The Y641C mutation of EZH2 alters substrate specificity for histone H3 lysine 27 methylation states

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A B S T R A C T

Mutations at tyrosine 641 (Y641F, Y641N, Y641S and Y641H) in the SET domain of EZH2 have been identified in patients with certain subtypes of non-Hodgkin lymphoma (NHL). These mutations were shown to alter the methylation pattern of lysine 27 on histone H3 (H3K27). An additional mutation at EZH2 Y641 to cysteine (Y641C) was also found in one patient with NHL and in SKM-1 cells derived from a patient with myelodisplastic syndrome (MDS). The Y641C mutation has been reported to dramatically reduce enzymatic activity. Here, we demonstrate that while the Y641C mutation ablates enzymatic activity against unmethylated and monomethylated H3K27, it is superior to wild-type in catalyzing the formation of trimethylated H3K27 from the dimethylated precursor.

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1. Introduction

EZH2 is the catalytic subunit of the Polycomb-Repressive Complex 2 (PRC2), which is responsible for the mono- through trimethylation of lysine 27 on histone H3 (H3K27) [1,2]. Of these methylation states, trimethylated H3K27 (H3K27me3) is associated with the repression of gene transcription. Physiologically, H3K27me3 establishment and maintenance is important during processes such as X-inactivation, germline development and stem cell pluripotency [3]. While PRC2 activity is fundamental to cellular development and differentiation, its misregulation has been implicated in oncogenesis and metastasis, and correlates negatively with patient survival. In particular, overexpression of EZH2 or other subunits of the PRC2 complex has been identified as a driver of lymphomagenesis [4,5] and has been linked to solid tumors of the prostate, breast, ovaries, colon, bladder, liver and skin [6–11]. In addition, loss-of-function mutations in the corresponding H3K27 demethylase, UTX, have also been reported in a variety of blood and solid cancers [12].

From a diverse set of studies, a common understanding of the role of PRC2 function in tumorigenesis has emerged; the overarching hypothesis today is that mechanisms leading to elevation of H3K27me3 levels result in transcriptional silencing of tumor suppressor genes that in turn result in malignancy. Additional support for this notion comes from a recent report of detailed biochemical characterization of heterozygous somatic mutations at tyrosine 641 within the catalytic SET domain of EZH2 (Y641F, Y641N, Y641S and Y641H) in follicular lymphoma (FL) [13]. These mutations were originally designated as loss-of-function mutations based on a marked reduction in the ability of the mutant enzymes to methylate an unmodified peptide substrate bracketing the K27 site of H3. Further analysis demonstrated that the Y641 mutants of EZH2 dramatically altered the substrate specificity of the enzyme with respect to methylation state of the H3K27 residue [2,14]. The wild-type (WT) enzyme is most proficient at performing the initial monomethylation reaction at H3K27. From a diverse set of studies, a common understanding of the role of PRC2 function in tumorigenesis has emerged; the overarching hypothesis today is that mechanisms leading to elevation of H3K27me3 levels result in transcriptional silencing of tumor suppressor genes that in turn result in malignancy. Additional support for this notion comes from a recent report of detailed biochemical characterization of heterozygous somatic mutations at tyrosine 641 within the catalytic SET domain of EZH2 (Y641F, Y641N, Y641S and Y641H) in follicular lymphoma (FL) [13]. These mutations were originally designated as loss-of-function mutations based on a marked reduction in the ability of the mutant enzymes to methylate an unmodified peptide substrate bracketing the K27 site of H3. Further analysis demonstrated that the Y641 mutants of EZH2 dramatically altered the substrate specificity of the enzyme with respect to methylation state of the H3K27 residue [2,14]. The wild-type (WT) enzyme is most proficient at performing the initial monomethylation reaction at H3K27 and is consequently less proficient at catalyzing the monomethyl to dimethyl and dimethyl to trimethyl reactions. In contrast, the NHL-associated mutant enzymes all displayed the opposite pattern of substrate utilization; these enzymes were essentially inactive as catalysts of the initial monomethylation

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Abbreviations: NHL, non-Hodgkin lymphoma; MDS, myelodisplastic syndrome; PRC2, polycomb repressive complex 2; FL, follicular lymphoma; GCB, germinal-center B-cell; DLBCL, diffuse large B-cell lymphoma; WT, wild-type; SAM, S-adenosylmethionine.
2. Materials and methods

Tyrosine 641 of EZH2 has been found to be mutated to cysteine (Y641C) in one patient with DLBCL [13]. This mutation was also observed to be heterozygous, but due to its low frequency (2%) relative to the other four mutations found (13–49%), it was not investigated further Recently, an EZH2 Y641C mutation was also found in SKM-1 cells derived from a patient afflicted with myelo-displastic syndrome (MDS) [15,16], and again enzyme assays of purified PRC2 incorporating this EZH2 mutant indicated that it had significantly reduced activity against an unmodified peptide substrate [15]. In light of the biochemical changes in activity seen with other Y641 mutations of EZH2, we were motivated to better understand the Y641C mutant. Here we report that the tyrosine to cysteine substitution at residue 641 of EZH2 prevents the ability of the enzyme to mono- or dimethylate the H3K27 residue. However the Y641C mutant demonstrates significant activity in catalyzing the final step of taking H3K27me2 and adding another methyl group to form the H3K27me3 product. This pattern of substrate utilization for the Y641C mutant is different from that observed for the EZH2 variants thus far identified in association with human disease.

2. Materials and methods

Recombinant 4-component PRC2 was produced in Spodoptera frugiperda (SF) cells using a baculovirus expression system. Wild-type EZH2 (NM_004456) was coexpressed with wild-type FLAG–EED (NM_003797), Suz12 (NM_015355), and RbAp48 (NM_005610). An N-terminal FLAG tag on the EED was used to purify active PRC2 complex from cell lysates. For production of mutant 4-component PRC2, EZH2 carrying the Y641C mutation was co-expressed with wild-type His–FLAG-tagged EED (NM_003797), His-tagged Suz12 (NM_015355) and His-tagged RbAp48 (NM_005610), again using the baculovirus system. The His- and FLAG-tags were used for purification. The purity of the final PRC2 preparations was assessed by SDS–PAGE with Coomasie blue staining and microfluidic capillary electrophoresis (Agilent Bioanalyzer). We have data (not shown in this paper) that the mutant complexes purified with the EED-FLAG or EED-FLAG-His, EZH2-His, Suz12-His and RbAp48-His tags have identical substrate specificity and enzyme kinetics. Peptide substrates representative of histone H3 residues 21–44 containing either un-, mono-, di- or trimethylated lysine 27 and a C-terminal biotin (appended to a C-terminal amide-capped lysine) were HPLC-purified to greater than 95% purity. The enzyme assay was performed as previously described [2]. K_{1/2} values for the peptide were calculated using GraphPad Prism 5.0.

The sequencing of SKM-1 cells was performed on 5 million cells in linear log growth phase that were collected, washed in PBS, and snap frozen. Genomic DNA was then extracted from the frozen cell pellets, and PCR amplified using optimized cycling conditions. Primer extension sequencing was performed by GENEWIZ, Inc (South Plainfield, NJ) using Applied Biosystems BigDye version 3.1. The reactions were then run on Applied Biosystem’s 3730x DNA Analyzer. Sequencing base calls were done using KB™ Basecaller (Applied Biosystems). Somatic Mutation calls were determined by Mutation Surveyor (SoftGenetics) and confirmed manually by aligning sequencing data with the corresponding reference sequence using Seqman (DNASTAR). Sequence alignment was done with DNADynamo software and WebLogo 3.

3. Results

The Y641C mutant of EZH2 was incorporated into a recombinant PRC2 complex as previously described for other EZH2 mutant enzymes [2], and was assessed for methyltransferase activity against a panel of peptide substrates present at a concentration of 1 μM with 200 nM S-adenosylmethionine (SAM) as the cofactor. The peptide substrates tested here represent the amino acid sequence of human histone H3 residues 21 through 44 with lysine 27 represented as the unmodified, monomethylated or dimethylated side chain amine. As a control, experiments with recombinant PRC2-containing WT EZH2 were run simultaneously under the same conditions as for the mutant enzyme. Fig. 1A illustrates the product progress curves for the WT enzyme against H3K27me0, H3K27me1 and H3K27me2 containing peptide substrates. As previously reported, the WT enzyme displays robust activity as a monomethyltransferase but wanes in activity when presented with the H3K27me1 and especially the H3K27me2 substrates. For comparison, the product progress curves for the Y641C mutant enzyme are illustrated in Fig. 1B. A distinctly different pattern of substrate utilization is seen for this mutant relative to the WT enzyme. For the mutant enzyme, only the final dimethyl- to trimethyl-lysine reaction is significantly catalyzed. The comparison of relative activities is best illustrated in Fig. 1C where the reaction velocities for each substrate with each enzyme are summarized.

To further understand the differences between wild-type and mutant enzyme activity, the methylation state substrate specificity was explored through steady-state enzyme kinetics using the same

Fig. 1. Progress curves for incorporation of a tritiated methyl groups into H3K27 peptides by a recombinant PRC2 complex containing (A) wild-type EZH2 and (B) Y641C EZH2. 8 nM total enzyme was used in the reactions and the biotinylated peptide substrates were captured in a scintillant-embedded microplate. The incorporation of tritium was measured in counts per minute (cpm). (C) Enzyme velocities of PRC2 complexes containing either wild-type or Y641C EZH2 are summarized. All plots contain error bars representing the standard deviation of three experiments.
H3K27 substrate peptides as described above, and is summarized in Table 1. The peptides displayed sigmoidal behavior and the data were fit using a $K_{1/2}$ calculation rather than classic Michaelis–Menten fits to determine the concentration of peptide resulting in half-maximal velocity [2,17]. In addition, the catalytic efficiency of the mutant and wild-type enzymes is compared by analysis of the ratio of $k_{cat}/K_{1/2}$ for each peptide substrate. The enzymes showed no activity on a peptide of the same length containing trimethylated lysine 27 (data not shown), ensuring that there was no methylation at a lysine other than H3K27 occurring. The $K_{1/2}$ values for the wild-type enzyme reported here are within range of previously reported values [2], but the $k_{cat}$ was consistently about 5-fold higher. We attribute this difference in turnover rate to a new preparation of the PRC2 complex having a higher proportion of catalytically active enzyme. There was no detectable activity of the Y641C mutant enzyme on the unmethylated peptide substrate, even at the highest concentrations of peptide tested (3 μM).

There is a discordance in the literature and the COSMIC database over the status of the Y641C mutation in the SKM-1 cell line; Ernst et al. describe it as being homozygous [15], while the COSMIC database indicates that it is heterozygous [16]. To rectify these conflicting results, we conducted an independent sequencing analysis of SKM-1 cells, and indeed confirmed that the Y641C mutation is heterozygous (Fig. 3). This finding is in agreement with the data seen previously with the other Y641 mutants [2,13] and supports the hypothesis that heterozygous mutations are required for the coordinated activities of wild-type and mutant enzymes in pathogenesis.

4. Discussion

The pattern of substrate utilization by the Y641C mutant is reminiscent of that seen for other Y641 mutations of EZH2, in that the first reaction is essentially not catalyzed by the mutant enzyme, the intermediate mono- to dimethyl reaction is modestly catalyzed and the final dimethyl to trimethyl reaction is robustly catalyzed. However, a subtle distinction is realized by inspection of Fig. 1C and Table 1. With the exception of EZH2 Y641F, the previously reported Y641 mutant EZH2 variants all demonstrated robust dimethyltransferase activity (i.e., catalyzing the H3K27me1 to H3K27me2 reaction at approximately 50% the velocity of WT enzyme) [2], while the Y641C mutant appears to be considerably weaker at catalyzing this intermediate reaction. However, despite the similarities in catalytic efficiencies for monomethylation and dimethylation that distinguish the Y641F and Y641C mutants from the others, they are differentiated by their ability to catalyze the trimethylation reaction. Like the other mutants, the Y641F mutant is markedly improved over wild-type as a trimethylase (>10-fold activity relative to wild-type), while the Y641C mutant is only roughly 2-fold improved over wild-type in this respect.

In Fig. 2, we examine how the catalytic efficiency of the mutant and wild-type enzymes is affected by the methylation status of the target lysine. The $K_{1/2}$, $k_{cat}$ and the $k_{cat}/K_{1/2}$ ratio are plotted as a function of the number of methyl groups on H3K27. Inspection of the $K_{1/2}$ plot reveals that the wild-type and Y641C mutant enzymes display similar affinities for the mono- and dimethyl peptide substrate as measured by the $K_{1/2}$ (Fig. 2A). Where the enzymes are noticeably different is in the rates of catalysis stimulated by the different methylation states of the substrate peptide. The enzymes have maximal reaction velocities trending in opposite directions as lysine 27 goes from me0 to me1 to me2. Whereas the wild-type enzyme shows a diminution in $k_{cat}$ as the peptide goes to the dimethyl state, the Y641C mutant displays increasing $k_{cat}$ (Fig. 2B). The catalytic efficiency, measured by the $K_{1/2}$ to $k_{cat}$ ratio also follows a pattern similar to the $k_{cat}$ (Fig. 2C), implying that like the other Y641 mutants, the Y641C mutant differs from the wild-type enzyme with respect to transition state recognition [2]. This is consistent with the concept that EZH2 Y641 mutations [2], and analogous SET domain mutations shown in SET7/9 [18] and G9a [19], alleviate steric crowding of the target lysine in the active site. This small change presumably accommodates a bulkier dimethyl lysine and induces a change of function that sees EZH2 act primarily as a trimethyltransferase rather than as a monomethyltransferase.

In addition, the Y641C steady-state $K_m$ for SAM utilization on the preferred substrate is about 2.7-fold greater (4 μM vs. 1.5 μM) than the measured $K_m$ values for the wild-type or other Y641 mutants, although the $k_{cat}$ values are within two-fold of each other. Taken together with the peptide kinetic data, the extent to which effective coupling between WT and Y641C mutant EZH2 may occur in a heterozygous lymphoma cell to elevate the production of the H3K27me3 product is unclear. This may explain the low frequency of occurrence of this mutant in lymphoma. Further studies will be required to assess the effectiveness of enzymatic coupling with this and other EZH2 mutants.

### Table 1

| Enzyme | Substrate H3K27 methylation status | $K_{1/2}$ (nM) | $k_{cat}$ (h⁻¹) | $k_{cat}/K_{1/2}$ (h⁻¹ nM⁻¹ × 10⁻⁴)
<table>
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<tbody>
<tr>
<td>Wild-type</td>
<td>0</td>
<td>157 ± 12</td>
<td>4.80 ± 0.20</td>
<td>305 ± 26</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>337 ± 26</td>
<td>3.33 ± 0.21</td>
<td>99 ± 9</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>144 ± 11</td>
<td>1.08 ± 0.04</td>
<td>75 ± 6</td>
</tr>
<tr>
<td>Y641C</td>
<td>0</td>
<td>N.M.⁺</td>
<td>N.M.⁺</td>
<td>N.M.⁺</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>279 ± 45</td>
<td>0.64 ± 0.03</td>
<td>23 ± 3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>185 ± 8</td>
<td>2.98 ± 0.25</td>
<td>161 ± 12</td>
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* All reactions were run with 8 nM PRC2. The WT reaction used 1.5 μM SAM and the Y641C reaction used 4 μM SAM.

⁺ N.M. denotes the activity was too low to quantify.

![Fig. 2](image-url) How different peptide substrate methylation states influences: (A) the $K_{1/2}$, the concentration of peptide stimulating half-maximal enzyme velocity, (B) the $k_{cat}$ and (C) the catalytic efficiency, defined by the $k_{cat}$ to $K_{1/2}$ ratio. Symbol legend: wild-type EZH2 (○) and Y641C EZH2 (■).
The results presented in this communication clearly demonstrate that, contrary to earlier reports, the Y641C mutant of EZH2 retains significant, albeit altered catalytic activity as an H3K27 methyltransferase. As with the previously studied Y641 mutants of EZH2, the Y641C mutant is most effective as a trimethyltransferase and least effective as a monomethyltransferase. The implications of this altered substrate specificity for EZH2 Y641C in human disease remain to be determined through further biochemical and cell biological characterization.

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References


