University of Cyprus
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EZH2: Role in breast cancer and potential therapeutic applications

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ABSTRACT

Human diseases, most notably cancer, are frequently associated with epigenetic dysregulation. Epigenetic modifications control gene function beyond the underlying sequence and are heritable but reversible alterations in histones or DNA. EZH2 is a critical epigenetic factor, member of the Polycomb group with H3K27me3 methyltransferase activity. Previous studies have demonstrated that EZH2 can function as an oncogene in a variety of breast cancer subtypes and that higher EZH2 expression levels are associated with more aggressive disease outcomes. By epigenetic interference with the WNT signaling pathway, EZH2 stimulates breast tumor development. Nowadays many EZH2 inhibitors are designed to specifically target either its catalytic or its non-catalytic function. This bibliographical review focuses on the significance of EZH2 in luminal B and Triple-Negative Breast cancer, as well as on two EZH2 inhibitors, namely Tazemetostat (EPZ6438) and GSK126, which target its catalytic methyltransferase activity. Targeting the catalytic domain results in a significant delay in tumor formation and inhibition of the metastatic capacity of the tumor, in models mimicking luminal B breast cancer and TNBC mouse models. EZH2 was also found to promote breast cancer bone metastases in vivo. This review also focuses on efforts to target the non-catalytic functions of EZH2, such as ZRANB1 deubiquitinase, targeting of which reduces cell migration and proliferation. Overall, EZH2 seems to be a very promising target to use in therapies against breast cancer, but further work is necessary to lead to clinical trial applications.

DEDICATION

This thesis is dedicated to my family who supported me throughout my education.

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COMPOSITION OF THE EXAMINATION COMMITTEE

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SEMINAR ANNOUNCEMENT

University of Cyprus
Department of Biological Sciences

BIO 680 Scientific Methodology in Molecular Biology

Student Presentation

Wednesday, 13 December 2023 at 10:30
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This seminar is open to the public

Maria Panayiotou

Thesis Supervisor: Special Teaching Staff, Dr. Annita Charalambous

"EZH2: Role in breast cancer and potential therapeutic applications"

Human diseases, most notably cancer, are frequently associated with epigenetic dysregulation. Epigenetic modifications control gene function beyond the underlying sequence and are heritable but reversible alterations in histones or DNA. EZH2 is a critical epigenetic factor, member of the Polycomb group with H3K27me3 methyltransferase activity. Previous studies have demonstrated that EZH2 can function as an oncogene in a variety of breast cancer subtypes and that higher EZH2 expression levels are associated with more aggressive disease outcomes. By

epigenetic interference with the WNT signaling pathway, EZH2 stimulates breast tumor development. Nowadays many EZH2 inhibitors are designed to specifically target either its catalytic or its non-catalytic function. This bibliographical review focuses on the significance of EZH2 in luminal B and Triple-Negative Breast cancer, as well as on two EZH2 inhibitors, namely Tazemetostat (EPZ6438) and GSK126, which target its catalytic methyltransferase activity. Targeting the catalytic domain results in a significant delay in tumor formation and inhibition of the metastatic capacity of the tumor, in models mimicking luminal B breast cancer and TNBC mouse models. EZH2 was also found to promote breast cancer bone metastases *in vivo*. This review also focuses on efforts to target the non-catalytic functions of EZH2, such as ZRANB1 deubiquitinase, targeting of which reduces cell migration and proliferation. Overall, EZH2 seems to be a very promising target to use in therapies against breast cancer, but further work is necessary to lead to clinical trial applications.

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INTRODUCTION

Breast cancer (BC) is the most common malignancy among women around the world. Currently, 1 in 8 cancer diagnoses globally are due to breast cancer, and there are 2.3 million new instances of the disease in both sexes combined (Arnold et al. 2022). Studies unraveling new prognostic tools and therapeutic targets are critical to the worldwide reduction of BC cases. As a consequence of the high heterogeneity that describes this disease, it is becoming more demanding to build tools that will synergistically improve the future of treatment. The treatment strategies may vary depending on the molecular subtype of cancer. There are a variety of pathways that lead to BC that still need further research. The basic molecular features that can describe breast cancer are the activation of human epidermal growth factor receptor 2 (HER2), the activation of hormone receptors (estrogen receptor and progesterone receptor), and BRCA mutations (Harbeck et al. 2019).

Historical Perspective of Breast Cancer

Humanity has known breast cancer for a long time. As breast cancer was first recorded by the ancient Egyptians and it has a very lengthy history (Bhushan et al. 2021). Although doctors back then were trying to unravel the mystery that causes this disease by assuming that it was linked with the end of menstruation. While their primary research was ongoing, they only performed surgical incisions to demolish the tumors. A more novel approach to breast cancer treatment started forming in the 19th century. William Halsted, an American surgeon, was the first to perform a radical mastectomy (Plesca et al. 2016). This set a milestone as the standard operation to treat breast cancer until the early 20th century. Improvements in mastectomy were set through the years until 1937 when scientists used for the first time the radiation therapy after a surgical operation to remove the breast. What was interesting is that after the removal of the breast and the near lymph nodes, there was a placement of a radioactive element, radium. About 40 years later, it was approved by the FDA (Food and Drug Administration) an antiestrogen drug, tamoxifen (Howell, Howell 2023). After the discovery of a new gene in rats, HER2 was linked with the aggressiveness of breast cancer and it was the main reason that this type of cancer wasn't responsive to treatments. In 1995 scientists discovered inherited mutations of BRCA1 and BRCA2 genes that were linked with increased risk of breast cancer (Godet, Gilkes 2017). A new drug that targets cells that are over-expressing HER2 was approved by the FDA in 1998, the drug was the Trastuzumab. A clearer scope of the five main

subtypes of breast cancer was given in 2013. After 6 years, in 2019, Enhertu (Trastuzumab deruxtecan) appeared to treat HER2-positive breast cancer that had metastasized, and it could not be removed with surgery. In 2020, Trodelvy (Sacituzumab govitecan) treated metastatic triple-negative breast cancer when the other available treatments weren't responsive (Goldman Rena 2020).

Symptoms

There are a variety of signs and symptoms of BC. The most common symptom patients tend to experience is a breast lump. A relatively recent study in the UK found out that 5 out of 6 women that are diagnosed with BC, tend to experience a breast lump (Koo et al. 2017). Women with non-lump breast symptoms experience nipple abnormalities, breast pain, and breast skin abnormalities. Another group of patients did not experience the common symptoms of BC, despite that they tend to experience a broader range of symptoms like axillary lump and musculoskeletal pain. Koo et al. in their study show that there is strong evidence that supports the fact that women with non-lump breast symptoms tend to elongate the diagnostic intervals (Koo et al. 2017).

Diagnosis

Since patients with smaller tumors at the time of diagnosis had a much-reduced mortality risk and greater survival rate, early detection of the disease is essential for treatment and a good prognosis (Duncan, Kerr 1976). Therefore, imaging of BC is critical to evaluate the type of therapeutic mechanisms that need to be employed. Accordingly, it's critical to note that the key to effective breast cancer treatment is early detection. (Bhushan et al. 2021). The detection, diagnosis, and clinical management of breast cancer tumors are evaluated by breast cancer imaging. Ultrasound constitutes the conventional method that plays a key role in the detection of BC, in image-guided biopsy, and lymph-node diagnosis. Furthermore, there is a variety of other regularly used methods to detect BC like mammography, scintimammography, ultrasonography, positron emission tomography (PET), magnetic resonance imaging (MRI), and single photon emission computed tomography (SPECT). If breast cancer is found, more tests are performed to determine whether cancer cells have spread within the breast or to other body areas, a process called staging of BC (Neumayer, Viscusi 2018).

Biomarkers of BC

Biomarkers are considered to be useful tools that have clinical and diagnostic potential, and their further goal is to provide and predict clinical outcomes (Strimbu, Tavel 2010). A critical aspect of breast cancer diagnosis and characterization are the marker proteins that act as biomarkers and are present within or on the surface of tumor cells. Human epidermal growth factor receptor 2 (HER2), estrogen receptor (ER), progesterone receptor (PR), and other biomarkers are crucial in the therapy of breast cancer. The accurate levels of these biomarkers can determine suitable treatments. Furthermore, the status of each biomarker is often assessed by histological examination of immunohistochemistry (IHC) stained tissue employing distinct IHC stains (Gamble et al. 2021). There are many other biomarkers that are associated with growth and proliferation (Ki-67, survivin, NGAL), invasion, metastasis (p53, EZH2, microRNAs miR-105, and miR126), EMT transition (WNT5A/B, Pea3), immune response (PD-L1), therapy resistance (HER2Δ16, pSTS3, KLK10) and survival (miR-574-3p, miR-660-5p, PIWIL3, PIWIL4) (Gamble et al. 2021).

Subtypes of BC

Gene expression profiling, which also considers the expression status of hormonal receptors like the estrogen receptor (ER) and progesterone receptor (PR), as well as human epidermal growth factor receptor 2 (HER2), and proliferation index (Ki-67), is the main basis for the molecular stratification of BC. As a result, BC is divided into five subtypes: basal-like, triple-negative, HER2-enriched, luminal ER-positive (luminal A and luminal B), and normal-like (Figure 1). Approximately 80% and 60–70% of breast carcinomas, respectively, express ER and PR. In ER-positive tumors, there is co-expression of ER and PR (ER+/PR+), and this type of tumor is more responsive to hormonal treatment than breast carcinomas that express ER+/PR- and ER-/PR+. The HER2 oncoprotein can also be detected by IHC and it is overexpressed in 20% of primary breast carcinomas (Turashvili, Brogi 2017). Despite the fact that HER2-positive breast cancer carcinomas have the worst early prognostic rate, they can be targeted and eliminated by an anti-HER2 targeted therapy. Another category of breast cancer carcinoma is the one that doesn't express ER, PR, and HER2 receptors and is referred to as

"Triple-negative" breast carcinomas (TNBC). These types of carcinomas have high heterogeneity both in histological and genetic aspects (Turashvili, Brogi 2017).

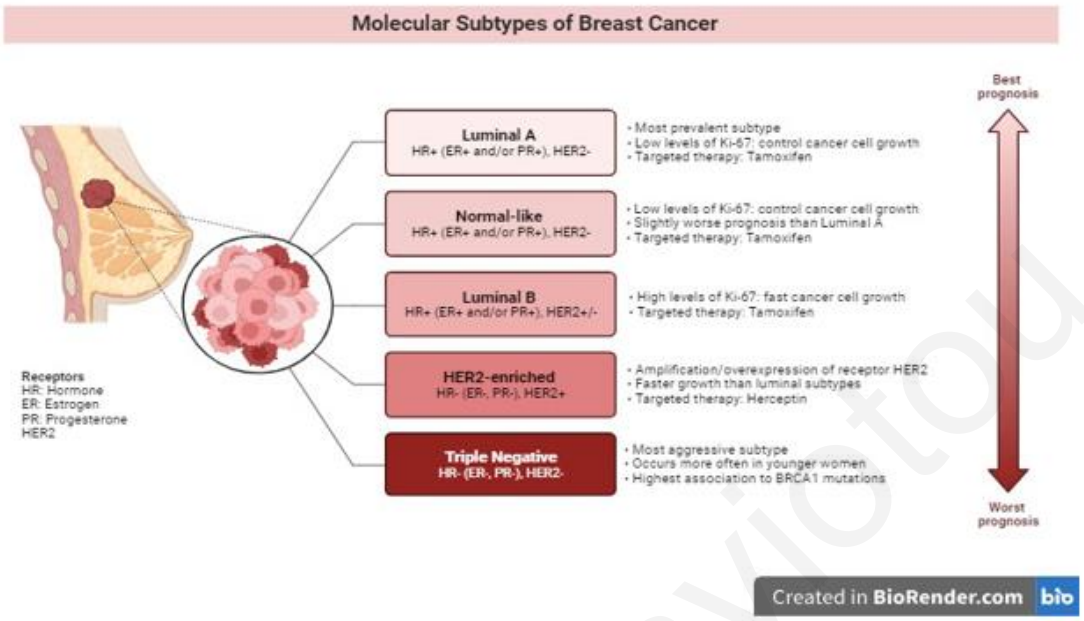

Figure 1 | Subtypes of BC are categorized based on the expression of hormone expression (ER, PR), the proliferation marker Ki-67 and the receptor tyrosine kinase HER2. The treatment options and prognosis vary among the different subtypes of this heterogeneous disease.

Genetic alterations that lead to BC

There are two significant genes that are associated with hereditary breast cancer, BRCA1 and BRCA2. BRCA 1, which is located in chromosome 17 was the first gene linked with BC. A few years after this discovery, BRCA2 was found on chromosome 13 and linked also with BC. A variety of mutations that can happen in each of these genes can alter the normal function of the wild-type gene. A large percentage of BC patients are carriers of these mutations (Shiovitz, Korde 2015). Although BRCA1 and BRCA2 aren't the only genes that can increase the risk of BC. Mutations in genes such as PTEN, TP53, CDH1, and STK11 can also lead to an 80% increase of lifetime risk of BC. A small percentage of BC cases, around 3%, result from mutations in more rare low-risk genes such as CHEK2, BRIP1, ATM, and PALB2 (Shiovitz, Korde 2015). The physiology of tumors that have arisen due to BRCA1 mutations differs. These types of tumors have a basal-like phenotype, and they have a high histologic grade, and are triple-negative tumors. Basal-like phenotypes are characterized by a high proliferative rate, aggressive growth, early recurrence after treatment, and a low chance of overall survival (Pazaiti, Fentiman 2011). On the other hand, tumors that have arisen due to BRCA2 mutations can lead to a more sporadic form of tumors. To be more precise, BRCA1 and BRCA2 genes

can act as tumor suppressor genes by being involved in double-stranded DNA (dsDNA) break repair and the mutations in these genes are inherited in an autosomal dominant manner (Pazaiti, Fentiman 2011).

Epigenetics alterations that lead to BC

Alterations at the level of gene expression, without changes in the DNA sequence, point towards epigenetic heterogeneity. Epigenetic modifications are heritable alterations in gene expression that aren't a consequence of changes in DNA sequence. Dysregulated epigenetic pathways might potentially be addressed by small molecule drugs, in contrast to genetic mutations, which are difficult to rectify (Garcia-Martinez et al. 2021). More specifically there is a variety of modifications that involve histones, the nuclear proteins that are useful for the packaging of DNA. There are many different types of epigenetic modifications such as DNA methylation, polycomb/trithorax associated proteins, short non-coding or short RNAs, and long non-coding RNAs(lncRNAs). The expression level of each gene that is being controlled by epigenetic modifications is pivotal in preserving homeostasis and more specifically in physiological development and growth. Relatively recent research discovered that breast cancer metastasis is affected and is being promoted by a series of lncRNAs, a type of non-coding RNAs. Moreover, another significant epigenetic modification that plays a key role in BC development is DNA methylation. To be more precise, DNA methylation in BC, first described by Hinshelwood and Clark, leads to enzyme-linked chemical modifications of the DNA sequence (Hinshelwood, Clark 2008). Two possible outcomes of DNA methylation in relation to the development of cancer are DNA hypomethylation, which can result in proto-oncogene overexpression, and DNA hypermethylation, which can result in suppression of tumor suppressor genes. This modification tends to be more common in specific areas of DNA in mammals, called CpG islands. In normal cells usually, the CpG islands are unmethylated whereas in cancer cells this fact changes, and they become methylated and that leads to the silencing of crucial genes like tumor suppressor genes. In breast cancer, the tumor suppressor genes that can be affected are p16INK4A, RASSF1A, ER, PR, and HER2 (Hinshelwood, Clark 2008).

The role and the levels of hypermethylation of tumor suppressor genes' (TSG) promoters are critical to study since that can lead to diverse BC clinical states. As a result of this hypermethylation is the lack of transcription and expression of the TSG products. Whereas this can lead to the development of a malignant phenotype. Although, due to the function of specific

enzymes, called Demethylases, DNA hypermethylation can be a reversible process. A new scope in the field of cancer therapy can be developed through the blockage of DNA hypermethylation and by this, re-expressing the silenced TSGs (Hinshelwood, Clark 2008).

To further understand the baseline and fundamental properties of epigenetic modifications is useful to note that DNA is packed into chromatin, forming nucleosomes, with the help of histone proteins. Histone modifications can interact with non-histone proteins, that result in the opening or closing of the chromatin states. The most fundamental part of chromatin is the nucleosome, which contains 146 base pairs of DNA wrapped around an octamer consisting of four core histones, an H3/H4 tetramer, and two H2A/H2B dimers (Li et al. 2014). There is widespread agreement that local chromatin architecture plays a key role in controlling gene expression. The N-terminal tails of histones are subject of posttranslational modifications like methylation, acetylation, phosphorylation, ubiquitination, sumoylation, ADP ribosylation, deamination, and proline isomerization. The alteration of the N-terminus of the histone tail can subsequently affect the nucleosome density and positioning and by this, makes the DNA accessible to DNA binding proteins through gene transcription initiation.

While hypoacetylation is linked to gene suppression, hyperacetylation is linked to enhanced transcriptional activity. HDAC (Histone deacetylases) has a variety of substrates like transcription-related proteins (p53, p73, E2F1, STAT1, GATA1, HMGB1, YY1, and NFkB), hormone response proteins (GR, ER, and AR), nuclear transporters (Importin7), WNT signaling proteins (catenin), DNA repairing proteins (Ku70) and heat shock proteins (HSP90). Another significant protein that holds a critical role in BC is CDH1, which is known as E-cadherin, which is a cell adhesion protein that has been described in many human malignancies' cases and among them in breast cancer. For breast cancer diagnosis, prognosis, and therapy to improve further, it is essential to comprehend all these epigenetic alterations and their function in breast carcinogenesis. There is a variety of breast cancer genes that are hypermethylated and included in Table 1. The discovery of long-range silenced genes caused by epigenetic changes has opened up new avenues for the study of epigenetic cancer (Li et al. 2014).

TABLE 1 Breast cancer genes that are hypermethylated.

Genes	Function
BRCA1 Wu et al. (2010)	DNA damage repair
APC Saelee and Pongtheerat, (2020)	Catenin, cell proliferation, migration, and adhesion inhibitor
GSTP1 Kanwal et al. (2014)	Prevention of oxidative DNA damage by conjugation to glutathione
Cyclin D2 Evron et al. (2001)	Regulators of CDK kinases
PTEN Ramadan and Hashmin, (2021)	Regulating the AKT/PBK signalling pathway negatively
$p16^{INK4\alpha}$ Hui et al. (2000)	Cyclin-dependent kinase inhibitor
RASSF1A Li et al. (2019)	Ras effector homologue, cell cycle arrest
RARβ Wang et al. (2020)	Retinoic acid receptor
ZMYND10 Wang et al. (2019)	Inhibitor of cancer cell colony formation

Estrogen subtypes and mechanisms of ERα signaling

Estrogen, a steroid hormone, is involved in different processes that affect the homeostasis of the body and mostly leads to reproductive maturation. More specifically estrogen correlates mitogenic and epigenetic mechanisms to control mammary gland development. The main estrogen subtypes are five. E1 that is converted to E2, the more biologically active form and they form the main estrogens in the body. E3 and E4 are distinguishable only during pregnancy. E1s is transformed easily into E1 and E2 with the help of steroid sulfatases (Garcia-Martinez et al. 2021). E2 is the main substrate of ERα. ERα consists of a number of useful domains influence its transcriptional and epigenetic activities inclusive of the N-terminal activation function 1 (AF1), hinge domain, activation function 2 (AF2) within the C-terminal ligand-binding domain (LBD), and the DNA-binding domain (DBD) (Figure 3a). The AF1 domain can be phosphorylated by mitogenic kinases in order to change ERα transcriptional activity. When the E2 substrate binds to the receptor conformational change at helix 12, it specifically binds to LBD where it also gets dimerized. AF2 is the domain that is responsible for the main transcriptional activation. The nuclear localization signal can be found in the hinge domain, between N-terminal AF1 and C-terminal LBD. This signal is useful in order for the ERα receptor to be properly localized to the nucleus. DBD makes possible the binding of ERα to a specific domain of DNA, known as estrogen response elements (EREs). Following E2 stimulation, ER homodimerization and recruitment to chromatin, either directly to EREs or indirectly by anchoring to transcription factors (e.g., JUN, SP1, FOS, and NF-kB) via AF domains, are the first steps in genomic-mediated processes (Garcia-Martinez et al. 2021)(Figure 2).When stimulated by E2, pioneer transcription factors (TFs) such as FOXA1, GATA3, PBX1, and AP-2 bind particular DNA target sequences in condensed chromatin and make ER-chromatin binding easier. In order to regulate the transcription or repression of genes,

activated ER may also recruit a group of coactivators or corepressors (Garcia-Martinez et al. 2021).

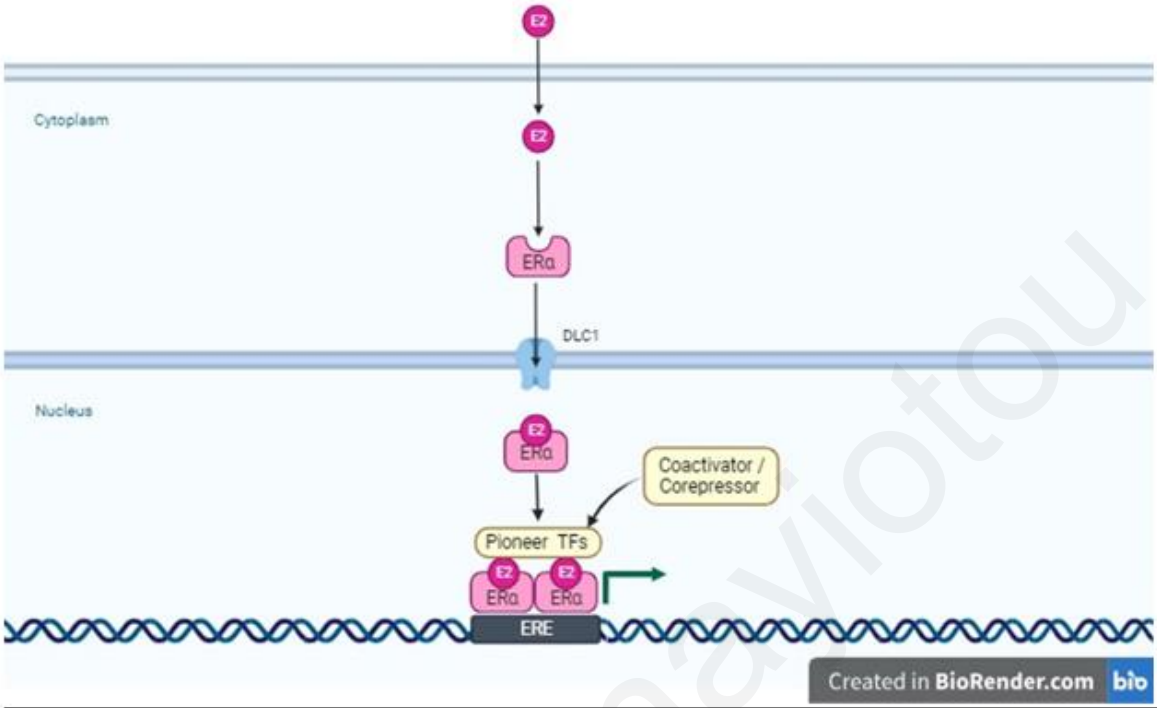

Figure 2 | Schematic representation of the E2 substrate binding to ERα receptor inducing a conformational change and finally getting dimerized. The recruitment of the complex to chromatin can be done directly to EREs or indirectly by anchoring to pioneer TFs. In order to regulate the transcription or repression of genes, activated ER may also recruit a group of coactivators or corepressors.

Epigenetic mechanisms underlying ERα signaling

To guarantee the correct transcriptional and repressive activity at ER target sites, hundreds of ER coregulators are recruited to the chromatin in a highly coordinated way upon E2 stimulation. ERα also has the ability to switch on and off the chromatin in a matter of hours and even minutes after the stimulation by its substrate, E2 (Garcia-Martinez et al. 2021). There are numerous ERα coactivators such as p160 family, P300/CBP, SWI/SNF complex, PRMTs, and the Mediator complex. Members of p160 family of activators, SRC-1, SRC-2, and SRC-3, can directly bind ERα and recruit chromatin remodeling complexes and activating enzymes in order to alter epigenetic profile at specific enhancers and promoters. An example of such protein that can be recruited specifically by SRC-3 is the P300 a histone acetyltransferase (HAT) that is responsible for H3K27ac (Figure 3b) (Garcia-Martinez et al. 2021).

ERα transcriptional activity corepressors including NCoR1, NCoR2, and LCoR are responsible for recruiting epigenetic repressors closer to ERα to proceed to the downregulation of E2-repressed genes. It's noteworthy that the most prominent ERα corepressor is the BRCA1. After BRCA1 binds to the AF2 domain of ER can subsequently target the receptor to lysosome for degradation by the addition of ubiquitin molecules on it (Garcia-Martinez et al. 2021). Pioneer TFs have the ability to bind condensed chromatin directly (Lemma et al. 2022) and are essential for E2-dependent ERα recruitment to chromatin. The depletion of these factors can lead to reduced E2-induced ERα chromatin binding. Relatively recent research has shown that PRC1 and PRC2 can do E2-dependent chromatin recruitment and can increase E2-induced ERα target gene expression in breast cancer cells (Garcia-Martinez et al. 2021).

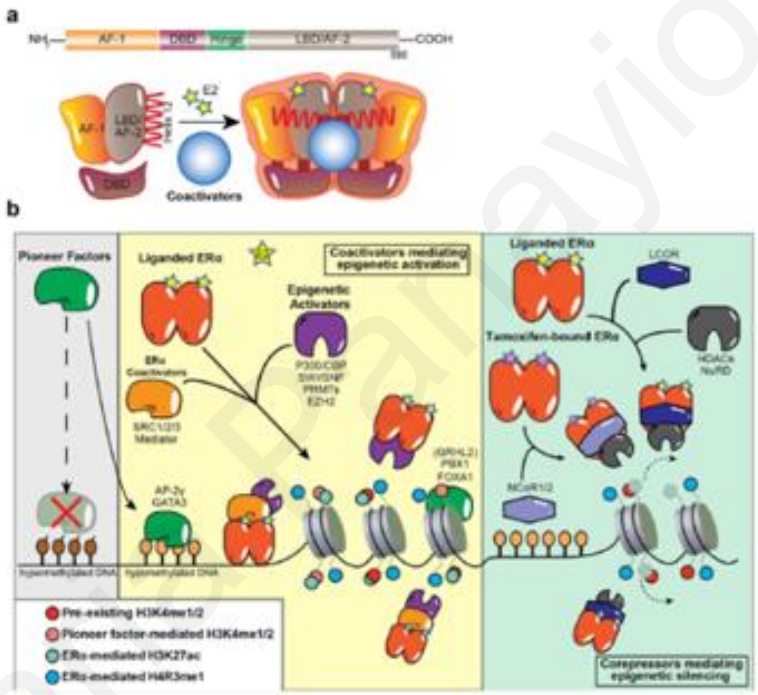

Figure 3 | ERα binding to the ER leads to a variety of epigenetic changes and interactions with co-regulators (co-activators or co-suppressors) and pioneer TFs.

a. Different structural domains of the estrogen receptor (ERα) are shown schematically. The LBD has surfaces for both coactivator binding and dimerization. Helix 12 in the LBD changes to an active shape upon binding E2, promoting ERα interaction with coactivators.

b. Pioneer factors that preferentially bind to hypomethylated genomic regions include FOXA1, GATA3, PBX1, and AP-2γ. Pioneer TFs help liganded ERα localize to the chromatin upon activation with the E2 ligand. ERα engages epigenetic activators such as P300/CBP, the SWI/SNF complex, PRMTs, and EZH2, which in turn activates gene expression.

Adapted from Garcia-Martinez et al. 2021.

Epidrugs in BC therapy

Epigenetic-based diagnostic and prognostic techniques are crucial to precision medicine. A variety of DNA methylation tests are being tested and some of them are already in use. Epidrugs, or drugs that target epigenetic modulators, were created as a result of precision oncology attempts to treat dysregulated epigenetic pathways. More specifically, epidrugs are chemical substances that modify the DNA and chromatin structures in order to activate tumor suppressors and DNA repair genes that have been epigenetically silenced (Kim et al. 2023). The two epigenetic mechanisms that are known and targeted by Epidrugs are the HDACs and the DNMTs. Drugs like these tend to inhibit the normal function of HDACs and DNMTs (Cheng et al.2019). To be more precise, DNMTis like azacitidine and decitabine which are approved by the FDA are broadly used in cancer therapeutics and they inhibit the methylation of CpG dinucleotides. The deacetylation of acetylated histone and by this the inhibition of gene transcription can be done by HDAC inhibitors (Kim et al. 2023). HDACs tend to overexpress in cancer cells, and they are responsible for the unwrap of specific regions of histones around DNA and this is leading to the suppression of many tumor suppression genes that are involved in aggressive proliferation, metastasis, and overall survival of cancer cells. Also, HDACis promote gene transcription by suppressing HDACs, which ultimately results in cell death. Only nine epidrugs were under the approval of the FDA for solid and hematological tumors (e.g., DNMTs, EZH2i and HDACis). It's noteworthy that in the ER+ breast cancer phase II clinical trials, scientists are exploring the efficacy of combinatorial use of Epidrugs with normal treatment in ER+ BC. The primary method used to treat ER+ BC in endocrine-based therapies like ER-blockade, estrogen synthesis inhibition, and selective ER degradation (Mathur et al. 2022).

Nowadays, studies also use the conventional therapy of tamoxifen in combination with HDACis to act synergistically to primarily induce apoptosis in ER-positive breast cancer cells. A specific HDACis Vorinostat resulted in the inhibition of TNBC cells by regulating the overexpression of miRNA expression that finally can lead to the expression of tumor suppression genes (Patra et al. 2022). Su et al. looked at the potential benefits of employing DNMTi to specifically target EMT for the treatment of TNBC. By preventing cell growth, such as by causing cell cycle arrest, DNMTi showed anticancer effects (Su et al. 2018).

While most breast cancers are expressing ERα the main therapeutic approaches are aiming to target this pathway. Even though endocrine therapy has a wide successful application in BC

treatment there are ongoing drawbacks like therapeutic resistance that leads to an unfortunate recurrence of the disease. This resistant phenotype is a result of alteration of many epigenetic factors and a switch in the chromatin landscape (Wang et al. 2023). This leads to the conclusion that epigenome plays a key role in hormone therapy response and epigenetic factors are promising targets to conquer these drawbacks in clinical therapy (Wang et al. 2023).

WNT signaling pathway in BC

Moving on to discuss the epigenetic processes in normal mammary gland development that are derailed in breast cancer. Until recently, it wasn't entirely clear what function WNT signaling plays in the development of the mammary gland and the onset, maintenance, and progression of breast cancer. Numerous signaling pathways, chromatin regulators, and hormonal factors cues that balance of self-renewal, differentiation, and tissue integrity all contribute to the development of the mammary gland (Xu et al. 2020). While the pubertal and reproductive phases are regulated by hormones, signaling pathways including WNT and Hedgehog (HH) coordinate the development of the embryonic mammary gland. Reactivation of developmental pathways is a characteristic of many cancer types and, in breast cancer, is intricately linked to the preservation of the mammary gland stem cell population. Abnormal WNT signaling activation can lead to the development of a number of cancer types as well as breast cancer. Breast tumorigenesis can be initiated by the epigenetic silencing of WNT antagonists (SFRP and DKK). The silencing of these genes is conducted via DNA methylation and is associated with poor prognosis. These modifications can lead to the activation of β-catenin emerging stem cell renewal and proliferation that can be related with the setback of the disease. Polycomb proteins connect the ER pathway to the WNT signaling pathway more specifically, EZH2 serves as a key link between the WNT and ER signaling pathways. It's interesting to note that using substances like 5-azacytidine and trichostatin A allows DKK3 expression to be recovered *in vitro*. However, clinical attempts to restore ER expression with hypomethylating drugs have been unsuccessful (Abreu de Oliveira et al. 2022).

Many features of tumorigenesis are very similar to embryonic development. The deregulation of developmental signaling pathways is frequent in several cancer types. It has been demonstrated that the intracellular reactions brought on by Wnt ligands may be divided into dependent signaling (the canonical WNT pathway) and independent signaling (the non-canonical WNT pathway). There are a variety of Wnt proteins that bind to specific receptors and can therefore activate downstream pathways. In this study, our goal is to study the

canonical WNT signaling pathway that has a crucial role in breast cancer proliferation and the preservation stemness profile that cancer cells are characterized by (Xu et al.2020).

Canonical WNT signaling pathway

The canonical WNT signaling depends on β-Catenin and T cell factor (TCF)/lymphoid enhancer factor (LEF). The receptors for Wnt proteins are 7 transmembrane proteins called Frizzleds (Fzds) and the coreceptors called lipoprotein receptor-related proteins (LRPs) are single-pass transmembrane proteins. WNT signaling can remain in a switched-off state in the absence of Wnt proteins (Xu et al.2020). The main element of canonical WNT signaling, β-Catenin, interacts with the cytoplasmic tail of E-cadherin to promote cell-cell adhesion. CK1 phosphorylates β -catenin at Ser45 initially, then GSK-3 phosphorylates it at Thr41, Ser37, and Ser33 residues. Ser33 and Ser37 are phosphorylated and act as docking sites for β-TrCP, which can be then degraded. Axin is made less stable by Tankyrase 1/2 (TNKS1/2), which makes it a desirable target for the control of WNT signaling. Furthermore, Siah-1 engages in an interaction with APC and facilitates the degradation of β-Catenin without the assistance of GSK-3-mediated phosphorylation or β-TrCP-mediated ubiquitin (Liu et al. 2022).

WNT signaling alterations in BC

Many of the components of the WNT signaling are modified in breast cancer cells. At the DNA level, there is a variety of modifications, among them mutations, deletions, and methylation of DNA sequences that have an impact on posttranscriptional modifications. These modifications hold a strong impact on the subcellular locations of β-Catenin can be found in. Furthermore, activation of WNT signaling plays a crucial role in the development of breast cancer mainly through epigenetic activation of Wnt proteins and inactivation of Wnt inhibitors (Xu et al. 2020). It has been found that the Wnt5a ligand is lost in breast cancer. Additionally, breast cancer patients with TNBC and basal-like breast cancer (BLBC) have higher levels of the majority of canonical and noncanonical Wnt receptors. In BLBC and TNBC, E-cadherin, a β-Catenin interacting protein, is commonly altered or silenced, allowing β-Catenin to escape the cytomembrane and enter the cytoplasm (Xu et al. 2020). The cytoplasmic destruction complex should carefully regulate cytoplasmic β-Catenin. However, the components of the destruction complex are commonly altered, such as by mutation, deletion, hypermethylation, or reduction, in breast cancer, increasing the stability of cytoplasmic β -Catenin and the likelihood that it will

reach the nucleus. In breast cancer, the majority of coactivators are expressed extensively. However, it's intriguing that breast cancer has higher levels of several corepressors (such TLE/GRG and CTBP). These data demonstrate that rather than β -Catenin or APC mutations, the activation of canonical WNT signaling in breast cancer is mostly caused by epigenetic changes in the constitutive components (Xu et al. 2020).

EZH2 as a novel epigenetic target in treating BC

Enhancer of zeste 2 polycomb repressive complex 2 subunit gene encodes the enhancer of zeste homolog 2 (EZH2) protein that is a member of Polycomb group (PcG) protein complexes. PcG protein complexes have the ability to methylate histones in order to repress transcription initiation. The PcG complexes are divided into two main groups in mammalian cells the PRC1 (Polycomb repressive complex 1) and PRC2 (Polycomb repressive complex 2). EZH1 or EZH2, EED (Embryonic Ectoderm Development), Zeste 12 inhibitor (Suppressor of Zeste 12, SUZ12), belong to PRC2 (Liu, Yang 2023).

More specifically the catalytic subunit of PRC2 is EZH2, which is mainly responsible for the methylation of H3 histone. The action modes of EZH2 can be divided into three main groups. First of all, as referred before it catalyzes the H3K27me3, a function that is PCR2 dependent that leads to transcriptional silencing. A second capacity that EZH2 has is that it can methylate many non-histone protein substrates like STAT3 and GATA4 resulting in either transcriptional activation or in transcriptional silencing. EZH2 co-activates transcription factors such as the AR-associated complex, NF-B signaling, TCF/β-catenin and PCNA, and β-catenin and ERα in a manner that is PRC2-independent (Gan et al. 2018).

The EZH2 gene, which has 20 exons and may code for 746 amino acids, is found on chromosome 7q35. Also, EZH2 consists of many domains critical for its function such as the EED interaction Domain (EID), Domain 1, Domain 2, Cysteine-rich domain (CXC Domain), and the Enhancer domain of zeste and trithorax (SET Domain). The methyltransferase active site can be found in the SET domain. Due to the ability of EZH2 to activate or inhibit the transcriptional activity of specific genes through histone methylation patterns, is usually linked to several human diseases such as cancer. More specifically EZH2 is responsible for the trimethylation of H3 histone at 27 lysine and by this, it can switch the structure of chromatin and finally, it can affect and eliminate gene transcription. Furthermore, EZH2 has a broad range of targets that can directly methylate such as GATA4, STAT3, β-catenin, and the lysines at

positions 510, 514, and 515 of PRC2. It can also interact with TCF, β-catenin, and ER to activate the c-Myc and cyclin D1 genes, which are located downstream. EZH2 also affects gene transcription via binding to the promoter region of target genes, which is frequently seen in the c-Myc and Notch1 genes (Figure 4). PRC2 regulates normal cell differentiation and proliferation and has been linked to context-dependent oncogenic and tumor-suppressive activities in cancer.

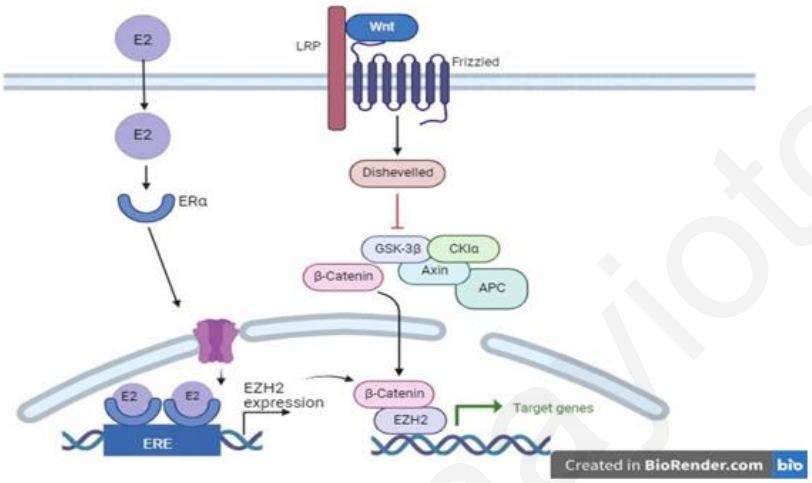

Figure 4 | Epigenetic mechanisms that are regulated by E2 and WNT signaling in BC. One of the key signaling routes in the development of tumors is WNT signaling. By attaching to the E2-estrogen receptor (ER), EZH2 is expressed and links WNT and estrogen signaling.

Genetically engineered mouse models (GEMMs) as BC models

To further discuss the BC mouse models this section will focus on GEMMs. Scientists designed a beneficial tool to study and investigate BC in mice, the GEMMs. The main goal of using traditional transgenic mice for breast cancer research is to overexpress a tumor-associated microRNA or the coding region of an oncogene (mutant or wild-type) under the control of a mammary-specific promoter. It is feasible to determine if the uncontrolled expression of these oncogenes is sufficient to trigger neoplastic transformation and the development of breast tumors by upregulating their levels in the mammary epithelium (Pfefferle et al. 2013). The degree of expression attained through the chosen promoter and the potency of the chosen oncogene can frequently affect tumor latency and penetrance (Usary et al. 2016).

Mammary tumor induction by specific promoters and CRE expression

In mice models of breast cancer, transgenic expression of proto-oncogenes or genetic deletion of tumor suppressors cause mammary carcinogenesis. Proto-oncogenes are expressed in

spontaneous breast cancer mice models under the guidance of mammary-specific promoters, including C3, β-lactoglobulin (BLG), whey acidic protein (WAP), and the long terminal repeat of the murine mammary tumor virus (MMTV-LTR) (Regua et al. 2021). Many oncogenes, including Myc, Ha-ras, Wnt, the polyomavirus middle T antigen (PyMT), and Her2/neu have been overexpressed using the MMTV-LTR. At specific latency periods, the expression of all these oncogenes leads to the development of breast tumors. One way to investigate the ongoing significance of transforming oncogenes in cancer progression is to combine animal models that allow for the temporally and spatially regulated regulation of transgenes. Tetracycline (Tet)-based methods have been effectively employed among other ligand-inducible transgenic technologies to regulate transgene expression in a variety of cell types and tissues *in vivo*, including the mammary gland. There are two different types of Tet-controlled transactivator systems, the Tet-OFF and the Tet-ON system. For the purposes of this study will only discuss the Tet-ON system. The reverse transactivator (rtTA), which is part of the Tet-ON system, is a mutant tetracycline repressor domain that becomes active when Tet or Dox (doxycycline)is present (Sakamoto et al. 2015).

Sakamoto et al. in their study tried to create a novel and enhanced transgenic mouse strain that allows transgenes to be expressed at certain times throughout the development of the mammary gland. Only the salivary gland and mammary epithelial cells display high levels of the tetracycline-regulatible transactivator (tTA), which is regulated by the mouse mammary tumor virus long terminal repeat (MMTV-LTR). Responder transgenes are transactivated over time in the new MMTV-tTA mouse strain, but this transactivation may be quickly reduced by administering doxycycline (Dox). According to their findings, exogenous proteins may be expressed in multipotent mammary progenitors from the very beginning of the formation of the mammary gland in order to evaluate their biological importance throughout mammogenesis using the innovative MMTV-tTA (Sakamoto et al. 2012).

Mammary tumor induction by virally-derived oncogenes

The formation of mammary tumors in GEMMs can be driven by the viral oncogene Polyomavirus middle T antigen (PyV mT). These tumors are similar to luminal B breast cancer. This model can highlight the activation of oncogenic signaling pathways involved in BC development like the PI3K-mTOR and Ras-MAPK pathways. The significance of these GEMMs is high of relevance due to their ability to mimic the premalignant stages of invasive and metastatic tumors exactly like a human breast tumor microenvironment (Liu et al. 2023).

Rao et al. in order to investigate Cre recombinase-mediated genetic alterations concurrently, have effectively developed a temporally controlled mouse model of PyV mT-mediated mammary tumorigenesis. The utility of this strain comes from the linking of PyV mT and Cre recombinase transgenes, which preserve all of the distinguishing characteristics of the well-established constitutive MMTV-PyV mT model. This forces mammary epithelial cells to couple PyV mT expression with conditional elimination of a specific gene (Rao et al. 2014).

OVERVIEW

It is well established that epigenetic dysregulation plays a key role in the contribution of epithelial to mesenchymal (EMT) transition and in tumor resistance. Even though the application of first-generation epigenetic drugs(epi-drugs) in solid tumors has been discouraging, there is mounting evidence that these epi-drugs can in combination with other therapeutic modalities play a significant role in cancer therapy (Wang et al. 2023). There are many improvements that need to be made in order for the epi-drugs to overcome the drawbacks of their usage. When it comes to breast cancer, mortality has decreased as a result of improved therapy regimens but there are still numerous challenges that need to be faced. Among them are severe side effects, the different tumor subtypes, and the intratumor heterogeneity. Eliminating cancer stem cells (CSCs), which are critical for metastasis and the emergence of multidrug resistance to treatment, is the toughest challenge in the fight against BC (Buocikova et al. 2020). Notably, many previous studies showed that epigenetic factor, EZH2 can act as an oncogene in many BC subtypes and the aggressiveness of the disease is linked to the elevated levels of expression of EZH2. The relevance of examining EZH2's function in each molecular subtype of breast cancer is highlighted by the varied biological characteristics of the many breast cancer subtypes (Liu et al. 2023).

Involvement of EZH2 in the Initiation of Breast Tumors

Liu et al. started first to genetically abolish the EZH2 factor to have a clearer view of the significance of this factor in tumor initiation. They crossed a conditional EZH2 knockout mice (Ezh2fl/fl) with MMTV-rtTA and the PyVmT-IRES- CRE (MIC) transgenic strains in order to ablate EZH2 expression in PyV mT-expressing cells (Figure 5A). Afterward, they confirmed the excision of EZH2 protein in MIC/Ezh2fl/fl (mutant) mammary epithelia. Whereas when control MIC mice were administered with doxycycline for 14 days there was huge expression of PyV mT and there was plenty of luminal filling in the mammary epithelium with proliferating PCNA-positive tumor cells (Figure 5B, C). As opposed to EZH2-proficient controls, genetic deletion of EZH2 dramatically decreased luminal filling and epithelial cell proliferation (Figure 5D) (Liu et al. 2023).

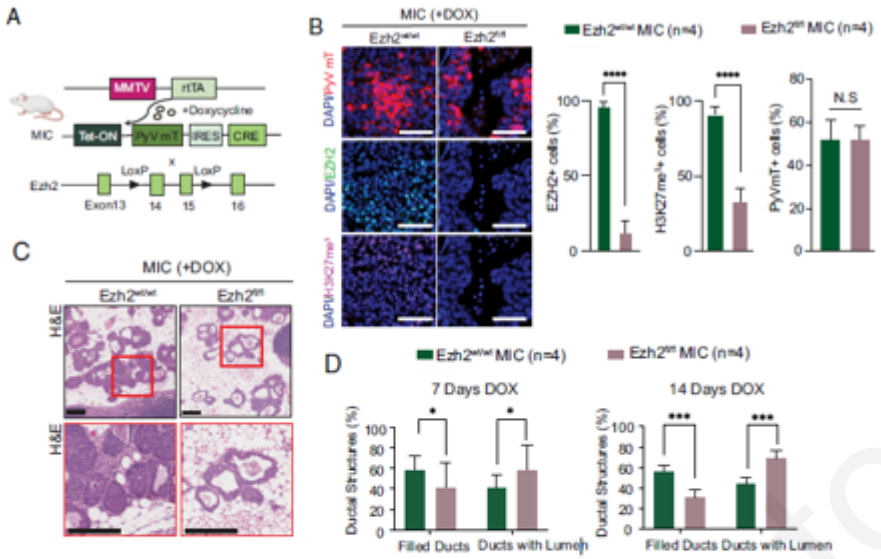

Figure 5 | Tumor initiation is inhibited by genetic deletion of EZH2, which increases tumor proliferation.

A. Diagram showing the conditional EZH2 model and the PyV mT-IRES-CRE (MIC strain) inducible transgenic mouse model with doxycycline (DOX).
B. Immunohistofluorescence panels and scale bars showing PyV mT, EZH2, H3K27me3, and DAPI staining (for nuclear staining) of MIC wild-type (Ezh2wt/wt) and Ezh2fl/fl mammary glands following two weeks of DOX induction.
C. After 14 days of DOX, representative hematoxylin and eosin staining pictures of the mammary glands in Ezh2wt/wt and Ezh2fl/fl.
D. Measurement of luminal fullness or retention in the ducts in Ezh2wt/wt and Ezh2fl/fl MIC mice at 7 and 14 days after DOX.

Adapted from Liu et al. 2023.

EZH2 methyltransferase activity in luminal B BC model

Then, Liu et al. developed an *in vitro* organoid culture system employing primary mammary epithelial cells that replicated the early phases of MIC tumor formation in order to directly evaluate the effect of epithelial ablation of EZH2 on mammary tumor progression (Figure 6A). In a similar manner, after the administration of doxycycline, the MIC organoids didn't express the EZH2 alleles in Ezh2fl/fl organoids. To further investigate the effect of abolishing EZH2 expression, they measured the diameter of organoids, their proliferative capacity, and their polarity and observed that these were significantly reduced. Their results were in agreement with the *in vivo* observations. They treated MIC/Ezh2wt/wt organoids with two chemically unrelated EZH2 methyltransferase inhibitors (GSK126 and EPZ6438) to see if this phenotype represented the loss of the methyltransferase activity of EZH2. This resulted in the reduction of H3K27me3 methylation, and the size of the organoid diameter decreased significantly (Figure 6D). These findings support the suggestion that EZH2's methyltransferase activity is necessary for tumor development in the luminal B BC model (Liu et al. 2023).

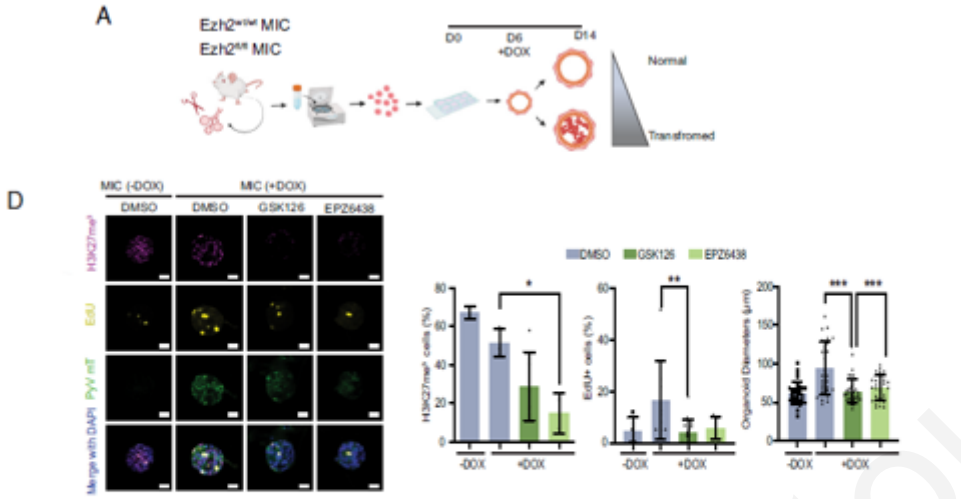

Figure 6 | Proliferation is suppressed by genetic or pharmacological suppression of EZH2.

A. Diagram showing the use of mammary epithelial cells taken from Ezh2wt/wt and Ezh2fl/fl MIC mice to create an *in vitro* 3D organoid model of tumor initiation.

D. H3K27me3, EdU, PyV mT, and DAPI were quantified (showed in bars) and stained (showed in panels) in immunofluorescence for Ezh2wt/wt MIC organoids treated with GSK126, EPZ6438, or DMSO for 8 days, with or without DOX.

Adapted from Liu et al. 2023.

EZH2 alterations in WNT signaling-related gene expression

After Liu et al. performed an RNA sequencing analysis on samples from EZH2-proficient and EZH2-deficient MIC organoids, a bioinformatic analysis was followed. Between the results of this analysis, it was found that a variety of genes were upregulated in EZH2-deficient MIC organoids and among them, regulators of WNT signaling, including SFRP1, SFRP2, and DKK2. Through a qRT-PCR validation of the results, the SFRP1 transcript was expressed at a much greater level in EZH2-deficient organoids whereas, in the same organoids, there was a significant decrease in Axin2 and c-Myc levels, in comparison with their EZH2-proficient counterparts (Figure 7C). After the pharmacological inhibition with GSK-126 and EPZ6436, the results also showed elevated levels of SFRP1 mRNA transcript. The same result was observed in Ezh2wt/wt MIC organoids that were pharmacologically treated with GSK126 and EPZ6438 (Figure 7D). From these results, we can conclude that SFRP1 is a direct epigenetically transcriptional target of EZH2 that is involved in tumor growth. *In vivo*, Liu et al. also saw that the major WNT pathway regulator, β-catenin, and the protein expression of c-Myc were both markedly downregulated (Liu et al. 2023).

In the same study, they next validated the previous results on primary mammary tissues from EZH2-proficient and EZH2-deficient MIC mice. Liu et al. demonstrated that, in comparison to

wild-type controls, the SFRP1 transcript was much higher in Ezh2-deficient hyperplasias using RNA fluorescence *in situ* hybridization (FISH) analysis (Figure 7F). Consequently, they also demonstrated that loss of H3K27me3 in mammary epithelial cells was connected with increased expression of Sfrp1 protein in Ezh2-deficient hyperplasias as opposed to Ezh2-proficient controls. According to these findings, SFRP1 is essential for controlling cell polarity, which is a fundamental feature of cellular transformation and is controlled by WNT signaling pathways in the development of the mammary gland and the development of cancer. Taken together, the findings of this study suggest that SFRP1 depletion occurs early in the course of breast cancer (Liu et al. 2023).

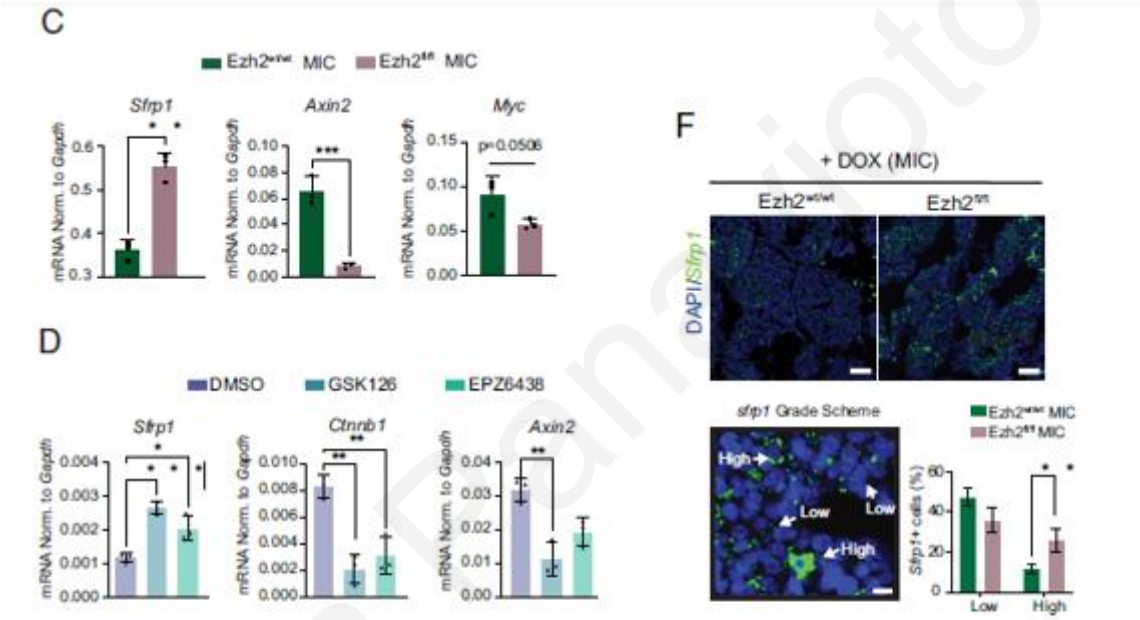

Figure 7 | WNT signaling pathways are changed by loss of EZH2 function.

C. Sfrp1, Axin2, and Myc (c-Myc) transcript levels in Ezh2wt/wt MIC and Ezh2fl/fl MIC organoids were measured using quantitative RT-PCR.

D. Sfrp1 and Axin2 transcript levels were measured by quantitative RT-PCR in Ezh2wt/wt MIC organoids treated for 8 days with GSK126, EPZ6438 or DMSO, and DOX.

F. Sfrp1 in Ezh2wt/wt MIC and Ezh2fl/fl MIC mammary glands was found using RNAscope in situ hybridization (ISH) following a two-week DOX induction stage.

Adapted from Liu et al. 2023.

EZH2 activity in TNBC cell invasive phenotype

After conducting a bioinformatics investigation of genes linked to epigenetic modifications in a sizable group of patients with breast cancer, Yomtoubian et al. discovered EZH2 as a putative cause of TNBC metastasis. To ascertain the causes and therapeutic potential of EZH2 in TNBC metastasis, they precisely suppressed EZH2 catalytic activity using selective pharmacological

agents and a precise complementary genetic strategy. According to their research, EZH2 inhibition prevents metastasis by transforming the metastatic phenotype of EZH2 high population into a phenotype more similar to that of a luminal (Yomtoubian et al. 2020).

Previous studies tried to abolish the EZH2 in breast cancer cells with DZNep (Kim, Roberts 2016). Nevertheless, these methods caused the PRC2 holoenzyme to become unstable, which makes it difficult to identify the precise roles that EZH2 HMT activity plays (Tan et al. 2007). For this reason, Yomtoubian et al. used GSK-126 as Liu et al. also used in their study. GSK-126 is a small molecule drug, which selectively targets only the EZH2 HMT and does not affect the stability of PRC2. Using the metastatic human TNBC cell line MDA-MB-231-LM2 (LM2), GSK-126 suppressed EZH2-mediated H3K27me3 in a dose-dependent manner without changing the amounts of EZH2 and H3K4me3 (Figure 8A). This inhibition of the EZH2 protein resulted also in decreased cell invasion and migration (Figure 8 B, C). These findings implied that EZH2 catalytic activity specifically suppresses TNBC cell invasiveness *in vitro* and may influence TNBC metastasis *in vivo* (Yomtoubian et al. 2020).

EZH2 involvement in distant metastasis in TNBC

Yomtoubian et al. in their study highlight that the inhibition of EZH2 protein influenced primary tumor growth and impeded distant metastasis. In order to ascertain how EZH2 inhibition affects TNBC development *in vivo*, they created luciferase and mCherry reporter transgene-expressing LM2 orthotopic tumors. After the GSK-126 administration, the drug engaged its target and led to a reduction of H3K27me3 in primary tumors but no effect on tumor size was observed (Figure 8D, E). Remarkably, there was a significant decrease in lung metastasis when compared to the control group (Figure 8F). These findings highlight the possibility that EZH2 inhibition targets a particular cellular compartment that may have invasive or metastatic capacity (Yomtoubian et al. 2020). Yomtoubian et al. also wanted to test the inhibition of EZH2 by producing a mutant form of EZH2, more specifically by using CRISPR/Cas9 to form a mutation in the catalytic center of EZH2, the SET domain. The conclusions gained from the catalytically inactive EZH2 mutant clones indicated that metastasis and EZH2 catalytic activity are closely related, and they also corroborated the specificity of the pharmacological findings (Yomtoubian et al. 2020).

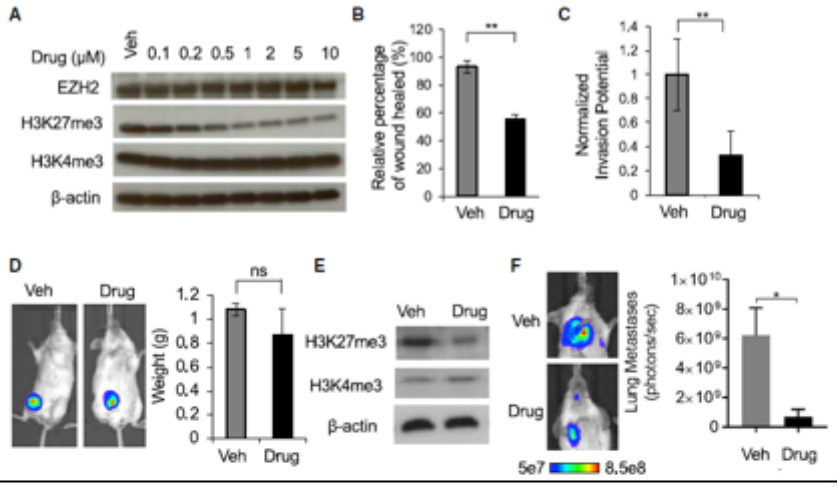

Figure 8 | TNBC Has Enhanced EZH2 Expression, which Aids in Invasion and Metastasis.

A. Western blot results demonstrating the medication GSK-126's dose-dependent impact on EZH2-mediated H3K27me3 in LM2 cells. b-actin was a loading control.

B. Quantitative evaluation of GSK-126 or vehicle-treated LM2 cells' migration.

C. Quantitative evaluation of LM2 cells treated with vehicle or GSK-126 for five days using matrix-coated transwell to determine their propensity for invasion.

D. BLI (Bioluminescence imaging) pictures of orthotopic LM2 primary tumors in severe combined immunodeficiency (SCID) mice receiving either GSK-126 or control treatment.

E. H3K27me3 and H3K4me3 Western blot analysis of primary tumors taken from D panel.

F. A sample of BLI photos showing lung metastases in mice from the D panel after treatment and pulmonary metastasis quantification.

Adapted from Yomtoubian et al. 2020.

Then the same group of scientists looked at tumor cell dissemination to better understand how EZH2 inhibition affects metastasis. The results implied that EZH2 plays a crucial role in TIC (Tumor Initiating Cells), also known as cancer stem cells (CSCs), maintenance. To find this they used a lentivirus-based stem cell reporter system in which repeats of stem cell TFs, SOX2 and OCT4, lead to the expression of the copepod GFP reporter (copGFP) transgene, which permits analysis of quick or transient promoter activity. GFP+ cells showed increased expression of SOX2, OCT4, NANOG, EZH2 and H3K27me3 (Figure 9C, D). Notably, the higher expression of EZH2 and H3K27me3 in GFP+ cells indicated that these cells could be part of a distinct subpopulation that is susceptible to EZH2 inhibition. Actually, inhibition of EZH2 by the GSK-126 drug led to the reduction of SOX2, OCT4, and NANOG in GFP+ cells. Altogether, this data suggested that the EZH2-mediated disseminating cell compartment in primary tumors is composed of SOX2/OCT4-GFP+ EZH2 high tumor cells and that EZH2 suppression leads to a decrease in these cells in the primary tumor, peripheral blood, and metastatic lungs (Yomtoubian et al. 2020).

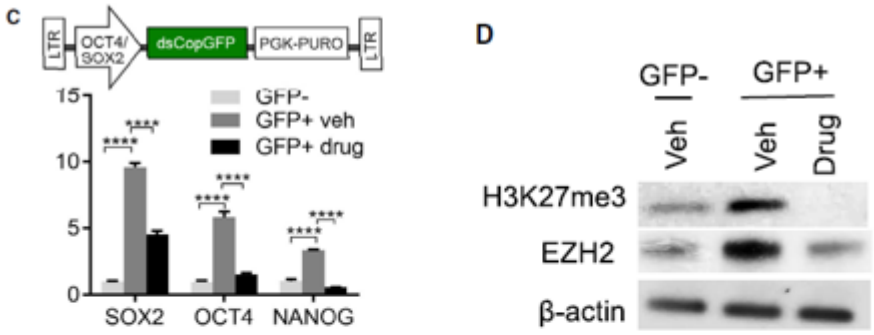

Figure 9 | An EZH2-Sensitive Population in TNBC Is Found Through Genetic Marking of TIC-like Cells.

C. Top panel: Diagram of the lentiviral vector containing the SOX2/OCT4-GFP promoter reporter system Bottom panel: qRT-PCR results of the TIC transcription regulators' mRNA gene expression (SOX2, OCT4, NANOG) in SOX2/OCT4-GFP+ vehicle-treated and GSK-126 drug-treated and GFP tumor cells.

D. EZH2 and H3K27me3 were analyzed using Western blot in GFP- and GFP+ cells. For five days, GFP+ cells were exposed to either vehicle or GSK-126 drug. β-actin is a loading control.

Adapted from Yomtoubian et al. 2020.

EZH2 inhibition and GATA3 Expression

Scientists sorted GFP+ cells and treated them with GSK-126 in order to ascertain the mechanisms through which EZH2 inhibition preferentially targets SOX2/OCT4-GFP+ cells. The downregulation of basal-associated genes and the overexpression of luminal-associated genes were the outcomes of EZH2 inhibition (Yomtoubian et al. 2020).

It is important to note that according to Asselin-Labat et al.' s study findings, GATA binding protein 3 (GATA3) plays a crucial role in the development of the mammary gland in both the embryo and the adult populations (Asselin-Labat et al. 2007). GATA3 expression in human BCs is positively correlated with ER expression. It is also necessary for the cell cycle progression of ER-positive cell lines. However, it has been discovered that GATA3 interacts with wild-type BRCA1 in ER-negative cell lines but cannot attach to mutant BRCA1 (Querzoli et al. 2021). Due to its established role as the main regulator of luminal differentiation, GATA3 was the primary focus in figuring out how EZH2 contributes to the basal-like phenotype (Visvader et al. 2007). ChIP-PCR study revealed that H3K27me3 on the GATA3 promoter was significantly diminished after GSK-126 drug treatment in LM2 cells (Figure 10C). In agreement with this finding, GATA3 expression was derepressed as a result of EZH2 inhibition (Figure 10D) (Yomtoubian et al. 2020).

Yomtoubian et al. also created orthotopic tumors that stably expressed shSCR or shGATA3 together with the SOX2/OCT4-GFP promoter-reporter in order to study the impact of GATA3 knockdown on SOX2/OCT4-GFP+ cells *in vivo*. All of these data pointed to the possibility that SOX2/OCT4/EZH2 high cellular pools, which aid in invasion and metastasis, are maintained and expanded through the repression of GATA3 gene expression by EZH2 HMT activity (Yomtoubian et al. 2020). In order to provide more insight into the differentiation-induced luminal-like phenotype, they assessed the expression levels of ERα and its downstream target, PR. The EZH2 inhibitor-treated GFP+ LM2 cells were seen to exhibit elevated expression of ERα (Figure 10J) and its downstream target PR (Figure 10K), implying the presence of intact and active ER signaling (Yomtoubian et al. 2020).

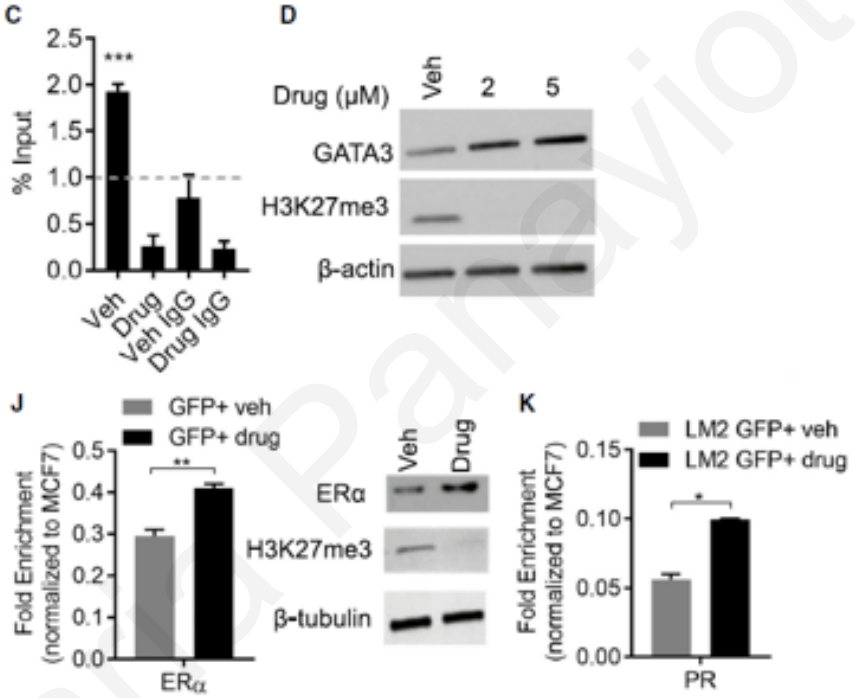

Figure 10 | EZH2 Inhibition activates GATA3 expression.

C. ChIP-qPCR against the control (IgG) or H3K27me3. The GATA3 promoter region in cells treated with either a vehicle or drug was used for testing.

D. GATA3 and H3K27me3 levels in LM2 cells after treatment with vehicle or GSK-126 drug were analyzed using Western blot.

J. Western blot analysis of ERα and H3K27me3 in LM2 GFP+ cells treated with vehicle or GSK-126 drug, adjusted to expression in MCF7 cells (right panel) shows the expression levels of ERα in these cells.

K. PR expression levels in LM2 GFP+ cells treated with a vehicle, or GSK-126 drug were normalized to MCF7 cell expression.

Adapted from Yomtoubian et al. 2020.

EZH2 influenced KRT14 overexpression in TNBC peritoneal metastasis

Verma et al. in their study they focused on a specific BC subtype, the TNBC that has a poor prognosis and unfavorable clinical results. The poor prognosis of TNBC has been linked to overexpression of EZH2. However, it is still unclear how much of EZH2's catalytic (H3K27me3) or non-catalytic (NC-EZH2) activity contributes to the advancement of TNBC (Verma et al. 2022). In their study, Verma et al. demonstrate how the metastatic landscape of TNBC is altered and its peritoneal metastasis, particularly splenic, is fostered by the preferential hyperactivation of functional EZH2 (H3K27me3) over NC-EZH2. It attenuated the binding of repressor SP1 to the promoter of KRT14, promoting transcription rather than H3K27me3-mediated suppression of gene expression. Moreover, KRT14 deletion dramatically lowered peritoneal metastasis, invasion, and migration of TNBC. H3K27me3 and KRT14 levels consistently showed a favorable connection in human TNBC metastasis. Lastly, TNBC peritoneal metastasis is decreased by EZH2 knockdown or H3K27me3 suppression by EPZ643, a selective inhibitor of EZH2 (Verma et al. 2022).

First of all, Verma et al.'s aim was to analyze the difference between NC-EZH2 and H3K27me3 in the development of TNBC. For this, they have created an animal model based on 4T-1 mammary carcinoma that closely resembles the course of TNBC in a basal-like manner in a preclinical context. The 4T1 mammary carcinoma tumor cell line is extremely invasive and tumorigenic. It has the ability to spontaneously spread from the main tumor in the mammary gland to several distant locations (Pulaski, Ostrand-Rosenberg 2001). More specifically, in order to investigate the distinct function of NC-EZH2 in comparison to the H3K27me3 function, they generated 4T-1 stable cells that either overexpressed catalytically hyperactive (Y641-F) or catalytically inactive (–ΔSET) EZH2 protein (Figure 11 A, B). After the subcutaneous inoculation of control and the 4T-1 cells into mice, they found that, in comparison to control tumor-bearing mice, Y641-F tumor-bearing animals have much smaller tumors in size, whereas EZH2 (–ΔSET) tumor-bearing mice show no discernible difference in tumor development (Figure 11C, D). There was also a significant loss of body weight in Y641-F tumor-bearing mice during tumor progression and that fact led the scientists to figure out the metastatic state of this group. Accordingly, the cause for the significant weight loss in Y641-F tumor-bearing mice might have arisen due to faster metastasis. In addition to this, scientists also found that Y641-F mutant cells are characterized by higher migratory potential and invasion. Overall, *in vivo* research has shown that TNBC metastasis is regulated by H3K27me3,

and not from NC-EZH2 protein. TNBC migration induction was reliant on H3K27me3. The FDA-approved medication Tazemetostat (EPZ6438), a pharmacological selective inhibitor of EZH2, was employed. It specifically suppresses the H3K27me3 activity of EZH2 without changing the protein's expression level (Figure 11R). Subsequently, they aimed to ascertain EPZ6438's impact on TNBC invasion and discovered that the drug's substantial reduction of TNBC invasion, even at a 10 µM dosage, was attributed to its specific inhibition of H3K27me3 (Figure 11T). Their comprehensive *in vivo* and *in vitro* studies collectively strongly implied that H3K27me3 stimulates TNBC invasion, migration, and metastasis (Verma et al. 2022).

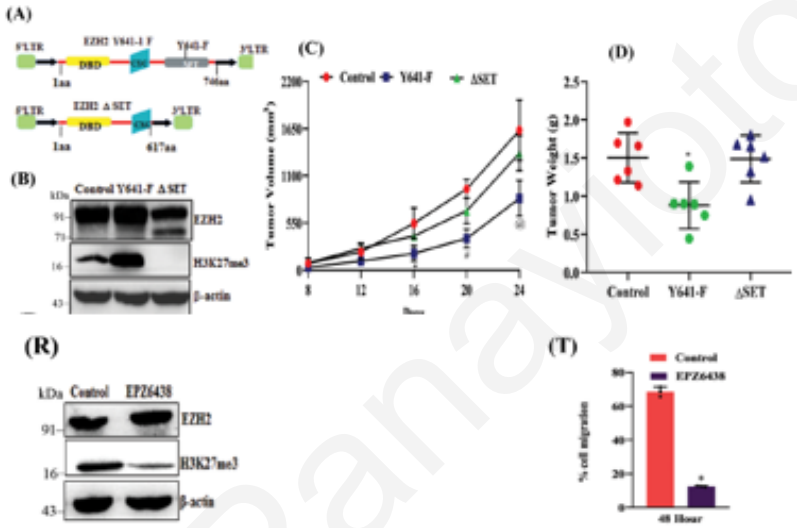

Figure 11 | TNBC metastasis is promoted by H3K27me3, but not by NC-EZH2 protein.

A. Diagrammatic illustration of the retroviral overexpression constructs EZH2 (Y641-F) and EZH2-ΔSET.

B. EZH2, H3K27me3, and β-actin expression in normal, stable EZH2 (Y641-F), and EZH2 (ΔSET) overexpressed 4T-1 cells were determined by immunoblot analysis.

C. The tumor growth curves for control, EZH2 (Y641-F), and EZH2 (ΔSET) are displayed.

D. The graph shows the average weight of the collected tumors in the control, EZH2 (Y641-F), and EZH2-ΔSET groups.

R. In order to examine the expression of EZH2, H3K27me3, and β-actin using immunoblotting, the 4T-1 cells were either treated with a vehicle or 10µM EPZ6438 drug.

T. The transwell chamber migration experiment quantitative bar graphs for control and EPZ6438 at 48 hours.

Adapted from Verma et al. 2022.

Verma et al.'s second goal was to ascertain how elevated H3K27me3 affected the TNBC metastatic pattern. In order to confirm their findings *in situ*, they used H&E (hematoxylin and eosin) to stain the lung, liver, and spleen tissues taken from tumor-bearing control and Y641-F mice. Their findings support that the most metastatic foci in the control mice are located in the

lung, while the majority of the metastases in the tumor-bearing Y641-F mice are found in the liver and spleen (Figure 12). All of these *in vivo* investigations collectively provide evidence that increased trimethylation influences TNBC peritoneal metastasis (Verma et al. 2022).

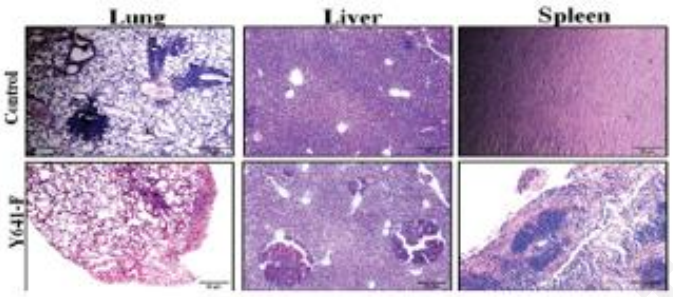

Figure 12 | H&E staining in the lung, liver, and spleen of tumor-bearing Y641-F and control mice under the microscope.

Adapted from Verma et al. 2022.

Strong alterations in the TNBC metastatic hallmark caused by H3K27me3 motivated scientists to find the underlying mechanistic understanding of the phenotype. For this, Verma et al. used transcriptome analysis in control and Y641-F cells. Through qRT-PCR, each of them independently confirmed that the targeted genes were expressed differently in Y641-F cells compared to control cells. Notably, it was discovered that, in comparison to control cells, the expression of NCAM1, AQP1, BNIP3, CBS, and NDRG1 was downregulated in Y641-F cells, whereas the expression of NIFK, ADAMTS1, CCN2, JAG1, SEMA3C, TM4SF1, CYP1B1, and KRT14 was found to be increased (Figure 13H). To determine the role of these modifications in determining peritoneal metastasis in TNBC at basal state, they then looked at the verified up- and down- regulated genes' mRNA expression in the metastatic cells that were separated from the original tumor, the lungs, the liver, and more. Further validation showed that, when compared to primary tumor cells, the cells separated from lung, liver, and splenic metastases had significantly higher expression of KRT14 with the strongest signal seen in the case of splenic metastasis (Figure 13I) (Verma et al. 2022).

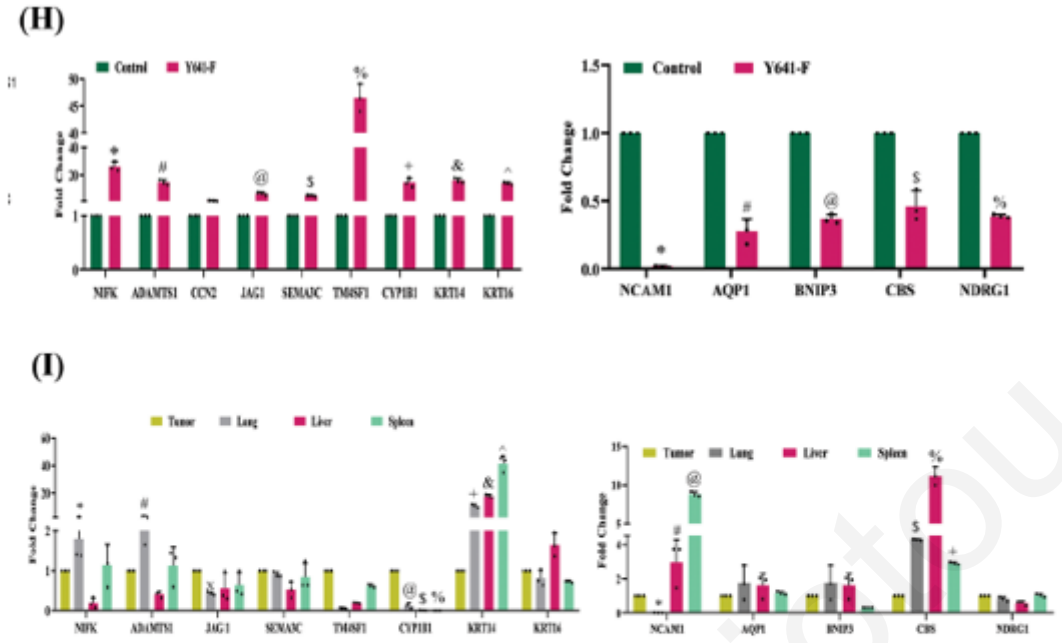

Figure 13 | KRT14 transcription is increased by overexpression of EZH2 catalytic activity (elevated H3K27me3).

H. Real-time PCR was used to analyze the expression of genes after RNA from control and EZH2 Y641-F 4T-1 cells were extracted.

I. In order to analyze gene expression, RNA from 4T-1 cells that were extracted from the main tumor, metastatic lung, liver, and spleen was submitted to real-time PCR.

Adapted from Verma et al. 2022.

Verma et al.'s next step was to confirm how H3K27me3 affects the control of KRT14 expression. KRT14 expression at the mRNA and protein levels was clearly decreased in response to Tazemetostat (EPZ6438) treatment in both normal and Y641-F cells (Figure 14D, E) (Verma et al. 2022). The preclinical animal model demonstrates a significant connection between H3K27me3 and KRT14 in TNBC splenic metastasis, as evidenced by the increased expression of both markers in splenic metastatic cells as compared to initial tumor cells (Figure 14J). The proliferation marker (Ki67) was not significantly differently expressed by original tumor cells and splenic metastatic cells, indicating that TNBC migration is the only function of elevated H3K27me3 levels (Figure 14K) (Verma et al. 2022).

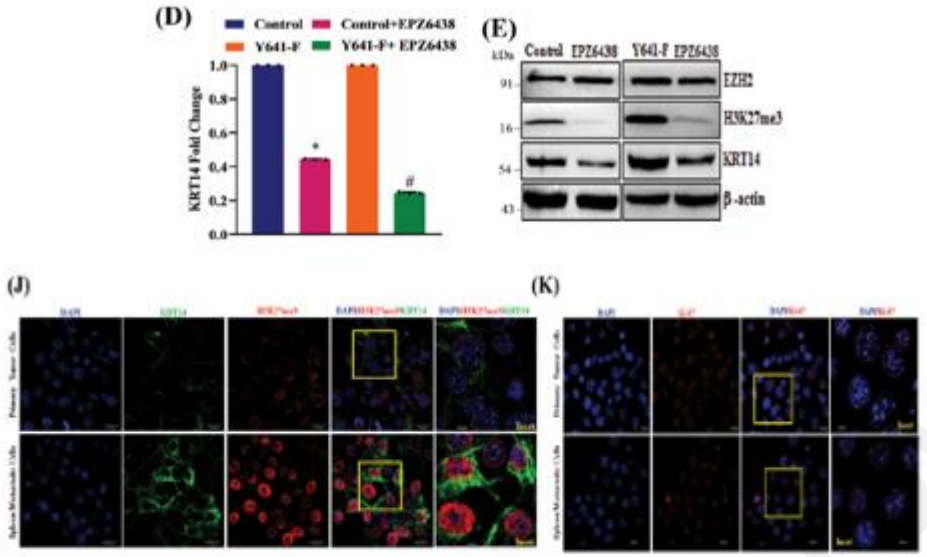

Figure 14 | The mRNA and protein levels of KRT14 expression are positively regulated by the H3K27me3 level.

D. After the treatment of the control and EZH2 Y641-F cells with a 10 μM concentration of EPZ6438 drug, the KRT14 expression was measured by qPCR.

E. After the 10 μM treatment of EPZ6438 drug to the control and EZH2 Y641-F cells, the expression of EZH2, H3K27me3, KRT14, and β-actin was examined by immunoblotting.

J& K. After isolating 4T-1 cells from original tumors and metastatic spleen, they were either single with H3K27me3 (red) and KRT14 (green) or co-stained (K) with red Ki-67 antibody and green KRT14 antibody, and then exposed to confocal imaging.

Adapted from Verma et al. 2022.

Furthermore Verma et al.'s goal was to determine if EZH2 functional inhibition ultimately restores the *in vivo* phenotype and to investigate the therapeutic significance of their findings in more detail. Using immunoblot, they verified EZH2 knockdown in 4T-1 cells. In fact, compared to the corresponding controls, EZH2 knockdown cells had decreased migratory (Figure 15C) and invasive capacity (Figure 15D, E) (Verma et al. 2022).

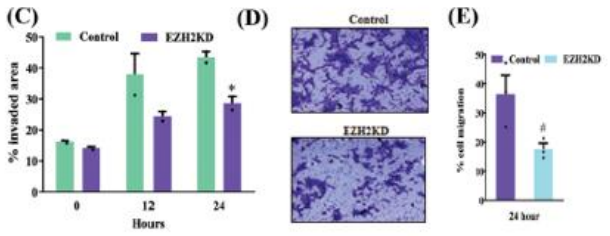

Figure 15 | EZH2 methyltransferase activity inhibition lowers peritoneal metastasis of TNBC.

C. The quantitative analysis of wound healing assay of control and EZH2KD4T-1 cells.

D. Images of the wound healing assay employed in control and EZH2KD4T-1 cells.

E. The quantitative analysis transwell assay of control and EZH2KD4T-1 cells.

Adapted from Verma et al. 2022.

The EZH2-H3K27me3-KRT14 axis may be one of the key regulators of TNBC peritoneal metastasis, according to their overall findings from *in vitro*, *in vivo*, and human patient samples. Tazemetostat, the FDA-approved EZH2 inhibitor may be a viable treatment approach to stop TNBC from progressing (Figure 16) (Verma et al. 2022).

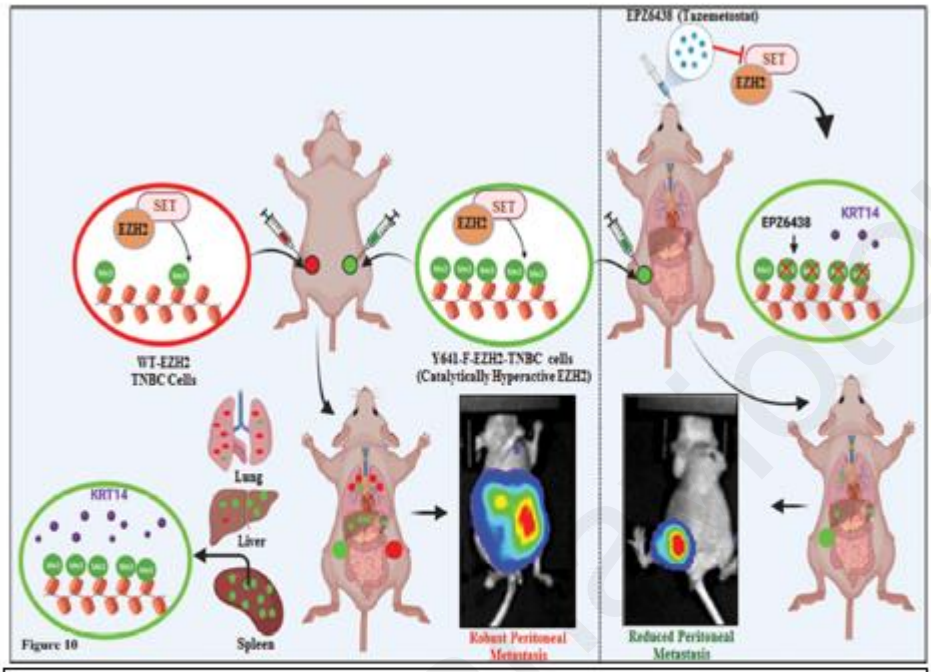

Figure 16| EZH2-H3K27me3 role and significance in TNBC metastasis.

Diagram illustrating the potential for TNBC peritoneal metastasis caused by catalytically hyperactive EZH2 (increased H3K27me3) and the therapeutic vulnerabilities associated with it when treated with the EZH2 inhibitor EPZ6438.

Adapted from Verma et al. 2022.

ZRANB1: a potential therapeutic target as deubiquitinase of EZH2

Even while EZH2-mutated lymphoma and ARID1A-mutated ovarian cancer have responded to EZH2 enzymatic inhibitors, many malignancies do not because EZH2 can promote cancer even in the absence of its histone methyltransferase activity (Bitler et al. 2015, Knutson et al. 2012). ZRANB1 is identified by Zhang et al. as the EZH2 deubiquitinase. More specifically, ZRANB1 stabilizes EZH2 by binding and deubiquitinating it. ZRANB1 depletion causes EZH2 instability and growth suppression in breast cancer cells. In preclinical models of TNBC, systemic administration of ZRANB1 small interfering RNA (siRNA) results in notable antitumor and antimetastatic effects. Interestingly, EZH2 is destabilized by a small-molecule inhibitor of ZRANB1, which prevents TNBC cell survival. ZRANB1 levels are correlated with EZH2 levels and poor survival in patients with BC. These results implied that targeting the EZH2 deubiquitinase, ZRANB1 might be therapeutic promising (Zhang et al. 2018).

Zhang et al. initially employed a panel of 46 Deubiquitinating enzymes (DUBs) fused to a triple-epitope tag, SFB, to screen for EZH2-interacting DUBs in order to identify the EZH2 DUB. They used S-protein beads to pull down the DUBs after co-transfecting each SFB-tagged DUB with MYC-tagged EZH2 into HEK293T cells. This allowed them to identify the physical link between EZH2 and six DUBs: USP22, USP39, USP44, USP49, USP53, and ZRANB1(Figure 17A). Zhang et al. transfected each of the six EZH2-interacting DUBs separately into HEK293T cells to investigate the effects on EZH2 ubiquitination and protein levels. They discovered that whereas USP22 and ZRANB1 reduced EZH2 polyubiquitination, only ZRANB1 increased endogenous EZH2 protein. In contrast, two separate siRNAs (2 and 5) suppressed ZRANB1 expression in MDA-MB-231 cells, resulting in a significant reduction in endogenous EZH2 protein levels (Figure 17B) (Zhang et al. 2018). Furthermore, co-expressing ZRANB1 siRNA might decrease the levels of endogenous EZH2 and H3K27 trimethylation that were raised by ZRANB1 overexpression in HEK293T cells (Figure 17C).

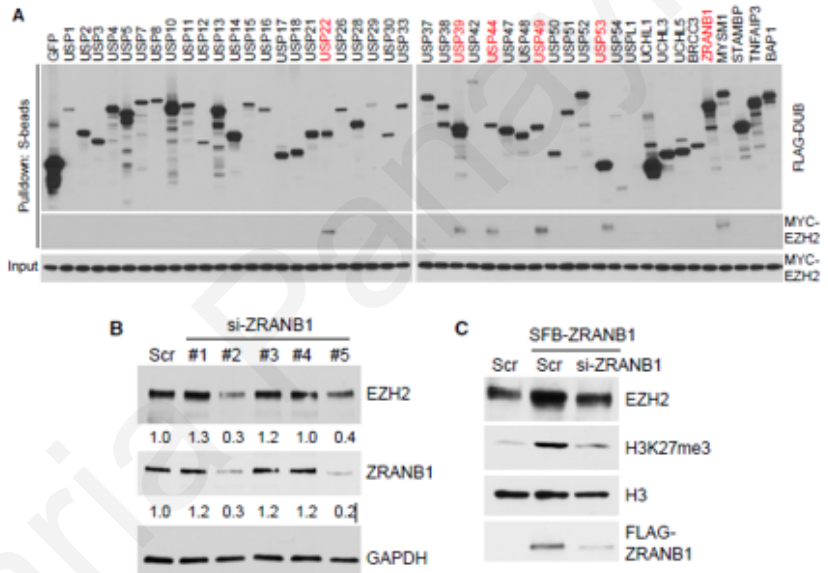

Figure 17 | ZRANB1 Controls the Level of EZH2 Protein.

A. EZH2 is physically associated with six out of 46 DUBs. After co-transfecting each SFB-tagged DUB into HEK293T cells along with MYC-tagged EZH2, the cells were pulled down using S-protein beads and immunoblotted using antibodies against FLAG and MYC.

B. ZRANB1 siRNA or a scramble control (Scr) transfected MDA-MB-231 cells were used for immunoblotting of EZH2, ZRANB1, and GAPDH.

C. HEK293T cells transfected with SFB-ZRANB1 and ZRANB1 siRNA, either separately or in combination, were used for the immunoblotting of EZH2, H3K27me3, H3, and FLAG-ZRANB1.

Adapted from Zhang et al. 2018.

Scientists believed that ZRANB1 deubiquitinates EZH2 to stabilize it. Indeed, EZH2 polyubiquitination was significantly decreased in ZRANB1-knockout HEK293A cells when ZRANB1 was overexpressed. It has been demonstrated that EZH2 stimulates mammary tumorigenesis and metastasis, although ZRANB1's role in cancer is uncertain. After transfecting MDA-MB-231 cells with two different ZRANB1 siRNAs, they observed that both siRNAs significantly reduced cell migration (Figure 18B) and proliferation (Figure 18A). Similar to this, two separate HEK293A cell ZRANB1 gRNA clones had a significant growth deficiency (Figure 18C). Nine more TNBC cell lines exhibited proliferative deficit and EZH2 downregulation as a result of siRNA-mediated knockdown of ZRANB1 (Zhang et al. 2018).

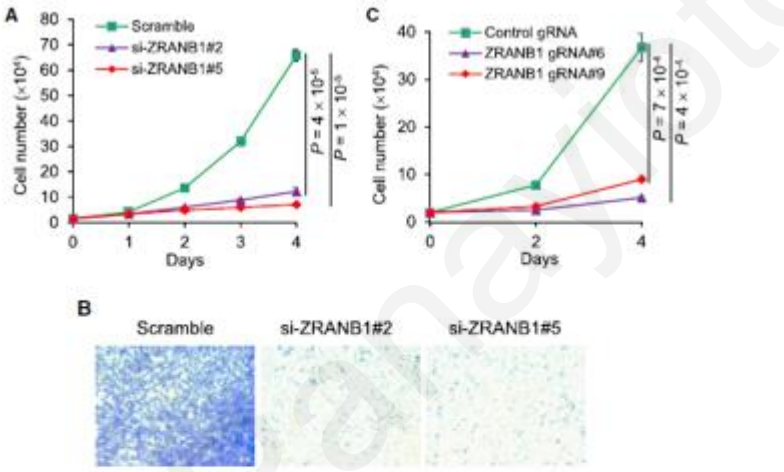

Figure 18 | ZRANB1 Is a Deubiquitinase That Promotes Growth.

A. MDA-MB-231 cell growth curves after transfection with ZRANB1 siRNA or a scramble control.

B. MDA-MB-231 cells transfected with ZRANB1 siRNA, or a scramble control were used in transwell migration experiments.

C. Growth curves of two separate CRISPR-Cas9-generated clones of ZRANB1-knockout HEK293A cells.

Adapted from Zhang et al. 2018.

The two separate ZRANB1 siRNAs caused a 60% reduction in tumor weight. As expected, the two ZRANB1 siRNA treatment groups significantly reduced the amount of EZH2 protein in the tumor in comparison to the vehicle group and the scramble control group (Figure 19C), showing that ZRANB1 siRNA was successfully destabilizing EZH2 *in vivo* (Zhang et al. 2018). Zhang et al also have administered the same dose schedule as in the tumor development research after injecting LM2 cells into NSG (NOD scid gamma) mice via the tail vein in order to evaluate the impact of ZRANB1 inhibition on lung metastatic colonization. They discovered that systemic administration of the two ZRANB1 siRNAs reduced human ZRANB1 mRNA

levels in lung in comparison to the scramble control group. This was achieved using a human ZRANB1-specific TaqMan test (Figure 19D). When mice treated with ZRANB1 siRNA encapsulated in DOPC were compared to mice treated with scramble RNA oligonucleotides encapsulated in DOPC, there was a consistent reduction in lung metastases, as demonstrated by bioluminescent imaging of living animals (Figures 19 E, F). In this TNBC model, the administration of nanoliposomal ZRANB1 siRNA resulted in significant anticancer and antimetastatic benefits (Zhang et al. 2018).

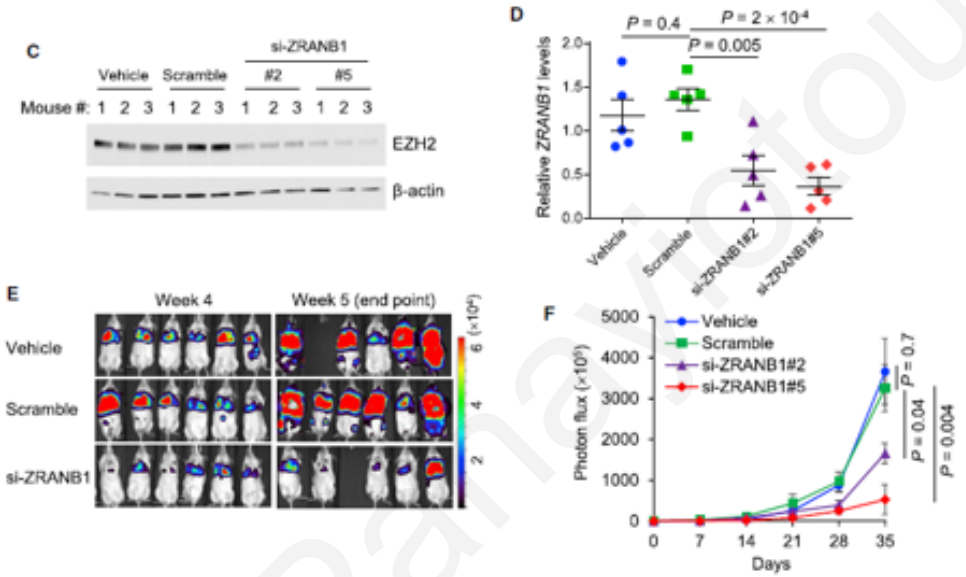

Figure 19| ZRANB1 Depletion Has Antitumor and Antimetastatic Properties.

C. EZH2 and β-actin immunoblotting in mouse tumor lysates.

D. ZRANB1 unique to humans detected by qPCR in lung tissues from NSG mice after LM2 cell injections into the tail vein. Beginning on day 3, mice were given intravenous injections of the vehicle, ZRANB1 siRNA encapsulated in DOPC, or scrambled oligonucleotides twice a week.

E. Mice's bioluminescent imaging.

F. Mice's photon flux measurement.

Adapted from Zhang et al. 2018.

EZH2 methyltransferase-independent mechanism in BC

Osteolytic lesions, which cause extensive bone resorption and bone fractures, are usually induced by breast cancer bone metastases. TGFβ has two functions in the development and spread of cancer. It can act as a tumor suppressor in premalignant cells and promotes angiogenesis, immunosuppression, and the epithelial-mesenchymal transition, which leads to breast cancer metastasis (Xu et al. 2015). Overexpression of EZH2 is thought to be a predictive

indicator of metastasis risk in women with early-stage hereditary breast cancer. Renal cell carcinoma tissues from individuals with bone metastases contained high levels of EZH2, indicating that EZH2 may facilitate the bone metastasis of cancer cells (Alford et al. 2012). It is uncertain, therefore, how EZH2 contributes to the breast cancer bone metastasis vicious cycle. Zhang et al. in their study found that *in vivo* the bone metastases of breast cancer were inhibited by EZH2 elimination. Two important effectors of the canonical TGFβ pathway, pS465/467-Smad2 and parathyroid hormone-like hormone (PTHLH) production were upregulated by EZH2 in response to TGFβ stimulation. EZH2 stimulates the transcription of ITGB1, which encodes integrin β1, activating focal adhesion kinase (FAK), a downstream effector. In order to initiate the TGFβ signaling pathway, activated FAK phosphorylates TGFβRI and increases TGFβRI's binding to TGFβRII. The study of Zhang et al. suggested that targeting FAK may be a useful therapy method for EZH2-induced breast cancer bone metastasis. It also highlighted the cooperative role of EZH2 and TGFβ signaling in driving bone metastasis of breast cancer through a methyltransferase-independent mechanism (Zhang et al. 2022). Zhang et al.'s first goal was to investigate the role of EZH2 in bone metastasis of breast cancer. For this, these scientists generated the EZH2-knockdown cell lines 1566.shEZH2 and its control cell line 1566.shScr by transfecting either EZH2 shRNA or control shRNA, respectively, into the MDA-MB-231 bone-seeking 231–1566 cell subline that expressed GFP and luciferase. Next, separate injections of the sublines 1566.shEZH2 and 1566.shScr were made into the left ventricles of nude mice. Compared to mice injected with 1566.shScr cells, mice injected with 1566.shEZH cells exhibited a considerably longer bone metastasis-free life as well as overall survival. After a variety of processes like Bioluminescence imaging (BLI), X-ray imaging, and hematoxylin and eosin (H&E) staining of bone lesions, results showed that the mice that were injected with 1566.shEZH cells had fewer bone metastases than mice that were injected with 1566.shScr cells (Figure 20) (Zhang et al. 2022).

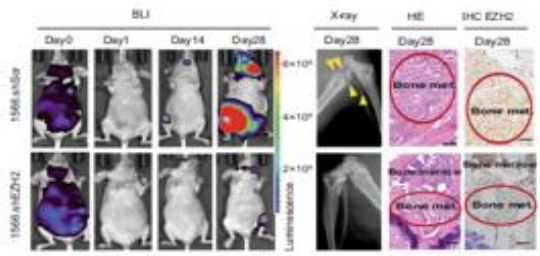

Figure 20 | An EZH2 methyltransferase inhibitor cannot stop EZH2, which increases breast cancer bone metastases. Illustrations of bone-metastatic lesions using bioluminescence (BLI), X-ray, and hematoxylin and eosin (H&E) staining.

Adapted from Zhang et al. 2022.

DISCUSSION

This study demonstrates an aspect of the epigenetic regulation of breast cancer initiation and metastasis. More specifically, we focused on the role of histone modifier and transcriptional repressor PRC2 in the initiation of different types of breast cancer, including luminal B breast cancer, and TNBC. Targeting the catalytic domain of the PRC2 complex, more specifically the H3K27 methyltransferase activity of EZH2, resulted in a delay of tumor formation, while the metastatic capacity of the tumor was blocked only in the PyV mT-expressing GEMM mimicking the luminal B subtype (Liu et al. 2023). When considered collectively, these findings suggest that EZH2 is essential for the development of the tumor in this GEMM of luminal B breast cancer. These results contrast with those of a study that targeted the mammary epithelial EZH2 gene in a BRCA1-deficient GEMM and found that deletion of EZH2 had no effect on tumor initiation (Bae et al. 2015). Another study previously supported that the loss of EZH2 function in a Notch-driven GEMM of breast cancer did not significantly affect the production of mammary tumors (Wassef et al. 2015).

Given the published literature, the findings of this study align with the established context-dependency of EZH2/PRC2's oncogenic function, which posits that PRC2's functional requirements during the progression of breast cancer are significantly influenced by the molecular subtype. To sum up the main findings of this review, the loss of EZH2 significantly impacted genes implicated in the WNT signaling pathway, according to transcriptomic and biochemical studies in EZH2-proficient and EZH2-deficient organoids (Liu et al. 2023). Liu et. al. verified that SFRP1, a strong inhibitor of WNT signaling, is a direct target of EZH2-mediated repression. Additionally, the same group demonstrated that via altering epithelial polarity, by CRISPR-Cas9-mediated ablation of SFRP1, the altered phenotype in EZH2 deficient MIC organoids was partially restored. It also has been shown in previous studies that EZH2 participates in WNT-dependent signaling in several tissue contexts. For instance, it has been shown that in breast tumors that express Wnt10a, EZH2 co-targets β-catenin-responsive genes, resulting in a chemoresistant phenotype (El Ayachi et al. 2019). The significance of WNT signaling in the PyV mT GEMM has been highlighted by recent data (Buechel et al. 2021), and by this, it would be interesting to investigate the possible role of WNT-dependent pathways in the development of luminal B breast cancer. Overall, the studies of luminal B BC models support that EZH2 is a master regulator of several oncogene-coupled signaling

pathways during the tumor initiation phase, influencing mRNA translation, cell proliferation, and cell polarity. The data included in this review study revealed that in luminal B BC, the expression of EZH2 targets SFRP1 and this is linked to improved clinical outcomes. These findings support the possibility of using EZH2 targeting as a treatment for specific breast cancer subtypes. EZH2-dependent transcriptional networks implicated in the development and progression of tumors in certain molecular subtypes may be further characterized. This will facilitate the development of treatment approaches that specifically target the essential roles of EZH2 signaling in BC (Liu et al. 2023).

Another significant study revealed the involvement of the epigenetic factor EZH2 in a different type of breast cancer, the TNBC .To be more precise, Yomtoubian et al. demonstrated that EZH2 HMT may be a key epigenetic driver of TNBC development. (Yomtoubian et al. 2020). Many studies like the study of Hussein et al. were using human tissue microarrays and by this, provided evidence that TNBC patients expressed more EZH2 than non-TNBC patients did (Hussein et al. 2012).

The inactivation of the EZH2 HMT domain inhibits distant metastasis without impacting primary tumor development by genetic modification and small-molecule suppression of EZH2 enzymatic activity. In the case of TNBC in comparison to luminal B BC, the role of EZH2 seems to be more significant in the metastatic ability of tumor cells as it can favor peritoneal metastasis. The pharmacological suppression of EZH2 HMT only decreased the invasion but had no effect on apoptosis or proliferation. In contrast, the results of previous studies showed that EZH2 ablation enhanced tumor development, but the more recent results indicate that tumors formed from EZH2 knockdown cells that were however considerably smaller (Gonzalez et al., 2014). Hirukawa et al.'s study examined the impact of EZH2 inhibition on two TNBC PDX (Patient-Derived Xenograft) models and discovered that EZH2 inhibition had no discernible influence on primary tumor development in either type. Nevertheless, in the same study, it was also demonstrated that metastasis was unaffected by EZH2 inhibition (Hirukawa et al. 2018). However, high EZH2 expression in the BL-1 and M, TNBC subtypes, corresponds with low GATA3 expression and sensitivity to EZH2 inhibition, highlighting the fact that the TNBC is made up of multiple molecular subtypes. It's possible that Hirukawa et al.'s study TNBC subtype is resistant to EZH2 inhibition. Collectively, these investigations highlighted how EZH2 functions differently depending on the subtypes of breast cancer (Comet et al. 2016).

The surprising discovery that EZH2 inhibition targets a distinct subpopulation of tumor cells that expresses EZH2 high OCT4, SOX2, and NANOG and that has increased invasion, dissemination, tumor initiation, and metastatic potential came from further research into the mechanisms by which EZH2 inhibition leads to metastasis suppression *in vivo*. This is a significant discovery as it implies a clear connection between EZH2 and metastasis, which is essential because the real metastatic component of TNBC has remained enigmatic. The findings of the studies are consistent with reports that back up a theory that TIC-like cells in the original tumor have the ability to spread and become invasive (Comet et al. 2016). Subsequent analysis showed that GATA3 suppression mediated by EZH2 is essential for TIC-like pools to be maintained. The transformation of TIC-like basal cells into a luminal-like phenotype was demonstrated to be aided by EZH2 inhibition, which raised GATA3 expression. This finding supports the idea that PRC2's catalytic activity acts as a barrier to cell fate transitions. This differentiation phenotype was linked to decreased invasion, dissemination, and metastasis as well as decreased TIC-like activity. Yomtoubian et al. conducted Chip-PCR research, and this revealed that GATA3 is a direct target of EZH2 methyltransferase and established the importance of the EZH2-GATA3 axis in modulating the propensity for invasiveness of SOX2/OCT4 high cells. Results implied that transcriptional stimulation of GATA3 may be the mechanism via which EZH2 inhibition provides a therapeutic advantage. These findings, taken together, provide light on a mechanism by which GATA3 is mediated by the catalytic activity of PRC2 to control the metastatic behavior of a certain basal-like population in TNBCs (Yomtoubian et al. 2020).

Clinical uses for EZH2 inhibition include the prevention of metastases in early illness stages. In advanced disease stages, on the other hand, EZH2 inhibition may distinguish TNBC from luminal-like breast cancer, which may be treated with existing endocrine treatments. Furthermore, Yomtoubian et al. research's findings might improve the field of epigenetic treatment for high-risk metastatic TNBC as EZH2 catalytic activity can be targeted using already available pharmacological-grade inhibitors such as GSK-126. In addition, Verma et.al.'s study indicated also that H3K27me3 plays a key role in basal-like TNBC development and progression in comparison to NC-EZH2 and found that H3K27me3 is essential for TNBC peritoneal metastasis (Verma et.al 2022). After conducting transcriptome analysis and validation scientists identified KRT14 as an H3K27me3 target. Rather than typical transcription inhibition mediated by H3K27me3, they found an elevated H3K27 tri-methylation mark for transcriptional activation of KRT14. Strong suppression of TNBC peritoneal metastasis,

especially splenic metastasis, was observed upon loss of EZH2, KRT14, or H3K27me3 function (Verma et.al 2022).

As discussed, the EZH2's function in tumor growth and progression is very context-dependent and, in certain cases, it even has the opposite impact in various forms of cancer. Because of EZH2's methyltransferase-independent roles in carcinogenesis, targeting it with methyltransferase inhibitors hasn't always been beneficial in clinical studies. Furthermore, several preclinical and clinical investigations indicate that Tazemetostat, an EZH2 inhibitor, demonstrated increased effectiveness and reduced therapeutic relapse mostly in lymphomas but not in solid tumors (Gulati et al. 2018). Tazemetostat or EPZ6438 specifically suppresses EZH2's H3K27me3 function, but not the amount of EZH2 protein overall. H3K27me3 function is essential for lymphomas to survive (Morin et al. 2021). Because certain mutations (Y646, A682, and A692) are prevalent in lymphomas but absent from solid tumors like TNBC, the efficacy of EZH2 inhibitors in lymphomas is often different from that in solid tumors. In about 30% of germinal center follicular lymphomas, these gain of function mutations produces catalytically hyperactive EZH2, rendering the tumors susceptible to EZH2 inhibitor treatment (Verma et.al 2022). Tazemetostat has thus shown to be particularly successful in lowering the growth of primary tumors in lymphomas, but in most solid tumors, Tazemetostat's selective loss of H3K27me3 activity does not affect the formation of primary tumors or the proliferation of tumor cells (Yomtoubian et al. 2020). Since Tazemetostat just recently acquired FDA clearance for the treatment of sarcoma, clinicians should carefully examine the EZH2 therapeutic window (Hoy 2020, Rothbart, Baylin 2020). Clinical trials with patients with BC haven’t been performed yet. Whereas clinical trials on patients with other malignancies like B cell non-Hodgkin's lymphoma and epithelioid sarcomas. started performed in 2020 alone or in combination with immunotherapy. Some of this research is still ongoing and results are going to be available by the end of each clinical trial (Eich et al. 2020).

However, EZH2's selective loss of H3K27me3 activity has a major inhibitory effect on the migration of cancer cells and the spread of tumors (Yomtoubian et al. 2020). The EZH2-H3K27me3-KRT14 axis is significantly overexpressed in human TNBC samples' peritoneal (liver) metastases in comparison to matched primary tumors, which is consistent with preclinical findings. In contrast, scientists observed a significant upregulation of H3K27me3 level in other secondary organ metastases, such as lung metastases, when compared to matched primary tumors. However, the EZH2 level on these metastatic sites did not correlate with H3K27me3 expression, indicating that EZH2-independent mechanisms might be at play in

these cases (Verma et.al 2022). Verma et al. concluded that KRT14 expression in the basal-like TNBC subtype is enhanced by the catalytic activity of EZH2. Mechanistically, H3K27me3 can regulate the TNBC peritoneal metastasis by upregulating the transcription of certain genes, such as KRT14, in place of the typical suppressive effect. Additionally, they also confirmed that the EZH2-H3K27me3-KRT14 axis plays a crucial role in the metastasis of TNBC in humans. Ultimately in the case of the most aggressive TNBC subtype, where targeted therapy is currently unknown, the EZH2 inhibitor medication Tazemetostat may represent a viable therapeutic alternative (Verma et.al 2022).

Another study showed an alternative way to indirectly target the EZH2 function is the ZRANB1 protein. The ubiquitination and degradation of EZH2 are facilitated by many ubiquitin ligases (Jin et al. 2017). Targeting EZH2's deubiquitinase may destabilize the protein, providing a different treatment strategy for EZH2-overexpressing cancers such as ovarian cancer and TNBC. In the study of Zhang et al., ZRANB1 was discovered to be the EZH2 deubiquitinase responsible for controlling the polyubiquitination and protein stability of EZH2. Not all of the non-TNBC cells rely on ZRANB1 and EZH2 to proliferate. However, in this case, scientists also present proof of principle that tiny compounds can block the EZH2 deubiquitinase, ZRANB1. This serves as the foundation for aiming for ZRANB1(Zhang et al. 2018). In clinical trials, targeting EZH2 with methyltransferase inhibitors hasn't always worked successfully. The results of Zhang et al. revealed that MDAMB-231 cell-induced bone metastases are not inhibited by small-molecule EZH2 inhibitors in an *in vivo* mouse model. On the other hand, clinically relevant kinase inhibitors that target the downstream effector FAK of EZH2 show remarkable results in preventing bone metastases of breast cancer. In mouse models of breast cancer, EZH2 inhibitors have been shown to prevent lung metastasis. However, in experimental models, the EZH2 inhibitor EPZ-6438 failed to prevent metastasis but instead encouraged bone metastasis (Zhang et al. 2022).

In Figure 21 there is a schematic representation of the main findings of the research included in this review as discussed in previous sections. From this review, we can conclude that EZH2 holds a key role both in tumor development and in its following metastasis in different subtypes of breast cancer. Further research might reveal many more pathways that lead to its dysregulation in BC in order to proceed to discover even more downstream targets of EZH2. The epigenetic therapeutic strategies with EZH2 inhibitors are well-promising factors whereas more precise research in vitro and in vivo is crucial. Also, the combinatorial use of such drugs

with the traditional already established therapies of BC needs further evaluation. This study provides evidence that EZH2 can be a very promising target in the future of BC treatment.

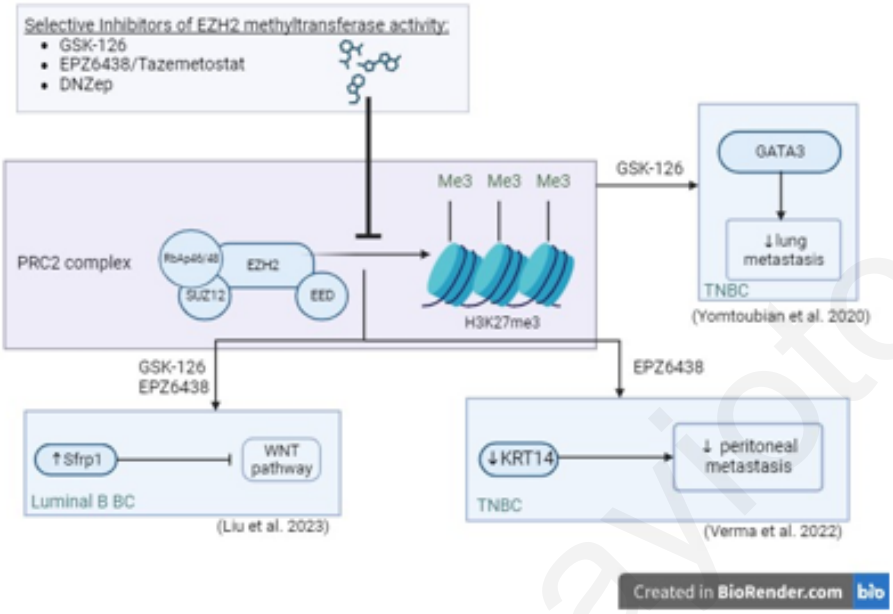

Figure 21 | Overview schematic representation of previously well-described studies that are linked with the catalytically dependent function (H3K27me3 methyltransferase activity) of EZH2 protein. EZH2 methyltransferase when inhibited by GSK-126 or EPZ6438 can lead to an increase of Sfrp1, an antagonist of Wnt proteins, and causes the inhibition of the WNT pathway in luminal B BC models (Liu et al 2023). When the catalytical dependent function of EZH2 is inhibited with EPZ6438 can lead to a decrease of KRT14 and consequently can lead to reduction of peritoneal metastasis in TNBC models (Verma et al. 2022). EZH2 methyltransferase activity inhibition by GSK-126 causes the reexpression of GATA3 protein that leads to the reduction in lung metastasis in TNBC cells (Yomtoubian et al. 2020).

ABBREVIATIONS

ABBREVIATION	MEANING
ADP	Adenosine Diphosphate
AF1	Activation Function 1
APC	Antigen-Presenting Cell
AR	Androgen Receptor
ARID1A	AT-Rich Interaction Domain 1A.
BC	Breast Cancer
BLBC	Basal-Like Breast Cancer
BLG	β-lactoglobulin
BRCA	Breast Cancer Gene
CDH1	CDC20 homolog 1
CHEK2, BRIP1, ATM, and PALB2	CHEK2, BRIP1, ATM, and PALB2
ChIP-PCR	Chromatin Immunoprecipitation quantitative real-time PCR
CK1	Creatine Kinase
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CSCs	Cancer Stem Cells
CXC Domain	Cysteine-rich domain

DAPI	4′,6-diamidino-2-phenylindole
DBD	DNA Binding Domain
DMSO	Dimethyl Sulfoxide
DNMT	DNA Methyltransferases
DOPC	1,2-dioleoyl-sn-glycero-3-phosphocholine
Dox	Doxycycline
dsDNA	double-stranded DNA
DUBs	Deubiquitinating enzymes
DZNep	3-Deazaneplanocin A
E2F1	E2F Transcription Factor 1
EED	Embryonic Ectoderm Development
EID	EED Interaction Domain
EMT	Epithelial to Mesenchymal Transition
ER	Estrogen Receptor
EREs	Estrogen Response Elements
EZH2	Enhancer of Zeste Homolog 2
FAK	Focal Adhesion Kinase
FDA	Food and Drug Administration
FISH	Fluorescence *in situ* Hybridization analysis
GEMMs	Genetically Engineered Mouse Models
GFP	Green Fluorescent Protein.

GR	Glucocorticoid Receptor
GSK-3	Glycogen Synthase Kinase 3
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylases
HEK293T	Human Embryonic Kidney cells 293T
HER2	Human Epidermal Growth Factor Receptor 2
HMGB1	High-Mobility Group protein 1
IHC	Immunohistochemistry
ISH	*in situ* Hybridization
LBD	Ligand Binding Domain
LCoR	Ligand-Dependent Corepressor
LEF	Lymphoid Enhancer Factor
lncRNAs	long non-coding RNAs
LRPs	Lipoprotein Receptor Related Proteins
MMTV-LTR	Murine Mammary Tumor Virus Long Terminal Repeat
MRI	Magnetic Resonance Imaging
NC-EZH2	Non-Catalytic Activity of EZH2
NCoR	Nuclear Receptor Corepressor
NFkB	Nuclear factor kappa B
NSG	NOD Scid Gamma mice
OCT4	Octamer-binding Transcription Factor 4

PCNA	Proliferating Cell Nuclear Antigen
PDX	Patient-Derived Xenograft
PET	Positron Emission Tomography
PR	Progesterone Receptor
PRC	Polycomb Repressive Complex
PRMTs	Protein arginine Methyltransferases
PTEN	Phosphatase and Tensin homolog
PyMT	Polyomavirus middle T antigen
RASSF1A	Ras Association domain-containing protein 1
RT-PCR	Reverse Transcription Polymerase Chain Reaction
rtTA	reverse tetracycline transactivator
SAM	S-Adenosyl Methionine
SCID mouse	Severe Combined Immunodeficiency mouse
SFRP	Secreted Frizzled Related Protein
siRNA	small interfering RNA
SOX2	Sex Determining region Y-box 2
SP1	Specificity Protein 1 TF
SPECT	Single Photon Emission Computed Tomography
SRC	Steroid Receptor Coactivator
STAT1	Signal Transducer and Activator of Transcription 1
STK11	Serine/Threonine Kinase 11

SUZ12	Suppressor of Zeste 12
TCF	T Cell Factor
Tet	Tetracycline
TFs	Transcription Factors
TGFβ	Transforming Growth Factor beta
TIC	Tumor Initiating Cells
TNBC	Triple Negative Breast Cancer
TNKS1/2	Tankyrase 1/2
TP53	Tumor Protein p53
TSG	Tumor Suppressor Gene
tTA	tetracycline-regulatible transactivator
USP22	Ubiquitin-Specific Protease 22
WAP	Whey Acidic Protein
WNT	Wingless-related integration site
YY1	Yin Yang 1
β-TrCP	β-Transducin repeat-containing protein

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