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TRIM28 INTERACTS WITH EZH2 TO REGULATE ITS ACTIVITY

Jing Li

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TRIM28 INTERACTS WITH EZH2 TO REGULATE ITS ACTIVITY

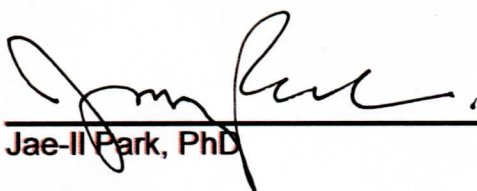
by

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APPROVED:



Advisory Professor, Michelle C. Barton, PhD



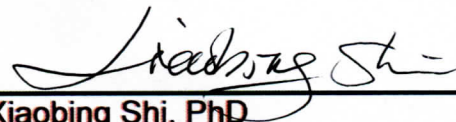
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TRIM28 INTERACTS WITH EZH2 TO REGULATE ITS ACTIVITY

A

DISSERTATION

Presented to the Faculty of
The University of Texas
Health Science Center at Houston
and
The University of Texas
MD Anderson Cancer Center
Graduate School of Biomedical Sciences
in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

by

Jing Li, B.M., M.S. Houston, Texas

Dec, 2015

DEDICATION

To My Family,

For their love and support

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First of all, I would like to extend my deepest gratitude to my mentor, Dr. Michelle Craig Barton, for the opportunity to learn from such an amazing and rewarding project in her laboratory, and especially for her support and guidance during my Ph.D. training. Shelley has opened up so many opportunities in my scientific career; she has guided, inspired and encouraged me tremendously in the past five years. I am thankful for her patience, encouragement, and the academic freedom to pursue my research interest in breast cancer epigenetics in her laboratory.

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TRIM28 INTERACTS WITH EZH2 TO REGULATE ITS ACTIVITY

Jing Li, B.M. M.S

Supervisory Professor: Michelle Barton, Ph.D.

In this dissertation, I discovered that TRIM28 interacts with EZH2 and regulates its activity in breast cancer cells. EZH2, the major H3K27 tri-methyltransferase, is over expressed in multiple subtypes of breast cancer; however, whether EZH2 plays an active role in breast cancer progression or treatment resistance is unknown. Here, we determined EZH2-interacting proteins in MCF7 cells by immunoprecipitation/mass spectrometry and detected TRIM28 with a high confidence score. TRIM28 is a transcription co-regulator with an N-terminal tri-partite RING finger, B-boxes and a coiled-coil domain (RBCC) and a C-terminal PHD/Bromo domain. Domain mapping revealed that the RBCC domain of TRIM28 interacts with EZH2, and the pre-SET region (Cysteine-rich domain) of EZH2 interacts with TRIM28. Transcriptome profiling and intersection of EZH2- and TRIM28-regulated genes indicated co-regulation of differentiation and morphogenesis pathways, as well as co-activation of the cytokine-cytokine receptor interaction pathway (CXCR4, CXCL12, EGFR, KIT). TRIM28 depletion in MCF7 cells decreased EZH2 recruitment and CXCR4 gene expression, in parallel with decreased mammosphere formation. TRIM28 regulation of EZH2 association with SWI/SNF complex might contribute to this process. Collectively these data indicate that EZH2 and TRIM28 regulate breast cancer stem cell maintenance by transcriptional regulation of CXCR4 gene expression.

In part two, TRIM28 is overexpressed in a human mammary epithelial cells (HMEC) model of breast cancer progression; interestingly, TRIM28 is heavily phosphorylated (pS824/pS473) in the more aggressive stages of this model. Furthermore, ectopic expression of TRIM28 WT and phosphorylation defective forms in HMEC cells indicates that TRIM28 promotes cell proliferation in a phosphorylation-dependent manner. Genome-wide TRIM28 binding profile analysis revealed transcription factor binding motifs, including TCF4 and RBP-J (a Notch effector), at TRIM28 binding sites. The promoter of *AXIN2*, which encompasses a TCF4 binding motif, is a direct transcriptional activation target of TRIM28. Collectively, we demonstrated a mechanism of regulation of breast cancer cell proliferation by TRIM28 in a phosphorylation dependent manner via a TCF4 binding motif.

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LIST OF ABBREVIATIONS

EZH2 – Enhancer Of Zeste Homolog 2

TRIM28 – TRIM28 tripartite motif containing 28, KAP-1, KAP1

TIF1-Beta - Transcription Intermediary Factor 1-Beta,

PRC2 – Polycomb Complex 2

PRC1 – Polycomb Complex 1

PcG - Polycomb-group (PcG) family

EED – Embryonic Ectoderm Development

SUZ12 – Suppressor Of Zeste 12

RBBP4 –(RbAp48, or NURF55) Retinoblastoma Binding Protein 4

TNBC - The Triple Negative Breast Cancer

ER- α – Estrogen Receptor alpha

PR – Progesterone Receptor

HER2 – Human Epidermal Growth Factor Receptor 2

CIS - Carcinoma In Situ

AJCC - American Joint Committee on Cancer

SWI/SNF Complex- SWItch/Sucose NonFermentable Complex

IHC – Immunohistochemistry

CyTOF – Mass Spectrometry Cell Time of Flight

qPCR - Quantitative Real-Time PCR

TIF I α - Transcription Intermediary Factor 1 Alpha

TRIM24 - Tripartite Motif Containing Protein 24

HSC – Hematopoietic Stem Cell

RBCC - a RING finger, b-boxes, a coiled-coil domain

RING - Really Interesting New Gene

RT – Reverse Transcriptase

PHD – Plant Homeo Domain

Bromo - Bromodomain

PHD/Br – PHD Finger-Bromodomain

HDAC– Histone Deacetylase

HMT – Histone Methyltransferase

CXCL/CCL12 – C-X-C Motif Chemokine 12, SDF-1

SDF-1 - The Stromal Cell-Derived Factor 1

CXCR4 - C-X-C Chemokine Receptor Type

H3K4me0 - unmethylated H3K4

H3K4me1 - Mono-methylated H3K4

H3K4me2 – Di-methylated H3K4

H3K4me3 – Tri-methylated histone H3K4

H3K27me0 – Un-methylated H3K27

H3K27me1 - Mono-methylated H3K27

H3K27me2 – Di-methylated H3K27

H3K27me3 – Tri-methylated histone H3K27

hESCs - Human Embryonic Stem Cells

eGFP - Enhanced Green Fluorescent Protein

DNMT1 - DNA Methyltransferase 1

co-IP - co-Immunoprecipitation

ChIP - Chromatin Immunoprecipitation

ChIP-Seq - ChIP-sequencing

HP1 α - Heterochromatin Protein 1 Alpha

MYC - Myelocytomatosis Viral Oncogene Homolog

EpCAM - epithelial cell adhesion molecule

ME - Myoepithelial

CK - Cytokeratins

NGFR - Nerve growth factor receptor

MYH11 – smooth muscle myosin heavy chain

SMA – Smooth Muscle Actin

MUC1 or EMA - epithelial membrane antigen

GATA3 – GATA Binding Protein 3

CHAPTER I

INTRODUCTION

AND RATIONALE FOR THE STUDY

1.1 Breast Cancer

Breast cancer is heterogeneous, and comprises a heterogeneous collection of diseases. It is the uncontrolled proliferation of mammary epithelial or myoepithelial cells from mammary gland. The smallest unit of mammary gland is terminal duct lobular unit complex. Lobules can generate milk, the thin tubes

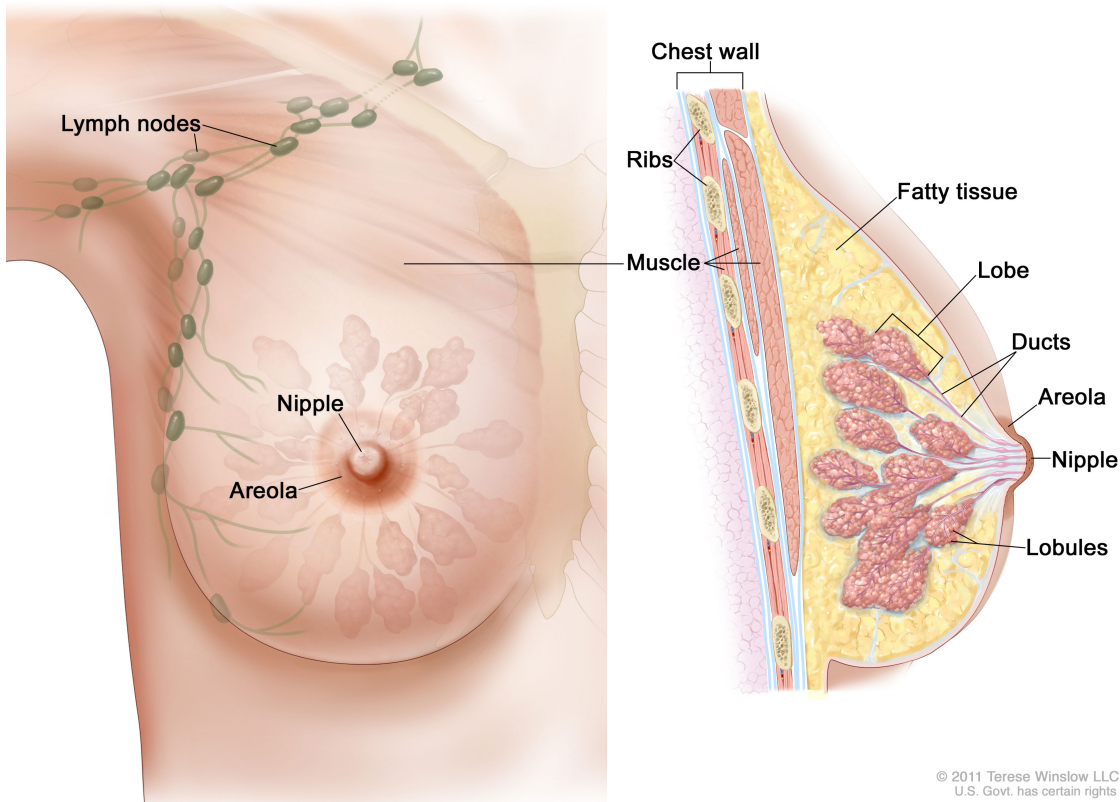


Diagram 1: Anatomy of the female breast.

The nipple, areola, lymph nodes, lobes, lobules, ducts, and other parts of the breast are shown. <http://www.cancer.gov/types/breast>

named ducts transport the milk from the lobules to the nipple. In addition, normal breast tissues also have fat tissue (white fat) and connective tissue, lymph nodes, and blood vessels. The anatomy of normal breast is illuminated in the diagram 1.

In general, cancer types are classified by tissue site of their origins. With the development of TCGA, molecular abnormalities may be eventually accepted as the new paradigm to name cancer types. The most common type of breast cancer is ductal carcinoma, which originates from the cells of the ducts. Breast lobular cancer is derived from the cells of the lobules; in general, the specific types of cancer are named by the anatomical location of tissues it originated from in the breast. Invasive breast cancer refers to breast cancer that has spread to surrounding tissue, lymph nodes, or organs.

Breast Cancer Anatomy

The following image (Diagram 2) (adapted from American Cancer Society, www.cancer.org) is an example of locations of early stage breast cancer.

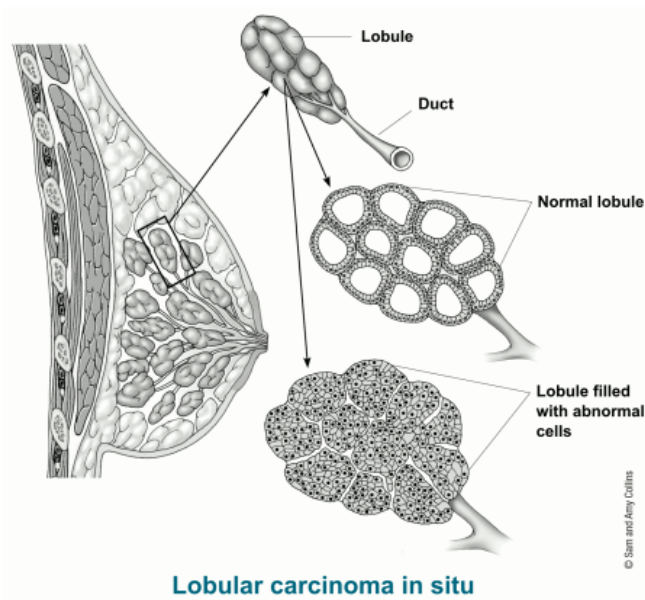


Diagram 2 Lobular carcinoma in situ
Adopted from www.cancer.org

Cell Types of the Mammary Gland and Luminal Epithelial Differentiation

The mammary gland is composed of a few types of cells, including epithelial, adipose, fibroblasts, immune, lymphatic and vascular cells. They collaborate to maintain a functional mammary gland ¹. The dysfunction of any one of these cell types contributes to breast carcinogenesis.

1) Mammary luminal epithelial cell

The markers of epithelial cells are listed in the table 1. Epithelial cell-derived mammary tumors constitute the largest percentage of breast cancer types. The vast majority of current breast cancer research focuses on the epithelial compartment. The epithelium has been shown to contain a stem cell population that exhibits considerable regenerative potential to develop within an entire fat pad ². Luminal progenitor cells were assessed regarding responsiveness to estrogen, and progesterone and found to be hormone insensitive ^{3,4}.

2) Myoepithelial cells

Myoepithelial (ME) cells are basally oriented in close contact with the basement membrane (BM). ME cells have some of the characteristics of both epithelial and smooth muscle (SM) cells. Developmental studies suggested that myoepithelial cells originated from luminal epithelial cells. The myoepithelial cells were described previously as the only cell able to repopulate the mammary gland and act as mammary stem cells ⁵. Since then, multi-potent basal stem cells have been identified within the myoepithelial compartment ^{6,7}.

3) Adipocytes

Adipocytes account for a large portion of the stromal fat pad. Adipocytes have been reported to secrete vascular endothelial growth factor (VEGF) and might mediate angiogenesis in the gland ⁸.

4) Fibroblasts

Stromal fibroblasts are supporting cells for both epithelial cell survival and morphogenesis in the fat pad. They regulate epithelial cell features and the epithelial cancer phenotype by synthesizing a number of ECM components, such as collagens, fibronectin, and matrix metalloproteinases ⁹.

5) Vascular and immune cells

There are many vascular and lymphatic networks throughout the fat pad. It was reported that Myoepithelial cell-derived VEGF drives lymph angiogenesis in the mammary gland ¹⁰.

In addition, GATA3, a luminal epithelial cell marker, is essential for mammary gland homeostasis and luminal epithelial differentiation ^{11,12}. Normal epithelial cells may exhibit a different ability to interact with basement membrane ¹³.

The identified cell markers of these two different cell types are summarized in the following table 1.

Luminal Epithelial Cell Markers		Myoepithelial Cell Markers	CK5
			CK14
	CK7		CK17
	CK8		SMA
	CK18		MYH11
	CK19		deltaNp63
	MUC1/EMA		Calponin
	Alpha6 Integrin		Caldesmon
	BCL2		β 4 Integrin
	ER		Laminin
	PgR		Maspin
	GATA3		CD10
	EpCAM		P-Cadherin
	Desmin		Caveolin1
			NGFR
			14-3-3 σ
			S-100

In some contexts, basal cells are also referred to as ME cells because of their location adjacent to the basement membrane and expression of basal cytokeratin. So, basal breast cells have two meanings in the context of normal mammary glands¹⁴: “(1) it has become a synonym for ME cells and (2) it defines a specific subpopulation of basal CK-expressing cells that may be found in either a luminal or basal location”¹⁴.

Molecular Subtypes of Breast Cancer

With the development of molecular biology, scientists and oncologists have classified six subtypes of breast cancer subtypes in terms of anatomy and molecular biology. Among all of the different types of breast cancers¹⁵: Luminal A, Luminal B, HER2, Basal, and Claudin low, the ER-positive Luminal breast cancers constitute the majority of all breast cancers and are generally treated with endocrine therapies¹⁶. Triple negative breast cancer (TNBC) generally refers to both Basal and Claudin low subtypes, which are the most malignant type of breast cancers in terms of clinical prognosis. Triple negative breast cancer was defined as estrogen receptor negative (ER- α^{-}), progesterone receptor negative (PgR $^{-}$), and human epidermal growth factor receptor 2 negative (HER2 $^{-}$), and has a more aggressive clinical course than other forms of breast cancer since it does not respond to anti-ER or anti-HER2 therapy¹⁷. The triple-negative breast cancer subtype is a heterogeneous group that represents 10%-20% of breast cancers¹⁸.

The following table 2 depicts the specific profiles of these six subtypes of breast cancer.

Table 2 Six Subtypes of Breast Cancer

Subtypes	These tumors tend to be*	Prevalence
Luminal A	ER+ and/or PR+, HER2-, low Ki67	42-59%
Luminal B	ER+ and/or PR+, HER2+ (or HER2- with high Ki67)	6-19%
Triple negative/basal-like	ER-, PR-, HER2-, cytokeratin 5/6 + and/or HER1+	14-20%
HER2+	ER-, PR-, HER2+	7-12%
Normal		
Claudin-low	often triple-negative, but distinct in that there is low expression of cell-cell junction proteins including E-cadherin and frequently there is infiltration with lymphocytes	
*These are the most common profiles for each subtype. However, not all tumors within each subtype will have all these features.		

Adapted from www.cancer.org

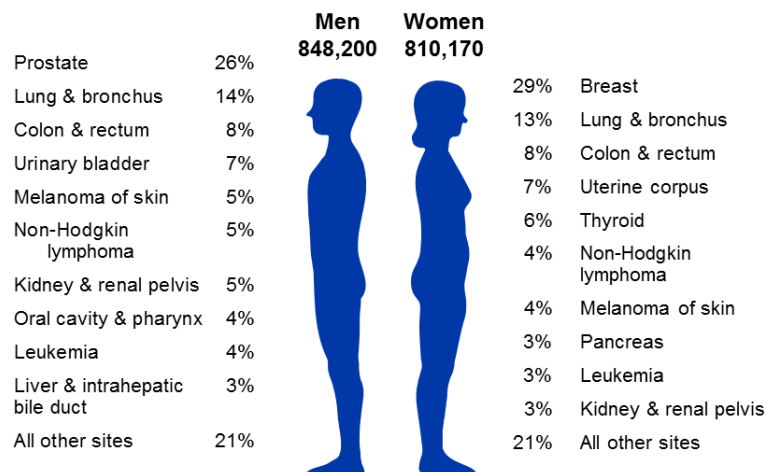
Clinically, triple negative breast cancer represents the most malignant subtype of breast cancer showing the worst prognosis among all of the subtypes^{15,19}.

Claudin-low type of breast cancer is relatively less studied compared to basal-like type of breast cancer. Basal-like breast cancer was believed to originate from myoepithelial cells, but the current clinical concept is that basal like breast cancer originated from both epithelial and myoepithelial cells. The gene-expression profile of basal-like tumors is reportedly similar to the transcriptome profile of tumors of BRCA1-germline mutation patients and also suggested its genetic signature is a less differentiated “stem/progenitor” cell phenotype or a stem cell origin for basal-like tumors¹⁷.

The Incidence (Prevalence) of Breast Cancer

According to American Cancer Society, Breast cancer is the most common cancer among American women. About 12% of women in the US will develop invasive breast cancer during their lifetime. The 2015 estimation is that about 231,840 new cases of invasive breast cancer will be diagnosed, and about 60,290 new cases of carcinoma in situ (CIS) will be diagnosed. In addition, the estimated breast cancer death is about 40,290 in the USA in 2015. Among all of the reasons for death, distant metastasis is responsible for 90% of deaths among breast cancer patients.

Estimated New Cancer Cases* in the US in 2015



*Excludes basal cell and squamous cell skin cancers and in situ carcinoma except urinary bladder.

Diagram 3 Estimated New Cancer Cases in the US in 2015
Adapted from www.cancer.org

Clinical Stages of Breast Cancer

The AJCC (American Joint Committee on Cancer) TNM system is the most common system used to describe the stages of breast cancer. The TNM pathological staging system classifies cancers based on their T, N, and M stages (www.cancer.org):

- The letter T followed by a number from 0 to 4 describes the tumor's size and spread to the skin or to the chest wall under the breast. Higher T numbers mean a larger tumor and/or wider spread to tissues near the breast.
- The letter N followed by a number from 0 to 3 indicates whether the cancer has spread to lymph nodes near the breast and, if so, how many lymph nodes are affected.
- The letter M followed by a 0 or 1 indicates whether the cancer has spread to distant organs -- for example, the lungs or bones.

Mortality (Survival Rate) of Breast Cancer Patients

The five-year overall survival rate of breast cancer is over 90%. The survival rates of breast cancer patients are correlated with clinical stages, the table of survival rate below was adopted from the National Cancer Institute's SEER database (<http://seer.cancer.gov/data/>).

Table 3 Survival Rates of Breast Cancer

Stage	5-year Relative Survival Rate
0	100%
I	100%
II	93%
III	72%
IV	22%

<http://seer.cancer.gov/data/>

Estimated Cancer Deaths in the US in 2015

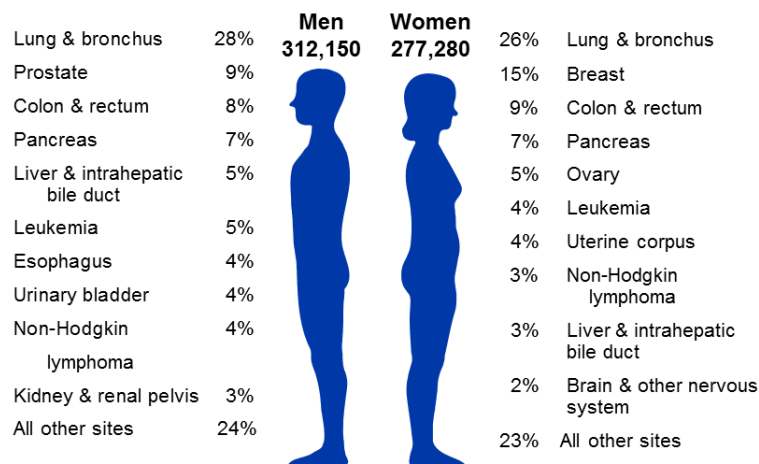


Diagram 4 Estimated Cancer Death in the US in 2015 Adopted from www.cancer.org

Among women, lung (26%), breast (15%), and colon & rectum (9%) cancers are the leading causes of cancer death. Thus, the development of better breast cancer therapy is greatly needed, and this will be facilitated by a deeper understanding of molecular mechanisms.

1.2 Breast Cancer Stem Cell

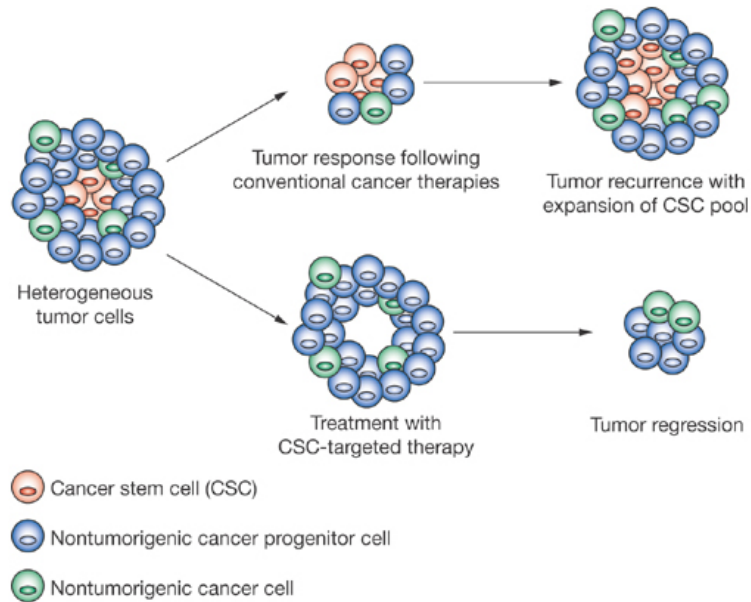
The cancer stem cell theory states that tumor proliferation originates from a rare subpopulation of tumor cells²⁰. This subpopulation of cancer stem/progenitor cell is believed to be responsible for cancer initiation, cancer progression, and cancer chemotherapy resistance. Cancer stem/progenitor cells are resistant to chemotherapy treatment^{21,22} and also contribute to one hallmark of cancer: heterogeneity²³. Cancer stem cells are characterized by the ability to undergo both self-renewal and differentiation, the differentiated cancer cells constitute the bulk of the tumor without tumorigenic capacity as they have a limited capacity to self-renewal and low proliferation potential^{24,25}.

The concept of cancer stem cells was first proposed almost 150 years ago^{20,25,26}. However, it was not supported by experimental evidence until 1997^{20,25,26}. The first experimental evidence showed that human leukemia is driven by a small number of leukemic stem cells that is able to reconstitute the leukemia in NOD/scid mice²⁷. Cancer stem cells in solid tumors were experimentally supported in 2003 using breast cancer cells²³. The breast cancer stem cell was

defined as a CD44⁺CD24⁻ population of breast cancer cells ²³. Other than CD44, CD24 breast cancer stem cell marker mentioned above, CD133 ²⁸, CXCR4 ²⁹, EpCAM ³⁰, and ALDH1 ^{25,31} were also reported to express on breast cancer stem cells and was suggested to serve as breast cancer stem cell markers. Additionally, Hoechst negative side population (SP) cells, defined by flow cytometry of hematopoietic malignancy cells, were also reported to exhibit self-renewal properties ³². Spheroid cultures of mammosphere were developed as a simple method to measure cancer stem cell populations ^{31,33}. Breast cancer stem cells have the capacity to be serial transplanted and expanded in immunodeficient mice. The following diagram 2 briefly explains the cancer stem cell concept and characteristics.

Figure 1- 1 Schematic representation of Cancer stem cell differentiation.

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1.3 The Link between Epigenetics, Cancer, and Breast Cancer

Epigenetics refer to heritable changes without alteration of genomic DNA sequence, including histone modifications, ATP-dependent chromatin remodeling, non-coding RNA-mediated gene silencing, and DNA methylation³⁴; histone modifications include acetylation, methylation, ubiquitination and others. Epigenetics is implicated in many biological processes such as genomic imprinting, X chromosome-inactivation, reprogramming in embryogenesis, and chromosomal structure. There has been an explosion of knowledge in our understanding of molecular mechanisms of epigenetics of late, especially with regards to histone methylation marking gene activation/repression. H3K4me2/3 is correlated with activated gene expression; whereas, H3K27me2/3 marks repressed gene expression. These histone modification markers are catalyzed by highly specific enzymes, for example, EZH2 writes H3K27me2/3 by adding methyl groups on histone H3 lysine 27 and LSD1 (KDM1A) erases H3K4me2/3 by removing methyl group from histone H3K4me2/3.

There are many misregulated epigenetics mechanisms uncovered in cancers, such as the following: BRD4, a bromodomain protein that “reads” and interacts with acetylated lysines³⁵, emerged as a therapeutic target in acute myeloid leukemia, due to its aberrant over expression. JQ1 was developed as a BRD4 inhibitor and has shown potential in different diseases³⁶. KDM2A promotes lung tumorigenesis by increasing ERK1/2 signaling activity³⁷. Somatic mutations of epigenetic molecule were discovered in cancers, such as the histone H3K27M mutation in paediatric glioblastoma, ATRX (α -thalassaemia/mental retardation

syndrome X-linked) and DAXX (death-domain associated protein) in pediatric glioblastoma ³⁸, methylcytosine hydroxylase TET2 in myelodysplasia and myeloid malignancies ³⁹, DNA methyltransferase DNMT3A ⁴⁰, and histone H2K27 methyltransferase EZH2 in various cancers ⁴¹.

lysine methyltransferases

SET domain catalyzes protein methylation, both histone and non-histone proteins. Histone H3K27 is methylated by EZH2/EZH1, and demethylated by UTX. SMYD3, SETD7, and MLLs catalyze H3K4 methylation⁴², SUV39 family add methyl group on H3K9; SET2 family methylates H3K9, H3K27, and H4K20. A new class of methyltransferases, PRDM family, catalyzes H3K9 methylation ⁴³.

1.4 Polycomb Repressive Complexes

Polycomb repressive complexes are transcriptional repressors governing development, pluripotency, senescence, and cancer ⁴⁴. There are two multiprotein PcGs complexes, PRC2 and PRC1.

1) PRC2 Complex - (Polycomb Repressive Complex2)

The canonical PRC2 complex is comprised of EZH2, EED, SUZ12, and RBBP4, its main function is transcriptional suppression by writing di and tri methyl group onto histone 3 lysine 27 ⁴⁵. Among the four core subunits, EZH2 is the catalytic unit, the major H3K27 tri-methyltransferase ⁴⁶⁻⁴⁹, writes H3K27me2/3 by adding methyl group on histone H3 lysine 27. PRC2 plays a critical role in various biological processes, ranging from differentiation, maintaining cell identity and proliferation, to stem-cell plasticity ⁵⁰. EZH2 must associate with RBBP4, SUZ12,

and EED to exhibit an intact histone 3 lysine 27 methyltransferase activity^{51,52}. In other word, all of the four core members of PRC2 are required for EZH2 to effectively methylate H3K27⁵³⁻⁵⁵. Once EZH2 no longer associates with its PRC2 partners, EZH2 can function as a transcriptional co-activator⁵⁶. In blood development, EZH1 is an alternative enzyme to catalyze histone 3 lysine 27^{49,57}. However, the core subunits of PRC2 complex are not sequence-specific DNA-binding proteins, and PRC2 must be recruited by other factors: JARID2^{58,59}, AEBP2⁶⁰, long non-coding RNAs⁵⁰, and more recently, ATRX⁶¹, are identified to recruit/direct the PRC2 complex to various target loci to silence gene expression. PRC2 preferentially occupies CpG-rich regions, as has been reported for PRC2 enrichment of binding at CpG sites in ES cells⁶². However, it is not known whether there is a similar recruitment mechanism in breast cancer development.

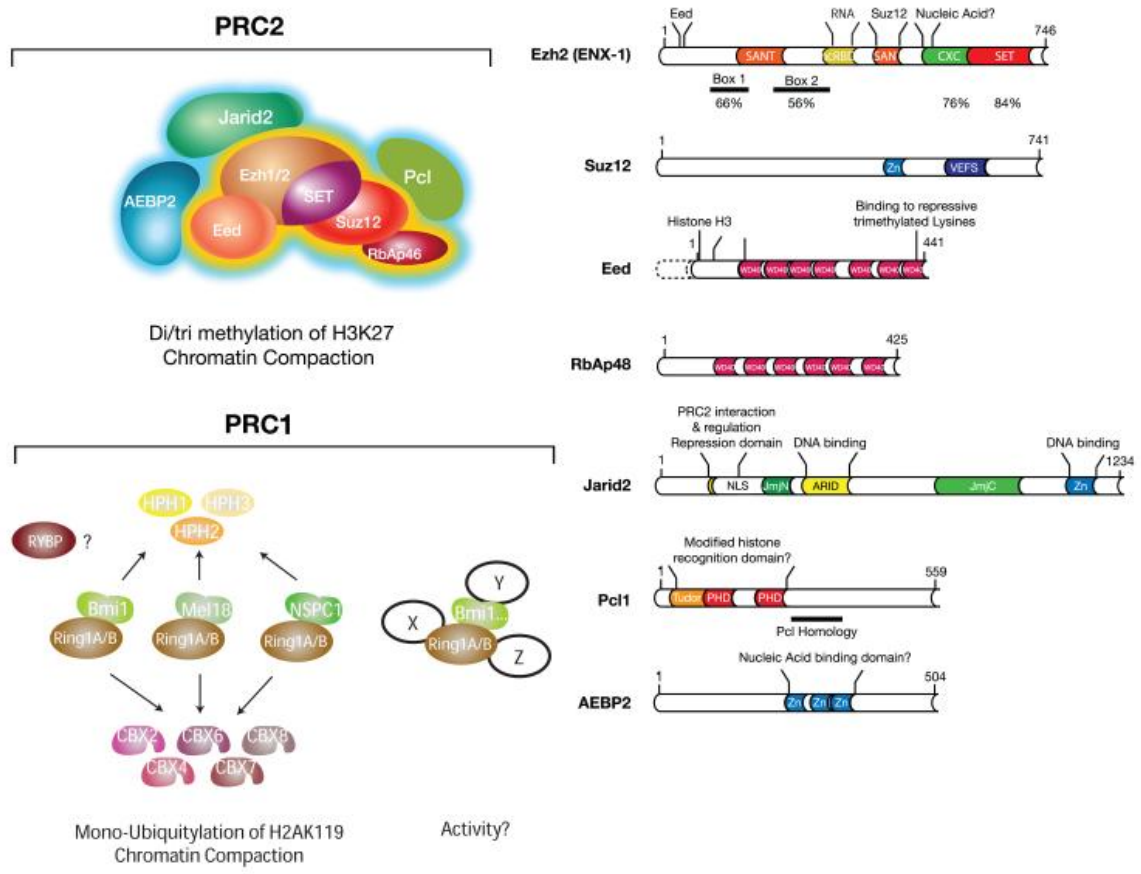
2) PRC1 Complex - (Polycomb Repressive Complex1)

Polycomb repressive complex 1 composition is quite complicated^{44,63}. The composition of different PRC1 complexes is programmed by the presence or absence of CBX proteins (canonical versus non-canonical) and some other proteins, such as PCGF (polycomb group factor), HPH (human polyhomeotic homolog), and the E3-ligase protein (RING) that catalyzes H2AK119ub⁴⁴. The canonical function of PRC1 is to repress gene expression dependent on its ubiquitination enzymatic activity⁶⁴, but PRC1 was shown to also activate transcription in the CNS⁴⁵.

Figure 1- 2 Schematic representation of Polycomb complex PRC1/2.

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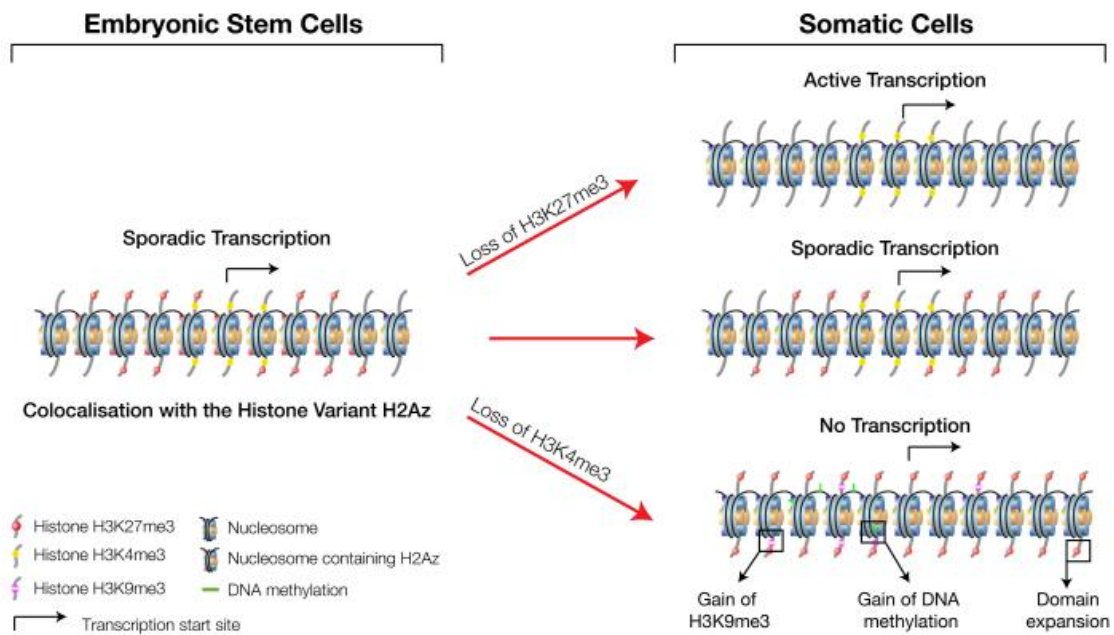
1.5 EZH2 - Enhancer of Zeste Homolog 2

EZH2 is over-expressed and act as an oncogene in various cancers such as melanoma, lymphoma, and poorly differentiated synovial sarcoma⁶⁵, and prostate and breast cancer.

EZH2 in Breast Cancer

EZH2 is a marker of aggressive breast cancer. Overexpression of EZH2 also promotes neoplastic transformation of normal prostatic cells and hyperplasia in breast epithelium. The transcriptional repressive role of EZH2 is associated with the entire intact PRC2 complex, which is described in the above. However, emerging evidences in cancer research indicate that EZH2 play an oncogenic role in various types of cancers, which is correlated with EZH2's transcriptional co-activator functions. In breast cancer, EZH2 was reported as a transcriptional co-activator by wiring estrogen pathway with WNT proliferation pathway in breast cancer⁶⁶, this raise a deeper question, whether EZH2 play a key role in drug resistant breast cancer. As an oncogene, EZH2 inhibitor as a promising anti-cancer drug is being extensively researched and developed in the pharmaceutical industry.

Figure 1- 3 Chromatin properties at PRC2 target genes in ES cells and differentiated cells. Schematic representation of chromatin at PcG target genes as a function of ES cell differentiation. Reprinted with permission from Nature. 2011 Jan 20; 469(7330): p.343-9 Copyright (2011) ⁵⁰.



Somatic Mutation of EZH2

EZH2 somatic mutation at Tyr641 occurs in 21.7% of GCB DLBCLs (GCB subtype of diffuse large B-cell lymphoma) and 7.2% of FLs (follicular lymphoma), but no mutations were found in ABC DLBCLs (activated B-cell) ⁶⁷. A gain of function for H3K27me3 mutations was first proposed and is now verified ^{67,68}. A longitudinal analysis of mutation status, during follicular lymphoma (FL) progression from FL to transformed FL, validated frequent mutation status of EZH2 in FL patients and also revealed that EZH2 hotspot mutation (3 recurrent mutation hot spots (Y646, A682, and A692)) is an early event in FL ⁶⁹.

Targeting Mutated EZH2

Therapeutically, the GSK (GSK-126) ⁷⁰ and Epizyme (EZH2 inhibitors: EPZ-6438) ⁷¹ are two pioneer inhibitors focused on targeting the catalytic SET domain of mutant EZH2 in patients with non-Hodgkin lymphomas, including germinal center diffuse large B-cell lymphoma (GCB-DLBCL), primary mediastinal B-cell lymphoma (PMBCL) and follicular lymphoma. This year, EPZ011989, a potent, selective, orally bioavailable inhibitor of EZH2 was developed with better characteristics of a therapeutic ⁷². Novartis is developing its own EZH2 SET domain targeting inhibitor named EI1⁷⁰, as is Pfizer ⁷³. Clearly there is considerable interest in EZH2 as a therapeutic target.

Targeting EZH2 in a mutated SWI/SNF Background

In both drosophila and mammals, the PRC2 complex was reported to genetically

antagonize SWI/SNF complex⁷⁴. But the functional interplay of these two complexes might be far more complicated in cancer biology. Immunologists showed that PRC2 may inhibit immune surveillance, and EZH2 could be targeted to reactivate immune surveillance in SWI/SNF deficient cancers⁷⁵.

Therapeutically, EPZ-7438 exhibits great efficacy in SMARCB1-deleted pediatric malignant rhabdoid tumors (MRT) cells⁷¹. EZH2 inhibition may also sensitize BRG1 and EGFR mutant small lung tumors to TopoII inhibitors⁷⁶. In ovarian cancer, ARID1A mutated in about 50%, and EZH2 inhibition in this genetic context exhibited synthetic lethal capacities⁷⁷.

EZH2 in Cancer Stem Cell

The first publication of EZH2 functioning in cancer stem cells was that EZH2 maintains glioblastoma stem cells⁷⁸. EZH2 was reported to function in promoting breast cancer initiating cell by activating RAF1- β -Catenin signaling⁷⁹. Another lab reported that EZH2 expanded a breast cancer stem cell population by activating NOTCH signaling in an H3K27me3 independent manner⁸⁰. In addition, EZH2 drives a cancer malignant phenotype in a polycomb-independent manner, but in a SET catalytic domain dependent way in castration-resistant prostate cancer⁵⁶. The authors proposed potential non-histone substrates of EZH2 are involved in this process.

EZH2 Catalyzes Non-Histone Substrates

There are at least three non-histone substrates of EZH2 identified to play critical roles in disease biology or cancer biology recently, for example: STAT3 in glioblastoma ⁸¹, ROR α ⁸² and GATA4 in heart development ⁸³.

The Post-Transcriptional Modification of EZH2

The high level of EZH2 protein in cancer may be due to decreased negative regulators, such as microRNAs. MicroRNA101 is the first identified post-transcriptional regulator of EZH2 and was identified in prostate cancer ⁸⁴. Later, the low level expression of microRNA101 is also observed in other cancers ^{85,86}. Further, more microRNAs are identified to directly regulate EZH2. MiR-26a ⁸⁷, miR214 ⁸⁸, and microRNA124 ⁸⁹ were experimentally shown to target EZH2.

The Post-Translational Modification of EZH2

EZH2 is phosphorylated at threonine residues 345 and 487 by CDK1 (cyclin-dependent kinase-1) to exhibit enhanced affinity with RNAs, such as HOTAIR and 5' end of Xist ^{90,91}; another group reported that CDK1 and CDK2 phosphorylate EZH2 at threonine 350 in an evolutionarily conserved motif that is critical for proper EZH2 recruitment and maintaining H3K27me3 mark ⁹⁰. Phosphorylation may also destabilize EZH2 by affecting proteasome dependent degradation ⁹². P300/CBP-associated factor (PCAF) was demonstrated to acetylate EZH2 at K348 to regulate its stability and function in lung carcinoma progression ⁹³.

1.6 Bivalent Genes

--The coexistence of H3K4me3 and H3K27me3 on nucleosomes

Almost 4000 promoters in human embryonic stem cells (2984 in mouse ESC) contain bivalent chromatin domains that combine the activating H3K4me3 mark and the repressive H3K27me3 mark within a single nucleosome^{94,95}. H3K27me3 is generally associated with decreased gene expression; however, the bivalency status of H3K27me3 does not correlate with gene expression in embryonic stem cells⁹⁴. These special chromatin structure domains poise expression of lineage-specific developmental genes in ESC to support timely activation in the presence of differentiation signals.

Figure 1- 4. Reprinted with permission from A double take on bivalent promoters Genes & Dev. 2013. 27: p.1318-1338 Copyright (2013)⁹⁶

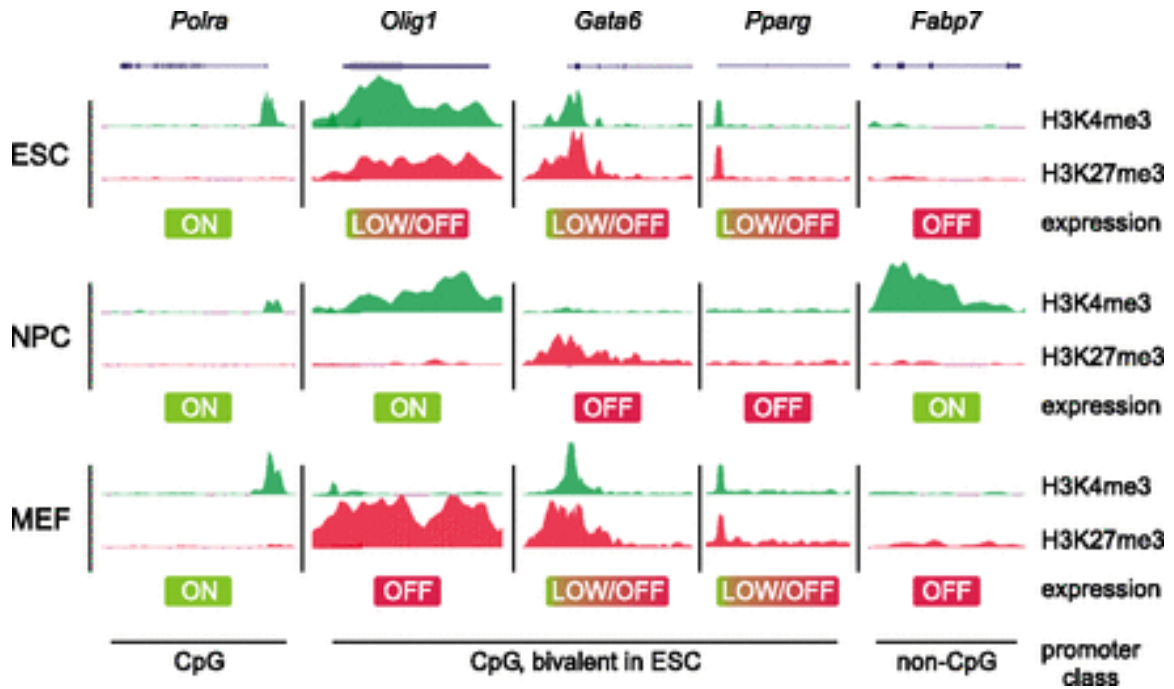
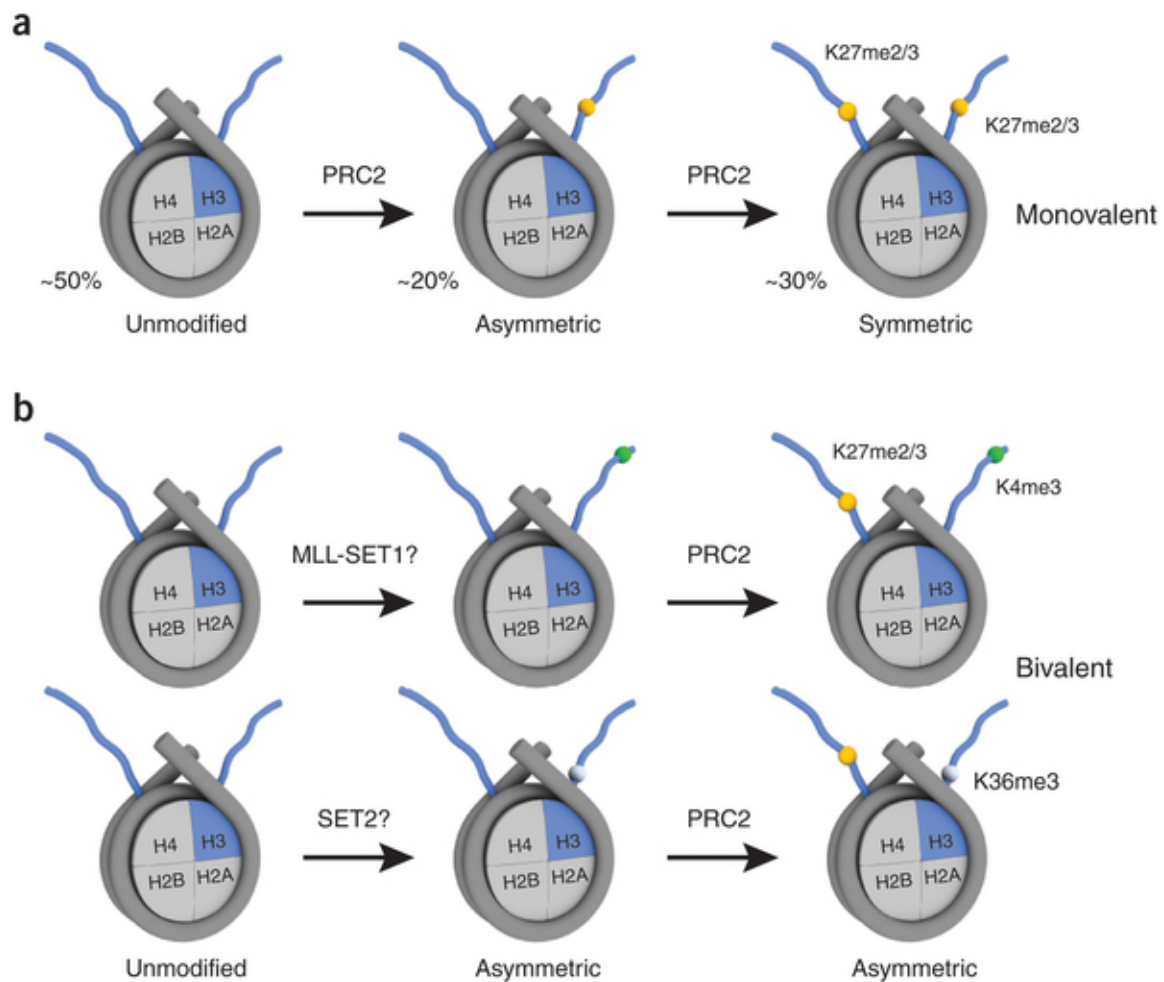


Figure 1- 5. Reprinted with permission from Asymmetrically modified nucleosomes expand the histone code *Nature Structural & Molecular Biology* 19, p.1064–106 Copyright (2012) ⁹⁷



The bivalent domains also exist in cancer cells and have been mapped at sites that partially overlap with bivalent genes in ES cells ^{98,99}. The bivalent genes in cancer tend to be predisposed for late DNA methylation ¹⁰⁰. Collectively, these

data support a stem cell origin for cancer with a possible link between bivalent genes and cancer stem cell characteristics¹⁰¹.

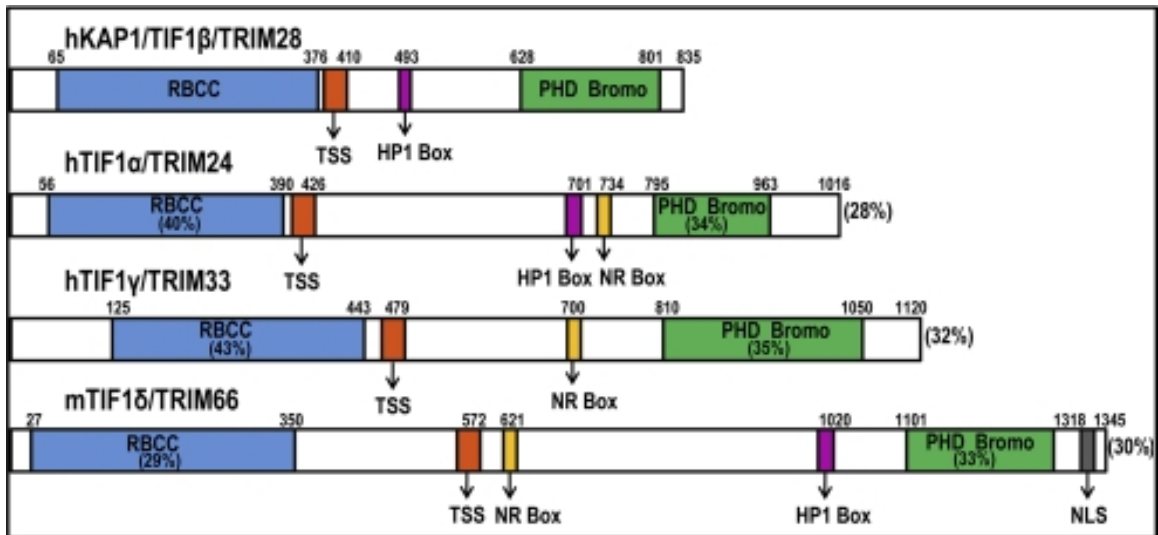
1.7 TRIM28 - Tripartite Motif Containing Protein 28, Transcription Intermediary Factor 1 β , KAP1

Transcriptional Repressor Functions of TRIM28

TRIM28 (KAP1, KAP-1, TIF1 β) is a heterochromatin protein that belongs to the tripartite motif superfamily. The tripartite motif is composed of a RING finger followed by two B-boxes and a coiled-coil domain, also called RBCC motif; TRIM28's C-terminal is composed of a bi-partite motif encompassing a PHD finger and a Bromo domain. TRIM28 was known as a universal transcription corepressor protein by interacting with Kruppel associated box (KRAB)-domain containing zinc finger proteins^{102,103}. TRIM28 binds and co-localizes with HP1 (heterochromatin protein 1) through a well-defined peptide motif, PxVxL. HP1 reads H3K9me3 (a repressive histone marker) with its chromo domain to play a pivotal role in heterochromatin mediated gene silencing¹⁰⁴. TRIM28 depletion leads to a lower level of global H3K9me3^{105,106}. TRIM28 binds and recruits SETDB1 complex to H3K9me3 genomic sites mediating heterochromatin silencing¹⁰⁷. TRIM28 has sumo E3 ligase activity and exhibits a self - sumoylation phenomena at K779, K804, and K554 via its PHD domain¹⁰⁸. SETDB1 and CHD3 were shown to bind SUMO-modified KAP1 via their functional SIM motifs^{108,109}. TRIM28 was purified by interacting with TRIM24 (TIF1 α) and TRIM33 (TIF1 γ), forming a macromolecular complex to regulate transcription. Somatic, hepatocyte-specific deletion of TRIM28, TRIM24, or TRIM33 causes mouse hepatocellular carcinoma¹¹⁰. The histone-binding

signature of TRIM28 remains elusive so far, though TRIM24 binds and reads un-methylated H3K4 and H3K23ac¹¹¹, TRIM33 reads K9me3 and K18ac¹¹².

Figure 1- 6 Schematics of the human TRIM28 protein (also called TIF1 β and KAP1) and other related proteins, including TIF1 α /TRIM24, TIF1 γ /TRIM33, and TIF1 δ /TRIM66. Reprinted with permission from Journal of Biological Chemistry. 2011 Jul 29; 286(30): p. 26267–26276, Copyright (2011)¹¹³.



TRIM28 and Stem Cell Pluripotency, Self-Renewal

Large genome wide RNAi screening revealed that TRIM28 is required for maintenance of mouse stem cell pluripotency and self-renewal ¹¹⁴. This pro-pluripotency function of TRIM28 acts in a phosphorylation (C-terminal serine 824) dependent manner ¹¹⁵. Phosphorylated form of S824 TRIM28 is the major form of TRIM28 in embryonic stem cell. pSer824 TRIM28 specifically interacts with OCT3/4, and subunits of the SWI/SNF2, such as Smarcd1, Brg-1, and BAF155 to induce ES cell-specific genes, such as SOX2, NANOG, OCT3/4, and maintain pluripotent status of mouse ES cells. In addition, TRIM28 dependent pluripotency in mouse stem cell may involve TRIM28-mediated repression of retrotransposon-based enhancers ¹¹⁶, as well as TRIM28 cooperative interactions with PRC1 to activate pluripotency-associated genes in embryonic stem cell ¹¹⁷.

TRIM28 with Endogenous Retroviral Elements

Endogenous retroelements account for more than 40% of the mammalian genome; and at least 25% of endogenous retroelements is comprised of endogenous retroviral elements (ERVs). TRIM28 is known to silence the transposable elements of endogenous retroviral elements in embryonic stem cells and during early embryonic development ^{118,119}. This process might involve recruitment of DAXX to ERVs along with ESET ¹¹⁹. A TRIM28 complex also was linked to epigenetic silencing of the retrovirus murine leukaemia virus (MLV) in embryonic cells ¹²⁰.

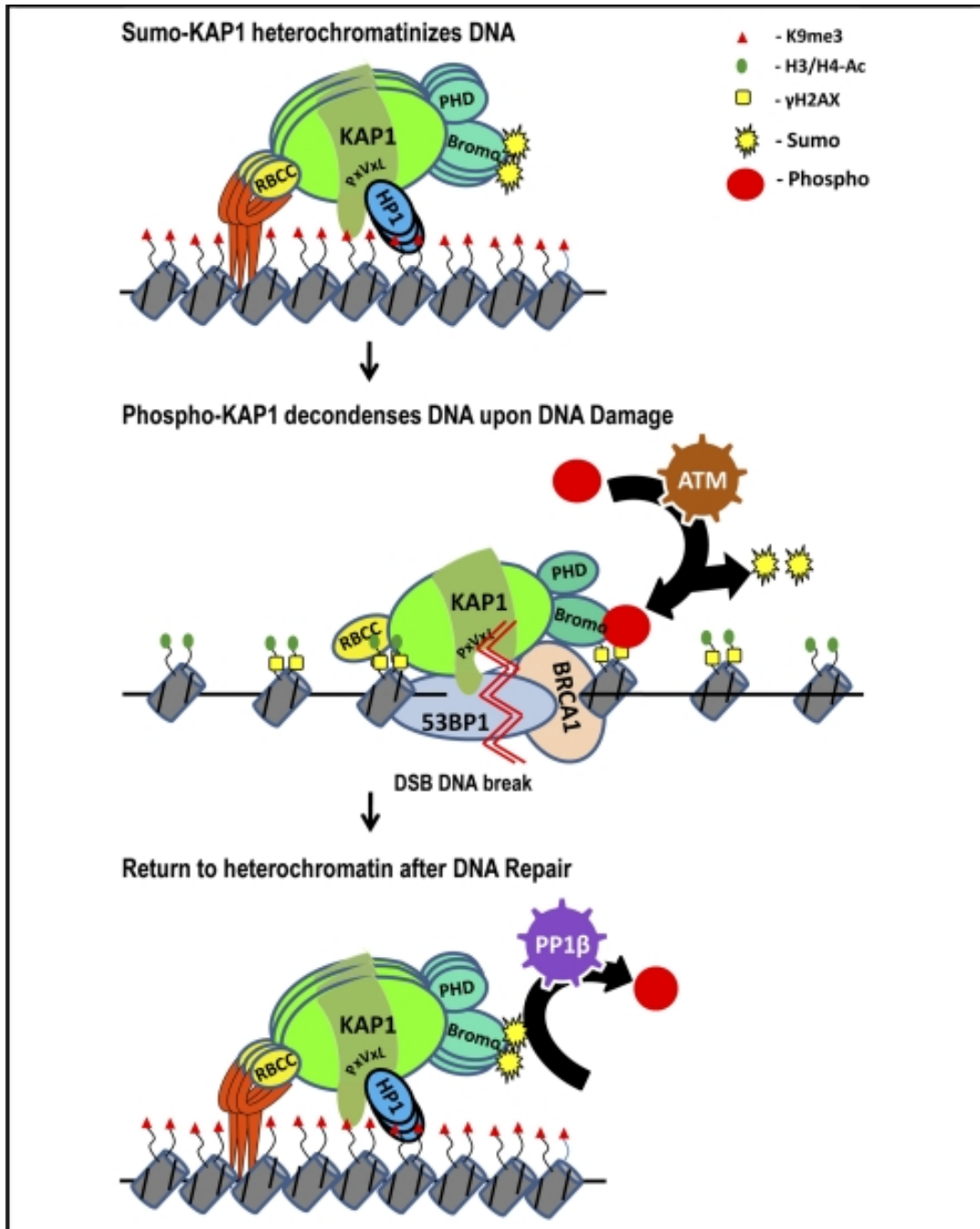
Transcriptional Activation and non-Transcription Roles of TRIM28

Despite the many studies of TRIM28 as a co-repressor of transcription, recent studies indicate that TRIM28 may also activate gene expression in specific contexts ^{113,121}. Using ChIP-sequencing technology, genome-wide binding profiles of TRIM28 indicates that TRIM28 plays a role distinct from transcriptional repression at the majority of its strongest binding sites ¹²¹.

TRIM28 and DNA DSB Repair

TRIM28 is phosphorylated at serine 824 at DNA double strands break sites by ATM/ATR to recruit DNA repair machinery ¹²². Phosphorylation of S824 TRIM28 was reported to colocalize with γ H2AX foci at damage sites and lead to constitutive chromatin relaxation in response of DNA double-strand breaks ¹²². TRIM28 is also phosphorylated at serine 473 and is diffusely localized in the nucleus by CHK1/2 to positively regulate DNA damage pathway ¹²³.

Figure 1- 7 **Model for TRIM28 involvement in DNA repair..** Reprinted with permission from Journal of Biological Chemistry. 2011 Jul 29; 286(30): p. 26267–26276, Copyright (2011) ¹¹³.



TRIM28 and Virus Infection

TRIM28 was also reported to inhibit HIV integration by interacting with acetylated integrase and inducing its deacetylation¹²⁴. Human cytomegalovirus (HCMV)¹²⁵ can establish life-long time latency infection in hematopoietic stem cell with the help of TRIM28 as a transcriptional repressor. As soon as TRIM28 is phosphorylated by pharmacological approach, HCMV latency is broken; HCMV is released from hematopoietic stem cell. There is a unique SUMO-interacting motif (LANA^{SIM}) in Kaposi's sarcoma-associated herpesvirus (KSHV) encoded LANA protein that binds with sumoylated TRIM28 to mediate viral episome maintenance and lytic gene silencing¹²⁶.

Phosphorylation Switch of TRIM28

Un-phosphorylated form of TRIM28 and phosphorylated form of TRIM28 appear to have different affinities for TRIM28 binding proteins. There is evidence for a phosphorylation switch of TRIM28 to regulate MyoD function. In myoblasts, TRIM28 serves as a scaffold to recruit not only coactivators such as p300 but also corepressors such as G9a and HDAC1 (histone deacetylase 1) to silence gene expression. Upon differentiation, phosphorylation of TRIM28 releases the corepressors from the scaffold, activating transcription to drive myogenesis¹²⁷.

TRIM28 and DNA Methylation in Imprinting Loci

Daniel Messerschmidt and Barbara Knowles reported that maternal Trim28 knockout results in a highly pleiotropic, 100% embryonic lethality. The underlying

mechanism in details is epigenetic misregulation of genomic imprinting loci in *TRIM28* null mice, mainly through the *H19/Igf2* cluster. *TRIM28* binding to differentially methylated regions (DMRs) is DNA methylation dependent¹²⁸. ZFP57 is required in *TRIM28* mediated DNA methylation at imprinted loci¹²⁹⁻¹³¹. A methylated hexanucleotide TGCCGC was discovered as the motif to be recognized by ZFP57/*TRIM28* in imprinting control regions (ICRs); H3K9me3 is also involved in this mechanism working together with DNA methylation.

1.8 Aims of the Work

The oncogenic role of EZH2 in breast cancer lacks detailed mechanisms, which dampen the development of EZH2 based therapy. In the studies described herein, we document the physical and functional interactions of TRIM28 with EZH2 and demonstrate that TRIM28 collaborate with EZH2 to regulate breast cancer stem cell maintenance. We found a specific interaction of EZH2 with TRIM28 and discovered that TRIM28 collaborates with EZH2 to regulate breast cancer stem cell maintenance by activating CXCR4 signaling pathway. Mechanistically, TRIM28 reprograms H3K27me3 distribution. Depletion of TRIM28 does not affect EZH2 expression levels in both MCF7 and HMEC cells, but decreases EZH2 recruitment at target genes, which is correlated with decreased gene expression. We also documented that TRIM28 is overexpressed in HMEC model of breast cancer progression; interestingly, TRIM28 is heavily phosphorylated (pS824) in aggressive breast cancer cells. Additionally, exogenous TRIM28 expression in HMEC cells increases cell proliferation in a phosphorylation dependent manner. Further analysis revealed that phosphorylated TRIM28 (pS824/pS473) does not bind to EZH2, suggesting a phosphorylation dependent precise signaling mechanism governing these interactions.

CHAPTER II

MATERIALS AND METHODS

2.1 Cell Culture, Vectors, and protein purification. The breast cancer cell line MCF7 and the immortalized human mammary epithelial cell line MCF10A were obtained from the American Type Culture Collection (ATCC) and grown in suggested media conditions. HMEC (Human Mammary Epithelial Cell) cells were gifts (<http://hmec.lbl.gov/mindex.html>) from Dr. Martha Stampfer who was a professor at Lawrence Berkeley National Laboratory. HA-TRIM28 CS2 backbone plasmid was a gift from Dr. Patrick Ryan Potts¹³². To clone TRIM28 into a flag tagged vector, full length TRIM28 was amplified with added restriction enzyme sites in the amplification primers. The amplicon was digested with the restriction enzyme for 2 hours, and subject to gel purification. The purified PCR product was incubated with vector for 1 hour at room temperature along with T4 ligase. And 2 ul of the reaction system was transformed into DH5a E Coli.. Colonies was picked up for sequencing for verification at MD Anderson Cancer Center DNA analysis core. MBP-EZH2 construct was generated in the lab, and purified from E.Coli. With MBP beads (NEB); GST-TRIM28 full length and RBCC fragment was also purified from *E.coli*.

2.2 Mutagenesis and Mutants

To generate various deletion and point mutants of HA-TRIM28 and Myc-EZH2, point mutagenesis protocol (Stratagene/Agilent) was used. Site-specific point mutations and serial domain deletions are introduced to HA-TRIM28 and Myc-EZH2 plasmid using QuickChange® Site-Directed Mutagenesis Kit (Stratagene/Agilent), according to manufacturer's suggestions with slightly

medication, KOD DNA polymerase (Millipore) was used to generate mutated plasmids instead of PfuUltra HF DNA polymerase. Dpn I was used to digest the template DNA for 2 hours. 2ul of amplicons were subject to transformation. And 2 ul of transformation is then plated on LB agar plates and incubated at 37°C 12-16 hours or overnight. DNA isolated from E. coli culture is purified using DNA Miniprep Kit (Qiagen). Specific mutations were confirmed with Sanger sequencing by M.D. Anderson DNA Analysis Core Facility.

2.3. Transient DNA Plasmids Transfection

To map the interaction domains of EZH2 and TRIM28, MCF7 or HEK-293T cells cultured in 10cm plates were co-transfected with HA-TRIM28 and Myc-EZH2/Flag-EZH2 using Effectene (Qiagen), according to manufacturer's instructions. Briefly, 2 µg of each DNA plasmids was re-suspended in 200µL EC Buffer, together with 16µL Enhancer and vortexed for 5 seconds. After 5 minutes of incubation at room temperature, 20µL of Effectene was added to the mixture, and vortexed for 10 seconds. After 10 minutes incubation at room temperature, medium is added to reach 800 µL volume in total and gently transferred onto the cells. Protein is harvested 30-48 hours after transfection.

2.4. Transient knockdown by siRNAs

MCF7 cells cultured at 6-well plates are transfected with siControl, siTRIM28 (ON-TARGETplus SMARTpool, Dharmacon) using Lipofectamine® RNAiMAX transfection reagent (Invitrogen), according to manufacturer's

instructions ¹¹¹. Briefly, for each transfection, 100 pmol of siRNA and 5 μ L Lipofectamine RNAiMAX was first separately re-suspended into 250 μ L completed medium and incubated at room temperature for 5 min. Then, siRNAs and Lipofectamine RNAiMAX are mixed together and further incubated at RT for 10 minutes. Cells covered with 250 μ L medium are then transfected with the siRNA-Lipofectamine RNAiMAX mixture. Medium is changed after 4 to 6 hours and cells are continually cultured for a total of 48-72 hours before harvest.

2.5 Lenti-Viral Infections, establishment of stable knockdown cell lines.

EZH2 knockdown was achieved by using stable pGIPZ short-hairpin interfering RNA in lentivirus (V2LHS_17507, open biosystems). A lentiviral scramble plasmid was used as control for transfection and virus packaging. The virus packaging protocol was reported previously ¹³³. Briefly, equal amount of shEZH2 plasmid was co-transfected with pPAX2, and pMAX into HEK293T cells to generate virus. After 48 hours, the supernatant was filtered and transferred into targeted cell lines for infection for two to three days. Then, 2ug/ml puromycin (Sigma) was added for selection for MCF7 cells, 1ug/ml for HMEC cells; puromycin selection lasts one to two weeks. And western blotting was used to test whether the cells were successfully knocked down. Once the knockdown efficiency is verified, maintenance concentration of 0.2 ug/ml of puromycin was used.

2.6 Western Blots. MCF7 cells were lysed in NTEP buffer (25mM Tris-HCl pH 7.5, 150mM NaCl, 5mM EDTA, 0.5% NP-40) for 30 min at cold room for western blot. Generally, 20ug protein lysate was loaded into various percentages of SDS-PAGE gels. PVDF membrane was used for transferring, 5% milk or BSA was used for blocking, 3% milk or BSA was used to dilute primary (dilute at 1:1000 to 1:5000) and secondary antibodies (dilute at 1:5000 to 1:10000) and incubate with membrane. Primary antibody was incubated with membrane for 2 hours at room temperature or overnight at cold room, and membrane was washed extensively for 3 times, 10 minutes each time. Secondary antibody was incubated with membrane for 45-60 minutes.

List of antibodies used in the project:

EZH2,	Active Motif, #39875, #39933, Cell Signaling, Rabbit mAb#5246
TRIM28,	Ab10483, Ab22533, Santa Cruz, SC-33186 (H300)
SUZ12,	Abcam, ab12073;
EED,	GeneTex, GTX628007;
RBBP4,	GeneTex, GTX62136;
TRIM33,	Abnova, #51992-M01;
HA,	Roche, 12CA5;
Myc,	Santa Cruz, SC-40 (9E10)
pS8244TRIM28,	Bethyl Laboratory, A300-767A
pS473TRIM28,	Biolegend, #644602

2.7 Immunoprecipitation, Mass Spec. MCF7 and HMEC cells were lysed in NTEP buffer (25mM Tris-HCl pH 7.5, 150mM NaCl, 5mM EDTA, 0.5% NP-40) for 30 minutes at cold room. The cell lysate were cleaned by high-speed centrifugation, 14,000rpm for 10 minutes. Protein concentration was measured with BCA assay. 500ug cell lysate was used to incubate with either EZH2, TRIM28, pS473TRIM, pS824TRIM28 antibodies overnight in the cold room. Then protein A Sepharose beads (GE Health) were added for additional 2 hours incubation. After extensive washing, 35ul SDS loading dye was added into the beads, the beads was boiled for 5-10 minutes to elute the protein complex. Elute was then subject to western blot in various percentage of SDS-PAGE gels. Gel was cut and sent out for mass spec at UTMB proteomics core.

2.8 Mammosphere Assay. Single-cell association for mammosphere formation assays was performed in six well plates following established protocols with MCF7 cells and derived mutant cell lines at a density of 1×10^4 cells/mL. Mammospheres were cultured in MammoCult Human Basal Medium with added proliferation Supplement (Stem Cell Technologies, BC, Canada) on Costar Ultra Low Attachment tissue culture plates. After 7 days, mammosphere sizes and numbers were determined using an inverted microscope at MD Anderson Cancer Center. Mammosphere sizes were measured as the widest diameter with the scale bar of the microscope. The mammosphere was counted only if its diameter is greater than 70 μ m. All experiments were done in triplicate.

2.9 RNA extraction, and real-time RT-PCR

RNA extraction, cDNA synthesis, Realtime RT-PCR is routinely used in our lab^{111,133}. Briefly, total RNA was extracted with Trizol (Invitrogen) according to manufacturer's suggestions. Then, 1ug of total RNA was used for reverse transcription into cDNA, Each real time RT-PCR reaction mix, containing 2μL of diluted cDNA (1:10 dilution), 5μL SYBR Green Reaction Mix (Applied Biosystems), 0.25μL forward primer (20μM), 0.25μL reverse primer (20μM), and 2.5μL sterile water, is set up in a 96-well plate and performed in triplicate using Applied Biosystems RT-PCR system at MD Anderson Cancer Center with SYBR Green Master Mix. Gene expression levels were calculated following normalization to Beta-Actin levels using the comparative Ct (cycle threshold) method. The primer sequences used for RT-PCR, and ChIP-PCR analyses are listed in Table 4.

2.10 RNA-Sequencing Analysis, DAVID pathway Analysis

Total RNA was extracted using Trizol from shControl, shEZH2, and shTRIM28 MCF7 cell lines. RNA-sequencing library preparation used Illumina True-Seq; and sequencing was performed by paired-end 150-mer sequencing. Raw reads were aligned to human reference genome (hg19) by Tophat v2.0.10¹³⁴, reads count for each transcript was obtained by htSeq¹³⁵. Differentially expressed gene lists were generated using edgeR¹³⁶, with adjusted p-value set to be less than 0.01. The differentiated gene lists was uploaded into online DAVID

functional annotation tool to analyze GO-function and biological biological pathways and annotations¹³⁷.

2.11 Chromatin Immunoprecipitation (ChIP-PCR), ChIP-Sequencing.

ChIP-PCR and ChIP-seq are routinely used in our lab^{111,133}. For chromatin immunoprecipitation, MCF7 cells are harvested and then cross-linked for 15 min, whereas HMEC cells are cross-linked for 10 minutes. Cells are lysed with 1 mL Cell Lysis Buffer (5mM PIPES pH 8.0, 85mM KCl, 0.5% NP-40, fresh protease inhibitors) for 15 minutes. After sonication, the tubes are spun. Supernatant is transferred to a new tube to check for fragment size. Lysates are divided and diluted using ChIP Lysis Buffer. Immunoprecipitation is performed overnight with specific antibodies. The next day, Protein A Sepharose beads (GE Health) are incubated with antibody/protein bound complexes for 2 hr at 4°C. Then, Protein A beads are washed and re-suspended and incubated with RNase A for 30 min at 37°C and then Protease K for 2 hours at 55°C. The crosslinks are reversed by incubating the samples at 65°C overnight. The next day, protein /antibody bound DNA fragments are extracted with Phenol/Chloroform. qPCR analyses are performed to analyze specific antibody- and protein-bound. DNA was sent out to University of Texas MD Anderson Cancer Center science park core sequencing facility. ChIP-seq raw reads were aligned to human reference genome (hg19) using Bowtie v1.0.0¹³⁸, allowing up to one mismatch per read. Only uniquely mapped reads were kept for downstream analysis. Peaks were called by MACS v1.3.7¹³⁹, using p-value cutoff 1e-8.

2.12 *In vitro* methylation assay

This method is performed as published in ¹⁴⁰. Briefly, recombinant GST-EZH2 (2 µg) was incubated with various amounts of EZH2 proteins or histone H3 peptides in methylation assay buffer (50mM Tris-HCl, pH 8.0, 10% glycerol, 20mM KCl, 5 mM MgCl₂, 1 mM DTT, 1 mM PMSF and 0.1 mM SAM or ³H-labeled SAM [GES Health Care]) at 30°C for 4 h. Reactions were stopped by adding SDS-PAGE sample buffer, and the methylation status was measured by Western blotting, autoradiography. Another simpler method to measure the methylation status is liquid scintillation counting.

2.13 IHC staining on TMA

Immunohistochemistry staining for TRIM28 was performed as before ¹¹¹. TRIM28 antibody was incubated with the breast cancer tissue array (Dr. Maciej Wiznerowicz's lab, Poznan University of Medical Sciences), followed by extensively wash, and then breast cancer tissue array was incubated with secondary antibody. The signal was visualized by adding DAB (3, 3'-diaminobenzidine).

2.14 CyTOF, Mass Cytometry Sample Processing and Analysis

MCF7 cells were washed with PBS then disassociated with 0.05% Trypsin (Sigma-Aldrich). Following disassociation, cells were washed once with serum free DMEM respectively then incubated in serum free media containing 10 µM 5-Iodouridine (Sigma-Aldrich) at 37°C for 10min. Live/dead cisplatin staining was

done according to the methods from [1]. Cells were washed twice with 1%BSA (Equitech Bio) in PBS and once with PBS. Cells were fixed with 2% paraformaldehyde (PFA) (Sigma-Aldrich) pH 6.9 at room temperature for 15min. Following fixation, 1×10^6 cells/mL were permeabilized with methanol overnight at 4°C and transferred to -80°C. Following permeabilization, cells were centrifuged at 300 x g for 5 minutes to remove methanol, then washed with 1 mL of 1% BSA in PBS for each mL of methanol used for permeabilization. Cells were washed once with Wash Buffer (0.5% BSA and 0.02% sodium azide in PBS) and incubated at a concentration of 4×10^5 cells/ μ l with CD24 and CD44 antibodies (Fluidigm). After antibody staining cells were washed twice with Wash Buffer, once with PBS, then fixed in 0.5mL of 1:2,000191/193 Iridium DNA intercalator (Fluidigm) in 4% PFA in PBS at room temp for 30 minutes. Cells were washed three times with Wash Buffer and analyzed using a CyTOF mass cytometer (Fluidigm). Cells were run at a concentration of 5×10^6 cells/mL. EQ Four Element Calibration Beads (Fluidigm) were added to the samples immediately prior to loading into a 96-well plate for autosampler running. Data were normalized based on bead signal using the included CyTOF software. Initial data processing and gating were performed using FlowJo vX10.0. Beads were gated off of the samples and data were gated on singlets, based upon Ir193 and Event Length parameters. Live/dead cell discrimination was performed based upon the Pt198 channel.

2.15 Statistical Analyses

GraphPad Prism6 software (GraphPad Software, Inc.) was used for statistical analysis of p -values. The two-tailed paired *student t-test* was employed to verify whether the difference between two groups is significant. P -values < 0.05 were considered to be statistically significant.

Table 4-1: Oligonucleotide sequences for Real Time qPCR analysis

hTRIM28-RT-F1	TTTCATGCGTGATAGTGGCAG
hTRIM28-RT-R1	GCCTCTACACAGGTCTCACAC
hTRIM28-RT-F2	CGGGATGGTGAACGTACTGTC
hTRIM28-RT-R2	GTCTCGGCAGGTGAGAGTATC
EZH2-RT-F2	GAGACTGGCGAAGAGCTGTTT
EZH2-RT-R2	GGCATCAGCCTGGCTGTATC
EZH2-RT-F3	CCCTGACCTCTGTCTTACTTGTGGA
EZH2-RT-R3	ACGTCAGATGGTGCCAGCAATA
β-Actin F	TCATGAAGTGTGACGTTGACATCCGT
β-Actin R	CCTAGAAGCACTTGCGGTGCACGATGGAG
hCXCR4-RT-F1	ACTACACCGAGGAAATGGGCT
hCXCR4-RT-R1	CCCACAATGCCAGTTAAGAAGA
hCXCR4-RT-F2	ACGCCACCAACAGTCAGAG
hCXCR4-RT-R2	AGTCGGGAATAGTCAGCAGGA
hAXIN2-RT-F	CAACACCAGGCGGAACGAA
hAXIN2-RT-R1	GCCCAATAAGGAGTGTAAAGGACT
hAXIN2-RT-F2	TACACTCCTTATTGGGCGATCA
hAXIN2-RT-R2	TTGGCTACTCGTAAAGTTTTGGT
HOXA1-F1	TCCTGGAATACCCCATACTTAGCA
HOXA1-R1	GCCGCCGCAACTGTTG

Table 4-2: Oligonucleotide sequences for Real Time qPCR analysis after ChIP analysis

CXCR4-ChIP-F1	CAGCCCATTCAGGAGGTAAA
CXCR4-ChIP-R1	TGCACCTGACCCTAGTGATG
AXIN2-ChIP-1-F	AAGAACCTACACCGCCTCCT
AXIN2-ChIP-1-R	GGCAGTCTCTCTGCTCCATC
AXIN2-ChIP-2-F	GCCCACTCCTGTCTGTTTCAT
AXIN2-ChIP-2-R	CACAATGCTAGGGTGGCTTT

CHAPTER III

TRIM28 INTERACTS WITH EZH2 TO REGULATE BREAST CANCER STEM CELL MAINTENANCE

CHAPTER III: TRIM28 Interacts with EZH2 to Regulate Breast Cancer Stem Cell Maintenance

Abstract:

EZH2, the major H3K27 tri-methyltransferase, is over expressed in multiple subtypes of breast cancer; however, whether EZH2 plays an active role in breast cancer progression or treatment resistance is unknown. Here, we determined EZH2-interacting proteins in MCF7 cells by immunoprecipitation/mass spectrometry and detected TRIM28 with a high confidence score. TRIM28 is a transcription co-regulator with an N-terminal tri-partite RING finger, B-boxes and a coiled-coil domain (RBCC) and a C-terminal PHD/Bromo domain. Domain mapping revealed that the RBCC domain of TRIM28 interacts with EZH2, and the pre-SET region (Cysteine-rich domain) of EZH2 interacts with TRIM28. Transcriptome profiling and intersection of EZH2- and TRIM28-regulated genes indicated co-regulation of differentiation and morphogenesis pathways, as well as co-activation of the cytokine-cytokine receptor interaction pathway (CXCR4, CXCL12, EGFR, KIT). TRIM28 depletion in MCF7 cells decreased EZH2 recruitment and CXCR4 gene expression, in parallel with decreased mammosphere formation. TRIM28 regulation of EZH2 association with SWI/SNF complex might contribute to this process. Collectively these data indicate that EZH2 and TRIM28 regulate breast cancer stem cell maintenance by transcriptional regulation of CXCR4 gene expression.

Introduction:

EZH2 (Enhancer of Zeste Homolog 2), the major, cellular H3K27 trimethyltransferase, catalyzes addition of methyl groups at lysine 27 of histone H3 (H3K27me3)⁴⁹. EZH2 is primarily characterized in repression of transcription, as a subunit of the canonical PRC2 complex (Polycomb Repressive Complex 2), along with EED, SUZ12, and RBBP4⁴⁹. The mammalian PRC2 complex does not bind genomic DNA directly in a sequence-specific manner; however, multiple DNA-binding proteins, including JARID2^{58,59}, AEBP2⁶⁰ and ATRX⁶¹, are implicated in recruitment of PRC2 complex to a variety of target gene loci to silence gene expression.

EZH2 is over-expressed in multiple cancers such as prostate, melanoma, lymphoma, endometrial and breast, and ectopic expression of EZH2 promotes neoplastic transformation of immortalized human breast epithelial cells¹⁴¹.

Multiple studies suggest that EZH2 is an attractive therapeutic target, and small molecule inhibitors that target its catalytic SET domain^{70,72} are now in clinical trials for patients with germinal center diffuse large B-cell lymphoma, primary mediastinal large B-cell lymphoma (PMBCL) and follicular lymphoma^{67,68}. EZH2 has been shown to drive a malignant cancer phenotype in cultured cells derived from castration-resistant prostate cancers and, although canonical PRC2 complex subunits were not required, the EZH2 SET catalytic domain was essential⁵⁶.

However, the catalytic activity of EZH2 may not be sufficient to account for all functions of EZH2 in cancer, and these may prove to be non-responsive to SET-domain inhibitors¹⁴². In contrast to its well-studied role in repression of transcription, EZH2 also acts as a transcriptional co-activator of estrogen- and WNT-regulated proliferation pathways⁶⁶. EZH2 over expression promotes expansion of a subpopulation of malignant cells, known as breast cancer initiating cells or breast cancer stem cells, by activating RAF1-b-Catenin signaling⁷⁹ or, as shown elsewhere, by activating NOTCH signaling in an H3K27me3-independent manner⁸⁰. Additionally, mammary gland-specific overexpression of EZH2 in transgenic mice induces hyperplasia of mammary epithelium but is not sufficient for tumor initiation and progression¹⁴³. Taken together, these studies suggest that EZH2 relies on multiple mechanisms to intersect with aberrant signaling pathways that play a role in tumorigenesis.

TRIM28/KAP1 is well studied as a co-repressor of transcription, acting in multiple pathways^{113,116,120}. Like EZH2, TRIM28 does not bind to DNA in a sequence-specific manner; it is generally recruited to DNA via sequence-specific binding proteins that have KAP1 repressor associated binding (KRAB) domains¹³⁰. TRIM28 expression was previously shown to promote breast cancer proliferation and metastatic progression, although the mechanisms were not delineated¹⁴⁴. Interestingly, RNAi screens revealed that TRIM28 is required for maintenance of mouse embryonic stem cell pluripotency and self-renewal¹¹⁴, which may involve TRIM28 interactions with OCT-4 and subunits of the

SWI/SNF2 chromatin remodeling complex, such as Smarcd1, Brg-1 and BAF155, to induce embryonic stem cell-specific genes ¹¹⁵.

The subpopulation of cancer stem/progenitor cell is believed to be responsible for cancer initiation, cancer progression, and cancer chemotherapy resistance, and is still alive after chemotherapy treatment ^{21,22}, and also contributes to one hallmark of cancer, heterogeneity ²³. Breast cancer stem cell was defined as CD44+CD24- population of breast cancer cells ²³, Spheroid culture of mammosphere was developed as a simple method to measure cancer stem cell populations ^{31,33}. In the studies described here, we show that TRIM28 interacts with SWI/SNF and EZH2, in a PRC2-independent manner, to coordinately regulate genes with roles in breast stem cell maintenance. A broader understanding of EZH2-mediated regulation and intersection with epigenetic regulators in cancers and stem cells is needed to effectively target therapy-resistant cancers and address the challenges of tumor heterogeneity.

RESULTS

The pre-SET domain of EZH2 interacts with RBCC domains of TRIM28

EZH2 promotes neoplastic transformation of breast epithelial cells ¹⁴¹; EZH2 expression level does not correlate with H3K27me3 level in human breast cancer TMA IHC staining (Figure 1 and 2). So, the regulatory mechanisms involved in

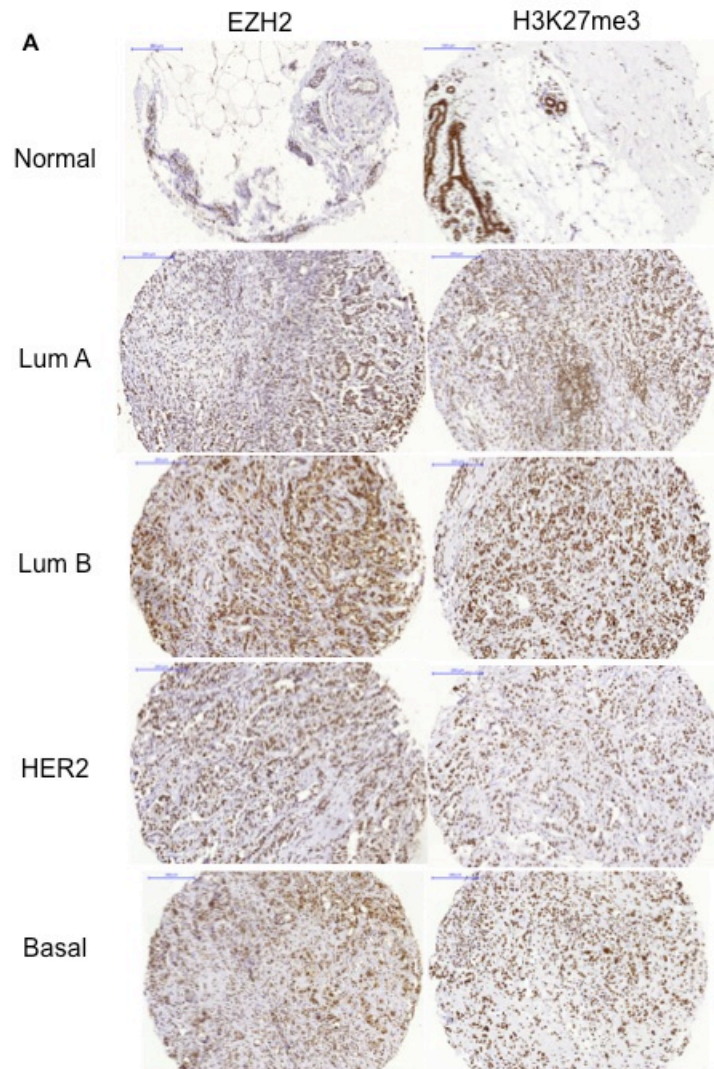


Figure 1 EZH2 expression level does not correlate with H3K27me3 level in breast cancer A. EZH2, H3K27me3 IHC staining of 5 subtypes of breast cancer TMA

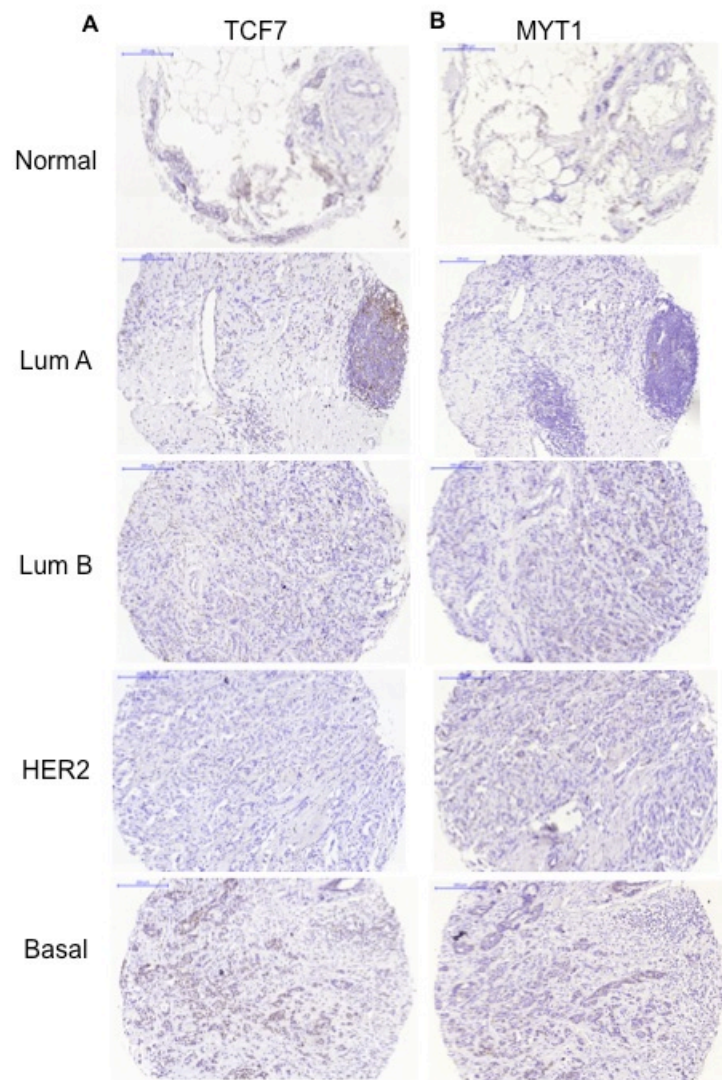


Figure 2 H3K27me3-dependent EZH2 target genes expression levels in breast cancer A. TCF7, MYT1, IHC staining of 5 subtypes of breast cancer TMA

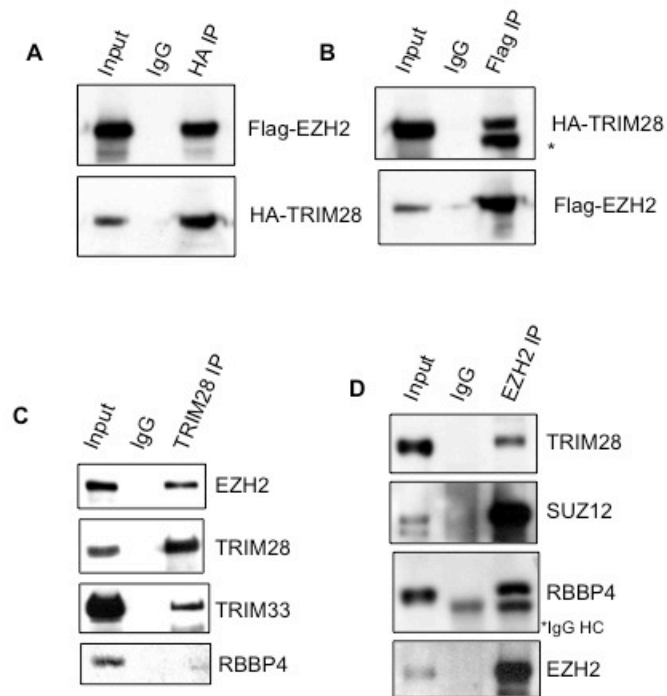


Figure3 TRIM28 Interacts with EZH2 A. Myc-EZH2 and HA-TRIM28 were co-transfected into HEK-293T cells, either Myc was immunoprecipitated (A) or HA was immunoprecipitated(B) C. TRIM28 was immunoprecipitated in MCF7 cell lysate, EZH2 was probed; D. EZH2 was immunoprecipitated in MCF7 cell lysate, TRIM28 was detected;

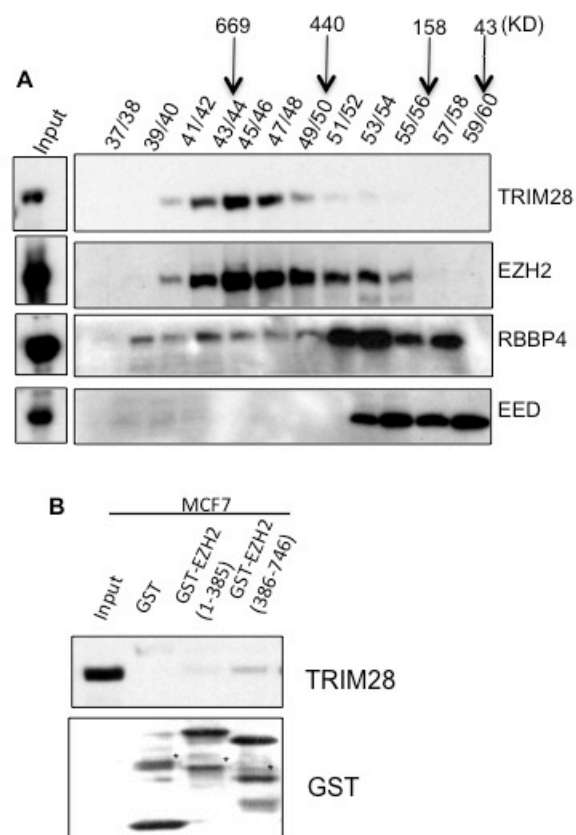


Figure 4 TRIM28 co-fractionates with EZH2 A. MCF7 WCL was fractionated in Superose 6 B. E.coli purified GST-EZH2 was incubated with MCF7 cell lysates, GST-pull down, TRIM28 was probed by western blotting

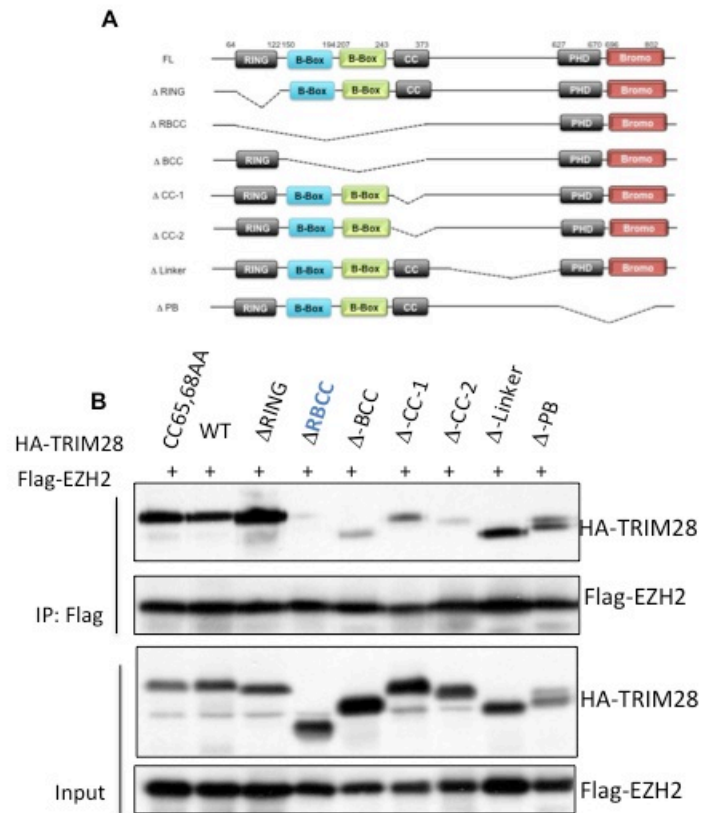


Figure5 Domain mapping of TRIM28 with EZH2. A. Deletion strategy for HA-TRIM28 plasmids B. After co-transfection of Myc-EZH2 and HA-TRIM28 wild type and various deletion mutants, Flag was immunoprecipitated, HA was probed.

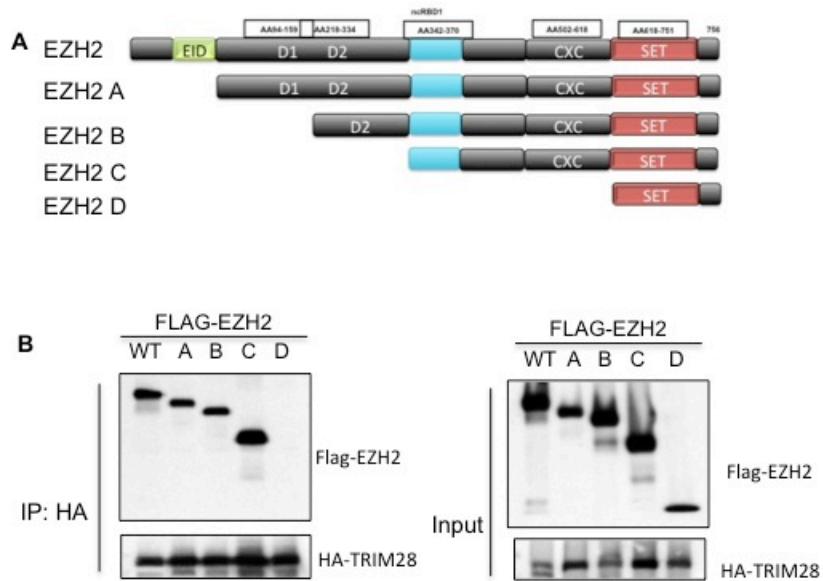


Figure 6 Domain mapping of EZH2 with TRIM28 A. Flag-EZH2 mutants, B. HA-TRIM28 was co-transfected with various Flag-EZH2 mutated plasmids. HA was immunoprecipitated, Flag was probed.

A Known binding partners and top 10 binding partners of EZH2 in MCF7

Known partners	top 10 binding partners
Gene Symbol	Gene Symbol
EZH2	STAU1
RBBP4	TRIM28
EED	CDAN1
	USP7
	USP10
	ASF1A/B
	MTDH
	H2A.X
	SHANK2
	PTPN11

Figure7 EZH2 purification 1 Lists of known binding partners and newly identified binding partners of EZH2

Known	#of Peptides	Unknown	#of Peptides	
SUZ12	6	TRIM28	6	
PHF1	4	ARID1A	12	
		BRG1(SMA		
AEBP2	4	RCA4)	4	
DNMT1	3	SMARCC2	5	SWI/SNF
EED	2	SMARCA5	5	
		PBRM1(BA		
		F180)	2	
		P300	5	
		ARID2	1	
		CHD4	8	NURD
		CHD3	1	
		MBD4	2	
		MTA1	1	
		PARP1	2	
		DICER1	3	
		ZNF687	1	
		GTF2I	4	
		KAT7	1	
		UHRF1	2	
		USP10	1	

Figure 8 EZH2 purification 2 Lists of known binding partners and newly identified binding partners of EZH2

EZH2-mediated transformation is not well understood. We used MCF7 cells, as a model of human estrogen receptor-positive, luminal-type breast cancers that comprise a majority of all breast cancers ¹⁶, and determined interacting protein partners of EZH2. Immunoprecipitation of endogenous EZH2, followed with LC-MS/MS analysis, identified known members of EZH2 complexes, such as SUZ12, EED, PHF1, AEBP2 and DNMT1 ⁵⁸, as well as multiple proteins not previously associated with PRC2 (Figure 7 and 8). Among these, co-purified proteins with 4 or more peptides identified by LC-MS/MS included factors with known functions in binding, remodeling and/or modification of chromatin structure, e.g. BRG1, SMARCC2, SMARCA5, BAF180, ARID1A, p300, CHD4 and TRIM28. Since EZH2 is primarily associated with repression of transcription, we assessed its potential functions as a partner of TRIM28, also known as KAP1 co-repressor protein ¹¹³. We validated interaction between EZH2 and TRIM28 proteins by co-immunoprecipitation of Myc-EZH2 and HA-TRIM28 in HEK293T cells (Figure 3A and 3B). Endogenous TRIM28, expressed in MCF7 cells, likewise interacts with endogenous EZH2, as well as TRIM33, previously reported as interacting with TRIM28 ¹¹⁰. Interestingly, although EZH2 associated with TRIM28, there was minimal interaction with PRC2 complex member RBBP4 (Figure 3C). Antibody enrichment of endogenous EZH2 also co-precipitated TRIM28, as well as previously reported PRC2 complex members SUZ12 and RBBP4 (Figure 3D). To assess protein complex composition, we performed gel filtration chromatography and found two distinct complexes of TRIM28 and

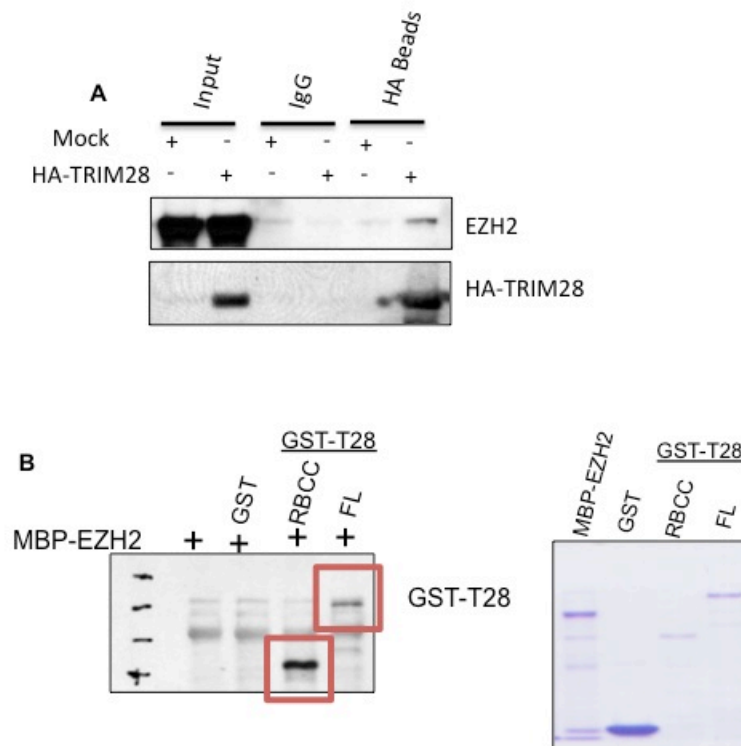


Figure 9 TRIM28 physical Interacts with EZH2 A. Immunoprecipitation assay, HA-TRIM28 was overexpressed in HEK-293T cells, HA beads were used to immunoprecipitate TRIM28, EZH2 antibody was analyzed by western blotting B. in vitro binding assay, E. coli. purified MBP-EZH2 was incubated with GST-TRIM28 full length, and RBCC fragment, MBP beads were used to pull down MBP-EZH2, GST antibody was analyzed by western blotting

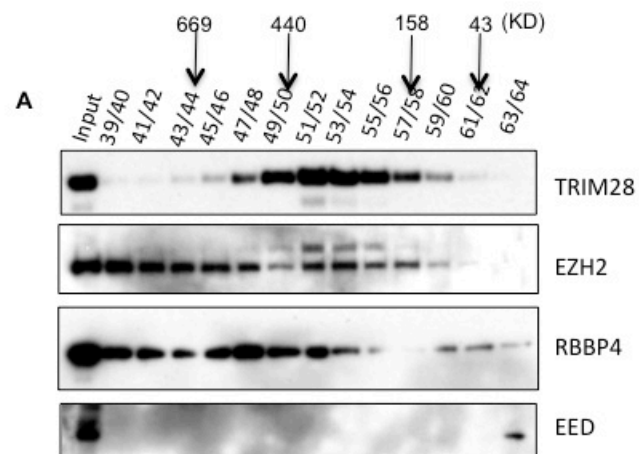


Figure10 TRIM28 is co-eluted with EZH2 A. MCF7 nuclear extract was fractionated in Superose 6

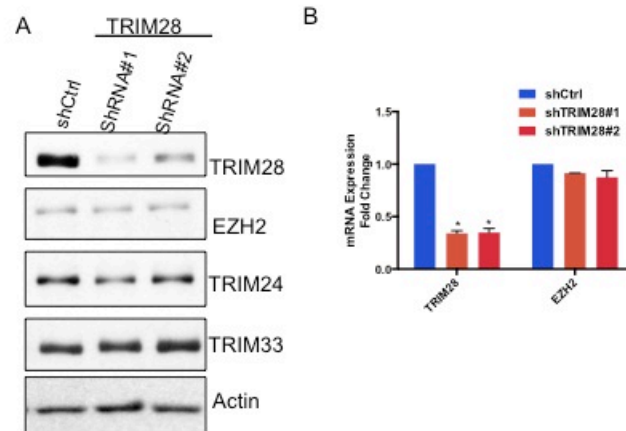


Figure11 TRIM28 does not regulate EZH2 stability A. EZH, TRIM28, TRIM24, and TRIM28, TRIM33 protein expression in TIM28 depleted cells
B. TRIM28, EZH2 mRNA expression in TRIM28 depleted MCF7 cells

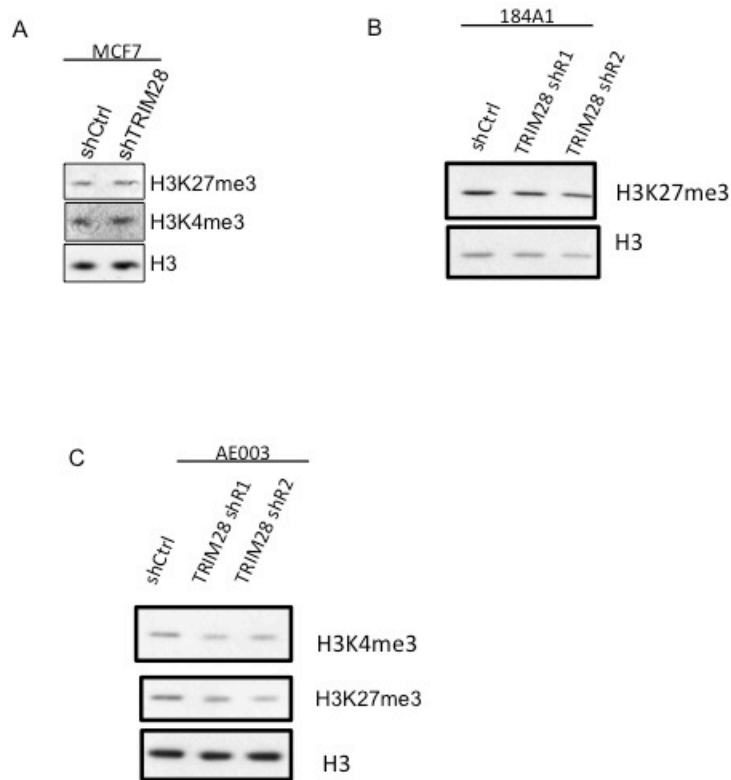


Figure 12 TRIM28 doesn't alter H3K4me3/K27me3 bulk levels A. histone was extracted with acid method from MCF7 cells, H3K27me3, H3K4me3, H3 was probed with western blotting B. . histone was extracted with acid method from 184A1 HEC cells, H3K27me3, H3 was probed with western blotting C. . histone was extracted with acid method from AE003 HMEC cells, H3K27me3, H3K4me3, H3 was probed with western blotting

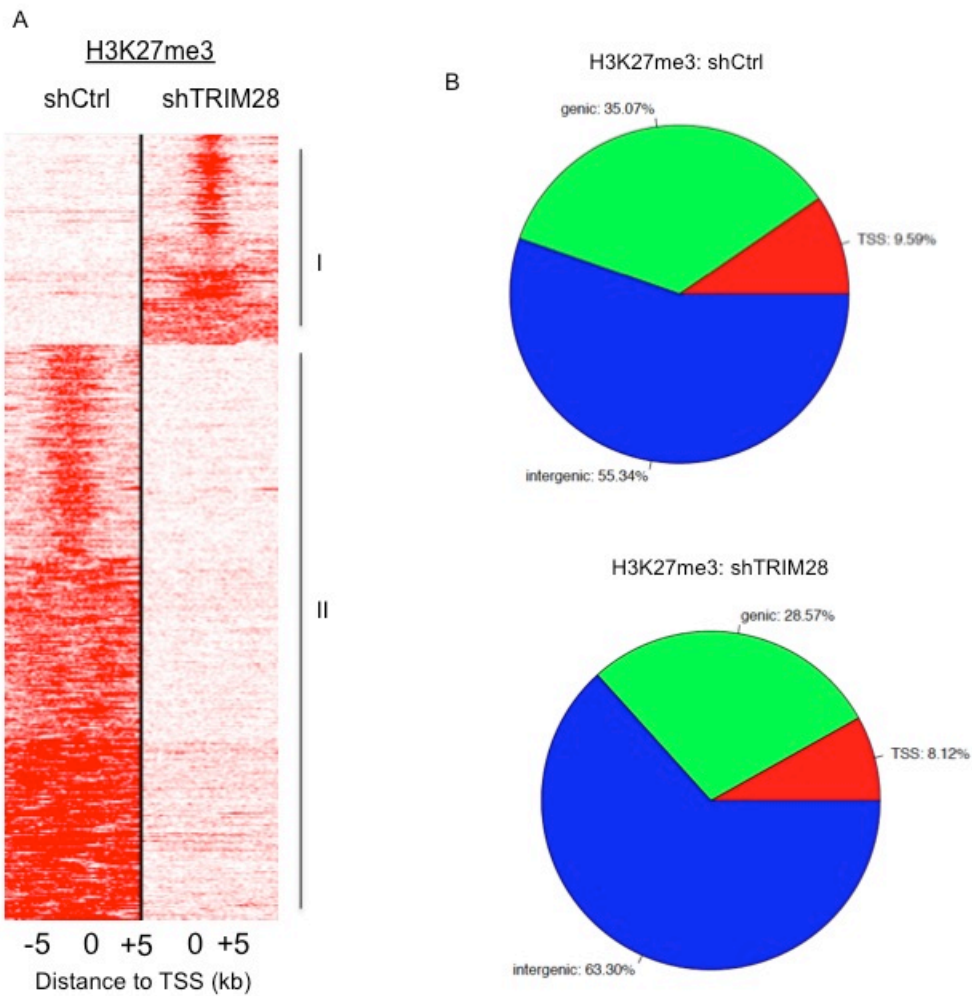


Figure 13 TRIM28 regulates H3K27me3 distribution A. Heatmap of H3K27me3 peaks in shCtrl and shTRIM28 MCF7 cells and clusters based on peaks height variation between shCtrl and shTRIM28 cells B. Distribution of H3K27me3 peaks in shCtrl, and shTRIM28 MCF7 cells. Peaks are categorized as TSS (+/- 3kb), genic, or intergenic.

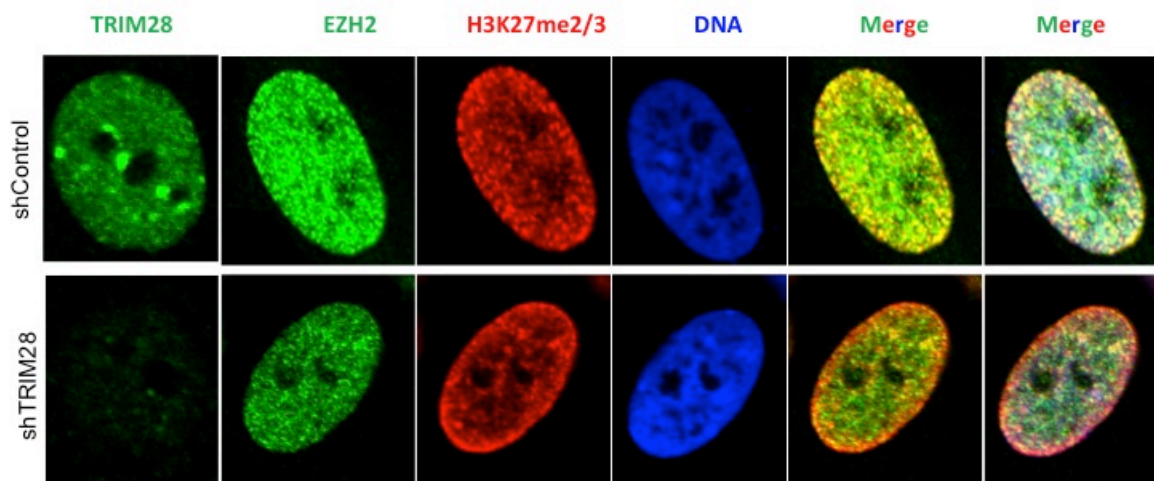


Figure 14 TRIM28 Depletion Alters H3K27me3 Distribution A. IF staining as indicated in the figure in shControl, shTRIM28 MCF7 cells

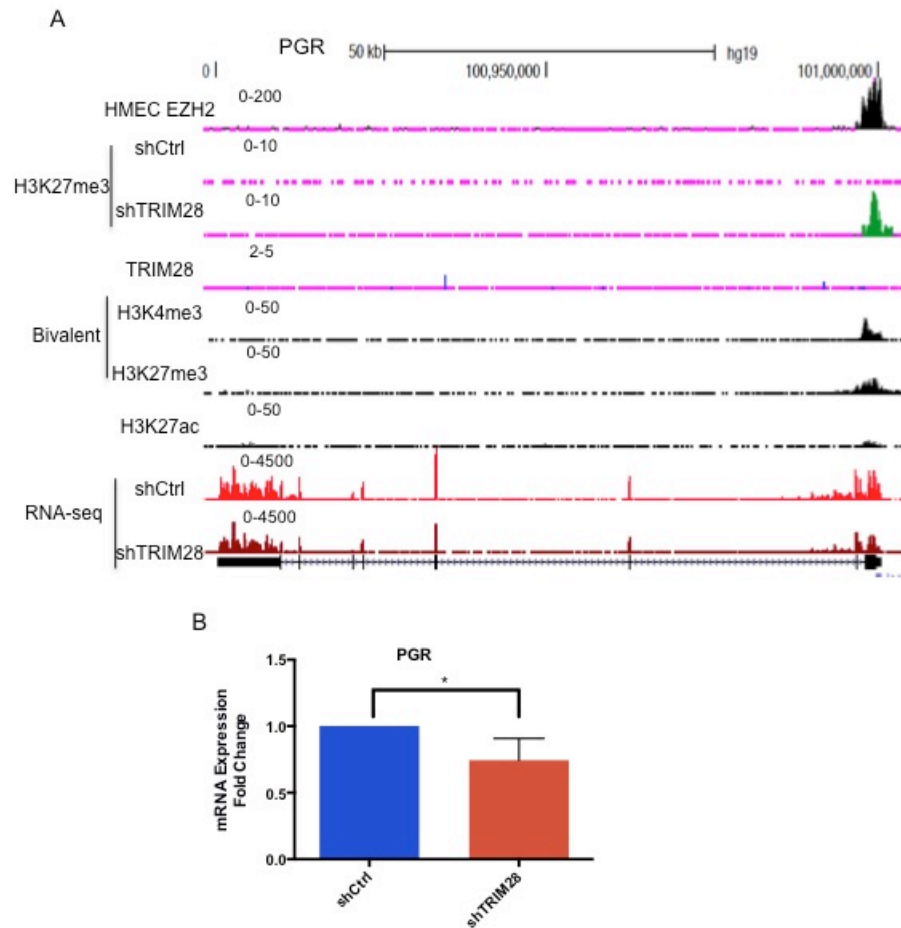


Figure 15 TRIM28 Alters H3K27me3 Distribution on the promoter of PGR A.

Screenshot of PGR promoter region from H3K27me3 ChIP-seq in shControl, shTRIM28 MCF7 cells, RNA-seq in shControl, shTRIM28 MCF7 cells B. Reatime RT-PCR of PGR in shControl, shTRIM28 MCF7 cells

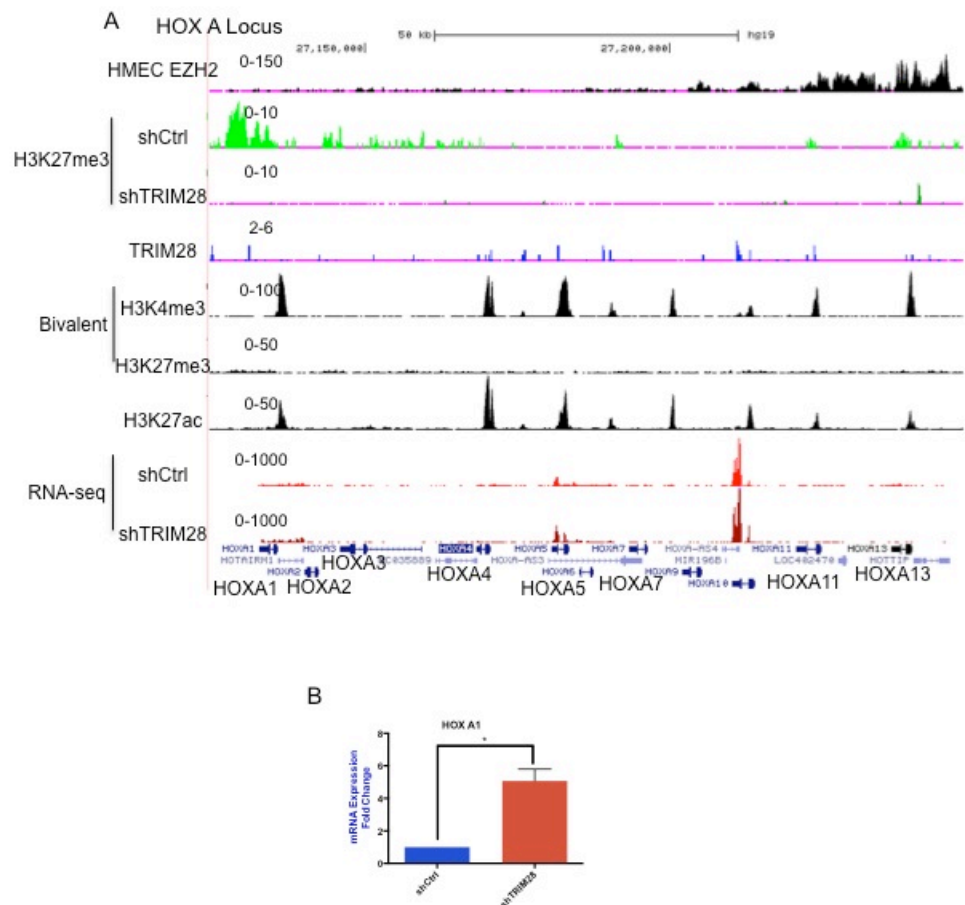


Figure 16 TRIM28 Alters H3K27me3 Distribution on the promoter of HOX A locus A. Screenshot of HOX A1 promoter region from H3K27me3 ChIP-seq in shControl, shTRIM28 MCF7 cells, RNA-seq in shControl, shTRIM28 MCF7 cells B. Realtime RT-PCR of HOX A1 in shControl, shTRIM28 MCF7 cells

RBBP4/EED that co-fractionated with EZH2 (Figure 4A and 10A). Although EED and RBBP4 are canonical PRC2 complex members, previously shown as essential for EZH2-mediated H3K27 methylation⁵³⁻⁵⁵, the majority of EZH2 in MCF7 cells co-fractionated with TRIM28 in a higher molecular weight complex (Figure 4A).

We determined that the interaction between EZH2 and TRIM28 was direct by pull-down assays with bacterially expressed, recombinant proteins MBP-EZH2 and GST-TRIM28 (Figure 9B). Further, we delineated the domains of interaction between EZH2 and TRIM28 by mutation and expression of Flag-EZH2 and HA-TRIM28 constructs. A series of TRIM28 deletion mutants, which removed the RING, RBCC, CC, B-BOX, PHD/bromo domains or the linker region (Figure 5A), were co-expressed with Flag-EZH2 in HEK-293T cells for affinity precipitation and analysis (Figure 5B). Removal of the multi-domain RBCC fragment led to loss of EZH2-TRIM28 interaction, while separation of the B-boxes and Coiled-Coil domains decreased binding of EZH2 and TRIM28, showing that multiple interactions with EZH2 likely occur across the RBCC domains. Similarly, EZH2 was serially fragmented as EZH2 A, B, C, D clones from amino- to carboxy-termini (as illustrated in Figure 6A). Expression and co-immunoprecipitation of these mutated forms of EZH2 with full length TRIM28 indicate that the SET domain of EZH2 is insufficient for TRIM28 interactions (Figure 6B). These results, combined with assays of GST-EZH2 and interaction with endogenous TRIM28 (Figure 4B), narrowed binding to the carboxyl-terminal amino acids of

385-618 that encompass the pre-SET domain. Thus, RBCC motif of TRIM28 physically interacts with the pre-SET region of EZH2.

TRIM28 Alters H3K27me3 Distribution

TRIM28 knockdown did not alter EZH2 expression in MCF7 cells in either protein or mRNA level (Figure 11A and B). This ruled out the possibility of TRIM28 altering EZH2 stability. Next we asked whether TRIM28 regulates H3K27me3 level. Histone extraction assay demonstrated that TRIM28 depletion does not alter the bulk levels of H3K27me3 and H3K4me3 in MCF7 cells (Figure 12). Surprisingly, H3K27me3 ChIP-sequencing results in shControl and shTRIM28 MCF7 cells demonstrated that the peak levels of H3K27me3 appear to be dramatically different in response to TRIM28 depletion. To investigate how TRIM28 altering H3K27me3 peaks distribution patterns, the differential H3K27me3 ChIP-seq peaks between wild type and TRIM28 depletion were compared and clustered. Immunofluorescent staining also showed the altered H3K27me3 distribution with TRIM28 depletion (Figure 14). In total, the differential H3K27me3 peaks (differential peaks >3 folds) between shCtrl and shTRIM28 cells were divided into two clusters (Figure 13A). Cluster 1 is the increased peak heights (>3 folds) of H3K27me3 upon TRIM28 depletion. In contrast, cluster 2 depicts the decreased H3K27me3 peak heights upon TRIM28 depletion in MCF7 cells. Progesterone receptor (PGR) (Figure 15A) is an exemplary molecule to demonstrate that TRIM28 depletion cause increased H3K27me3 binding on the

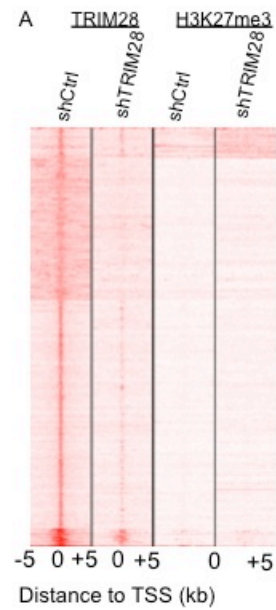


Figure 17 TRIM28 binding does not correlate with H3K27me localization

A. Heatmap showing correlation of TRIM28 binding and H3K27me3 binding on gene promoters

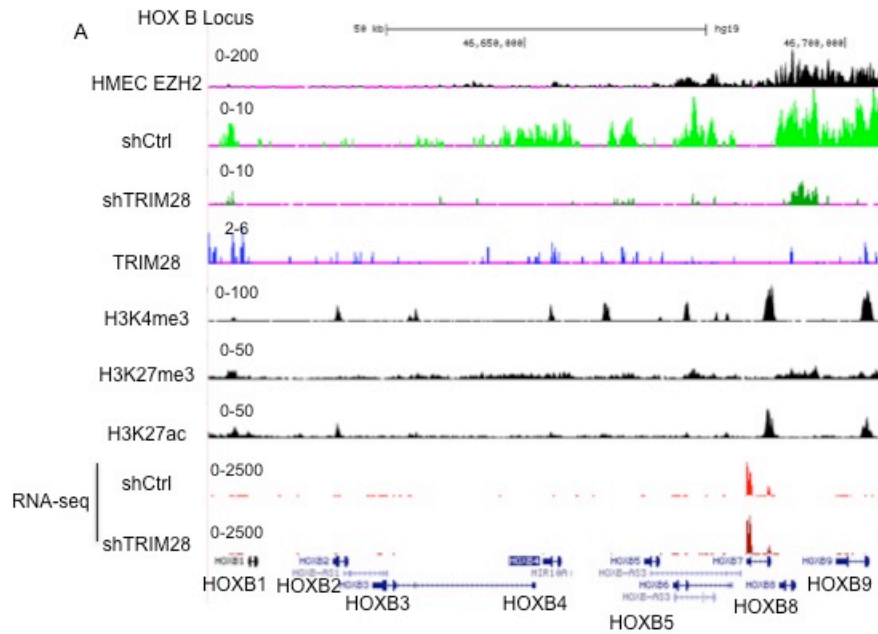


Figure 18 TRIM28 Alters H3K27me3 Distribution on the promoter of *HOX B* locus A. Screenshot of *HOX B* locus from H3K27me3 ChIP-seq in shControl, shTRIM28 MCF7 cells, RNA-seq in shControl, shTRIM28 MCF7 cells

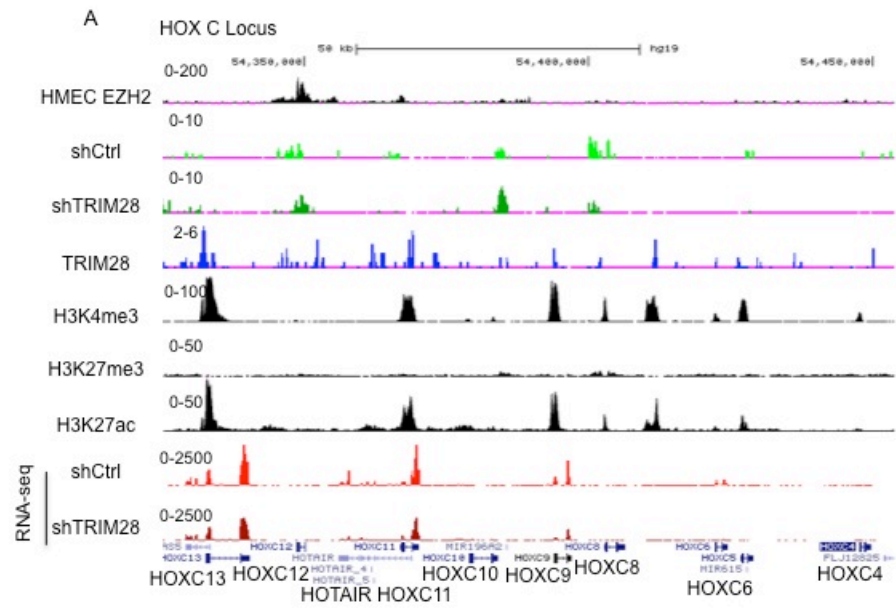


Figure 19 TRIM28 Alters H3K27me3 Distribution on the promoter of *HOX C* locus A. Screenshot of *HOX C* locus from H3K27me3 ChIP-seq in shControl, shTRIM28 MCF7 cells, RNA-seq in shControl, shTRIM28 MCF7 cells

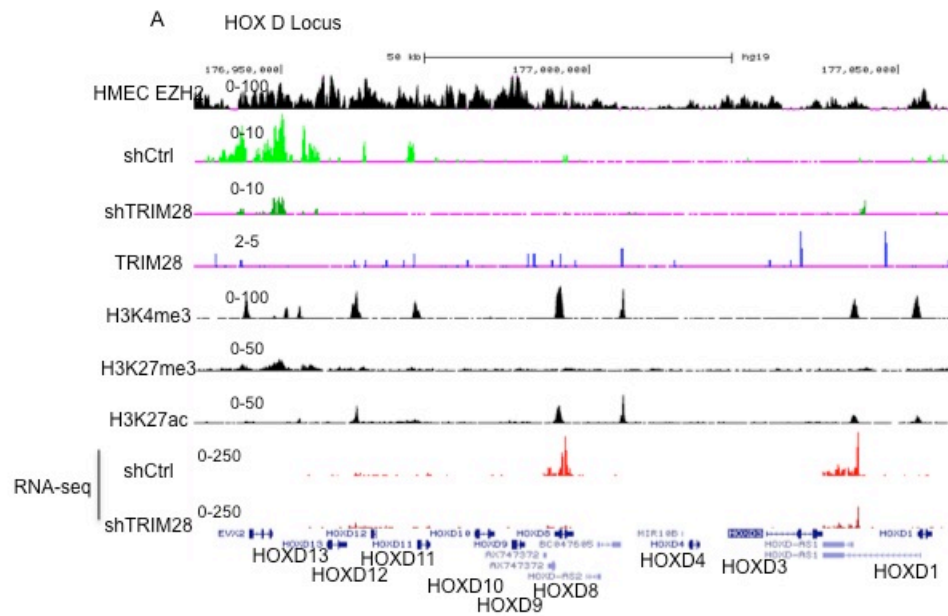


Figure 20 TRIM28 Alters H3K27me3 Distribution on the promoter of *HOX D* locus A. Screenshot of *HOX D* locus from H3K27me3 ChIP-seq in shControl, shTRIM28 MCF7 cells, RNA-seq in shControl, shTRIM28 MCF7 cells

A

Genes with H3K27me3 peak increased upon TRIM28 depletion				
Identifier	GO Term	Genes in the List	Total Genes	PValue
GO:0006811	ion transport	8	768	0.017840204
GO:0030182	neuron differentiation	6	438	0.018990099
GO:0030001	metal ion transport	6	465	0.023892598
GO:0048666	neuron development	5	339	0.031762499
GO:0000902	cell morphogenesis	5	356	0.037022215

B

Genes with H3K27me3 peak increased upon TRIM28 depletion				
Identifier	GO Term	Genes in the List	Total Genes	PValue
hsa04020	Calcium signaling pathway	5	176	0.001403292

C

Genes with H3K27me3 peak decreased upon TRIM28 depletion				
Identifier	GO Term	Genes in the List	Total Genes	PValue
GO:0006835	dicarboxylic acid transport	4	15	3.08E-04
GO:0046942	carboxylic acid transport	8	147	3.85E-04
GO:0015849	organic acid transport	8	148	4.01E-04
GO:0015807	L-amino acid transport	4	23	0.001137337
GO:0015837	amine transport	6	118	0.004425328

D

Genes with H3K27me3 peak decreased upon TRIM28 depletion				
Identifier	GO Term	Genes in the List	Total Genes	PValue
hsa04730	Long-term depression	5	69	0.002357203
hsa04530	Tight junction	6	134	0.00458421
hsa04510	Focal adhesion	6	201	0.023799558
hsa05412	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	4	76	0.024269601
hsa04512	ECM-receptor interaction	4	84	0.031439033

Figure 21 GO term analysis of altered H3K27me3 peaks by TRIM28 A. GO term analysis of genes with H3K27me3 peak increased upon TRIM28 depletion B. KEGG pathway analysis of genes with H3K27me3 peak increased upon TRIM28 depletion C. GO term analysis of genes with H3K27me3 peak decreased upon TRIM28 depletion D. KEGG pathway analysis of genes with H3K27me3 peak decreased upon TRIM28 depletion

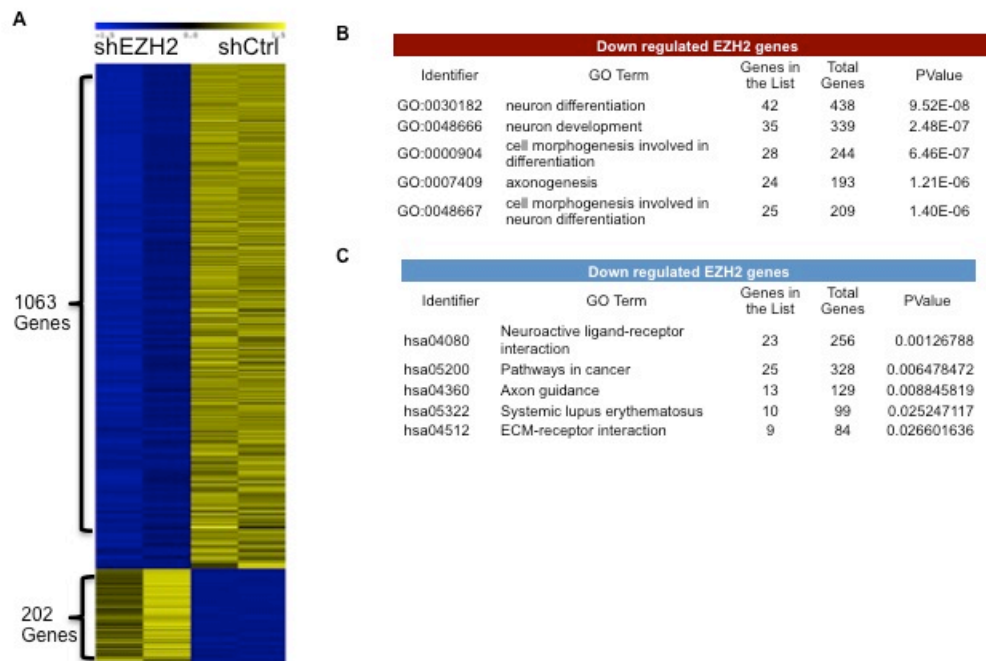


Figure 22 EZH2 mainly acts as a transcriptional co-activator in breast cancer cells A. Heatmap of differentially expressed genes of shCtrl, shEZH2 RNA-seq in MCF7 cells B. Biological process analysis of down-regulated genes in shEZH2 cells C. KEGG pathway analysis of down-regulated genes in shEZH2 RNA-seq

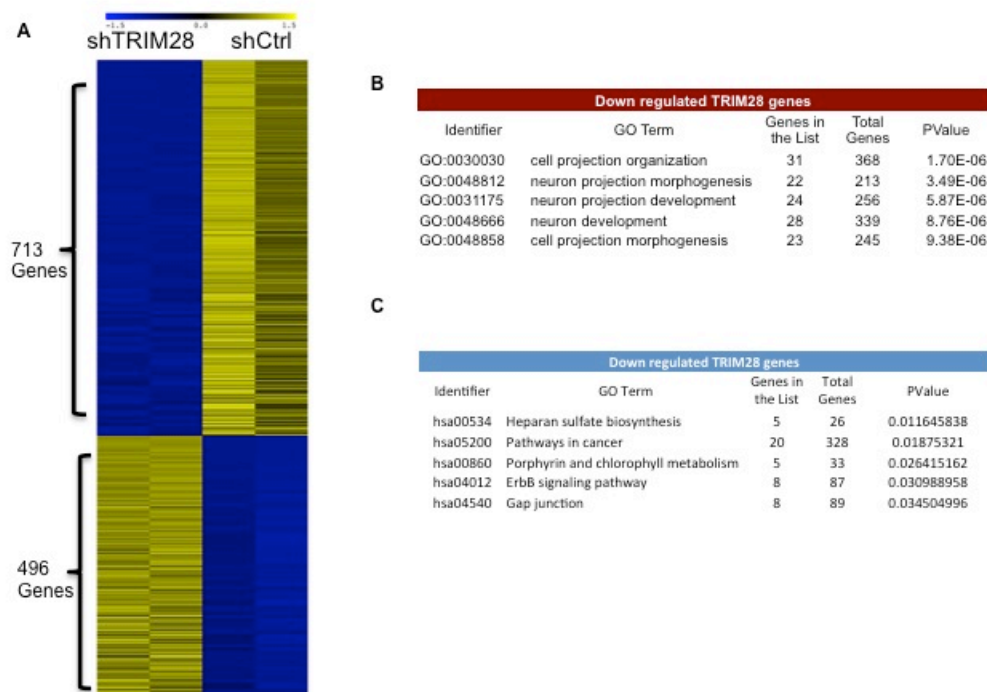


Figure 23 TRIM28 Regulated Transcriptome A. Heatmap of RNA-Seq data from shCtrl, shTRIM28 MCF7 cells. B. Biological Process analysis of down-regulated genes in shTRIM28 transcriptome C. KEGG pathway analysis of down-regulated genes in shTRIM28 RNA-seq

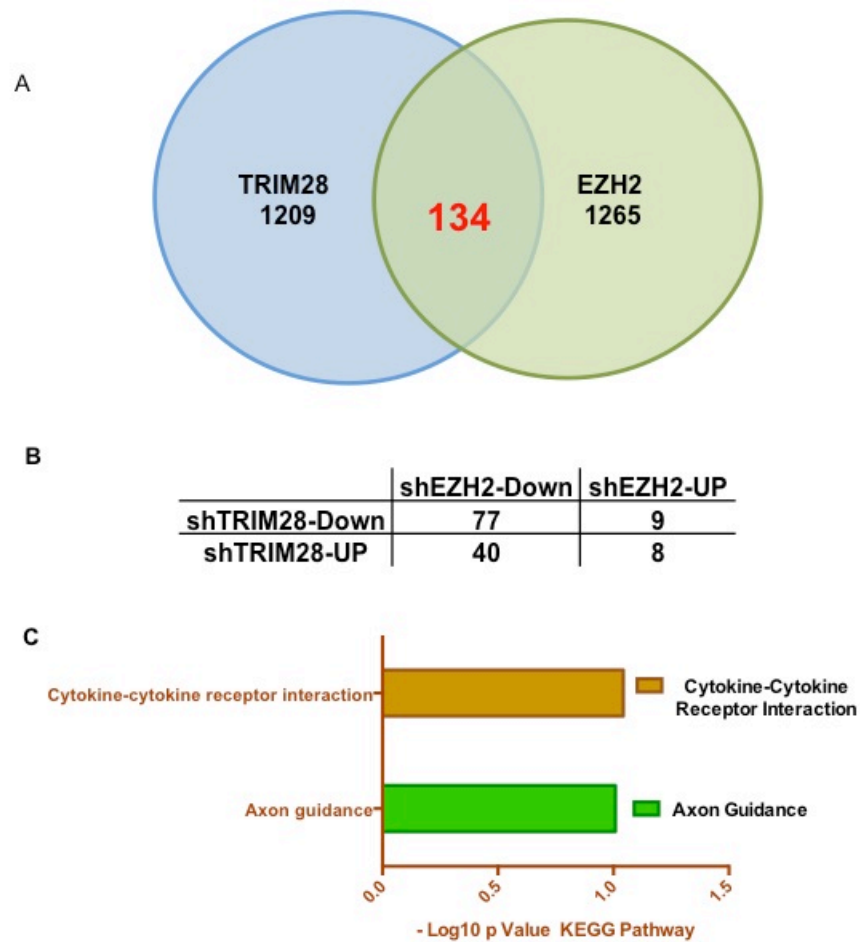


Figure 24 EZH2/TRIM28 Co-regulate Cytokine-Cytokine Receptor Interaction Pathway A. TRIM28/EZH2 RNA-seq intersection reveals 134 co-regulated genes, and B. the numbers of genes showing details of regulation directions by TRIM28/EZH2 C. KEGG pathway analysis of 134 co-regulated genes by TRIM28/EZH2

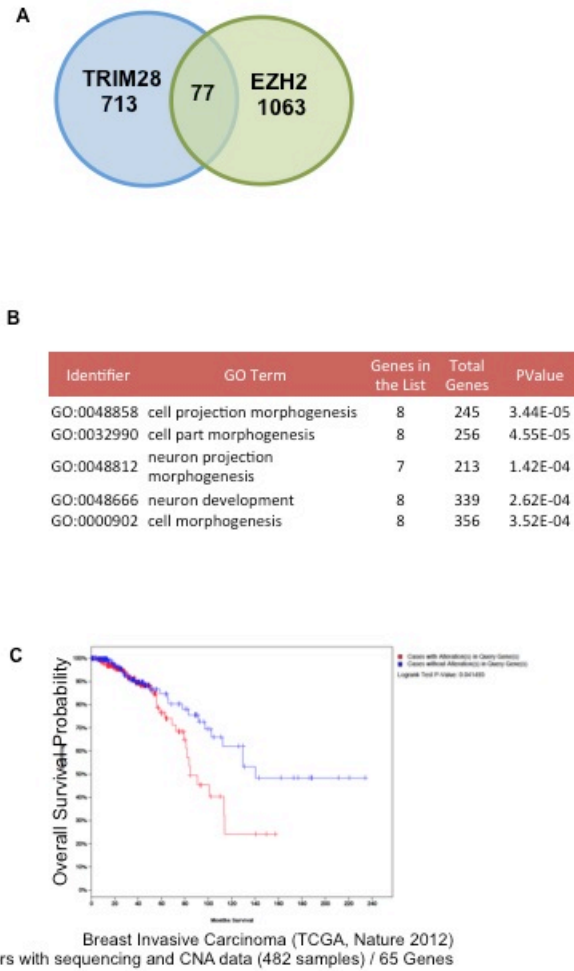


Figure 25 TRIM28/EZH2 Co-regulated Genes A. TRIM28/EZH2 RNA-seq intersection reveals 77 co-activated genes, B. GO term analysis of these co-down-regulated differentially expressed genes by EZH2 and TRIM28 C. Overall survival curve of these co-down-regulated differentially expressed genes

Enriched Protein Domains in EZH2, TRIM28 Co-Down-Regulated Genes			
Identifier	INTRERPRO Term-Name	P-Value	Fold Enrichment
IPR000742	EGF-like, type 3	0.021510452	6.605471848
IPR006210	EGF-like	0.023584476	6.37543054
IPR013032	EGF-like region, conserved site	0.060469222	4.373588868

Top Canonical Pathways		
Name	p-value	Overlap
Proline Degradation	6.49E-03	50.0 % 1/2
Atherosclerosis Signaling	7.67E-03	2.4 % 3/124
Protein Citrullination	1.61E-02	20.0 % 1/5
Chemokine Signaling	2.24E-02	2.8 % 2/71
Ephrin B Signaling	2.35E-02	2.7 % 2/73

Figure 26 A. Introdomain Analysis of EZH2/TRIM28 Co-regulated Genes B. Top canonical pathway revealed by IPA analysis for EZH2/TRIM28 co-regulated genes

promoter of PGR, this correlates with decreased PGR gene expression (Figure 15B). In contrast, in the HOX loci, H3K27me3 binding is depleted in the response to TRIM28 depletion (Figure 16A, Figure 18, 19, and 20), H3K27me3 reduction on HOXA1 correlates with increased HOX A1 gene expression (Figure 16B). Altogether, these data demonstrate that loss of TRIM28 causes improper EZH2 targeting in MCF7 cells, re-targeting H3K27me3 from HOX loci to different genomic loci. Thus, we conclude that TRIM28 is a specificity determinant for H3K27me3 localization and function. Examination of global H3K27me3 ChIP-seq patterns revealed an unexpected shift to intergenic regions (Figure 13B). These data suggested that TRIM28 is required to specify H3K27me3 localization to bind its target genes.

DAVID annotation suggests that the cluster 1 related biological processes are ion transport, differentiation, development, and cell morphogenesis (Figure 21A); KEGG pathway analysis shows that the related pathway is calcium-signaling pathway (Figure 21B). Functional analysis of genes from cluster 2 suggests that the cluster 2 related biological processes are dicarboxylic acid transport, carboxylic acid transport, organic acid transport, L-amino acid transport, and amine transport (Figure 21C). The KEGG pathway analysis shows that the related pathways are long term depression, tight junction, focal adhesion, arrhythmogenic right ventricular cardiomyopathy (ARVC), and ECM-receptor interaction (Figure 21D).

EZH2 and TRIM28 co-regulate genes associated with differentiation and morphogenesis

To understand the impact of EZH2 and TRIM28 on gene expression in ER-positive, breast cancer cells, we profiled EZH2- and TRIM28-dependent transcriptomes (RNA-sequencing) in MCF7 cells. EZH2 was depleted in shRNA-stable MCF7 lines at both protein (Figure 27A) and mRNA (Figure 27B) levels. Genome wide expression profiling showed that EZH2 activates considerably more genes (1063 genes) than it represses (202 genes) in MCF7 cells (Figure 22A). Biological process analyses (gene ontology or GO functions) revealed that EZH2 activates differentiation and morphogenesis-related genes (Figure 22B), which are associated by KEGG pathway analysis with ligand-receptor interaction molecules and pathways in cancer (Figure 22C). The top biological processes (GO functions) of genes repressed by EZH2 are collectively associated with the unfolded protein response (Figure 28A).

RNA-sequencing of stable shTRIM28 MCF7 cells (Figure 23A) showed that TRIM28 depletion led to increased expression of 713 genes and decreased expression of 496 genes (Figure 23A). The GO functions of genes activated by TRIM28 (repressed in shTRIM28) include cell projection organization, morphogenesis, and development (Figure 23A), while those repressed by TRIM28 (up-regulated in shTRIM28) are associated with extracellular matrix organization, structure, secretion, and adhesion (Figure 29A).

To determine whether EZH2 and TRIM28 regulate a shared set of genes in MCF7 cells, we intersected EZH2 and TRIM28 transcriptome data. This

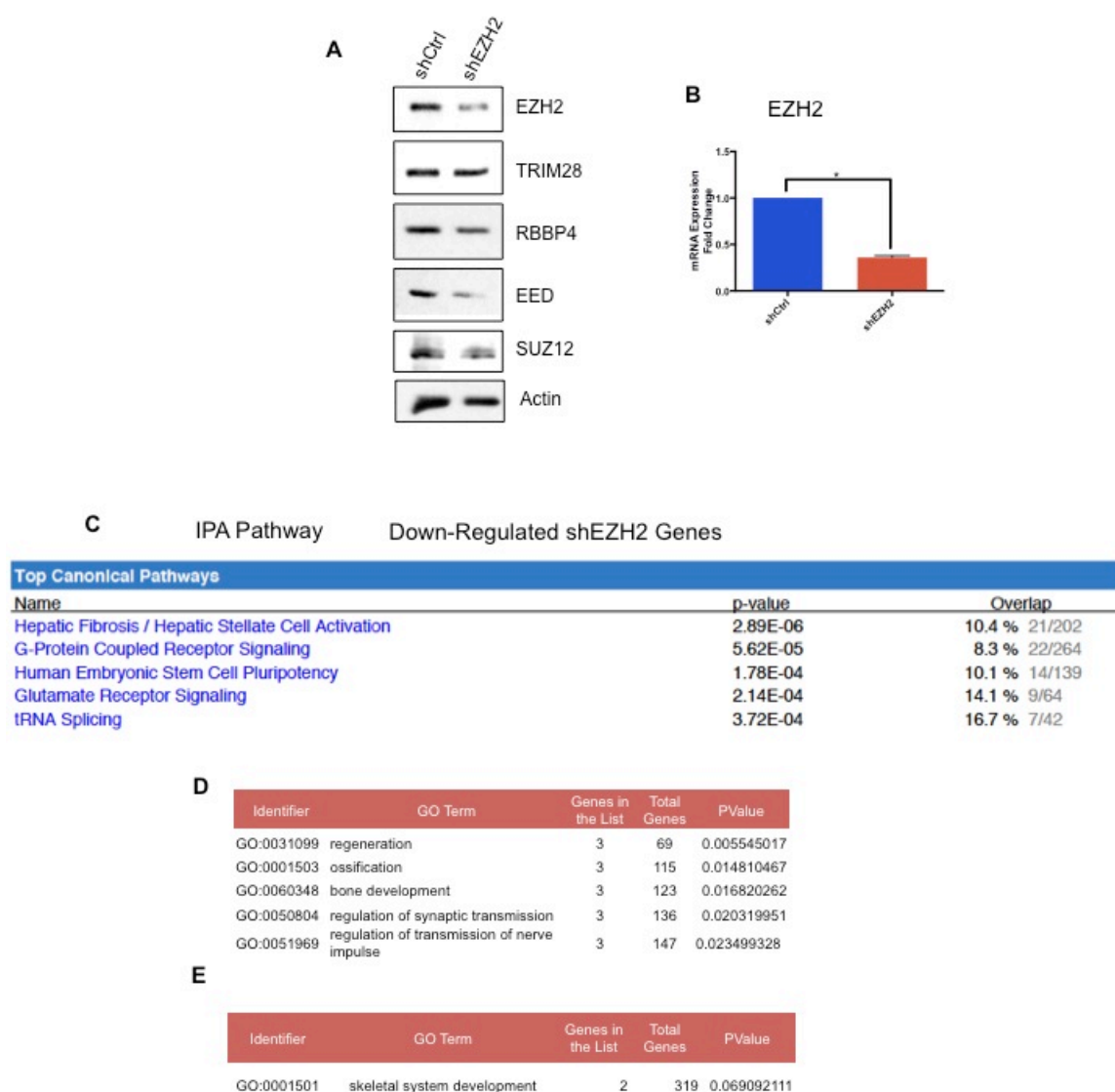


Figure 27 EZH2 Depletion Efficiency and Destabilize PRC2 Complex A. EZH2, TRIM28, RBBP4, EED, SUZ 12 protein expression in shControl and shEZH2 MCF7 cells B. EZH2 mRNA expression level in shControl, shEZH2 MCF7 cells C. . Top canonical pathway revealed by IPA analysis for EZH2 activated genes

A

Up regulated EZH2 genes				
Identifier	GO Term	Genes in the List	Total Genes	PValue
GO:0006986	response to unfolded protein	6	71	6.49E-05
GO:0051789	response to protein stimulus	6	107	4.48E-04
GO:0006457	protein folding	7	177	6.64E-04
GO:0010033	response to organic substance	10	721	0.028026513
GO:0045683	negative regulation of epidermis development	2	5	0.029585857

B

Up regulated EZH2 genes				
Identifier	GO Term	Genes in the List	Total Genes	PValue
hsa04612	Antigen processing and presentation	3	83	0.08048029

C

IPA Pathway up-Regulated shEZH2 Genes

Top Canonical Pathways		
Name	p-value	Overlap
Aldosterone Signaling in Epithelial Cells	2.01E-06	5.3 % 8/152
Protein Ubiquitination Pathway	8.52E-05	3.1 % 8/255
NRF2-mediated Oxidative Stress Response	4.89E-04	3.3 % 6/180
Glucocorticoid Receptor Signaling	8.33E-04	2.5 % 7/275
Huntington's Disease Signaling	1.74E-03	2.6 % 6/230

Figure 28 GO term analysis of EZH2 regulated genes A. GO term analysis of EZH2 repressed genes in breast cancer MCF7 cells B. KEGG pathway analysis of EZH2 repressed genes in breast cancer MCF7 cells C. IPA analysis of EZH2 repressed genes in breast cancer MCF7 cells

A

Up regulated TRIM28 genes				
Identifier	GO Term	Genes in the List	Total Genes	PValue
GO:0030198	extracellular matrix organization	10	104	3.39E-05
GO:0043062	extracellular structure organization	12	163	4.72E-05
GO:0051046	regulation of secretion	11	202	0.001223544
GO:0007155	cell adhesion	23	700	0.001303948
GO:0022610	biological adhesion	23	701	0.001327914

B

IPA Pathway Down-regulated genes in shTRIM28 MCF7

Top Canonical Pathways		
Name	p-value	Overlap
Xenobiotic Metabolism Signaling	1.51E-06	8.5 % 23/271
Dermatan Sulfate Biosynthesis (Late Stages)	2.08E-05	18.2 % 8/44
Chondroitin Sulfate Biosynthesis (Late Stages)	2.92E-05	17.4 % 8/46
LPS/IL-1 Mediated Inhibition of RXR Function	3.16E-05	8.2 % 18/219
Heparan Sulfate Biosynthesis (Late Stages)	5.47E-05	16.0 % 8/50

C

Up regulated TRIM28 genes				
Identifier	GO Term	Genes in the List	Total Genes	PValue
hsa04512	ECM-receptor interaction	5	84	0.01494689
hsa04080	Neuroactive ligand-receptor interaction	8	256	0.025255523
hsa04020	Calcium signaling pathway	6	176	0.049121955

D

IPA Pathway Up-regulated genes in shTRIM28 MCF7

Top Canonical Pathways		
Name	p-value	Overlap
Eicosanoid Signaling	1.32E-02	6.2 % 4/64
Methylglyoxal Degradation VI	1.43E-02	100.0 % 1/1
Hepatic Fibrosis / Hepatic Stellate Cell Activation	2.39E-02	3.5 % 7/198
Adenosine Nucleotides Degradation II	2.40E-02	11.8 % 2/17
Glycine Degradation (Creatine Biosynthesis)	2.83E-02	50.0 % 1/2

Figure 29 GO term analysis of TRIM28 regulated genes A. GO term analysis of TRIM28 repressed genes in breast cancer MCF7 cells B. IPA analysis of TRIM28 activated genes in MCF7 cells C. KEGG pathway analysis of TRIM28 repressed genes in breast cancer MCF7 cells D. IPA analysis of TRIM28 repressed genes in breast cancer MCF7 cells

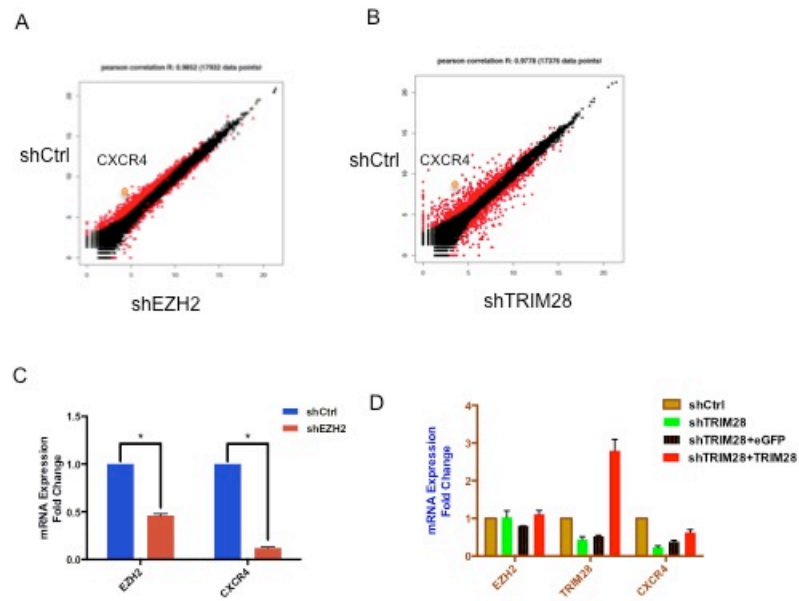


Figure 30 EZH2/TRIM28 co-activates CXCR4 gene expression A. scatter plot of EZH2 transcriptome B. scatter plot of TRIM28 transcriptome C. Realtime RT-PCR validation of CXCR4 mRNA expression in shEZH2 MCF7 cells D. Realtime RT-PCR validation of CXCR4 mRNA expression in shTRIM28 MCF7 cells

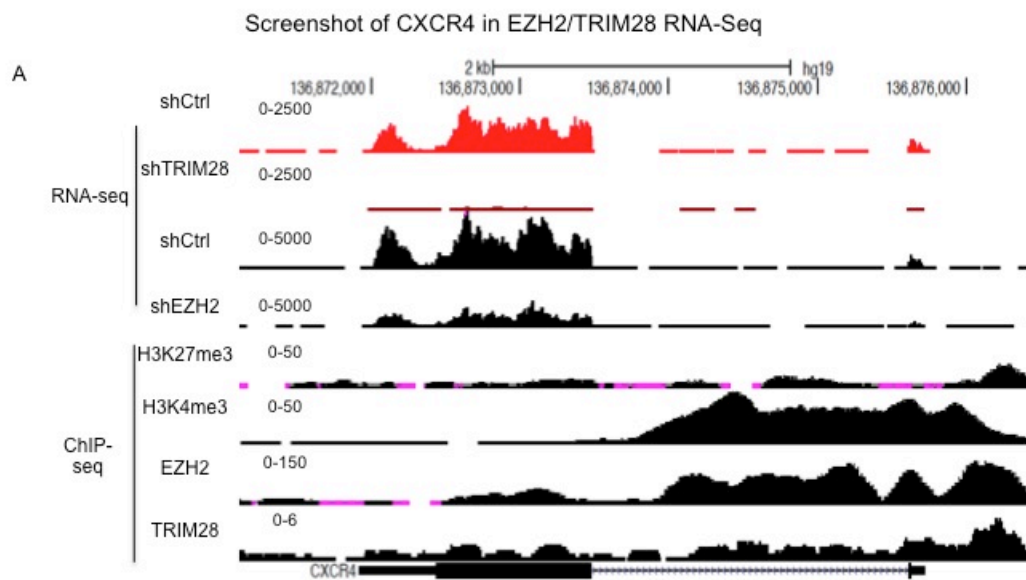


Figure 31 Screenshot of EZH2/TRIM28 co-activation of *CXCR4* A. Screenshot of *CXCR4* promoter region from H3K27me3 ChIP-seq in shControl, shTRIM28 MCF7 cells, RNA-seq in shControl, shTRIM28 MCF7 cells

intersection revealed 134 genes that are differentially expressed in both shEZH2 and shTRIM28 MCF7 cells (Figure 24A). There are 40 EZH2-activated genes that are repressed by TRIM28 (Figure 27D), and only 8 genes are co-repressed by both EZH2 and TRIM28 (Figure 27E). Of the 134 differentially regulated EZH2/TRIM28 genes, a subset of 77 is down regulated in both shTRIM28 and shEZH2 stable cell lines (Figure 28A). Biological process analysis revealed that these 77 genes, activated by both TRIM28 and EZH2, share GO functions of cell projection, morphogenesis and development (Figure 24B). Interestingly, the EZH2/TRIM28 activated gene set is associated with worse overall survival of breast cancer patients by TCGA breast cancer patient data analysis (Figure 25C).

To identify the directly regulated, target genes of TRIM28 in breast cancer cells, we performed TRIM28 ChIP-Seq and integrated these data with differentially expressed genes in shTRIM28 MCF7 cells (Figure 32A). Of the 227 TRIM28 target genes differentially regulated by TRIM28, 16 were activated by both TRIM28 and EZH2 (Figure 32A). Further mining of these data, intersection with ENCODE EZH2 ChIP-Seq ¹⁴⁵, and validation by real-time RT-PCR of RNA isolated from shTRIM28, shEZH2 and shControl MCF7 cells, respectively, revealed 7 genes that are directly activated and bound by both EZH2 and TRIM28 (Figure 32B, 32C, 32D and 32E). ChIP-PCR showed that TRIM28 was essential for EZH2 association with chromatin at the regulatory regions of these

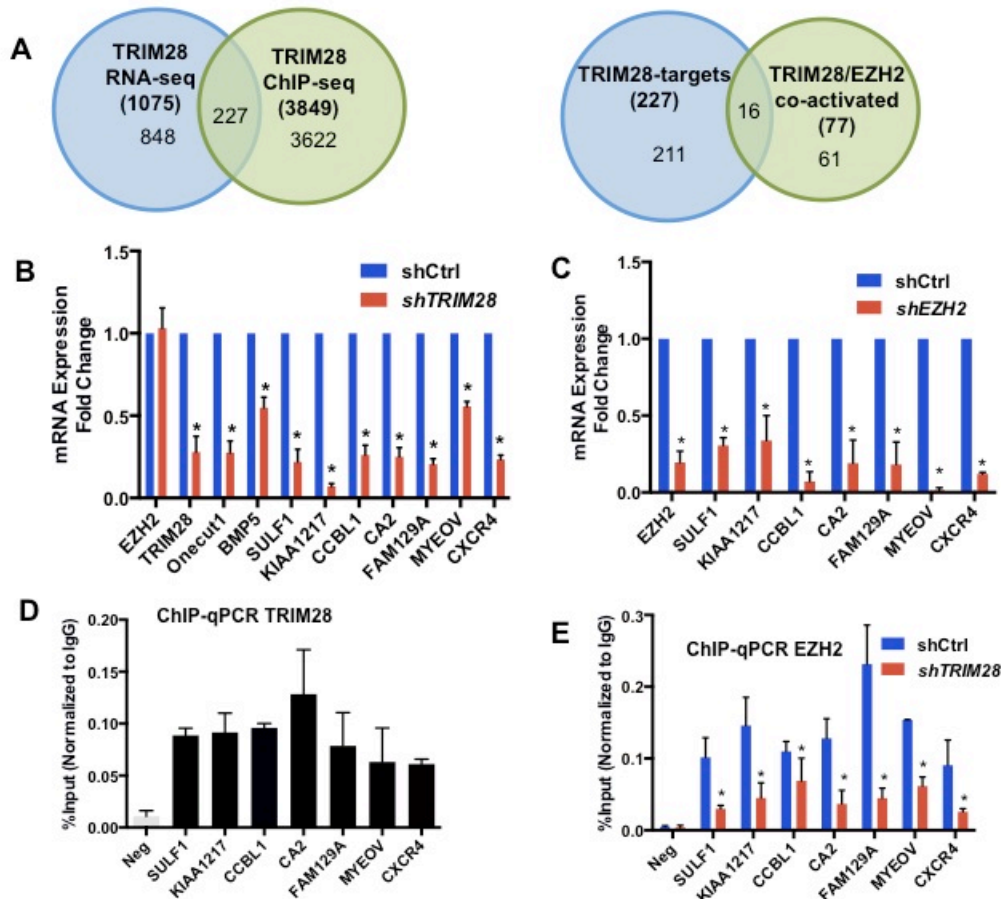


Figure 32 EZH2/TRIM28 directly co-activates a subset of genes A. Ven Diagram showing number of TRIM28 direct target genes by integrating TRIM28 ChIP-Seq with RNA-Seq. Ven Diagram showing the overlapping TRIM28 direct target genes with TRIM28/EZH2 co-activated genes. B. RT-qPCR analysis of *EZH2*, *TRIM28*, *Onecut1*, *BMP5*, *SULF1*, *KIAA1217*, *CCBL1*, *CA2*, *FAM129A*, *MYEOV*, and *CXCR4* in shCtrl and shTRIM28 MCF7 cells. Error bars represent SEM from three independent experiments (* $P < 0.05$). C. RT-qPCR analysis of *EZH2*, *SULF1*, *KIAA1217*, *CCBL1*, *CA2*, *FAM129A*, *MYEOV*, and *CXCR4* in shCtrl and shEZH2 MCF7 cells. Error bars represent SEM from three independent experiments (* $P < 0.05$). D. ChIP-qPCR analysis of TRIM28 occupancy at *SULF1*, *KIAA1217*, *CCBL1*, *CA2*, *FAM129A*, *MYEOV*, and *CXCR4* promoter in MCF7 cells. Error bars represent SEM from three independent experiments. E. ChIP-qPCR analysis of EZH2 occupancy at *SULF1*, *KIAA1217*, *CCBL1*, *CA2*, *FAM129A*, *MYEOV*, and *CXCR4* promoter in shCtrl and shTRIM28 MCF7 cells. Error bars represent SEM from three independent experiments (* $P < 0.05$).

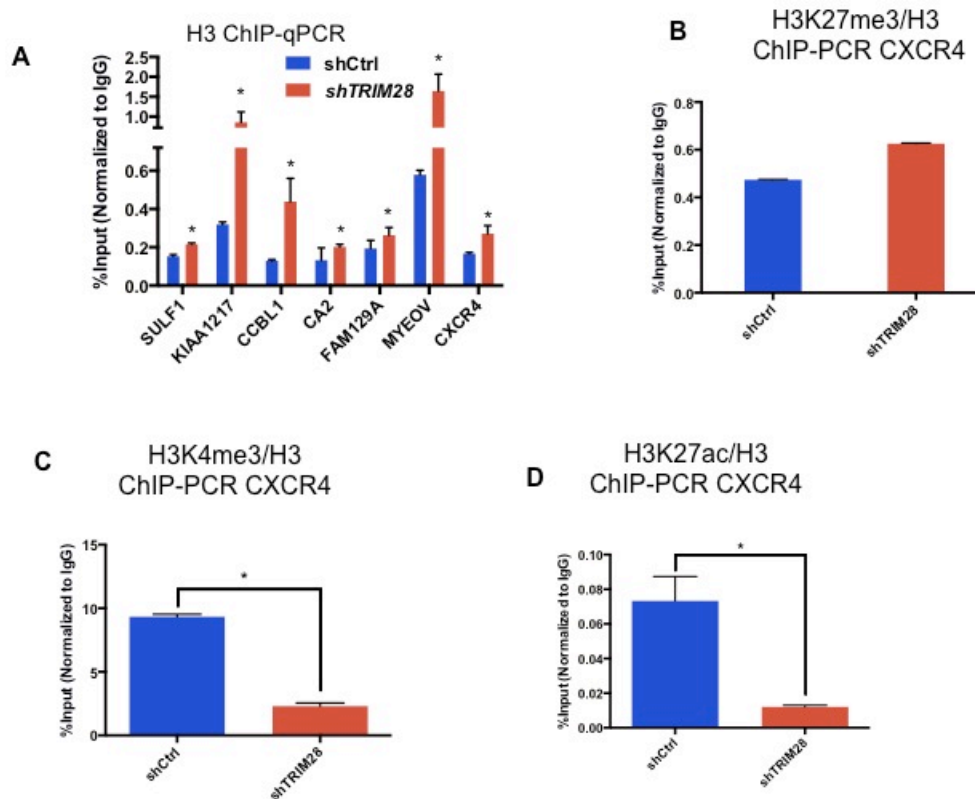


Figure 33 TRIM28 alters histone modification of CXCR4 promoter A. ChIP-qPCR analysis of histone H3 occupancy at *SULF1*, *KIAA1217*, *CCBL1*, *CA2*, *FAM129A*, *MYEOV*, and *CXCR4* promoter in shCtrl and shTRIM28 MCF7 cells. Error bars represent SEM from three independent experiments (* $P < 0.05$). B. H3K27me3 ChIP-PCR on the promoter of CXCR4 in shControl, shTRIM28 MCF7 cells C. H3K4me3 ChIP-PCR on the promoter of CXCR4 in shControl, shTRIM28 MCF7 cells . D. H3K4me3 ChIP-PCR on the promoter of CXCR4 in shControl, shTRIM28 MCF7 cells

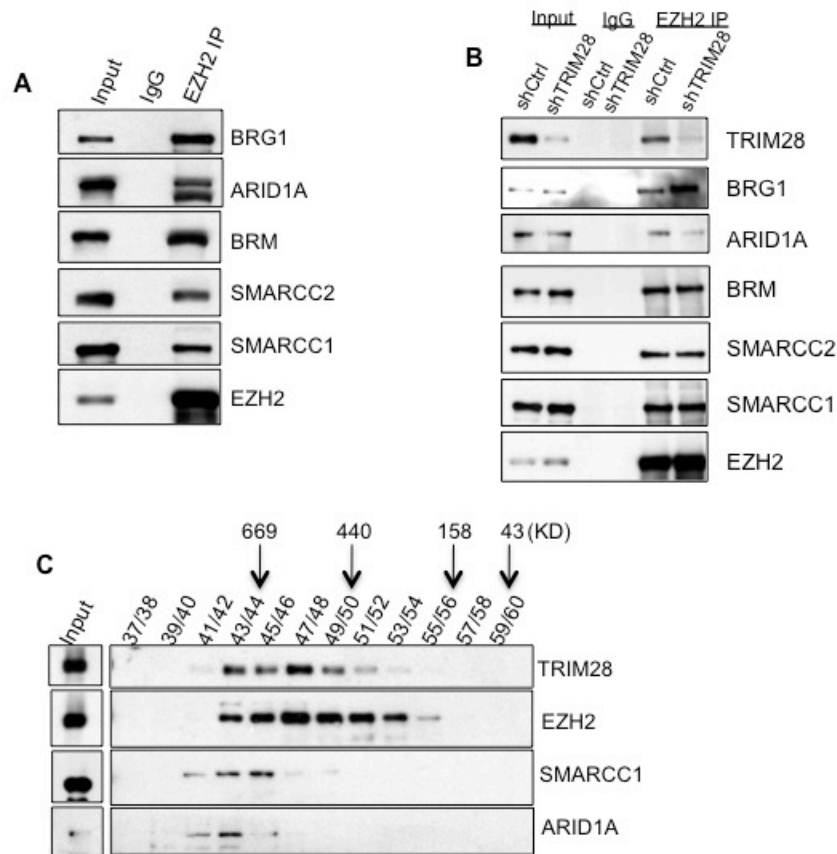


Figure 34 TRIM28 Regulates EZH2 association with SWI/SNF complex A.

EZH2 was immunoprecipitated from MCF7 cell lysate, BRG1, ARID1A, BRM, SMARCC2, SMARCC1, EZH2 was detected with western blotting B. EZH2 was immunoprecipitated from shControl, shTRIM28 MCF7 cell lysate, TRIM28, BRG1, ARID1A, BRM, SMARCC2, SMARCC1, EZH2 was detected with western blotting C. Gel filtration assay analysis of SWI/SNF complex with EZH2, TRIM28, and MCF7 lysate was fractionated by Superose 6 chromatography, followed by immunoblotting for the antigens indicated.

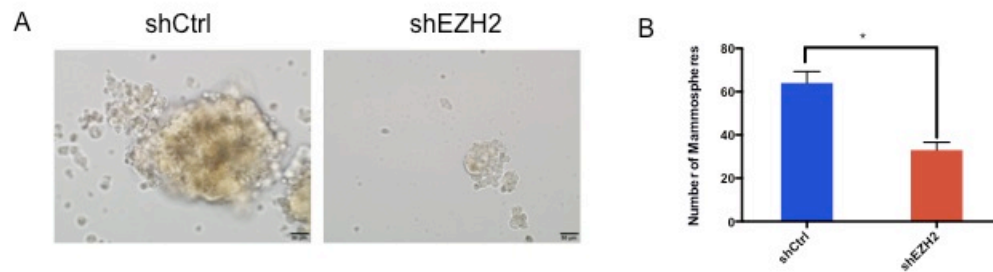


Figure 35 EZH2 Regulates Breast Cancer Stem Cell Maintenance A. mammosphere assay in shControl, shEZH2 MCF7 cells, and B. Quantification

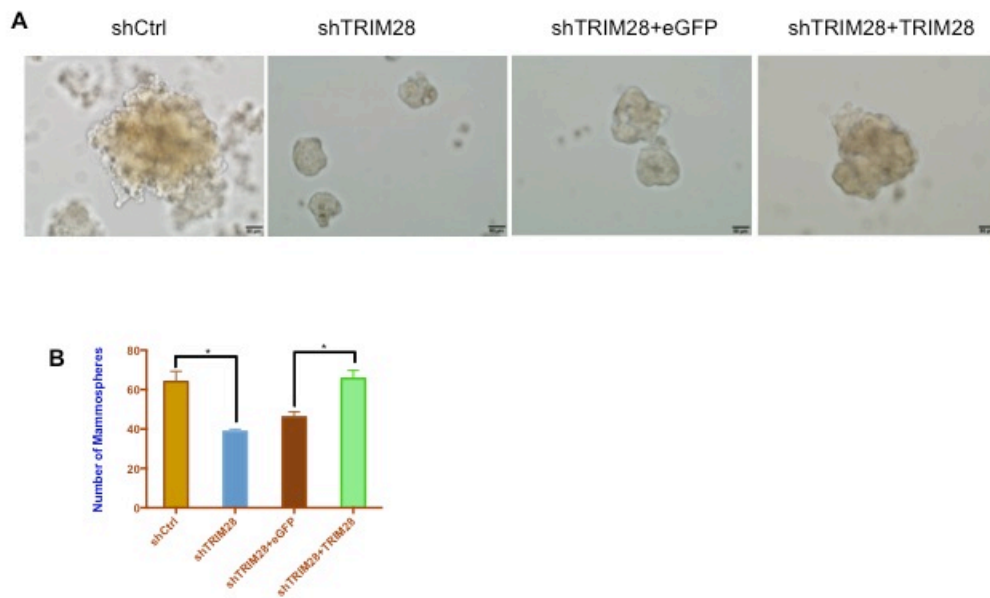


Figure 36 TRIM28 Depletion Regulate Breast Cancer Stem Cell

Maintenance A. mammosphere assay in shControl, shTRIM28, shTRIM28 plus eGFP, shTRIM28 plus TRIM28 MCF7 cells, and B. Quantification

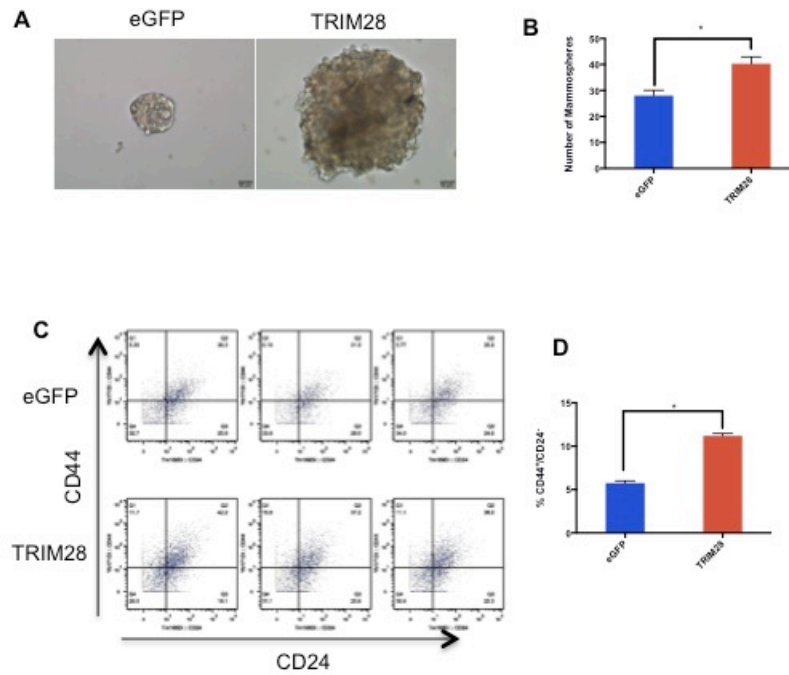


Figure 37 TRIM28 Ectopic Expression Regulate Breast Cancer Stem Cell Maintenance A. mammosphere assay in eGFP, TRIM28 overexpressing MCF7 cells, and B. Quantification C. CyTOF assay to measure CD44⁺ CD24⁻ population in eGFP, TRIM28 overexpressing MCF7 cells

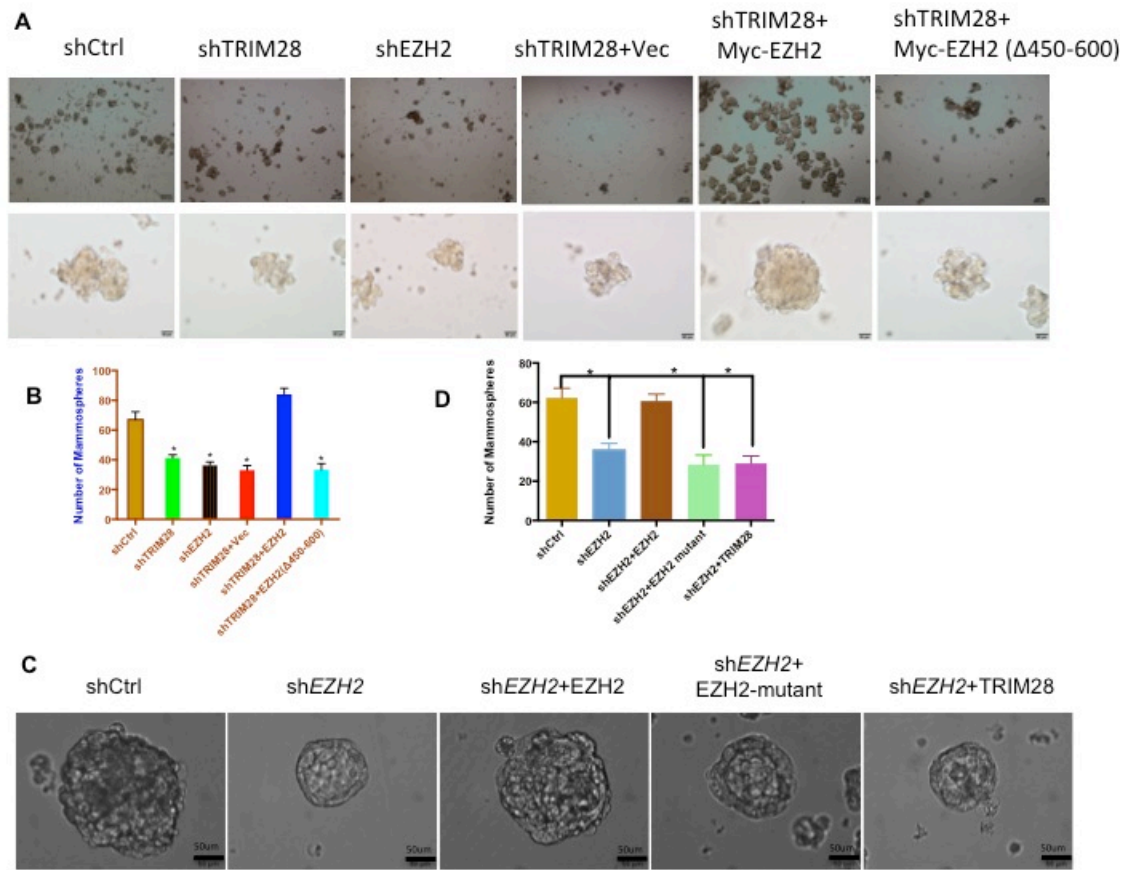


Figure 38 TRIM28 and EZH2 are epistatic in mammosphere formation, and dependent on the interaction region. A. mammosphere assay in shControl, shTRIM28, shEZH2, shTRIM28 plus Vector, shTRIM28 plus EZH2 wt, shTRIM28 plus interaction mutant, MCF7 cells, and B. Quantification C. EZH2 depletion inhibited MCF7 mammosphere formation, which is rescued by expression of shEZH2-resistant EZH2, but not interaction mutant EZH2 and wt TRIM28; results were also quantified.

EZH2/TRIM28 co-activated genes, which were previously associated in various aspects of cancer promotion, metastasis and stem cells (Figure 32E).

EZH2 and TRIM28 orchestrates a CXCR4 signaling pathway

KEGG pathway analysis of the coordinately TRIM28/EZH2-regulated 77 genes showed that the cytokine-cytokine receptor interaction pathway (CXCR4, CXCL12, EGFR, KIT) is the top pathway (Figure 24C). Each TRIM28/EZH2-regulated gene in this pathway, CXCR4^{146,147}, CXCL12^{146,147}, EGFR¹⁴⁸, KIT²⁰), has been implicated as a regulator of cancer stem cells. We examined the gene expression mechanisms of EZH2/TRIM28-mediated regulation using CXCR4 as our model. Scatter plots of EZH2 and TRIM28 transcriptomes show that CXCR4 is a highly differentially regulated gene when either EZH2 or TRIM28 is depleted in MCF7 cells (Figure 30A and 30B). Real-time RT-PCR of CXCR4 confirmed that EZH2 and TRIM28 depletion significantly decreased the mRNA expression levels of CXCR4 in MCF7 cells (Figure 30C and 30D). ChIP-PCR analysis of EZH2 and TRIM28 binding to chromatin in MCF7 cells showed that both EZH2 and TRIM28 bind the promoter of the CXCR4 gene (Figure 31A, 32A and B). With depletion of TRIM28 in shTRIM28 MCF7 cells, EZH2 recruitment to the promoter of CXCR4 was significantly decreased (Figure 32B).

Our analysis of EZH2 protein complexes in MCF7 cells showed that TRIM28 and canonical PRC2 subunits, EED and RBBP4, associated with EZH2 as separate entities (Fig. 4A, and 10A). Interestingly, we found that H3K27me3 enrichment at the CXCR4 promoter was unchanged when TRIM28 was depleted

(Figure 33B), but H3K4me3 and H3K27ac (Figure 33C and D) decrease in parallel with TRIM28 and EZH2 binding in shTRIM28 MCF7 cells. Hence, we conclude that TRIM28 recruits EZH2 to the promoter of CXCR4, concomitant with activated CXCR4 gene expression, independently of EZH2-mediated H3K27me3.

TRIM28 Regulates EZH2 Association with SWI/SNF Complex

In our analysis of binding partners of EZH2 in MCF7 cell, we identified multiple subunits of the SWI/SNF complex: BRG1, SMARCC2, SMARCA5, BAF180, ARID1A, as well as p300 (Figure 8A). We determined whether SWI/SNF acted in transcriptional activation of CXCR4, as a mechanism of EZH2/TRIM28 activation of transcription in breast cancer cells. The association of EZH2 and SWI/SNF complex subunits was validated, along with TRIM28 (Figure 34A). Interestingly, when TRIM28 was depleted from MCF7 cells (shTRIM28), we observed that EZH2 association with the ARID1A subunit of the SWI/SNF association was disrupted (Figure 34B). ARID1A is critical for SWI/SNF complex stability, mutated in a number of cancers and associated with cellular proliferation and DNA repair^{149,150}. Gel filtration and size fractionation of MCF7 extracts showed that SWI/SNF subunits co-elute with TRIM28 and EZH2 as a likely sub-complex of proteins associated with EZH2 (Figure 34C). TRIM28 may act as a switch to modulate EZH2 and SWI/SNF association and regulate EZH2-activated genes, such as CXCR4. SWI/SNF chromatin remodeling complexes function in positioning, sliding, exchanging or evicting nucleosomes, which impact chromatin

A

Down regulated TRIM28 target genes				
Identifier	GO Term	Genes in the List	Total Genes	PValue
GO:0030030	cell projection organization	13	368	7.02E-06
GO:0048858	cell projection morphogenesis	10	245	4.25E-05
GO:0007409	axonogenesis	9	193	4.96E-05
GO:0032990	cell part morphogenesis	10	256	5.98E-05
GO:0048667	cell morphogenesis involved in neuron differentiation	9	209	8.69E-05

B

Down regulated TRIM28 target genes				
Identifier	GO Term	Genes in the List	Total Genes	PValue
hsa04360	Axon guidance	6	129	0.003501873
hsa04340	Hedgehog signaling pathway	3	56	0.074343572

Figure 39 Annotation of TRIM28 Activated Genes A. GO term analysis of TRIM28 activated genes B. KEGG pathway analysis of TRIM28 activated genes

A

Up regulated TRIM28 target genes				
Identifier	GO Term	Genes in the List	Total Genes	PValue
GO:0006355	regulation of transcription, DNA-dependent	25	1773	3.47E-06
GO:0051252	regulation of RNA metabolic process	25	1813	5.15E-06
GO:0045449	regulation of transcription	30	2601	1.08E-05
GO:0006350	transcription	24	2101	1.86E-04
GO:0007155	cell adhesion	9	700	0.025533536

B

Up regulated TRIM28 target genes				
Identifier	GO Term	Genes in the List	Total Genes	PValue
hsa02010	ABC transporters	2	44	0.083307214
hsa04080	Neuroactive ligand-receptor interaction	3	256	0.087006263

Figure 40 Annotation of TRIM28 Repressed Genes A. GO term analysis of TRIM28 Repressed genes B. KEGG pathway analysis of TRIM28 Repressed genes

A	TRIM28 bound genes				
	Identifier	GO Term	Genes in the List	Total Genes	PValue
	GO:0006355	regulation of transcription, DNA-dependent	283	1773	7.19E-109
	GO:0051252	regulation of RNA metabolic process	283	1813	2.68E-106
	GO:0006350	transcription	302	2101	9.28E-106
	GO:0045449	regulation of transcription	324	2601	4.17E-98
	GO:0006688	glycosphingolipid biosynthetic process	3	11	0.070949711

B	TRIM28 bound genes				
	Identifier	GO Term	Genes in the List	Total Genes	PValue
	hsa04070	Phosphatidylinositol signaling system	8	74	0.001232893
	hsa04722	Neurotrophin signaling pathway	10	124	0.001712112
	hsa04660	T cell receptor signaling pathway	9	108	0.002722329
	hsa04012	ErbB signaling pathway	6	87	0.044809723
	hsa04910	Insulin signaling pathway	7	135	0.081585133

Figure 41 Annotation of TRIM28 bound target Genes A. GO term analysis of TRIM28 bound target genes B. KEGG pathway analysis of TRIM28 bound target genes

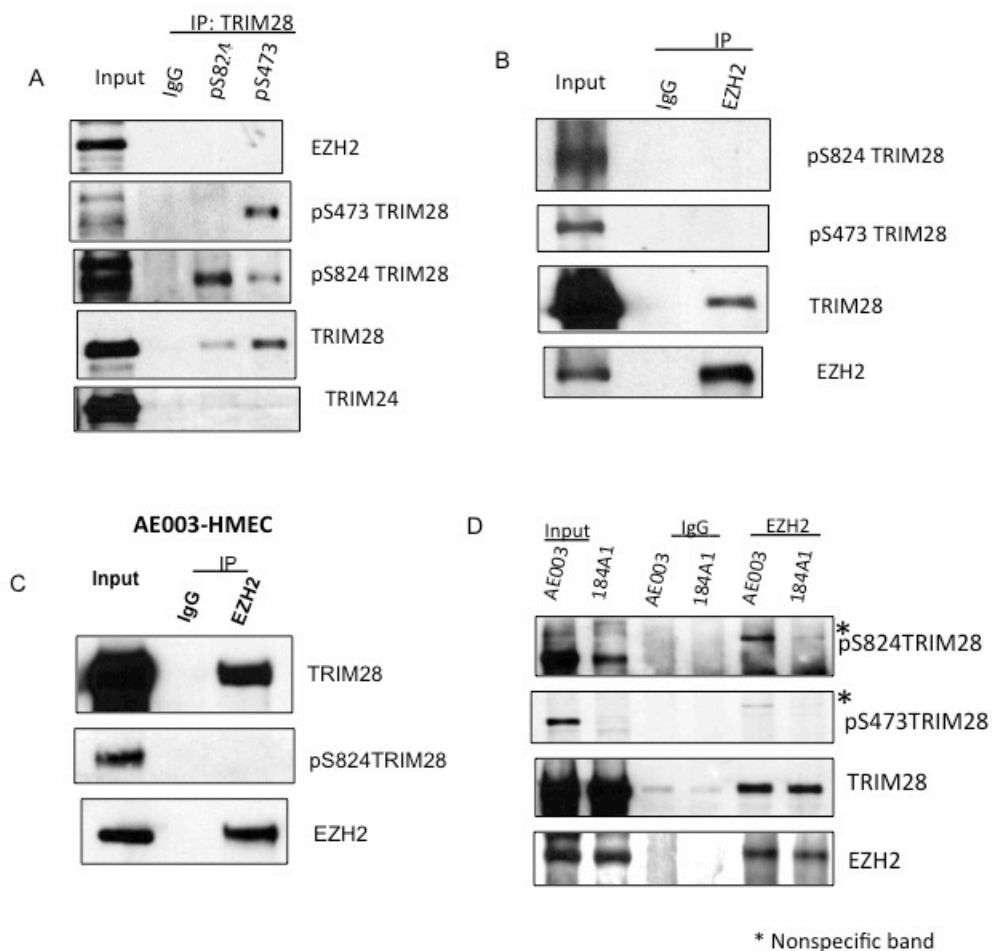


Figure 42 Phosphorylation interferes with EZH2/TRIM28 interaction with endogenous immunoprecipitation A. pS824/pS473TRIM28 was immunoprecipitated in MCF7 cell lysate, EZH2, pS473TRIM28, pS824TRIM28, TRIM28 were probed; C. EZH2 was immunoprecipitated in MCF7 cell lysate, TRIM28, pS473TRIM28, pS824TRIM28, EZH2 were detected; C. EZH2 was immunoprecipitated in AE003 HMEC cell lysate, TRIM28, pS824TRIM28, EZH2 were detected D. EZH2 was immunoprecipitated in AE003 HMEC cell lysate, TRIM28, pS473TRIM28, pS824TRIM28, EZH2 were detected

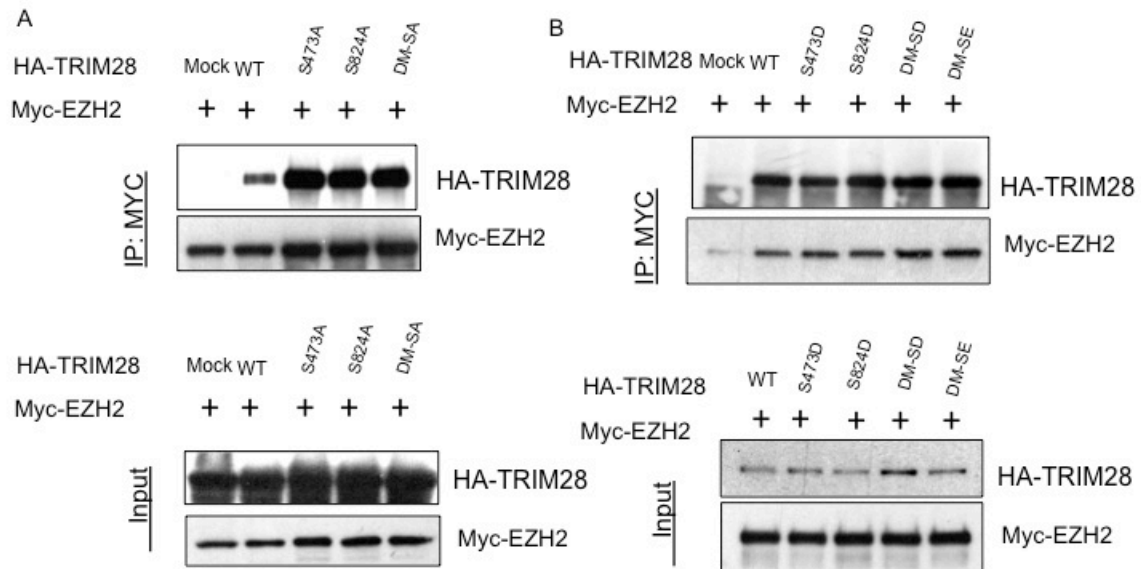


Figure 43 Phosphorylation interferes with EZH2/TRIM28 interaction with TRIM28 SA mutants co-transfection A. Myc-EZH2 and HA-TRIM28 wt, SA mutants were co-transfected into HEK-293T cells, either Myc was immunoprecipitated, HA was probed. A. Myc-EZH2 and HA-TRIM28 wt, SD mutants were co-transfected into HEK-293T cells, either Myc was immunoprecipitated, HA was probed

structure-dependent processes, including transcription, replication and DNA repair essential for cellular homeostasis ¹⁵¹. We analyzed histone H3 enrichment by ChIP-PCR as a gross assessment of nucleosome occupancy across the sites of TRIM28/EZH2 interactions at their differentially regulated target genes in MCF7 cells (Figure 33A). These findings suggest that TRIM28/EZH2-mediated interactions with SWI/SNF complexes alter chromatin structure of their shared, activated target genes.

TRIM28 Regulates MCF7 Mammosphere Formation and Stem Cell

Maintenance

Previous studies of tumor-derived cells and mouse models showed that the PRC2 complex has opposing functions to the SWI/SNF complex in oncogenic transformation and cancer-associated stem cells ¹⁵². How EZH2 may function with regards to SWI/SNF in non-PRC2 dependent activities, as we define here in MCF7 cells, is unknown. Given previous association of SWI/SNF with cancer stem cells and our finding that CXCR4, a marker of cancer stem cells, is a target of EZH2/TRIM28 activation, we used MCF7 sphere-forming or mammosphere assays ^{31,153}, as a surrogate for cancer stem cell assessment and to determine if a TRIM28/EZH2 axis had a role in breast stem cell maintenance.

First, we assessed whether EZH2 and/or TRIM28 played roles in mammosphere formation using shControl, shEZH2 and shTRIM28 MCF7 cells (Figure 35A). The number of mammospheres formed was significantly decreased by depletion of either EZH2 (Figure 35A) or TRIM28 (Figure 36A). MCF7 mammosphere numbers were greatly reduced in shTRIM28 cells and rescued by

TRIM28 expression (Figure 36A and B). To further assess the effects of varying levels of TRIM28, we relied on a different vector and clone selection method to over express TRIM28 in MCF7 cells and saw increased efficiency of mammosphere formation, as well as increases in size of the formed mammospheres (Figure 37A). These data confirm previous reports that EZH2 plays a role in maintenance of breast cancer stem cells^{79,80}, using this assay system, and suggest that TRIM28 likewise regulates MCF7 mammosphere formation and stem cell maintenance.

Breast stem cell populations are characterized by cell surface marker expression patterns of CD44⁺ and CD24⁻ or low^{23,33}. We determined levels of these indicators by mass cytometry, or CyTOF (Fluidigm)¹⁵⁴, of MCF7 cells with ectopic expression of TRIM28 or eGFP (Figure 37C). The population of CD44⁺/CD24⁻ cells in TRIM28 overexpressing MCF7 cells is two-fold greater than control MCF7 plus eGFP cells, (Figure 37C). Further rescue assays of ectopic eGFP, TRIM28 and EZH2 expression in shControl and shTRIM28, showed that TRIM28 and EZH2 are epistatic in mammosphere formation (Figure 38A). Overexpression of EZH2 was sufficient for significant rescue of mammosphere formation and required EZH2/TRIM28 interaction domain, even when TRIM28 was depleted. A pre-SET domain deletion mutant EZH2 construct failed to rescue EZH2 dependant mammosphere formation (Figure 38C), which indicates that the interaction of TRIM28 with EZH2 is critical for EZH2 mediated mammosphere formation. Full length TRIM28 failed to rescue EZH2 dependant mammosphere formation (Figure 38C), which indicates that TRIM28 is upstream

of EZH2 (Figure 38C). Taken together with our global expression analyses, which indicated that TRIM28 and EZH2 coordinately regulated expression of genes with biological functions in differentiation and morphogenesis (Figure 25B), our findings support a role for TRIM28 in facilitating EZH2-mediated functions in stem cell maintenance.

pS473/824 TRIM28 does not interact with EZH2 in breast cancer cells

To investigate how the phosphorylation of TRIM28 affects its interaction with EZH2. Surprisingly, S473/824TRIM28 showed diminished interaction with EZH2 (Figure 42). In other words, S473, S824 phosphorylation interferes EZH2 interaction with TRIM28. In addition, SA mutants of TRIM28 show huge increased amount of binding with EZH2 compared with wild type TRIM28 (Figure 43).

DISCUSSION:

Here we report that the pre-SET domain of EZH2 interacts with TRIM28 in a RBCC domain dependent manner in breast cancer cells. Further study indicates that TRIM28 regulates EZH2 association with SWI/SNF complex in breast cancer cells. The interaction of TRIM28 with EZH2 may contribute to breast cancer stem cell phenotype maintenance through transcriptionally activating CXCR4 cancer stem cell signaling pathway. So, targeting the highly expressed EZH2 in breast cancer might provide a valuable strategy to overcome resistance of breast cancer chemotherapy by eliminating breast cancer stem cell. The deeper

understanding of EZH2 regulation by further study is of interest for breast cancer patients.

The newly identified interaction of TRIM28 with EZH2 might contribute to the polycomb-independent role of EZH2 in breast cancer, and this polycomb-independent role of EZH2 was also reported in prostate cancer ¹⁵⁵. The specific region of EZH2 interacting with TRIM28 is the pre-SET domain region (containing CXC domain); the pre-SET domain region of EZH2 was reported to direct SET domain activity in drosophila¹⁵⁶. In mammal study, the pre-SET domain interacts with AEBP2 ¹⁵⁷, SUZ12 ¹⁵⁷, and PAF ¹⁵⁸. Historically, the name of “CXC domain” was originally coined to refer to the pre-SET motif of EZH2¹⁵⁹ in drosophila, but now, the pre-SET domain of EZH2 refers to more than CXC domain ¹⁵⁸. The TRIM28 regulation of pre-SET domain (CXC) region of EZH2 in mammalian system suggests that TRIM28 might also direct SET domain activity, switching EZH2’s affinity between histone substrate and non-histone substrates. Thus, targeting EZH2/TRIM28 interaction could be a better approach to dampen breast cancer progression than targeting SET domain. The current EZH2 inhibitors (Epizeme, GSK, etc) were designed to target SET domain, Stuart Orkin’s antiproliferative experiments using GSK-126 on MDA-MB-231 cells appear no effect at all¹⁴². This knowledge in combination with our study here indicates that an effective EZH2 inhibitor design should target the pre-SET domain of EZH2.

Functionally, through transcriptome study, TRIM28 mediated EZH2 activity was pinpointed to maintenance the breast cancer stem cells phenotype via transactivating CXCR4 signaling pathway. We report to identify CXCR4/CXCL12 pathway as EZH2's H3K27me3 independent targets. TRIM28 also directly targets CXCR4/CXCL12 pathway through regulating recruitment of EZH2 onto the CXCR4 promoter. Whereas, a very recent publication⁸⁰ of investigating mechanism of EZH2 expanding breast cancer stem cell discovered NOTCH signaling as EZH2's H3K27me3 independent targets. The possible reason of this discrepancy is probably due to the technical limitation of specific stem cell signaling focused PCR array this group employed in this specific study, while we used RNA-sequencing with better coverage of the whole genome.

This is the very first time to report the function of TRIM28 in breast cancer stem cell biology. There are at least two publications highlighted that TRIM28 implicated in embryonic stem cell biology^{114,115}. In general, cancer stem cell tends to phenocopy embryonic stem cell, if we link SOX2 from stem cell to cancer stem cell¹⁶⁰. Additionally, our data indicate that mutant EZH2 of deleting the interaction domain with TRIM28 shows very limited capacity to form mammosphere. This data show that EZH2/TRIM28 interaction is critical for breast cancer stem cell maintenance. Thus, targeting EZH2/TRIM28 interaction is a very useful approach to dampen breast cancer progression. Breast cancer stem cell represents the novel direction of drug resistant breast cancer therapy development²⁰⁻²². The newly identified breast cancer stem cell regulatory axis

could be a potential therapeutic target. Thus, this study brings unparalleled value to the community.

Moreover, our data indicates that TRIM28 maintains breast cancer stem cell population. Previously, TRIM28 was published to promote breast cancer proliferation and metastatic progression ¹⁴⁴, we did invasion and metastasis assays in our lab, the TRIM28 mediated effect of metastatic progression is relatively marginal in MCF7 cells. However, Douglas Cress published that TRIM28 suppress MDA-MB-231 cells proliferation ¹⁶¹, and proposed a model that TRIM28 is a tumor suppressor in early transformation stages, but in later stages it functions as an oncogene in lung cancer ¹⁶². In breast cancer, It is also possible that the proliferation regulation of TRIM28 could be cell line dependent effect, or post-translational modification dependent effect of TRIM28 ¹²⁷. These questions can be answered by mouse genetics methods, and further biochemical characterization of TRIM28 protein *in vivo*. And another question still remains whether TRIM28 mediated breast cancer promotion are dependent on the expansion, differentiation of breast cancer stem cell or some other mechanisms?

EZH2 was proposed to contribute to tumor progression via its H3K27me3 dependent transcriptional repressive program for a long time ^{163,164}. But there are arguments that the oncogenic function of EZH2 is independent of H3K27me3 ¹⁶⁵. We observed that TRIM28 regulates cancer stem cell stemness by directly regulating CXCR4 signaling in an H3K27me3 independent manner. This

indicates the possible existence of non-histone substrates of EZH2 as a methyltransferase in breast cancer ¹⁶⁵, this concept is also in agreement with other types of cancers, such as castration resistant prostate cancer⁵⁶, glioblastoma ¹⁶⁶. This study is the further extension of some of the previous publications revealing the transactivation or polycomb-independent role of EZH2 in breast cancer^{66,80}, prostate cancer⁵⁶, and potentially other types of cancers.

Our transcriptome study indicates that a set of 134 genes is co-regulated by both EZH2 and TRIM28. Among these gene list, 77 genes are co-activated genes, these 77 genes enriched cytokine cytokine receptor interaction pathway ¹³⁷, which is implicated in regulating cancer stem cell stemness ¹⁴⁶. Some of genes in these 77 genes are not well studied in either stem cell, or cancer biology yet; it is very likely there are more genes in this list that are uncharacterized regulatory proteins/factors for breast cancer stem cell self-renewal and differentiation, or even other types of cancer stem cells.

In summary, TRIM28 interacts with EZH2 to regulate breast cancer stem cell maintenance. TRIM28 and EZH2 axis contributes to activation of CXCR4 gene expression, and then render to be an effective therapeutic target for optimized effective EZH2 therapy in breast cancer, and other types of breast cancer.

CHAPTER IV

TRIM28 PHOSPHORYLATION SWITCH REGULATES BREAST CANCER CELL PROLIFERATION

CHAPTER IV: TRIM28 Phosphorylation Switch Regulates Breast Cancer

Cell Proliferation

Abstract:

TRIM28 (KAP1, TIF1 β) is broadly associated with transcription repression, facultative heterochromatin functions, embryonic stem cell renewal and, more recently, breast cancer metastasis. TRIM28's functions in breast cancer progression remain largely unclear. TRIM28 is overexpressed in HMEC model of breast cancer progression; interestingly, TRIM28 is heavily phosphorylated (pS824/pS473) in aggressive breast cancer cells. Furthermore, ectopic expression of TRIM28 WT and phosphorylation defective form in HMEC cells indicates that TRIM28 promotes cell proliferation in a phosphorylation dependent manner. Genome-wide TRIM28 binding profile analysis unveils a novel binding motif, highly similar with TCF4 motif. The promoter of *AXIN2* encompass one of this kind binding motif of TRIM28, is a direct transcriptional target of TRIM28. TRIM28 specifically express in the epithelial cells of mammary gland, but not myoepithelial cells. Collectively, we demonstrated a mechanism of regulation of breast cancer cell proliferation by TRIM28 in a phosphorylation dependent manner via a TCF4 motif. *AXIN2* gene expression might be involved in this process.

Introduction:

TRIM28 (KAP1, KAP-1, TIF1 β) connects heterochromatin and euchromatin, is one of known facultative heterochromatin proteins^{107,167}, and structurally belongs to tripartite motif superfamily. The tripartite motif is composed of a RING finger followed by two B-boxes and a coiled-coil domain, also called RBCC motif; TRIM28's C-terminal is composed of a bi-partite motif encompassing a PHD finger and a Bromo domain. TRIM28 was known as a universal transcription corepressor protein by interacting with the Kruppel associated box (KRAB)-domain containing zinc finger proteins^{102,103}. TRIM28 binds and co-localizes with HP1 (heterochromatin protein 1) through a well-defined peptide motif, PxVxL. HP1 reads H3K9me3 (a repressive histone marker) with its chromo domain to play a pivotal role in heterochromatin mediated gene silencing¹⁰⁴. TRIM28 depletion leads to lower level of global H3K9me3^{105,106}. TRIM28 binds and recruits SETDB1 complex to H3K9me3 genomic sites mediating heterochromatin silencing¹⁰⁷. TRIM28 is phosphorylated at S824 by ATM/ATR to form foci within DNA double strand break sites¹²², and phosphorylated at S473 by CHK1/2 during DNA repair¹²³ in the response of DNA damage. Phosphorylation of TRIM28 might affect the ability of TRIM28 to condense chromatin^{122,168}. Both Serine 473 and 824 residuals phosphorylation play an important role in DNA damage/repair pathway. TRIM28 was recently reported to promote proliferation and metastatic progression of breast cancer cells¹⁶⁹, and acting as an oncogenic protein. However, the exact role of TRIM28 in breast cancer progression remains largely unclear.

In the study described herein, we document that TRIM28 exhibits a novel-binding motif in breast cancer cells, which is distinct from its counterpart in embryonic stem cell; TRIM28 is overexpressed in HMEC model of breast cancer progression; interestingly, TRIM28 is heavily phosphorylated (pS824/pS473) in aggressive breast cancer cells. Additionally, ectopic TRIM28 expression in HMEC cells promotes cell proliferation in a phosphorylation dependent manner. Further analysis unveiled that phosphorylated TRIM28 (pS824/pS473) is required for breast cancer stem cell maintenance, suggesting a phosphorylation dependent precision mechanism governing breast cancer stem cell fate and proliferation.

Results

pS473/824 TRIM28 is overexpressed in breast cancer cells

To determine TRIM28 expression profiles in five different subtypes of breast cancer cells, we profiled proteins expression level of TRIM28 in HMEC system. Surprisingly, TRIM28 is overexpressed in HMEC systems (Figure 44A, B, and C). mRNA level of TRIM28 also slightly increased along with breast cancer progression (Figure 44D). Interestingly, the total TRIM28 protein expression level between 184A1 and AE003 HMEC remains the same (Figure 44A, B, and C), however, the dramatic difference of S473/824 TRIM28 expression between 184A1 and AE003 HMEC cells is observed, which indicates a potential oncogenic role of S473/824TRIM28 in breast cancer. Based on their expression

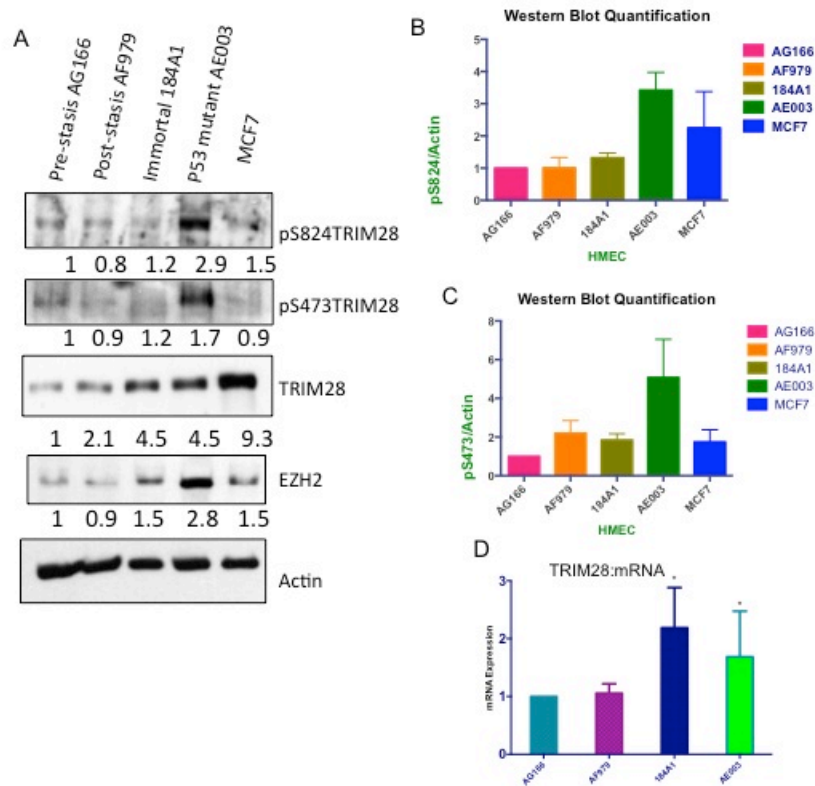


Figure 44 P-TRIM28 expression Level in HMEC system

A. TRIM28, p-TRIM28 protein expression profiles in HMEC system B. pS824TRIM28 protein expression quantification C. pS473 TRIM28 protein expression quantification D. TRIM28 mRNA expression in HMEC system

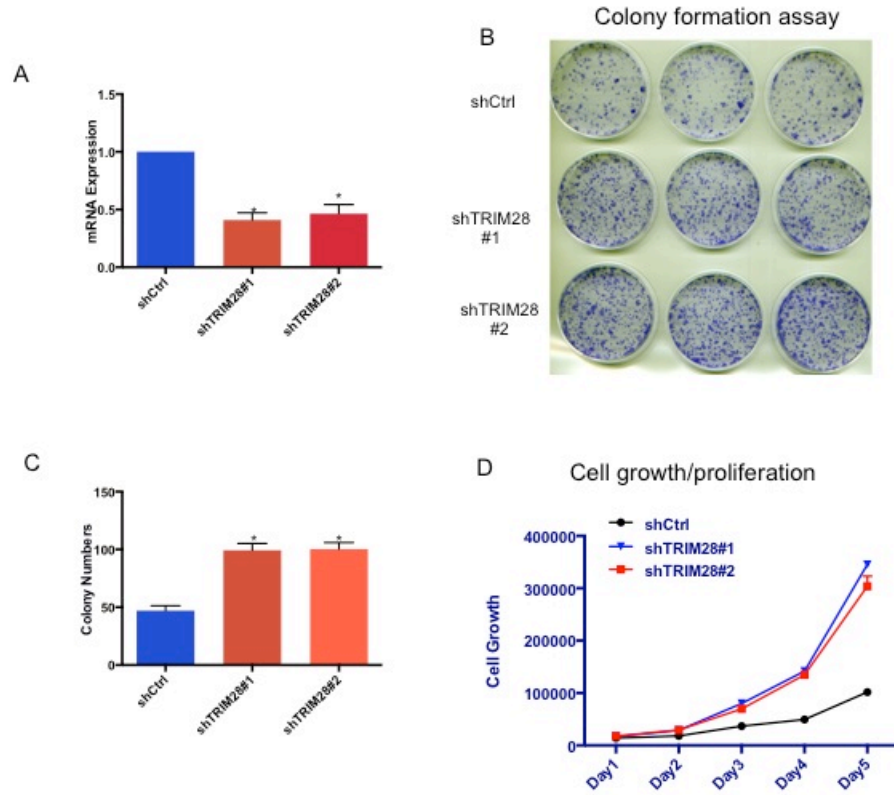


Figure 45 TRIM28 Inhibits HMEC cell proliferation and survival in shTRIM28 184A1 (pS824^{low}/pS473^{low}) HMEC cells A. mRNA expression level of TRIM28 in shControl, shTRIM28#1, #2 184A1 cells. B. Colony formation assay of shCtrl, shTRIM28 184A1 cells C. Quantification of colony formation assay of (B) D. 184A1 cell proliferation in shCtrl, shTRIM28 cells

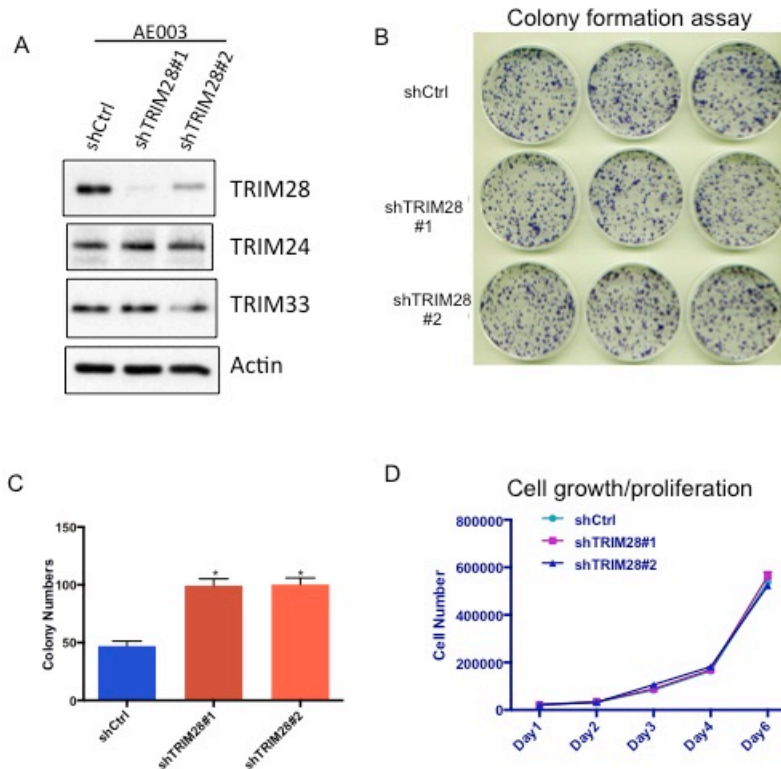


Figure 46 TRIM28 does not regulate shTRIM28 AE003 (pS824high/pS473high) HMEC cell Proliferation and Survival A. protein expression level of TRIM28 in shCtrl, shTRIM28 AE003 cells B. Colony formation assay of shCtrl, shTRIM28 AE003 cells C. Quantification of B. D. Cell proliferation of shCtrl, shTRIM28 AE003 cells

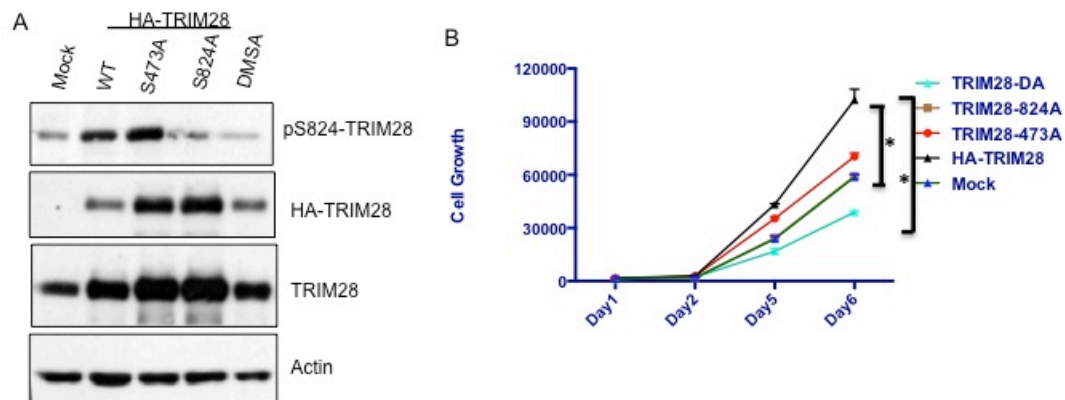


Figure 47 Depletion of TRIM28 Impacts Cell Proliferation in a Phosphorylation Dependent Manner A. western blot of TRIM28, p-TRIM28 showing the transfection efficiency of TRIM28 wt, mutated plasmids B. cell proliferation after TRIM28 wt, mutated plasmids transfection

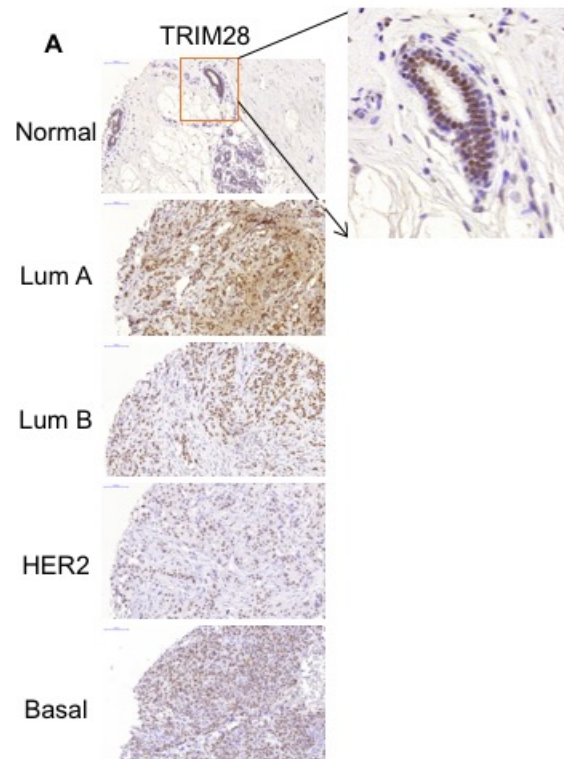


Figure 48 TRIM28 Express in Epithelial, But not Myoepithelial Cells A. IHC staining of TRIM28 in 5 subtypes of breast cancer tissue array

profile, we designate 184A1 HMEC cells as pS824^{low}/pS473^{low}184A1-HMEC cells; AE003 HMEC cells as pS824^{high}/pS473^{high}AE003-HMEC cells.

TRIM28 negatively regulates breast cancer growth in a phosphorylation dependent manner

These data raises a question whether phosphorylation of TRIM28 might play a pivotal role in breast cancer progression. To investigate the role of phosphorylated form of TRIM28, first, we knocked down TRIM28 in pS824^{low}/pS473^{low}184A1-HMEC cells to test their proliferation and colony formation. As expected, TRIM28 negatively regulate pS824^{low}/pS473^{low}184A1-HMEC cells proliferation by counting cell numbers (Figure 45D). TRIM28 knockdown efficiency was shown (Figure 45A). Colony formation assay also indicates that TRIM28 significantly inhibits HMEC cell colony formation (Figure 45B, C). The data are in agreement with a previous research report¹⁷⁰. So, we decided to test this result in another HMEC cell line, AE003 that represents an advanced stage of breast cancer with higher level of pS824/pS473 TRIM28. TRIM28 knockdown efficiency was shown (Figure 46A). Surprisingly, there is no growth effect for shTRIM28 pS824^{high}/pS473^{high}AE003-HMEC cells (Figure 46B, C, Figure 46D). Thus, these data demonstrates that there is phosphorylation-dependent proliferation phenotype of TRIM28. This further suggests a phosphorylation dependent proliferation effect of TRIM28 in breast cancer cells.

To further validate the phosphorylation-dependent proliferation phenotype of TRIM28, TRIM28 wild type, S473A, S824A, and double serine alanine mutants were transfected into AE003 cells, a dramatic proliferation phenotype was observed (Figure 47B). The transfection efficiency was verified in figure 47B, S824A and double SA mutants exhibited lower phosphorylation levels of TRIM28 when they were detected with phosphorylation specific antibodies (Figure 47B). Thus, these data demonstrated that there is phosphorylation-dependent proliferation phenotype of TRIM28 in breast cancer.

TRIM28 is specifically expressed in the epithelial cells, but not in the myoepithelial cells

To determine the expression profile of TRIM28 in human breast cancer patients, IHC staining of human normal breast tissues indicates that TRIM28 is mainly expressed in the epithelial cells, but not in the myoepithelial cells (Fig 48A). For luminal A, luminal B, HER2 positive, and basal type of breast cancers, TRIM28 express on both of the epithelial and myoepithelial cells in human breast cancer patients. This suggests that TRIM28 not only regulates myoepithelial lineage in normal mammary tissue, but also promotes bi-lineage identify in breast cancer tissues. TRIM28 deregulation in breast cancer might play a critical role in epithelial and myoepithelial lineages transition.

TRIM28 and Phosphorylation-TRIM28 level increases in malignant types of breast cancer

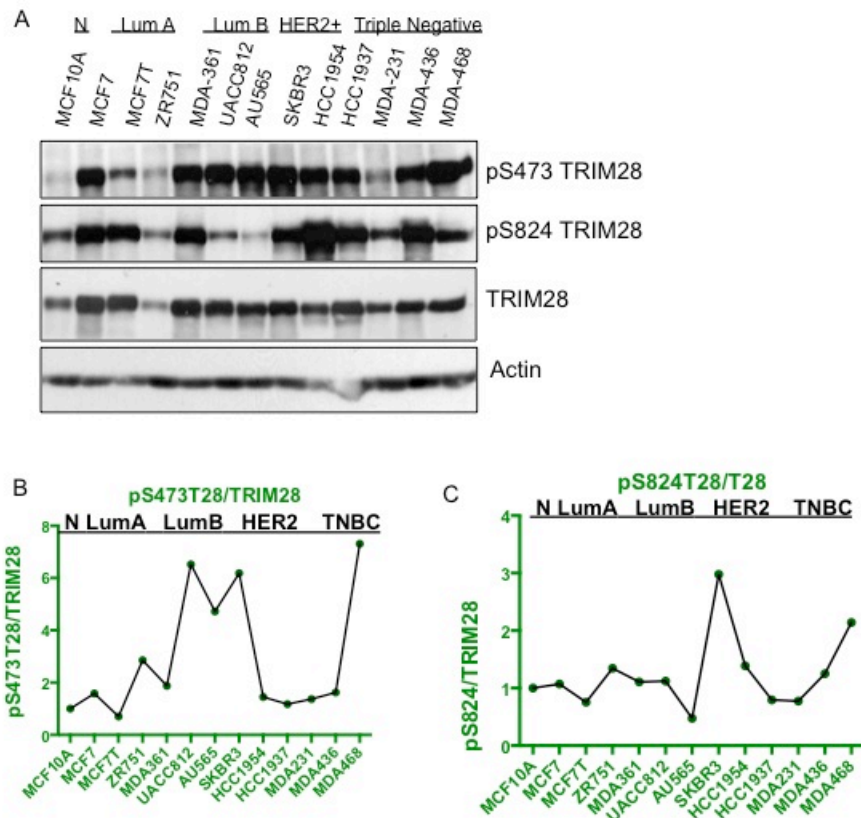


Figure 49 P-TRIM28 expression Level in Breast Cancer Cell

Lines A. TRIM28, p-TRIM28 protein expression profiles in breast cancer cell lines B. pS824TRIM28 protein expression quantification C. pS473 TRIM28 protein expression quantification

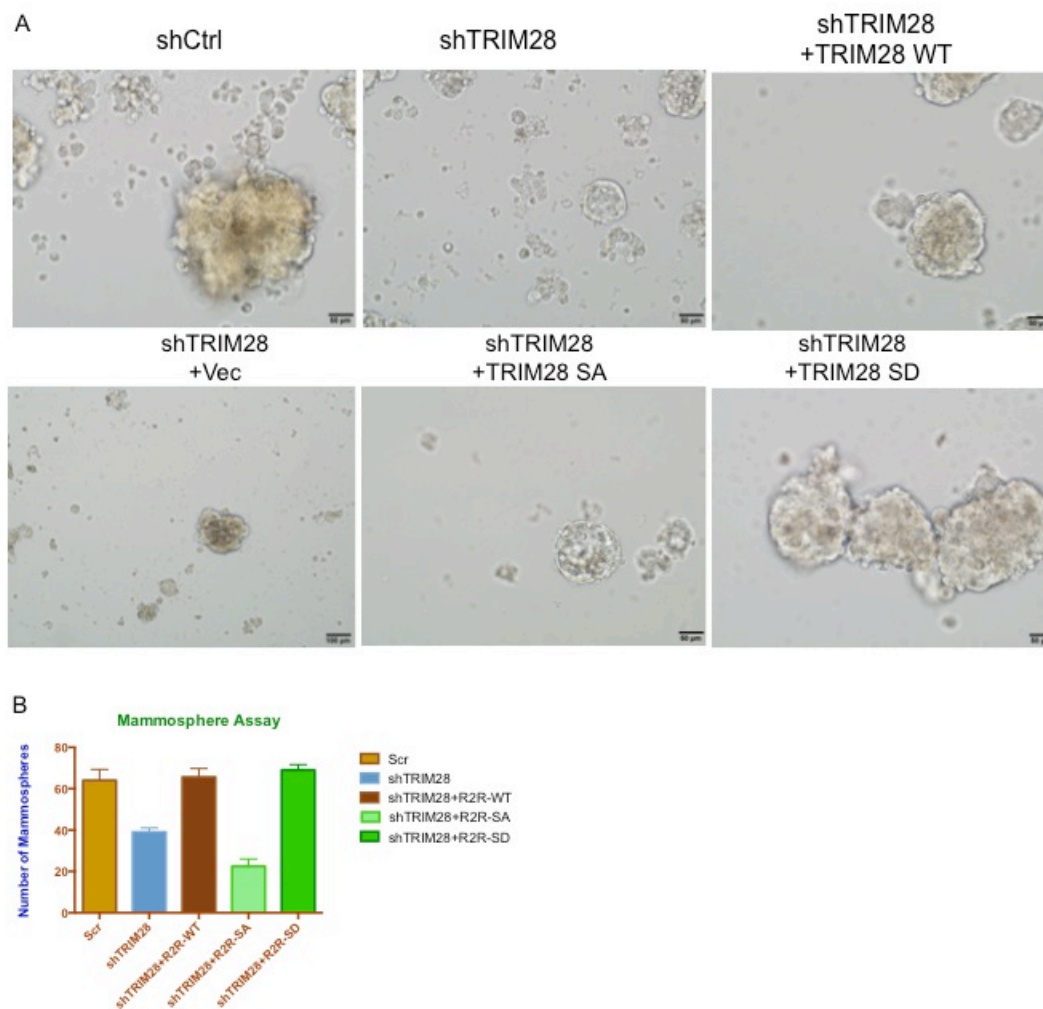


Figure 50 TRIM28 maintains breast cancer stem cell in a phosphorylation dependent manner A. mammosphere assay of shCtrl, shTRIM28, shTRIM28 plus TRIM28, shTRIM28 plus vector, shTRIM28 plus double SA mutated TRIM28, and shTRIM28 plus double SD mutated TRIM28 B. Quantification of A.

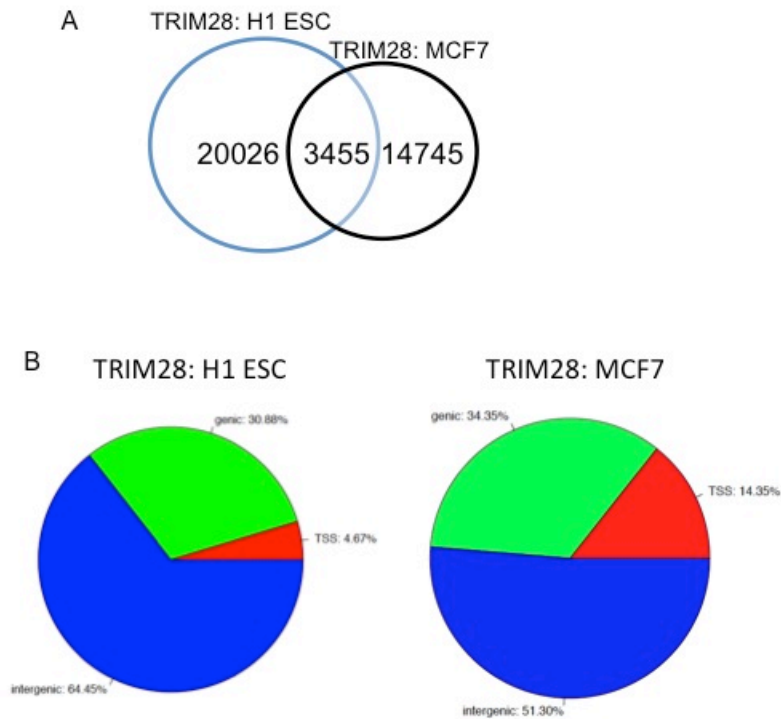


Figure 51 TRIM28 global binding profile comparison between hESC and MCF7 cells A. Van Diagram of Intersection of Number of peaks TRIM28 binding in H1 ESC and MCF7 cells. B. Distribution of TRIM28 peaks in MCF7 cells. Peaks are categorized as TSS (\pm 3kb), genic, or intergenic.

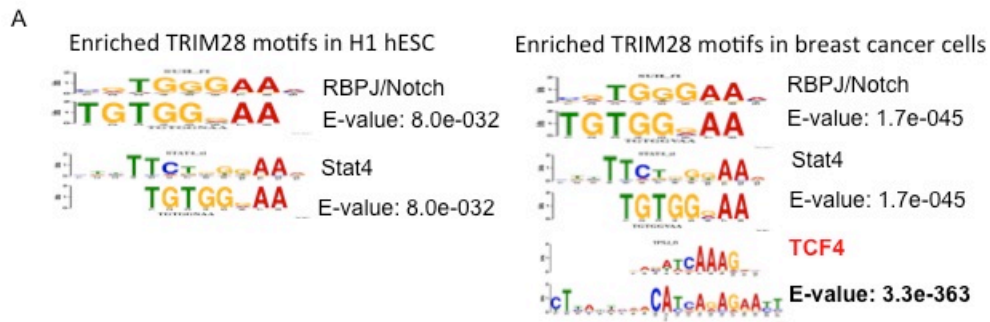


Figure 52 Genome-wide Mapping of TRIM28 Reveal a Novel Binding Motif, TCF4 motif A. TRIM28 binding motif in H1 hESC and MCF7 cells with MEME analysis

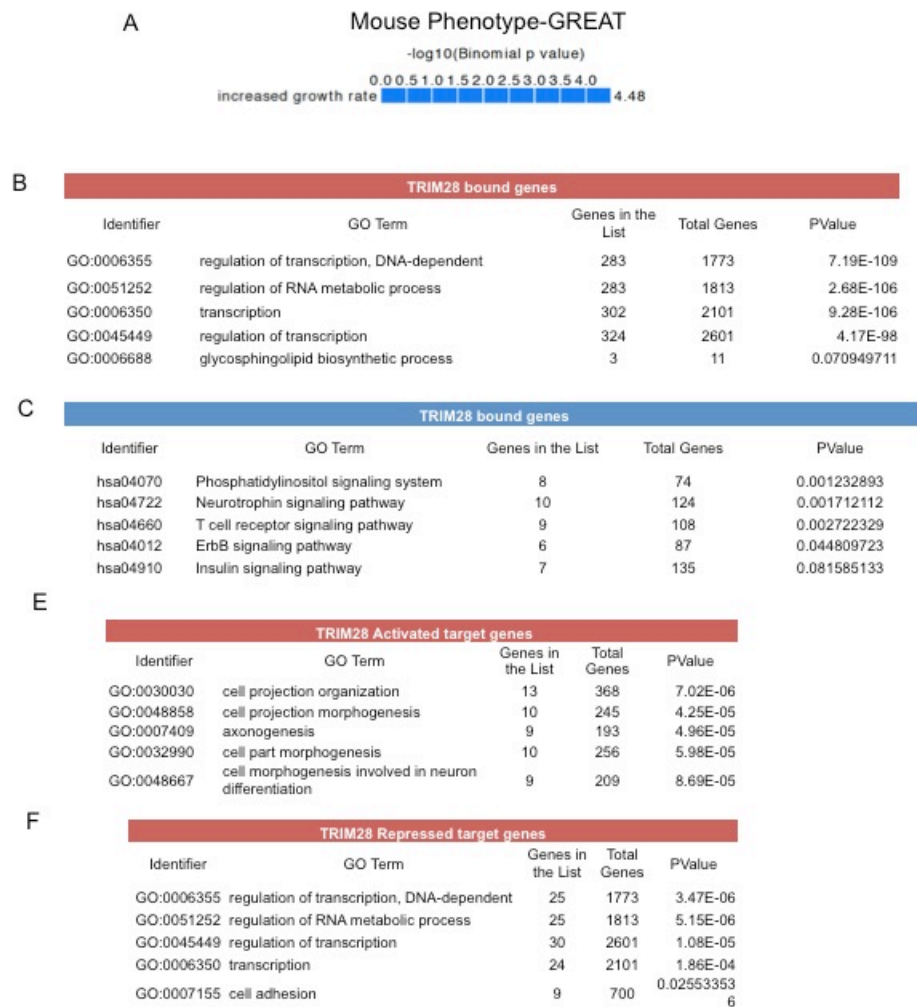


Figure 53 GO term analysis of TRIM28 bound and target genes A. GREAT mouse phenotype analysis of TRIM28 binding peaks in MCF7 cells B. GO terms analysis of TRIM28 bound genes C. KEGG pathway analysis of TRIM28 bound genes E. GO terms analysis of TRIM28 directly activated genes that is intersected between TRIM28 ChIP-seq and RNA-seq F. GO terms analysis of TRIM28 directly repressed genes

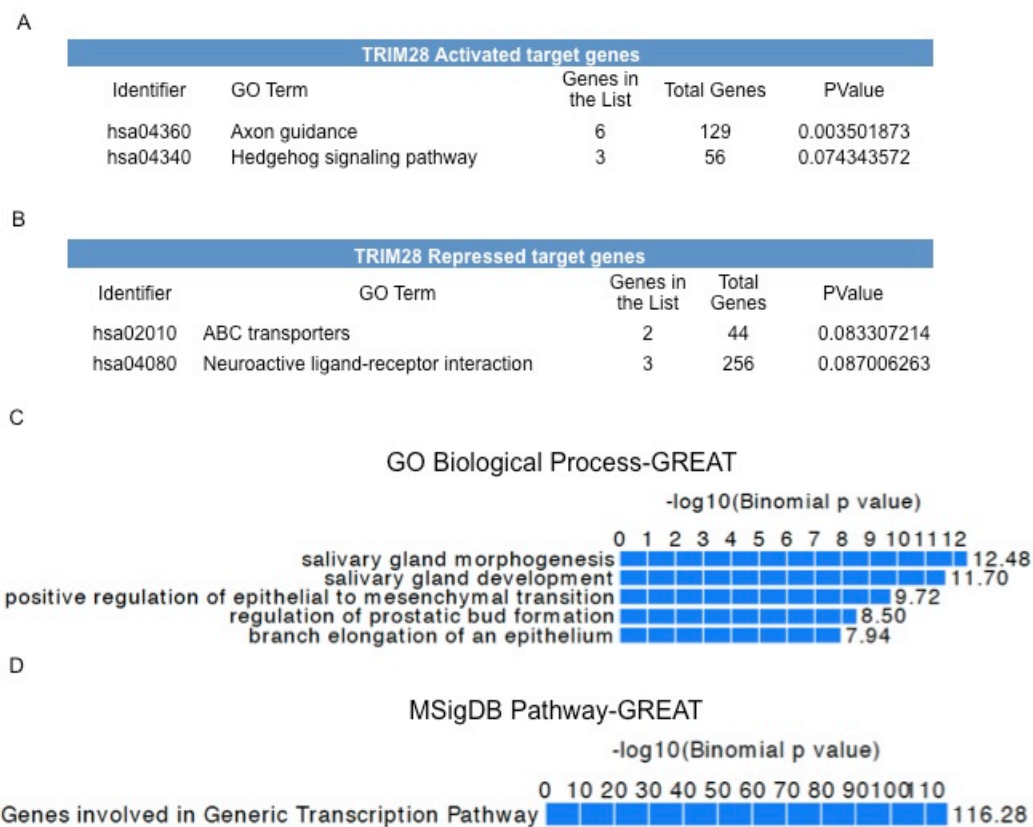


Figure 54 GO term analysis of TRIM28 target genes A. KEGG pathway analysis of TRIM28 activated target genes B. KEGG pathway analysis of TRIM28 repressed target genes that is intersected between TRIM28 ChIP-seq and RNA-seq C. GO term analysis of TRIM28 direct target genes with GREAT online tool D. MSigDB Pathway analysis of TRIM28 direct target genes

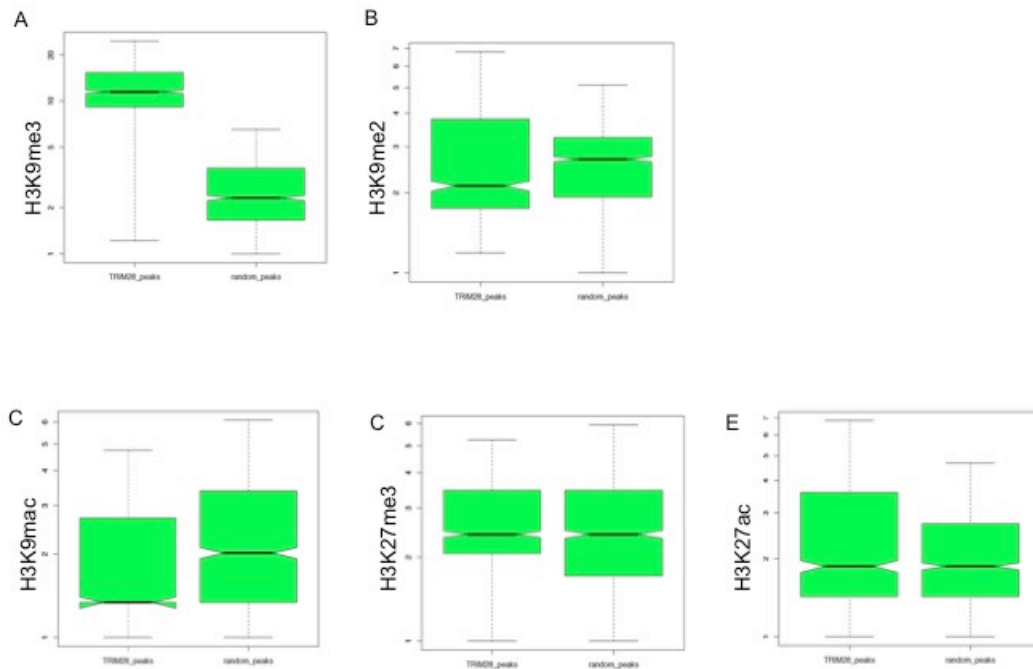


Figure 55 TRIM28 global binding profile Correlates with H3K9me3 in MCF7 cells A. Global TRIM28 binding profile correlates with H3K9me3 in MCF7 cells. B. Global TRIM28 binding profile does not correlates with H3K9me2 in MCF7 cells. C. Global TRIM28 binding profile does not correlates with H3K9ac in MCF7 cells. D. Global TRIM28 binding profile does not correlates with H3K27me3 in MCF7 cells. E. Global TRIM28 binding profile does not correlates with H3K27ac in MCF7 cells.

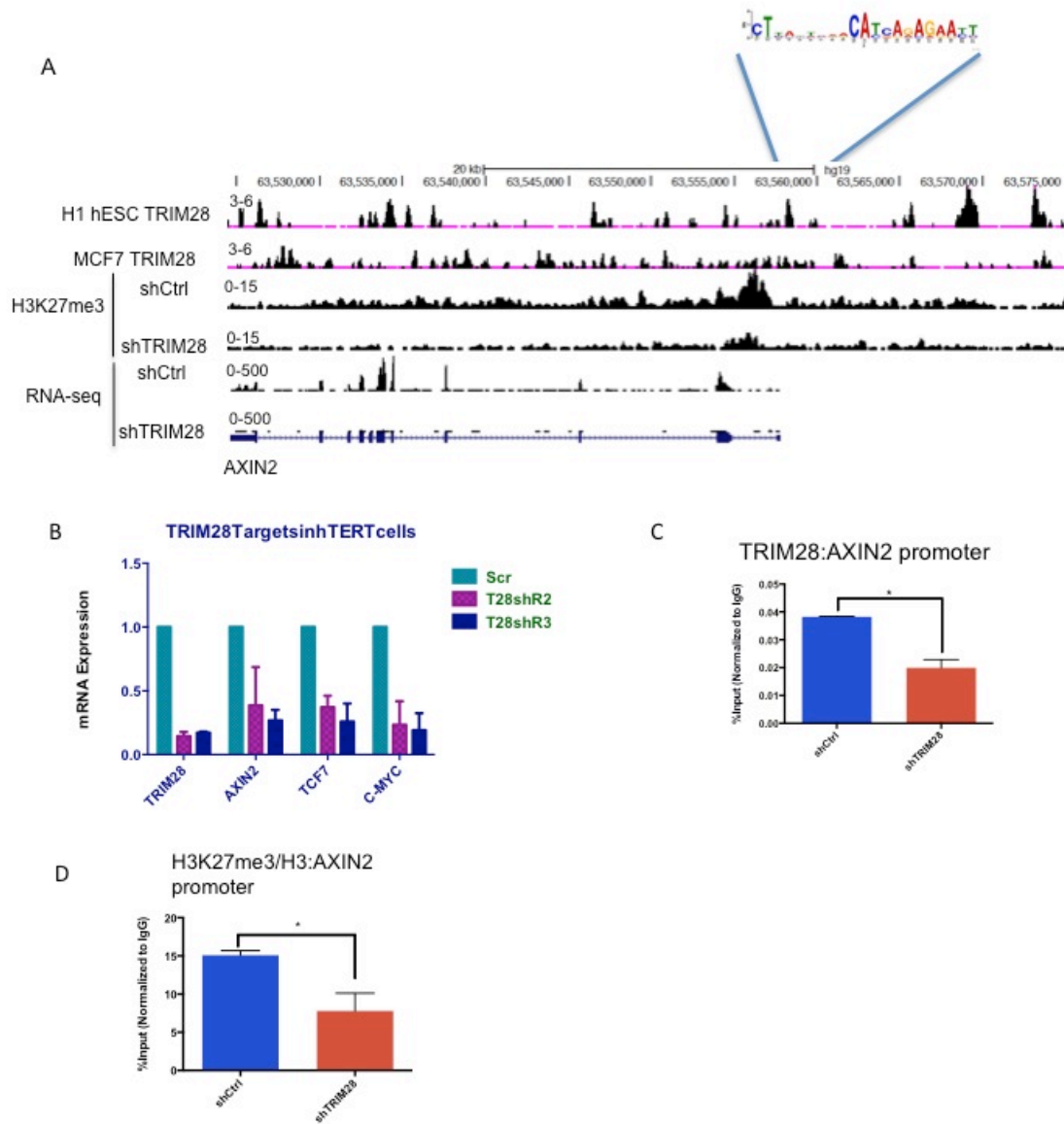


Figure 56 TRIM28 Directly Binds on the Promoter of *AXIN2* to Activate *AXIN2* Expression in Breast Cancer Cells A. Screenshot of *AXIN2* gene of TRIM28 binding in H1 ESC and MCF7 cells, H3K27me3 ChIP-seq, and RNA-seq in shCtrl and shTRIM28 MCF7 cells B. mRNA expression level of *AXIN2* in shCtrl, shTRIM28 MCF7 cells C. ChIP-PCR of TRIM28 on the promoter of *AXIN2* D. H3K27me3 ChIP-PCR on the promoter of *AXIN2* in shCtrl, shTRIM28 HMEC cells

To rule out the possibility that this is cell line dependent effect, profiling the expression of TRIM28 in human established breast cancer cell lines that represent five subtypes of breast cancers reveals that TRIM28 express higher in breast cancer compared with normal cell line. However, the overall survival of breast cancer patients with deregulated TRIM28 show no difference, which suggests that post-translational modification of TRIM28 might be the functional form to contribute to breast cancer progression, specifically S473/824 TRIM28 phosphorylation. Surprisingly, S473/824 TRIM28 is overexpressed in high malignant breast cancer cell lines, especially in basal/triple negative breast cancer cell lines (Figure 49A, B, and C).

Phosphorylation of TRIM28 is required for regulation of cancer stem cell fate

To understand whether the phosphorylation dependent of breast cancer cell proliferation TRIM28 is due to phosphorylation dependent regulation of breast cancer stem cell fate, we overexpressed SA and SD mutants of TRIM28 in shTRIM28 MCF7 cells and performed the mammosphere assay. As expected, SA mutants of TRIM28 could not rescue shTRIM28 phenotype (Figure 50A, and B), but SD mutants of TRIM28 perfectly rescued shTRIM28 mediated mammosphere size decrease (Figure 50A, and B). This data indicate that phosphorylation of TRIM28 is required for maintenance of breast cancer stem cell. Thus, we conclude that the phosphorylation dependent of breast cancer cell

proliferation TRIM28 is due to phosphorylation dependent regulation of breast cancer stem cell fate.

TRIM28 exhibits a Novel TCF4 Binding Motif in breast cancer MCF7 cells

In order to investigate the mechanism and functions of TRIM28-mediated proliferation regulation in human breast cancer cells, we performed TRIM28-specific chromatin immunoprecipitation with deep sequencing (ChIP-seq) in MCF7 cells, because MCF7 cells express very high level of TRIM28 (Figure 49A, B, and C). These analyses revealed 21274 TRIM28-bound peaks in MCF7 cells (Figure 51A). GO term analysis demonstrate that TRIM28 bound genes exhibits a prominent role of positive regulation of epithelial to mesenchymal transition (EMT) in breast cancer cells (Figure 53B), as well as transcription (Figure 53B), RNA metabolic process.

To further understand the global binding distribution of TRIM28, we did TRIM28 global binding profiles comparison between human embryonic stem cells ¹⁷¹ and breast cancer MCF7 cells. The global binding profiles comparison reveals that the TSS (+/-3KB) binding sites is robustly increased from 4.67 percent to 14.35 percent (Figure 51B), nearly three folds enrichment to the transcription start site, as TRIM28 binding profile from undifferentiated cells (hESC) to differentiated cells (MCF7). This suggests that a subset of genes is gaining TRIM28 binding on their promoter, which might correlate with TRIM28 mediated transcriptional repression/activation.

We next compared TRIM28 global binding motif between hESC and MCF7 cells. In details, TRIM28 ChIP-sequencing in H1 human embryonic stem cell (a public dataset ¹⁷¹) exhibits 20026 unique TRIM28 bound peaks with our re-analysis of the public dataset ¹⁷¹; whereas TRIM28 ChIP-sequencing in breast cancer MCF7 cells reveals 14745 unique TRIM28 bound peaks. The integration of TRIM28 ChIP-sequencing between embryonic stem cell and MCF7 cells yields only 3455 peaks shared between hESC and MCF7 cells. This suggests that TRIM28 global binding profile and potential transcriptional program is dramatically different between hESC and MCF7 cells.

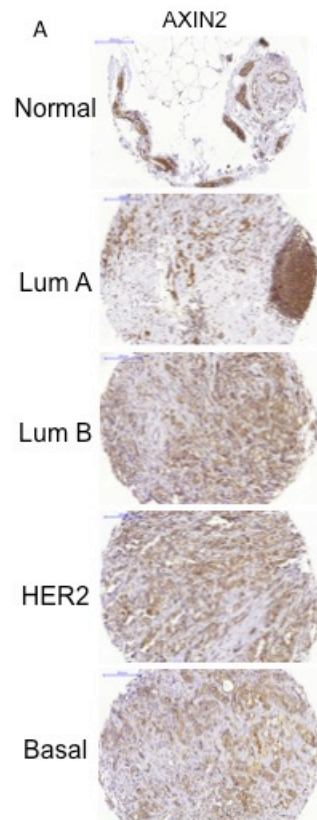


Figure 57 AXIN2 expression in breast cancer TMA A. IHC staining of AXIN2 in 5 subtypes of breast cancer tissue array

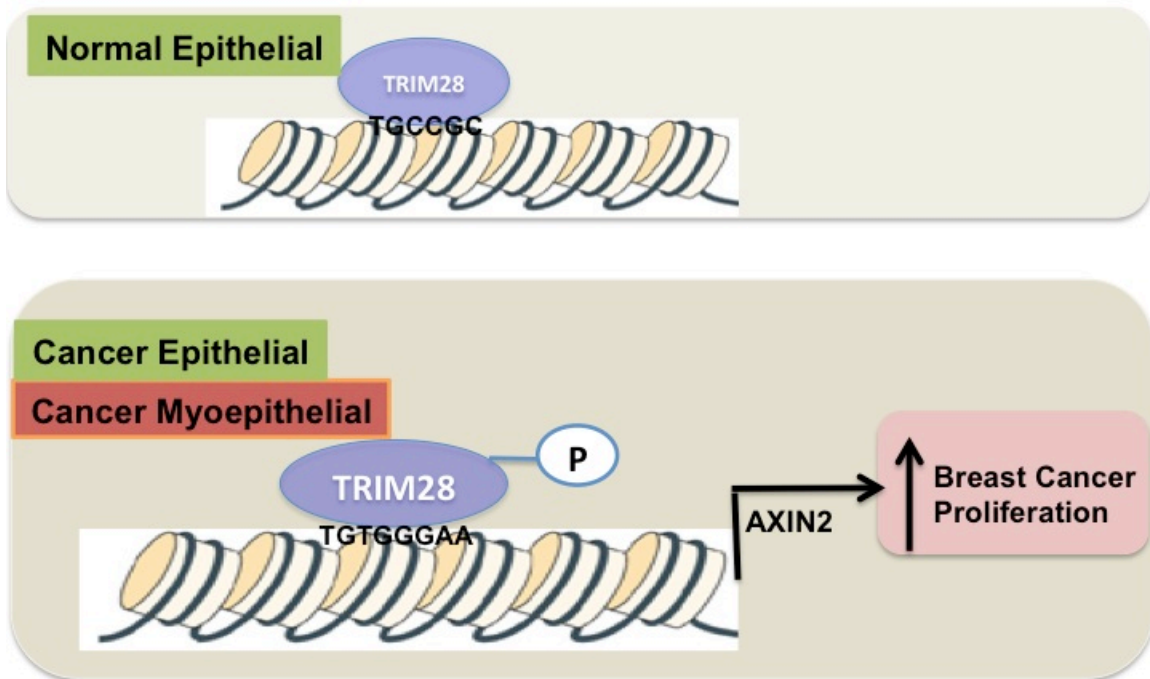


Figure 58 Epithelial Specific Expressed TRIM28 Regulates Breast Cancer Cell Proliferation in a Phosphorylation Dependent Manner

Enriched motif analysis revealed the different binding mechanisms of TRIM28 in hESC and MCF7 cells. The ChIP-seq we performed in MCF7 cells unveils a

TCF4 binding motif, RBP-J and Stat4 binding motif. Whereas, the ChIP-seq data re-analysis from H1 hESC cells reveals that the top TRIM28 binding motif is RBP-J and Stat4 binding motif (Figure 52A). No TCF4 binding motif is enriched in human ES cells indicated that TCF4 motif is breast cancer specific (Figure 52A), about 2844 out of 21274 TRIM28 bound peaks (>10%) enriched with TCF4 binding motif (Figure 52A). This demonstrated a potential unique interplay between TRIM28 and TCF4, a sequence specific transcription factor containing a basic helix-loop-helix (bHLH) ¹⁷², and potentially Wnt signaling in breast cancer cells. Thus, the different binding profile between hESC and MCF7 suggests that TRIM28 may be recruited to chromatin by different DNA-binding factors in breast cancer cells than human embryonic stem cells. In other words, TRIM28 controls a breast cancer specific subset of genes that are 2844 peaks TRIM28 bound, enriched with TCF4 motif.

TRIM28 direct target genes in breast cancer is associated with Growth, Morphogenesis

We examined TRIM28 directly targeted genes list that was generated by integrating TRIM28 ChIP-seq and RNA-seq in MCF7 cells. TRIM28 direct target genes were firstly analyzed in GREAT online annotation tool ¹⁷³, the mouse phenotype shows “increased growth rate” (Figure 53A). This suggests that the main function of TRIM28 in breast cancer cells might be involved in cell growth, proliferation. Further, functional annotation study revealed that the function of TRIM28 activated target genes is cell projection organization, morphogenesis,

and axonogenesis (Figure 53E). The function of TRIM28 repressed target genes is associated with transcription regulations (Figure 53F), related to ABC transporters (Figure 54B). Surprisingly, KEGG pathway analysis shows that TRIM28 activated target genes are associated with Hedgehog signaling pathway, which were linked with breast cancer stemness¹⁷⁴ (Figure 54A).

Genome-wide Binding Profile of TRIM28 Highly Correlates with H3K9me3

TRIM28 was previously known to correlate with histone marker H3K9me3. But there is no direct evidence whether TRIM28 global binding profile still correlates with H3K9me3 in breast cancer cells. The correlation analysis by comparing TRIM28 ChIP-seq of MCF7 cells with H3K9me3 ChIP-seq demonstrated that TRIM28 binding events highly correlate with H3K9me3 (Figure 55A), but not H3K9me2 (Figure 55B), H3K9ac (Figure 55C), H3K27me3 (Figure 55D), and H3K27ac (Figure 55E). In conclusion, this data indicates that TRIM28 binding events specifically correlate with H3K9me3 in breast cancer, not other kinds of histone modifications.

AXIN2 is a direct target gene of TRIM28 in breast cancer cells

Binding motif analysis of TRIM28 ChIP-seq unveils a unique TCF4 binding motif in breast cancer cells (Figure 52A), which suggests a potential direct transcriptional activation/repression role of TRIM28 on a subset of TRIM28 bound genes containing TCF4 binding motif. A gene that encompass TCF4 binding motif could be a direct target gene of TRIM28, may implicate in breast

cancer tumorigenesis. To identify transcriptional target genes of TRIM28 in breast cancer cells, we examined TRIM28 directly targeted genes list that was generated by integrating TRIM28 ChIP-seq and RNA-seq in MCF7 cells. AXIN2 was the top hit (Figure 56A), since 1) it is a Wnt signaling target gene; 2) contains a TCF4 binding motif we identified on the promoter region (\pm 3KB of TSS). This suggests that TRIM28 might transcriptionally regulate AXIN2 gene expression on a direct transcriptional level.

To further test whether AXIN2 is a transcriptional target gene of TRIM28, AXIN2 mRNA level was examined in the TRIM28 depleted cells; it suggests that AXIN2 gene expression was reduced upon TRIM28 depletion. (Figure 56B) TRIM28 ChIP was performed on the promoter of AXIN2. Data indicate that TRIM28 binds on the promoter of AXIN2 (Figure 56C). To understand the histone modification status of TRIM28 mediated AXIN2 gene expression, we examined the H3K27me3 ChIP-seq profiles in both shCtrl and shTRIM28 MCF7 cells (Figure 56A), we found a decreased level of H3K27me3 on the promoter region of AXIN2, ChIP-PCR was performed to verify the reduced H3K27me3 level on the promoter of AXIN2 upon TRIM28 depletion (Figure 56D). Thus, we conclude that AXIN2 is a direct target gene of TRIM28 in breast cancer cells.

Discussion:

In this paper, we presented that epithelial cell specifically expressed TRIM28 promotes breast cancer cell proliferation in a phosphorylation dependent manner. Using a unique HMEC system ¹⁷⁵, we demonstrated that TRIM28 and phosphorylation of TRIM28 is overexpressed in malignant types of breast cancer, TRIM28 depletion promotes pS824^{low} /pS473^{low} 184A1-HMEC cell proliferation, but no effect for pS824^{high} /pS473^{high} AE003-HMEC cells. Ectopic expression of TRIM28 phosphorylation defective SA mutants inhibited breast cancer cell proliferation. We identified a novel unique TRIM28 binding motif in breast cancer cells, showing high similarity with TCF4 binding motif. Then, we identified that AXIN2 contains TCF4 binding motif and serve as a directly transcriptional target gene of TRIM28 in breast cancer cells. It is very likely that the tumor regulating effect of TRIM28 is context dependent. Our unique HMEC system ¹⁷⁵ contributes to this interesting question related to different contexts.

The investigation of overcoming resistance of breast cancer treatment is in extremely high demand. Epigenetics has emerged as intriguing field to possess a lot of opportunities to overcome treating difficult breast cancer patients. Deeper understanding of cancer or breast cancer specific epigenetics is a key step to discover novel therapies. TRIM28 was implicated in embryonic stem cell ¹¹⁴ and cancers^{169,176}. Priscilla Turelli & Didier Trono reported ¹⁷¹ that more than 57,000 TRIM28 peaks were called in hESC, close to three-quarters of which were on EREs (endogenous retroelements), which is in line with our data of Figure 1B. Though we used a different peak calling approach, the TRIM28 global binding

profile is similar, since we called 23481 TRIM28 binding peaks in H1 hESC cells, 15133 peaks are in the intergenic region. They failed to present the rest of over one-quarter of TRIM28 bound peaks. So, at least one-quarter of TRIM28 bound peaks are regulating non-ERE gene expression in hES cells. Our re-analysis of the TRIM28 bound peaks in hESC indicates that TRIM28 might regulate NOTCH signaling pathway not only in hESC, but also in breast cancer cells. The shared regulatory characteristic of NOTCH signaling in both model cell system suggest the potential critical role of TRIM28 in connecting NOTCH signaling with breast cancer stem cells.

Our reanalysis of published TRIM28 ChIP-seq data demonstrated that TRIM28 binds with RBP-J motif in human ES cells. In contrast, TRIM28 exhibits a novel unique TCF4 binding motif in breast cancer cells on top of RBP-J motif, which suggests a more profound role of TRIM28 in breast cancer, which might involve in Wnt signaling, and NOTCH signaling. The role of TRIM28 in breast cancer remains largely unclear. Previous publications showed the controversial role of TRIM28 in cancer progression in a context dependent manner. TRIM28 acts as an oncogenic protein to promote breast cancer progression¹⁶⁹. TRIM28 global binding profiling ChIP-sequencing revealed TRIM28 has 2844 peaks out of total 21274 TRIM28 binding peaks sharing similarity with TCF4 binding motif, implying its role in mediating Wnt signaling in breast cancer.

Similarly, there are 2698 TRIM28 bound peaks that share binding motif with RBP-J binding motif, which suggests that TRIM28 might also have a role in NOTCH signaling pathway in breast cancer cells, since RBP-J is a major Notch effector protein ¹⁷⁷. Notch signaling pathway is prominent in the field of cancer biology and cancer stem cell, emerging as an attractive target of cancer therapy ¹⁷⁸. This implies that TRIM28 might deeply involve in NOTCH signaling in breast cancer, maybe in other types of cancer as well. The novel TRIM28 binding motif exhibits the tremendous interests to the breast cancer field, because of its high similarity with RBP-J motif, a relatively well studies transcription factor. RBP-J remains as a transcriptional repressor by recruiting chromatin remodelers in the absence of NOTCH signaling, it transform into a transcriptional activator through forming a complex with NOTCH1 in the presence of NOTCH signaling.

It was reported that Wnt/ β -catenin is a central downstream effector of Notch signaling in cardiac progenitor differentiation ¹⁷⁹, in this chapter, we reported that TRIM28 transcriptionally activates *AXIN2* gene expression, which is a target gene of WNT signaling pathway. So, it is possible that TRIM28 activates *AXIN2* through binding TCF4 binding motif, since there are multiple TCF4 binding motif around the promoter region of *AXIN2*. This question might need to be further validated in the future.

The deeper understanding of TRIM28 regulation by further study is of interest for cancer patients. This research will set a fundamental stage for instructing

TRIM28 inhibitor development against chemotherapy resistant breast cancer.

TRIM28 inhibitor could be a promising anti-breast cancer drug, which may still need extensive research and develop in the academia and pharmaceutical industry.

Therapeutically, one of the most obvious questions is which domain of TRIM28 needs to be targeted, considering design a TRIM28 inhibitor. The inhibition of Bromo domain of TRIM28 is particularly interesting because bromo domain inhibitors are in the hot trend of the whole pharmaceutical industry. However, whether it is a real case for TRIM28 remains unknown. The RBCC motif has a lot of impact for cells in various aspects, first, it could act as an E3 ubiquitin ligase collaborate with MAGE A3/A6 to degrade p53^{132,180}, and collaborate with MAGE A3/A6 to degrade AMPK in a cancer specific manner since MAGE proteins are X chromosome specific, be turned on in cancer cells¹⁸¹, second, it could serve as a sumo E3 ligase to add sumo moiety onto IRF7¹⁸², NPM1¹⁸³, and Vps34¹⁸⁴. Clear understanding of this mechanism is prerequisite to develop a rational TRIM28 inhibitor. The breast cancer, mammary gland specific feature of TRIM28 also needs to be understood in order to design a TRIM28 inhibitor for breast cancer.

It is extremely intriguing that TRIM28 express in the epithelial cells, but not in the myoepithelial cells indicated by IHC staining of human normal breast, but express at both myoepithelial and epithelial cells in luminal A, luminal B, HER2,

Basal type breast cancers; since myoepithelial cells is reported to originate from epithelial cells^{185,186}, the unique expression pattern of TRIM28 suggests that TRIM28 might play an important role in epithelial cells' differentiation into myoepithelial cells. In addition, basal lineage breast cancer is derived from myoepithelial cells^{14,187}; this raises a possibility that TRIM28 might involve in the programming of luminal type breast cancer into basal type breast cancer. This suggests that TRIM28 might only regulate myoepithelial lineage in normal mammary tissue, but promotes bi-lineage identify in breast cancer tissues.

Phosphorylated form of TRIM28 (pS824) is involved in mouse embryonic stem cell self-renewal¹¹⁵. The mechanism is that pS824 TRIM28 specifically activates pluripotent genes. However, I could not repeat this data in breast cancer cells, which suggests that the significant differences between embryonic stem cells and breast cancer cells. This is in line with our genome wide TRIM28 binding profiles comparison data in Figure 51. In the future study, it would be very interesting whether and how TRIM28 coordinates Wnt and Notch signaling as a chromatin platform, because TRIM28 binds with both TCF4 motif and RBP-J motif.

CHAPTER V

DISCUSSION AND FUTURE DIRECTIONS

In this dissertation, we discovered that EZH2 interact with TRIM28 in a RBCC domain dependent manner in breast cancer cells. Further study indicates that TRIM28 mediates EZH2 recruitment on the promoter of CXCR4, one of the breast cancer stem cell marker genes. This interaction of TRIM28 with EZH2 may contribute to breast cancer stem cell phenotype through collaboratively transcriptional activating CXCR4 cancer stem cell signaling pathway. Targeting the highly expressed EZH2 in breast cancer might provide a useful strategy to overcome chemotherapy resistance of breast cancer. Collectively, our data demonstrated that targeting EZH2/TRIM28 interaction is a better approach to dampen breast cancer progression (Figure 59).

The potential link of cancer epigenetics with cancer immunotherapy in the perspective of EZH2 study

EZH2 is known to promote cancer progression, invasion, and cancer stem cell maintenance, thus serve as a promising therapeutic target for various types of cancer. However, EZH2 is also essential for immune system homeostasis. So, the administration of EZH2 into cancer patients has the potential to further inhibit the weakened cancer immune system of patients¹⁸⁸, then harm the patients' overall survival and quality of life. Thus deeper understanding of EZH2 inhibition considering not only epigenetics but tumor surrounding immune system is required for the future.

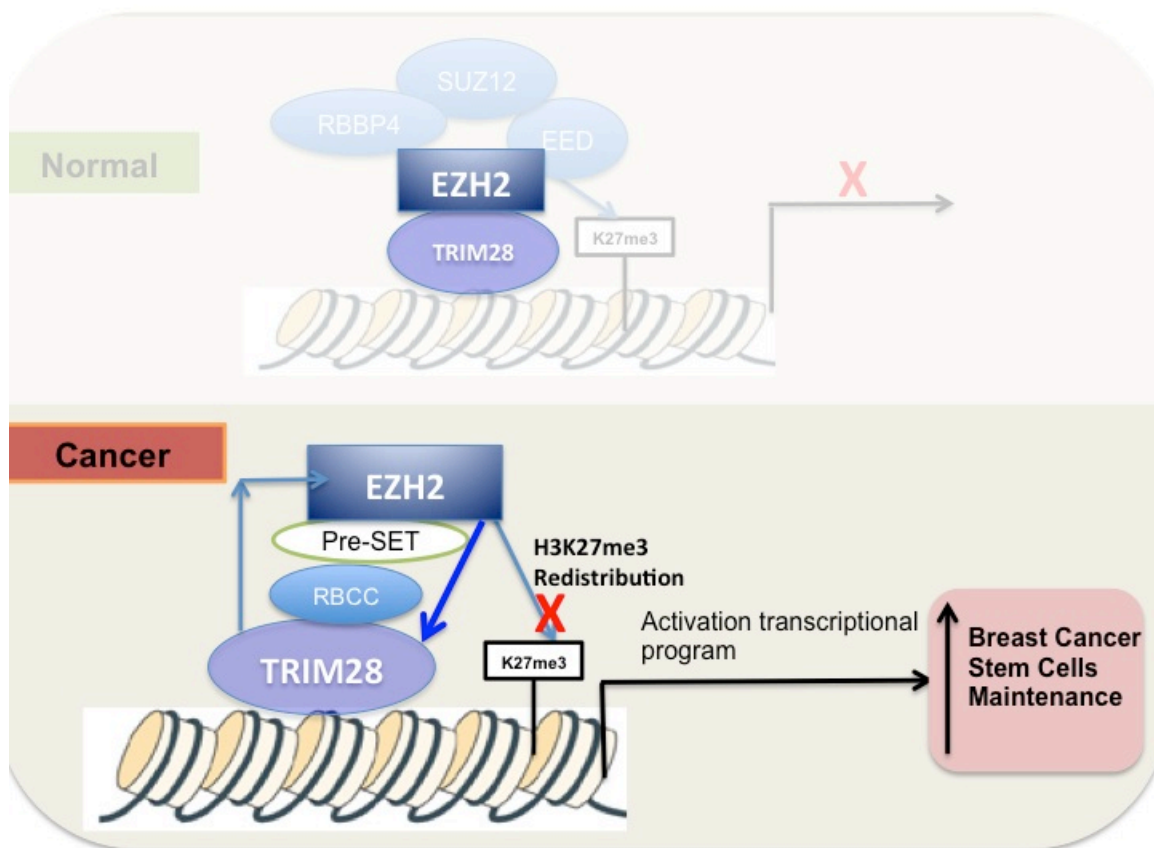


Figure 59 TRIM28 Interacts with EZH2 and Regulates its Activity

The transcriptional co-activator role of EZH2 in breast cancer

Previously, EZH2 was believed to contribute to tumor progression via its H3K27me3 dependent transcriptional repressive program for a fairly long time¹⁶³. But, most recently, the oncogenic function of EZH2 in castrate resistant prostate cancer is independent of H3K27me3¹⁶⁵. We observed the similar results of inconsistent correlation of EZH2 and H3K27me3 levels in breast cancer in human breast cancer tissue array. This suggests the potential existence of non-histone substrates of EZH2 as a lysine methyltransferase in breast cancer. This is the further validation of some of the previous publications revealing the transactivation or polycomb-independent role of EZH2 in breast cancer^{66,80}, and prostate cancer⁵⁶.

The therapeutic potential of targeting pre-SET region of EZH2 in breast cancer

The newly identified interaction of TRIM28 and EZH2 might contribute to the polycomb-independent role of EZH2 in breast cancer. The specific region of EZH2 interacting with TRIM28 is the pre-SET domain region (containing CXC domain); the pre-SET domain region of EZH2 was reported to direct SET domain activity in drosophila¹⁵⁶. And the name of “CXC domain” was originally coined to refer to the pre-SET motif of EZH2¹⁵⁹ in drosophila. A previous structural study indicates that pre-SET region of EZH2 is critical to associate with SUZ12, and AEBP2¹⁵⁷. The TRIM28 interaction with pre-SET domain (CXC) region of EZH2 in mammalian cells indicates that TRIM28 might also direct SET domain activity, global H3K27me3 binding profiling indicates that TRIM28 regulates H3K27me3

localization in breast cancer cells, might potentiate chromatin status to promote breast cancer progression.

Further, TRIM28 might potentially switch EZH2's affinity to histone substrate or non-histone substrates. Thus, targeting EZH2/TRIM28 interaction could be a better approach to dampen breast cancer progression than targeting SET domain along. This new knowledge collaborates with our study here may inspire a new strategy of EZH2 inhibitor design that should more efficiently target the activation function domain of EZH2. More precisely, there is potential involvement of sumoylation pathway in TRIM28/EZH2 interaction in breast cancer cells. Previously, sumoylation pathway was reported to be required in breast cancer^{182,189}.

A very recent publication⁸⁰ of investigating mechanism of EZH2 expanding breast cancer stem cell discovered NOTCH signaling as EZH2's H3K27me3 independent targets. Here we report to identify CXCR4/CXCL12 pathway as EZH2's H3K27me3 independent targets. The possible reason of this discrepancy is probably due to the technical limitation of specific stem cell signaling focused PCR array they employed.

The positive role of TRIM28 in breast cancer stem cell maintenance

This dissertation reports the function of TRIM28 in breast cancer stem cell biology. On the other hand, there are at least two publications highlighted that

TRIM28 implicated in embryonic stem cell biology^{114,115}. In general, cancer stem cell tends to phenocopy embryonic stem cell, if we link SOX2 from stem cell to cancer stem cell¹⁶⁰. Additionally, our data indicate that mutant EZH2 of deleting the interaction domain with TRIM28 show very limited capacity to form mammosphere. This data show that EZH2/TRIM28 interaction might be critical for breast cancer stem cell maintenance. Thus, targeting EZH2/TRIM28 interaction is a very useful approach to dampen breast cancer progression. Breast cancer stem cell represents the novel direction of drug resistant breast cancer therapy development. The newly identified breast cancer stem cell regulatory molecule could be a potential therapeutic target. Thus, this study brings unparalleled value to the community.

On the other hand, TRIM28 was published to promote breast cancer proliferation and metastatic progression¹⁴⁴, we reported here that TRIM28 regulated breast cancer proliferation is actually phosphorylation dependent. But whether the metastasis regulation of TRIM28 is also dependent on its phosphorylation status remains to be investigated.

There is huge potential of TRIM28 in breast cancer research since TRIM28 express in the epithelial cells, but not in the myoepithelial cells indicated by IHC staining of human normal breast. However TRIM28 express at both myoepithelial and epithelial cells in luminal A, luminal B, HER2, Basal type breast cancers. So, for myoepithelial cells, TRIM28 protein expression is gradually increased from

normal cells to malignant breast cancer cells, which is the classical characteristic of oncogenic protein. Most of malignant types of breast cancer, especially basal type breast cancer are mixed lineages with cells originated from both epithelial and myoepithelial cells¹⁴. So, it is reasonable to speculate that inhibition of TRIM28 in mixed lineage type of breast cancer might be able to reverse the myoepithelial originated cancer cells back to normal.

From the perspective of development biology, myoepithelial cells are reported to originate from epithelial cells^{185,186}, the unique expression pattern of TRIM28 suggests that TRIM28 might play a critical role in epithelial cells' differentiation into myoepithelial cells. In addition, basal lineage breast cancer is derived from myoepithelial cells^{14,187}; this raises a possibility that TRIM28 might involve in the programming of luminal type breast cancer into basal type breast cancer.

The existence of non-histone substrates of EZH2 in breast cancer is high likely. There are potentially more unknown substrates in breast cancer to contribute to tumorigenesis; our proteomics study in breast cancer cells provided a few candidate substrates, the SWI/SNF complex members are high likely to be one or more of the non-histone substrates of EZH2.

The potential application of TRIM28/EZH2 axis in X chromosome inactivation

Since EZH2 is involved in X chromosome inactivation¹⁹⁰, most of PRC2 associated proteins and long noncoding RNAs play a critical role in XCI. So, it is

possible that TRIM28, as a specific associated protein of EZH2, might also contribute to X chromosome inactivation. EZH2 is known to directly regulate DNA methylation in whole genome wide¹⁹¹; there is a report that TRIM28 KO displayed reprogramming defect of embryo through single cell DNA methylation analysis¹⁹². It is possible that TRIM28/EZH2 coordinately involve in early epigenetic reprogramming during embryo development, but the details need to be further investigated.

The investigation of overcoming resistance of breast cancer treatment is in extremely high demand. Previous publications showed that EZH2 is over-expressed in various cancers such as melanoma, lymphoma, and prostate and breast cancer. EZH2 is a marker of aggressive breast cancer. Overexpression of EZH2 also promotes neoplastic transformation of normal prostatic cells and hyperplasia in breast epithelium. As an oncogene, EZH2 inhibitor as a promising anti-cancer drug is being extensively researched and developed in the pharmaceutical industry. However, this depth of knowledge is essential for rational development of EZH2-targeted therapeutics of various subtypes of breast cancers. The deeper understanding of EZH2 regulation by further study is of interest for cancer patients. This research will set a fundamental stage for instructing EZH2 inhibitor development against resistant breast cancer.

It is extremely intriguing that TRIM28 express in the epithelial cells, but not in the myoepithelial cells indicated by IHC staining of human normal breast, but

express at both myoepithelial and epithelial cells in luminal A, luminal B, HER2, Basal type breast cancers; since myoepithelial cells is reported to originate from epithelial cells^{185,186}, the unique expression pattern of TRIM28 suggests that TRIM28 might play an important role in epithelial cells' differentiation into myoepithelial cells. In addition, basal lineage breast cancer is derived from myoepithelial cells^{14,187}; this raises a possibility that TRIM28 might involve in the programming of luminal type breast cancer into basal type breast cancer.

This suggests that TRIM28 might only regulate myoepithelial lineage in normal mammary tissue, but promotes bi-lineage identity in breast cancer tissues. However, EZH2 was reported to promote a bi-lineage identity in basal-like breast cancer cells¹⁹³.

The potential role of EZH2 as a lineage factor, not the real cancer stem cell self-renewal maintenance factor?

Unphosphorylated form of TRIM28 interact with EZH2 reveals the EZH2 dependent TRIM28 regulatory role in cancer stem cell maintenance. However, the phosphorylated form of TRIM28 loses interaction with EZH2, and still promotes mammosphere formation, in comparison with the phosphorylation defected form of TRIM28. This leads us to hypothesize phosph-TRIM28 govern cancer stem cell maintenance through an EZH2 independent pathway. If this is true, p-TRIM28 and EZH2 are regulating two parallel, two different pathways, these two different signals could not both involved in cancer stem cell

maintenance. If phosphorylation-TRIM28 is regulating cancer stem cell maintenance, EZH2 should be less pluripotent, and then might be a lineage factor.

The potential link between H3K27me3 and H3K9me3

We showed that TRIM28 interacts with EZH2 to regulate H3K27me3 distribution here, which may correlate with bivalent chromatin signature, a feature of embryonic stem cell⁹⁴. This leads us to examine the role of TRIM28 in cancer stem cell maintenance. The global histone modification profiling analysis indicates that TRIM28 global binding profile is highly correlated with H3K9me3 binding profile. Thus, TRIM28 links H3K9me3 with H3K27me3 histone modifications in breast cancer cells. To my knowledge, this is the first direct experimental evidence to show the crosstalk between H3K27me3 and H3K9me3. It was reported that there are a couple of indirect observations that link H3K9me3 with H3K27me3 before in drosophila¹⁹⁴. SUZ112 links HP1 α with EZH2 in fly¹⁹⁵, which imply the crosstalk between H3K9me3 and H3K27me3. More recently, EZH2 and SUZ12 were showed to be required for HP1 α stability in mouse tissue¹⁹⁶. In vitro peptides binding assay demonstrates that HP1 $\alpha/\beta/\gamma$ binding affinity with H3K9me3 was greatly increased with the presence of H3K27me3 peptide. So, it is likely that a cooperative mechanism of H3K9 and H3K27 residues may exist, connected by HP1 α anchorage at chromatin¹⁹⁶.

Future Directions:

The following questions might be able to guild us to think deeper.

1. Does EZH2/TRIM28 mediated transcriptional activation involves p300 recruitment? Because p300 appear in the dataset of the latest EZH2 purification.

2. Which Zinc Finger protein recruited EZH2/TRIM28?

Would it be RBP-J, Notch pathway, or TCF4, Wnt pathway? Since Motif analysis of TRIM28 ChIP-seq identified these two binding motifs.

3. Are cluster 1 genes (including some of the lincRNAs) functionally similar to ESR1, PgR? Mediating luminal epithelial differentiation?

4. Does H3K9me3 connect with H3K27me3 in breast cancer? And how?

5. EZH2 interacts with SWI/SNF, NURD complex directly or indirectly?

6. If the interaction is indirect, does TRIM28 act as a scaffold protein linking EZH2 and SWI/SNF complex?

7. Does EZH2 methylate itself? Does EZH2 methylate PBRM1? BRG1? Dicer1?

8. How TRIM28 destabilize the interaction of EZH2 and BRG1?

9. Does TRIM28 sumoylate EZH2?

10. Whether EZH2 only interact with sumoylated TRIM28? Or both unsumoylated and sumoylated form of TRIM28?

11. RBCC motif binds KRAB Zinc Finger proteins, and EZH2, does ZFP compete with EZH2 to bind with TRIM28?

12. Is there more Cancer Stem Cell marker genes in the gene list jointly down-regulated by EZH2/TRIM28? Does EZH2/TRIM28 co-mark a gene set which is very critical for cancer stem cell?

Chapter Six: Conclusion

Targeting the highly expressed EZH2 in breast cancer might provide a useful strategy to overcome resistance of breast cancer. However, this depth of knowledge is essential for rational development of EZH2-targeted therapeutics of various subtypes of breast cancers. The deeper understanding of EZH2 regulation by further study is of interest for cancer patients. EZH2 was believed to contribute to tumor progression via its H3K27me3 dependent transcriptional repressive program for a long time. But there are argument claiming that the oncogenic function of EZH2 is independent of H3K27me3. We observed the similar results of inconsistent correlation of EZH2 and H3K27me3 levels in breast cancer in both cell lines and human breast cancer tissue array. This indicates the possible existence of non-histone substrates of EZH2 as a methyltransferase in breast cancer. This is the further validation of some of the previous publications revealing the transactivation or polycomb-independent role of EZH2 in breast cancer, and prostate cancer.

In the first part of my dissertation, I demonstrated that the newly identified interaction of TRIM28 and EZH2 might contribute to the polycomb-independent role of EZH2 in breast cancer, and this interaction was also reported in prostate cancer. The specific region of EZH2 interacting with TRIM28 is the pre-SET domain region (containing CXC domain). The H3K27me3 redistribution was observed in TRIM28 depleted MCF7 cells. Three clusters of genes with H3K27me3 gains, loss, and flat upon TRIM28 depletion was described.

Functionally, through transcriptome study, TRIM28 mediated EZH2 function was pinpointed to maintenance of the breast cancer stem cells via transcriptionally activation of CXCR4. We identify CXCR4/CXCL12 pathway as EZH2's H3K27me3 independent targets in cancer cells. The pre-SET domain region of EZH2 was reported to direct SET domain activity in drosophila. The TRIM28 regulation pre-SET domain (CXC) region of EZH2 in mammalian system indicates that targeting EZH2/TRIM28 interaction could be a better approach to dampen breast cancer progression than targeting SET domain. This new knowledge collaborates with our study here may inspire a new strategy of EZH2 inhibitor design that should target the activation function domain of EZH2.

In the second part of my dissertation, I demonstrated that unphosphorylated form of TRIM28 interact with EZH2 which reveals the EZH2 dependent TRIM28 regulatory role in cancer stem cell maintenance. However, the phosphorylated form of TRIM28 loses interaction with EZH2, and still promotes mammosphere formation, in comparison with the phosphorylation defected form of TRIM28. Phosph-TRIM28 might govern breast cancer stem cell maintenance through an EZH2 independent pathway. Previously, TRIM28 was published to promote breast cancer proliferation and metastatic progression, we did invasion and metastasis assays in our lab, the TRIM28 mediated effect of metastatic progression is relatively marginal in MCF7 cells, compared with the regulation of CSC feature with TRIM28. And the proliferation regulation function part of it is still controversial, as Douglas Cress published that TRIM28 actually suppress MDA-

MB-231 cells proliferation. So the proliferation regulation of TRIM28 could be cell line dependent effect, or post-translational modification dependent effect of TRIM28. The post-translational modification of TRIM28 effect on breast cancer progression needs to be further characterized. And another question still remains whether TRIM28 mediated breast cancer promotion is dependent on the expansion of breast cancer stem cell or some other mechanisms?

TRIM28 genomically binds on the same motif of RBPJ, this might directly repress Wnt/AXIN2 pathway. It is extremely intriguing that TRIM28 express in the epithelial cells, but not in the myoepithelial cells indicated by IHC staining of human normal breast, but express at both myoepithelial and epithelial cells in luminal A, luminal B, HER2, Basal type breast cancers; since myoepithelial cells is reported to originate from epithelial cells, the unique expression pattern of TRIM28 suggests that TRIM28 might play an important role in epithelial cells' differentiation into myoepithelial cells. In addition, basal lineage breast cancer is derived from myoepithelial cells. This suggests that TRIM28 might only regulate myoepithelial lineage in normal mammary tissue, but promotes bi-lineage identity in breast cancer tissues. In contrast, EZH2 was reported to promote a bi-lineage identity in basal-like breast cancer cells.

Additionally, the marginal effect of GSK126 inhibition of mammosphere formation in MCF7 suggests the existence of non-histone substrates of EZH2 in luminal type breast cancer. Whereas Stuart Orkin's antiproliferative experiments of GSK-

126 on MDA-MB-231 cells appear no effect at all. The discrepancy here indicates the anti-breast cancer effect of GSK-126 is cell line dependent, as two very recent publications demonstrated. Another possibility is that there are potentially more unknown substrates in breast cancer to contribute to tumorigenesis; the SWI/SNF complex members are high likely to be one of the non-histone substrates.

Collectively, in my dissertation, I reported that EZH2 interacts with TRIM28 in a catalytic domain dependent manner in breast cancer cells. Further study indicates that TRIM28 reprogram H3K27me3 distribution in breast cancer cells. TRIM28 interacts with EZH2 to maintain breast cancer stem cell phenotype through collaboratively transcriptional activation of CXCR4 cancer stem cell signaling pathway. This is the very first data to report the function of TRIM28 in breast cancer stem cell biology. In the other hand, there are at least two publications highlighted that TRIM28 implicated in embryonic stem cell biology. In general, cancer stem cell tends to phenocopy embryonic stem cell, if we link SOX2 from stem cell to cancer stem cell. Additionally, our data indicate that mutant EZH2 of deleting the interaction domain with TRIM28 show very limited capacity to form mammosphere. The deeper understanding of EZH2 regulation by further study is of interest for cancer patients. This data show that EZH2/TRIM28 interaction is critical for breast cancer stem cell maintenance. Thus, targeting EZH2/TRIM28 interaction is a very useful approach to dampen breast cancer progression. Breast cancer stem cell represents the novel direction

of drug resistant breast cancer therapy development. The newly identified breast cancer stem cell regulatory axis could be a potential therapeutic target. Thus, this study brings unparalleled value to the community. This research will set a fundamental stage for instructing EZH2 inhibitor development against resistant breast cancer.

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Vita

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