EZH2 Methyltransferase and H3K27 Methylation in Breast Cancer

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Abstract

Histone modifications are thought to control the regulation of genetic programs in normal physiology and cancer. Methylation (mono-, di-, and tri-methylation) on histone H3 lysine (K) 27 induces transcriptional repression, and thereby participates in controlling gene expression patterns. Enhancer of zeste (EZH) 2, a methyltransferase and component of the polycomb repressive complex 2 (PRC2), plays an essential role in the epigenetic maintenance of the H3K27me3 repressive chromatin mark. Abnormal EZH2 expression has been associated with various cancers including breast cancer. Here, we discuss the contribution of EZH2 and the PRC2 complex in controlling the H3K27 methylation status and subsequent consequences on genomic instability and the cell cycle in breast cancer cells. We also discuss distinct molecular mechanisms used by EZH2 to suppress BRCA1 functions.

Key words: EZH2, H3K27 methylation, Breast cancer, BRCA1

Transcriptional regulation modulated by the H3K27 methylation status

Post-translational modifications of the N-terminal tails of core histones, including methylation, acetylation, ubiquitylation and phosphorylation, influence chromatin configuration, which can modulate accessibility of transcription factors and transcriptional activity of nearby genes [1]. Four lysine residues in the N-terminal tail of histone H3, K4, K9, K27 and K36, are primary targets of specific histone methyltransferases and demethylases. Methylated H3K27 is abundant, and three different statues (mono-, di- or tri-methylation) exist. For example, based on quantitative mass spectrometry 50% of H3K27 in embryonic stem (ES) cells is dimethylated, 15% trimethylated and 15% monomethylated [2].

H3K27 di- and tri-methylation are characteristic of PcG (Polycomb group) target genes and are associated with transcriptional repression [3]. PRC1 (Polycomb repressive complex 1) and PRC2 are associated with chromatin condensation. PRC1 catalyzes the monoubiquitylation of histone H2A and PRC2 contributes to the methylation of H3K27 [4]. PRC2 is composed of several proteins, including EED, SUZ12, RbAP46/48 and either EZH1 or EZH2. EZH1 and 2 are characterized by a SET domain, which functions as a histone-lysine N-methyltransferases [5-8]. PRC2 complexes containing EZH1 or EZH2 affect repression of gene expression. While PRC2 complexes containing EZH2 are primarily responsible for H3K27 methylation, PRC2 complexes containing EZH1 can also catalyze H3K27 methylation [9], or compact chromatin through mechanisms other than histone methylation. However, these mechanisms remain to be fully explored (Figure 1A) [10].

Experiments with cell-specific Ezh2 knockout mice have demonstrated that EZH2 influences proliferation and differentiation of stem cells and progenitor cells in three distinct cell types, adipocytes, keratinocytes, and neurons [11-13]. Experimental evidence from several systems suggests that H3K27 methylation is mainly achieved by EZH2 and to a
lesser extent by EZH1. The critical importance of EZH2 is reflected by the fact that Ezh2-null mice die in utero, while Ezh1-null mice have no overt defects, possibly due to compensatory functions of EZH2. However, EZH1 can also compensate to some extent for EZH2 as loss of both EZH1 and 2 evoke more severe consequences than loss of EZH2 alone [14]. In addition, contribution to H3K27 monomethylation by G9a, a well-known H3K9 methyltransferase, was observed in vitro and in vivo [15, 16]. Although no changes in H3K27 di- and tri-methylation were observed between wild type and G9a-/- ES cells, the extent of monomethylation was significantly decreased in G9a +/- ES cells. It is feasible that G9a activity partially compensates for the loss of EZH2. It is possible that G9a introduces a monomethyl group on H3K27 in the absence of EZH2 and that this is sufficient to bind by Embryonic Ectoderm Development (EED), a component of the PRC2 complex, which contains the methyl-lysine histone-binding domain WD40.

JMJD3 (KDM6B, (jumonji-domain containing protein 3)) and UTX (KDM6A (Ubiquitously transcribed tetratricopeptide repeat gene on the X chromosome)) are demethylases acting on H3K27 (Figure 1B). They remove the gene-inactivating H3K27 di-methyl and tri-methyl marks and thereby presumably contribute to the maintenance of gene expression. Aberrant of UTX and JMJD3 levels have been associated with cancer and defective differentiation programs [17].

**EZH2 and human breast cancer**

Excessive EZH2 concentrations have been reported as a marker of aggressive breast cancer [18, 19] and associated with invasion and cancer progression. Chinnaiyan and colleagues tested EZH2 protein levels in 280 breast cancer patients using high-density tissue microarray [20]. EZH2 levels were elevated in patients with invasive breast carcinoma relative to normal or atypical hyperplasia. Notably, increased expression was already observed in ductal carcinoma in situ (DCIS), a precursor of invasive carcinoma [21].

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![Figure 1. Transcriptional regulation through H3K27 methylation.](image-url)  
(A) Transcriptional repression regulated by the polycomb repressive complex 2 (PRC2) including EZH1 or EZH2. PRC2 complexes, which include EED, SUZ12, RbAP46/48 and EZH2, catalyze H3K27 di- and tri-methylation. This in turn leads to a more condensed chromatin state and transcriptional repression. PRC2 complexes containing EZH1 can also catalyze H3K27 methylation, or compact chromatin through other mechanism. G9a has H3K27 methyltransferase activity and also affects gene silencing. (B) Transcriptional activation regulated by UTX and JMJD3. UTX and JMJD3 are demethylases that form complexes with MLL, RbBP5 and WDR5. The removal of methyl groups from H3K27me3 leads to transcriptionally active chromatin.
Figure 2. The biology of EZH2 in breast cancer. EZH2 gene expression is regulated by the HIF, E2F, and RAF/ERK/Elk pathways. Increased levels of EZH2 have been linked to H3K27 trimethylation (H3K27me3) on promoter regions of several genes (RAD51, RUNX3, CDKN1C (p57KIP2), FOXC1, and CDH1 (E-cadherin)) whose expression is decreased. Reduced levels of RAD51 lead to the activation of Raf1/ERK and β-catenin signaling. Transcription of the CDKN1A gene is under RUNX3 control and lower concentrations of RUNX3 result in lower p21WAF/Cip1 levels, which fail to fully block the cell cycle. The CDKN1C gene encodes the cyclin-dependent kinase inhibitor p57KIP2, a strong inhibitor of cyclinE1 and CDK2 complexes. Expression of the CDKN1C gene is regulated through H3K27me3 of its promoter. Reduction of p57KIP2 leads to accelerated G1-S transition, which has been suggested to support breast cancer progression. Accumulation of H3K27me3 in the promoters of the FOXC1 gene, a fork head family transcription factor used in developmental programs, and the CDH1 gene, that encodes E-cadherin, has been observed in breast cancer cell lines. A decrease of FOXC1 and E-cadherin would be in agreement with enhanced cell invasion and metastasis.

Dynamic signaling regulated by EZH2

Elevated EZH2 concentration has been associated with increased tumor cell proliferation in various cancers including breast, prostate and melanoma [22]. The two-layered regulatory loop includes transcriptional regulation of the EZH2 gene and ensuing consequences of associated H3K27me3 (Figure 2). Three well-defined transcription factors have been invoked in the regulation of EZH2. E2F, a target of the retinoblastoma protein (pRB), plays a critical role in regulating cell cycle progression through activating genes that control entry into the S phase and genes associated with DNA replication [23]. Upon phosphorylation of pRB, activated E2F binds to promoter regions of EZH2 and EED, and controls their expres-
sion. However, Helin and colleagues confirmed that the interrupt of EZH2 and EED expression was not enough to increase expression of negative regulators of cell cycle including p14ARF, but positive regulators of cell proliferation, cyclinD1 (CCND1), cyclinE1 (CCNE1), cyclinA2 (CCNA2) and cyclinB1 (CCNB1), significantly decreased in EZH2 lacking cells. They concluded that the PRC complex, including EZH2, is required for the activation or maintenance of the activated state of certain genes in proliferating cells through H3K27 methylation [24].

EZH2 expression is also controlled by hypoxia through HIF response elements (HRE) in the EZH2 gene promoter [25]. In hypoxic microenvironment, EZH2 expression is increased and thereby promotes proliferation of breast tumor initiating cells. Lastly, MEK/ERK/Elk pathway can also lead to EZH2 overexpression in ERBB2-overexpressing breast cancer cell lines [26].

A set of genes associated with cell proliferation and invasion is regulated by EZH2. Among them RAD51, RUNX3, and CDKN1C (p57kip2) regulate cell proliferation, and FOXC1 and CDH1 (E-cadherin) have been linked to metastasis. The runt-related (RUNX) family is associated with normal development and neoplasia and RUNX3 is an established tumor suppressor gene in gastric cancer [46]. Inactivation of RUNX3 expression through DNA hypermethylation has been reported in various cancers, including those of prostate, lung and pancreas. Notably, upon reducing EZH2 expression in the MCF-7 breast cancer cell line, RUNX3 expression was recovered. RUNX3 expression also increased upon treatment with the deacetylase inhibitor TSA [27]. It has been suggested that RUNX3 down-regulation is controlled by H3K27me3 through EZH2 and histone deacetylation through HDAC1. Even though DNA methylation affects repression of RUNX3, H3K27me3 by EZH2 has a critical role in down-regulation of RUNX3. RUNX3 reduction leads to the decrease of the cyclin-dependent kinase inhibitor p21WAF/Cip1 expression, which results in the induction of cell proliferation in breast cancer [28].

FOXC1, a member of the Forkhead box transcription factor family, plays an important role in differentiation. Increased EZH2 influences transcriptional repression of FOXC1 through the accumulation of H3K27me3 and diminished acetylation of H3/H4 in MDA-MB-231 cell lines. Elevated FOXC1 concentrations lead to reduced migration and invasion in vitro and in vivo [29]. The cyclin-dependent kinase (CDK) inhibitor, CDKN1C (p57kip2) encodes the tumor suppressor p57kip2 protein. Reduced CDKN1C expression in various breast cancer cell lines was associated with EZH2 overexpression and increased H3K27me3 [30]. Upon treatment with the histone methylation inhibitor 3-deazaneplanocin A (DZNep), recovery of CDKN1C expression was observed suggesting a direct link between H3K27me3 and transcriptional suppression. The transmembrane receptor E-cadherin maintains epithelial cellular adhesion and integrity and it has been proposed that its down-regulation leads to enhanced invasion in cancer. In EZH2-overexpressing breast epithelial cells, reduced CDH1 (E-cadherin) expression correlated with H3K27me3 levels in the promoter. However, elevated expression of EZH2 was not sufficient to increase enzymatic activity for H3K27me3 at the CDH1 promoter, which also required HDAC activity. Increased HDAC activity leads to the removal of acetyl groups from H3K27, a prerequisite for EZH2-controlled histone methylation. Increased EZH2 induces reduction of CDH1 expression, and it leads to metastasis and invasion in breast cancer as well as pancreatic cancer and ovarian cancer [31-33].

EZH2 also appears to regulate breast tumor initiating cells (BTICs) through suppressing RAD51 expression. PHO and GAGA motifs, which are targets for PRC2 complexes, are located in the RAD51 promoter and H3K27me3 coincides with these sites. Reduction of RAD51 affects chromosome breaks, deletion and translocation as well as increase of centrosome number and genome instability through activated RAF/pERK/β-catenin signaling [25].

A BRCA1-EZH2 connection

Mutations in BRCA1 are a cause of basal like breast carcinomas that are ER, PR and Her2-neu negative (triple negative) and increased EZH2 concentrations were found mainly in basal type breast carcinomas [34]. Germline Brca1 mutations predispose women to develop breast cancer, and patients have a poor prognosis, in part because BRCA1’s role in DNA repair and genomic stability [35]. Even though the majority of basal breast cancers do not carry Brca1 germline mutations, reduced concentration of BRCA1 protein and mRNA levels were reported in these patients [36]. It has been speculated that the underlying cause is promoter methylation or transcriptional repression. However, the mechanisms causing decreased BRCA1 expression in basal like carcinomas remains to be established. Figure 3 proposes mechanisms linking EZH2 overexpression and BRCA1 in basal like breast cancer.
EZH2 is frequently overexpressed in ER-negative breast cancer cell lines, and a reduction of EZH2 results in suppressed cell proliferation. Biological effects of EZH2 knockdowns were observed in vivo and in vitro system using xenograft transplantations. Reduced tumor growth was confirmed in xenograft mouse models injected with EZH2 knockdown cells [37]. EZH2 overexpression inhibits BRCA1 phosphorylation (Ser1423) and thereby promotes an increase of Cdc25C, an essential player for G2/M checkpoint control. Once Cdc25C is activated, it induces the activity of the Cdc2/cyclinB1 complex. In summary, elevated EZH2 concentrations in ER-negative breast cancer cell lines confer increased cell proliferation in part possibly through the inhibition of BRCA1 phosphorylation [37].

BRCA1 is a nuclear protein involved in numerous activities, including DNA repair and estrogen receptor modulation. It has been proposed that Akt-1 (Ser473), but not Akt-2 and 3, are phosphorylated and activated by EZH2 in the EZH2 overexpressing breast cancer cell line CAL51, and nuclear location of BRCA1 protein is suppressed in these cells by a yet to be determined mechanism (Figure 3). This in turn results in aberrant mitoses with extra centrosomes, and genomic instability [38]. Elevated EZH2 concentrations were observed in mouse mammary epithelium from which the Brca1 gene had been deleted. As a result gene expression related to differentiation was suppressed and excessive cell proliferation was promoted. DZNep, a well known as EZH2 inhibitor [47], affects EZH2 concentrations. It was confirmed that

Figure 3. Molecular interplay between EZH2 and BRCA1 in basal like breast cancer. In ER-negative breast cancer cells, elevated EZH2 concentrations block the phosphorylation of BRCA1 (Ser1423). Once the tyrosine phosphatase Cdc25C is phosphorylated, it dephosphorylates the cyclinB-bound Cdc2 (CDK1) and thereby triggers entry into mitosis. Accumulation of Cdc2 and cyclinB1 results in increased cell proliferation. In addition to its canonical function as a H3K27 methyltransferase, EZH2 most likely also has non-canonical functions. EZH2 can phosphorylate Akt-1 (Ser473), but not Akt-2 and -3, and interact with Akt-1. It leads to reduced nuclear retention of BRCA1. In breast cancer cell lines this leads to increased centrosome numbers and genome instability. The EZH2 inhibitor DZNep is more effective on BRCA1-deficient cells, and can thus serve as one arm in a therapeutic regimen.
BRCA1-deficient cells are more sensitive to DZNep, a potential treatment, when compared with BRCA1-proficient cells [39].

**Genome-wide approaches to explore PRC**

Genome-wide approaches have been applied to identify genomic localizations and target genes of PRC complexes in human and mouse ES cells. This system has been explored using a combination of chromatin immunoprecipitation (ChIP), ChIP on CHIP and microarray analyses [40-43]. Transcription factors related with developmental regulation, including homeobox protein (HOX), GATA binding protein (GATA), and runt related transcription factor (RUNX) are regulated by the PRC2 complex in human ES cells [42]. In mouse ES cells, 512 genes regulated by PRC1 and 2 were identified. These include genes encoding the transcription factor families FOX, SOX, GATA and TBX [40]. Moreover, enriched H3K27me3 was found in proximal promoter regions of these genes, which were also derepressed in Suz12- and Eed-null cells [40, 42] supporting the notion that their suppression is dependent on the PRC complex. Moreover, the differentiation of ES cells resulted in the activation of these genes. ChIP-seq analyses with antibodies against EZH2, Ring1B, H3K4me3 and H3K27me3 have been performed to identify the conservation of chromatin states in orthologous genomic loci in human and mouse ES cells [44]. Bivalent promoters, which carry H3K4me3 and H3K27me3 marks, are highly conserved between mouse and human ES cells. In addition to PRC2, PRC1 binding was identified in the above genes. In general, H3K27me3 retention was most pronounced in regions that were occupied by both PRC1 and 2, while poor retention was detected on sequences occupied by PRC2 only. These findings suggest that PRC2 catalyzes and regulates H3K27 methylation but is not sufficient to maintain the methylation status of respective target genes.

Genome-wide approaches have been used to predict the importance of the PRC2 complex in the regulation of developmental transcription through H3K27 methylation. However, it is not clear what concentration of EZH2 is required to regulate H3K27 methylation, and whether excess EZH2 has other functions besides its methyltransferase activity.

**Moving forward using mouse genetics**

The suggestion that excess EZH2, and thereby altered histone modifications, leads to a changed epigenetic genomic landscape, and thereby contributes to cancer progression, is mainly based on correlative studies and cell culture investigations. Although biochemical studies are capable of linking elevated EZH2 concentrations with altered genetic programs in cancer, clear genetic evidence is scarce. It will be necessary to conduct decisive genetic experiments in mice to link EZH2 to the H3K27 methylation status and subsequent genetic programs in relevant breast cancer models, such as mice carrying mutant Brca1 [45]. It will be necessary to explore breast cancer initiation and progression in relevant mouse models in which H3K27 methylation is disrupted. By ablating the Ezh1 and Ezh2 genes in mammary epithelium the mammary genome will lack H3K27me3 marks and upon deletion of the Utx and Jmjd3 genes a persistent H3K27me3 should be expected. Such experiments, although labor intensive and time-consuming, shall provide definitive answers on the role of H3K27 methylation in development and the etiology of cancer.

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**Conflict of Interests**

The authors have declared that no conflict of interest exists.

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