PRC2 is recurrently inactivated through EED or SUZ12 loss in malignant peripheral nerve sheath tumors

William Lee1,2,17, Sewit Teckie2,3,17, Thomas Wiesner3,17, Leili Ran3,17, Carlos N Prieto Granada4, Mingyan Lin5, Sinan Zhu3, Zhen Cao3, Yupu Liang3, Andrea Sbone6–8, William D Tap9,10, Jonathan A Fletcher11, Kety H Huberman12, Li-Xuan Qin13, Agnes Viale12, Samuel Singer14, Deyou Zheng5,15,16, Michael F Berger3,4, Yu Chen3,9,10, Cristina R Antonescu4 & Ping Chi3,9,10

Malignant peripheral nerve sheath tumors (MPNSTs) represent a group of highly aggressive soft-tissue sarcomas that may occur sporadically, in association with neurofibromatosis type I (NF1 associated) or after radiotherapy and chemotherapy. Conventional MPNSTs present in sporadic, 70% of NF1-associated and 90% of radiotherapy-associated MPNSTs. MPNSTs with PRC2 loss showed complete loss of methylation at lysine 27 of histone H3 (H3K27me3) and aberrant transcriptional activation of multiple PRC2-repressed homeobox master regulators and their regulated developmental pathways. Introduction of the lost PRC2 component in a PRC2-deficient MPNST cell line restored H3K27me3 levels and decreased cell growth. Additionally, we identified frequent somatic alterations of the Polycomb repressive complex 2 (PRC2) components (EED or SUZ12) in 92% of sporadic, 70% of NF1-associated and 90% of radiotherapy-associated MPNSTs. MPNSTs with PRC2 loss showed complete loss of trimethylation at lysine 27 of histone H3 (H3K27me3) and aberrant transcriptional activation of multiple PRC2-repressed homeobox master regulators and their regulated developmental pathways. Introduction of the lost PRC2 component in a PRC2-deficient MPNST cell line restored H3K27me3 levels and decreased cell growth. Additionally, we identified frequent somatic alterations of the Polycomb repressive complex 2 (PRC2) components (EED or SUZ12) in 92% of sporadic, 70% of NF1-associated and 90% of radiotherapy-associated MPNSTs. MPNSTs with PRC2 loss showed complete loss of trimethylation at lysine 27 of histone H3 (H3K27me3) and aberrant transcriptional activation of multiple PRC2-repressed homeobox master regulators and their regulated developmental pathways.

MPNSTs arise from peripheral nerves and associated cellular components and represent a highly aggressive subtype of soft-tissue sarcoma1. MPNSTs metastasize early and are often resistant to radiotherapy and chemotherapy. Conventional MPNSTs present in three distinct clinical settings: sporadically, in association with neurofibromatosis type I (NF1 associated) or in association with previous radiotherapy (radiotherapy associated), accounting for approximately 45%, 45% and 10% of cases, respectively2,4,5. Histologically, MPNSTs are characterized by intersecting fascicles of monotonous spindle cells with hyperchromatic nuclei and high mitotic counts with focal areas of necrosis, but accurate diagnosis remains challenging because of the lack of specific immunohistochemical and molecular biomarkers5,6. Among individuals with NF1, loss of the non-mutant allele of NF1 is thought to be the key driver in benign NF1-associated neurofibroma5. Little is known of the genetic alterations that mediate progression from neurofibroma to MPNST in individuals with NF1 or of the molecular pathogenesis of sporadic and radiotherapy-associated MPNST.

To investigate the molecular basis of MPNST, we performed whole-exome sequencing, DNA copy number and loss-of-heterozygosity (LOH) profiling, and whole-transcriptome sequencing (RNA-seq) on a discovery cohort consisting of paired normal-tumor tissues for 15 MPNSTs from 12 individuals (6 NF1 associated, 4 sporadic, 4 radiotherapy associated and 1 epithelioid) (Supplementary Tables 1 and 2). Epithelioid MPNST is a rare histological variant of MPNST that is composed of exclusively epithelioid malignant cells with diffuse immunoreactivity for the S100 protein and is not associated with NF1 (ref. 6).

We identified four frameshift mutations and one splice-site mutation in EED (Fig. 1a, Supplementary Fig. 1). RNA-seq validated aberrant EED splicing in the sample with the mutated splice site (Supplementary Fig. 2a). All five samples showed LOH at the EED locus, three samples (11T, 12T and 14T) by copy-neutral LOH and two samples (15T and 16T) by copy-neutral LOH (Supplementary Fig. 2b). These data suggest that samples with EED mutation have complete loss of EED function.

---

1Computational Biology Program, Memorial Sloan Kettering Cancer Center, New York, New York, USA. 2Department of Radiation Oncology, Memorial Sloan Kettering Cancer Center, New York, New York, USA. 3Department of Pathology, Memorial Sloan Kettering Cancer Center, New York, New York, USA. 4Department of Genetics, Albert Einstein College of Medicine, Bronx, New York, USA. 5Department of Pathology and Laboratory Medicine, Weill Cornell Medical College, New York, New York, USA. 6Department of Pathology and Laboratory Medicine, Weill Cornell Medical College, New York, New York, USA. 7Institute for Precision Medicine, Weill Cornell Medical College, New York, New York, USA. 8Genomics Core Laboratory, Memorial Sloan Kettering Cancer Center, New York, New York, USA. 9Department of Surgery, Memorial Sloan Kettering Cancer Center, New York, New York, USA. 10Department of Pathology, Brigham and Women’s Hospital, Boston, Massachusetts, USA. 11Department of Epidemiology and Biostatistics, Memorial Sloan Kettering Cancer Center, New York, New York, USA. 12Department of Neuroscience, Albert Einstein College of Medicine, Bronx, New York, USA. 13Department of Oncology, Albert Einstein College of Medicine, Bronx, New York, USA. 14Department of Pathology, Memorial Sloan Kettering Cancer Center, New York, New York, USA. 15Department of Neurology, Albert Einstein College of Medicine, Bronx, New York, USA. 16Department of Neuroscience, Albert Einstein College of Medicine, Bronx, New York, USA. 17These authors contributed equally to this work.

Received 21 April; accepted 26 August; published online 21 September 2014; doi:10.1038/ng.3095
We further identified two homozygous and five heterozygous deletions of SUZ12 (Fig. 1a,c and Supplementary Figs. 1 and 3a). We examined RNA-seq profiles for the SUZ12 transcript among the five samples with heterozygous loss of the gene. Two samples, 9T and 10T, with homozygous deletion, expressed full-length SUZ12 transcript among the five samples, suggesting that the remaining copy is intact (Supplementary Fig. 1b and data not shown). Remarkably, the other three samples displayed structural alterations of the SUZ12 transcript, starting at exon 6, exon 10 and exon 4 in samples 2T, 7T and 13T, respectively (Supplementary Fig. 3b–d). These structural changes are likely due to local genomic rearrangements of the remaining copy, which were not identified by standard whole-exome sequencing. Indeed, for 7T and 18T, derived from two tumors from the same individual, there was a DNA break in exon 10 identified through manual examination of whole-exome sequencing data (Supplementary Fig. 3c).

We designated these cases as having structural variation and heterozygous loss at the SUZ12 locus, and, intriguingly, all constituted radiotherapy-associated MPNSTs (Fig. 1a).

EED and SUZ12 are the core components of PRC2 and together with EZH1 and EZH2, establish and maintain the di- and trimethylated state of H3K27, which is required for the transcriptional repression of target genes. In line with this, we observed homozygous deletion and radiotherapy-associated MPNSTs (Fig. 1a) and EED and SUZ12 genetic alteration suggested that they significantly co-occur: \( P = 0.001 \).
heterozygous loss of the CDKN2A locus in 73% (11/15) and 13% (2/15) of MPNSTs, respectively. We also observed nonsynonymous mutations and heterozygous loss of TP53 in 13% (2/15) and 20% (3/15) of MPNSTs. We did not identify other recurrent somatic alterations with relatively high frequency (Supplementary Table 3).

Next, we used a targeted sequencing approach (IMPACT 13; Supplementary Table 2) to characterize a validation cohort of (2/15) of MPNSTs, respectively. We also observed nonsynonymous alterations in PRC2 components. (from whole-exome sequencing data, except for samples 21 and 22, which are colored on the basis of manual examination of the RNA-seq data for mutations (Figure 2). Genetic alterations in PRC2 WT (PRC2 loss PRC2 WT loss (●●●) loss segregate by principal component 1 (PC1). Each sample is colored on the basis of its corresponding PRC2 mutational status derived from whole-exome sequencing data, except for samples 21 and 22, which are colored on the basis of manual examination of the RNA-seq data for mutations (Supplementary Table 3). Genetic alterations in PRC2 WT (PRC2 loss PRC2 WT loss (●●●) loss segregate by principal component 1 (PC1). Each sample is colored on the basis of its corresponding PRC2 mutational status derived from whole-exome sequencing data, except for samples 21 and 22, which are colored on the basis of manual examination of the RNA-seq data for mutations (Figure 2).

To understand the effect of PRC2 loss, we performed gene expression analysis in 16 MPNSTs (Supplementary Table 1). In principal-component analysis (PCA), all samples with EED mutation or SUZ12 homozygous deletion clustered together and were separated from the other samples by the first principal component (PC1) (Figs. 1a and 2a). Of the five samples with heterozygous loss of SUZ12, three with structural variation of the remaining SUZ12 copy (2T, 7T and 13T) and one with an SUZ12 mutation encoding p.His213fs (12T) clustered with the group with PRC2 loss and one with an intact SUZ12 transcript (9T) clustered with the group with wild-type PRC2. These observations highlight the complexity in identifying structural variations that accurately determine PRC2 status in cases of heterozygous EED loss.

To explore the transcriptional consequences of PRC2 loss, we generated a gene set composed of genes that were differentially expressed in MPNSTs with loss of PRC2 and those with wild-type PRC2 (Supplementary Table 5). Hierarchical clustering of these genes robustly separated the MPNSTs with loss of PRC2 and those with wild-type PRC2. The vast majority of differentially expressed genes (455/479; 95%) were upregulated in MPNSTs with PRC2 loss, consistent with the role of PRC2 in transcriptional repression (Fig. 2b). In Gene Ontology (GO) analysis, known PRC2-suppressed targets, including homeobox transcription factors and genes associated with development and morphogenesis, were highly enriched.
among the genes upregulated in MPNSTs with PRC2 loss (Fig. 2c and Supplementary Table 6). The expression of several prototypical PRC2-suppressed genes confirmed this difference (Fig. 2d). In Gene Set Enrichment Analysis (GSEA), the most significantly enriched gene sets upregulated in MPNSTs with PRC2 loss included the ‘PRC2 module’ defined by genes bound by PRC2 components in mouse embryonic stem (ES) cells15 (Fig. 2e and Supplementary Table 7) and H3K27me3 target genes in neural precursor cells16 (Fig. 2f) and brain tissue17 (Fig. 2g). These data indicate that loss of PRC2 function results in distinct transcriptome changes, including activation of developmentally regulated master regulators and imprinted genes (for example, IGF2).

We evaluated H3K27me3 levels by immunohistochemistry in formalin-fixed, paraffin-embedded samples of MPNST and neurofibroma. Whereas MPNSTs with wild-type PRC2 showed robust staining for H3K27me3, MPNSTs with PRC2 loss showed complete loss of H3K27me3 in tumor cells and preservation of H3K27me3 staining in stromal cells (Fig. 3a). Positive and negative immunostaining for H3K27me3 were highly concordant with a genetic status of wild-type PRC2 and homozygous PRC2 loss, respectively (Fig. 3b). However, heterozygous loss of PRC2 components was not predictive of H3K27me3 staining in immunohistochemistry (Fig. 3b). Among the samples with heterozygous loss for which associated RNA-seq data were available, clustering by transcriptional pattern matched H3K27me3 immunohistochemistry results. This finding suggests that DNA sequencing (exome or IMPACT) alone cannot predict PRC2 functional status in all MPNSTs and that H3K27me3 immunohistochemistry might be more accurate.

All neurofibromas (7/7), which were wild type for PRC2 except for one sample with heterozygous loss of SUZ12, retained H3K27me3 immunostaining (Figs. 1b and 3c,d). In specimens that contained the interface of MPNSTs arising from preexisting benign plexiform neurofibromas, we observed a transition from robust H3K27me3 staining in the plexiform neurofibroma to a clear loss of this staining in the MPNST. These data suggest that PRC2 loss is involved in the malignant progression of benign plexiform neurofibroma into MPNST. Indeed, 56% (19/34) of the NF1-associated MPNSTs had lost H3K27me3 (Fig. 3d). Curiously, a significantly greater percentage (>90%) of sporadic and radiotherapy-associated MPNSTs had lost H3K27me3 staining (Fig. 3d), suggesting that the progression of disease and sequence of genetic inactivation of NF1, CDKN2A and PRC2 components might be different in MPNSTs that arise in distinct clinical settings. Unlike NF1-associated MPNSTs that universally arise from preexisting neurofibromas, sporadic and radiotherapy-associated MPNSTs rarely have identifiable preexisting benign nerve sheath tumors6. In one sporadic MPNST sample (16T), the presence of nonsynonymous mutations in both NF1 (encoding a p.Asp1237splice alteration) and EED (encoding p.Glu249fs) allowed us to use the prevalence of these mutations to infer the sequence of genetic events18. The largest subpopulation of cells (84%) contained the NF1 mutation, whereas a smaller subpopulation (57%) contained the EED mutation, suggesting that the NF1 mutation occurred first during progression of this sporadic MPNST (Supplementary Fig. 4). The sequence of NF1, PRC2 component and CDKN2A inactivation described here is largely correlative. The precise sequence of events will require experimental validation with sequential inactivation of each pathway in cell line and mouse models.

To determine whether PRC2 loss is required for MPNST oncogenesis, we screened available human MPNST cell lines using immunoblotting for H3K27me3. We identified one MPNST cell line (ST88-14; derived from an NF1-associated MPNST) that had lost H3K27me3 marks. RNA-seq analysis showed that ST88-14 cells had lost expression of SUZ12, and immunoblotting confirmed loss of the SUZ12 protein (Fig. 4a,b and Supplementary Fig. 5). We next introduced Flag-HA–tagged wild-type SUZ12 (FH-SUZ12) or EED (FH-EED) into the ST88-14 cell line and into an MPNST cell line with wild-type PRC2 (MPNST724) that maintained H3K27me3 levels (Fig. 4a,b). FH-SUZ12 but not FH-EED restored H3K27me3 levels in ST88-14 cells and substantially decreased cell growth (Fig. 4a,c). In MPNST724 cells, there was a mild increase in H3K27me3 levels with the introduction of either FH-SUZ12 or FH-EED (Fig. 4a), but neither had any effect on cell growth (Fig. 4c). These data suggest that PRC2 loss contributes to oncogenesis at least in part by promoting cell proliferation and growth.
Figure 4 PRC2 loss promotes cell proliferation and growth in MPNST with PRC2 loss. (a) Immunoblots demonstrating SUZ12 loss and corresponding loss of H3K27me3 in ST88-14 cells, a cell line derived from NF1-associated human MPNST, in comparison to a cell line derived from sporadic human MPNST, MPNST724, with intact PRC2 and retained H3K27me3 levels. Introduction of exogenous Flag-HA–tagged SUZ12 (FH-SUZ12) but not Flag-HA–tagged EED (FH-EED) in ST88-14 cells restores H3K27me3 protein levels. *, exogenous FH-SUZ12 or FH-EED; **, endogenous SUZ12 or EED. (b) Immunofluorescence of H3K27me3 demonstrating the restoration of H3K27me3 levels at the cellular level by introducing FH-SUZ12 in the ST88-14 MPNST cell line with SUZ12 loss. Scale bars, 100 µm. DAPI, 4',6-diamidino-2-phenylindole. (c) Representative growth curves for the MPNST724 and ST88-14 cell lines demonstrating that the introduction of FH-SUZ12 but not FH-EED in SUZ12-deficient ST88-14 cells leads to growth retardation, whereas it had no effect in MPNST724 cells. Similar results have been obtained in at least three independent experiments. (d) ST88-14 and MPNST724 cells were infected with vector control virus or virus expressing FH-SUZ12. Plots of expression determined by quantitative RT-PCR (expressed as percent input) of SUZ12, EZH2, H3K27me3, H3K4me3, H3K27ac and IgG control for the FOXN4, IGF2, PAX2 and TLX1 genes are shown. Error bars, s.e.m. n = 3 technical replicates.

We next examined the transcriptional and chromatin changes in ST88-14 and MPNST724 cells after the introduction of FH-SUZ12, focusing on several known PRC2-regulated genes (FOXN4, IGF2, PAX2 and TLX1) that are significantly upregulated in MPNST samples with PRC2 loss in comparison to MPNST samples with wild-type PRC2 (Fig. 2c). At baseline, ST88-14 cells exhibited increased expression of FOXN4, IGF2, PAX2 and TLX1 accompanied by loss of PRC2 components (SUZ12 and EZH2) and the PRC2-repressive mark (H3K27me3) and reciprocal gain of the activation marks trimethylation at lysine 4 of histone H3 (H3K4me3) and acetylation of lysine 27 of histone H3 (H3K27ac) at their promoters (Fig. 4d and Supplementary Fig. 6). After the introduction of FH-SUZ12 in ST88-14 cells, FH-SUZ12 localized to the promoters of these genes. This localization was accompanied by increased levels of EZH2 and H3K27me3 and decreased levels of H3K4me3 and H3K27ac at the promoter regions, as well as by decreased transcript levels of these PRC2 target genes. These data suggest that PRC2 loss has a direct impact on transcriptional regulation, and introduction of the missing PRC2 component has the ability to at least partially restore PRC2 function.

MPNSTs often exhibit divergent differentiation, including rhabdomyoblastic, glandular, squamous and neuroendocrine elements6. Our study identified a high frequency of loss-of-function genetic alterations in NF1, CDKN2A and PRC2 components (EED or SUZ12), demonstrating that MPNSTs share common molecular pathogenic pathways despite clinical and histological diversity. PRC2 loss activates multiple developmentally suppressed pathways, which might explain the frequent observation of divergent differentiation in MPNST. The high frequency of PRC2 loss suggests that PRC2 mutational status and, more specifically, H3K27me3 immunohistochemistry could be used as biomarkers for the more acute diagnosis of MPNST.

PRC2 was initially thought to be oncogenic: PRC2 components have higher expression in dividing cells and are important in maintaining stemness. EZH2 is overexpressed in a variety of cancers19,20, and activating EZH2 mutations are found in a subset of lymphomas21. Paradoxically, recent work suggests that PRC2 can be tumor suppressive in distinct contexts, with loss-of-function genetic alterations found in up to 25% of myeloid disorders and T cell acute lymphoblastic leukemia (ALL)22–24 and 42% of early T cell ALL25. Notably, the majority of the loss-of-function alterations are found in EZH2 (refs. 22–25). Cellular studies and mouse models have shown that Ezh1 can maintain suppression of Polycomb-regulated genes in the setting of Ezh2 loss, and combined Ezh1 and Ezh2 loss or individual loss of Eed or Suz12 derepresses Polycomb-regulated genes and causes Cdkn2a-mediated growth arrest26–29. These findings suggest that MPNST is unique in
that complete loss of PRC2 function is important for tumorigenesis, and loss of CDKN2A might be a critical cooperative event in addition to NF1 loss.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Accession codes.** Exome sequencing, RNA-seq and DNA copy number data have been deposited in the database of Genotypes and Phenotypes (dbGaP) under accession phs000792.v1.p1.

**ACKNOWLEDGMENTS**

Next-generation sequencing and SNP6.0 analysis were performed at the Memorial Sloan Kettering Cancer Center (MSKCC) Genomics Core Facility, Center for Molecular Oncology (CMO). The authors thank members of the cBioPortal team (J. Gao, B. Gross, N. Schultz and C. Sander) for assistance with data analysis and visualization. The authors also thank T. Chan (MSKCC) and HOPP informatics for assistance with data analysis. This work was supported in part by a grant from the US National Institutes of Health (NIH) to W.L. (NCI U24-CA143840), the Harry J. Lloyd–Translational Research grant to T.W., the Charles H. Revson Senior Fellowship to T.W., Jubilaeumsfonds of the Oesterreichische Nationalbank to T.W., grants from the US NIH to PC. (P50CA140146, Career Development Award and Developmental Research Project.; DP2CA174499; K08CA151660), Y.C. (K08CA140946), L.-X.Q. (P50CA140146), C.R.A. (P50CA140146) and S.S. (P50CA140146), an award from the Sidney Kimmel Foundation to PC. (Kimmel Scholar Award) and funding from the Cycle for Survival Fund to PC.

**AUTHOR CONTRIBUTIONS**

Project planning and experimental design: PC., C.R.A., Y.C., T.W., W.L., S.T. and L.R. Sample collection, clinical database and cell lines: S.T., S.S., C.R.A., C.N.P.G., J.A.F. and W.D.T. Pathology review: C.R.A. and C.N.P.G. Preparation of DNA, RNA and next-generation sequencing libraries: T.W., K.H.H., S.T. and A.V. Sequence data analysis: W.L., Y.C., S.T., PC., M.E.B., M.L., D.Z., Y.L. and A.S. Immunohistochemistry: T.W. Protein blots, immunofluorescence, growth curves and all cellular assays: L.R. and S.Z. Generation of the expression vectors: Z.C. and L.R. Biostatistics: L.-X.Q. Manuscript writing: PC., Y.C., W.L., T.W. and L.R. The final manuscript was reviewed by all authors.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.
ONLINE METHODS

Human tumor tissue collection. Biospecimens were collected during surgical resection from patients with pathologic diagnosis of MPNSTs or neurofibromas. Material was collected under institutional review board (IRB)-approved protocols (IRB 06-107) at MSKCC. All patients provided informed consent. Pathologic diagnosis was confirmed by at least two pathologists using diagnostic formalin-fixed and paraffin-embedded sections to select cases with an estimated tumor content of >70%. The majority of the tumors were collected from different patients. In some cases, more than one tumor was selected from the same patient, and these tumors were resected from distinct anatomical locations in different surgeries. Sample annotation is shown in Supplementary Table 1.

For the discovery cohort, the goal was to obtain normal DNA and tumor DNA for whole-exome sequencing and SNP6.0 array analysis and to obtain tumor RNA for RNA-seq analysis. A total of 15 fresh-frozen paired MPNST tumor–normal samples were identified. Tumor and adjacent normal tissue specimens were embedded in optimal cutting temperature (OCT) medium, and a histological section was obtained for review. Cryomolds of both tumor and normal tissues were macrodissected to minimize contamination before RNA and DNA preparation. Sample processing was designed to secure samples and minimize the availability of identifying information. Specimens with insufficient tissue amount or severely degraded nucleic acids were excluded.

The IMPACT assay is a hybridization capture, next-generation sequencing platform amenable to DNA from both fresh-frozen and formalin-fixed, paraffin-embedded samples for targeted sequencing. The panel includes NF1, SUZ12, EED, CDKN2A and TP53 (ref. 13). We validated the same mutational findings from the discovery cohort by performing IMPACT assays on the same DNA isolated from tumor tissue. In addition, we performed IMPACT assays on DNA from formalin-fixed, paraffin-embedded samples derived from a second cohort of 37 MPNSTs and 7 neurofibromas from individuals with NF1 who were diagnosed with concurrent MPNST (Supplementary Table 1).

Sample preparation and quality control. RNA was extracted from tumor and normal tissues using a modification of the protocol for the DNA/RNA AllPrep kit (Qiagen). DNA from fresh-frozen tissues was extracted from tumor and normal tissue specimens using the DNeasy Blood and Tissue kit (Qiagen). DNA from formalin-fixed, paraffin-embedded samples was isolated using the QIAamp DNA FFPE Tissue kit (Qiagen). DNA from each specimen was initially quantified using the NanoDrop UV spectrophotometer and was further quantified with the Bioanalyzer assay (Agilent Technologies).

RNA sequencing and analysis. The isolated RNA was processed using the TrueSeq RNA Sample Prep kit (15026495, Illumina) according to the manufacturer’s protocol. Briefly, RNA was polyA selected and reverse transcribed, and the cDNA obtained underwent end repair, A-tailing, ligation of the indexes and adapters, and PCR enrichment. Libraries were sequenced on the Illumina HiSeq 2500 platform with 51-bp paired-end reads to obtain a minimum yield of 40 million reads per sample. Sequence data were processed and mapped to the human reference genome (hg19) using STAR (v2.3)30. Gene expression levels were quantified with htsseq-count11 and normalized using DESeq212. Variance in expression levels was calculated for all genes across samples, and the 75th percentile was set as a cutoff. PCA was performed on the set of genes with variance greater than this cutoff. We used ANOVA to define differentially expressed genes between samples with PRC2 loss and wild-type PRC2. Genes that showed a >8-fold difference in expression and a corrected FDR of <0.05 (479 genes) were used for clustering and gene ontology analysis. Hierarchical clustering was performed using Pearson correlation in GENE-E software, and heat maps were displayed using GENE-E. Gene ontology analysis was performed using DAVID to discover enriched pathways and gene ontologies133. GSEA to discover gene sets enriched among upregulated genes in samples with PRC2 loss was performed using the JAVA GSEA 2.0 program134. The gene sets used were Broad Molecular Signatures Database gene sets 2 (curated gene sets), c5 (gene ontology gene sets), c6 (oncogenic signatures) and c7 (immunologic signatures) as well as the additional sets ‘PRC2_Module’ and ‘ES_Core’ (ref. 15), totaling 6,886 gene sets.

DNA sequencing and analysis. Whole-exome sequencing of DNA from fresh-frozen tissue used 1,000 ng (or 500 ng in select cases) of DNA from either tumor or normal samples. DNA was subjected to shearing, end repair, phosphorylation and ligation to barcoded sequencing adapters according to the manufacturer’s guidelines. Ligated DNA was size selected for fragments between 200 and 400 bp in length. These fragments were multiplexed and underwent exonic hybrid capture with SureSelect V4+UTRs exome bait (Agilent Technologies). Captured DNA was sequenced on the Illumina HiSeq 2500 platform with 75-bp paired-end reads. Raw sequences were aligned to the human genome reference sequence (hg19) using Burrows-Wheeler Aligner (BWA)135. Total read count and coverage depth are shown in Supplementary Table 2. Aligned data were further processed by removing duplicates using Picard followed by indel realignment and base quality score recalibration with the Gnome Analysis Toolkit (GATK)36. Single-nucleotide somatic mutations and somatic indels were called by taking the union of the calls made by MuTect17, Strelka38 and VarScan and applying a set of heuristic filters as described in the VarScan 2 report39. Mutations were further filtered to remove variants that were present in dbSNP137 but not in the Catalogue of Somatic Mutations in Cancer (COSMIC, v64)40. The data were further analyzed and visualized using the BioPortal41.

For IMPACT assays, library construction and sequencing was performed by the MSKCC Genomics Core Facility. Alignment and SNV and indel calling were performed as described above. Copy number analysis was performed as previously described13.

SNP6.0 arrays and analysis. A total of 500 ng of DNA from each tumor or normal tissue sample was hybridized to the Affymetrix SNP6.0 array using protocols from the Genomics Core Laboratory at MSKCC. Allele-specific copy number for each tumor-normal pair of arrays was calculated using Copynumber42 in the Aroma package.

Histology and immunohistochemistry. Tissue processing, embedding, sectioning and staining with hematoxylin and eosin were performed by the MSKCC Department of Pathology. Photographs were taken using an Olympus DP21 camera. Immunohistochemistry was performed on archival formalin-fixed, paraffin-embedded tumors using a standard multimer/DAB detection protocol on a Discovery Ultra system (Ventana Medical Systems) with appropriate negative and positive controls. For H3K27me3 staining, we diluted an antibody to H3K27me3 (07-449, Millipore) 1:250 in SignalStain Antibody Diluent (8112, Cell Signaling Technology).

Cell lines and in vitro analysis. The MPNST724 and ST88-14 human MPNST cell lines were obtained from the laboratory of J.A.F. (Dana-Farber Cancer Institute) and by testing have been determined to be mycoplasma free. MPNST724 cells were grown in RPMI supplemented with 10% FBS and ST88-14 cells were grown in RPMI supplemented with 15% FBS. cDNAs for wild-type human EED and SUZ12 in pDONR vectors were obtained from Harvard PlasmidID and cloned into an murine stem cell virus (MSCV)-based retroviral vector with a sequence encoding a Flag-HA (FH) tag (Addgene plasmid 41033)44 using Gateway technology. To generate cell lines stably expressing these constructs, MPNST724 and ST88-14 cells were infected with empty vector, MSCV-FH-EED or MSCV-FH-SUZ12 and selected using puromycin (2 μg/ml for 72 h). Growth curve analysis of the infected cells was performed using Alamar blue cell viability reagent (DAL1100, Life Technologies).

For immunofluorescence of infected cell lines, cells were fixed in 4% paraformaldehyde for 10 min, permeabilized in 0.1% Triton X-100 and blocked for 1 h using 10% goat serum. Cells were then incubated for 2 h in primary antibody (H3K27me3: 9733, Cell Signaling Technology, 1:400 dilution) followed by secondary antibody (Alexa Fluor 594–conjugated goat anti-rabbit, Invitrogen). Slides were mounted using Prolong Gold with DAPI (Invitrogen). Photographs were taken on a Nikon microscope using a Rofer Scientific camera.

Protein blotting. Cell lysates were prepared in RIPA buffer (9806, Cell Signaling Technology) supplemented with Halt protease and phosphatase inhibitor cocktail (78440, Thermo Scientific). Equal amounts of protein, as measured by BCA protein assay (23225, Thermo Scientific), were resolved on NuPAGE Novex 4–12% Bis-Tris Protein Gels (NP0321BOX, Life Technologies) and transferred electrophoretically onto a nitrocellulose 0.45-μm membrane.
Membranes were blocked for 1 h at room temperature in 5% BSA in TBST before being incubated overnight at 4 °C with the primary antibodies diluted to 1:1,000 in 5% BSA in TBST (20 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween-20). Antibodies to NF1 (sc-67, Santa Cruz Biotechnology; 1:500 dilution), EZH1 (ab13665, Abcam; 1:1,000 dilution), EZH2 (5246, Cell Signaling Technology; 1:1,000 dilution), SUZ12 (3737, Cell Signaling Technology; 1:1,000 dilution), EED (ab4469, Abcam; 1:1,000 dilution), H3K27me3 (9733, Cell Signaling Technology; 1:1,000 dilution), H3K27ac (ab4729, Abcam; 1:1,000 dilution), total histone H3 (HRP conjugated, 12648, Cell Signaling Technology; 1:1,000 dilution) and actin (HRP conjugated, ab49900, Abcam; 1:5,000 dilution) were used.

Chromatin immunoprecipitation and quantitative PCR. Chromatin isolation from MPNST724 and ST88-14 cells under different experimental conditions was performed as previously described 44. For SUZ12 re-expression with associated vector control (encoding Flag-HA–tagged β-glucuronidase (GUS)), chromatin was isolated approximately 2 weeks after lentiviral infection. One microgram of antibody to EZH2 (5246, Cell Signaling Technology), SUZ12 (3737, Cell Signaling Technology), H3K27me3 (9733, Cell Signaling Technology), H3K27ac (ab4729, Abcam) and H3K4me3 (39159, Active Motif) was used for each ChIP. The primer pair sequences for human ChIP–quantitative PCR are listed in Supplementary Table 8.

RNA isolation and quantitative RT-PCR. For cells from tissue culture, RNA was isolated using the E.Z.N.A. Total RNA kit (Omega). For quantitative RT-PCR, RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription kit (ABI), and PCR was run using Power SYBR Master Mix (ABI) on a Realplex machine (Eppendorf). Expression was normalized to the transcript amount of the ribosomal protein RPL27. The primer pairs used are listed in Supplementary Table 8.

Statistical analysis. Fleiss’ κ statistic was used to assess the strength of co-occurrence for NF1 mutation, PRC2 component mutation and CDKN2A mutation. The R package irr was used to calculate the statistic and P value.14

30. Dobin, A. et al. STAR: ultrafast universal RNA-seq aligner. Bioinformatics 29, 15–21 (2013).
31. Anders, S., Pyl, P.T. & Huber, W. HTSeq—a Python framework to work with high-throughput sequencing data. bioRxiv doi:10.1101/002824 (2014).
32. Anders, S. & Huber, W. Differential expression analysis for sequence count data. Genome Biol. 11, R106 (2010).
33. Huang, D.W., Sherman, B.T. & Lempicki, R.A. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat. Protoc. 4, 44–57 (2009).
34. Subramanian, A. et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc. Natl. Acad. Sci. USA 102, 15545–15550 (2005).
35. Li, H. & Durbin, R. Fast and accurate long-read alignment with Burrows-Wheeler transform. Bioinformatics 26, 589–595 (2010).
36. McKenna, A. et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res. 20, 1297–1303 (2010).
37. Cibulskis, K. et al. Sensitive detection of somatic point mutations in impure and heterogeneous cancer samples. Nat. Biotechnol. 31, 213–219 (2013).
38. Saunders, C.T. et al. The Catalogue of Somatic Mutations in Cancer (COSMIC). Nat. Rev. Genet. 13, 843–849 (2012).
39. Koboldt, D.C. et al. VarScan 2: somatic mutation and copy number alteration discovery in cancer by exome sequencing. Genome Res. 22, 568–576 (2012).
40. Forbes, S.A. et al. TumorBoost: normalization of allele-specific tumor copy numbers from a single pair of tumor-normal genotyping microarrays. BMC Bioinformatics 11, 245 (2010).
41. Sowa, M.E., Bennett, E.J., Gygi, S.P. & Harper, J.W. Defining the human deubiquitinating enzyme interaction landscape. Cell 138, 389–403 (2009).
42. Chi, P. et al. ETV1 is a lineage survival factor that cooperates with KIT in gastrointestinal stromal tumours. Nature 467, 849–853 (2010).