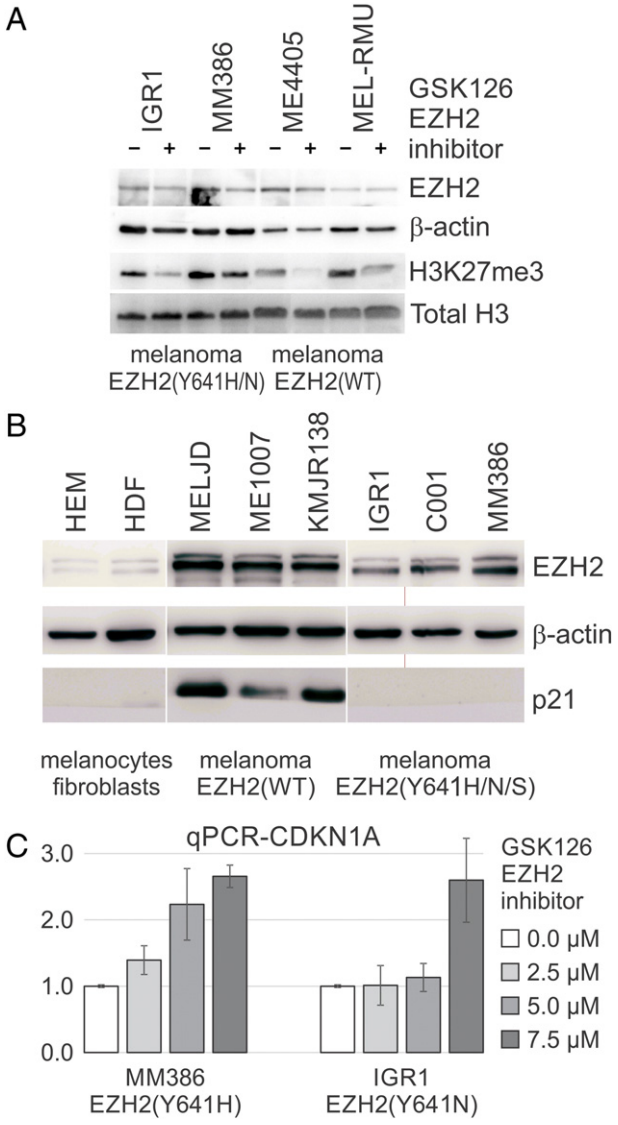

Figure 7. Histone methylation of H3K27 is reversible and responsive to EZH2 inhibition in melanoma cells. (A) Western blot analysis of EZH2 and H3K27me3 levels in melanoma cell lines with WT EZH2 status (right) and melanoma cell lines with activating Y641 mutations (left) in absence or presence of 48 hour treatment with 7.5 μM GSK126, a small molecule inhibitor of EZH2 activity. Hypermethylation of H3K27 of melanoma cells is reversible and responsive to EZH2 inhibitor treatment. (B) Activating mutations of EZH2 are associated with silenced CDKN1A expression in melanoma cell lines. Western blot analysis of EZH2 and CDKN1A expression in normal melanocytes and fibroblasts (left), melanoma cell lines with WT EZH2 status (middle), melanoma cell lines with activating Y641 mutations (right). Blots are from separate membranes developed at the same time. (C) Expression of CDKN1A following 48 h of treatment with increasing doses of EZH2 inhibitor GSK126. RT-qPCR values are relative to vehicle treated control (DMSO) normalized to 1.

activating mutations of EZH2 before and after treatment with the GSK126 inhibitor were a valuable resource to identify genes linked to EZH2 activation in the TCGA data set and allowed for detailed study of their epigenetic DNA makeup in SKCM patients. The test group of silenced candidate target genes recapitulated EZH2 status in unsupervised hierarchical clustering of their DNA methylation pattern and revealed accumulation of methylation sites at the transcription start site of candidate target genes.

The tissue-specific effector genes of the histone modifier EZH2 vary greatly. *In vitro* studies of cancer cell lines recapitulating activating Y641 mutations show a more than three-fold increase of tri-methylation of H3K27me3, a histone mark connected with gene silencing [11–14]. Activated EZH2 is associated with decreased transcription of tumor suppressor genes as well as antigen presentation in melanoma patients. Gene enrichment analysis of the genes combined from the GSK126 inhibitor studies with that of genes repressed in the TCGA dataset identified genes with well-known tumor suppressor activity, regulation of cell division, invasion and metastases. JUN, JUND, FOS, and FOSB implicated in our study are part of the AP-1 transcription factor complex that induces differentiation. EZH2 knockout studies in mice showed that in early development, EZH2 in the skin prevents premature AP-1 binding and maintains the epidermal progenitors until the precise moment differentiation is appropriate, sparked by a decline in EZH2 [26,27]. It has been suggested that aberrant EZH2 activity in cancer maintains the cells in a stem like state, therefore EZH2 inhibitors may represent a strategy to induce differentiation and repress tumor growth. Transcription of putative targets CDKN1A, BCL6, BOK, NDRG1, and NFKB is significantly reduced in SKCM patients with EZH2 activation. Although involved in different signaling axes—often with feed-back loops and dual impact in tumor promotion and suppression—these important regulators have in common that they can promote proliferative events, if suppressed [28].

We were able to confirm transcriptional reactivation of the tumor suppressor CDKN1A in EZH2 Y641 mutated melanoma cells, using an EZH2 inhibitor. CDKN1A encodes the cyclin dependent kinase inhibitor p21, which activates multiple tumor suppressor pathways including cell cycle arrest, differentiation and cellular senescence. These findings are consistent with a previous report that demonstrated EZH2 depletion in melanoma lead to reactivation of p21 and inhibited the growth of xenografts in mice [17,19]. Patients with activating EZH2 mutations, show hypermethylation and transcriptional silencing of the tumor suppressor CDKN1A resulting in absence of p21, the gene product of CDKN1A. Inhibition of EZH2 by small molecule drugs reactivates CDKN1A and illustrates how epigenetic control by EZH2 can regulate the plasticity of melanoma.

An unexpected finding was the repression of many genes associated with immune responses in patients with EZH2 dysregulation. However, this is consistent with increasing appreciation that activated EZH2 is associated with decreased transcription of genes involved in antigen presentation in melanoma patients. Recent data suggests that specific oncogenic signals can mediate cancer immune evasion and resistance to immunotherapies [29] and specifically EZH2 can dampen the anti-tumor immune response via repression of MHC-II genes [30,31]. In support of this we identified downregulation of CD74 [GeneBank:972], an important chaperone that associates with MHC-II to regulate antigen presentation for immune responses, in addition to chemokines/chemokine receptors, CCL28 [GeneBank:56477], CCL3L1 [GeneBank:6349] and CCR7 [GeneBank:1236], known to activate T cells and B cells [29]. Modulation of immune potentiation via epigenetic signals may point toward new candidate targets for melanoma treatment. A group of human leukocyte antigen (HLA) genes stood out as enriched cluster in transcriptomic and epigenomic analysis. HLA genes controlled by epigenetic pattern may contribute to observed regulation and diversity of neoantigens in melanoma [32].

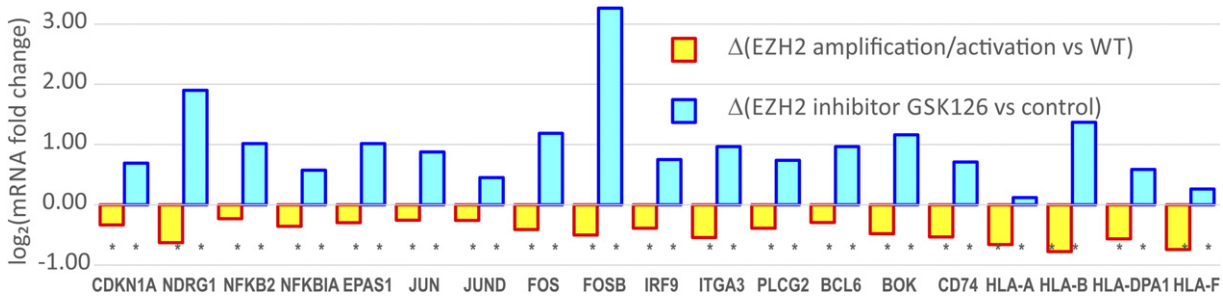

Figure 8. Inhibition of EZH2 with GSK126 reverses transcriptional silencing caused by somatic amplification or activating mutations of EZH2 in melanoma. Transcriptional suppression is observed in RNASeq data of SKCM patients with EZH2 amplification or activation (red frame, negative log2 fold changes). Presented genes showed increased levels of DNA methylation. Transcriptional suppression is reversed in microarray data of melanoma cell lines with activating Y641 mutations following treatment with EZH2 inhibitor (blue frame, positive log2 fold changes). Asterisk symbol below transcriptional data indicates significant deregulation of log2 fold changes with adjusted p-values below 0.05 threshold.

Conclusion

In summary these studies indicate that dysregulation of EZH2 is a relatively common occurrence in patients with melanoma and that this has negative implications for patient survival. Somatic events across different omics levels were associated with repression of genes involved in suppression of tumors as well as immune responses against cancers. The isoform-specific RNASeq analysis unambiguously identifies the major transcript of EZH2 and the copy number analysis shows functional amplification events of EZH2 across melanoma patients. The data also suggests that the previously described association of BRAF V600 mutations with methylation of DNA may be linked to overexpression of closely associated regions on chromosome 7. Repression of the EZH2 target genes appears to be largely due to both tri-methylation of H3K27 as well as methylation of DNA and this provides a basis for investigating combinations of both EZH2 and DNA methyltransferase inhibitors in patients with over expressed EZH2. Taken together, the PRC2 displays enhanced activity in melanoma. Hyperactivation of EZH2 by somatic copy number amplification, activating somatic mutations, or transcriptional upregulation correlates with DNA methylation and epigenetic silencing of genes involved in tumor suppression and immune responses in melanoma. Further studies are needed to determine whether inhibitors of EZH2 may also have a role immunotherapy with checkpoint inhibitors. Sophisticated chromatin immunoprecipitation experiments will closely decipher interaction of EZH2 with its target genes, chromatin modifiers and DNA methyltransferases. Taken together, EZH2 and its associated chromatin remodeling machinery represent a promising opportunity for therapeutic intervention in melanoma.

Competing Interest

The authors declare that there is no competing interest as part of the submission process of this manuscript.

Authors' Contributions

J.C.T., S.W. contributed equally to the work. J.C.T., S.W., S.J.G., P.H., F.V.F. designed the study, wrote the text, conducted experiments, performed the data analysis, and reviewed the final manuscript.

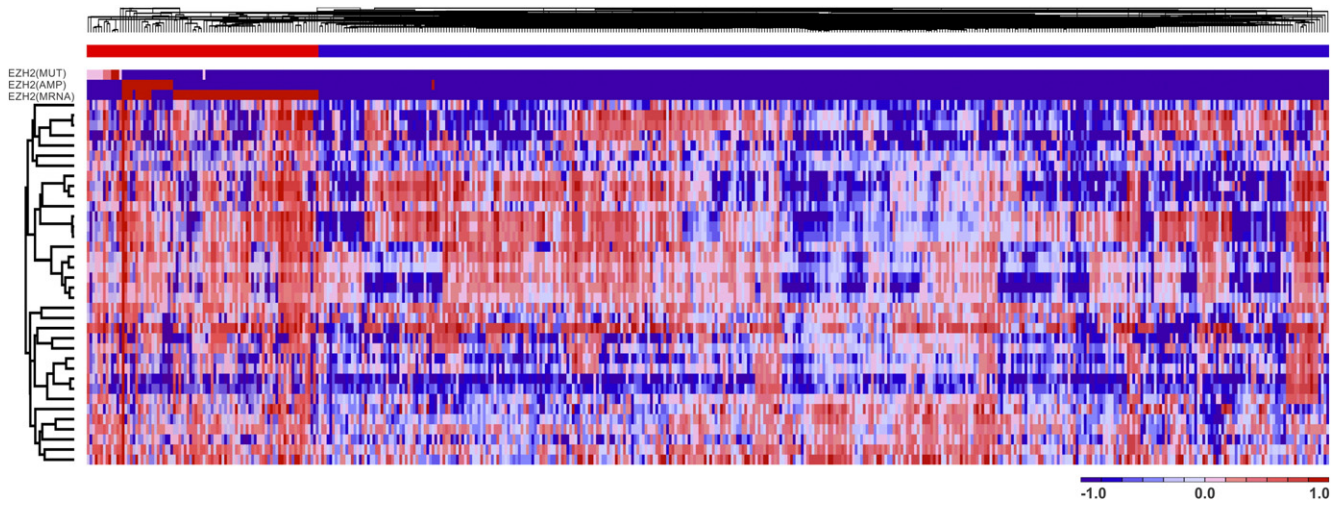

Figure 9. CG methylation markers recapitulate EZH2 activation patterns. Unsupervised hierarchical clustering of methylation markers of 461 patients aligns with EZH2 status. Presence of EZH2 activation is indicated as red bars on top of plot. EZH2 mutations, amplifications or mRNA upregulation are labelled in the first three rows.

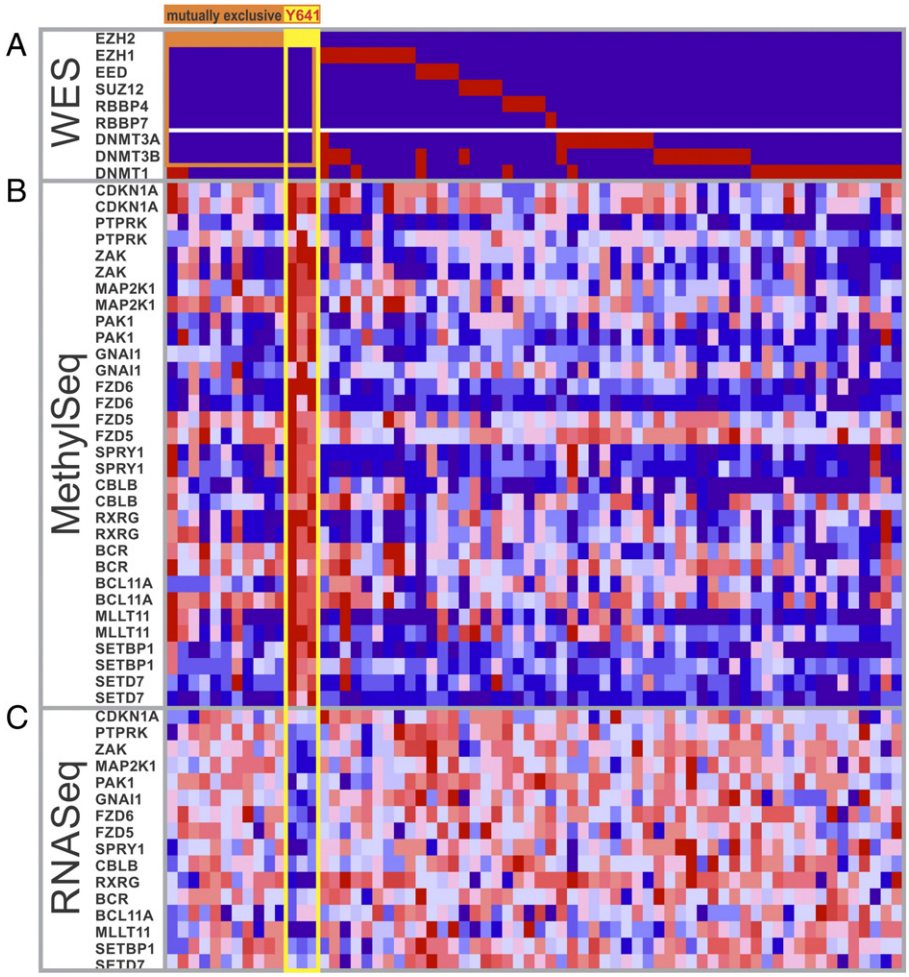

Figure 10. Somatic mutation of Y641 in EZH2 is associated with increased methylation of CpG markers and decreased transcription of tumor suppressors and epigenetic remodelers in melanoma patients. (A) Somatic mutations of EZH2 occur in a mutually exclusive setting in members of the polycomb repressive complex 2, or in *de novo* DNA methyltransferases. The activating somatic mutations of Y641 in EZH2 are marked in yellow in the first row. Transcriptomic and epigenomic data from patients with Y641F or Y641N (2x) are boxed in yellow. Other mutations of EZH2 are marked in orange in the first row. Mutually exclusive setting of EZH2 with EZH1, EED, SUZ12, RBBP4, RBBP7, or *de novo* DNA methyltransferases DNMT3A or DNMT3B is boxed in orange. (B) Representative CpG markers of candidate target genes show enhanced methylation in patients with mutated Y641 status. (C) RNASeq transcription levels of same candidate target genes are reduced.

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Appendix A. Supplementary data

The supplementary information contains tables on somatic mutations and whole-exome sequencing patient cohort (Supplementary Table 1), somatic copy number aberrations (Supplementary Table 2), transcriptomic data of identified target genes (Supplementary Table 3), methylome data of identified target genes (Supplementary Table 4), and pathway enrichment scores (Supplementary Table 5). Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neo.2016.01.003.

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