A687V EZH2 Is a Driver of Histone H3 Lysine 27 (H3K27) Hypertrimethylation

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Abstract

The EZH2 methyltransferase silences gene expression through methylation of histone H3 on lysine 27 (H3K27). Recently, EZH2 mutations have been reported at Y641, A677, and A687 in non-Hodgkin lymphoma. Although the Y641F/N/S/H/C and A677G mutations exhibit clearly increased activity with substrates dimethylated at lysine 27 (H3K27me2), the A687V mutant has been shown to prefer a monomethylated lysine 27 (H3K27me1) with little gain of activity toward H3K27me2. Herein, we demonstrate that despite this unique substrate preference, A687V EZH2 still drives increased H3K27me3 when transiently expressed in cells. However, unlike the previously described mutants that dramatically deplete global H3K27me2 levels, A687V EZH2 retains normal levels of H3K27me2. Sequencing of B-cell–derived cancer cell lines identified an acute lymphoblastic leukemia cell line harboring this mutation. Similar to exogenous expression of A687V EZH2, this cell line exhibited elevated H3K27me3 while possessing H3K27me2 levels higher than Y641- or A677-mutant lines. Treatment of A687V EZH2-mutant cells with GSK126, a selective EZH2 inhibitor, was associated with a global decrease in H3K27me3, robust gene activation, caspase activation, and decreased proliferation. Structural modeling of the A687V EZH2 active site suggests that the increased catalytic activity with H3K27me1 may be due to a weakened interaction with an active site water molecule that must be displaced for dimethylation to occur. These findings suggest that A687V EZH2 likely increases global H3K27me3 indirectly through increased catalytic activity with H3K27me1 and cells harboring this mutation are highly dependent on EZH2 activity for their survival. Mol Cancer Ther; 13(12); 1–12. ©2014 AACR.

Introduction

EZH2 is the catalytically active methyltransferase component of the polycomb-repressive complex 2 (PRC2) and functions to methylate histone H3 on lysine 27 (H3K27; refs. 1, 2). Trimethylation of H3K27 (H3K27me3) is associated with chromatin condensation and transcriptional repression of genes involved in development and differentiation (3–5). EZH2 is overexpressed in many human tumor types, including prostate, breast, neuroendocrine lung, renal, and others (6–11). Diverse mechanisms underlie EZH2 overexpression, including amplification (12, 13), E2F activation (12), and loss of repressive microRNAs (miR101 and miR26a; refs. 14, 15). EZH2 is also recurrently mutated at specific residues in human non-Hodgkin lymphoma. EZH2 tyrosine 641 (Y641) is mutated in 14% to 22% of germinal center B-cell (GCB) diffuse large B-cell lymphomas (DLBCL) and 7% to 22% of follicular lymphomas (FL; refs. 16–18). In addition, alanine 677 of EZH2 is mutated to a glycine (A677G) in roughly 1% to 2% of DLBCLs (19, 20).

These recurrent EZH2 mutations affect residues located within the lysine binding pocket that are critical for the positioning of the K27 substrate during the methyl transfer reaction. The lysine binding pocket of wild-type (WT) EZH2 is designed to stabilize the K27me0 substrate, but is also limited by steric hindrance with the larger K27me2 substrate. Mutations of Y641 to smaller residues (Y641N/F/S/H/C) relieve the steric hindrance with the larger K27me2 substrate. Mutations of Y641 to smaller residues (Y641N/F/S/H/C) relieve the steric hindrance with the larger K27me2 substrate. Mutations of Y641 to smaller residues (Y641N/F/S/H/C) relieve the steric hindrance with the larger K27me2 substrate.

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The A677 residue of EZH2 is not located directly in the lysine binding pocket, but rather resides behind the Y641 residue (19). The mutation of A677 to the smaller glycine residue increases activity with an H3K27me2 substrate by enlarging the lysine binding pocket. Importantly, however, because this mutant retains the WT Y641 residue, it does not compromise the key interactions for positioning K27me0 and me1. Therefore, the A677G mutant is an efficient methyltransferase with any of the H3K27 substrates (H3K27me0/1/2; ref. 19). These mutations provide a mechanism for lymphoma cells to dramatically increase global H3K27me3 and potentially contribute to aberrant transcriptional silencing of several genes involved in normal cellular homeostasis.

Recently, recurrent mutation of EZH2 A687 (residue numbering based on NM_001203247/NP_001190176; A692 in NM_004456/NP_004447) to a valine (A687V) has been reported in several large-scale sequencing studies of non-Hodgkin lymphoma (20, 23, 24). Biochemical characterization of A687V EZH2 demonstrated that this mutant exhibited a substrate preference unique from that of WT, Y641X, and A677G EZH2. A687V EZH2 exhibited greatly reduced activity with an H3K27me0 substrate, 4-fold increased activity with H3K27me1, and little change with H3K27me2 (25). Herein, we demonstrate that although A687V EZH2 does not have increased activity with H3K27me2 substrates, it promotes hypertrimethylation of H3K27 and does so without dramatic depletion of H3K27me2 like other previously reported Y641 and A677 mutants. In addition, we identify this mutation in a B-cell acute lymphoblastic leukemia (ALL) cell line possessing elevated H3K27me3 levels and using GSK126 (26), a selective inhibitor of EZH2 catalytic activity, we demonstrate that this A687V EZH2-mutant cell line is highly dependent on EZH2 function for survival.

Materials and Methods

Structural modeling of EZH2

As previously described (19), a homology model of EZH2 was built using GLP/EHMT1 bound to an H3K9me2 peptide substrate (Protein Data Bank ID = 2RFI) as a primary template and structurally compared with other related SET domain containing histone lysine methyltransferases with determined crystal structures.

Cloning, expression, and purification of 5-member PRC2 complexes

Five-member PRC2 complexes were prepared as previously described (19). For A687V EZH2, human EZH2 in pENTR/TEV/D-TOPO was mutagenized by site-directed mutagenesis (QuikChange II XL; Agilent Technologies) using primers described in Supplementary Table S1, the entire coding region of all mutants was confirmed by double-stranded DNA sequencing, and subcloned into pDEST8 with an N-terminal FLAG epitope tag. Individual baculovirus stocks were generated for expression of EED, SUZ12, RbAp48, AEBP2, and FLAG–tev–EZH2 and PRC2 complexes were purified using anti-FLAG M2 resin (Sigma) as previously described (19). For mammalian expression studies, WT human EZH2 was subcloned into pIRE2-ZsGreen1 (Clontech) and site-directed mutagenesis was used as described above to obtain the A687V mutant. All components and EZH2 mutations were confirmed by peptide mapping analysis.

Biochemical evaluation of methyltransferase activity

Unless otherwise stated, all reagents were obtained from Sigma and were at a minimum of reagent grade. Peptides contained within the library (Supplementary Table S2) were acquired from 21st Century Biochemicals, AnaSpec, or Alta Bioscience. Library peptides all contain a terminal biotin tag and range in purity from crude to 97%. Streptavidin SPA beads (RPNQ0261) and [\(^{3}H\)-S-adenosyl]-methionine (SAM) were purchased from PerkinElmer.

All reactions were evaluated at ambient temperature in assay buffer containing 50 mmol/L Tris-HCl (pH 8.0), 2 mmol/L MgCl2, 4 mmol/L DTT, and 0.001% Tween-20. The peptide library screen was run as 10 μL reactions in Greiner 384-well plates that were pre-stamped with 100 nL peptide (1 μmol/L final) in 100% DMSO. [\(^{3}H\)]-SAM (200 nmol/L, 0.16 μCi/mL final) was added to the plate followed by the addition of WT or mutant PRC2 (16 nmol/L final). Reactions were quenched after 1 hour via the addition of unlabeled SAM and streptavidin-coated SPA imaging beads at 0.5 mmol/L and 1.5 mg/mL final concentrations, respectively. Activity was analyzed by reading the plates on a Wallac ViewLux CCD Imager (613/55 emission filter).

Cell culture

The SUP-B8 ALL cell line that harbors a heterozygous A687V EZH2 mutation was kindly provided by Dr. Ronald Levy (Stanford University, Stanford, CA) and was maintained in RPMI-1640 media (MediaTech) supplemented with 10% FBS (Sigma-Aldrich). All other cell lines were obtained from either the ATCC or DSMZ, maintained in the recommended cell culture media, and authenticated via short tandem repeat analysis before storage in the GlaxoSmithKline cell repository.

Transient expression of WT and mutant EZH2 proteins in cells

MCF-7 breast cancer cells (3 × 10^5) were seeded into 6-well tissue culture plates in RPMI-1640 media supplemented with 10% FBS the day before transfection. Following the manufacturer’s recommendations, 2 μg plasmid DNA and 6 μL Lipofectamine 2000 (Invitrogen) were combined in 500 μL Opti-MEM (Invitrogen) and incubated for 20 minutes at room temperature before being added to cells. Cells were then incubated for 72 hours at 37°C with 5% CO2 and harvested for protein lysates.
Western blot analysis

Cell lysate preparation and Western blotting were performed as previously described (19). Antibodies used included: EZH2 (BD Transduction Laboratories), histone H3 (Abcam), H3K27me1 (ActiveMotif), H3K27me2 (Cell Signaling Technology), H3K27me3 (Cell Signaling Technology), EED (Santa Cruz Biotechnology), SUZ12 (Cell Signaling Technology), or Actin (Sigma). Histone methylation antibody specificity was previously confirmed using full-length recombinant methylated histones harboring mono-, di-, or trimethylation at H3K4, H3K9, H3K27, H3K36, and H3K79 (19). The abundance of H3K27 methylation states was quantified using LI-COR Odyssey software and a standard curve of recombinant methylated histones, including H3K27me1 (Active Motif #31214), H3K27me2 (Active Motif #31215), H3K27me3 (Active Motif #31216), or total histone H3 (Active Motif #31207; Supplementary Fig. S1).

Analysis of H3K27 methylation by mass spectrometry

WT EZH2 NALM-6 cells were labeled with heavy (isotopic) [U-13C6, 15N4]-Arginine and [U-13C6]-L-Lysine (Invitrogen) for six population doublings before harvest and lysis. Acid-extracted cell lysates from the EZH2-mutant cell lines SUP-B8 and VAL were mixed 1:1 based on protein concentration with labeled NALM-6 lysate. Proteinase ArgC (Sigma) was added at a 10:1 enzyme to the protein ratio and the samples were digested for 2.5 hours at 37°C. Digestion was stopped with formic acid. Peptide mixtures were separated on 100 μm inner diameter x 5-cm RP-PSDVB monolithic columns (Dionex) using an acetonitrile:water:formic acid gradient at 500 μl/min. The column was interfaced directly to an LTQ-Orbitrap Velos Pro (ThermoFisher Scientific) mass spectrometer. Peptide identifications and SILAC ratios were determined by data-dependent LC/MS-MS using a resolution of 30,000 for the MS spectrum and MS/MS in the ion trap. To quantify H3K27 methylation, targeted, full-time LC/MS-MS spectra were acquired using HCD with a resolution of 7,500 in the Orbitrap on precursor ions for the H3 peptide 27 to 40 containing one through five methyl groups. No evidence was found for the peptide containing six methyl groups. Histones H3.1 and H3.2 have the same amino acid sequence through the 27 to 40 region, whereas H3.3 has an alanine to serine switch at position 31. Given the rather low abundance of H3.3 it was not included in the analysis. In addition, given the very low abundance of acetylation on K27 and K36, we also did not include peptides, which might contain acetylation in this region. Global methylation profiles for K27 were obtained by extracting the ion intensity for the b5 N-terminal fragment ion containing 0, 1, 2, and 3 methyl groups from each of the five methyl containing precursors and totaling the values. Because the b5 ion contains only the three N-terminal amino acids of the peptide, there is no contribution from methylation on K36 in the intensities.

Sanger sequencing of EZH2

Isolation of genomic DNA and Sanger sequencing of full-length EZH2, including exons 16 (Y641) and 18 (A677, A687), was performed as previously described (19).

Cell proliferation assay

Cells were evaluated for sensitivity to GSK126 in a 6-day proliferation assay as described previously (26). Briefly, cells were seeded into 384-well plates at a density that permitted proliferation for 6 days. Cells were then treated in duplicate with a 20-point 2-fold dilution series of GSK126 or 0.147% DMSO. After incubation with compound for 6 days, cell proliferation was evaluated using CellTiter-Glo (Promega) according to the manufacturer’s specifications. Data were fit with a 4-parameter equation to generate a concentration response curve and to determine the concentration of GSK126 required to inhibit 50% of growth (gIC50).

Caspase 3/7 assay

For detection of caspase 3/7 activity, Caspase-Glo 3/7 (Promega) was used according to the manufacturer’s directions. Values were normalized to CellTiter-Glo (Promega) levels at each time point and expressed as a percentage of vehicle-treated control.

Gene-expression profiling

SUP-B8 and NALM-6 cells (2 × 10⁵/well) were seeded into 6-well tissue culture plates 24 hours before treatment with 0.1% DMSO or 500 nmol/L GSK126 for 72 hours. Cells were collected into TRIzol reagent (Invitrogen) and total RNA was isolated via phenol:chloroform extraction and the RNeasy Kit (Qiagen) according to the manufacturer’s instructions. Total RNA was labeled and hybridized to Affymetrix Human Genome U133 Plus 2.0 oligonucleotide microarrays according to the manufacturer’s instructions (Affymetrix). CEL files were processed and differentially expressed probe sets were determined as described previously (26). Briefly, significant probe sets were identified after filtering for detection, an average fold change >2 or <−2, and P values adjusted for multiple testing correction by FDR (Benjamini Hochberg) < 0.1. Data are available through the Gene Expression Omnibus under accession GSE56954.

Gene ontology and gene set enrichment analysis

Functional analyses of differentially expressed probe sets were performed using DAVID (27). Significantly overrepresented GO Biological Process and Molecular Function terms (levels 3–5) were filtered for terms with at least five significantly altered probes and EASE P value of <0.01. For GSEA (28), log₂-transformed microarray data were filtered (100% present calls and signal >6 in at least one cohort). In total, 25,059 probesets were included. Gene set permutations were used to identify significantly enriched gene sets from the 2 (curated gene sets) and c3 (motif gene sets) MSigDB V3.0 databases (29).
Results

**EZH2 A687V is mutated in human cancers and affects substrate specificity**

The A687V EZH2 mutation has been observed in two independent studies of DLBCLs with incidences of 1 of 49 and 1 of 127 (20, 23). Although the incidence of this mutation appears to be quite low (\( \sim 1\% - 2\% \)), it is similar to what has been described for the A677G EZH2 mutation (19, 20). A687 is located within the catalytic SET domain of EZH2 (Fig. 1A) and lies adjacent to the highly conserved NH5 motif found in most SET domain methyltransferases (Fig. 1B). Interestingly, although the residues equivalent to EZH2 A687 in human EZH1 and Drosophila E(z) are both alanine, this residue is most frequently either valine (31%) or isoleucine (35%) in other SET domain methyltransferases (Fig. 1B and Supplementary Fig. S2).

Recent biochemical studies (25) demonstrated that A687V EZH2 exhibits 4-fold increased catalytic efficiency with a monomethylated K27 substrate. To further evaluate whether this was the only change in substrate specificity, we assessed PRC2 complexes containing WT or A687V-mutant EZH2 with a library of 602 peptides representing sequences within histones H2A, H2B, H3, or H4 and possessing up to five posttranslational modifications, such as lysine and/or arginine methylation; lysine acetylation; or phosphorylation of serine, tyrosine, and/or threonine (Supplementary Table S2). Supporting the previous report (25), this global analysis revealed that A687V EZH2 exhibited enhanced activity for three peptides that showed little activity with WT EZH2 (Fig. 1C and Supplementary Fig. S3). These three peptides derived from histone H3 all harbored monomethylation at either K27 (\( n = 2 \)) or K9 (\( n = 1 \)). Methylation of H3K9-containing peptides is a known biochemical artifact for the PRC2 complex that only occurs in vitro (1, 2). Thus, the A687V EZH2 complex is unique from both WT and previously reported Y641 and A677 mutants in that it gains activity with a monomethylated, and not dimethylated, substrate.

**Transient expression of A687V EZH2 increases global H3K27me3, while maintaining H3K27me2 levels**

To explore the effect of the A687V EZH2 mutant on H3K27 methylation levels, WT and mutant versions of EZH2 were transiently expressed in MCF-7 cells. MCF-7 cells possess a WT EZH2 and have previously been shown to exhibit increased H3K27me3 and decreased H3K27me2 in response to transient expression of Y641 and A677 EZH2 mutants (19). Interestingly, although A687V EZH2 exhibits increased efficiency in vitro for the dimethylation reaction, but not the trimethylation reaction, exogenous expression of A687V EZH2 reproducibly increased H3K27me3 levels relative to the control (Fig. 2A and B). This represents approximately half of the effect observed with the A677G, Y641F, or Y641N EZH2 mutants. However, in contrast with these mutants that induced depletion of global H3K27me2 in the process of generating H3K27me3, A687V EZH2 maintained H3K27me2 at levels equivalent to that of WT EZH2. H3K27me1 levels were minimally affected by either WT or mutant EZH2. These observations suggest that the increased dimethylation activity of A687V EZH2 may increase H3K27me3 through the law of mass action and over time equilibrium is achieved with increased H3K27me3 and near WT levels of H3K27me2.

**Identification of a B-cell ALL cell line harboring heterozygous A687V EZH2**

To identify a cell line possessing the A687V EZH2 mutation, a panel of cancer cell lines of B-cell origin was sequenced for the A687 codon located in exon 18 of EZH2. A single cell line, SUP-B8, was identified to harbor a
heterozygous A687V EZH2 mutation (Supplementary Fig. S4). The SUP-B8 cell line was established in 1988 from a 13-year-old female with B-cell ALL (30). As this was to the best of our knowledge the first observation of a potentially activating EZH2 mutation in B-cell ALL, we performed full-length sequencing of EZH2 in a panel of 11 cell lines reported to derive from B-cell ALL. Although no other A687V EZH2 mutations were identified, a heterozygous Y641N EZH2 mutation was identified in VAL, a cell line derived from the bone marrow of a 50-year-old female diagnosed with B-cell ALL (31).

Because activating mutations at EZH2 Y641 and A677 in DLBCL cell lines lead to an imbalance of H3K27 methylation states with increased H3K27me3 and dramatically reduced H3K27me2 (19, 21), we examined the impact of the A687V and Y641N EZH2 mutations in the context of B-cell ALL cell lines by Western blot and mass spectrometry approaches. Consistent with Y641 EZH2-mutant DLBCL cell lines, the Y641N EZH2-mutant VAL cell line exhibited elevated H3K27me3 and marked loss of H3K27me2 (Fig. 3A and B). Interestingly, although the A687V EZH2-mutant SUP-B8 cell line exhibited high H3K27me3, its H3K27me2 levels were intermediate between those of EZH2 WT and Y641/A677-mutant cell lines. Thus, these findings from a panel of B-cell ALL cell lines confirm the transient overexpression studies and demonstrate that A687V EZH2 promotes an imbalance in H3K27 methylation states different from Y641 or A677 EZH2 mutants.

Cells harboring the A687V EZH2 mutation are dependent upon EZH2 activity for growth and survival

To examine the extent to which A687V EZH2-mutant cells are dependent upon EZH2 activity for their survival, we used a highly selective small-molecule inhibitor of EZH2, GSK126. GSK126 has been shown to effectively inhibit the proliferation of many Y641 and A677 EZH2-mutant DLBCL cell lines (26). Among a panel of eight B-cell ALL cell lines, the A687V EZH2-mutant SUP-B8 cell line exhibited the greatest sensitivity to GSK126 with a
examined for cell proliferation and caspase 3/7 activity. The results from a greater dependence of these cells on EZH2 with the most sensitive EZH2 WT cell line, NALM-6 (Fig. 4A). EZH2 catalytic activity and loss of H3K27me3 in A687V EZH2 cell line (Supplementary Fig. S6). Thus, inhibition of EZH2 catalytic activity and loss of H3K27me3 in A687V EZH2 cells induce a program of transcriptional activation for genes associated with lymphocyte activation and cell death, suggesting a greater dependence on EZH2 in A687V EZH2-mutant cells.

Figure 3. A B-cell ALL cell line harboring A687V EZH2 exhibits elevated H3K27me3 levels. A, Western blot analysis was performed with antibodies specific for H3K27me3, H3K27me2, H3K27me1, total histone H3, EZH2, EED, and SUZ12, and actin using protein lysates from a panel of B-cell ALL and DLBCL cell lines. Actin and total histone H3 serve as loading controls. EZH2 mutation status as determined from full-length Sanger sequencing is indicated. B, relative levels of histone H3K27me0, me1, me2, and me3 were determined using mass spectrometry and heavy isotope amino acid labeling. Because contributions from the histone H3 variant and acetylation on the histone H3 AA27-40 peptide were negligible based on analysis of the total histone H3 population, these are not included in this figure.

dose of 441 nmol/L being required to inhibit 50% of cell proliferation (growth IC50; Fig. 4A). EZH2 WT cell lines, on the other hand, exhibited a range of sensitivity to GSK126 with growth IC50 values ranging from 2.5 to 7.8 µmol/L. This increased sensitivity observed for SUP-B8 cells was comparable with that seen with A677G- and Y641N/F-mutant DLBCLs. In addition, sensitivity was not correlated with proliferation rate (Fig. 4A) or mechanistic potency as SUP-B8 exhibited comparable, or slightly reduced, demethylation of H3K27me3 when compared with the most sensitive EZH2 WT cell line, NALM-6 (Fig. 4B). These data suggest that SUP-B8 sensitivity likely results from a greater dependence of these cells on EZH2 activity.

To evaluate the kinetics of growth inhibition and mechanism of cell death, SUP-B8 and NALM-6 cells were examined for cell proliferation and caspase 3/7 activity over a 6-day time course. Although little growth inhibition and no activation of caspases 3 or 7 were observed through 6 days in NALM-6 cells (Fig. 5A and B), a dose-dependent growth inhibition was observed in SUP-B8 cells after 4 days of treatment and was maximal on day 6 when cell number losses were evident for all doses above 100 nmol/L GSK126, suggesting induction of cell death mechanisms (Fig. 5C). Consistent with these observations, caspase 3/7 activity was increased in a dose-dependent fashion beginning on day 5 (Fig. 5D) demonstrating that GSK126 potently induces apoptotic cell death in A687V EZH2-mutant cells.

**Discussion**

Examination of Y641 and A677 EZH2 mutants suggests that alterations to the lysine binding pocket architecture can affect substrate preferences leading to global hypertrimethylation of H3K27 (19, 21, 22). In addition to these
two mutated residues, numerous mutations have now been reported throughout the entire EZH2-coding sequence. Most of these mutations observed in myeloid cancers, however, appear to inactivate enzymatic activity likely reflecting a tumor-suppressive role for EZH2 and/or PRC2 in myeloid cells (33). It is necessary, therefore, to...

Figure 4. A687V EZH2-mutant ALL is highly dependent on EZH2 activity. A, sensitivity of ALL and DLBCL cell lines to EZH2 inhibition with GSK126. Cells were treated with a 20-point 2-fold dilution series of GSK126 (range, 36 nmol/L–70 pmol/L) for 6 days and cell growth was measured using CellTiter-Glo (Promega). Data are represented as the concentration of GSK126 required to inhibit 50% of growth (growth IC50). The growth of each cell line is indicated below the graph as the number of population doublings for vehicle-treated controls for each cell line. B, Western blot analysis of H3K27me3, H3K27me2, H3K27me1, and total histone H3 following treatment of NALM-6 and SUP-B8 cell lines with varying concentrations of GSK126 for 72 hours.

Figure 5. A687V EZH2-mutant cells exhibit growth inhibition and caspase activation in response to EZH2 inhibition. Temporal kinetics of GSK126-induced growth inhibition (A and C) and caspase 3/7 activation (B and D) in NALM-6 (A and B) and SUP-B8 (C and D) over a 6-day time course using CellTiter-Glo or Caspase-Glo 3/7, respectively.
consider that EZH2 mutations may be activating or inactivating and a thorough understanding of the biochemical and cellular consequences of each mutation may help clarify the oncogenic or tumor-suppressive activities of EZH2 in different contexts. To this end, we have characterized the cellular effects of A687V EZH2 and demonstrate that this mutant increases global H3K27me3, but unlike Y641 and A687V EZH2 mutants, retains near normal levels of H3K27me2. Importantly, these cells appear to be highly dependent on EZH2 activity for their survival.

Because EZH2 and PRC2 crystal structures have remained elusive, a homology model of the EZH2 SET domain was constructed to understand the location of A687 within the catalytic domain. This previously described model is based on the protein sequence of EZH2 and a crystal structure of GLP/EHMT1 bound to a histone H3 peptide containing H3K9me2 (19). In this model, A687 resides at the interface of the SAM- and peptide-binding pockets (Fig. 7A). The A687 side chain is buried from solvent and points away from the SAM pocket toward I684 and F724, the phenylalanine–tyrosine switch residue of EZH2. The backbone carbonyl of A687 points into the active site and forms H-bonds with both Y726 and a conserved active site water molecule. While Y726 appears to contribute to efficient binding of SAM/SAH (S-adenosyl homocysteine) as well as proper folding of the SET domain, the active site water molecule is stabilized by the carbonyl groups of A687 and I684 and likely helps coordinate the lysine substrate. Thus, A687 appears to occupy a highly conserved position within the SET domain and may function in multiple roles to regulate binding of both SAM and lysine substrates.

Based upon study of our EZH2 active site model, related SET domain containing methyltransferases, and various SET domain mutants, different enzyme-substrate interactions appear to be required for mono-, di-, and trimethylation reactions. For the me0→me1 reaction, the key requirement appears to be proper positioning of the lysine substrate’s ε-amine at the methyl transfer pore. This is accomplished in large part by the highly conserved tyrosine residue (Y641 in EZH2, Y245 in SET7/9; Supplementary Fig. S7) and the active site water molecule as evidenced by the loss of activity with unmethylated lysine substrates in the EZH2 Y641N/F/S/H/C and SET7/9 Y245A mutants (19, 21, 22, 34). For the me1→me2 reaction, the lysine substrate must be oriented with the Kme1 methyl group rotated away from the methyl transfer pore so that a second methyl group may be transferred (Fig. 7B). This requires that the conserved active site water molecule be displaced from the lysine binding pocket or relocated within the enzyme to make room for the larger substrate. Finally, the me2→me3 reaction is accomplished when the Kme2 substrate is oriented with both methyl groups positioned away from the methyl transfer pore. This step is limited by steric hindrance between the large Kme2 substrate and EZH2 Y641, SET7/9 Y245, or G9a Y1067. Mutation of this restrictive tyrosine to a smaller residue converts these enzymes into efficient trimethyltransferases (19, 21, 22, 34, 35).

The active site water molecule in EZH2 appears to be coordinated by A687, I684, and the lysine substrate (Fig. 7A). In SET domain enzymes in which the F/Y switch residue is a tyrosine, the hydroxyl group of the tyrosine creates a fourth stabilizing interaction for this water (see SET8 and SET7/9 in Supplementary Fig. S7). On the basis of the role of the active site water molecule in regulating product specificity and the positioning of this water in part by EZH2 A687, we propose that mutation of A687 to the larger valine residue results in expansion of the lysine
substrate pocket such that binding of the water is destabilized relative to the WT enzyme (Fig. 7C). In this scenario, it would be easier for the methyl group of the H3K27me1 substrate to be accommodated in the active site of A687V EZH2 through displacement of the less tightly bound water, thereby enhancing turnover with an H3K27me1 substrate (Fig. 7D).

Although there has not been much work focused on EZH2 A687, or the equivalent residue in other enzymes, this proposal is supported by extensive work with SET domain F/Y switch residues that also coordinate the active site water molecule. In SET7/9, this residue is a tyrosine (Y305) and mutation to a phenylalanine increases the amount of Kme2 product (34). Conversely, in G9A, this residue is a phenylalanine (F1152) and mutation to a tyrosine reduces the amount of Kme2 product (36). The mechanism by which the F/Y switch residue affects product specificity is through the strength of hydrogen bonding to the active site water molecule. When a tyrosine occupies this position, the water molecule is bound less tightly and can be displaced resulting in the increased ability to form Kme2 product. Thus, even though EZH2 contains a phenylalanine in the switch position and can accomplish dimethylation, the A687V mutation appears to further weaken the binding of the active site water and facilitates a further increase in the rate of dimethylation.

Despite the fact that the predominant biochemical alteration is a gain in activity with an H3K27me1 substrate (25), when A687V EZH2 is transiently expressed in cells or found to occur naturally in an ALL cell line, the observed phenotype is increased trimethylation of H3K27, and not increased dimethylation (Figs. 2 and 3). The data presented herein and in the literature suggest that the Y641, A677, and A687 EZH2 mutants all stimulate hypertrimethylation of H3K27, but vary in the extent to which they induce hypodimethylation of H3K27. Unfortunately, the relevance of this difference is unknown because little is understood about the distinct roles played by H3K27me2 and H3K27me3. Global studies of histone marks from

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**Figure 7.** EZH2 A687 coordinates an active site water molecule required for the stabilization of the H3K27me0 substrate. A homology model of EZH2 was generated using the crystal structure of GLP/EHMT1 bound to an H3K9me2 peptide substrate. SAM is colored with orange carbons, WT EZH2 residues are colored with gray carbons in A and B and red carbons in C and D, A687V EZH2 residues are colored with gray carbons in C and D, H3K27 is colored with cyan carbons, and water is indicated by a red sphere. Distances between atoms are indicated in angstroms. Represented are WT EZH2 with H3K27me0 (A), WT EZH2 with H3K27me1 (B), A687V EZH2 with H3K27me0 (C), and A687V EZH2 with H3K27me1 (D).
CD4\(^+\) T cells suggest that while H3K27me1 is enriched at actively transcribed genes, both H3K27me2 and H3K27me3 are present at genes that are silenced or lowly expressed (37). Work from Sarma and colleagues (38) suggests that H3K27me2 is associated with lowly expressed or poised genes, whereas the conversion to H3K27me3 may more completely silence gene expression. Interestingly, the transition from di- to trimethylation appears to be stimulated by the presence of PHF1, a Polycomblike (Pcl) family member, known to coimmunoprecipitate with a fraction of cellular PRC2 (38–40). The Drosophila Pcl protein has three human homologs, including PHF1, MTF2, and PHF19, and all include at least one PHD (Plant Homeo Domain) finger domain and a TUDOR domain. The existence of specific biological mechanisms for the regulation of H3K27me2 and H3K27me3 suggests that there may be distinct biological requirements for these two marks and perhaps study of these EZH2-mutant cell lines can provide some insight to their functions.

Considering that mutations of EZH2 Y641 are found in up to 22% GCB DLBCL and FL, it is somewhat surprising that mutations of A677 and A687 appear to be so rare (~1%–2%) because they too increase H3K27me3. We have previously proposed for the A677G EZH2 mutation that this is due in part to the fact that glycine appears to be the only amino acid that when substituted for A677 will promote hypertrimethylation and that when considering single-nucleotide mutations of the A677 codon only a single mutation is capable of generating a glycine (19). The low incidence of the A687V EZH2 mutation may be similarly explained. At the nucleotide level, only one of nine single-nucleotide mutations within the A687 codon will result in a valine residue. Other amino acids that can be generated by single-nucleotide mutations include glutamic acid, proline, threonine, serine, and glycine. We hypothesize that glutamic acid and proline mutations may disrupt proper enzyme folding due to their dramatically different properties when compared with the WT alanine. Serine and threonine may be tolerated on the basis of their size alone; however, the polar side chains of these residues might also alter enzyme folding. Finally, glycine, a slightly smaller residue, may be tolerated, but may not change the WT product specificity as the active site water molecule would likely still be sufficiently coordinated with this substitution. Therefore, it appears that the frequency of these mutations may relate to multiple independent factors that can be rationalized when placed within the context of the enzyme’s active site and the various interactions required for proper functioning.

Although activating mutations in EZH2 (Y641F/N/S/H/C, A677G, and A687V) have thus far been found primarily in GCB DLBCL and FL (16–20, 23), this study identified cases of Y641N and A687V EZH2 mutations in B-cell ALL cell lines. Although much additional screening is required to determine the incidence of these mutations in B-cell ALL, this observation raises the question whether EZH2 may be uniquely required for homeostasis in the cell types from which these tumors arise. GCB DLBCL and FL arise from centroblasts or centrocytes, respectively (41), and common B-cell ALL arises from pre–pro-B cells or pro-B cells (42). EZH2 expression appears to be tightly regulated throughout B-cell differentiation. EZH2 levels are high in pro-B cells and then decrease in pre-B cells and are nearly undetectable in immature naive B cells (43, 44). EZH2 is then upregulated during the germline center reaction in centrocytes and centroblasts before decreasing again in mature recirculating B cells (43, 44). Thus, it appears that activating mutations of EZH2 are found in cancer cells arising from cell types that highly express EZH2. This may suggest that these tumors select for cells harboring preexisting activating mutations in EZH2 because they provide an opportunity for the mutant EZH2 to be expressed. Alternatively, it may be that silencing of EZH2 is critical for progression to the next stage of differentiation and that activated EZH2 establishes an altered epigenetic state that impedes further differentiation and promotes transformation in the presence of additional oncogenic mutations. Further work with mouse models engineered to overexpress mutant EZH2 at various stages of B-cell differentiation may provide insight into these critical questions.

This study has demonstrated that the A687V EZH2 mutation is capable of increasing global H3K27me3 in cells and that a cancer cell line harboring this mutation is dependent on EZH2 activity for its survival. Thus, there are now three EZH2 residues, when mutated in human cancers, alter the default substrate preferences leading to aberrant methylation of H3K27. Our studies with a specific EZH2 inhibitor demonstrate that targeting of EZH2 in these mutant cell lines is an efficient strategy for inducing growth inhibition and cell killing. These studies and others (26, 45, 46) provide encouraging data supporting the use of EZH2 inhibitors in patients harboring activating mutations of EZH2.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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