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## GSK3 $\beta$ -MEDIATED EZH2 PHOSPHORYLATION SUPPRESSES METHYLATION OF H3K27 AND EZH2'S ONCOGENIC FUNCTIONS

How-Wen Ko

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SUPPRESSES METHYLATION OF H3K27 AND EZH2'S  
ONCOGENIC FUNCTIONS**

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A  
DISSERTATION

Presented to the Faculty of  
The University of Texas Health Science Center at Houston  
and  
The University of Texas M. D. Anderson Cancer Center  
Graduate School of Biomedical Sciences  
in Partial Fulfillment  
of the Requirements  
for the Degree of

DOCTOR OF PHILOSOPHY

by

How-Wen Ko, M.D.

Houston, Texas

May 2016

## **DEDICATION**

To my parents, Chin-Cheng Ko and Su-Chun Tseng, my wife, Yu-Chen Wang, my daughter, Sunny, and my sons, Max and Daniel for their unconditional love and support.

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Advisory Professor: Mien-Chie Hung, Ph. D.

During the process of tumorigenesis, inactivation of tumor suppressors is a critical step. Enhancer of zeste homolog 2 (EZH2), a histone methyltransferase and the enzymatic core subunit of polycomb repressive complex 2 (PRC2), promotes cell growth and migration through catalyzing trimethylation of histone H3 at Lys 27 (H3K27me3) and plays an important role in tumorigenesis. Its expression can be controlled by phosphorylation. However, the regulation of EZH2 activity by tumor suppressor kinase is not well understood. Glycogen synthase kinase 3 beta (GSK3 $\beta$ ), a multifunctional serine/threonine kinase, is involved in many cellular processes. GSK3 $\beta$  also participates in neoplastic transformation, tumor development and regulate cancer cell metastasis. Inactivation of GSK3 $\beta$  contributes to tumor development in certain types of cancers, such as breast cancer. In this study, we found that GSK3 $\beta$  negatively regulates H3K27 trimethylation. We also validated that GSK3 $\beta$  physically interacts with EZH2 and their interaction mainly exists in the cytosol. GSK3 $\beta$  phosphorylates EZH2 at Ser363 and Thr367 *in vitro*, and activating GSK3 $\beta$  upregulates Thr367 phosphorylation *in vivo*. Cells expressing mutant EZH2 to block phosphorylation by

GSK3 $\beta$  have higher H3K27 trimethylation and enhanced ability of cell migration and anchorage-independent growth. Inactivation of GSK3 $\beta$  as measured by its phosphorylation at Ser9 is positively correlated with higher level of H3K27 trimethylation in breast cancer patients. Our study indicates that GSK3 $\beta$  has a critical role in regulating EZH2-mediated oncogenesis.

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## Abbreviations

3'-UTR	three prime untranslated region
AEBP2	Adipocyte Enhancer-Binding Protein 2
AR	Androgen receptor
ATP	Adenosine-5'-triphosphate
BSA	Bovine serum albumin
CDH1	E-cadherin gene
CDK	cyclin-dependent kinase
DMEM	Dulbecco's modified Eagle's medium
DNMT	DNA methyltransferase
E2F	Electro-acoustic 2 Factor (Transcription factor E2F)
EED	Embryonic ectoderm development
EZH1	Enhancer of zeste homolog 1
EZH2	Enhancer of zeste homolog 2
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
FBS	Fetal bovine serum
GBM	Glioblastoma multiforme
GSK3 $\alpha$	Glycogen synthase kinase 3 alpha
GSK3 $\beta$	Glycogen synthase kinase 3 beta
H2AK119ub1	Monoubiquitylation of histone H2A at Lysine 119
H3K4me3	Trimethylation of histone H3 at Lysine 4



H3K27me1	Monomethylation of histone H3 at Lysine 27
H3K27me2	Dimethylation of histone H3 at Lysine 27
H3K27me3	Trimethylation of histone H3 at Lysine 27
HDAC	Histone deacetylases
HOTAIR	HOX transcript antisense RNA
HOX	Homeobox
INK4	Inhibitor of cyclin dependent kinase 4
JARID2	Jumonji AT Rich Interactive Domain 2
Kip2	p57, cyclin-dependent kinase inhibitor 1C
Lys	Lysine
MAPK	Mitogen-activated protein kinase
MCL-1	Myeloid Cell Leukemia 1
miR	MicroRNAs
ncRNA	Non-coding RNAs
NSCLC	Non-small cell lung cancer
p90RSK	p90 ribosomal S6 kinase
PcG	Polycomb group proteins
PRC1	Polycomb repressive complex 1
PRC2	Polycomb repressive complex 1
PBS	Phosphate-buffered saline
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C

RAD51	DNA repair protein RAD51 homolog 1
RB	Retinoblastoma
RbAp46/48	Retinoblastoma suppressor associated protein 46/48
RNase	Ribonuclease
S6K1	p70 ribosomal S6 kinase
SAH	S-adenosyl-L-homocysteine
SAM	S-adenosyl-methionine
SCLC	Small cell lung cancer
Ser	Serine
SET domain	Su(var)3-9, Enhancer-of-zeste and Trithorax domain
STAT3	Signal transducer and activator of transcription 3
SUZ12	Suppressor of zeste 12
Thr	Threonine
Tyr	Tyrosine
TrxG	Trithorax group proteins
YY1	Ying Yang 1

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# **Chapter 1**

## Introduction

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## **1-1 Epigenetic regulations and cancer**

Epigenetic regulation is an important mechanism in mediating gene expressions through modifying chromatin structure without changing DNA sequence. It is critical in DNA-based processes, such as transcription, DNA replication and repair, and essential for cell fate specification and normal tissue development. Chromatin consists of DNA and histone proteins, providing the platform for package of whole genome. The basic unit of chromatin is the nucleosome, which contains 147 base pairs of DNA wrapped around a histone octomer. Epigenetic mechanisms include DNA methylation, histone modifications, nucleosome remodeling and incorporation of histone variants, and non-coding RNAs (Henikoff and Ahmad 2005, Kouzarides 2007, Suzuki and Bird 2008, Jiang and Pugh 2009, Esteller 2011). All these mechanisms can work together to regulate gene expressions by altering compactness and accessibility of chromatin. The interaction between these modifications demonstrates an “epigenetic landscape”, and determines and maintains tissue-specific gene expression patterns. Aberrations in the processes alter normal gene expressions, cause activation or inactivation of regular signaling pathway, and lead to disease states like cancer (Sharma, Kelly et al. 2010).

Cancer is a disease arising from the accumulation of genetic abnormalities. Evidence has revealed that epigenetic abnormalities contribute to cancer initiation and progression. Epigenetic deregulations can cause silencing of tumor suppressor genes or activation of oncogenes, resulting in uncontrolled cell growth. These deregulations result from alterations in the patterns of DNA methylation and histone

modifications as well as abnormal expressions or mutations of chromatin-modifying enzymes. These genetic and epigenetic abnormalities can interact with each other to promote tumorigenesis. Unlike genetic mutations in cancers, epigenetic alterations are potentially reversible. Because of this character, therapies against epigenetic aberrations have been developed for specific cancer management and some of them have been approved (Dawson and Kouzarides 2012).

### **1-2 Polycomb group proteins, PRC2 complex and EZH2**

Polycomb group (PcG) proteins control gene silencing through histone modifications and are essential epigenetic regulators in cell differentiation and tissue development. Their deregulations have been implicated in cancer development. Polycomb group proteins were originally discovered in *Drosophila melanogaster* as transcriptional repressors of *HOX* genes, a gene family controlling development of anterior-posterior body segmentation. They have later been recognized as key mediators in cell fate determination and play an essential role in epigenetic regulation (Sauvageau and Sauvageau 2010). In vertebrates, they have similar effects on maintenance of homeotic gene expression patterns (Schuettengruber, Chourrout et al. 2007). Polycomb group proteins are well conserved between *Drosophila* and mammals. In mammals, two major multiprotein complexes have been identified, polycomb repressive complex 1 and 2 (PRC1 and PRC2). The compositions of these two complexes may be variable among organisms and cell context-dependent, but each complex contains common core components (Sauvageau and Sauvageau 2010). Core components of PRC1 complex include ring finger protein RING1A/B, chromobox homolog (CBX), B

lymphoma Mo-MLV insertion region 1 (BMI1), polyhomeotic homolog (PH), and SCML subunits. The PRC1 complex monoubiquitylates histone H2A at Lys 119 (H2AK119ub1) through its catalytic subunit, ubiquitin E3 ligase RING1 protein, to generate a repressive mark in chromatin structure. Core components of PRC2 complex contain enhancer of zeste homolog 2 (EZH2), embryonic ectoderm development (EED), suppressor of zeste 12 (SUZ12) and retinoblastoma suppressor associated protein 46/48 (RbAp46/48). The PRC2 complex catalyzes trimethylation of histone H3 at Lys 27 (H3K27me3) to epigenetically silencing specific gene expressions via enzymatic core subunit, lysine histone methyltransferase EZH2. PRC1 and PRC2 work together to repress gene transcription (Margueron and Reinberg 2011). Once the PRC2 complex is recruited to chromatin, EZH2 catalyzes histone H3 to generate H3K27me3 mark. Subsequently, the PRC1 complex is recruited to interact with H3K27me3 through its chromodomain-containing subunit, CBX, thereby catalyzing H2AK119ub1 to impede RNA polymerase II-dependent transcriptional elongation, thereafter repressing gene transcription. However, both complexes have independent functions in transcription repression (Eskeland, Leeb et al. 2010). Another groups of proteins, Trithorax group (TrxG) proteins, work as the functional counter parts of polycomb group proteins. They were also initially identified in *Drosophila* and are known to be crucial in cell differentiation and tissue development. Trithorax group proteins modify chromatin structure through catalyzing trimethylation of histone H3 at Lys 4 (H3K4me3) and serve as transcriptional activators of gene expression (Schuettengruber, Martinez et al. 2011). The H3K27me3 and H3K4me3 act as

repressive and active marks in epigenetic regulation. Active histone marks barely co-exist with H3K27me3 on the same histone tail (Schmitges, Prusty et al. 2011, Voigt, LeRoy et al. 2012). In certain context like embryonic stem (ES) cells, these two histone marks occur concurrently on the same nucleosome as “bivalent domains” to regulate the expressions of developmentally important genes (Bernstein, Mikkelsen et al. 2006, Margueron and Reinberg 2011).

In many types of solid cancers, increased expressions of PRC2 components have been reported (Varambally, Dhanasekaran et al. 2002, Kleer, Cao et al. 2003, Bachmann, Halvorsen et al. 2006, Li, Cai et al. 2012, Yu, Simons et al. 2012, Sato, Kaneda et al. 2013, Xia, Jin et al. 2015). Furthermore, regions within chromatin structure that are hypermethylated in cancers are often found to be pre-marked by H3K27me3 in embryonic stem cells (Ohm, McGarvey et al. 2007, Schlesinger, Straussman et al. 2007). This evidence has linked the developmental regulation to tumorigenesis and suggests an important role of PRC2 complex in cancer.

Human EZH2 gene was mapped to chromosome 7q35, which encodes protein containing 746 amino acids (Cardoso, Mignon et al. 2000). The functional domains of EZH2 include: WD-40 binding domain (WDB), domains I & II, two SWI3, ADA2, N-CoR and TFIIB (SANT) domains, cysteine-rich CXC domain and evolutionarily conserved SET domain. WD-40 binding domain is EED binding region, thus also called EED interacting domain (EID). Domain II is area for interaction with SUZ12. SANT domains are responsible for interaction with histone peptides. SET domain, located at C-terminal region of EZH2, is required for histone methyltransferase activity of EZH2/PRC2 complex (Margueron and Reinberg 2011, Li 2014, Tan, Yan

et al. 2014). Another enhancer of zesta homolog (EZH) is EZH1. EZH1 mainly exists in differentiated cells, whereas EZH2 is predominately expressed in proliferative tissues. PRC2 complex containing either EZH2 or EZH1 possesses histone methyltransferase activity but the complex of PRC2-EZH1 has markedly reduced enzymatic activity and different chromatin binding properties (Margueron, Li et al. 2008).

### **1-3 Enzymatic activity and functions of EZH2**

The best characterized enzymatic activity of EZH2/PRC2 complex is the trimethylation of H3K27 (Cao, Wang et al. 2002, Kuzmichev, Nishioka et al. 2002). In mammals, PRC2 complex is probably the only enzyme known to catalyze the methylation of H3K27 and it post-translationally modifies this residue in a successive manner from the monomethylation to the trimethylation (H3K27me1, H3K27me2, H3K27me3) (Kadoch, Copeland et al. 2016). Steady state kinetic studies of PRC2 containing EZH2 demonstrated that PRC2 complex preferentially catalyze the first methylation reaction (H3K27me1 as product) rather than the second and third reactions (H3K27me2 and H3K27me3 are products, respectively) (Sneeringer, Scott et al. 2010). The enzymatic activity of lysine methyltransferases depends on a catalytic domain, SET domain that consists of an S-adenosylmethionine (SAM)-binding site and a methyl acceptor lysine-binding site (Copeland, Solomon et al. 2009). These protein enzymes catalyzes methylation by the addition of methyl groups to their substrates at specific amino acids. EZH2 is the catalytic core subunit of PRC2 complex, which contains a SET domain.



However, EZH2 alone lacks of enzymatic activity and it requires a minimum of two additional PRC2 subunits, EED and SUZ12, for its robust histone lysine methyltransferase activity (Cao and Zhang 2004, Pasini, Bracken et al. 2004, Montgomery, Yee et al. 2005). EED is a WD-repeat protein, with a doughnut-like structure that offers a scaffold for association with its interacting proteins, such as EZH2 and H3K27me3. The region of EZH2 responsible for EED binding is N-terminal fragment (around residues 35-70) (Han, Xing et al. 2007, Tan, Yan et al. 2014). In addition, EED can directly bind H3K27me3, which recruits PRC2 complex to regions within chromatin for further repression (Hansen, Bracken et al. 2008, Margueron, Justin et al. 2009). It has also been reported that binding of EED to H3K27me3 can stimulate EZH2 enzymatic activity (Margueron, Justin et al. 2009). SUZ12 is also necessary for the histone methyltransferase activity of PRC2 complex, but how it contributes to the enzymatic action remains elusive. SUZ12 has been shown to be able to stabilize the PRC2 complex (Cao and Zhang 2004, Pasini, Bracken et al. 2004, Jiao and Liu 2015). A study has demonstrated that dense chromatin can activate PRC2 activity to regulate H3K27me3, which is in part mediated by SUZ12 (Yuan, Wu et al. 2012). SUZ12 is a zinc-finger protein, which contains a conserved VRN2-EMF2-FIS2-SUZ12 (VEFS) domain at its C-terminal region. Deletion of this domain disrupts SUZ12-EZH2 interaction and leads to dissociation of PRC2 complex (Yamamoto, Sonoda et al. 2004). Mutations in this domain do not affect the assembly of PRC2 complex but reduce its enzymatic activity (Rai, Vargas et al. 2013). Furthermore, it has been shown that C-terminal region of SUZ12 harboring VEFS domain is the minimal requirement for the histone

methyltransferase activity of PRC2 complex (Schmitges, Prusty et al. 2011). Other PRC2 components, such as RbAp46/48, AEBP2, and JARID2, also contribute to PRC2's enzymatic activity (Margueron and Reinberg 2011). However, they function as accessory rather than essential factors.

Although the composition of PRC2 complex may vary, all PRC2 either containing EZH1 or EZH2 primarily catalyzes methylation of H3K27. The reaction of methylation is stepwise as aforementioned. In mammalian cells, methylated H3K27 is abundant and all three forms exist. Mass spectrometry (Peters, Kubicek et al. 2003) and proteomic analyses (Ferrari, Scelfo et al. 2014) revealed that in mouse embryonic stem cells most H3K27 is methylated (around 80%) and the majority of methylated H3K27 is dimethylation form (50~70%). Monomethylation and trimethylation of H3K27 occur in about 5% and 10%, respectively. H3K27me3 is a stable mark and its enrichment is related to gene silencing. In contrast, H3K27me2 seems to be less important in gene repression, yet it may be a critical intermediate product. It has been proposed that H3K27me2 is a substrate for subsequent formation of H3K27me3 mark via monomethylation and it may also facilitate the action of PRC2 by prevention of H3K27 acetylation (Margueron and Reinberg 2011). Both H3K27me3 and H3K27me2 are associated with gene silencing, while the enrichment of H3K27me1 throughout the gene bodies has been found to be related to actively transcribed genes (Ferrari, Scelfo et al. 2014). Despite H3K27me1 is produced by PRC2 complex through catalyzing monomethylation of H3K27, it is still detected in cells expressing non-functional

PRC2 (Schoeftner, Sengupta et al. 2006). How is H3K27me1 generated in cells is not fully understood.

Genome-wide screening for the distribution of PRC2 complex and H3K27me3 have been explored in human and mouse embryonic stem cells, in which H3K27me3 has been found to be enriched in the promoter regions where are also occupied by PRC2 complex (Pan, Tian et al. 2007, Zhao, Han et al. 2007). Several target genes, such as HOX genes and other developmental regulators, have been identified (Boyer, Plath et al. 2006, Lee, Jenner et al. 2006). On the other hand, the interaction of PRC2 complex with other factors facilitates PRC2 recruitment to its specific loci. Many of transcription factors, including Ying Yang 1 (YY1), interact with EZH2 to promote recruitment of PRC2 complex to target site for gene silencing (Palacios, Mozzetta et al. 2010). Non-coding RNAs (ncRNA), such as HOX transcript antisense RNA (HOTAIR), also interact with PRC2 complex and play an important in the recruitment of PRC2 complex the specific loci (Tsai, Manor et al. 2010).

EZH2 also cooperates with other epigenetic regulators to modulate gene expression. It has been shown that EZH2 physically interacts with and recruits DNA methyltransferases, DNMT1, DNMT3A and DNMT3B, to methylate target genes and to establish a more deeply silenced chromatin state (Vire, Brenner et al. 2006). In addition, histone deacetylases (HDAC) also contribute to the methylation of PRC2. Lys 27 of histone H3 can be acetylated. If H3K27 is acetylated, a HDAC is required before the action of PRC2 complex. HDAC modifies the local histone code of genes prepared for silencing through deacetylation of H3K27, thereby making

H3K27 available for methylation by PRC2 (van der Vlag and Otte 1999, Tie, Furuyama et al. 2001). The interaction between EZH2 and DNMT, or EZH2 and HDAC, has also been found to contribute to tumor development (Tong, Cai et al. 2012, Ning, Shi et al. 2015).

Recently, EZH2 has been shown to methylate substrates other than histone. EZH2 interacts with and methylates a cardiac transcription factor, GATA4 at Lys 299. PRC2-mediated methylation of GATA4 disrupts GATA4's interaction with p300, which suppresses GATA4's transcription activity (He, Shen et al. 2012). EZH2 also monomethylates retinoic acid-related orphan nuclear receptor  $\alpha$  (ROR $\alpha$ ) at Lys 38 (Lee, Lee et al. 2012). Monomethylated ROR $\alpha$  is recognized and ubiquitinated by the DCAF1/DDB1/CUL4 E3 ubiquitin ligase complex. EZH2-mediated methylation leads to ROR $\alpha$ 's degradation and represses its target gene expression. These studies provide evidence showing that PRC2 or EZH2 can repress gene transcription through mediating non-histone targets. Moreover, it has been demonstrated that EZH2 transcriptionally activates androgen receptor (AR)'s target genes through interaction with and methylation of AR or AR-associated proteins (Xu, Wu et al. 2012). EZH2 also binds to and methylates signal transducer and activator of transcription 3 (STAT3) at Lys 180, which increases STAT3 activity and leads to transcription activation (Kim, Kim et al. 2013). These evidence indicates that EZH2 can activate gene expression through its methyltransferase activity in a polycomb-independent manner.

#### **1-4 Regulation of EZH2 expression and activity**

EZH2 level is low in differentiated cells (Varambally, Dhanasekaran et al. 2002, Kleer, Cao et al. 2003) but is relatively high in embryonic stem cells or undifferentiated progenitors of adult tissues (Su, Basavaraj et al. 2003), which suggests that EZH2 is essential in cell differentiation. In cancer, EZH2 is frequently overexpressed and has been found to be implicated in cancer cell growth and survival (Yamaguchi and Hung 2014). Thus, the regulators of EZH2 expression are critical for these cellular processes and understanding the underlying molecular mechanisms is important. Several transcription factors or signaling pathways regulate the expression of EZH2. pRB-E2F signaling has been demonstrated to be involved in EZH2 overexpression (Bracken, Pasini et al. 2003). EZH2 is downstream of this pathway and E2F positively controls EZH2 expression. Phosphorylation of retinoblastoma (RB) dissociates E2F from the pRB-E2F complex. The binding of dissociated E2F to the EZH2 promoter up-regulates EZH2 transcription. Deregulation of this pathway or E2F overexpression is correlated with higher expression of EZH2 in breast cancer and small cell lung cancer (Bracken, Pasini et al. 2003, Byers, Wang et al. 2012, Coe, Thu et al. 2013). EZH2 has also been identified as downstream of MEK-ERK-ELK1 pathway (Fujii, Tokita et al. 2011). Phosphorylation of transcription factor ELK1 by ERK leads to ELK1 binding to the promoter of EZH2 gene, thereby activating EZH2 transcription. Regulation of EZH2 overexpression by this pathway has been found in aggressive breast cancer subtypes, such as triple-negative and HER2-overexpressing breast cancers (Fujii, Tokita et al. 2011). Furthermore, hypoxia-inducible factor-1 $\alpha$  (HIF $\alpha$ ) directly

regulates EZH2 transcription (Chang, Yang et al. 2011). Hypoxic condition in tumor microenvironment stimulates HIF $\alpha$  binding to HIF $\alpha$  responsive element (HRE) within the EZH2 gene promoter to upregulate EZH2 expression. Myc activates EZH2 transcription through either binding to EZH2 promoter (Koh, Iwata et al. 2011) or downregulating microRNAs (miR-26a and miR-101) (Sander, Bullinger et al. 2008, Koh, Iwata et al. 2011, Wang, Zhang et al. 2014). Transcription factors ETS, NF- $\kappa$ B, STAT3, and fusion oncoprotein EWS-FLI1 also directly regulates EZH2 transcription in prostate, ovarian, colorectal cancers, and Ewing's sarcoma, respectively (Richter, Plehm et al. 2009, Kunderfranco, Mello-Grand et al. 2010, Garipov, Li et al. 2013, Lin, Ren et al. 2013).

EZH2 expression are also regulated by post-transcriptionally (Yamaguchi and Hung 2014). Several microRNAs (miRs) are responsible for this regulation. These microRNAs directly downregulate EZH2 transcription through binding to the three prime untranslated region (3'-UTR) of EZH2 gene. The increased expression of EZH2 led by the loss of these microRNAs has been found in various cancers and seems to be associated with aggressive disease. miR-101 is the first microRNA identified to regulate EZH2 expression (Varambally, Cao et al. 2008). This regulation was initially detected in prostate cancer and has later been found to be involved in many other cancers, such as transitional cell carcinoma of bladder, glioblastoma multiforme, and non-small cell lung cancer (Friedman, Liang et al. 2009, Smits, Nilsson et al. 2010, Zhang, Guo et al. 2011). Other microRNAs mediating EZH2 expression in cancer include miR-25, -26a, -30d, -124, -137, -138, -144, -214 and let-7 (Volkel, Dupret et al. 2015).

In addition to transcription and post-transcription regulations, emerging evidence shows that protein level and enzymatic activity of EZH2 are regulated by post-translational modifications, such as phosphorylation and ubiquitination (Yamaguchi and Hung 2014). These modifications lead to EZH2 degradation and are critical for its protein stability and enzymatic activity. The first discovery of this regulation is AKT1-mediated phosphorylation, in which EZH2 is phosphorylated at Ser21 and phosphorylated EZH2 has altered affinity with histone H3 (Cha, Zhou et al. 2005). EZH2 phosphorylation at Ser21 by AKT1 has later been shown to have increased enzymatic activity in control of non-histone substrates, androgen receptor and STAT3 (Xu, Wu et al. 2012, Kim, Kim et al. 2013). Cell cycle regulators, cyclin-dependent kinase 1 and 2 (CDK1 and CDK2), phosphorylate EZH2 at Thr487 and Thr416, respectively (Wei, Chen et al. 2011, Yang, LaBaff et al. 2015). EZH2 phosphorylation at Thr478 by CDK1 disrupts PRC2 assembly and decreases EZH2/PRC2 histone methyltransferase activity (Wei, Chen et al. 2011). This regulation not only suppresses cell invasion and migration, but also enhances osteogenic differentiation of human mesenchymal stem cells. CDK2-mediated Thr416 phosphorylation augments cell migration, invasion and tumor growth, and higher phosphorylation correlates with poor survival in triple-negative breast cancer patients (Yang, LaBaff et al. 2015). CDK1/2-mediated phosphorylation of EZH2 has also been identified by other groups with findings of different sites and effects (Chen, Bohrer et al. 2010, Kaneko, Li et al. 2010, Wu and Zhang 2011, Minnebo, Gornemann et al. 2013). Other kinases, including mitogen-activated protein kinase p38 $\alpha$ , Janus Kinase 2 (JAK2) and ataxia telangiectasia mutated (ATM), have been

identified to phosphorylate EZH2 at different sites and are critical for the protein stability, enzymatic activity and biological functions of EZH2 (Palacios, Mozzetta et al. 2010, Li, Hart et al. 2013, Sahasrabuddhe, Chen et al. 2015). Several inhibitors targeting kinases have been developed to treat human diseases like cancer. The identification of EZH2 phosphorylation is significant not only because this regulation controls EZH2 activity but also because it may provide clues for the development of novel therapeutic strategies. Thus, to better understand how EZH2 is regulated by phosphorylation is important.

### **1-5 Role of EZH2 in tumorigenesis**

A number of studies have revealed that EZH2 is widespread implicated in tumor development. The most manifest evidence is that high expression of EZH2 has been reported in multiple cancer types, including prostate cancer, breast cancer, small cell and non-small cell lung cancers, endometrial carcinoma, ovarian cancer, bladder cancer, renal cell carcinoma, gastric cancer, pancreatic cancer, esophageal cancer, hepatocellular carcinoma, brain tumors and melanoma (Varambally, Dhanasekaran et al. 2002, Kleer, Cao et al. 2003, Raman, Mongan et al. 2005, Bachmann, Halvorsen et al. 2006, Matsukawa, Semba et al. 2006, Kondo, Shen et al. 2007, McHugh, Fullen et al. 2007, Ougolkov, Bilim et al. 2008, Crea, Hurt et al. 2010, Rao, Cai et al. 2010, Yamada, Fujii et al. 2011, Byers, Wang et al. 2012, Lee and Choe 2012, Behrens, Solis et al. 2013, Murai, Koinuma et al. 2015). Higher expression of EZH2 is associated with aggressive and advanced diseases and poor outcome in many of them (Varambally, Dhanasekaran et al. 2002, Kleer, Cao et al. 2003, Bachmann, Halvorsen et al. 2006, Matsukawa, Semba et al. 2006,



Crea, Hurt et al. 2010, Yamada, Fujii et al. 2011). Indeed, several studies have demonstrated that EZH2 contributes to tumorigenesis through promoting malignant transformation, cancer cell growth, invasion and migration. In noncancerous epithelial cell lines, overexpression of EZH2 enhances *in vitro* anchorage-independent colony formation (Kleer, Cao et al. 2003, Karanikolas, Figueiredo et al. 2009). In many types of cancer cells, EZH2 augments their invasion/migration ability (Kleer, Cao et al. 2003, Rao, Cai et al. 2010, Wei, Chen et al. 2011, Moore, Gonzalez et al. 2013, Xu, Hou et al. 2013, Han, Jiao et al. 2016), and some studies have reported that EZH2 mediates invasion and/or migration by epigenetically repressing tumor suppressor gene expressions through catalyzing trimethylation of H3K27 (Yu, Cao et al. 2007, Cao, Yu et al. 2008, Du, Li et al. 2012, Ren, Baritaki et al. 2012). Furthermore, in a tumorigenic mouse experiment using the mouse mammary tumor virus (MMTV) long terminal repeat, EZH2 overexpression in mammary epithelial cells induces intraductal epithelial hyperplasia phenotype (Li, Gonzalez et al. 2009). Similarly, overexpression of EZH2 in benign prostate epithelial cells leads to tumor growth *in vivo* (Karanikolas, Figueiredo et al. 2009). All these investigations underline the critical role of EZH2 in tumorigenesis.

Gain-of-function mutations of EZH2 have been identified in B-cell lymphoma (Morin, Johnson et al. 2010, Majer, Jin et al. 2012, McCabe, Graves et al. 2012). Around 22% of diffuse large B-cell lymphoma and 7% of follicular lymphoma harbor the recurrent somatic mutations of EZH2 at tyrosine 641 (Tyr641), which is located within the catalytic SET domain (Morin, Johnson et al. 2010). In contrast to wild-type EZH2, Tyr641 mutant has higher methyltransferase activity to catalyze

dimethylation and trimethylation of H3K27 but has lower activity to generate H3K27 monomethylation (Sneeringer, Scott et al. 2010, Yap, Chu et al. 2011). Subsequent experiments using transgenic mice expressing EZH2<sup>Y641F</sup> in lymphocytes demonstrated a significantly higher H3K27me3 level in spleen cells and developed lymphomas when combined with Eμ-Myc expression, which validated the observations and the oncogenic roles of this mutant (Berg, Thoene et al. 2014). Additional studies have also identified A677G and A687V activating mutations with higher enzymatic activity for trimethylation of H3K27 in B-cell lymphoma (Majer, Jin et al. 2012, McCabe, Graves et al. 2012). These evidence further supports the oncogenic role of EZH2 and activating mutations functioning as EZH2 overexpression highlights the importance of its histone methyltransferase activity in EZH2-mediated oncogenesis.

Cancer stem cell (CSC) hypothesis has been proposed for decades (Dick 2008). Cancer stem cells represent a small portion of tumor population, which are postulated to be responsible for cancer initiation, progression and metastasis. They share features of normal stem cells, including self-renewal and multiple lineage differentiation capacity, and may account for drug resistance. EZH2 has been demonstrated to be essential in the maintenance of self-renewal and other properties of embryonic and adult stem cells (Cao and Zhang 2004, Ezhkova, Pasolli et al. 2009). Several studies have also found that EZH2 is not only frequently overexpressed in a wide range of cancers, but also associated with an aggressive and advanced disease, and has been linked to neoplastic transformation, cancer cell migration/invasion and tumor development as aforementioned. An integrative

analysis using genome-wide screening approach displayed that a significant group of PRC2 target genes in cancer are also PRC2 targets in embryonic stem cells, and repression of these genes correlates with worse outcome (Yu, Cao et al. 2007). It has therefore been speculated that EZH2 is implicated in cancer stem cell biology. Indeed, in breast tumor initiating cells, increased EZH2 expands cell population through repressing RAD51 expression while knockdown of EZH2 reduces the repression of RAD51 transcription by decreasing H3K27 trimethylation (Chang, Yang et al. 2011). Similarly, EZH2 has been revealed to promote stem-like cell properties in glioblastoma multiforme and skin cancer, which are related to its enzymatic activity or increased H3K27 trimethylation (Kim, Kim et al. 2013, Adhikary, Grun et al. 2015).

HOX genes are well-known EZH2 target genes, which are vital in embryonic development (Boyer, Plath et al. 2006, Lee, Jenner et al. 2006). In addition to HOX genes, several genes have been identified in cancer and the majority of them are tumor suppressors as EZH2 functions primarily as a tumor promoter. The INK4A-ARF-INK4B locus, encoding tumor suppressor p16<sup>INK4A</sup>, is controlled by EZH2/PRC2 as well as BMI1. Its downregulation increases cell proliferation and is critical in stem cell biology and cancer (Bracken, Kleine-Kohlbrecher et al. 2007, Ezhkova, Pasolli et al. 2009). E-cadherin, a key mediator in epithelial-mesenchymal transition (EMT) and metastasis, is also regulated by EZH2 and repression of E-cadherin gene expression (CDH1) enhances cell migration and metastasis in several types of cancers (Cao, Yu et al. 2008, Fujii and Ochiai 2008, Wang, Liu et al. 2013). In nasopharyngeal carcinoma, EZH2 forms a complex with Snail and

HDAC1/HDAC2 to repress E-cadherin transcription, thereby promoting cell aggressiveness (Tong, Cai et al. 2012). Moreover, there are several EZH2 target genes involved in EZH2-mediated cancer progression, many of them are related to invasion or migration. These include FOXC1 and RKIP in breast cancer, DAB2IP, SLIT2, ADRBP2, TIMP-2 and TIMP-3 in prostate cancer, and their expression is repressed by EZH2-mediated H3K27 trimethylation (Chen, Tu et al. 2005, Yu, Cao et al. 2007, Yu, Cao et al. 2010, Du, Li et al. 2012, Ren, Baritaki et al. 2012, Shin and Kim 2012). In addition, RAD51, CDKN1C (p57<sup>KIP2</sup>), RUNX3, KLF2 and IKK $\alpha$  genes are also targeted by EZH2, and their repression mediated by H3K27me3 is involved in cell proliferation, growth and differentiation in distinct cancer types, respectively (Fujii, Ito et al. 2008, Yang, Karuturi et al. 2009, Chang, Yang et al. 2011, Taniguchi, Jacinto et al. 2012, Yan, Zhang et al. 2014).

## **1-6 EZH2 and breast cancer**

Breast cancer is the most prevalent malignancy and the second leading cause of cancer-related mortality among women in the United States. Based on most recent statistics, approximately 246,660 of new invasive breast cancer patients and 40,450 breast cancer mortality are expected among women in 2016 (Siegel, Miller et al. 2016). The mortality rate of breast cancer has been declining since 1989 and the 5-year survival rate is 89% at present in all stages combined; however, in breast cancer patients with distant metastasis, the 5-year survival rate falls to 26% (Siegel, Miller et al. 2016). Almost all cancer deaths result from local invasion and distant metastasis of tumor and patients with cancer metastasis is

undoubtedly associated with worse prognosis. Therefore, a better understanding of metastasis is critical.

As previously described, higher EZH2 expression has been correlated with aggressive disease and poor outcome in breast cancer patients (Kleer, Cao et al. 2003). Ectopic expression of EZH2 in transgenic mouse model induces mammary epithelial hyperplasia (Li, Gonzalez et al. 2009). Several studies indicated that EZH2 promotes invasive and metastatic potential in breast cancer, many of them validates that this regulation is mediated by repression of tumor suppressors through EZH2 catalyzing trimethylation of H3K27, such as E-cadherin, FOXC1 and RKIP (Cao, Yu et al. 2008, Du, Li et al. 2012, Ren, Baritaki et al. 2012). Indeed, an investigation of immunohistochemical staining in human breast tumor specimens also showed that EZH2 expression is upregulated in metastatic tissues (Moore, Gonzalez et al. 2013). These evidence suggests that EZH2 acts as a tumor promoter in breast cancer, and plays an important role in breast cancer metastasis.

### **1-7 EZH2 is a potential substrate of GSK3 $\beta$**

In the process of tumorigenesis, inactivation or loss of function of tumor suppressors is a crucial step. As mentioned previously, EZH2 activity can be mediated kinases. However, the regulation of EZH2 activity by tumor suppressor kinase in cancer is not fully understood. Interestingly, we noticed that EZH2 amino acid sequence contains several GSK3 $\beta$  phosphorylation motifs (Ser/Thr-X-X-X-Ser/Thr, where X represents any amino acid) (Doble and Woodgett 2003), suggesting that EZH2 might be regulated by GSK3 $\beta$  (Figure 1-1).

**Figure 1-1. GSK3 $\beta$  consensus motif in EZH2 amino acid sequence**

EZH2 AMINO ACID SEQUENCE:

1	MGQ	TGKKSEK	GPVCWRKRVK	SEYMRLRQLK	RFRRADDEVK	S	MFSS	NRQKIL	ERTEILNQEW
61	KQRRIQPVHI	L	TSVSSLRGT	RECSVTSDDL	FPTQVIPLKT	LNAVASVPIM	YWSPLQQNF		
121	MVEDETIVLHN	IPYMGDEVLD	QDGTFFIEELI	KNYDGKVHGD	RECGFINDEI	FVELVNALGQ			
181	YNDDDDDDDG	DDPEEREKQ	KDLEDHRDDK	ESRPPRKFP	DKIFEAISSM	FDPKGTAEEEL			
241	KEYKELTEQ	QLPGALPPEC	TPNIDGPNK	SVQREQSLHS	FHTLFCRRCF	KYDCFLHPFH			
301	ATPNTYKRKN	TETALDNKPC	GPQCYQHLEG	AKEFAAALTA	ERIKTPPKRP	GGRRRGRLPN			
361	N	SSRPSTPTI	NVLESKDIDS	DREAGTETGG	ENNDKEEEEK	KDE	TSSSSEA	NSRCQTP	IKM
421	KPNIEPPENV	EWSGAEASMF	RVLIGTYIDN	FCAIARLIGT	KTCRQVYEFR	VKESSIIAPA			
481	PAEDVDTPPR	KKKRKHRLWA	AHCRKIQLKK	DGSSNHVYNY	QPCDHPRQPC	DSSCPCVIAQ			
541	NFCEKFCQCS	SECQNRFPGC	RCKAQCNKQ	CPCYLAVREC	DPDLCLTCGA	ADHWD	S	KNVS	
601	CKNCISQGRS	KKHLLAPSD	VAGWGIFIKD	PVQKNEFISE	YCCEIISQDE	ADRRGKVYDK			
661	YMCSFLFNLN	NDFVVDATRK	GNKIRFANHS	VNPNCYAKVM	MVNGDHRIGI	FAKRAIQTGE			
721	ELFFDYRYSQ	ADALKYVGIE	REMEIP						

GSK3B PHOSPHORYLATION MOTIF: S/T-X-X-X-S/T

### 1-1. EZH2 amino acid sequence contains GSK3 $\beta$ phosphorylation motif.

The region of amino acid sequence of EZH2 containing GSK3 $\beta$  phosphorylation motif is highlighted in yellow, in which possible phosphorylation serine/threonine residues is marked in red.

## 1-8 GSK3 $\beta$ : its regulation and action

Glycogen synthase kinase 3 beta (GSK3 $\beta$ ) is a multifunctional serine/threonine kinase. GSK3 $\beta$  was originally found in mammalian skeletal muscle, responsible for phosphorylation of glycogen synthase, leading to its inactivation in glycogen metabolism (Frame and Cohen 2001). It has later been identified as a critical mediator of various signaling pathways involved in multiple physiological processes, such as transcription, protein synthesis, cell cycle, apoptosis, cell motility, and immune cell regulation (Grimes and Jope 2001, Doble and Woodgett 2003, Sutherland 2011). In addition to GSK3 $\beta$ , there is an isoform in human, GSK3 $\alpha$ . GSK3 $\alpha$  and GSK3 $\beta$  are 51 and 47 KDa proteins, encoded by *GSK3A* and *GSK3B* genes, respectively. Although these two isoforms have similar structure and overlapping functions, they are not redundant (McCubrey, Steelman et al. 2014). GSK3 $\beta$  knockout mice are embryonically lethal, whereas GSK3 $\alpha$  knockout mice are not (Hoeftlich, Luo et al. 2000, MacAulay, Doble et al. 2007). Owing to its extensive involvement in cellular functions, the deregulation of GSK3 $\beta$  has been implicated in a wide range of human diseases, including neurodegenerative disorder, cardiovascular disease, diabetes mellitus, bipolar disorder, and inflammation/immune disease (Grimes and Jope 2001, Sutherland 2011). Aberrations in GSK3 $\beta$  regulation also contribute to cancer initiation and progression (Luo 2009, McCubrey, Steelman et al. 2014).

GSK3 $\beta$  is generally active in resting cells but becomes inactive upon external signals (Grimes and Jope 2001). The activity of GSK3 $\beta$  is controlled by site-specific phosphorylation through extracellular stimuli or signaling pathway (Medina and

Wandosell 2011). GSK3 $\beta$  phosphorylation at Tyr216 associates with an increase of its enzymatic activity. Tyr216 phosphorylation can be mediated by kinases such as proline-rich tyrosine kinase 2 (PYK2), but may be due to autophosphorylation as GSK3 $\beta$  is constitutively active at rest (Hartigan, Xiong et al. 2001, Cole, Frame et al. 2004). GSK3 $\beta$  phosphorylation at Ser9 results in its inactivation via proteasomal degradation (Medina and Wandosell 2011). Several kinases mediating this regulation include protein kinase A (PKA), protein kinase B (also known as Akt), certain isoform of protein kinase C (PKC), p90 ribosomal S6 kinase (p90RSK), and p70 ribosomal S6 kinase (S6K1) (McCubrey, Steelman et al. 2014). A number of factors, such as insulin, epidermal growth factor and transforming growth factor, and some signaling pathways, like insulin signaling, mitogen-activated protein kinase (MAPK)/p90RSK, or mTOR/S6K pathways can inactivate GSK3 $\beta$  through this modulation (Ding, He et al. 2007). Our previous work also found that ERK contributes this regulation (Ding, Xia et al. 2005). ERK phosphorylates GSK3 $\beta$  at Thr43 residue, which facilitates Ser9 phosphorylation by p90RSK, leading to its inactivation. These studies suggest that Ser9 phosphorylation is probably the most important mechanism to control GSK3 $\beta$  activity (Luo 2009). In addition, this modification has also been utilized as a measurement for GSK3 $\beta$  inactivation (Ding, He et al. 2007).

Numerous proteins, such as EMT regulator SNAIL (Zhou, Deng et al. 2004) and antiapoptotic protein MCL-1 (Ding, He et al. 2007), have been identified as substrates of GSK3 $\beta$  and these substrates are implicated in an extensive spectrum of cellular functions, suggesting that GSK3 $\beta$  is a fundamental regulator in cells



(Sutherland 2011). GSK3 $\beta$  usually suppresses the function or activity of its substrates via phosphorylation, meaning that these substrates are inactive or inaccessible under unstimulated conditions (Grimes and Jope 2001, Sutherland 2011). One of the well-known substrate of GSK3 $\beta$  is  $\beta$ -catenin.  $\beta$ -catenin is the key molecule of WNT/ $\beta$ -catenin signaling pathway (Wu and Pan 2010). Without stimulation,  $\beta$ -catenin exists in the cytosol and forms a complex with Axin, adenomatous polyposis coli (APC) and GSK3 $\beta$ . GSK3 $\beta$  phosphorylates  $\beta$ -catenin, thereby resulting in degradation and inactivation of  $\beta$ -catenin. Once the pathway is stimulated, GSK3 $\beta$  is inactivated, which in turn prevents  $\beta$ -catenin phosphorylation and degradation in the cytosol, and subsequently leading to its nuclear translocation and downstream target gene activation. Not all substrates of GSK3 $\beta$  are degraded after being phosphorylated, but most of them are inactivated through this regulation. In addition, the amino acid sequence of GSK3 $\beta$ 's substrates usually contains a phosphorylation motif, that is Ser/Thr-X-X-X-Ser/Thr, where X represents any amino acid (Sutherland 2011). This motif is found in several substrates of GSK3 $\beta$ , such as glycogen synthase,  $\beta$ -catenin, SNAIL and MCL-1 (Frame and Cohen 2001, Liu, Li et al. 2002, Zhou, Deng et al. 2004, Ding, He et al. 2007).

### **1-9 Role of GSK3 $\beta$ in tumorigenesis**

GSK3 $\beta$  has been shown to phosphorylate and inactivate many oncoproteins or cell cycle regulators, and is a negative mediator of WNT/ $\beta$ -catenin signaling pathways, suggesting that GSK3 $\beta$  functions as a tumor suppressor in carcinogenesis (Luo 2009). In an in vitro soft agar assay and an in vivo model of

skin tumorigenesis, overexpression of wild-type or active form (S9A mutant) GSK3 $\beta$  reduces anchorage-independent growth of cells combined with treatment of epidermal growth factor or chemicals and attenuates tumorigenicity; in contrast, kinase-inactive form GSK3 $\beta$  enhances colony formation and tumor growth (Ma, Wang et al. 2007). Similarly, this effect has been observed in mammary tumor development. Overexpression of kinase-inactive form GSK3 $\beta$  in transgenic mice using the mouse mammary tumor virus-long terminal repeat promotes mammary tumorigenesis (Farago, Dominguez et al. 2005). Our group also demonstrated that in tumor-bearing mice, activation of GSK3 $\beta$  by intratumor injection of the liposome complex with GSK3 $\beta$  reduces mammary tumor growth (Ding, He et al. 2007). These results imply that inactivation of GSK3 $\beta$  is a significant contribution factor for the process of tumor development in these cancers. In addition to its role in neoplastic cell transformation and tumor development, GSK3 $\beta$  is also involved in cancer metastasis. Epithelial—mesenchymal transition (EMT), an embryonically developmental process, has been shown to play an essential role in promoting metastasis in epithelial cancer (Tsai and Yang 2013). In non-small cell lung cancer, GSK3 $\beta$  controls EMT and cancer metastasis via phosphorylation and degradation of an EMT regulator, SLUG (Kao, Wang et al. 2014). Our previous work also found that GSK3 $\beta$  negatively regulates EMT by phosphorylation of another EMT mediator, SNAIL, in breast cancer (Zhou, Deng et al. 2004). Together, these evidence indicates that GSK3 $\beta$  is an important regulator for tumorigenesis and cancer metastasis.

A number of studies have revealed that WNT/ $\beta$ -catenin signaling pathway is pivotal in control of embryonic development and adult cell fate and perturbations of this pathway has been associated with maintenance of cancer stem cells and subsequent tumorigenesis (Clevers 2006). Since GSK3 $\beta$  is a key regulator of WNT/ $\beta$ -catenin signaling pathway (Wu and Pan 2010), one would predict that GSK3 $\beta$  is also involved in the regulation of cancer stem cell population (Benoit, Guezguez et al. 2014). In chronic myeloid leukemia, a nonfunctional form of GSK3 $\beta$  has been detected in a subset of leukemia stem cells with aberrant activation of  $\beta$ -catenin, which contributes to leukemia stem cell generation and cancer progression (Abrahamsson, Geron et al. 2009). This evidence links GSK3 $\beta$  to cancer stem cells and further supports the importance of GSK3 $\beta$  inactivation in cancer. However, studies investigating the direct role of GSK3 $\beta$  in cancer stem cell biology are limited. Although aberrant WNT/ $\beta$ -catenin signaling pathway has been shown to be related to cancer epigenetics, the relationship between GSK3 $\beta$  and epigenetic regulators in cancer remains unclear (Benoit, Guezguez et al. 2014).

### **1-10 Rationale for this study**

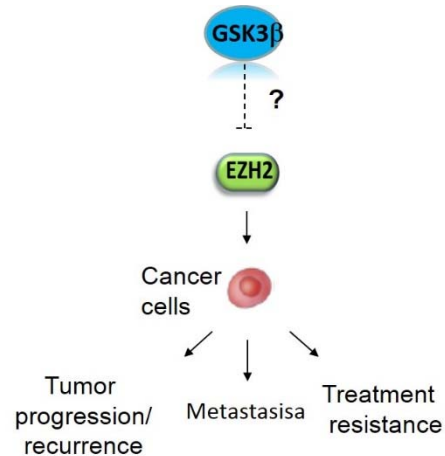
Alterations in epigenetic mechanism, such as aberrant expression or activation of epigenetic regulators, often induce neoplastic cell transformation and tumor growth. EZH2, a histone methyltransferase, is the enzymatic core subunit of PRC2 complex, which is responsible for catalyzing trimethylation of H3K27, leading to epigenetically silencing of specific gene expression. Overexpression or activation of EZH2 has been associated with several types of cancers and is related to

aggressive disease and poor outcome. Studies have also demonstrated that EZH2 contributes to tumorigenesis through promoting malignant transformation, cell proliferation, invasion and migration. The histone methyltransferase activity of EZH2 plays a critical role in these processes.

In the process of tumor development, inactivation of tumor suppressors is always an important step. EZH2 expression can be controlled by phosphorylation. However, the regulation of EZH2 activity by tumor suppressor kinase is not well understood. Interestingly, we noticed that EZH2 amino acid sequence contains GSK3 $\beta$  phosphorylation motifs, suggesting that EZH2 might be regulated by GSK3 $\beta$ .

GSK3 $\beta$  is a serine/threonine kinase. It also participates in neoplastic transformation and tumor development. In breast cancer, it functions as a tumor suppressor. Studies have shown that inactivation of GSK3 $\beta$  promotes tumor growth. GSK3 $\beta$  also regulates epithelial-mesenchymal transition and cancer cell metastasis. However, little is known about the role of GSK3 $\beta$  in epigenetic regulation during tumor development. It is not clear whether GSK3 $\beta$  regulates EZH2 activity and functions. In this study, we proposed that GSK3 $\beta$  attenuates tumorigenesis through phosphorylation of EZH2 and suppression its enzymatic activity (Figure 1-2).

**Figure 1-2. Hypothesis: GSK3 $\beta$  may regulate tumorigenesis through phosphorylation of EZH2**



**1-2 GSK3 $\beta$  may regulate EZH2's oncogenic functions through phosphorylation of EZH2**

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## **Chapter 2**

### Materials and Methods

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## **2-1 Cell culture**

All cell lines were obtained from ATCC (Manassas, VA) and their validation was performed in Characterized Cell Line Core Facility, MD Anderson Cancer Center. The cell lines used in this study include MDA-MB-231, BT549, MDA-MB-435S, MDA-MB-468, MCF7, MCF12A, HeLa and HEK 293T cells. All cells except MCF12A were grown in Dulbecco's Modified Eagle's Medium/F12 supplement (DMEM/F12) supplemented with 10% heat inactivated fetal bovine serum (FBS), and Penicillin/Streptomycin (100 U, 100 µg/ml) at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. MCF12A cells were cultured in DMEM/F12 media supplemented with 5% horse serum, Penicillin/Streptomycin (100 U, 100 µg/ml), EGF (20ng/ml), Hydrocortisone (0.5mg/ml), Cholera toxin (100ng/ml) and insulin (10µg/ml). For inhibiting or activating GSK3 $\beta$  activity, cells were treated with 40 mM lithium chloride (Sigma) for 16 hr or 0.1 µM staurosporine (Sigma) for 2 hr.

## **2-2 Transfection**

Transfection of cells with DNA was performed with liposome. Cells were seeded in 10-cm culture dish or 6-well plate one day before transfection. Upon transfection, plasmid DNA and liposome were mixed in a 1:2 (w/v) ratio and diluted in OPTI-MEM. The plasmid DNA-liposome mixture were then added into media in cell culture dish or plate. After incubation for 4-6 hrs, media containing DNA-liposome mixture were replaced with fresh regular media. Transfected cells were harvested for analysis or exploited for other experiments 48 hrs after transfection. For generation of stable transfectants, the lentiviral-based pCDH or shRNA was

used. To generate lentivirus expressing pCDH for EZH2 expression or shRNA for GSK3 $\beta$  knockdown, HEK 293T cells were transfected with pCMV-  $\Delta$ R8.92, pCMV-VSVG plus EZH2, GSK3 $\beta$  or vector control plasmids. Media were change 6 hrs after transfection, then were collected 48 hr and 72 hr after first media change. The collected media containing lentivirus were filtered with 0.45- $\mu$ m filters to remove cell debris. Target cells were prepared before infection and collected media were added into these cells at around 50% density. The media of infected cells were changed 24 hr after infection. Infected cells were selected by either G418 (Fisher) or puromycin (Invivogen). EZH2 expressing and GSK3 $\beta$  knockdown stable transfectants were validated by western blot analysis.

### **2-3 Whole cell lysis and subcellular fractionation**

For whole cell lysis, 80 ~ 90% confluent cells were washed with cold PBS buffer twice and lyzed with modified RIPA buffer (50 mM Tris-HCl at pH 7.6, 150 mM NaCl, 1% NP-40, 0.1% SDS) supplemented with 25 mM NaF, 2 mM Na<sub>3</sub>VO<sub>4</sub> and protease inhibitor cocktail (1:100 dilution, Biotool). Lyzed cells were then harvested, sonicated and centrifuged. The supernatant after centrifugation was collected as cell lyate and its protein concentration was determined by Bio-Rad protein assay kit (Pierce) using bovine serum albumin (BSA) as a standard.

For subcellular fractionation, cells were washed and firstly lyzed with Nori buffer (20 mM HEPES at pH 7.0, 10 mM KCl, 2 mM MgCl<sub>2</sub>, 0.5% NP-40) supplemented with 25 mM NaF, 2 mM Na<sub>3</sub>VO<sub>4</sub> and protease inhibitor cocktail (1:100 dilution, Biotool). After incubation for 30 min, the lyzed cells were



homogenized by Dounce homogenizer for 30 strokes. Homogenized samples were then centrifuged. The supernatant part was saved as non-nuclear (or cytosolic) fraction. The rest part was further lysed with modified RIPA buffer (50 mM Tris-HCl at pH 7.6, 150 mM NaCl, 1% NP-40, 0.1% SDS) supplemented with 25 mM NaF, 2 mM Na<sub>3</sub>VO<sub>4</sub> and protease inhibitor cocktail. The nuclear fraction was harvested after sonication and centrifugation. Protein concentration was then determined.

#### **2-4 Western blot and immunoprecipitation**

For western blot analysis, cell lysate samples were diluted in sample buffer and denatured by heating at 95 °C for 5 min. Equal amount of cell lysates in same volume was subjected into SDS-polyacrylamide gel electrophoresis. Proteins separated by SDS-PAGE were then transferred onto PVDF membrane. After blocking with non-fat 5% milk/TBST, PVDF membrane was incubated with primary antibodies at 4 °C for overnight and subsequently incubated with HRP secondary antibody at room temperature for 1hr. After wash, PVDF membrane was shortly immersed in enhanced chemiluminescence (ECL) solution mixture. The signals on PVDF membrane were visualized by developing on autography film. Quantification of the band density of target proteins in western blot experiments was analyzed by free software ImageJ.

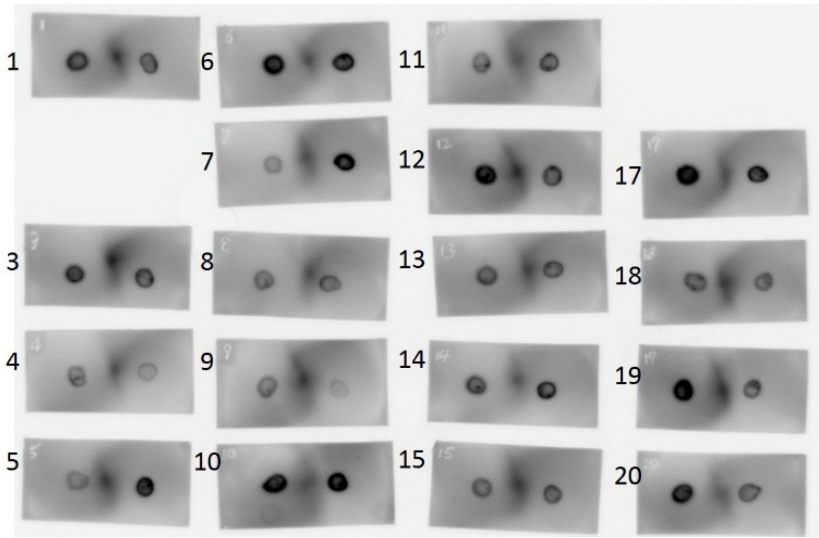
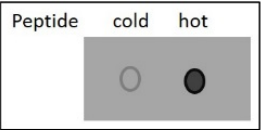
For immunoprecipitation assay, the protein amount of cell lysates and antibody were used at a ratio of 1mg: 5µg for each reaction. Cell lysates were incubated with immunoprecipitation antibodies at 4°C overnight, followed by addition with 50 µl of 50% protein G sepharose beads per 1 mg cell lysates and

continuous incubation for 4 hr. After washed with modified RIPA buffer without 0.1% SDS three to five times, beads were suspended in sample buffer and boiled at 95 °C for 5 min. Immunoprecipitates were then subjected into SDS-polyacrylamide gel and analyzed by western blotting.

## **2-5 Antibodies and phosphorylation antibody generation / screening**

The following antibodies were used in western blotting and immunoprecipitation: EZH2, phospho-GSK3 $\beta$  Ser9, trimethyl-H3K27, dimethyl-H3K27 and histone H3 (Cell Signaling Technology); monomethyl-H3K27 (EMD Millipore), GSK3 $\beta$  (BD Biosciences); dephospho- $\beta$ -catenin (Ezno Life Sciences); Tubulin and Actin (Sigma); Lamin B1 (Abcam); Myc and HA (Roche). The mouse phospho-EZH2 Ser363 and phospho-EZH2 Thr367 antibodies were produced against the synthetic peptides RLPNNS(pS)RPSTPTI and SRPS(pT)PTINVLESKD at China Medical University Hospital in Taiwan, respectively. The synthetic peptides were obtained from LifeTein LLC. Twenty mice were immunized for each phosphorylation antibody generation. Each mouse has been injected with phosphopeptide (hot peptide) for five times. After immunization, eighteen mouse sera against Ser363 phosphorylation and twenty sera against EZH2 Thr367 phosphorylation were obtained. All mouse sera were screened by dot blot analysis using corresponding phosphorylated (hot) and nonphosphorylated (cold) peptide (Figure 2-1 and 2-2).

**Figure 2-1. Dot blot analysis of mouse antisera generated for Ser363 phosphorylation polyclonal antibody**

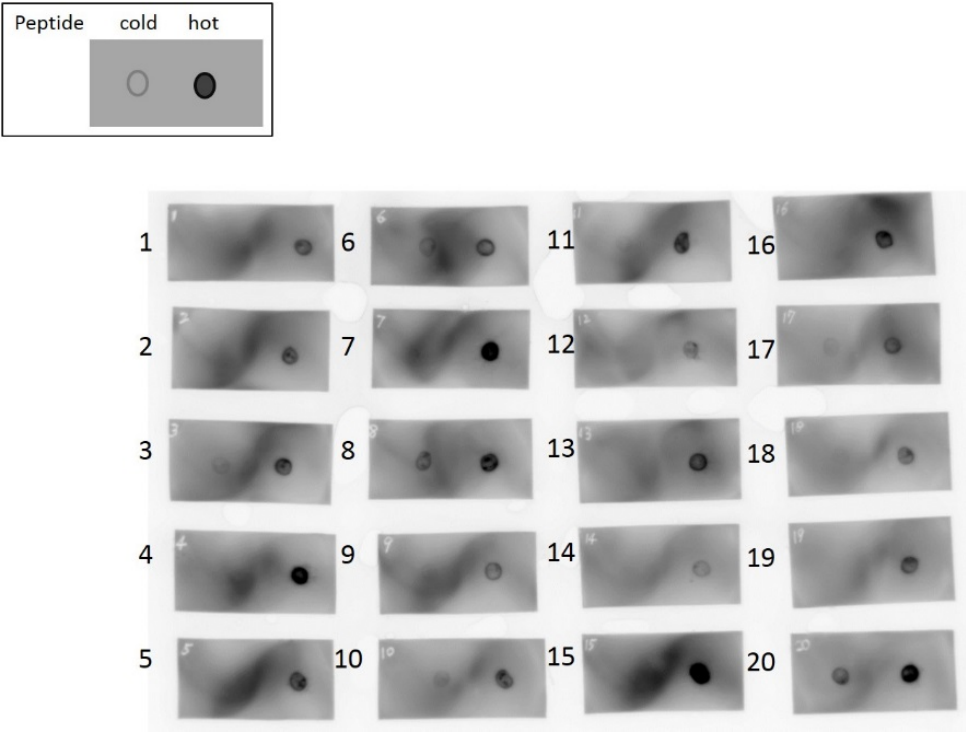


**Figure 2-1. Dot blot screening for mouse sera against phospho-EZH2 Ser363 peptide.**

Dot blot analysis was performed using PVDF membrane. Phospho-EZH2 at Ser363 (hot) and non-phospho-EZH2 at Ser363 (cold) peptides (100ng/5ul/each) were spotted on the membrane as illustrated. Each membrane containing hot and cold peptides was incubated with various mouse antiserum (1:2000 dilution in 5% BSA/TBST) as marked at 4 °C overnight. After incubation with mouse secondary antibody and ECL, the signals on PVDF membrane were acquired using the ImageQuant LAS 4000 mini system (GE Healthcare Life Science, Pittsburgh, PA).

In this screening, only #5 and #7 mouse sera were able to distinguish hot peptide from cold one. These two antisera were tested in a subsequent *in vitro* kinase assay. Mouse antiserum #7 nonspecifically recognized phosphorylated wild-type GST-EZH2, GST-EZH2<sup>S363A</sup> and GST-EZH2<sup>T367A</sup> (data not shown). The test result of #5 antiserum is shown in Figure 3-2J.

**Figure 2-2. Dot blot analysis of mouse antisera generated for Thr367 phosphorylation polyclonal antibody**



## **2-2. Dot blot screening for mouse sera against phospho-EZH2 Thr367 peptide.**

Dot blot analysis was performed using PVDF membrane. Phospho-EZH2 at Thr367 (hot) and non-phospho-EZH2 at Thr367 (cold) peptides (100ng/5ul/each) were spotted on the membrane as illustrated. Each membrane containing hot and cold peptides was incubated with various mouse antiserum (1:2000 dilution in 5% BSA/TBST) as marked at 4 °C overnight. After incubation with mouse secondary antibody and ECL, the signals on PVDF membrane were acquired using the ImageQuant LAS 4000 mini system.

The screening shows that many of mouse sera were able to distinguish Thr 367 EZH2 hot peptide from cold one. Some of them were tested in an *in vitro* kinase assay. Mouse antiserum #7 was the best one and its test result is shown in Figure 3-2K.

## **2-6 qRT-PCR**

Total RNA was extracted from cells for qRT-PCR assays. Cells were washed with PBS twice and lyzed in TRIzol (Life Technologies). Phase separation of lyzed sample was carried out by chloroform and colorless aqueous phase of sample were collected. RNA was then precipitated by isopropyl alcohol, washed with 75% ethanol and dissolved in DEPC-treated water. cDNA was synthesized for measurement of mRNA expression from extracted total RNA using SuperScript III First-strand synthesis system by random hexamers (Life Technologies) following the manufacturer's protocol. Quantitative real-time PCR (qRT-PCR) was performed using SYBR Green dye on a qRT-PCR machine (iQ5, BioRad, CA, USA). GAPDH mRNA expression was used as internal control. The primer sequences used for analysis of HOXA gene expression was listed in Table 2-1.

**Table 2-1. Primers for qRT-PCR**

<b>Table 2-1. Primers for qRT-PCR</b>		
<b>Gene name</b>	<b>Forward primer</b>	<b>Reverse primer</b>
HOXA2	ACAGCGAAGGGAAATGTAAAAGC	GGGCCCCAGAGACGCTAA
HOXA3	TGCAAAAAGCGACCTACTACGA	CGTCGGCGCCCAAAG
HOXA7	CAAAATGCCGAGCCGACTT	TAGCCGGACGCAAAGGG
HOXA9	CCGAGAGGCAGGTCAAGATC	AAATAAGCCCAAATGGCATCA
HOXA13	AAATGTACTGCCCCAAAGAGCA	ATCCGAGGGATGGGAGACC
GAPDH	TCCACTGGCGTCTTCACC	GGCAGAGATGATGACCCTTTT



## 2-7 Plasmids

Plasmids of HA-GSK3 $\beta$ -WT (wild-type), HA- GSK3 $\beta$ -CA (constitutively active, S9A GSK3 $\beta$ ), and HA- GSK3 $\beta$ -KD (kinase dead, K85R GSK3 $\beta$ ) were described previously (Ding, He et al. 2007). pCDNA3-Myc-EZH2 was a gift from A. Chinnaiyan. For EZH2 stable transfection, two EZH2 constructs were generated. Myc-EZH2 was subcloned into the vector of pCDH-CMV-MCS-EF1-Puro (System Biosciences), and Flag-EZH2 was subcloned into the vector of pCDH-CMV-MCS-EF1-Neo (System Biosciences). To generate constructs for bacterial expression of GST-tagged EZH2, two truncations were made in GST-EZH2 fusion protein format in pGEX-6P1 vector (Amersham Biosciences or GE Healthcare). One EZH2 truncation, N-terminal fragment, was from amino acid residues 1-385, and another, C-terminal fragment, was from 386-746. Site-directed mutagenesis was performed to generate mutant EZH2 according to a protocol (Liu and Naismith 2008). Primers used for mutagenesis were provided in Table 2-2. GSK3 $\beta$  knockdown was carried out by pGIPZ-shRNA with the target sequence of 5'-TACTTGACAGTTCTTGAGT-3' (CDS, Clone ID V3LHS\_309039, shRNA core facility, MD Anderson Cancer Center).

**Table 2-2. Primers for site-directed mutagenesis**

Table 2-2. Primers for mutagenesis		
Target	Forward primer	Reverse primer
S362A	CAATAACGCTAGCAGGCCAGCACCCCCAC	CCTGCTAGCGTTATTGGGAAGCCGTCCTCTTC
S363A	CAATAACAGTGCCAGGCCAGCACCCCCAC	CCTGGCACTGTTATTGGGAAGCCGTCCTCTTC
S366A	CCCGCCACCCCCACCATTAATGTGCTGGAATC	GGGGGTGGCGGGCCTGCTACTGTTATTGGG
T367A	CCCAGCGCCCCACCATTAATGTGCTGGAATC	GGGGGCGCTGGGCCTGCTACTGTTATTGGG
2A	GCCAGGCCAGCGCCCCACCATTAATGTGCTG	GGCGCTGGGCCTGGCACTGTTATTGGGAAGCCGTC
S363E	CAATAACAGTGAAAGGCCAGCACCCC	CCTTTCAGTGTTATTGGGAAGCCGT
T367E	CCCAGCGAACCACCATTAATGTGCTG	GGGTTCGCTGGGCCTGCTACTG
2E	GAAAGGCCAGCGAACCACCATTAATGTGCTGG	TTGCTGGGCCTTTCAGTGTTATTGGGAAGCCGTC
S363D	CAATAACAGTGACAGGCCAGCACCCC	CCTGTCACTGTTATTGGGAAGCCGT
T367D	CCCAGCGACCCACCATTAATGTGCT	GGGGTCGCTGGGCCTGCTACTG
2D	GACAGGCCAGCGACCCACCATTAATGTGCTG	TCGCTGGGCCTGTCACTGTTATTGGGAAGCCGTC

## **2-8 *in vitro* kinase assay**

Recombinant, active GSK3 $\beta$  kinase was obtained from Life Technologies and glutathione-S-transferase (GST)-fused full length EZH2 was purchased from BPS Bioscience. GST-EZH2 C-terminal, wild-type and mutant GST-EZH2 N-terminal fragments were purified from bacteria. For the GSK3 $\beta$  *in vitro* kinase assay, active GSK3 $\beta$  kinase was incubated with wild-type or mutant GST-EZH2 purified proteins in kinase buffer (50 mM Tris-HCl at pH 7.6, 10 mM MgCl<sub>2</sub>, 2 mM DTT and 0.1 mM EDTA) in the presence of 5  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P] ATP and 50  $\mu$ M cold ATP with substrates at 30 °C for 30 min. Reaction mixtures were then subjected to SDS-PAGE, and <sup>32</sup>P-labelled proteins were detected by autoradiography.

## **2-9 GST protein purification**

GST-EZH2 N-terminal and C-terminal fragment proteins were purified using BL21 E.coli. BL21 competent cells were transformed with plasmids encoding GST-EZH2 N-terminal or C-terminal fragments. After heat shock, transformed cells were plated on Ampicillin-containing LB-Agar plate and incubated at 37 °C for overnight. Next day, one single colony from each GST-EZH2 transforming clone growing on LB-Agar plate was picked up and continued to grow in one 10-cm culture tube with 5 ml of Ampicillin-containing LB medium at 37 °C and 200 rpm shaking for overnight. The 5-ml bacterial culture was diluted into 45 ml of LB medium next day and continued to grow in the same condition until an OD value of 0.5 is reached. IPTG Isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) was then added into 50-ml bacterial culture at a concentration of 0.2mM for protein induction. The bacterial

culture was incubated at 30 °C and 200 rpm. After 3 hr, the bacterial culture was centrifuged at 6000 rpm for 15 min. The bacterial pellets were collected and lysed in RIPA buffer containing cocktail protease inhibitor, 1mM lysozyme, 1mM DNase and 1mM DTT. After sonication and centrifugation, the supernatant from lysed bacterial pellets was collected and protein concentration was determined by protein assay kit using BSA as a standard. To extract GST proteins, the supernatant was incubated with GST beads at a ratio of 100ul/5mg (beads/proteins) at 4°C for 2 hr. GST-EZH2 proteins were eluted from GST beads with 20mM glutathione at 4°C. The eluents were subjected into SDS-PAGE, followed by Coomassie blue staining for quantification.

## **2-10 Mass spectrometry analysis**

To identify phosphorylation sites of EZH2, mass spectrometry analysis was performed as previously described (Wei, Chen et al. 2011). Briefly, truncated GST-EZH2 purified proteins, GST-EZH2 N-terminal (a.a. 1-385) and C-terminal (a.a. 386-746) fragments, were incubated with GSK3 $\beta$  kinase in a kinase reaction mixture at 30 °C for 30 min. After being resolved by SDS-PAGE, the protein band corresponding to EZH2 was excised and subjected to digestion with trypsin. The phosphopeptides were then isolated by immobilized metal affinity chromatography. The micro-liquid chromatography/tandem mass spectrometry (LC–MS/MS) was used to analyze the phosphopeptides through an Ultimate capillary LC system (LC Packings) coupled with a time-of-flight (TOF) mass spectrometer (Applied Biosystems). Carbamidomethyl cysteine was used as the control modification,

whereas serine, threonine, and tyrosine phosphorylation were set as variables. The identified phosphopeptides were further validated via manual interpretation of the product ion spectra from LC–MS/MS.

### **2-11 *in vitro* methylation assay**

Lysates of cells stably expressing Flag-tagged wild-type EZH2, nonphosphorylatable mutants, and phospho-mimic mutants were harvested. Flag-tagged EZH2 proteins were purified by monoclonal anti-Flag M2 affinity agarose beads (Sigma). For *in vitro* methylation assay, 30ul of beads containing Flag-EZH2 were mixed with 2ug of recombinant histone H3 (New England Biolabs) or nucleosome (BPS Bioscience) and 1 mM of S-adenosylmethionine (SAM; New England Biolabs) as methyl group donors in methylation buffer (50 mM Tris-HCl at pH 8.0 and 10 mM DTT) and incubated at 30 °C for 1 hr. The supernatant of reaction mixtures were resolved by 15% SDS-PAGE gel and immunoblotting with antibodies against methylation of histone H3. The rest beads were suspended in sample buffer, boiled at 95 °C for 5 min, and subjected to SDS-PAGE for analysis of EZH2 protein expression.

### **2-12 3D soft agar and 2D clonogenic assays**

For the soft agar colony formation assay,  $2.5 \times 10^4$  cells were seeded in 1 ml of regular medium with 0.5% low melting point agarose and overlaid on 1 ml of medium with 1% agarose in each well of a six-well plate. After 3 weeks, colonies

were fixed and stained with crystal violet. Colonies larger than 100  $\mu\text{m}$  in diameter were counted under a microscope for quantitation.

For the clonogenic assay, 200 cells per well were seeded in a 6-well plate and grown in regular medium for 10 days. Cell colonies were fixed and stained with crystal violet, and then counted for quantitation.

### **2-13 Cell migration assay**

The wound healing assay was performed using a culture-insert (ibidi GmbH, Germany) according to manufacturer's instruction. The culture-insert had two cell culture reservoirs, which were separated by a 500  $\mu\text{m}$ -thick wall. Same numbers of cells were seeded in the culture-insert. After 24 hr, the culture-insert was removed, which left a cell-free "wound" of around 500  $\mu\text{m}$  in width. The wound closure was observed by a time lapse microscopy (Zeiss, Germany) and images were obtained at 1 h interval for 24 hr. The area of wound was analyzed using the ImageJ software program.

Migration abilities were also determined using 24-well Boyden chamber plates with an 8  $\mu\text{m}$  pore size polycarbonate filter (BD Biosciences).  $1 \times 10^5$  cells were seeded into upper chambers with serum-free medium. Lower chambers contained 10% FBS medium. After incubation at 37 °C for 24 h, chamber filters were fixed and stained. Cells on the top of filters were removed. Numbers of migrated cells were counted under a light microscopy.

## **2-14 Animal experiment**

Tumorigenesis assay was carried out using an orthotopic mouse model. MCF12A cells with stable expression of EZH2-WT, EZH2-S363A, EZH2-T367A, EZH2-2A, or EZH2-2E were exploited in the experiment. Two millions of each stable line were injected into the mammary fat pads of six-week-old nude mice (five mice per group). Tumor size was measured twice a week with calipers, and tumor volume was determined using the formula  $0.5 \times L \times W^2$ , where L is the longest diameter and W is the shortest diameter. The animal protocol was reviewed with the approval of the Institutional Animal Care and Use Committee (IACUC) at The University of Texas MD Anderson Cancer Center. All animal procedures were performed according to the regulations of the Division of Laboratory Animal Medicine at The University of Texas MD Anderson Cancer Center.

## **2-15 Immunohistochemical staining**

Immunohistochemical (IHC) staining was performed using an immunoperoxidase-based staining method. One hundred and ten human breast cancer tissue specimens were obtained from the Department of Pathology, Shanghai East Breast Disease Hospital, Shanghai, P.R. China. Tissue microarray (TMA) slides were prepared from the paraffin-embedded breast tumor samples. Each specimen was stained with specific antibodies against pS9-GSK3 $\beta$  (Cell Signaling Technology) and H3K27 trimethylation (EMD Millipore) and scored by an H-score method as previously described (Ding, He et al. 2007). The intensity of

staining based on histological scoring was ranked into: high (score 3), medium (score 2), low (score 1), and negative (score 0). High and medium intensity of staining were grouped into “high expression”. Low and negative were “low expression”.

## **2-16 Statistical analysis**

SPSS software (version 22, IBM) was used for statistical analysis. To analyze categorical variables between the two groups, a Pearson  $\chi$ -squared test was used to examine the relationship between pS9-GSK3 $\beta$  expression and H3K27 trimethylation levels. A p-value of less than 0.05 is considered statistically significant.



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## **Chapter 3**

### Results

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### **3-1 GSK3 $\beta$ negatively regulates H3K27 trimethylation**

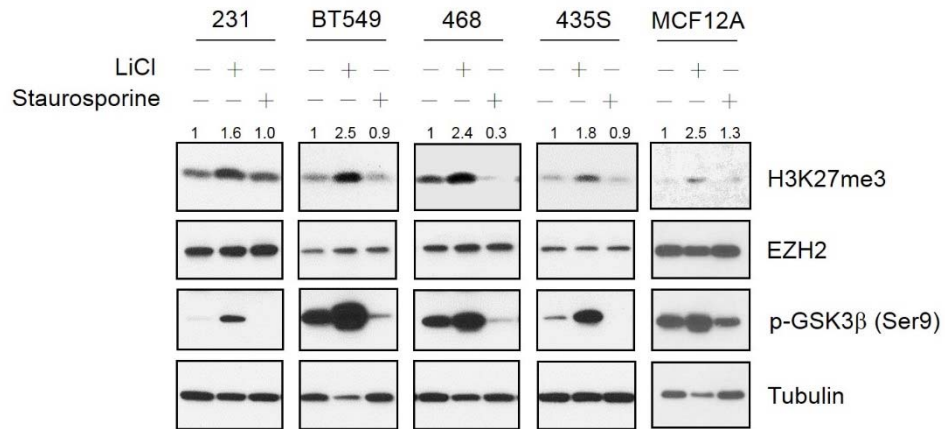
EZH2 is a histone methyltransferase and has been reported to modulate tumor initiating cell expansion and cancer cell invasion, migration through catalyzing trimethylation of H3K27 (Chang, Yang et al. 2011, Ren, Baritaki et al. 2012). EZH2 is highly expressed in many solid tumors (Bachmann, Halvorsen et al. 2006). Overexpression of EZH2 promotes malignant transformation and tumor growth (Kleer, Cao et al. 2003, Karanikolas, Figueiredo et al. 2009). These evidence suggests that EZH2 functions as a tumor promoter in carcinogenesis. In addition, GSK3 $\beta$ , a serine/threonine kinase, has been demonstrated to negatively regulate neoplastic transformation and tumorigenesis (Luo 2009). It has also been shown to control cancer cell metastasis through mediating inactivation of EMT regulators (Zhou, Deng et al. 2004). Since EZH2 and GSK3 $\beta$  have common functions in tumorigenesis, we speculated whether GSK3 $\beta$  regulates EZH2 expression or activity.

To investigate the regulation of EZH2 activity by GSK3 $\beta$ , we first determined whether alteration of GSK3 $\beta$  activity affects H3K27 trimethylation. We found that inhibition of GSK3 $\beta$  by lithium chloride, a GSK3 $\beta$  inhibitor, increased H3K27 trimethylation expression in breast cancer and mammary epithelial cells, and conversely, activation of GSK3 $\beta$  using the anticancer drug staurosporine reduced the H3K27 trimethylation level in MDA-MB-468, MDA-MB-435S and BT549 cells (Figure 3-1A). There was no change in EZH2 level. Consistently, knockdown of GSK3 $\beta$  by small hairpin RNA enhanced trimethylation of H3K27 in HeLa cells (Figure 3-1B). Exogenous expression of the wild type or constitutively active form

GSK3 $\beta$  decreased H3K27 trimethylation expression in HeLa cells (Figure 3-1C). HOX genes are well-known EZH2 target genes (Lee, Jenner et al. 2006). We therefore examined the expression of EZH2 target genes in the HOXA family. We found that lithium chloride downregulated the expression of many of HOXA genes (Figure 3-1D). Together, these results suggested that GSK3 $\beta$  negatively regulates the expression of H3K27 trimethylation and EZH2-targeted genes.

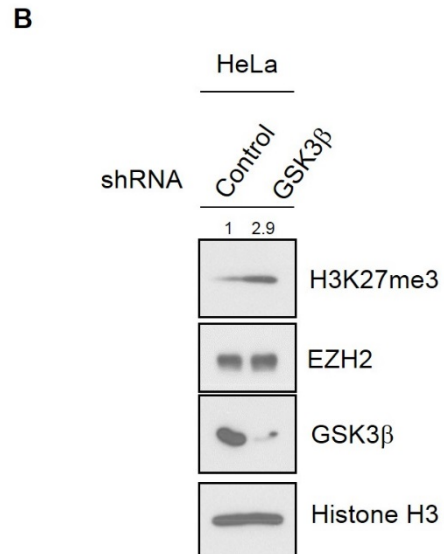
**Figure 3-1. GSK3 $\beta$  downregulates H3K27 trimethylation and EZH2 targeted genes, HOXA genes.**

**A.**



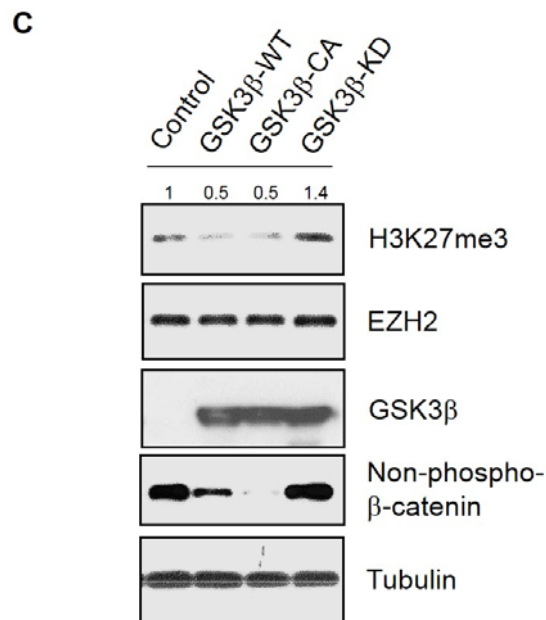
**A. Alteration of GSK3 $\beta$  activity changes H3K27 trimethylation.** MDA-MB-231, BT549, MDA-MB-468, MDA-MB-435S, and MCF12A cells were treated with lithium chloride (LiCl), staurosporine (STS) as indicated. Cell lysates were subjected to western blot analysis with the indicated antibodies. The intensities of H3K27me3 bands from treated cells were compared to those from untreated cells and the relative ratios are shown.

**Figure 3-1**



**B. Inhibiting GSK3 $\beta$  by knockdown of GSK3 $\beta$  upregulates H3K27 trimethylation.** HeLa cells were infected with lentiviruses expressing control or GSK3 $\beta$  shRNA. Cells were lysed and analyzed by immunoblot with antibodies against indicated proteins. Relative intensities of H3K27me3 bands are shown.

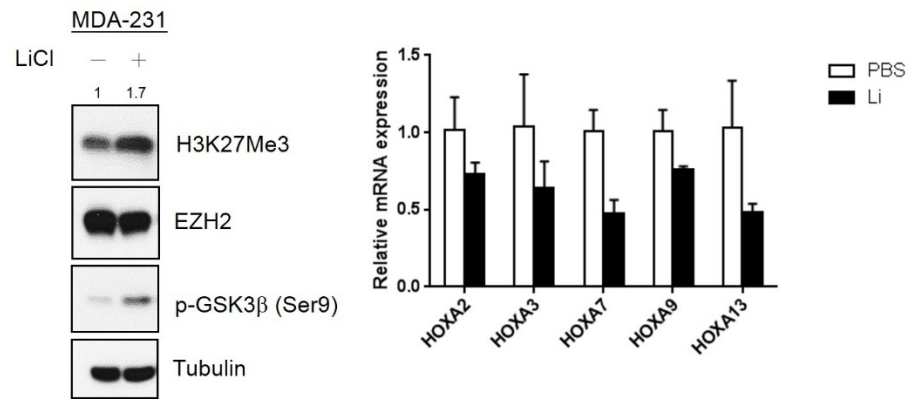
**Figure 3-1**



**C. Activating GSK3 $\beta$  activity by overexpression of wild-type of active GSK3 $\beta$  reduces H3K27 trimethylation.** HeLa cells were transfected with the plasmids encoding wild-type (WT), constitutively active (CA), kinase-dead (KD) GSK3 $\beta$  or empty vector control. Equal amounts of cell lysates were analyzed by western blot using antibodies against specific proteins. Relative intensities of H3K27me3 bands are shown, normalized to the intensity of H3K27me3 band from HeLa cells transfected with control plasmid.

**Figure 3-1**

**D**



**D. Inhibiting GSK3 $\beta$  activity by lithium chloride downregulates HOXA genes.**

Left: Western blot analysis of lysates from MDA-MB-231 cells treated with PBS or lithium chloride. Lysates were immunoblotted with the indicated antibodies. Relative intensities of H3K27me3 bands are shown. Right: qPR-PCR analysis of relative mRNA expression of HOXA family genes in lysates from MDA-MB-231 cells treated with PBS or lithium chloride. Data are expressed as mean  $\pm$  s.d. (n = 3).

### 3-2 GSK3 $\beta$ interacts with and phosphorylates EZH2

Several proteins have been found to be phosphorylated by GSK3 $\beta$  and GSK3 $\beta$  usually attenuates the activity of its substrates by this regulation. GSK3 $\beta$  is a serine/threonine kinase and has a preference for its substrates through recognizing a phosphorylation motif, Ser/Thr-X-X-X-Ser/Thr, where X represents any amino acid proteins (Sutherland 2011). Interestingly, we noticed that EZH2 amino acid sequence contains this motifs, suggesting that EZH2 is a potential substrate of GSK3 $\beta$ . Since altering GSK3 $\beta$  activity affects the trimethylation of H3K27 and the expression of EZH2-targeted genes, we next investigated whether GSK3 $\beta$  interacts with and phosphorylates EZH2. To address this possibility, we first performed a co-immunoprecipitation experiment, which demonstrated an association between endogenous GSK3 $\beta$  and EZH2 in MCF12A cells (Figure 3-2A). Because EZH2 functions primarily in nucleus and GSK3 $\beta$  exists mainly in the cytosol, we further examined subcellular localization of their interaction. We performed subcellular fractionation and found, indeed, EZH2 is predominantly present in the nucleus, whereas GSK3 $\beta$  is found mostly in the cytosol (Figure 3-2B, right panel). Surprisingly, we detected the GSK3 $\beta$ -EZH2 interaction mainly in the cytosol (Figure 3-2B, left panel). These results suggested that GSK3 $\beta$  physically interacts with EZH2 in the cytosol.

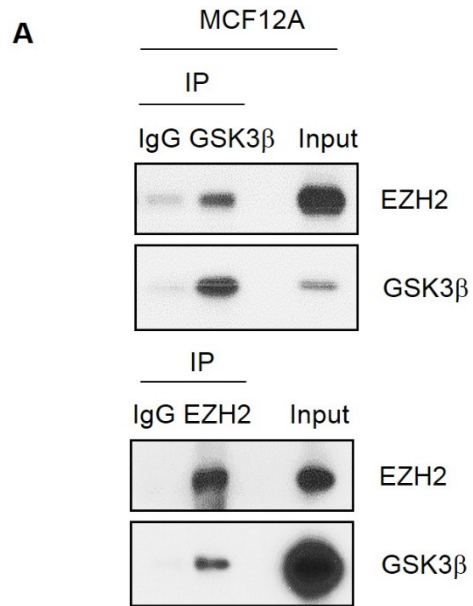
Next, we examined whether GSK3 $\beta$  can phosphorylate EZH2. An *in vitro* kinase assay revealed that GSK3 $\beta$  catalyzed the phosphorylation of EZH2 but not glutathione-S-transferase (GST) (Figure 3-2C, lane 1-3). Mass spectrometry



analysis using truncated GST-EZH2 N-terminal and C-terminal fragments identified 4 phosphorylation sites. On N-terminal fragment, two phosphorylation sites were identified, one is either Ser362 or Ser363; another is Ser366 or Thr367 (Figure 3-2D). The rest 2 other sites were identified on C-terminal fragment, one is Thr386 or Thr388 (Figure 3-2E), the other is Ser620 (Figure 3-2F). Since this phosphorylation was catalyzed primarily on EZH2's N-terminal fragment, rather than its C-terminal fragment (Figure 3-2C, lane 4-6), we then focused on investigating the possible 4 sites identified on N-terminal fragment. We replaced these 4 residues with alanine individually. An *in vitro* kinase assay demonstrated that the phosphorylation catalyzed by GSK3 $\beta$  was reduced at the Ser363A mutant and was nearly undetectable at the Thr367A mutant (Figure 3-2G), which suggests that these two residues are GSK3 $\beta$  phosphorylation sites on EZH2. As Thr367A mutant totally abolished GSK3 $\beta$  phosphorylation but Ser363 did not, we reasoned that Thr367 phosphorylation may be required for Ser363 phosphorylation. To test this hypothesis, we generated phosphomimic Thr367 mutants, Thr367D and Thr367E. We also generated a double A (Ser363A & Thr367A) mutant for further validation. In the experiment of *in vitro* kinase assay, we did not observe the phosphorylation catalyzed by GSK3 $\beta$  at the Thr367D and Thr367E mutants, but consistently, the phosphorylation catalyzed by GSK3 $\beta$  was virtually undetectable in double A mutant (Figure 3-2H). A search of National Center for Biotechnology Information (NCBI) database using Basic Local Alignment Search Tool (BLAST) revealed that Ser363 and Thr367 of EZH2 are highly conserved across species (Figure 3-2I).

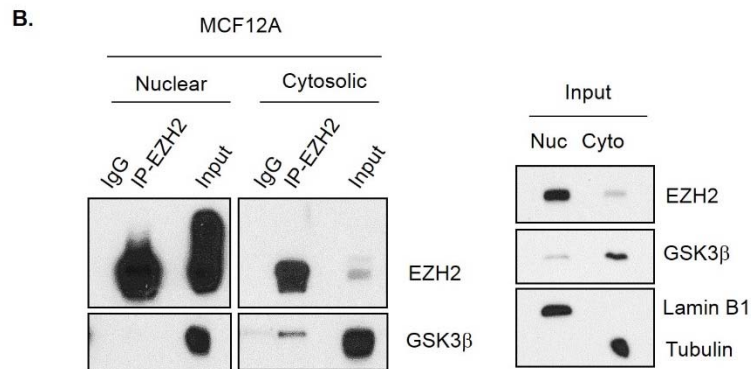
To confirm the phosphorylation of EZH2 by GSK3 $\beta$  *in vivo*, we generated specific mouse antisera against the phosphorylated EZH2 at Ser363 and Thr367 separately. After dot blot screening (Figure 2-1 and 2-2), we validated the specificity of the antibody by an *in vitro* kinase assay using cold ATP. The mouse antiserum generated against Ser363 phosphorylation recognized GSK3 $\beta$ -catalyzed phosphorylation on wild-type GST-EZH2; however, it also identified non-phosphorylated wild-type GST-EZH2 or GST-EZH2<sup>S363A</sup> mutant (Figure 3-2J), suggesting that its specificity for Ser363 phosphorylation recognition is not good. This antibody did not work in the following experiments, neither. For mouse antisera against Thr367 phosphorylation, the antibody recognized the GSK3 $\beta$ -catalyzed phosphorylation on wild-type GST-EZH2 or GST-EZH2<sup>S363A</sup> mutant, but not the nonphosphorylatable GST-EZH2<sup>T367A</sup> mutant (Figure 3-2K), indicating that it can specifically identify Thr367 phosphorylation of EZH2. Using this antibody, we found that GSK3 $\beta$  enhanced the endogenous level of the phosphorylated EZH2 at Thr367 when GSK3 $\beta$  was overexpressed in HeLa cells (Figure 3-2L). Taken together, these data indicated that GSK3 $\beta$  phosphorylates EZH2 at Ser363 and Thr367.

**Figure 3-2. GSK3 $\beta$  physically associates with and phosphorylates EZH2**



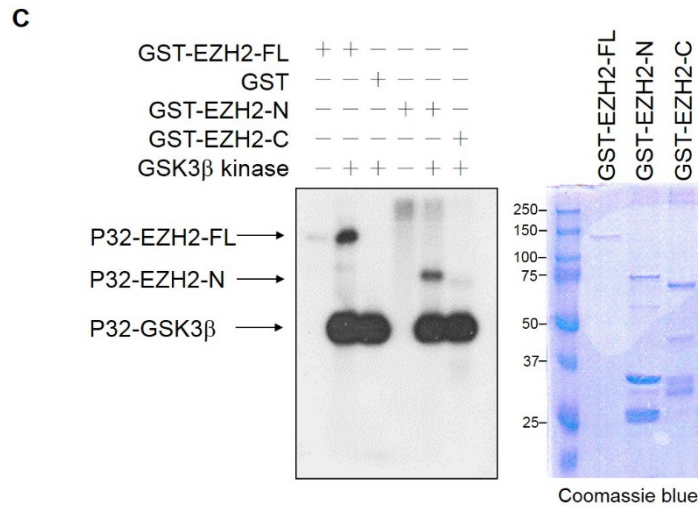
**A. Endogenous interaction between GSK3 $\beta$  and EZH2.** Cell lysates from MCF12A cells were immunoprecipitated by either anti-GSK3 $\beta$  (right panel) or anti-EZH2 (left panel) antibodies, then immunoblotted with indicated proteins.

**Figure 3-2**



**B. GSK3β interacts with EZH2 mainly in cytosol.** MCF12A cells were lysed and followed by cellular fractionation. Nuclear (Nuc) and cytosolic (Cyto) fractions were immunoprecipitated with anti-EZH2 antibody and immunoblotted by antibodies against EZH2 and GSK3β. Lamin B1 and Tubulin were used as markers for nuclear and cytosolic fractions respectively.

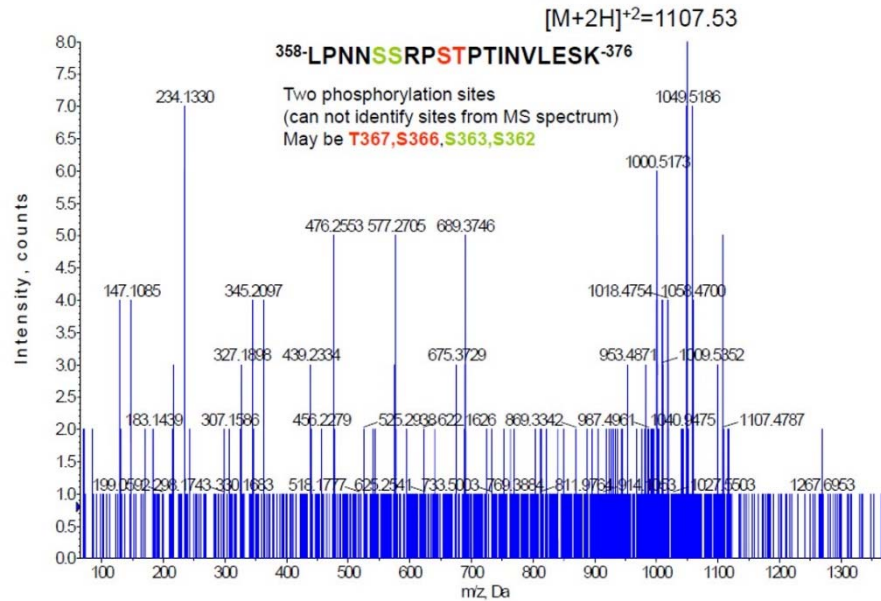
**Figure 3-2**



**C. GSK3 $\beta$  phosphorylates EZH2 *in vitro*, and GSK3 $\beta$  phosphorylation of EZH2 is primarily on EZH2 N-terminal fragment.** *in vitro* kinase assay was performed with recombinant, active GSK3 $\beta$  kinase and full-length GST-EZH2 (FL), GST-EZH2 N-terminal (a.a. 1-385; GST-EZH2-N), or C-terminal (a.a. 386-746; GST-EZH2-C) fragment. The phosphorylation was detected by autoradiography. Loading amount of different EZH2 proteins was accessed by coomassie blue staining.

Figure 3-2

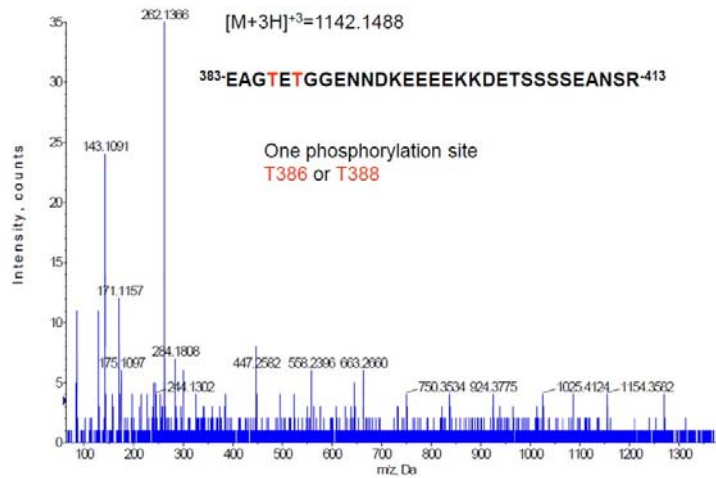
D.



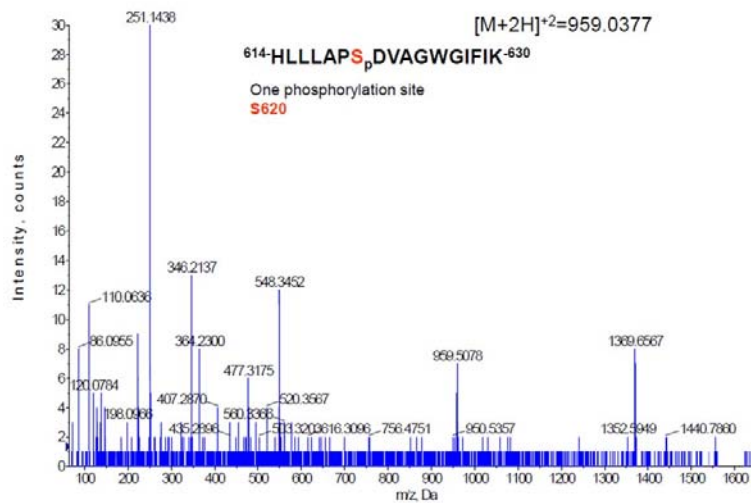
**D. Mass spectrometry analysis of GSK3 $\beta$ -phosphorylated GST-EZH2 N-terminal fragment.** The samples from an *in vitro* kinase assay with GSK3 $\beta$  kinase and GST-EZH2-N (a.a. 1-385) were used for mass spectrometry analysis. The spectrum shows that two phosphorylation sites were identified; one is either T367 or S366 (marked in red), another is S363 or S362 (marked in green).

Figure 3-2

E.



F.

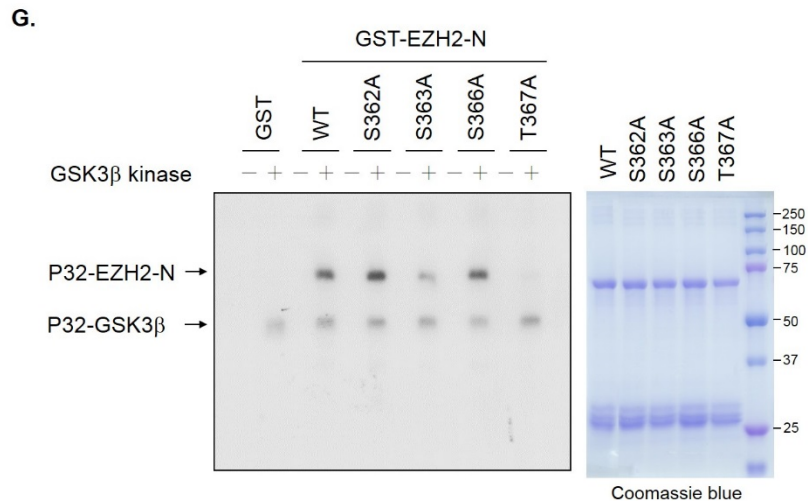


**E & F. Mass spectrometry analysis of GSK3 $\beta$ -phosphorylated GST-EZH2 C-terminal fragment.** The samples from *in vitro* kinase assays with GSK3 $\beta$  kinase

and GST-EZH2-C (a.a. 386-746) were used for analysis. This analysis identified two phosphorylation sites; one is either T386 or T388 (**E**), another is S620 (**F**).

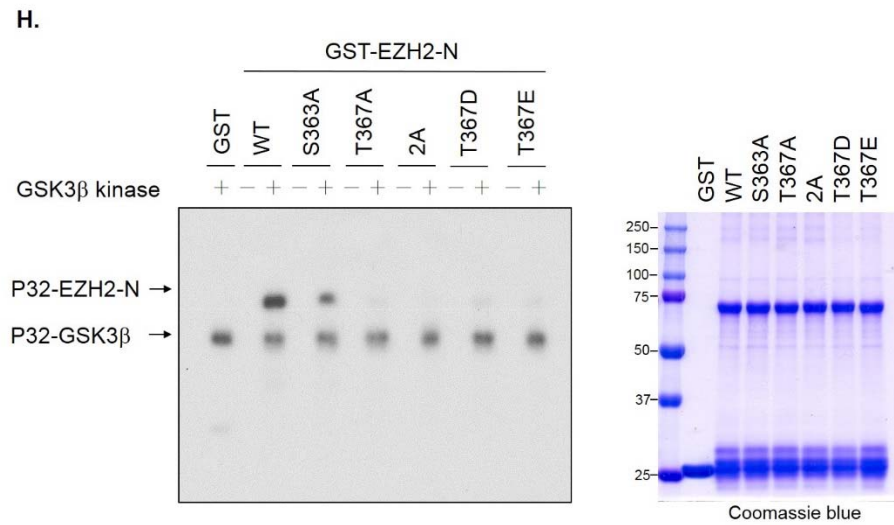


**Figure 3-2**



**G. GSK3 $\beta$  phosphorylates EZH2 at Ser363 and Thr367.** *in vitro* kinase assay was carried out with active GSK3 $\beta$  kinase and wild-type GST-EZH2 N-terminal fragment (WT), EZH2<sup>S362A</sup>-N, EZH2<sup>S363A</sup>-N, EZH2<sup>S366A</sup>-N, or EZH2<sup>T367A</sup>-N. The phosphorylation was examined by autoradiography. Loading of GST-EZH2 N-fragment was accessed by coomassie blue staining.

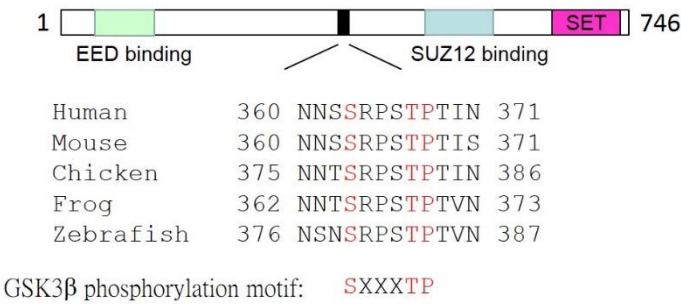
**Figure 3-2**



**H. Phospho-mimic T367-EZH2 does not facilitate GSK3 $\beta$  phosphorylation of EZH2 *in vitro*.** *in vitro* kinase assay was performed with active GSK3 $\beta$  kinase and wild-type (WT) or mutant GST-EZH2 N-terminal fragments as indicated. The phosphorylation was examined by autoradiography. Loading of EZH2 proteins was accessed by coomassie blue staining. 2A represents S363A and T367A mutant.

**Figure 3-2**

**I.**



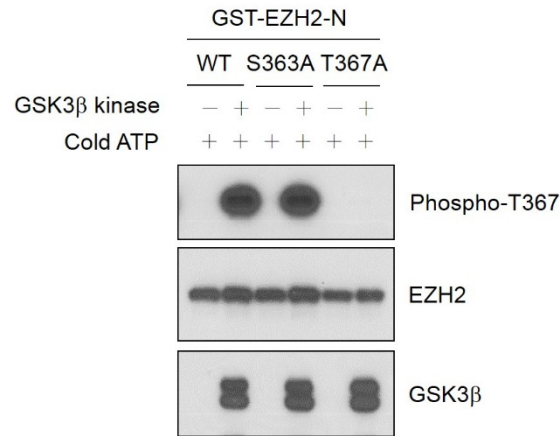
**I. Comparison of GSK3β phosphorylation sites of EZH2 among various species.**

**Figure 3-2**



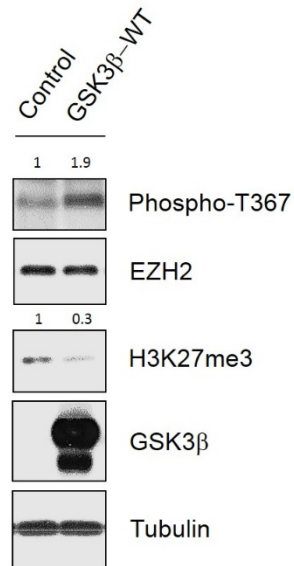
**J. S363 phosphorylation antibody recognizes non-phosphorylated EZH2 in addition to Ser363 phosphorylation.** Ser363 phosphorylation antibody was tested using the samples from an *in vitro* kinase assay with active GSK3 $\beta$  kinase and purified wild-type GST-EZH2-N (WT), GST-EZH2<sup>S363A</sup>-N or GST-EZH2<sup>T367A</sup>-N in the presence of cold ATP at 30°C for 30 min. Reaction mixtures were analyzed by western blot with mouse serum against Ser363 phosphorylation of EZH2 or antibodies as indicated. Eighteen mouse sera against Ser363 phosphorylation were screened by dot blot analysis. Two of them were able to distinguish phosphorylated (hot) peptide from nonphosphorylated (cold) one. Only one can recognize Ser363 phosphorylation, which is shown here.

**Figure 3-2**



**K. Validation of Thr367 phosphorylation antibody.** Thr367 phosphorylation antibody was tested using the samples from an *in vitro* kinase assay with active GSK3 $\beta$  kinase and purified wild-type GST-EZH2-N (WT), GST-EZH2<sup>S363A</sup>-N or GST-EZH2<sup>T367A</sup>-N in the presence of cold ATP at 30°C for 30 min. Reaction mixtures were analyzed by western blot with mouse serum against Thr367 phosphorylation of EZH2 or antibodies as indicated.

**Figure 3-2**



**L. GSK3 $\beta$  enhances endogenous T367 phosphorylation of EZH2.** Cell lysates from HeLa cells transfected with control or wild-type GSK3 $\beta$  were immunoblotted with Thr367 phosphorylation antibody or specific proteins. Relative intensities of Thr367 phosphorylation and H3K27me3 bands are shown, normalized to those from HeLa cells transfected with control plasmid.

### 3-3 GSK3 $\beta$ -mediated phosphorylation of EZH2 downregulates H3K27 trimethylation

Since GSK3 $\beta$  negatively regulates H3K27 trimethylation and phosphorylates EZH2 at Ser363 and Thr367, we next investigated whether GSK3 $\beta$  phosphorylation sites on EZH2 affects the expression of H3K27 trimethylation. MDA-MB-231 and MCF12A cells were transfected with plasmids expressing the wild-type EZH2, nonphosphorylatable, or phospho-mimic mutants. In MDA-MB-231 cells, we transfected with wild-type EZH2, EZH2<sup>2A</sup> or EZH2<sup>2E</sup> and found that EZH2<sup>2A</sup> increased H3K27 trimethylation levels and EZH2<sup>2E</sup> decreased its expression (Figure 3-3A). To examine the effects of individual phosphorylation sites on H3K27 trimethylation levels, MCF12A cells were expressed with EZH2<sup>S363A</sup>, EZH2<sup>T367A</sup>, EZH2<sup>2A</sup>, or wild-type EZH2. In MCF12A stable cells, trimethylation of H3K27 was upregulated in all nonphosphorylatable mutants (Figure 3-3B). Consistently, we observed the similar effects of phospho-mimic mutants on H3K27 trimethylation levels in MCF7 (Figure 3-3C) and MCF12A (Figure 3-3D) cells expressing EZH2<sup>S363E</sup>, EZH2<sup>T367E</sup>, or EZH2<sup>2E</sup>. These data suggests that GSK3 $\beta$  phosphorylation sites on EZH2 inversely affects the expression of H3K27 trimethylation.

To validate whether these phosphorylation sites' effect on the expression of H3K27 trimethylation is modulated by GSK3 $\beta$  activity, HeLa cells were co-transfected with plasmids encoding constitutively active GSK3 $\beta$  plus wild-type EZH2, or nonphosphorylatable mutant EZH2 (EZH2<sup>2A</sup>, EZH2<sup>S363A</sup> and EZH2<sup>T367A</sup>).

We found that and GSK3 $\beta$  activation decreased the trimethylation of H3K27 in wild-type EZH2 but not in mutants (Figure 3-3E).

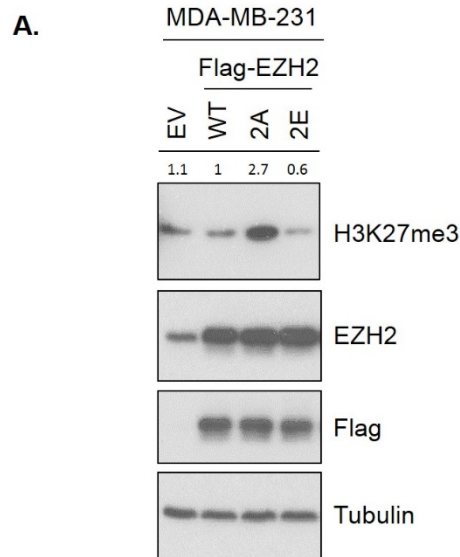
EZH2 is a histone methyltransferase and responsible for catalyzing trimethylation of H3K27. To further examine whether EZH2 phosphorylation by GSK3 $\beta$  changes the histone methyltransferase activity of EZH2, we performed an *in vitro* methylation assay using recombinant histone H3 peptides as substrates. Stable cells expressing wild-type and mutant Myc-EZH2 have been used for *in vitro* methylation experiments but they did not work. Flag-tagged EZH2 construct was then generated. NIH-3T3 cells were transfected with plasmids encoding wild-type Flag-EZH2, nonphosphorylatable (EZH2<sup>2A</sup>, EZH2<sup>S363A</sup> and EZH2<sup>T367A</sup>) or phospho-mimic mutants (EZH2<sup>2E</sup>, EZH2<sup>S363E</sup> and EZH2<sup>T367E</sup>). In these EZH2 stable lines, H3K27 trimethylation was consistently reduced in all phospho-mimic mutant EZH2 cells, and was, to a lesser extent, increased in nonphosphorylatable mutants (Figure 3-3F, right panel). *in vitro* methylation assay revealed that monomethylation of H3K27 catalyzed by phospho-mimic mutant EZH2 was reduced compared to that by wild-type EZH2, and dimethylation (data not shown) or trimethylation of H3K27 catalyzed by either wild-type or mutant EZH2 was similar (Figure 3-3F, left panel). EZH2 is able to catalyze methylation of histone H3 from monomethylation to trimethylation step by step. This data may not be drawn a conclusion, but provides a hint that GSK3 $\beta$  phosphorylation of EZH2 might suppress EZH2's histone methyltransferase activity.

As aforementioned, the association of EZH2 with SUZ12 and EED affects EZH2's histone methyltransferase activity. However, we did not observe an



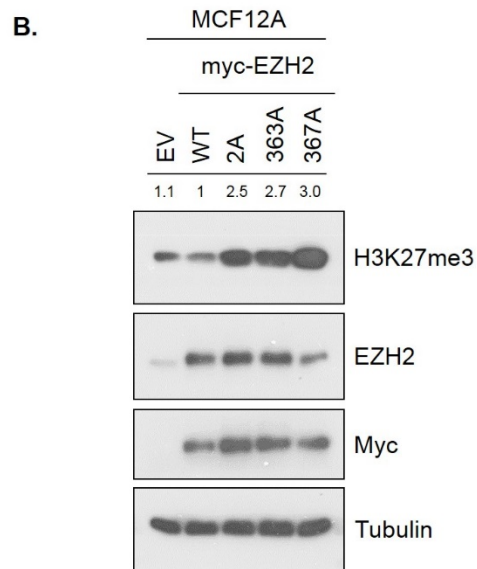
increased binding of EZH2 to SUZ12 or EED in MCF12A cells expressing nonphosphorylatable EZH2 (Figure 3-3G). Collectively, these data suggested that GSK3 $\beta$ -mediated phosphorylation of EZH2 negatively mediates H3K27 trimethylation, implying that this regulation may affect EZH2's enzymatic activity.

**Figure 3-3 GSK3 $\beta$ -mediated phosphorylation of EZH2 suppresses H3K27 trimethylation.**



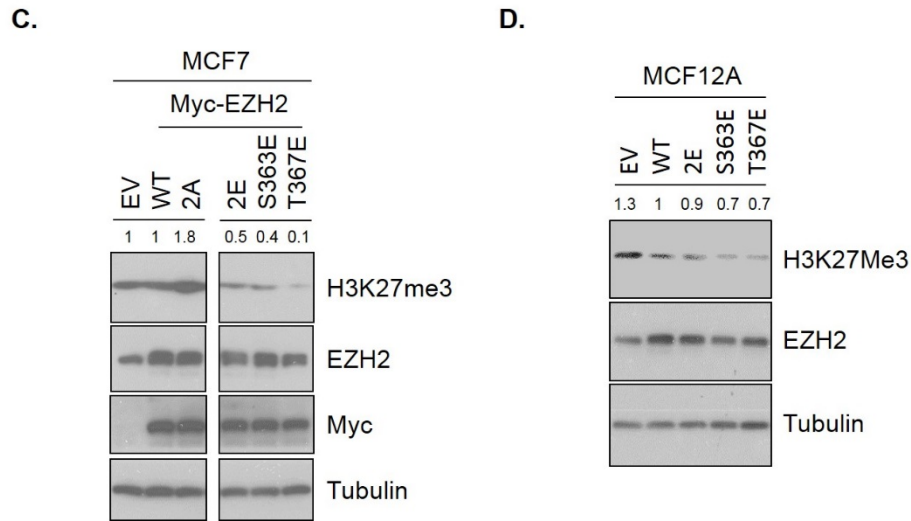
**A. GSK3 $\beta$  nonphosphorylatable 2A mutant increases H3K27me3 level and phospho-mimic 2E mutant decreases it in MDA-MB-231 cells.** MDA-MB-231 cells were stably transfected with plasmids encoding wild-type EZH2, EZH2<sup>2A</sup>, EZH2<sup>2E</sup> or empty vector control. Cell lysates were subjected to western blot analysis using indicated antibodies. Relative intensities of H3K27me3 bands are shown, compared to those from cells expressing wild-type EZH2. 2E represents Ser363E and Thr367E.

**Figure 3-3**



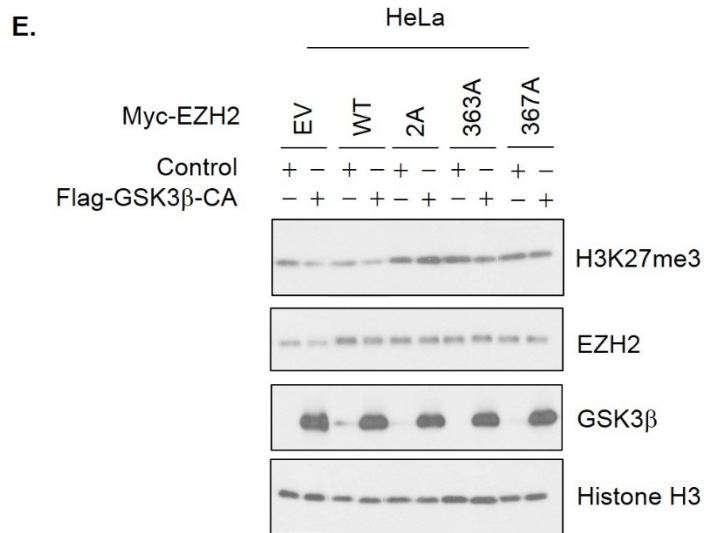
**B. GSK3 $\beta$  nonphosphorylatable mutants upregulates H3K27me3 level in MCF12A cells.** MCF12A cells were stably transfected with plasmids encoding wild-type EZH2, EZH2<sup>2A</sup>, EZH2<sup>S363A</sup>, EZH2<sup>S367A</sup>, or control. Cell lysates were immunoblotted with specific antibodies. Relative intensities of H3K27me3 bands are presented.

**Figure 3-3**



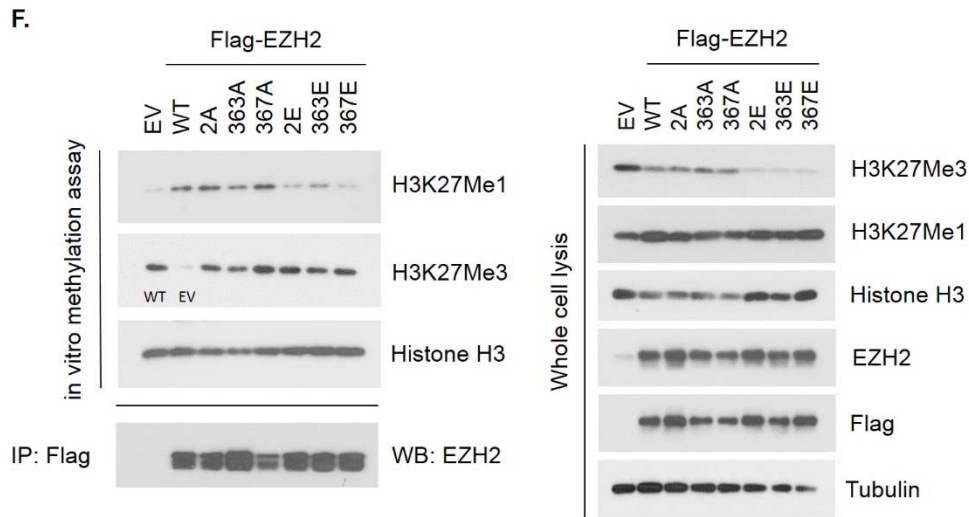
**C & D. GSK3 $\beta$  nonphosphorylatable 2A mutant enhances H3K27me3 level and phospho-mimic mutants reduces it in MCF7 (C) and MCF12A cells (D).** MCF7 and MCF12A cells were stably transfected with plasmids encoding wild-type EZH2, EZH2<sup>2A</sup>, EZH2<sup>2E</sup>, EZH2<sup>S363E</sup>, EZH2<sup>T367E</sup>, or vector control. Cell lysates were subjected to western blot analysis using indicated antibodies. Relative intensities of H3K27me3 bands are shown, compared to those from cells expressing wild-type EZH2. 2E represents Ser363E and Thr367E.

**Figure 3-3**



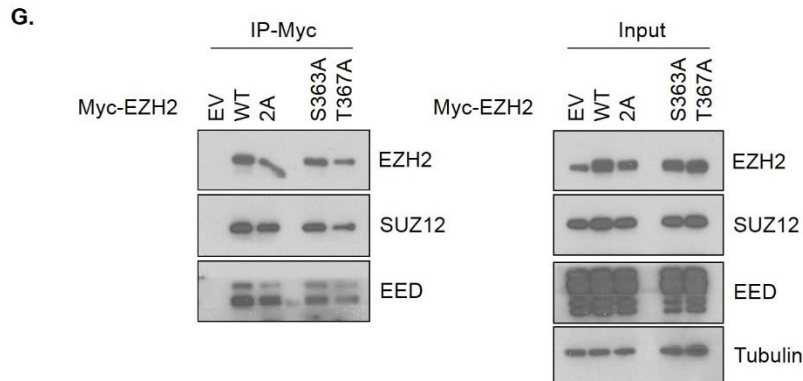
**E. GSK3 $\beta$  activation suppresses H3K27 trimethylation in wild-type EZH2 but not in mutants.** HeLa cells were co-transfected with plasmids encoding wild-type or nonphosphorylatable mutant Myc-EZH2 plus constitutively active form Flag-GSK3 $\beta$ . Cell lysates were subjected to western blot analysis with the indicated antibodies.

**Figure 3-3**



**F. GSK3 $\beta$  phospho-mimic mutant EZH2 might have relatively lower histone methyltransferase activity.** NIH-3T3 cells were transfected with plasmids as indicated. Whole cell lysates were collected and immunoblotted with specific antibodies. For *in vitro* methylation assay, Flag-tagged EZH2 proteins were purified by monoclonal anti-Flag M2 affinity agarose beads. *in vitro* methylation assay was performed using purified Flag-EZH2 protein incubated with recombinant histone H3 and S-adenosylmethionine (SAM) as methyl group donors in methylation buffer at 30 °C for 1 hr. The supernatant of reaction mixtures were resolved by 15% SDS-PAGE gel and immunoblotting with antibodies against methylation of histone H3. The immunoprecipitate from the rest beads were subjected to SDS-PAGE for EZH2 expression.

**Figure 3-3**



**G. GSK3 $\beta$  phosphorylation of EZH2 does not affect the association of EZH2 with SUZ12 and EED.** Cell lysates from MCF12A stable cells expressing wild-type EZH2, EZH2<sup>2A</sup>, EZH2<sup>S363A</sup>, EZH2<sup>T367A</sup>, or vector control were immunoprecipitated with anti-Myc antibody, followed by immunoblotted with EZH2, SUZ12 and EED antibodies (left panel). Input lysate was analyzed to detect the expression levels of the indicated proteins (right panel).

### **3-4 GSK3 $\beta$ nonphosphorylable mutants enhances EZH2's oncogenic functions**

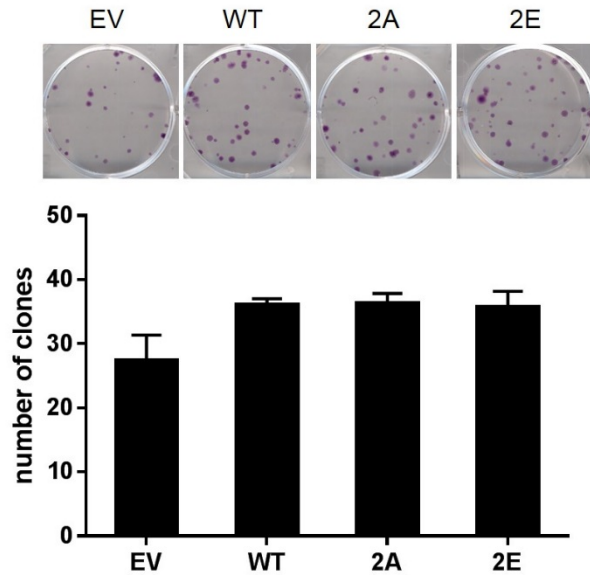
Because EZH2 is known to promote cell transformation and migration (Kleer, Cao et al. 2003, Moore, Gonzalez et al. 2013), and GSK3 $\beta$ -mediated phosphorylation of EZH2 downregulates H3K27 trimethylation, we next studied the effect of nonphosphorylable and phospho-mimic mutant EZH2 on EZH2-regulated biological functions. As described previously, GSK3 $\beta$  tends to be constitutively active in quiescent state and becomes inactive in response to extracellular signals or in exciting state (Luo 2009). In contrast to noncancerous cells, GSK3 $\beta$  might be relatively inactive in cancerous cells. To investigate the tumor promoting role of EZH2 mediated by GSK3 $\beta$ , we chose noncancerous mammary epithelial MCF12A cells for following function assays in addition to breast cancer MDA-MB-231 cells.

Colony formation abilities and cell migration potentials were determined in MDA-MB-231 stably transfected with wild-type EZH2, nonphosphorylable 2A or phospho-mimic 2E mutant. There was no significant difference in cell proliferation among all transfectants (Figure 3-4A). A soft agar assay revealed that nonphosphorylable EZH2 (EZH2<sup>2A</sup>) increased cell growth whereas phospho-mimic mutant (EZH2<sup>2E</sup>) had similar colony formation with wild-type EZH2 (Figure 3-4B). Consistently, a wound healing assay monitored by time-lapse microscopy demonstrated that nonphosphorylable mutant (EZH2<sup>2A</sup>) enhanced cell migration while EZH2<sup>2E</sup> had similar rate of wound closure, compared to wild-type EZH2 (Figure 3-4C). To further determine the effect of individual single nonphosphorylable mutants on the EZH2's biological functions, we performed a soft



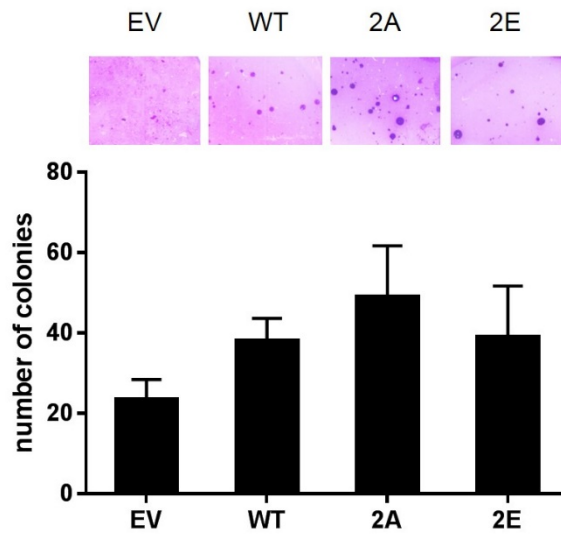
agar and a wound healing experiments in noncancerous MCF12A mammary epithelial cells stably expressing EZH2<sup>S363A</sup>, EZH2<sup>T367A</sup>, EZH2<sup>2A</sup> mutants. The results revealed that all nonphosphorylatable EZH2 promoted cell growth in an anchorage-independent manner as well as cell migration (Figure 3-4D and 3-4E). A Boyden chamber migration assay also supported our previous observations (Figure 3-4F). These results suggested that GSK3 $\beta$  inactivation promotes EZH2's oncogenic functions.

**Figure 3-4. GSK3 $\beta$  nonphosphorylable mutant EZH2 promotes anchorage-independent growth and cell migration.**



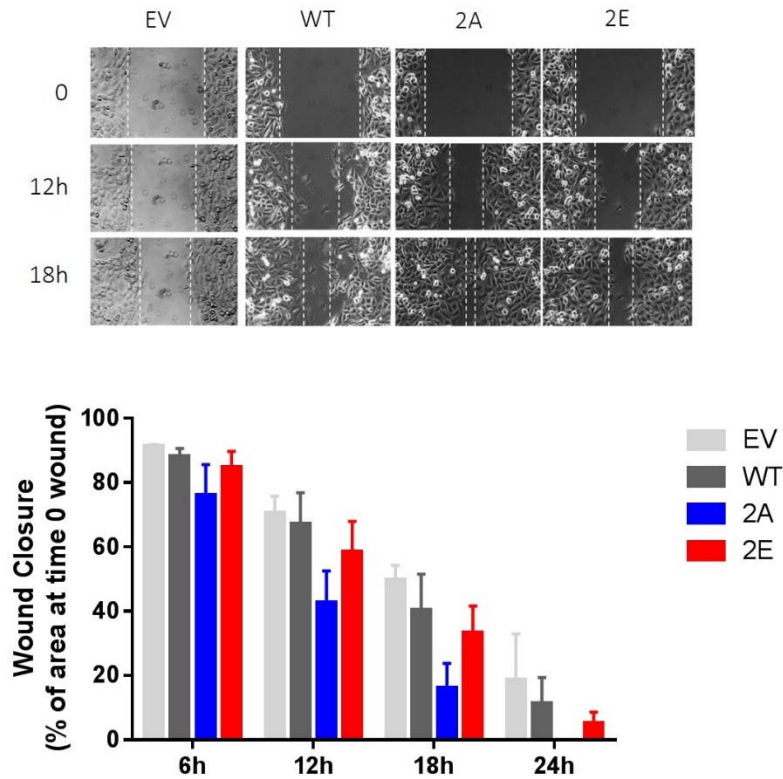
**A. GSK3 $\beta$  nonphosphorylable or phospho-mimic mutant EZH2 does not affect cell growth in 2D culture condition in MDA-MB-231 cells.** Colony formation abilities of MDA-MB-231 stable cell lines were determined using 2D clonogenic assay. Cells were seeded in 6-well plates as described. The number of colonies counted in one well of 6-well plate is shown as bar graphs. Data are expressed as mean  $\pm$  s.d. from three independent experiments. Representative images are shown at the top of each bar graph.

**Figure 3-4**



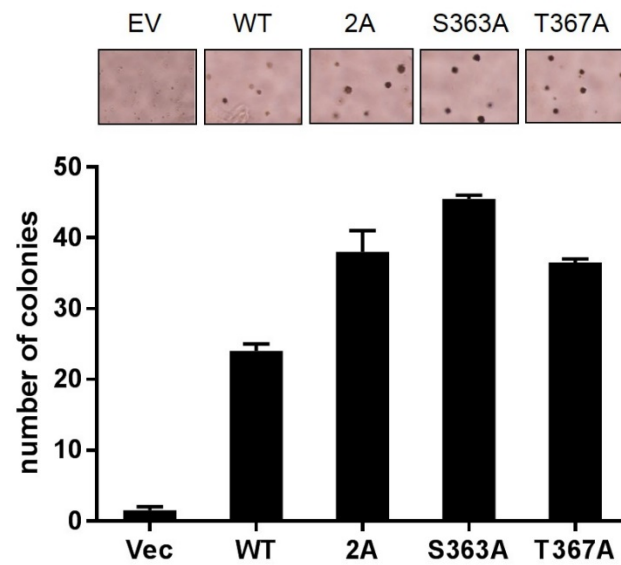
**B. GSK3 $\beta$  nonphosphorylable 2A mutant promotes anchorage-independent cell growth in MDA-MB-231 cells.** Colony formation abilities of MDA-MB-231 stable cell lines were determined using soft agar assay. Cells were seeded in 6-well plates as described. The number of colonies counted in one well of 6-well plate is shown as bar graphs. Data are expressed as mean  $\pm$  s.d. from three independent experiments. Representative images are shown at the top of each bar graph.

**Figure 3-4**



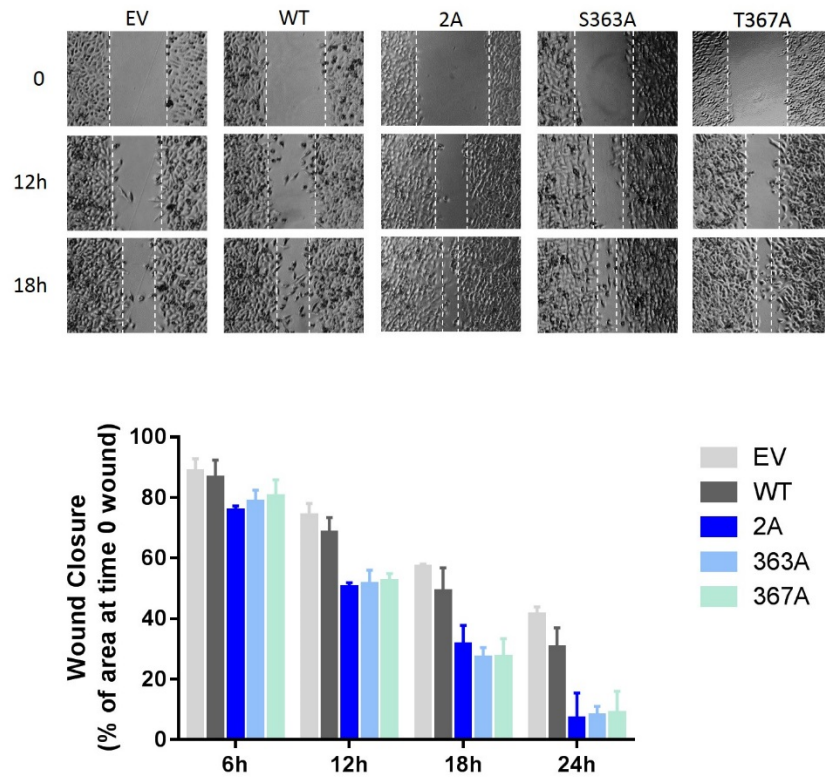
**C. GSK3 $\beta$  nonphosphorylable 2A mutant enhances cell migration in MDA-MB-231 cells.** Migration potential of MDA-MB-231 stable cell lines were measured by wound healing assay. Cells were seeded in culture inserts and migration was observed by time-lapse microscope as described. Representative images of each line are shown immediately (time 0), 12 h, and 18 h after removal of culture inserts. The areas of wound gap at the indicated time points were determined using the ImageJ software program and normalized to the area of wound gap at time 0. Wound closures were calculated and are plotted as bar graphs. Data are mean  $\pm$  s.d. from three independent experiments.

**Figure 3-4**



**D. GSK3 $\beta$  nonphosphorylatable mutants promotes anchorage-independent cell growth in MCF12A cells.** The same experiments as described in A were performed in MCF12A stable cell lines.

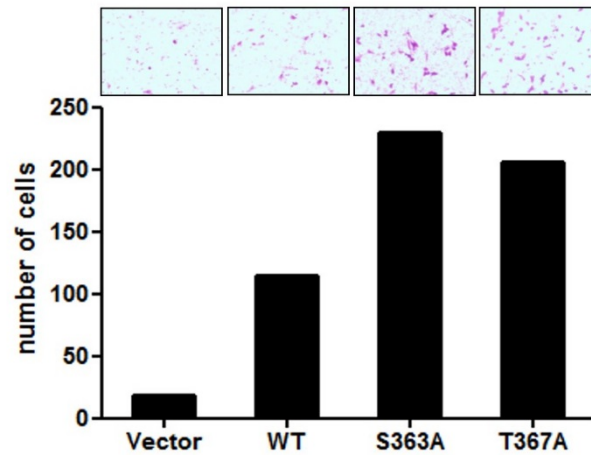
**Figure 3-4**



**E. GSK3 $\beta$  nonphosphorylatable 2A mutant enhances cell migration in MCF12A cells.** The same experiments as described in B were performed in MCF12A stable cell lines.

**Figure 3-4**

**E.**



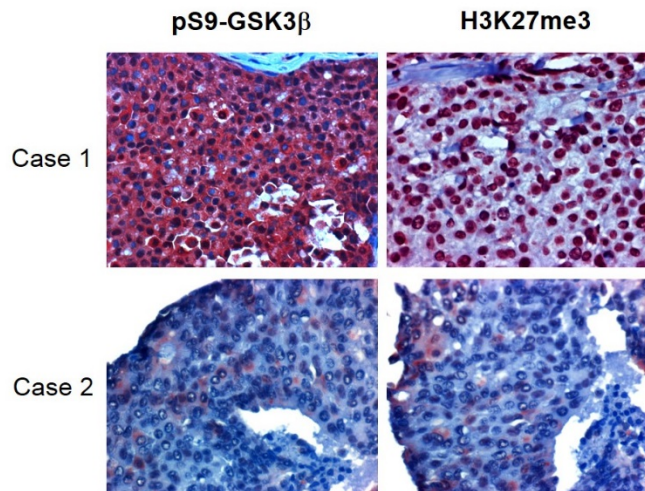
**F. GSK3 $\beta$  nonphosphorylatable mutants enhances cell migration in MCF12A cells determined by Boyden chamber migration assay.** Migration abilities of MCF12A cells expressing wild-type EZH2 (WT), EZH2<sup>S363A</sup>, EZH2<sup>T367A</sup>, or vector control were determined using 24-well Boyden chamber plates with an 8  $\mu$ m pore size polycarbonate filter (BD Biosciences) as described. Numbers of migrated cells were counted under a light microscopy and are shown as bar graph. Representative images are shown at the top.

### **3-5 H3K27 trimethylation is inversely correlated with GSK3 $\beta$ activity in breast cancer patients**

To examine the pathological relevance of EZH2 regulation by GSK3 $\beta$ , we analyzed correlation between the activity of GSK3 $\beta$  and the enzymatic activity of EZH2 in human breast tumor specimens. Since GSK3 $\beta$ -mediated phosphorylation of EZH2 did not affect EZH2 expression level but reduce H3K27 trimethylation, and it is known that Ser9 phosphorylation can inactivate GSK3 $\beta$  activity and the measurement of Ser9 phosphorylation can be used to determine GSK3 $\beta$  inactivation (Ding, He et al. 2007), we compared expression of H3K27 trimethylation with level of GSK3 $\beta$  phosphorylation at Ser9 in tumor tissue samples from 110 breast cancer patients. Consistently, immunohistochemical staining revealed that the level of GSK3 $\beta$  phosphorylation at Ser9 was positively correlated with the expression of H3K27 trimethylation ( $p = 0.006$ ; Table 3-1 and Figure. 3-5). This result suggested that GSK3 $\beta$  activity is inversely related to EZH2 activity in breast cancer tissues.



**Figure 3-5. Trimethylation of H3K27 is positively correlated with the expression of Ser9 GSK3 $\beta$  phosphorylation.**



**A. Representative cases of IHC staining for pS9-GSK3 $\beta$  expression and H3K27 trimethylation.** One hundred ten breast tumor tissue samples were subjected to immunohistochemical staining with antibodies specific to phosphorylated GSK3 $\beta$  at Ser9 and H3K27 trimethylation, respectively. Case 1 shows a representative specimen with high expression of Ser9 phosphorylation of GSK3 $\beta$  and H3K27 trimethylation. Case 2 is a sample with low expression of pSer9-GSK3 $\beta$  and H3K27me3.

**Table 3-1.**

**Relationship between H3K27me3 and pS9-GSK3 $\beta$  expression in surgical specimens of breast cancer**

	H3K27me3			<i>p</i> value
	Low	High	Total	
pS9-GSK3 $\beta$				
Low	13	10	23	
High	23	64	87	
Total	36	74	110	
				0.006

Low: includes negative (-) and score 1 (+)

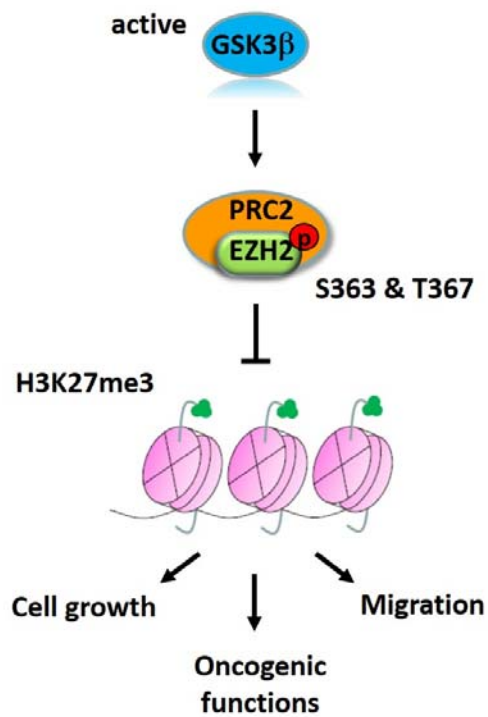
High: includes score 2 (++) and 3 (+++)

\*Correlation between H3K27 trimethylation and pS9-GSK3 $\beta$  was analyzed using the Pearson Chi-Square test. P value of less than 0.05 was set as the criterion for statistical significance.

### 3-6 Summary

In this study, we identified an interesting regulatory mechanism by which GSK3 $\beta$  regulates EZH2 activity via direct phosphorylation. Our findings demonstrated that alteration of GSK3 $\beta$  activity inversely changed H3K27 trimethylation without alteration of EZH2 protein level. We confirmed that GSK3 $\beta$  physically interacts with and found that their interaction is mainly in the cytosol. GSK3 $\beta$  phosphorylates EZH2 at Ser363 and Thr367 *in vitro*, and activation of GSK3 $\beta$  increases Thr367 phosphorylation *in vivo*. Moreover, the GSK3 $\beta$  phosphorylation sites are evolutionarily conserved among various species. Consistently, these modifications do not alter EZH2 protein expression nor affect EZH2's association with SUZ12 and EED, but suppresses H3K27 trimethylation. Through functional assays, we found that nonphosphorylatable mutant EZH2 enhanced cell migration and cell growth in an anchorage-independent manner, indicating that GSK3 $\beta$ 's regulation is critical for the oncogenic functions of EZH2. Notably, the immunohistochemical staining results revealed that inactivation of GSK3 $\beta$  is significantly correlated with higher level of H3K27 trimethylation in breast cancer patients. Therefore, we proposed that GSK3 $\beta$  regulates tumorigenesis via directly mediating EZH2 phosphorylation (Figure 3-6).

**Figure 3-6. Proposed model.**



**Figure 3-6. Proposed model of GSK3 $\beta$ -mediated regulation of EZH2.** GSK3 $\beta$  phosphorylates EZH2 at Ser363 and Thr367, which suppresses H3K27 trimethylation and EZH2 oncogenic functions.

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## **Chapter 4**

### Discussion

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Epigenetic regulation, including histone modification, is an essential mechanism in control of cell differentiation and tissue development through mediating gene expression without modifying DNA sequence. Alterations in this regulation, such as aberrant expression or activation of epigenetic regulators, undoubtedly change regular gene expression, which often induce neoplastic cell transformation and tumor growth. EZH2, a histone methyltransferase, is a key player in normal differentiation and development by catalyzing trimethylation of H3K27, leading to epigenetically silencing of specific gene expression. Overexpression or activation of EZH2 has been associated with several types of cancers and is related to aggressive disease and poor outcome. Studies have also demonstrated that EZH2 contributes to tumorigenesis through promoting malignant transformation, cell proliferation, invasion and migration. The histone methyltransferase activity of EZH2 plays a critical role in these processes by repressing specific gene expressions, such as RAD51, CDH1 (E-cadherin), FOXC1, CDKN1C (p57<sup>KIP2</sup>), through trimethylation of H3K27 in these target gene promoters. The results in the present study suggested that H3K27 trimethylation is negatively regulated by GSK3 $\beta$  phosphorylation of EZH2. GSK3 $\beta$  phospho-mimic mutant EZH2 reduces H3K27 trimethylation and vice versa. Consistently, this observation was supported by our immunohistochemical staining analysis which revealed that inactivation of GSK3 $\beta$  is significantly correlated with higher level of H3K27 trimethylation in 110 breast cancer patients. GSK3 $\beta$  is frequently inactivated and inactivation of GSK3 $\beta$  contributes to tumor development in certain types of cancers. Our study provides a link between GSK3 $\beta$  and epigenetic regulator and a

plausible explanation for the tumor promoting events controlled by EZH2-H3K27me3 pathway. These findings that EZH2 is subjected to GSK3 $\beta$  regulation are consistent with a recent study showing that GSK3 $\beta$  negatively regulates EZH2 expression in nasopharyngeal cancer cells and that the level of GSK3 $\beta$  phosphorylation at Ser9 is associated with higher EZH2 protein expression in patients with nasopharyngeal carcinoma (Ma, Wei et al. 2013).

GSK3 $\beta$  is known to be involved in tumor development, but its role in tumorigenesis remains controversial: in some types of cancer like pancreatic cancer, it contributes to tumorigenesis; in others such as breast cancer, it functions as a tumor suppressor (Luo 2009). In our study, we demonstrated that GSK3 $\beta$  nonphosphorylatable mutant EZH2 (EZH2<sup>2A</sup>, EZH2<sup>S363A</sup>, EZH2<sup>T367A</sup>) enhances cell migration and cell growth in breast cancer and mammary epithelial cells. Together with previously published reports, our results further strengthened the role of GSK3 $\beta$  as a tumor suppressor in breast cancer and implied that inactivation of GSK3 $\beta$  is one of the mechanisms enhancing EZH2 activity in cancer.

In many substrates, GSK3 $\beta$  interaction is associated with the switch of their subcellular localization. A well-known example is  $\beta$ -catenin. GSK3 $\beta$  physically interacts with and phosphorylates  $\beta$ -catenin in the cytosol, which leads to  $\beta$ -catenin inactivation. Once GSK3 $\beta$  is inactivated,  $\beta$ -catenin translocates into nucleus and activates its target genes. In this study, we found that GSK3 $\beta$  mainly interacts with EZH2 in the cytosol and GSK3 $\beta$  phosphorylation does not result in EZH2 degradation. EZH2 has been shown to regulate cell migration through interaction with a cytosolic and cytoskeletal protein, talin, suggesting that EZH2 has a cytosolic

function. Our finding implies that cytosolic interaction between GSK3 $\beta$  and EZH2 may lead to inhibition of EZH2 activity in the nucleus. Their cytosolic interaction does not affect EZH2 protein level, making it possible that this interaction may enhance EZH2 cytosolic function. Further investigation is needed to clarify the role of their interaction in the cytosol.

Studies have shown that EZH2 can be translationally modified by phosphorylation. Previous phosphoproteomic analyses of EZH2 have identified many phosphorylation residues in mouse tissues and human cell lines, including Ser362, Ser363, Ser366 and Thr367 (Mayya, Lundgren et al. 2009, Huttlin, Jedrychowski et al. 2010). Our work confirmed that GSK3 $\beta$  phosphorylates EZH2 at Ser363 and Thr367. As mentioned earlier, GSK3 $\beta$  has a preference for its substrates by recognizing a phosphorylation motif. Interestingly, the identified phosphorylation sites on EZH2 are compatible with this consensus motif. Furthermore, these two phosphorylation sites are highly conserved residues, implying that this regulation could be functional in other organisms. NIH-3T3 cells expressing phospho-mimic mutant EZH2 have lower H3K27 trimethylation, supporting this notion. Previously, a study revealed that EZH2 can be phosphorylated at Thr367 by p38 $\alpha$  in muscle stem cells (Palacios, Mozzetta et al. 2010). p38 $\alpha$ -mediated EZH2 phosphorylation leads to *Pax7* repression through trimethylation of H3K27 in its promoter while inhibition of p38 $\alpha$ -EZH2 pathway promotes muscle stem cell proliferation. In our study, we observed that GSK3 $\beta$  nonphosphorylatable mutant, EZH2<sup>T367A</sup>, increased global H3K27 trimethylation in mammary epithelial cells and enhanced cell growth and migration. Activation of



GSK3 $\beta$  upregulated Thr367 phosphorylation *in vivo*. Our findings and previous report suggested that the biological significance of EZH2 phosphorylation at Thr367 is cell context-dependent.

Unlike other kinases, GSK3 $\beta$  prefers a pre-phosphorylated substrate for priming phosphorylation. In this modification, GSK3 $\beta$  phosphorylates a substrate at target serine/threonine residue after this substrate has been pre-phosphorylated at another serine/threonine 4 amino acids C-terminal to the target site by other kinase. Apart from priming phosphorylation, GSK3 $\beta$  can phosphorylate its substrate at more than one sites. For example, SNAIL contains several consecutive phosphorylation motifs and GSK3 $\beta$  phosphorylates SNAIL at 6 serines in a sequential manner starting from the residue closest to C-terminus (Zhou, Deng et al. 2004). Our *in vitro* kinase assay revealed that GSK3 $\beta$  phosphorylates EZH2 without primed phosphorylation. Moreover, Thr367A mutant totally abolished GSK3 $\beta$  phosphorylation but Ser363 did not. This promoted us to speculate the possibility of sequential phosphorylation. Although a subsequent *in vitro* kinase assay did not prove it, the possibility cannot be excluded.

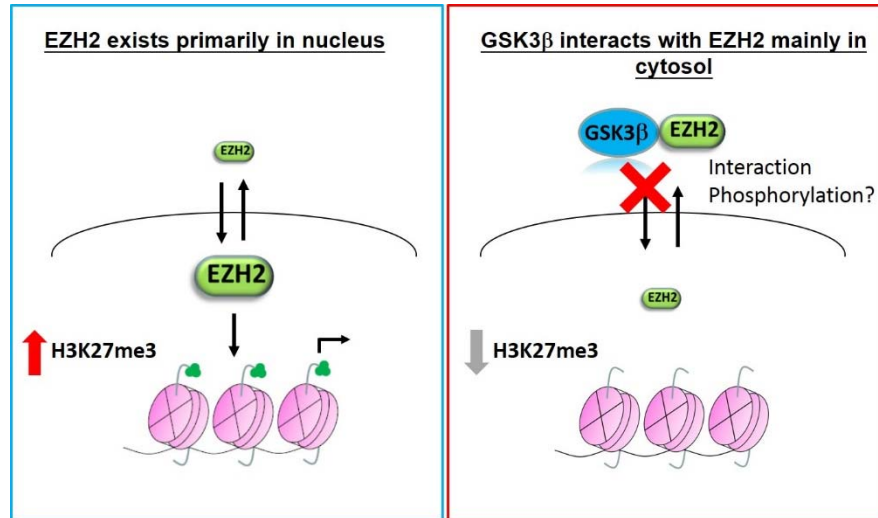
EZH2 is a histone methyltransferase and its enzymatic activity requires the association with SUZ12 and EED. Methylation of H3K27 is catalyzed by EZH2/PRC2 complex in a progressive manner, in which EZH2/PRC2 exerts its action on its substrates, histone H3, H3K27me1, H3K27me2, to convert them into H3K27me1, H3K27me2 and H3K27me3 by addition of one methyl group, respectively. In the present study, an *in vitro* methylation assay using recombinant histone H3 protein as substrate revealed that monomethylation of H3K27 catalyzed

by phospho-mimic mutant EZH2 was reduced but dimethylation (data not shown) or trimethylation of H3K27 was not. As the substrate used in this assay was recombinant histone H3 protein which has no modification on H3K27 and the methylation of H3K27 catalyzed by EZH2 is a successive process from mono-, di- to trimethylation, the result suggested that phospho-mimic mutant EZH2 exhibits lower histone methyltransferase activity than that of wild-type EZH2. In this experiment, the signals of dimethylation and trimethylation of H3K27 were similar in wild-type and mutant EZH2 but were not detectable in control vector, I reasoned that they might be background signals occurring due to immunoprecipitation of Flag-EZH2.

The histone methyltransferase activity of EZH2 can be regulated by site-specific phosphorylations in different mechanisms. For example, JAK2 phosphorylates EZH2 at Tyr641, which is located on the catalytic SET domain, promotes EZH2's interaction with  $\beta$ -TrCP and leads to its degradation (Sahasrabudhe, Chen et al. 2015). Thr487 phosphorylation by CDK1 disrupts PRC2 assembly and reduces H3K27 trimethylation (Wei, Chen et al. 2011). In the current study, these two phosphorylation sites we identified are neither located on the SET domain nor in the regions of SUZ12 or EED binding. Compatible with the observation, we and others (Palacios, Mozzetta et al. 2010) did not find that mutations at these sites (EZH2<sup>2A</sup>, EZH2<sup>S363A</sup>, EZH2<sup>T367A</sup>) change the association of EZH2 with EED or SUZ12. Furthermore, we did not detect alteration in EZH2 protein expression upon this regulation. However, our work revealed that GSK3 $\beta$  interacts with EZH2 and, unexpectedly, their interaction is mainly in the cytosol. As

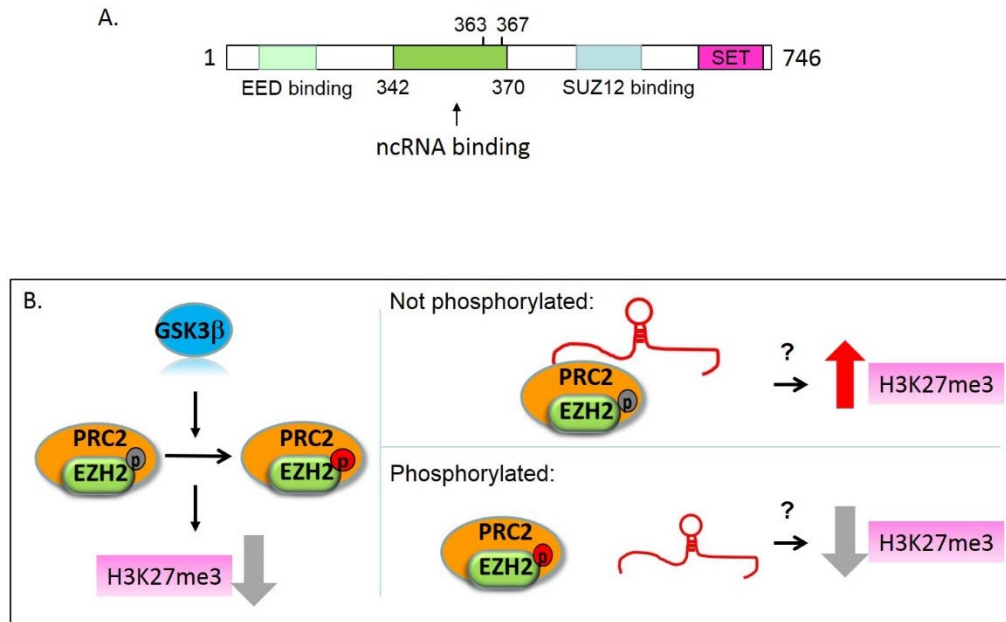
mentioned previously, EZH2 is able to interact with cytosolic protein (Gunawan, Venkatesan et al. 2015). Thus, we hypothesized that GSK3 $\beta$  regulates H3K27 trimethylation by mediating EZH2's localization (Figure 4-1). Further investigation is needed to verify this hypothesis. In addition to the possibility of switch in EZH2's subcellular localization, GSK3 $\beta$  phosphorylation may suppress the enzymatic activity of EZH2 via modulating its interaction with other factors. EZH2 and PRC2 complex has been shown to interact with noncoding RNA (ncRNA) and this interaction regulates their function. One well-known example is X-chromosome inactivation, which is an important developmental process in mammals. X inactivation induces the expression of an ncRNA, X-inactive specific transcript (XIST), which helps to recruit EZH2/PRC2 complex to trimethylate H3K27 on inactivated X-chromosome (Margueron and Reinberg 2011). Similarly, another ncRNA, HOTAIR, has been demonstrated to be able to interact with EZH2. Notably, phosphorylation of EZH2 at Thr345 promotes its binding to HOTAIR and an ncRNA-binding domain between residues 342 and 370 has been identified (Kaneko, Li et al. 2010). Interestingly, GSK3 $\beta$  phosphorylation sites on EZH2, Ser363 and Thr367, is located on this domain. Whether GSK3 $\beta$  phosphorylation mediates EZH2 interaction with ncRNA and consequently suppresses H3K27 trimethylation needs further studies (Figure 4-2).

**Figure 4-1. Cytosolic interaction of GSK3 $\beta$  and EZH2 may lead to inactivation of EZH2's enzymatic activity**



**4-1. Hypothesis: GSK3 $\beta$  may trap EZH2 in the cytosol.** The majority of EZH2 proteins and functions are in the nucleus. A recent study reported that EZH2 can interact with cytosolic protein and has cytosolic function. Once GSK3 $\beta$  binds to EZH2, EZH2 may stay in the cytosol, thereby leading to reduce its HMT activity and H3K27me3 level.

**Figure 4-2. GSK3 $\beta$  phosphorylation may affect EZH2 interaction with ncRNA**



**A. The ncRNA binding region of EZH2.** EZH2 can interact with ncRNA and its binding domain has been identified between residues 342 and 370. GSK3 $\beta$  phosphorylation sites, Ser363 and Thr367, are located within this region.

**B. Hypothesis: GSK3 $\beta$  phosphorylation may regulate EZH2 association with ncRNA to suppress H3K27 trimethylation.**

PRC2 complex is essential in regulating self-renewal capacity in embryonic and adult stem cells and EZH2 has been reported to promote expansion of tumor initiating cell population. At the beginning of this study, we aimed to study the role of GSK3 $\beta$ -mediated EZH2 regulation in cancer stem cells. However, we did not observe differences between wild-type and GSK3 $\beta$  nonphosphorylatable mutant EZH2 stable cells using flow cytometry analysis and mammosphere formation assay. Interestingly, we found that GSK3 $\beta$  nonphosphorylatable mutants enhance cell growth in an anchorage-independent manner but not affect cell proliferation in 2D culture. Furthermore, these mutants increase cell migration ability. EZH2 has been shown to promote colony formation and cell migration. Our observation is compatible with previous studies and GSK3 $\beta$  inactivation augments these EZH2's oncogenic functions. EZH2 has also been demonstrated to regulate cell growth and migration through repressing tumor suppressor expression by catalyzing trimethylation of H3K27. It is possible that GSK3 $\beta$  mediates these EZH2's oncogenic functions through modulating these target gene repression via H3K27 trimethylation. On the other hand, GSK3 $\beta$  is a multi-tasking kinase involved in many signaling pathways, such as AKT, ERK and WNT/ $\beta$ -catenin pathway. The regulation of EZH2 by GSK3 $\beta$  suggests that these oncogenic pathways could control cell growth and migration by mediating GSK3 $\beta$  activity. Recently, an interesting study reported that specific KRAS mutation regulates EZH2 protein expression through the PI3K/AKT and/or MEK/ERK signaling pathways in lung cancer (Riquelme, Behrens et al. 2016). EZH2 inhibition enhances the sensitivity to MEK-ERK or PI3K/AKT targeted therapies in specific KRAS-mutant lung cancer

cells and tumors. Since GSK3 $\beta$  is known to be inactivated by AKT or ERK, our work suggests a direct role of the GSK3 $\beta$ -EZH2 pathway in this scenario and offers a rationale for enhancing GSK3 $\beta$  activity and/or targeting EZH2 in anti-cancer therapy.

Since GSK3 $\beta$  is widely involved in several cellular processes, its deregulation has been found in many diseases, such as neuropsychiatric disorder, or diabetes mellitus. Inhibitors targeting GSK3 $\beta$  has been developed and used to treat these diseases. As mentioned earlier, GSK3 $\beta$  has tumor promoting effect in certain types of cancer. GSK3 $\beta$  inhibitors have also been tested in the anticancer treatment, particularly in treating hematological malignancy. However, inactivation of GSK3 $\beta$  by these inhibitors may cause a concern for tumor development like breast cancer as GSK3 $\beta$  inhibition can activate EZH2-H3K27me3 pathway and enhance its oncogenic functions.

Because of EZH2's importance in tumorigenesis, several inhibitors targeting EZH2 have been developed and tested in preclinical and clinical trials. These inhibitors are summarized in Table 4-1 (Kim and Roberts 2016). Most of them are studied in non-Hodgkin lymphoma, few are in solid tumors. All of these compounds inhibit EZH2's enzymatic activity. Because GSK3 $\beta$  attenuates EZH2's enzymatic activity, inactivation of GSK3 $\beta$  may be a biomarker for EZH2 targeting therapy. Moreover, therapies enhancing GSK3 $\beta$  activity in combination with EZH2 inhibitor may be a novel therapeutic strategy in anti-cancer management.

**Table 4-1. EZH2 inhibitors in development**

Table 4-1. EZH2 inhibitors in development			
Compound	Mechanism	Cancer type	Trial status
DZNep	SAH hydrolase inhibitor		Pre-clinical
EI1	SAM-competitive inhibitor		Pre-clinical
EPZ005687	SAM-competitive inhibitor		Pre-clinical
GSK343	SAM-competitive inhibitor		Pre-clinical
UNC1999	SAM-competitive inhibitor		Pre-clinical
SAH-EZH2	Disrupt the protein interaction between EZH2 and EED		Pre-clinical
GSK126	SAM-competitive inhibitor	Non-Hodgin lymphoma	Phase I
CPI-1205	SAM-competitive inhibitor	Non-Hodgin lymphoma	Phase I
EPZ-6438	SAM-competitive inhibitor	Non-Hodgin lymphoma; Soft tissue sarcoma	Phase I/II



**Conclusion:**

The results in this study indicates that GSK3 $\beta$  negatively regulates EZH2 activity through phosphorylation. We confirmed that GSK3 $\beta$  physically associates with EZH2 and their interaction is mainly in the cytosol. We found that GSK3 $\beta$  phosphorylates EZH2 at Ser363 and Thr367 and activation of GSK3 $\beta$  enhances T367 phosphorylation *in vivo*. Cells expressing mutant EZH2 to block phosphorylation by GSK3 $\beta$  have higher H3K27 trimethylation and enhanced ability of cell migration and anchorage-independent growth, which suggests that inactivation of GSK3 $\beta$  augments EZH2's oncogenic functions. Furthermore, our results indicate that inactivation of GSK3 $\beta$  measured by GSK3 $\beta$  phosphorylation at Ser9 may lead to higher EZH2 activity characterized by excessive expression of H3K27 trimethylation in human breast cancer tissues, suggesting the clinical significance of this regulation in tumorigenesis. Taken together, our study contributes to a better understanding of tumor progression and will be helpful to develop therapeutic strategies for future anti-cancer management.

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## **Chapter 5**

### Future Work and Direction

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This study leaves several questions: First, it is important to investigate what target genes of EZH2 and H3K27me3 are affected by GSK3 $\beta$  regulation. As mentioned earlier, EZH2 can regulate cell proliferation, growth and migration through repression of several target genes by catalyzing H3K27 trimethylation, such as RAD51, CDH1 (E-cadherin), FOXC1 and RKIP. Chromatin immunoprecipitation (ChIP) can be performed to examine the binding of EZH2 and/or H3K27me3 to these gene promoters in cells expressing wild-type, nonphosphorylatable or phospho-mimic mutant EZH2 or in cells with or without activation of GSK3 $\beta$ , or both. The mRNA expression of these genes can also be measured by qRT-PCR in these cells. However, EZH2 binding to these genes and their expression may not be affected by GSK3 $\beta$  regulation. ChIP sequencing can be utilized to identify potential target genes which will be important in GSK3 $\beta$ -mediated EZH2's oncogenic functions.

Second, what is the role of cytosolic interaction between GSK3 $\beta$  and EZH2 is not clear. As described in discussion, their cytosolic interaction may “trap” EZH2 in the cytosol, leading to a reduction of nuclear EZH2 amount, thereby downregulating H3K27 trimethylation. To verify this hypothesis, nuclear and cytosolic fraction of EZH2 protein amount can be examined in cells with or without activation of GSK3 $\beta$ . To explore whether GSK3 $\beta$  phosphorylation of EZH2 switches its subcellular localization, nuclear and cytosolic EZH2 can be analyzed in cells transfected with wild-type, nonphosphorylatable or phospho-mimic mutant EZH2. Because their cytosolic interaction does not affect EZH2 protein level, it might be possible that this interaction enhance EZH2 cytosolic function. Whether EZH2 gains

its cytosolic function by GSK3 $\beta$  phosphorylation is worthwhile to be further investigated.

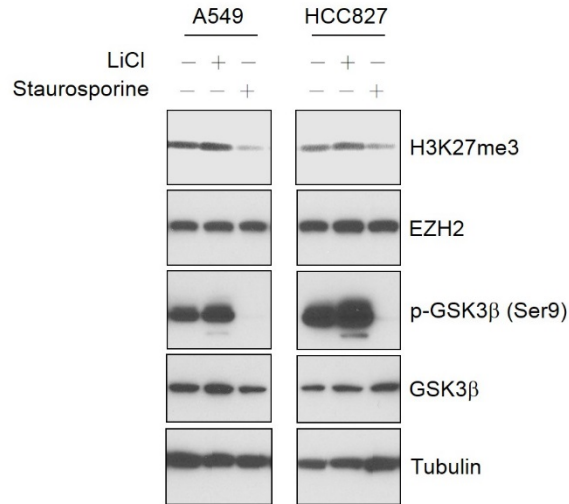
Moreover, GSK3 $\beta$  phosphorylation sites locate on the ncRNA binding region of EZH2. Actually, EZH2 is an RNA binding protein. A sucrose gradient experiment can be performed for preliminary understanding whether GSK3 $\beta$  phosphorylation affects EZH2 association with RNA using wild-type and mutant EZH2 stable cells with or without RNase treatment. EZH2 is known to interact with several ncRNAs, such as Rep A, HOTAIR and Metastasis Associated Lung Adenocarcinoma Transcript 1 (MALAT-1). An RNA immunoprecipitation (RIP) can be utilized to specifically analyze the affinity of wild-type and mutant EZH2 to these ncRNAs. The ncRNAs interacting with EZH2 can be knocked down to determine if their interaction modulate H3K27 trimethylation.

In addition to these plans, I would like to test this regulation in lung cancer. Lung cancer is still the leading cause of cancer-related death in the United States and worldwide in spite of advances in anti-cancer treatment. Non-small cell lung cancer (NSCLC) accounts for about 85% of all cases. In patients with NSCLC, higher expression of EZH2 is a poor prognostic marker and associated with a metastatic disease (Behrens, Solis et al. 2013, Wang, Zhao et al. 2016). Studies has also demonstrated that overexpression of EZH2 contributes to cancer cell migration and tumorigenesis and EZH2 knockdown reduces cell proliferation and growth in NSCLC (Cao, Ribeiro Rde et al. 2012, Xu, Hou et al. 2013, Kim, Kim et al. 2015, Serresi, Gargiulo et al. 2016), part of these studies have shown that EZH2 regulation is through catalyzing trimethylation of H3K27. Recently, a study reported

that EZH2 inhibitor, GSK126, sensitizes NSCLC cell lines with *BRG1* or *EGFR* mutation to topoisomerase II inhibitor Etoposide treatment (Fillmore, Xu et al. 2015). These evidence suggests that EZH2 play an important role in NSCLC tumorigenesis and EZH2 could be a therapeutic target in selective patients with NSCLC. On the other hand, GSK3 $\beta$  expression and activity are relatively low in NSCLC, in contrast to its counterpart, small cell lung cancer (SCLC) (Zheng, Saito et al. 2007, Byers, Wang et al. 2012). A significant portion of NSCLC patients harbors *KRAS* or *EGFR* mutation, both can lead to inactivation of GSK3 $\beta$ . Thus, the regulation identified in this study potentially provides a therapeutic strategy that GSK3 $\beta$  inactivation may be a useful marker to guide anti-EZH2 treatment in NSCLC patients if this signaling pathway exists in NSCLC. To examine whether this regulation occurs in NSCLC cell lines, we used the same approach to alter GSK3 $\beta$  activity by treating cells with lithium chloride and staurosporine. The preliminary result reveals that, similar with previous findings in breast cancer and mammary epithelial cell lines, lithium chloride treatment increases H3K27 trimethylation and, more significantly, staurosporine reduces its level in *KRAS*-mutant A549 and *EGFR*-mutant HCC827 NSCLC cell lines (Figure 5-1), implying that this regulation may exist in NSCLC. An immunohistochemical staining analysis can be used to further evaluate the clinical relevance and significance of this regulation in NSCLC cancer patients. To investigate whether inactivation of GSK3 $\beta$  can be utilized to guide EZH2 targeting therapy in NSCLC, the sensitivity (IC<sub>50</sub>) of NSCLC cell lines to EZH2 inhibitors can be measured to correlate with GSK3 $\beta$  activity determined by the level of GSK3 $\beta$  phosphorylation at Ser9. Furthermore, increasing GSK3 $\beta$

activity in cells with relatively inactivated GSK3 $\beta$  status by expressing constitutively active GSK3 $\beta$  can be performed to determine whether activation of GSK3 $\beta$  enhances efficacy of EZH2 inhibitor. Alternatively, GSK3 $\beta$  activity can be enhanced by chemotherapeutic drugs or EGFR-TKI. A combination therapy with EZH2 inhibitor plus a drug enhancing GSK3 $\beta$  activity can be tested according to this regulation.

**Figure 5-1. GSK3 $\beta$  negatively regulates H3K27 trimethylation in NSCLC cell lines.**



**5-1. Alteration of GSK3 $\beta$  activity changes H3K27 trimethylation in NSCLC cell lines.** NSCLC cell lines, A549 and HCC827 cells were treated with lithium chloride (LiCl), staurosporine as indicated. Cell lysates were subjected to western blot analysis with the indicated antibodies.

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