Published in final edited form as:

Oncogene. 2018 January 11; 37(2): 208–217. doi:10.1038/onc.2017.311.

EZH2 contributes to the response to PARP inhibitors through its PARP-mediated poly-ADP ribosylation in breast cancer

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Abstract

Inhibitors against poly (ADP-ribose) polymerase (PARP) are promising targeted agents currently used to treat *BRCA*-mutant ovarian cancer and are in clinical trials for other cancer types, including *BRCA*-mutant breast cancer. To enhance the clinical response to PARP inhibitors (PARPi), understanding the mechanisms underlying PARP inhibitor sensitivity is urgently needed. Here, we show enhancer of zeste homolog 2 (EZH2), an enzyme which catalyzes H3 lysine trimethylation and associates with oncogenic function, contributes to PARPi sensitivity in breast cancer cells. Mechanistically, upon oxidative stress or alkylating DNA damage, PARP1 interacts with and attaches poly ADP-ribose (PAR) chains to EZH2. PARylation of EZH2 by PARP1 then induces PRC2 complex dissociation and EZH2 downregulation, which in turn reduces EZH2-mediated H3 tri-methylation. In contrast, inhibition of PARP by PARPi attenuates alkylating DNA damage-induced EZH2 downregulation, thereby promoting EZH2-mediated gene silencing and cancer stem cell property compared to PARPi-untreated cells. Moreover, the addition of an EZH2 inhibitor sensitizes the *BRCA*-mutant breast cells to PARPi. Thus, these results may provide a

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Supplementary Information accompanies this paper on the *Oncogene* website

Conflicts of Interest

The authors declare no conflict of interest.

rationale for combining PARP and EZH2 inhibition as a therapeutic strategy for *BRCA*-mutated breast and ovarian cancers.

Introduction

Poly (ADP-ribose) polymerase (PARP) catalyzes the attachment of ADP-ribose units to itself and its target proteins to recruit the DNA repair machinery to the site of DNA damage. PARP1 also functions in other cellular processes, including transcriptional regulation and cell cycles¹⁹. In DNA single strand break (SSB) repair, inhibition of PARP causes the replication fork to stall during DNA replication and induces double strand break (DSB)²³. DSB is then repaired via the homologous recombination (HR) repair pathway, requiring *BRCA* and Rad51²². Studies have indicated women who have germline mutations in *BRCA* genes (*BRCA1* or *BRCA2*) are predisposed to breast and ovarian cancer. In cancer cells with *BRCA* deficiency, inhibition of PARP leads to synthetic lethality^{3, 18}. Hence, PARP inhibitors (PARPi) are promising targeted agents for cancers with defects in the HR pathway. Various PARPi have been developed and tested in clinical trials⁵³, which led to the approval of olaparib (Lynparza, AstraZeneca) in 2014 by the U.S. Food and Drug Administration for treatment of *BRCA*-deficient ovarian cancer²⁹. However, *BRCA*-deficient tumors in a significant number of patients do not respond to PARPi treatment, and tumors from those who demonstrated response to PARPi treatment ultimately acquire resistance^{29, 53}. Thus, it is urgent to better understand the mechanisms underlying PARPi resistance and develop more effective therapeutic approaches.

Epigenetic regulators are potential targets for cancer therapy, and several drugs have been approved by the FDA or are in clinical trials, including inhibitors against enhancer of zeste homolog 2 (EZH2). EZH2, an enzymatic subunit of PRC2 complex, catalyzes histone H3 (H3) lysine 27 trimethylation (K27-me3), which is essential in multiple gene silencing. EZH2 is frequently overexpressed or mutated in cancers⁶³. For example, EZH2 is overexpressed in breast, ovarian, prostate, bladder, lung, liver, brain, kidney, gastric, esophageal, liver, and pancreatic cancers and melanoma, and EZH2-activating mutations are found in several subtypes of B-cell lymphoma^{40, 52}. Many studies have shown that EZH2 promotes cancer cell proliferation, tumor growth, cancer stem cell expansion, and metastasis^{7, 31, 35, 46, 64}. Thus, EZH2 is considered as a promising drug target. Currently, several inhibitors against EZH2 (EZH2i) are under investigation in clinical trials²⁶.

The PRC complexes are recruited to UV-induced DNA damage sites in a PARP-dependent manner⁹. Through a non-biased proteomics approach, PARP1 was identified as one of the many potential EZH2-binding proteins¹. Hence, we sought to investigate the role of EZH2 in PARPi resistance. Our results indicated that PARylation of EZH2 by PARP1 inhibits its functions, and inhibition of PARP1 leads to increased EZH2 activity. Because EZH2 is oncogenic, the PARPi-enhanced EZH2 activity may diminish the anti-tumor activity of PARPi. Thus, EZH2i is expected to sensitize PARPi treatment. Indeed, we found that EZH2i sensitized PARPi effect in *BRCA*-defective cancer cells *in vitro* and *in vivo*, suggesting that combined inhibition of EZH2 and PARP may be worthwhile to test in clinical trials for patients with *BRCA*-defective tumors.

Results

Activation of PARP1 enhances its interaction with EZH2

To determine whether PARP1 regulates EZH2 activity, we first validated their interaction by performing a GST-pull down assay using GST-EZH2 and His-PARP1 protein. As shown Figure 1a, a direct interaction between PARP1 and EZH2 was detected, which is consistent with prior studies^{1,9}. We then treated triple-negative MDA-MB-231 (231) and *BRCA*-mutant SUM149 breast cancer cells with methyl methanesulfonate (MMS), a DNA alkylating agent, to activate PARP (Figure 1b, Supplementary Figure S1a). The association between PARP1 and EZH2 increased upon PARP activation as detected by co-immunoprecipitation. Similar results were obtained when 231 cells were treated with H₂O₂, which induces oxidative stress leading to PARP activation, (Figure 1c). The endogenous interaction between PARP1 and EZH2 *in vivo* was validated by Duolink *in situ* PLA assays in 231, SUM149, and MDA-MB-436 cells (Figure 1d, Supplementary Figure S1b). H₂O₂ treatment further enhanced the PARP1-EZH2 interaction (Figure 1d). EZH2 and PARP1 predominantly localize in the nucleus, but several reports demonstrated that both proteins also localized in the cytoplasm^{13, 45, 49, 54}. Interestingly, we found that EZH2 interacted with PARP1 in the cytoplasm by Duolink assay (Figure 1d). To validate this result, we performed subcellular fractionation followed by co-immunoprecipitation. Consistently, the EZH2-PARP1 interaction was detected in both the cytosol and nucleus, which was enhanced by H₂O₂ treatment (Supplementary Figure S1c). Next, to ask whether other components of the PRC2 complex, e.g. SUZ12 and EED, are involved in the interaction between EZH2 and PARP1, we knocked down *SUZ12* and *EED* by specific siRNA and shRNA. *SUZ12*- and *EED*-knockdown 231 cells were then treated with MMS and subjected to immunoprecipitation followed by Western blotting using an EZH2 antibody. Knocking down both *SUZ12* and *EED* gene expression as expected slightly downregulated EZH2⁴⁴, but remained well associated with PARP1 (Supplementary Figure S2). Together, the results suggested that activation of PARP1 enhances interaction of PARP1 and EZH2 without requiring SUZ12 or EED.

PARP induces EZH2 PARylation in cell free and cellular assays

Given the association between PARP1 and EZH2, we next asked whether PARP1 poly (ADP-ribosyl)ates (PARylates) EZH2. To this end, we first immunoprecipitated EZH2 with EZH2 antibody and blotted against poly (ADP-ribose) polymer (PAR) in MDA-MB-453 (453) cells (Figure 2a, left). We also immunoprecipitated PAR with PAR antibody followed by Western blotting to detect EZH2 (Figure 2a middle panels). PARylated EZH2 was detected in both immunoprecipitates and disappeared when the cells were pretreated with PARPi. Consistently, 231 and SUM149 cells treated with H₂O₂ demonstrated enhanced PARP1-EZH2 interaction (Figure 1b and c) and PARylated EZH2 (lane 2 vs. 1, Figure 2b), which disappeared when the cells were pretreated with PARPi. Similarly, EZH2 PARylation was not observed in PARP1-knockdown cells (Supplementary Figure S3). These results suggest that activated PARP1 interacts with and PARylates EZH2. To further validate PARylation of EZH2 by PARP1, we performed a cell free ribosylation assay using recombinant PARP1 and immunoprecipitated EZH2. We detected strong signals from the immuocomplexes incubated with PARP1 and NAD⁺ (Figure 2c), supporting that EZH2 is

PARylated in the cell free assay. Since the immunocomplexes may include EZH2 binding proteins, such as EED and SUZ12, we also performed cell free PARylation assay using purified GST-EZH2 protein. As shown in Figure 2d, GST-EZH2 but not GST was PARylated in the cell free assay. Next, to identify the sites on EZH2 PARylated by PARP1, PARylated GST-EZH2 by the cell free assay was subjected to mass spectrometric analysis⁶⁶. Multiple PARylation sites were identified, which included EED binding sites (aa39-68), Suz12 binding site (aa218-334), two SANT domains (SANT1: aa159-250, SANT2: 428-476), and SET domain (catalytic domain, aa611-738). However, no apparent consensus PARylation motif was found in EZH2 from mass spectrometric analysis. Among them, two (D233 and E239) were strongly PARylated (Supplementary Table S1). Thus, we performed the cell free immunocomplex PARylation assay in using the D233A/E239A (AA) mutant EZH2 and demonstrated that PARylation of mutant EZH2 was significantly reduced compared wild-type EZH2 (Figure 2e). These results indicated that EZH2 is a target of PARP1-mediated PARylation.

PARP regulates EZH2 methyltransferase activity

Since EZH2 is modified by PARP, we next determined whether PARP regulates EZH2 methyltransferase activity, which is the major function of EZH2. We first examined the effects of PARP activation on H3 K27-me3 by treating 231 and SUM149 cells with PARPi and/or MMS, followed by Western blot analysis with K27-trimethylated H3 antibody. As shown in Figure 3A, MMS induced downregulation of H3-K27-me3, which was reversed by PARPi. We next performed chromatin immunoprecipitation (ChIP) assay to examine the levels of H3-K27-me3 on EZH2 target genes, HOXA9 and DAB2IP, in breast cancer cells^{47, 55}. In the presence of MMS, H3 K27-me3 levels were decreased but increased in the presence of PARPi (Figure 3b). Consistently, mRNA levels of HOXA9 and DAB2IP were upregulated by MMS treatment but downregulated by PARPi (Figure 3c). Moreover, the effects of PARPi and MMS on H3K27-me3 and EZH2 target gene expression were impaired in EZH2-knockdown cells (Supplementary Figure S4). Finally, we investigated whether EZH2 activity toward H3 methylation also decreases after its PARylation *in vitro*, by subjecting immunoprecipitated EZH2 to *in vitro* PARylation assay, followed by *in vitro* methylation assay with a chromatin complex as a substrate. Indeed, after PARylation, the EZH2 activity was significantly attenuated as indicated by the reduction in H3 K27-me3 (Figure 3d). Together, these results suggested that PARP negatively regulates EZH2 function through PARylation of EZH2.

PARP inhibits EZH2 functions in breast cancer cells

Since PARP1 interacts with and PARylates EZH2, we next asked whether PARP inhibition affects EZH2-mediated oncogenic functions in cancer cells. One of the most important oncogenic functions of EZH2 is the promotion of expansion of cancer stem cells (CSC, also known as tumor initiating cells, TIC)⁷. Thus, we first asked whether PARP inhibition (olaparib) affects the expansion of CSC population. To this end, we carried out sphere formation assay to measure CSC population using MCF7 cells, which have low CSC populations. Olaparib-treated cells had significantly higher sphere-forming ability compared with untreated cells (Supplementary Figure S2). Addition of EZH2 inhibitor (EZH2i), GSK343, decreased the number of spheres in PARPi-treated cells compared with those

without EZH2i treatment (Supplementary Figure S5a). To validate this result, we also assayed the aldehyde dehydrogenase (ALDH) activity, an indicator of cancer stem cells²⁰. Consistent with the results from sphere formation assay, PARPi treatment increased the population of ALDH-positive cells, which was reversed by the EZH2 inhibitor (Supplementary Figure S5b). While chemotherapeutic drugs are frequently used in combination with PARPi in clinical trials, they have also been reported to enrich CSC populations^{15, 34, 57, 59}. Therefore, we examined whether inhibition of PARP further increases CSC property when combined with chemodrugs. To explore this possibility, we prepared 231 cells with PARP1 knockdown, PARP1 knockdown and re-expression of PARP1, or control vectors and treated them with cisplatin. Then, we performed sphere formation assay. Knockdown of PARP1 substantially enhanced sphere formation, which was attenuated by re-expression of PARP1 (Figure 4a). Moreover, EZH2i also inhibited sphere formation in all cells (Figure 4a). To further validate that the PARP-EZH2 pathway regulates CSC property, we treated 231 cells with PARPi in the presence of EZH2i, and subjected cells to Hoechst 33342 dye staining followed by flow cytometric analysis to determine the Hoechst side population, which represents CSCs. Similar to the results from sphere formation assay, we observed higher Hoechst side population after olaparib treatment, which was reversed by the addition of GSK343 (Figure 4b). This result was further validated by measuring the ALDH-positive population (Supplementary Figure S5c). We also treated *BRCA*-deficient PARPi-sensitive SUM149 cells with olaparib for 10 days and expanded the survived (resistant) population. We then examined stem cell population in both parental and resistant cells, and found that higher stem cell population in the resistant cells (Figure 4c and Supplementary Figure S5d). Moreover, Western blot analysis indicated that the resistant population had higher EZH2 protein expression and H3-K27-me3 levels, suggesting that EZH2 plays a role in PARPi resistance (Figure 4d). Together, these results suggest that PARP inhibition increases CSC property through EZH2 activation.

PARylation of EZH2 leads to its dissociation from the PRC2 complex and subsequent degradation

To understand the mechanism of the PARP-suppressed EZH2 activities, we asked whether PARP negatively regulates expression of EZH2 protein because PARylation was reported to regulate the stability of some proteins¹⁹. We treated PARP1 knockdown and parental cells with PARP activator MMS, and determined EZH2 protein expression under PARP activation. Consistently, after MMS treatment, EZH2 protein levels were significantly reduced (Figure 5a) but remained relatively unchanged by PARP1 knockdown, suggesting that MMS-activated PARP1 is required for EZH2 downregulation. Consistently, inhibition of PARP1 blocked MMS-induced decrease in EZH2 protein half-life (Figure 5b). A study recently demonstrated that PARP1 downregulates EZH2 mRNA expression through inhibition of E2F4 transcription factor in lymphoblastoid B cells³⁷. However, we did not observe EZH2 mRNA downregulation after PARP inhibition in breast cancer cells (Supplementary Figure S3a), suggesting this may be cell type dependent. MMS-induced reduction of EZH2 protein was attenuated in the presence of proteasome inhibitor MG132 (Figure 5c), indicating that proteasome-mediated protein degradation plays a critical role in PARP-regulated EZH2 expression in breast cancer cells. Indeed, EZH2 and cellular PAR

levels (represent PARP activity) were negatively correlated in various breast cancer cell lines (Supplementary Figure S3b).

Since protein PARylation affects protein-protein interactions, we asked whether EZH2 PARylation interferes with PRC2 complex formation. Moreover, because EZH2 protein stability was reduced after knocking down SUZ12 and EED⁴⁴ (Supplementary Figure S1b), we reasoned that the PARylation of EZH2 leads to its dissociation from PRC2 complex which may enhance its degradation. PRC2 complexes were co-immunoprecipitated via EZH2 antibody, and the immunocomplexes were subjected to *in vitro* PARylation assay. Binding of SUZ12 and EED to EZH2 was substantially reduced (by 80%) following PARylation of EZH2 (Figure 5d). In the presence of PARPi, the binding of SUZ12 and EED to EZH2 *in vivo* was about 2.3 and 4.4 times higher than without PARPi treatment (Figure 5e). We also compared the EZH2-SUZ12 and EZH2-EED binding between wild-type EZH2 and AA mutant EZH2 after *in vitro* PARylation assay. Consistent with the results from Figure 2e, AA mutant EZH2 had less reduction in SUZ12 and EED binding compared with wild-type EZH2 after PARylation (Figure 5f). Together, we identified the novel regulatory mechanism of EZH2 by PARP1, namely, PARylation of EZH2 by PARP1 dissociates the PRC2 complex, resulting in EZH2 downregulation and subsequently reducing EZH2 activity. Since PARPi is used in clinic, the mechanism identified raises the question of whether PARPi treatment ultimately activates oncogenic PRC2 complex, which may affect the response to PARPi.

Inhibition of EZH2 sensitizes BRCA-mutant cancers to PARPi in vitro and in vivo

The above results indicated that EZH2-mediated upregulation of CSCs was enhanced by PARP inhibition. Therefore, we investigated whether the inhibition of EZH2 enhances the therapeutic effects of PARPi. Since PARPi is approved for *BRCA*-mutant ovarian cancer cells in the clinic and is being investigated in many clinical trials for both breast and ovarian *BRCA*-mutant cancers, we treated breast cancer cells harboring *BRCA* mutation (SUM149) and epigenetically silenced *BRCA* (HCC38), and ovarian cancer cells carrying *BRCA* deletion (UWB1.289) with olaparib in the presence or absence of EZH2i, GSK343. Inhibition of EZH2 by GSK343 alone reduced cell growth, but the effects were different among cell lines, which may be due to the differential activity of EZH2 in these cells. Thus, we selected a working concentration of GSK343 for each cell line for GSK343 alone and in combination with olaparib. All cells were sensitive to olaparib, and GSK343 enhanced the inhibitory effect of olaparib on colony formation (Figure 6a; representative images shown in Supplementary Figure S7a). However, the effects of GSK343 on PARPi in UWB1.289 cells were not significant ($p = 0.073$, Supplementary Figure S7b). PARP1 trapping has been reported to play an important role in determining tumor cell cytotoxicity caused by PARP inhibitors. We also assessed another effective PARP1 trapper, talazoparib, and a poor PARP1 trapper, veliparib, in combination with GSK343 in SUM149 cells. The results indicated that EZH2i induced similar effects on both types of PARPi (Figure 6b and Supplementary Figure S7c). Moreover, similar results were observed using another combination of rucaparib (PARPi) and EPZ6438 (EZH2i) (Supplementary Figure S8). We evaluated the effects of this combination by calculating the combination index (CI) via the Chou-Talalay method and performing a Bliss independence drug interaction analysis. The Chou-Talalay CI index

analysis revealed a synergistic effect for the rucaparib and EPZ6438 combination ($CI < 1$), but the results from the Bliss independence analysis indicated an additive effect for the combination. We also compared the effects of GSK343 and temozolomide, an alkylating agent that is frequently used for combination therapy with PARPi in clinical trials, on PARPi by colony formation assay, and found that effects of both drugs were comparable (Supplementary Figure S9). The inhibitory effects of the olaparib and GSK343 on colony formation were further validated by soft agar assay (Figure 6c), and the result showed that the combination inhibited colony formation more effectively than the single drug treatment. To further verify the role of EZH2 in PARPi sensitivity in *BRCA*-mutant breast cancer cells, we ectopically expressed EZH2 in SUM149 cells followed by olaparib treatment. These cells were less sensitive to olaparib (Figure 6d, Supplementary Fig S7d). In contrast, cells became more sensitive to olaparib when we knocked down EZH2 in HCC38 cells (Supplementary Figure S10). In a SUM149 xenograft mouse model, we showed that tumor growth was significantly inhibited by the combination of olaparib and GSK343 compared with either agent alone (Figure 6e). Together, our results suggested that combined inhibition of EZH2 and PARP may be a potential therapeutic strategy for *BRCA*-mutant cancers.

Discussion

PARPi is a new class of anti-cancer drugs, and currently used for treatment of advanced *BRCA*-mutant ovarian cancer. In addition to *BRCA*-mutant cancers, preclinical and clinical studies have shown that several cancers with deficiency in DNA damage repair pathways also respond well to PARP inhibition^{38, 41, 56}. However, the underlying mechanism of PARPi resistance is not yet well established. Several studies have reported possible resistance mechanisms underlying PARPi resistance. First, restoration of BRCA proteins observed in resistance to platinum drug in *BRCA*-mutant ovarian cancer was implicated in PARPi resistance^{2, 42}. Moreover, in *Brcal*-deficient mouse mammary tumors, the loss of 53BP1 was shown to induce PARP inhibitor resistance²⁴. Activation of the P-glycoprotein drug efflux transporter was also shown to contribute to PARPi resistance *in vivo*⁴⁸. Recently, we identified a new mechanism of PARPi resistance in which oxidative DNA damage induces c-Met kinase-mediated phosphorylation of PARP1, resulting in PARP1 activation and PARPi resistance¹⁴. In the current study, we revealed another new possible mechanism of PARPi resistance attributed to PARPi-induced stabilization of PRC2 complex. Byers et al. previously reported that small cell lung cancer cells express high levels of PARP and EZH2, and are more sensitive to PARPi than non-small cell lung cancer cells and suggested future clinical studies to evaluate PARP and EZH2 inhibition together with chemotherapy or other agents⁴. In the current study, we reported our findings of the combination of PARPi and EZH2i in breast cancer cells.

Following DNA damage, cell cycles halt and transcription profiles are altered. In addition to DNA damage, PARP1 also regulates transcription and chromatin dynamics²⁷. The relationship between DNA damage and epigenetics is further exemplified by studies showing that PARP1 regulates ISWI chromatin remodeling complex⁵¹ and that PARP1 inactivates H3-K4 demethylase KDM5 through PARylation, which in turn alters the chromatin structures and activates transcription of KDM5 target genes²⁸. PARP1 was previously reported to regulate gene expression via downregulation of EZH2 mRNA in

lymphoblastoid B cell line³⁷. However, we did not observe upregulation of EZH2 mRNA by PARPi treatment in breast cancer cells, suggesting that the mechanism may be cell type dependent. Nonetheless, our findings provide a newly identified link between DNA damage and chromatin dynamics.

EZH2 is regulated by multiple posttranslational modifications. Phosphorylation is the best-studied modification of EZH2 by many kinases, such as AKT, CDK1, CDK2, ATM, p38, and JAK2^{6, 8, 25, 32, 43, 50, 61}. These kinases have been shown to positively and/or negatively regulate EZH2 activity, depending on cell types and specific stimulation⁶³. In addition to phosphorylation, EZH2 is also modified by ubiquitination and O-GlcNAcylation, both of which are involved in EZH2 stability^{10, 50, 65}. In the current study, we demonstrated PARylated EZH2 by PARP negatively regulates EZH2 activity, disrupting its binding to the components of the PRC2 complex, and subsequently its protein stability. Indeed, it has previously been shown that knockdown of Suz12 causes EZH2 downregulation, which is blocked by MG132⁴⁴. Similarly, knockout of EED reduces EZH2 expression³⁹. Consistently, we observed that knockdown of EED and Suz12 induced downregulation of EZH2 (Supplementary Fig S2). Although we showed that proteasome inhibitor MG132 inhibits EZH2 degradation, which suggested a ubiquitin-mediated proteasome degradation pathway may be involved, the specific E3 ligase responsible for degradation of PARylated EZH2 has yet to be determined. CHFR and RNF146 E3 ligases have been shown to ubiquitinate PARylated target proteins for degradation^{30, 36}, but it has yet to be determined whether EZH2 could also be regulated by those PAR-dependent E3 ligases. In addition, there may be a potential interplay between EZH2 PARylation and other modifications such that EZH2 phosphorylation may inhibit or facilitate the interaction between PARP1 and EZH2 to reduce or increase EZH2 PARylation⁶⁰.

EZH2 is also involved in the DNA repair pathways through various mechanisms. For example, EZH2 downregulates Rad51 and BRCA1 in breast cancer cells^{7, 21}, both of which are critical for the HR pathway. In addition, BRCA1 is known to interact with EZH2, resulting in the inhibition of EZH2-HOTAIR non-coding RNA interaction and EZH2 function⁵⁸. In contrast, EZH2 induces nuclear export of BRCA1 and inhibits its function³³, indicating that EZH2 antagonizes BRCA in *BRCA*-positive cells. Moreover, cell cycle checkpoint protein, ataxia-telangiectasia-mutated, which is activated by double strand DNA damage, can phosphorylate and inhibit EZH2 in neurons³². In glioblastoma cells, depletion of EZH2 increases DNA damage repair, suggesting that EZH2 is a negative regulator of DNA damage¹¹. However, EZH2 inhibition has been reported to enhance DSB repair efficacy and IR-induced cell death in U2OS osteosarcoma cells⁵. Therefore, the role of EZH2 in DNA damage repair may be cell type and DNA damage dependent. More studies will be required to dissect mechanisms underlying cell type-specific role of EZH2 in DNA damage repair machinery.

In summary, we discovered a novel molecular interplay between EZH2 and PARP1 in which PARP1 induces PARylation of EZH2, resulting in downregulation of EZH2 and the EZH2-mediated CSC property. Since PARPi has been used to treat cancer patients, this newly identified activity raises the question of whether PARPi-induced EZH2 activity increases CSC population, which could attenuate the therapeutic efficacy of the PARPi. In particular,

temozolomide has been frequently used in combination with PARPi in clinical trials. Since temozolomide is an alkylating agent like MMS, it may also inhibit EZH2 via PARP activation. Because PARP-mediated inhibition of EZH2 is attenuated by PARPi, the results of the current study suggested an approach to overcome this issue by adding EZH2i to PARPi therapy (Supplementary Figure S11). However, we did not observe an apparent synergistic effect of the combination of PARPi and EZH2i in several experiments. Further preclinical studies are warranted to analyze various PARPi and EZH2i combinations in multiple cell lines and mouse models to determine their future clinical applications.

Materials and Methods

Plasmids, antibodies, chemicals, and recombinant proteins

Expression of EZH2 protein and shRNA against EZH2 were described previously⁶¹. Point mutations were introduced by site-directed mutagenesis as described previously¹⁶. EED shRNA expression plasmids and siRNAs against *SUZ12* were obtained from Thermo Fisher Scientific. The following antibodies were used: EZH2 (#5246, Cell Signaling Technology; 612667, BD Biosciences), EED (sc-28701, Santa Cruz Biotechnology; 09-774, EMD Millipore), SUZ12 (#3737, Cell Signaling Technology), PAR (4335-MC-100-AC, Trevigen; 551813, BD Biosciences), PARP1 (#9532, Cell Signaling Technology; sc-7150, Santa Cruz Biotechnology), HA (11666606001, Sigma-Aldrich; sc-805, Santa Cruz Biotechnology), Histone H3 (ab1791, Abcam; #3638, Cell Signaling Technology), H3-K27-me3 (ab6002, ab108245, Abcam), GST (sc-53909, Santa Cruz Biotechnology), γ -H2AX (#05-636, EMD Millipore), Tubulin (T5168, Sigma-Aldrich). Purified PARP protein and GST-EZH2 were purchased from Abcam and BPS Bioscience, respectively. Olaparib, EPZ-6438, GSK132 were purchased from Selleck Chemicals, and AG014699 from ChemieTek.

Cell culture and lentivirus production

All breast cancer cell lines were obtained from ATCC and cultured in DMEM/F12 medium supplemented with fetal bovine serum (FBS) and antibiotics except for SUM149 cells, which were cultured in RPMI1640 with FBS and antibiotics. Ovarian cancer cell lines were kindly provided by Dr. Anil Sood (MD Anderson Cancer Center) and cultured in RPMI1640/MEGM supplemented with FBS and antibiotics. We used the following BRCA mutant cell lines in this study; SUM149 cells (BRCA1 2288delT mutation), MDA-MD-436 (BRCA1 5396 + 1G>A mutation), and UWB1.289 (BRCA1 2594delC mutation)^{12, 17}. We also used HCC383 cells, which exhibit low BRCA1 expression due to BRCA1 promoter methylation⁶². For lentivirus production, the lentivirus expression plasmids were co-transfected with pCMV- 8.91 and pCMV-VSVG into 293T cells. Cells were placed in fresh DMEM for 16 hour following transfection. The culture media containing the lentiviral particles were then harvested after 48-hour incubation. Lentivirus infection was performed by incubating cells with the virus-containing media with polybrene for 16 hours. Stable cells were then selected on puromycin (1 μ g/ml).

ChIP assay, qPCR and RT-qPCR

ChIP assay was performed using EZ-ChIP kit (Millipore) according to the manufacturer's instructions. In brief, 231 cells were fixed with 3.7 % of formaldehyde, lysed with the lysis

buffer, and sonicated. Immunoprecipitation was then performed with H3 and H3-K27me3 antibodies (Abcam). Purified DNA fragments were analyzed by qPCR using iQ™ SYBR® Green Supermix (Bio-Rad, Hercules, CA) and CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). RNA extraction was performed using Trizol reagent (Thermo Fisher Scientific), and cDNA was prepared using SuperScript® VILO™ cDNA Synthesis Kit (Thermo Fisher Scientific). The primers used for ChIP assay and qPCR are as follows: *HOXA9*-ChIP (F: TCCACCTTTCTCTCGACAGCAC; R: GTGGGAGGCTCAGGATGGAAG), *DAB2IP*-ChIP (F: CCTGCTTTCTGTTTCCTTCTCCTG; R: TTGAACCACCTCCTCCTCCCTCTC), *HOXA9*-qPCR (F: TTGGAGGAAATGAATGCTGA; R: TGGTCAGTAGGCCTTGAGGT), *DAB2IP*-qPCR (S: AGCGGGATAAGTGGATGGAG; R: CGGGCATAGAGCACATCGTC).

GST pull-down, Western blotting and co-immunoprecipitation (co-IP) assay

GST-EZH2 or GST proteins (0.5 µg) were immobilized onto glutathione sepharose beads. The beads were then incubated with 0.5 µg of His-PARP1 protein in 1 ml of PBS with 1% NP-40 at 4 °C for 2 hours. Bound proteins were washed with 1% NP-40/PBS, extracted with SDS sample buffer from the beads, and subjected to Western blotting or SDS-PAGE followed by Coomassie blue staining. For Western blotting, cells were lysed in NP-40 lysis buffer (150 mM NaCl, 10 mM Tris-HCl pH7.4, and 1% NP-40, protease and phosphatase inhibitors cocktails) and sonicated. For histone H3 Western blotting, cells were lysed in RIPA buffer and sonicated. For co-IP, 1 mg of cell lysates was incubated with 2 µg of antibodies or control IgG in 500 µl of NP-40 lysis buffer at 4 °C for overnight. Protein G or A beads (15 µl) were added to the mixture, which were further incubated at 4 °C for 4 hours. The beads were then washed. Total proteins or immunocomplexes (20 µg) from IP were separately on SDS-PAGE followed by Western blotting with the indicated primary and corresponding secondary antibodies. Signals were detected with ECL substrate (Bio-Rad) and captured on x-ray films, and densities were analyzed using ImageJ (NIH, Bethesda, MD).

Colony formation assay and soft agar assay

For colony formation assay, 1,000 cells were seeded in 24-well plates and treated with the indicated drugs. After 10–20 days, cells were washed with phosphate buffered saline (PBS), fixed with 3.7 % formaldehyde, and stained with 0.5% crystal violet solution. Relative cell densities were analyzed using Image J. For soft agar assay, 2,000 cells are mixed with 0.3% agar in DMEM/F12 and then were laid on top of 0.5% solidified agar in DMEM/F12 in 12-well plates. Culture media containing the indicated drugs was added to each well. 4 weeks later, cells were stained with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution, and the number of colonies no smaller than 50 µm was counted under a microscope.

Duolink assay in situ proximity ligation assay

Duolink assay was performed using the Duolink Assay Kit (Sigma-Aldrich). Briefly, cells treated with or without H₂O₂ were fixed, permeabilized, and incubated with primary antibodies, followed by secondary antibodies conjugated to PLA probes. Ligation and amplification were performed according to the manufacturer's instructions. Signals were

then captured by confocal microscope. The mean $\pm$ s.d. of PLA signal intensity from 20 cells in each treatment group was calculated.

Animal studies

All animal procedures were conducted under the approval of the Institutional Animal Care and Use Committee (IACUC) at The University of Texas MD Anderson Cancer Center (protocol number 00001334-RN00). SUM149 (2×10^6) cells were injected into the mammary fat pads of female nude (Swiss Nu/Nu) mice of 6–8 weeks of age (Department of Experimental Radiation Oncology Breeding Core, The University of Texas MD Anderson Cancer Center). When the tumor volume reached $\sim 50 \text{ mm}^3$, olaparib (5 mg/kg) and GSK343 (10 mg/kg) were orally administered to mice five times per week alone or in combination for the number of days specified in the figure legend. Tumor was measured at the indicated time points, and tumor volume was calculated by the formula $\pi/6 \times \text{length} \times \text{width}^2$.

In vitro PARylation assay and methylation assay

GST-EZH2 protein or EZH2 immunoprecipitated from cells was incubated with 50 ng of activated DNA, 0.5 mM of NAD, and 100 ng PARP protein in total 20 μl of 1x assay buffer from a commercial assay kit (EMD Millipore Cat# 17-10149) at room temperature for 30 min. For GST-EZH2, the samples were diluted with PBS and GST-beads were then added to them. After 16 hour incubation at 4 $^{\circ}\text{C}$, the beads were extensively washed and subjected to Western blotting, Coomassie blue staining, and mass spectrometry⁶⁶. For the EZH2 immunocomplex from cells, EZH2 was overexpressed in 293T cells and immunoprecipitated with anti-EZH2 antibody or control IgG. After extensive washing, the beads were incubated with the purified PARP protein in the presence or absence of NAD⁺. Then, the samples were extensively washed with RIPA buffer, and subjected to Western blotting or *in vitro* methylation assay. *In vitro* methylation assay was performed as described previously⁶¹ using H3-K27-me3 antibody for detection.

Determination of Hoechst side-population and sphere formation assay

Cells were trypsinized, washed with PBS, and single cells were incubated in medium containing 5 $\mu\text{g/ml}$ Hoechst 33342 at 37 $^{\circ}\text{C}$ for 2 hours. Control cells were treated with 1 μM of P-glycoprotein inhibitor Tariguidar (Selleckchem) plus Hoechst 33342. Hoechst-positive population was analyzed by flow cytometry, and the side population gating was set based on Tariguidar-treated cells. Tumorsphere assay was performed in low adhesive plate with complete Mammocult medium (Stem Cell Technology). Single cells were seeded at density of 500–1,000 per well in a 6-well dish and cultured for 7 days. Spheres larger than 100 μm was counted under a microscope.

Statistical Analysis

Data in bar graphs represents mean fold change relative to untreated or control groups with standard deviation unless indicated otherwise. Statistical analyses were performed using SPSS (Ver. 20, SPSS, Chicago, IL). Student's *t* test or ANOVA was performed for experimental data. A *P* value < 0.05 was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported in part by the following: the National Institutes of Health (CA109311, CA099031, and CCSG CA016672); Patel Memorial Breast Cancer Endowment Fund; National Breast Cancer Foundation, Inc.; Breast Cancer Research Foundation; The University of Texas MD Anderson Cancer Center-China Medical University and Hospital Sister Institution Fund; Ministry of Science and Technology; International Research-intensive Centers of Excellence in Taiwan (I-RiCE; MOST 105-2911-I-002-302); Ministry of Health and Welfare, China Medical University Hospital Cancer Research Center of Excellence (MOHW105-TDU-B-212-134003); Center for Biological Pathways; The UT Southwestern Endowed Scholar Program, the Welch Foundation (I-1800), and National Institutes of Health (GM114160) to Y.Yu. Dr. Yonghao Yu is a Virginia Murchison Linthicum Scholar in Medical Research and a CPRIT Scholar in Cancer Research.

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Figure 1. PARP1 directly interacts with EZH2, and their interaction is upregulated by PARP1 activation

a, GST-pull down assay with GST-EZH2 and His-PARP1. **b**, MDA-MB-231 cells were treated with 0.5 mM MMS (DNA alkylating agent, a PARP activator) for 4 hours and subjected to co-immunoprecipitation, followed by Western blotting with the indicated antibodies. **c**, MDA-MB-231 cells were treated with 20 mM H₂O₂ for 30 min and subjected immunoprecipitation and Western blot analysis. **d**, MDA-MB-231 cells were treated with 20 mM H₂O₂ and subjected to Duolink assay. Signals were quantified (right). *P < 0.01, Student's *t*-test.

Figure 2. EZH2 is PARylated by PARP *in vitro* and *in vivo*

a, MDA-MB-453 cells were treated with 5 μ M olaparib for 24 hours and subjected to immunoprecipitation with EZH2 (left panels) and PAR (right panels) antibodies, followed by Western blotting with the indicated antibodies. **b**, MDA-MB-231 cells were treated with H₂O₂ in the presence or absence of 5 μ M olaparib, and subjected to IP with PAR antibody, followed by Western blotting with EZH2 antibody. **c**, EZH2 proteins were immunoprecipitated with EZH2 antibody, and the immunocomplex were incubated with purified PARP1 protein *in vitro*. The asterisk indicates the PARylation signals from non-specific proteins. **d**, GST-EZH2 or GST protein were incubated with purified PARP1 protein *in vitro*, and subjected to Western blotting with PAR antibody. Coomassie blue staining is shown on right. **e**, Wild-type or D233A/E239A mutant (see Supplementary Figure S1) EZH2 were expressed in 293T cells and immunoprecipitated with an HA antibody. The immunocomplexes were incubated with purified PARP1 protein *in vitro*.

Figure 3. PARP inhibitor and activator affect EZH2 methyltransferase activity

a, MDA-MB-231 (231) and SUM149 cells were pretreated with or without 5 μ M and 1 μ M of olaparib and further cultured for 6 hours in the presence of 0.5 mM of MMS. The levels of H3-K27me3 were determined by Western blotting using the indicated antibody. **b** and **c**, 231 cells were treated with olaparib and/or MMS as described in (**a**), and the levels of H3-K27me3 on EZH2 target genes and their mRNA expression levels were determined by ChIP assay (**b**) and quantitative PCR (**c**), respectively. * $P < 0.01$, ** $P < 0.05$, control vs. MMS or PARPi treatment, Student's *t*-test. **d**, PRC2 complexes were immunoprecipitated and the immunocomplexes incubated with purified PARP1 protein *in vitro*. After washing the beads, the chromatin complex was added to the beads and incubated in the presence of SAM. Histone H3 methylation was determined by Western blotting with H3-K27me3 antibody.

Figure 4. PARP inhibitors affect EZH2-mediated cancer stem cell upregulation

(a) MDA-MB-231 vector control, shPARP1, and shPARP1 in PARP1 re-expressing cells were treated with 5 μM of cisplatin for 5 days. Cells that survived were then subjected to sphere formation assay in the presence or absence of 2 μM of GSK343. * $P < 0.01$, control *vs.* PARP1 re-expressing cells, Student's *t*-test. (b) 231 cells were treated with or without 5 μM of olaparib in the presence or absence of 2 μM of GSK343 for 5 days. The cancer stem cell population (CSC) was determined by Hoechst side population (SP) staining. * $P < 0.01$, number of CSC in PARPi-treated cells in control *vs.* EZH2i-treated cells, Student's *t*-test. **c**, **d**, SUM149 cells were treated with 0.2 μM olaparib for 10 days. Cancer stem cell population (CSC) in survived cells (resistant population) and parental cells was determined by Hoechst side population (SP) analysis (**c**). Simultaneously, EZH2 and H3-K27me3 levels were determined by immunoblotting (**d**).

Figure 5. EZH2 PARylation induces dissociation of PRC2 complex and EZH2 degradation

a, MDA-MB-231 wild-type and PARP1-knockdown cells were cultured in the presence of 0.2 mM MMS for 12 hours, and EZH2 expression was determined by Western blotting. **b**, MDA-MB-231 cells were treated with 0.5 mM MMS or 0.5 mM MMS + 5 μ M olaparib in the presence of 50 μ g/ml of cycloheximide (CHX), and EZH2 protein half-life was determined by Western blot. Quantification of the Western blot is shown in the upper graph. **c**, MDA-MB-231 cells were treated with MMS and MG132 (10 μ M) or DMSO in the presence of cycloheximide (CHX) for 4 hours. The effect of MG132 on the EZH2 protein level was determined by immunoblotting. **d**, PRC2 complexes were immunoprecipitated and the immunocomplexes incubated with purified PARP1 protein *in vitro*. After washing the beads, the binding of SUZ12 and EED to EZH2 was determined by Western blotting. **e**, MDA-MB-231 cells were treated with 5 μ M olaparib for 48 hours and subjected to immunoprecipitation with EZH2 antibody, followed by Western blotting with EED and SUZ12 antibodies. **f**, Wild-type or D233A/E239A mutant EZH2 were expressed in 293T cells and immunoprecipitated with an HA antibody. The immunocomplexes were incubated with purified PARP1 protein *in vitro*. After washing the beads, SUZ12 and EED binding to EZH2 were determined by Western blotting.

Figure 6. EZH2 inhibitor increases PARPi sensitivity in *BRCA*-deficient breast and ovarian cancer cells

a, *BRCA*-deficient SUM149, HCC38, and UWB1.289 cancer cells were subjected to colony formation assay in the presence of the indicated concentrations of PARPi (olaparib) and EZH2i (GSK343). Representative images and statistical analysis of data by ANOVA are shown in Supplementary Figure S7a and S7b, respectively. **b**, SUM149 cells were subjected to colony formation assay in the presence of the indicated concentrations of PARPi (veliparib or talazoparib) and EZH2i (GSK343). Representative images and statistical analysis of data by ANOVA are shown in Supplementary Figure S7c. **c**, SUM149 cells were subjected to soft agar assay in the presence of the PARPi (olaparib; olap, 50 nM) and EZH2i (GSK343; GSK, 5 μM). * $P < 0.01$, combination vs. single treatment, Student's *t*-test. **d**, EZH2 was stably overexpressed in SUM149 cells, and the effects of olaparib were determined by colony formation assay. Representative images and statistical analysis of data by ANOVA are shown in Supplementary Figure S7d. **e**, The effects of olaparib and GSK343 alone or in combination in a SUM149 orthotopic xenograft model. * $P < 0.01$, combination vs. GSK343 or olaparib, ANOVA.