The Role of EZH2 in Breast Cancer Progression and Metastasis

by

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DEDICATION

Kay Lavon Dickson

1943 – 2012

I dedicate my thesis in loving memory of my aunt Kay Dickson. Kay passed away in October of 2012 from pancreatic cancer and her memory reminds me every day why the research I conduct is so very important. Kay overflowed with love and warmth and she generously shared these with those who were lucky enough to have known her.

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I wish the absolute best to all I have encountered throughout this journey!

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LIST OF ABBREVIATIONS

AH	Atypical Hyperplasia
ALDH1	Aldehyde Dehydrogenase 1
BM	Basement Membrane
ChIP	Chromatin Immunoprecipitation
CSC	Cancer Stem Cell
CTC	Circulating Tumor Cell
DCIS	Ductal Carcinoma *in situ*
DNMT	DNA Methyltransferase
DOX	Doxycycline
DZNeP	3-deazaneplanocin A
EED	Embryonic Ectoderm Development
EMT	Epithelial-to-Mesenchymal Transition
ER$^-$	Estrogen Receptor Negative
EZH2	Enhancer of Zeste Homolog 2
GSI	γ-Secretase Inhibitor
HDAC	Histone Deacetylase
H3K27me3	trimethylation of histone H3 at lysine 27
IP	Immunoprecipitation
LCIS	Lobular Carcinoma *in situ*
MAPK	Mitogen-Activated Protein Kinase
MET	Mesenchymal-to-Epithelial Transition
MMP	Matrix Metalloproteinase
MMTV	Mouse Mammary Tumor Virus
PcG	Polycomb Group
PRC1	Polycomb Repressive Complex 1
PRC2	Polycomb Repressive Complex 2
shEZH2	EZH2-targeted short hairpin RNA
shRNA	short hairpin RNA
SUZ12	Suppressor of Zeste 12
TDLU	Terminal-Ductal-Lobular-Unit

ABSTRACT

The Role of EZH2 in Breast Cancer Progression and Metastasis

Breast cancer is the second leading cause of cancer-related deaths for women in the United States, with the majority due to the development of distant metastasis. Understanding how breast cancer cells disseminate and metastasize is essential to develop more efficacious treatments and to improve survival. Enhancer of Zeste Homolog 2 (EZH2) is a Polycomb group protein which functions mainly as a transcriptional repressor through histone trimethylation. Our laboratory has found that EZH2 overexpression in clinical samples of invasive breast carcinomas is associated with worse survival. Here, we have focused our work on elucidating the functions and mechanisms by which EZH2 promotes aggressive breast carcinomas with metastatic potential. We have found that EZH2 regulates two important processes for metastasis: the epithelial-to-mesenchymal transition and migration, and the numbers of breast cancer stem cells.

We discovered that downregulation of EZH2 in aggressive and metastasizing breast cancer cells promotes a mesenchymal-to-epithelial transition and reduces motility and invasion. *In vivo*, EZH2 knockdown in breast cancer cells decreased spontaneous metastasis to the lungs. We uncovered an unexpected role of EZH2 in inducing the p38 signaling pathway, a known regulator of breast cancer invasion and metastasis. EZH2 was demonstrated to bind phosphorylated-p38 (p-p38) in association

with other core members of the Polycomb Repressive Complex 2 (PRC2). Moreover, the effect of p-p38 was confirmed *in vivo* and correlated with decreased spontaneous metastasis. Through analysis of invasive human breast cancers, we found that EZH2 expression was upregulated in all cases, and that EZH2 and p-p38 were co-expressed in 63% of cases, consistent with the functional results.

In our studies on the role of EZH2 in breast cancer stem cell biology, we found that EZH2 expression levels regulate stem cell numbers in nontumorigenic and malignant breast cells. Mechanistically, we revealed a novel role of EZH2 in activating Notch1 signaling through binding of the *Notch1* promoter. Binding was independent of its catalytic methyltransferase activity and PRC2, and correlated instead with transcriptional activation. Notch1 inhibition was sufficient in preventing the EZH2-induced expansion of the stem cell population. In a transgenic mouse model with targeted EZH2 overexpression, we found that EZH2 promoted earlier breast cancer initiation and correlated with Notch1 expression. Additionally, EZH2, Notch1 and stem cell markers were found to correlate in human breast cancer. Taken together, these findings reveal important and novel functional links between EZH2, stem cells and breast cancer migration and invasion, and their underlying mechanisms involving EZH2-mediated regulation of p38 and Notch1 signaling pathways. Our work establishes EZH2 as a regulator of breast cancer progression and metastasis, and identifies potential targets for treatment of this deadly malignancy.

CHAPTER 1

Introduction

1-1. The Human Mammary Gland and Breast Cancer Development

Mammals are distinguished from all other animal groups by the presence of a unique organ, the mammary gland, which functions in secreting milk to nourish young. The development of the mammary gland is divided into three stages: embryonic, pubertal and reproductive [1]. Proper maintenance and control of these temporal stages are essential for correct development and tissue homeostasis. Unfortunately, when aberrations in these developmental processes transpire during the postnatal life of a female, uncontrolled cell growth and subsequent breast cancer may result.

For women in the United States, breast cancer is the most common malignancy and is also the second most common cause of cancer-related deaths behind lung cancer [2]. It is currently estimated that a women living in the United States has a 1 in 8 lifetime risk of being diagnosed with breast cancer. Better screening and treatment strategies have resulted in improved survival and quality of life in breast cancer patients as demonstrated in a yearly 2.2% decrease in breast cancer death rates since 1990 [2]. However, although death rates are decreasing, approximately 40,000 women were expected to die from breast cancer in 2011 alone [2]. Even though breast cancer incidence rates have remained relatively stable since 2003, approximately 288,000 new cases of *in situ* and invasive breast cancer were expected to be diagnosed among

women in 2011 [2]. Research, ranging from basic to clinical, into the mechanisms behind breast cancer development and progression are responsible for the improving trend in breast cancer statistics, but more research is needed to see further reductions in incidence and death rates.

It is important to note that breast cancer cannot be considered a single disease. It is rather a heterogeneous mix of breast malignancies that exhibit various genetic, epigenetic and genomic alterations, which can have diverse histological presentations and outcomes in patients. This diversity has led to the subdivision of breast cancer into four main molecular classes based on gene expression profiling: luminal A, luminal B, basal-like or triple-negative and HER2/ERBB2-overexpressing [3, 4]. Our current theory on breast cancer is that it develops along a continuum within the epithelium of breast [5]. Breast ducts and acini exhibit a bilayer of two cell types present in roughly equal numbers: an outer layer of elongated, myoepithelial cells surrounding an inner lining of polarized, luminal epithelial cells [1, 6-8]. The bilayered ducts form a branching structure throughout the breast that end in clusters of small secretory acini that compose the terminal-ductal-lobular-units (TDLUs), or lobules. By birth, a female has a rudimentary ductal tree that grows isometrically to the rest of the body up until puberty. During puberty and pregnancy, the epithelium undergoes great proliferation and expansion, but the mammary glands do not reach full maturity until pregnancy. During pregnancy, the luminal cells within the TDLUs become alveolar cells that produce milk proteins. Functionally, the myoepithelial cells contract upon hormone stimulation during lactation allowing for release of milk from the luminal cells into the lumen, which travels through the ducts to the nipple. Following pregnancy, the TDLUs involute decreasing in number,

but the cycle of proliferation and expansion can be repeated with subsequent pregnancies until a woman is no longer able to conceive. At all stages, the ducts and lobules are enclosed by a continuous, laminin-rich basement membrane (BM), which separates the epithelium from the collagenous stroma. The tree-like structure of the epithelium is supported by the stroma, which is also referred to as the mammary fat pad, extending its branches throughout this tissue. The stroma is composed of adipocytes, blood vessels, nerves, fibroblasts and immune cells, all of which help in the development and maintenance of the mammary gland.

The linear continuum of breast cancer development postulates that epithelial cells within the TDLUs progress through stages of alterations that may eventually advance into invasive breast carcinoma and metastasis (Figure 1-1, A) [5, 9]. Breast cancer may initiate with epithelial hyperplasia in the TDLUs. A small proportion of these hyperplastic cells may develop atypia and progress into atypical hyperplasia (AH), either ductal or lobular, if the hyperplastic cells begin to layer and take on an abnormal appearance. AH is considered a non-obligate precursor to cancer as only about 20% of patients with AH will go on to develop to cancer within 15 years of diagnosis [10]. AH may progress into ductal carcinoma *in situ* (DCIS) or lobular carcinoma *in situ* (LCIS), which are defined as noninvasive malignant lesions. Of the estimated 58,000 *in situ* cases diagnosed yearly, the majority, approximately 83%, are classified as DCIS, while LCIS accounts for about 11% of *in situ* cancer cases [2]. As DCIS and LCIS lesions are in most cases excised, there are limited available data on the percentage of *in situ* carcinomas that would be expected to progress to invasive breast cancer. However, data indicate that the 10-year mortality rate for patients with DCIS is less than 2% after

excision or mastectomy [11-13]. A diagnosis of invasive breast cancer is confirmed when the malignant epithelial cells escape the confines of the duct or lobule by breaking through the surrounding BM into the stroma. Once invasive breast cancer develops, the risk for developing metastasis significantly increases. The five-year survival rate for women with regional breast cancer metastasis, meaning it has mainly spread to the axillary lymph nodes, is 84%, but unfortunately, the same rate for women with metastasis to distant organs is a mere 23% [2]. Even with progress in research, however, metastatic breast cancer is considered essentially incurable and most deaths from breast cancer occur as a result of metastasis. More research aiming to understand the mechanisms involved in the progression of breast cancer from *in situ* to invasive and metastatic stages is needed for the development of better treatment strategies and survival.

The linear model of tumor progression holds to the traditional view that a single cell of origin gains genetic variability and a clonal expansion of more aggressive cells evolves with time [5, 9]. This generally implies that most primary cells have low metastatic potential and that metastasis-driving mutations are acquired during later stages of tumorigenesis. With this model, metastatic dissemination is expected to occur as a late process and to correlate with primary tumor size, and this does hold true in most cases [14]. Further support for this model is seen in studies showing that primary tumors and their matched metastases have similar molecular signatures [15-17]. In addition, mutations associated with driving metastasis are more likely to be seen in metastatic cells versus cells from the primary tumor site [18, 19].

It is important to recognize that the linear progression for breast cancer development described above is only a model and deviations do occur. For instance, observations have suggested that breast cancer cells may acquire the ability to disseminate early during tumor progression, perhaps even during a premalignant stage [20]. This parallel model for metastasis implies that metastatic breast cancer cells may evolve independently from the primary tumor [20, 21]. Indeed, clinical analysis of a large cohort of breast cancer patients indicated that metastasis may be initiated before diagnosis of the primary tumor and that survival following metastasis was almost unrelated to primary tumor size [22]. Husemann and colleagues demonstrated that malignant cells were detected in the circulation and in the bone marrow of patients with DCIS [23]. In further support, research has shown that disseminated cancer cells from breast cancer patients display fewer genetic alterations when compared to cells isolated from the primary tumor [21, 23-25]. In reference to this model, attention should be paid to delineating the genetic alterations in disseminated cancer cells, as presence of these cells in the bone marrow of breast cancer patients has been shown to be an independent indicator of recurrence [26]. It is possible to characterize breast cancer cells circulating in the peripheral blood or disseminated to the bone marrow, and these characterizations may provide important information on predicting response to therapies in metastatic breast cancer [27]. In addition, genetic studies on disseminated tumor cells may allow for earlier detection of metastasis. Taken together, there is no defined set of standards on how, when or where breast cancer progresses to a metastatic state. The linear and parallel models should remain as no more than broad guidelines being

applied on an individual basis as metastatic capability may arise early or late during breast cancer progression.

1-2. The Breast Cancer Metastatic Cascade

The metastatic cascade collectively describes the complex multistep process that allows tumor cells to leave the primary site and establish a distant colony (Figure 1-1, B). The major stages breast cancer cells undergo in this succession of biological events are characterized as (1) local invasion through the basement membrane into surrounding stromal tissues, (2) intravasation into blood vessels, (3) survival during dissemination through vasculature, (4) arrest and extravasation at distant sites, (5) survival in new microenvironment and (6) re-initiation of proliferative capabilities at the metastatic site [18, 20]. These basic steps will be described in more detail in the following sections.

Invasion, Migration and the Role of the Epithelial-to-Mesenchymal Transition

As previously mentioned, progression from carcinoma *in situ* to invasive breast carcinoma occurs when tumor cells gain access to the stroma by breaking through the well-confined barrier of the surrounding BM. One of the first alterations required of tumor cells to invade is the loss of cell-to-cell adhesion and adhesion to the BM. Studies have shown that tumor cells may utilize the process of an epithelial-to-mesenchymal transition (EMT), where epithelial cells undergo transdifferentiation to mesenchymal cells to move and invade [28-32]. Expanding on the previous definition, breast epithelial cells are tightly bound together through cell-to-cell adhesion complexes forming a sheet

of columnar cells that display an apico-basal cell polarity. In contrast, mesenchymal cells throughout the body lack intercellular junctions, act individually and possess motile abilities. Thus, a switch of tumorigenic epithelial cells to a mesenchymal phenotype might enable migration and invasion.

EMT events were first described in nontumorigenic cells [29, 30]. Type 1 EMT occurs during embryogenesis and is first observed at gastrulation with the formation of the primitive streak. Type 2 EMT takes place during instances of inflammation, such as at times of wound healing and tissue regeneration. Focus on metastasis research has led to the development of Type 3 EMT, or oncogenic EMT, where carcinoma cells may gain characteristics of mesenchymal cells allowing for detachment from the primary tumor, invasion and intravasation. Recent studies in mouse models and in human tumors provide evidence for oncogenic EMT [33-35]. In a study where almost 500 invasive breast carcinomas were analyzed for different markers, protein expression patterns associated with a mesenchymal phenotype were found to associate in basal-like tumors, the most aggressive breast cancer molecular subtype, signifying a relationship between mesenchymal differentiation and metastatic capabilities [36]. In most cases of oncogenic EMT, the transition is considered only partial, as tumor cells may not fully lose epithelial characteristics [30]. Also, as metastases usually appear histologically similar to their primary tumor counterparts, it has been suggested that tumor cells at a metastatic site may undergo a mesenchymal-to-epithelial transition (MET) emphasizing the plasticity of these transitions [37].

E-cadherin, considered a marker protein of epithelial cells, is well documented in mediating cell-to-cell junctions and its downregulation has been associated with

metastasis and poor prognosis in breast cancer [28, 38-40]. Several mesenchymal-related transcription factors, such as Snail1, Snail2, Twist1, ZEB1 and ZEB2, have been shown to regulate EMT, with some even directly repressing levels of E-cadherin, and their upregulation is also associated with poor prognosis [18, 28-30]. Moreover, several signaling pathways, including TGF-β, EGF, Wnt, Notch, and Hedgehog, have all been found to induce EMT in breast cancer, sometimes activating the above transcription factors [41-46]. For example, the induction of EMT in mammary epithelial cells through TGF-β and active Ras leads to the phosphorylation of Twist1 by p38 mitogen activated protein kinase (MAPK) and promotes invasiveness [47].

When a loss in cell adhesion occurs through reprogramming to a mesenchymal-like protein expression pattern, a change in polarity from apico-basal to front-rear is observed, which initiates invasion and motility with cytoskeleton remodeling [28, 48]. Specifically, activation of p38 signaling, especially of the p38γ isoform, through overexpression of RhoC GTPase has been found to be important in breast cancer cell motility and invasion [49, 50]. The induction of proteases, especially matrix metalloproteinases (MMPs), which degrade BM components, is also associated with EMT, and it has been demonstrated that levels of these enzymes are highest at the *in situ* stage prior to invasion [51-53]. It is believed that cancer cell motility is enhanced with these BM degrading enzymes as they may make channels through the BM and stroma allowing for movement. In breast cancer cells, p38α has been shown to be important in orchestrating motility and invasion as it mediates the expression of several MMPs [54]. Once free from the constraints of the BM, research suggests that cells within the stroma may enhance the aggressive behaviors of the cancer cells and

promote further motility and invasion [25, 55-57]. For example, secretion of IL-6 by adipocytes can stimulate invasion in breast cancer cells [25], and secretion of IL-4 by breast cancer cells can promote protease activity in macrophages [57]. A feedback loop may occur between carcinoma and stromal cells as carcinoma cells may create stroma with attributes associated with inflammation, and reactive stroma may then enhance aggressive traits within the carcinoma cells.

Besides the need to lose cell-to-cell contacts and degrade the BM, breast tumor cells may also have to breach the outer layer of myoepithelial cells within the epithelium before even encountering the BM. Normal myoepithelial cells form a physical border between tumorigenic cells within the lumen and the underlying basement membrane. Studies suggest that myoepithelial cells can influence tumorigenic cells by blocking proliferation through promotion of growth arrest and apoptosis, and by blocking invasion through inhibition of angiogenesis and basement membrane degradation [58-60]. Thus, differentiated myoepithelial cells act as natural tumor suppressors, and it is not surprising then that gene expression profiles in myoepithelial cells surrounding a DCIS show significant differences from myoepithelial cells surrounding a healthy gland [61]. DCIS-associated myoepithelial cells exhibit overexpression of several chemokines, which boost proliferation, migration, and invasion of neighboring epithelial cells [61]. Moreover, the same cells show overexpression of enzymes implicated in degradation of the basement membrane and extracellular matrix, such as MMPs [61]. Interestingly, once a tumor is classified as invasive, the adjacent myoepithelial layer can no longer be found [60]. The mechanism behind this disappearance remains unknown but may be due to degradation of the myoepithelial cells by the very proteolytic enzymes they were

shown to overexpress. Mammary stem cells may also prevent myoepithelial differentiation or selective apoptosis may be responsible [62, 63]. How myoepithelial cells are eliminated needs to be deduced, but once they are gone, tumorigenic cells gain easier access to the basement membrane and stroma. Before their complete disappearance, myoepithelial cells play an important role in invasion influencing the tumor microenvironment through secretion of key proteins.

Intravasation and Survival in the Circulation

Intravasation occurs when invasive breast cancer cells enter the lumen of lymphatic or blood vessels. Although invasion of tumor cells into regional lymph nodes classifies a tumor as metastatic, it is believed that tumor cell dissemination via blood circulation is primarily responsible for distant metastasis [19]. Many aspects of EMT are believed to be important in intravasation as carcinoma cells are required to break through vessel walls. For instance, TGF-β signaling, a pathway known to induce EMT, has been shown to enhance breast cancer intravasation [64]. Outside of EMT, Wyckoff and colleagues have demonstrated that perivascular macrophages are associated with breast cancer intravasation implying the importance of the associated microenvironment [65]. Tumor cells may also initiate neoangiogenesis and create new blood vessels within their local microenvironment [18]. These vessels, unlike normal blood vessels, are characterized as being in a constant state of reconfiguration and are susceptible to leaks, which allows for easier intravasation [66, 67].

Once successful intravasation occurs, breast carcinoma cells have the ability to widely travel throughout the blood circulation system and are termed circulating tumor

cells (CTCs). Recent advances have allowed for detection and characterization of CTCs in the bloodstream of patients [27, 68-70], and studies have revealed a correlation between the number of CTCs and patient survival [71-74]. In order for a successful metastasis to occur, CTCs must survive or evade certain stressors, such as anoikis caused by matrix detachment and detection by the immune system. The formation of large platelet-coated emboli has been shown to allow for survival by providing a shield from vascular turbulence and immune cells [75, 76].

Even though invasion and migration might be considered effective processes, metastatic outgrowth is deemed quite inefficient as only 0.01% of CTCs are able to produce a single bone metastasis [77, 78]. Likewise, CTCs have been detected in disease-free breast cancer patients up to 22 years after treatment implicating that CTCs in the bloodstream are required, but not sufficient, for distant metastasis [21]. In addition, how long a CTC can persist in the bloodstream remains an unanswered question. As carcinoma cells at 20-30μm in diameter outsize the diameter of capillaries, it is expected that CTCs would become trapped quickly after intravasation, and this short time in circulation may allow for anoikis evasion by CTCs [18].

Arrest and Extravasation at Distant Sites

CTCs, if they survive circulation, do eventually arrest at a distant site and the most common metastatic organs for breast CTCs are the brain, bone, liver and lungs [18]. It is unknown whether this organotropism is due to the structure and size restrictions of capillaries in certain organs or an ability to selectively target to specific organs. It is confounding when carcinoma cells arrest in a distant organ that is

downstream of capillary beds whose diameters should have not allowed passage of CTCs. But, this does occur, and the plasticity of CTCs may be accountable and allow for entrapment at more distal sites. In contrast, CTCs may exhibit features that allow them to home to specific organs, implying that distinct adaptive programs may be developed for each metastatic site [20]. For instance, the expression of metadherin in breast cancer cells may allow for specific binding to the pulmonary vasculature [79]. Research has also found that chemokines may be involved in tissue tropism as breast cancer cells highly express chemokine receptor 4 (CXCR4), while its respective ligand, CXCL12, is highly expressed in lymph nodes, lung, liver and bone, but weakly expressed in other sites such as the kidney and skeletal muscle [80]. Several other studies in human breast cancer have described gene expression signatures associated with specific metastasis to bone, lung, liver and brain [81-84].

How CTCs become lodged at a distant site requires further research, but the process of CTCs invading through the luminal wall of a vessel and gaining access to the stromal tissue of a distant organ is known as extravasation [18]. Alternately, CTCs may become trapped and form a microcolony, and with additional growth, the microcolony may burst vessel walls and allow access to stromal tissue [85]. Although both mechanisms are possible, the following section will only focus on extravasation.

It would seem logical that extravasation would be the reverse of intravasation, but the specific microenvironments at metastatic sites greatly differ from those surrounding the primary tumor site. As previously mentioned, blood vessels created by carcinoma cells within the primary tumor via neoangiogenesis are quite permeable and allow for easier intravasation. In contrast, blood vessels at the distant metastatic site are

expected to be normal and functional, and much less penetrable. This may partially be why nearly all CTCs arrested at a distant site die shortly after lodging [86, 87]. However, factors secreted by the primary tumor may create a pre-metastatic niche that aids in surmounting the vessel wall barrier by inducing vascular hyper-permeability. Secreted factors, such as Angptl4, MMP1 and MMP4, have been demonstrated to upset cell-to-cell junctions in the pulmonary vasculature [67, 88]. Additionally, secretion of VEGF promotes extravasation of breast cancer cells through recruitment of inflammatory monocytes to pulmonary metastatic sites [89]. Taken together, the specific microenvironment present at possible distant sites plays a significant role in extravasation and metastasis formation.

Survival and Metastatic Colonization

Disseminated cancer cells are most likely poorly adapted to the microenvironment present at the metastatic site as the types of stromal cells and extracellular matrix components may differ from those present at the primary tumor site. Survival in the foreign microenvironment might be accomplished through establishment of the aforementioned pre-metastatic niche [90, 91]. This model proposes that cells from the primary tumor release factors prior to the arrival of CTCs that prime metastatic sites for colonization. These factors may include those previously mentioned that aid in organotropism and extravasation. Further evidence for a pre-metastatic niche has been illustrated through clustering of hematopoietic progenitor cells positive for the receptor VEGFR-1 to metastatic sites [91]. These hematopoietic progenitor cells work to modify the local microenvironment through release of MMP9, which may free the cancer cell

chemoattractant SDF-1. Survival may also be promoted through cell-autonomous programs and has been demonstrated in the activation of Src tyrosine kinase signaling in breast carcinoma cells disseminated to bone marrow. Although Src signaling was deemed dispensible for tissue homing, Src signaling provided survival and outgrowth signals for the tumor cells [92].

Survival in a foreign microenvironment does not guarantee the formation of large, proliferating metastases (macrometastases) as breast cancer patients may go years or even decades without relapse after mastectomy [93]. In fact, disseminated cancer cells may undergo periods of dormancy, which may be due to a lack of growth signals or an incompatibility with the microenvironment [94, 95]. This quiescent state of mammary carcinoma cells has been shown to be dependent on a lack of integrin β1 signaling at distant sites [96-98]. Additionally, cell-nonautonomous programs may be necessary for metastatic proliferation. Breast cancer cells may stimulate the mobilization and recruitment of bone marrow-derived cells to a metastatic site through secretion of factors, such as osteopontin and SDF-1, and they may trigger outgrowth [99, 100]. Conversely, proliferation of cancer cells may occur freely in a metastatic site, but a net gain in size may not be seen and may be due to a high rate of apoptosis or a failure in neoangiogenesis [95].

Ongoing research has delineated many of the steps and mechanisms involved in breast cancer metastasis as demonstrated in the previous sections, but much remains to be discovered. Hopefully, ongoing research will help to make a diagnosis of metastatic breast cancer not so devastating and better treatment strategies will be developed.

1-3. Breast Cancer and the Cancer Stem Cell Hypothesis

The cancer stem cell (CSC) hypothesis postulates that malignant tumors are initiated and maintained by a subpopulation of neoplastic cells that possess similar properties to normal adult stem cells. In order to qualify as a CSC, three functional characteristics must be exhibited [101, 102]. First, CSCs must have the ability to initiate a phenocopy of the primary tumor in immunocompromised or syngeneic mice, which explains why CSCs are interchangeably referred to as tumor-initiating cells. Second, a capacity for self-renewal must be demonstrated in secondary mice. Cells from newly formed tumors initiated by potential CSCs, when serially transplanted, must form additional tumors that recapitulate the primary tumor. Third, CSCs must have the capacity to form tumors that contain the original heterogeneity of cell types found in the bulk of the primary tumor from which they were derived. This characteristic shows a capacity for pluripotency as CSCs must be able to differentiate into a population of non-self-renewing cells, which may constitute the majority of a tumor.

Two prominent theories aim to describe the origin of CSCs [103]. One model builds on the linear progression of cancer described earlier where cancers are believed to develop through an accumulation of mutations over a longer period of time. As adult stem cells are long-lived and have a high proliferative capacity, they have the potential to acquire the numerous mutations that lead to a malignant state. Indirect evidence supporting this model has been demonstrated *in vitro* as adult stem cells were shown to spontaneously transform into tumorigenic stem cells [104-108]. Alternately, the other model proposes that CSCs result from mutations in lineage-committed cells, which lead to dedifferentiation and the acquisition of self-renewal capacity. Takahashi and

colleagues showed that pluripotent stem cells could be induced in differentiated cells through expression of a small subset of transcription factors [109]. Although both models are possible, they each are consistent with the concept of the CSC hypothesis in that tumorigenic cells exhibit stem-like properties in propagating a malignancy.

The profound expansion and proliferation of the mammary gland during puberty and pregnancy implies the existence of adult breast stem cells. Indeed, early studies in mice demonstrated that an entire mammary gland could be generated *in vivo* in a cleared fat pad from serially transplanted portions of mammary epithelium [110-113]. Building on this work, other researchers have found that a functional mammary gland could be produced from transplantation of a single cell in mice [114, 115]. Kuperwasser and colleagues extended these studies to a human relevance through development of a xenograft model in which functional mammary glands from human epithelial cells were generated in mouse fat pads “humanized” by injections with irradiated human fibroblasts [116]. The use of these *in vivo* transplant assays has allowed for the identification of breast stem cell markers. For example, human breast stem cells have found to be enriched in $CD49f^{hi}EpCAM^{-}$ fractions and in fractions positive for Aldehyde Dehydrogenase 1 ($ALDH1^{+}$) activity [117-119].

Evidence for the existence of mammary stem cells has been accumulating since the 1950s; however, the identification of human breast CSCs has been a more recent event. Al-Hajj and colleagues were the first in 2003 to show that a minority of breast cancer cells had the ability to form new tumors when serially transplanted in NOD/SCID mice, and these breast CSCs were identified and isolated as being $CD44^{+}/CD24^{-/low}$ [120]. In another study, an invasiveness gene expression profile was generated for

$CD44^{+}/CD24^{-/low}$ breast CSCs compared to cells of normal breast epithelium, and researchers found that this profile predicted shorter metastasis-free survival in patients [121]. The $CD44^{+}/CD24^{-/low}$ profile has also been associated with CSCs in other solid tumors, including gastric and prostate [122, 123]. However, the cell surface profile of breast CSCs has been extended to include ESA, ALDH1, and CD133, amongst others [103, 118]. Additionally, the development of an *in vitro* cultivation system, known as the mammosphere assay, has shown that the ability to form non-adherent spheres in culture is a property of breast stem cells and CSCs and allows for enrichment of these populations [118, 124-126]. More, established breast cancer cell lines have been shown to contain subpopulations of CSCs identified by ALDH1 or $CD44^{+}/CD24^{-/low}$ [126-128]. In all, CSCs expressing these markers have been found to display enhanced invasive and metastatic capabilities and to associate with poor clinical outcome in breast cancer [118, 125-127, 129]. Recent studies also suggest that CSCs may be more resistant to radiation and chemotherapy implying that these treatments mainly target non-CSCs, while CSCs may remain behind to proliferate and repopulate the tumor [130-132].

Recently, an interesting link between CSCs and EMT has been put forth. Two independent studies have found that induction of EMT in differentiated human mammary epithelial cells increases the number of cells that express surface stem cell markers and that form mammospheres [133, 134]. It was also observed that stem-like cells isolated from normal human mammary glands and breast carcinomas expressed EMT markers at higher levels when compared to non-stem cells [133]. When EMT was induced in transformed human mammary epithelial cells, a CSC phenotype was induced as these cells formed mammospheres and tumors more efficiently. In addition,

the acquisition of a stem cell-like gene expression signature has been associated with poor prognosis and high grade estrogen receptor negative (ER^-) breast cancers, often of the aggressive basal-like subtype [102]. As previously mentioned, a mesenchymal phenotype was found to associate in basal-like tumors, signifying that the relationship between mesenchymal differentiation and metastatic capabilities may be extended to include a stem cell-like gene expression [36]. These findings revealed for the first time a direct relationship between EMT and CSC properties, and imply that CSCs may play an important role in breast cancer metastasis.

It is not surprising that many signaling pathways, such as Wnt, Hedgehog and Notch, which are important in the regulation of EMT have been implicated in CSCs [101, 103]. Specifically, in the normal mouse mammary gland, Notch was found to regulate the expansion of stem cells and differentiation to a luminal lineage establishing a role for Notch in normal breast development [135, 136]. Many studies have implicated a correlation between the expression of Notch receptors and ligands in breast cancer progression, which associates with poor prognosis and survival [137-144]. Research utilizing pre-clinical models of DCIS, Notch signaling was shown to have a role in DCIS acini growth and mammosphere formation through treatment with the Notch inhibitor DAPT [145]. In a study of breast CSCs, Notch4 signaling activity was shown to be increased when compared to differentiated cells and that inhibition of Notch4 reduced stem cell activity and tumor formation [146]. A number of recent studies investigating Notch1 have established its role in breast CSCs as inhibition of Notch1 through several methods reduces CSC populations, tumor incidence and the formation of metastasis [147-152]. In further support, knockdown of nicastrin, a component of the γ-secretase

protein complex that is responsible for the release of the activated intracellular domain of Notch1, in breast cancer cells led to a decrease in CSCs and invasion accompanied by a morphological change to an epithelial-like phenotype *in vitro* and decreased tumorigenicity *in vivo* [153]. Mao and colleagues found that Notch1 may play a critical role in the resistance of CSCs to chemotherapy as knockdown of Notch1 in breast cells treated with paclitaxel decreased CSC populations and tumor growth [154]. Further research delineating the signaling pathways involved in CSCs will aid in drug discovery and hopefully help in targeting the cells that may be responsible for a good portion of tumor propagation.

1-4. The Tumorigenic Role of EZH2

Overexpression of the epigenetic regulator Enhancer of Zeste Homolog 2 (EZH2) in a wide range of malignancies has been established in cancer research. EZH2 was first associated with aggressive and metastatic prostate cancer through analyses of gene expression in human tumor microarrays [155]. Through similar microarray profiling and other studies, EZH2 expression was found to strongly correlate with breast cancer aggressiveness acting as an independent predictor of recurrence and survival [156-159]. EZH2 was also found increased in histologically normal breast epithelium with a higher risk of developing cancer, indicating that EZH2 may prove as a valuable marker for detecting preneoplastic lesions [160, 161]. Elevated EZH2 expression has since been described in other types of cancers: bladder [162-164], liver [165], colon [166-168], lung [169], gastric [170], enodometrial [157], skin [157, 171], lymphoma [172-175], pancreatic [176], Ewing's sarcoma [177, 178], and myeloma [179]. In all reported cancer studies, the common discovery is that EZH2 expression is increased in cancer

compared to normal tissues, being the highest in the most advanced stages of cancer, and correlates with poor prognosis in patients.

Polycomb-Mediated Epigenetic Silencing

EZH2 is classified as a member of the Polycomb-group (PcG) family of proteins. PcG proteins were initially identified as regulators of body patterning by silencing homoeotic (Hox) genes in *Drosophila melanogaster* [180-182]. Upon mutation of PcG genes, flies displayed defects in body segmentation in the anterior-posterior axis that were attributed to the expression of Hox genes outside their normal spatial regions. Repressive functions of PcG proteins have been conserved through vertebrates as several PcG mutants exhibit skeletal malformations [183-186]. In addition, the crucial role of PcG proteins in mammalian development is emphasized by studies showing that deletion of some genes encoding for PcG proteins leads to early embryonic lethality in mice [187-191]. Our current understanding of the role PcG proteins play in transcription regulation has expanded to include genes outside the *Hox* family. Through numerous genome wide chromatin immunoprecipitation (ChIP) studies and other approaches, PcG proteins have been found to accumulate at hundreds of target genes in *Drosophila* [192-197] and mammalian cells [198-202]. These reports indicate that PcG proteins in flies and vertebrates regulate approximately 1.3% and 3-4% of genes, respectively, and that the most prevalent target genes are transcription factors. Interestingly, several studies have demonstrated differences in PcG protein binding profiles signifying that PcG gene regulation can vary in different cell types and at different stages of development [193, 199, 202]. With the wide spectrum of target genes identified thus far, it is not surprising

that PcG proteins have emerged as regulators of key processes such as multicellular development, cell fate determination and tumor formation [203-205].

At the molecular level, PcG proteins are mainly divided into two distinct groups dependent on their formation of multimeric complexes, termed Polycomb Repressive Complex 1 (PRC1) and PRC2 (Figure 1-2) [203, 205-208]. PRC2, which is conserved from *Drosophila* to mammals, consists of three core mammalian members that are required for catalytic activity: EZH2, Suppressor of Zeste 12 (Suz12), and Embryonic Ectoderm Development (EED). Other proteins have been shown to transiently interact with PRC2 include RbAp46/48 [209-211], AEBP2 [211-213], JARID2 [214-218], the mammalian orthologs of *Drosophila* PCL proteins (PHF1, MTF2, & PHF19) [214, 219-223], SIRT1 [224], and EPC1 [121]. Although these factors are not required for PRC2 enzymatic activity *in vitro*, they generally confer modulating and/or recruiting functions as they have been shown to be necessary for optimum PRC2 transcriptional repression. The core composition of PRC1 is much more variable and contains one subunit of the CBX, RING1, SCML, PHC, and PCGF paralog protein groups, with many of these paralogs exhibiting overlapping and redundant functions [132, 203-205, 225]. Altogether, multiple versions of PRC1 and PRC2 exist in mammalian systems, and the differing configurations may confer distinct functions.

Functionally, PRC2 is responsible for initiating gene repression and occurs when the catalytically active member EZH2 methylates histone H3 at lysine 27 [126, 209, 211, 226]. Even though EZH2 is capable of adding three methyl groups to the ε-amino group of the lysine side chain, the trimethylated form, H3K27me3, is predominant and considered to convey gene repression as its genome-wide distribution coincides with

PcG complexes. Furthermore, the C-terminal cysteine-rich and SET domains of EZH2 were shown to be required for this catalytic activity. Studies suggest that once methylated, the H3K27me3 mark is specifically recognized by the chromodomain of a CBX protein within PRC1 [227]. The recruitment of PRC1 leads to monoubiquitination of histone H2A on lysine 119 (H2AK119ub) by the E3 ubiquitin ligases RING1 [228-230]. This monoubiquitination is believed to block binding of transcription factors, inhibit transcription initiation by RNA polymerase II and compact chromatin [231, 232]. Although it is generally accepted that PRC1 functions downstream of PRC2 as outlined above, this may not always be the case. Studies have demonstrated that some genes targeted by PRC2 lack PRC1 or H2AK119ub, and conversely, some genes targeted by PRC1 do so in the absence of PRC2 [233-235]. Generally, though, both of the post-translational modifications rendered by PRC1 and PRC2 are often required for gene repression.

Recent studies in human cells have demonstrated physical and functional links between PcG repression and other epigenetic modifications. Vire and colleagues found PRC2 regulates DNA methylation as EZH2 co-immunoprecipitated with three DNA methyltransferases (DNMTs), which resulted in recruitment of DNMTs to PRC2 target genes and subsequent methylation [236]. Additionally, BMI1and two CBX paralogs, members of PRC1, have been shown to interact with DNMTs [237-239]. It is estimated that approximately 47% of genes regulated by DNMT3B are also bound by PRC1 and PRC2 in colon cancer cells [240]. The link between EZH2 and DNA methylation has been expanded as PRC2 and H3K27me3 have been found at gene promoters containing aberrant CpG island hypermethylation in cancer cells [241-243]. These

hypermethylated gene promoters were seen to correlate with sites displaying H3K27me3 during normal development suggesting that these genes may be tagged to undergo hypermethylation during transformation. In support, another study by Ku and colleagues showed that >97% of genes bound by EZH2 in embryonic stem cells associated with CG-rich DNA sequences or CpG islands [233]. In relation to other histone methylating marks, researchers have found that the H3K4 demethylase RBP2 associates with PRC2 at a number of PcG target genes in mouse embryonic stem cells [210]. As trimethylation of H3K4 is normally associated with active gene transcription, this mechanism suggests a coordinated regulation of H3K4 demethylation and H3K27 trimethylation during development. Lastly, histone deacteylase (HDAC) activity has been shown to be required for mediating gene repression by PcG proteins, and EED directly interacts with HDAC1 [155, 156, 244, 245].

In order to better understand how PcG proteins regulate gene expression, a better comprehension of how PcG complexes are recruited to specific target genes is required. In *Drosophila*, PcG proteins are recruited to specific DNA sequences upstream of target genes, which are defined as Polycomb Repressive Elements [205, 207]. These elements contain several hundred base pairs, can be distally located away from the transcription start sites of target genes and contain DNA-binding consensus sites for transcription factors [246, 247]. Until recently, truly similar motifs had yet to be identified in vertebrates. A 3 kb murine PRE, termed PRE-kr, was found by Sing and colleagues to regulate expression of the mouse *MafB/Kreisler* gene [234]. PRE-kr contains a palindromic double PHO-binding site, which is not present in the human PRE-kr sequence. Interestingly, PRC1 bound the PRE-kr with higher affinity than PRC2,

which indicated that PRC1 gene recruitment may not be completely dependent upon PRC2. Even more recently, a potential 1.8 kb PRE has been identified in human embryonic stem cells [248]. Located between the *HOXD11* and *HOXD12* loci, the region contains binding sites for YY1, a transcriptional regulator whose knockdown removes EZH2 and H3K27me3 from target genes in mouse myoblasts [248, 249]. YY1, in addition to PRC1 and PRC2, was found recruited to this PRE.

Recruitment of PcG complexes may also occur through intermediary molecules. For instance, DNA-binding protein JARID2 has been demonstrated to form a stable complex with PRC2 and was shown to be required for recruitment of PRC2 to target genes [217]. The phosphatase NIPP1 also has been found to complex with PRC2 on chromatin and they silence a common set of genes [250]. NIPP1 knockdown results in the dissociation of EZH2 from some target genes whereas NIPP1 overexpression causes a redistribution of EZH2 between target genes [251]. In another example, Gupta and colleagues found that overexpression of the long noncoding RNA HOTAIR in epithelial cancer cells retargeted PRC2 genome-wide to specific targets, silencing metastasis suppressor genes [252]. Expression of HOTAIR was found to be associated with aggressive breast cancer and the authors observed that loss of HOTAIR inhibited cancer invasiveness, suggesting that lincRNAs may have a modulating role in recruiting PRC2 to genes involved in cancer progression. In another study, HOTAIR was found to target PRC2 to the human *HOXD* cluster as its depletion led to decreased H3K27me3 and the re-expression of genes in this locus [253]. Lastly, leukemic fusion proteins PML-RARα, PLZF-RARα and TMPRSS2-ERG have the capability to recruit PcG proteins to

specific target loci implying oncogenes may play a role in PcG-related carcinogenesis [254-256].

EZH2 and Cancer

As previously mentioned, EZH2 is overexpressed and associated with poor prognosis and metastasis in numerous types of cancer. EZH2 has been established as an oncogene as its overexpression in cancer cells *in vitro* leads to increased cell proliferation, invasion and colony formation [155, 156, 257, 258]. Additionally, overexpression of EZH2 induces tumor growth in xenograft mouse models [179, 258, 259]. Conversely, when EZH2 is downregulated in cancer cells, a concomitant decrease in cell proliferation *in vitro* and in tumor growth *in vivo* is observed [155, 179, 259-261].

Multiple studies have indicated that overexpression of EZH2 in cancer can occur at various levels. At the transcriptional level, Bracken and colleagues have revealed that the pRB-E2F pathway regulates the expression of EZH2 and EED through direct binding of E2F transcriptions factors to *EZH2* and *EED* promoters, which leads to activation and proliferation [257]. In Ewing's sarcoma, the EWS/FLI1 fusion protein binds to the EZH2 promoter and induces expression in tumor cells and mesenchymal stem cells [178]. Loss of the SNF5 tumor suppressor in prostate cancer leads to increased expression of EZH2 and activation of stem cell-associated gene expression signatures [262]. Additionally, micro-RNAs have been shown to regulate EZH2 levels as loss of miR-26a and miR-101 in lymphoma and prostate cancer cells, respectively, leads to EZH2 overexpression [263-265]. Recent work in lymphoma has also identified a heterozygous missense mutation in EZH2 at amino acid Y641, within the SET domain

[266, 267]. Wild type EZH2 has a high catalytic activity for monomethylation of H3K27, whereas Y641 mutants have enhanced catalytic activity for di- and trimethylation of H3K27. Therefore, the combination of the two catalytic activities of mutant and wild type EZH2 resulted in increased gene repression through H3K27me3 in a mechanism equivalent to EZH2 overexpression.

EZH2 plays a multi-faceted role in cancer through regulation of a plethora of genes, many of which have been implicated in development of metastasis. For example, Cao and colleagues have found in prostate cancer cells that EZH2 promotes EMT by repression of E-cadherin expression through interaction with Snail1, and this influence on E-cadherin has since been demonstrated in many other types of cancer cells [268]. In addition, EZH2 has been found in breast cancer cells to directly repress *ADAMTS1*, a gene encoding for a metallopeptidase which degrades proteins in the extracellular matrix disrupting cell adhesion and promoting migration [269]. EZH2 has also been found to be a direct driver of tumor initiation and metastasis in prostate cancer cells through silencing of *DAB2IP*, a RasGap gene known to regulate Ras and NF-κB [270, 271]. Moreover, repression of *FOXC1* by EZH2 in breast cancer cells was seen to inhibit differentiation, whereas stable overexpression of FOXC1 protein reduced migration and invasion *in vitro* and metastasis *in vivo* [272]. Ren and colleagues have reported that EZH2 directly represses the metastasis suppressor RKIP in breast and prostate cancer cells leading to increased invasion through an interaction with Snail1 [273]. Likewise, in hepatocarcinoma cells, EZH2 has been found to epigenetically repress several micro-RNAs characterized as tumor suppressors for their anti-tumor or anti-metastatic established roles [274]. In addition, EZH2 has been implicated in

promoting tumor angiogenesis and ovarian cancer growth *in vivo* as VEGF-stimulated overexpression of EZH2 leads to the repression of *VASH1*, a negative regulator of angiogenesis [275]. Our laboratory has discovered a novel link between EZH2 and DNA repair mechanisms as overexpression of EZH2 in breast cells impaired the formation of RAD51 repair foci at sites of DNA damage leading to decreased cell survival [276]. In another recent study, EZH2 expression-mediated downregulation of RAD51 led to *RAF1* gene amplification and an expansion in breast tumor-initiating cells, linking EZH2 to regulation of CSCs [277]. Taken together, these studies confirm the essential roles EZH2 plays in tumor progression and suggest that blocking EZH2 expression or activity may have therapeutic implications.

Although primarily known as a gene silencer, evidence has emerged indicating EZH2 in activating functions. In genome-wide mapping ChIP experiments, 10-20% of PcG target genes were found actively transcribed in embryonic stem cells, and 2% of genes bound by PcG proteins were also bound by RNA Polymerase II [200, 201]. Indeed, EZH2 has been demonstrated to be required in the expression of several genes important in cell cycle regulation providing a proliferative advantage [257]. In another study using breast cancer cells, EZH2 was demonstrated to directly activate the transcription of *c-myc* and *Cyclin D1*, genes commonly targeted by Wnt signaling, through interaction with Estrogen Receptor α and β-catenin [278]. This activity was found to be independent of the SET domain and instead required the two N-terminal homology domains of EZH2. Another study utilizing glioma CSCs and ChIP experiments also revealed that *c-myc* is a positively regulated direct target of EZH2 as *c-myc* expression was strongly repressed upon EZH2 downregulation [279]. Su and

colleagues have reported the existence of cytosolic EZH2, which still forms a complex with SUZ12 and EED [280]. They found EZH2 to regulate Actin polymerization, and suggested post-translational lysine methylation by EZH2 as a novel function in signal transduction. In further support, Lee and colleagues demonstrated that EZH2 physically interacts with RelA and RelB proteins to promote the expression of NF-κB target genes in basal-like breast cancer cells, independent of the SET domain [281]. Moreover, EZH2 has been indicated to activate the transcription of *AXL*, a gene encoding for a receptor tyrosine kinase implicated in mesenchymal development, in a methylation independent manner in glioma cells [282]. Another recent study demonstrated in prostate cancer cells that EZH2 binding to transcriptionally active gene sites occurred independent of PRC2 complex members and H3K27me3 [283]. Although independent of its H3K27me3, the authors found the SET domain to be required for gene activation. Taken together, these studies establish context-specific, non-repressive roles for EZH2, and promote the need for further research into understanding all functions of EZH2.

* * *

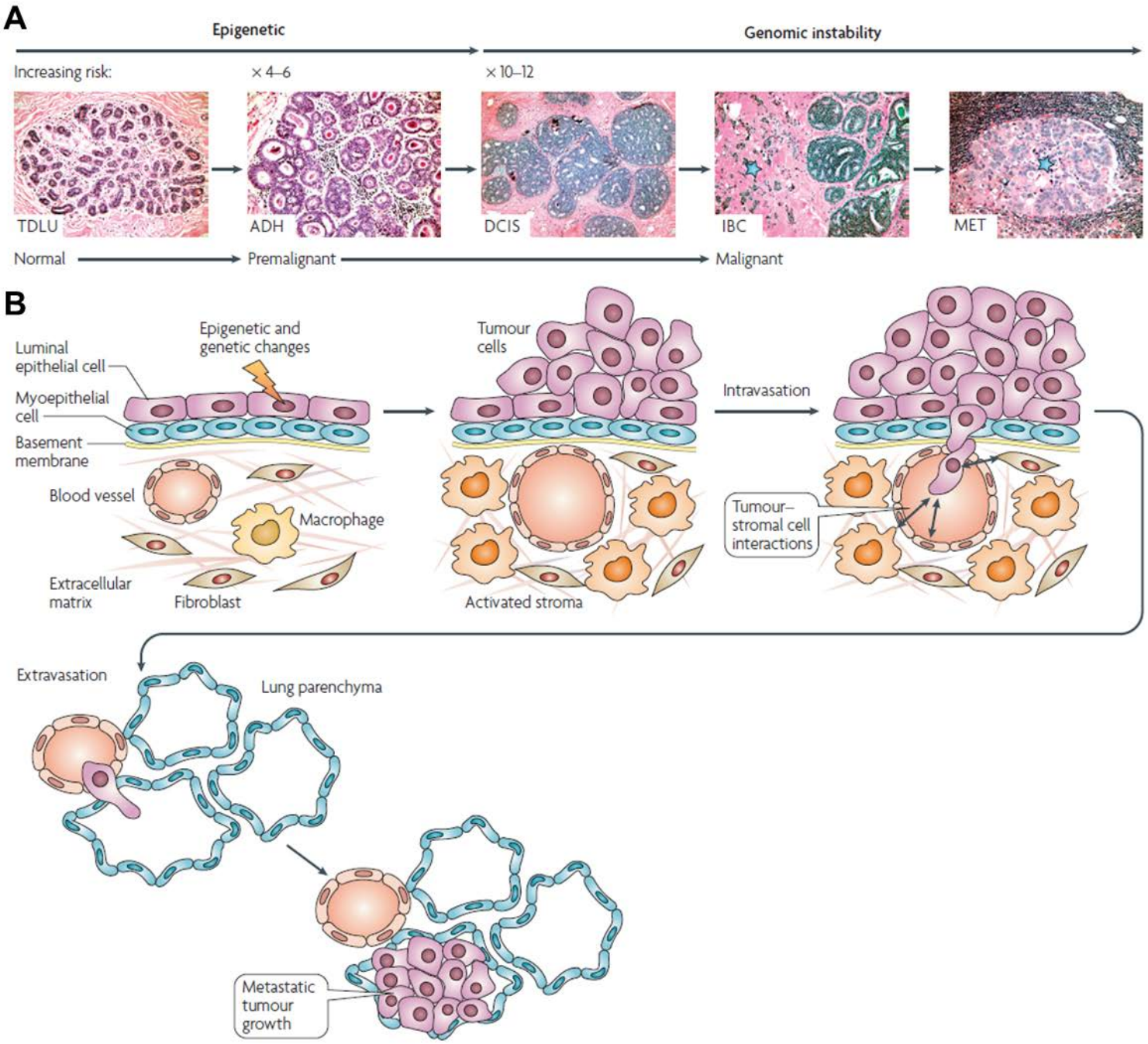
It is well known that EZH2 is vital in a number of key cellular processes, such as embryonic and adult stem cell maintenance and tumor progression. Although an association between EZH2 and breast cancer metastasis has been established, a causal relationship has not. The over-arching hypothesis of this thesis is that EZH2 overexpression in cancer cells is required for breast cancer metastasis. In Chapter 2, the regulation of EZH2 on key steps in metastasis, such as EMT, invasion and motility, and the underlying mechanisms will be more thoroughly examined *in vitro*. Using a

xenograft mouse model system, we will directly address whether knockdown of EZH2 in breast cancer cells affects metastatic burden *in vivo*. In Chapter 3, the regulation of EZH2 on CSCs, which are implicated in metastasis, will be studied in both tumorigenic and malignant breast cells. Do the levels of EZH2 influence the numbers and tumorigenicity of CSCs? And, if so, what signaling pathways are essential for this function? These important questions will be the primary focus in the following chapters.

1-5. Figures

Figure 1-1 The continuum of breast cancer progression and the metastatic cascade.
(A) Breast cancer is a believed to develop along a continuum. The normal breast terminal ductal lobular unit (TDLU) contains lobules and ducts that consist of a bilayered epithelium of luminal and myoepithelial cells. Atypical ductal hyperplasia (ADH) is a premalignant lesion characterized by abnormal cell layers within the duct or lobule. ADH is thought to be the precursor of ductal carcinoma in situ (DCIS), which is a non-invasive lesion that contains abnormal cells still confined within the duct. With each stage, the risk of developing malignant or invasive breast cancer (IBC) increases. DCIS may give rise to IBC (indicated by a blue star adjacent to a DCIS lesion), although how to predict which lesions will progress is still unknown. Once cells have invaded through the surrounding basement membrane into the stroma, the risk for developing metastasis significantly increases. The lymph nodes are the primary site for breast cancer metastasis (indicated by a blue star). **(B)** A schematic of breast cancer progression is shown. The transformation of breast epithelial cells to metastatic breast cancer is an accumulation of epigenetic and genetic changes and aberrant interactions within the microenvironment. During this multistage process, control of proliferation, survival, differentiation and migration become deregulated, and aberrant tumor–stromal cell interactions facilitate this process. To form metastases, cells must invade through the basement membrane, enter the vasculature through intravasation, survive in the absence of adhesion in the circulatory system, exit the vasculature through extravasation, and establish a new tumor in a foreign microenvironment.
(From Vargo-Gogola and Rosen, Nature Reviews Cancer, 2007)

(Figure on following page)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Luminal epithelial cell
Myoepithelial cell
Basement membrane
Blood vessel
Macrophage
Extracellular matrix
Fibroblast
Epigenetic and genetic changes
Tumour cells
Activated stroma
Intravasation
Tumour–stromal cell interactions
Extravasation
Lung parenchyma
Metastatic tumour growth

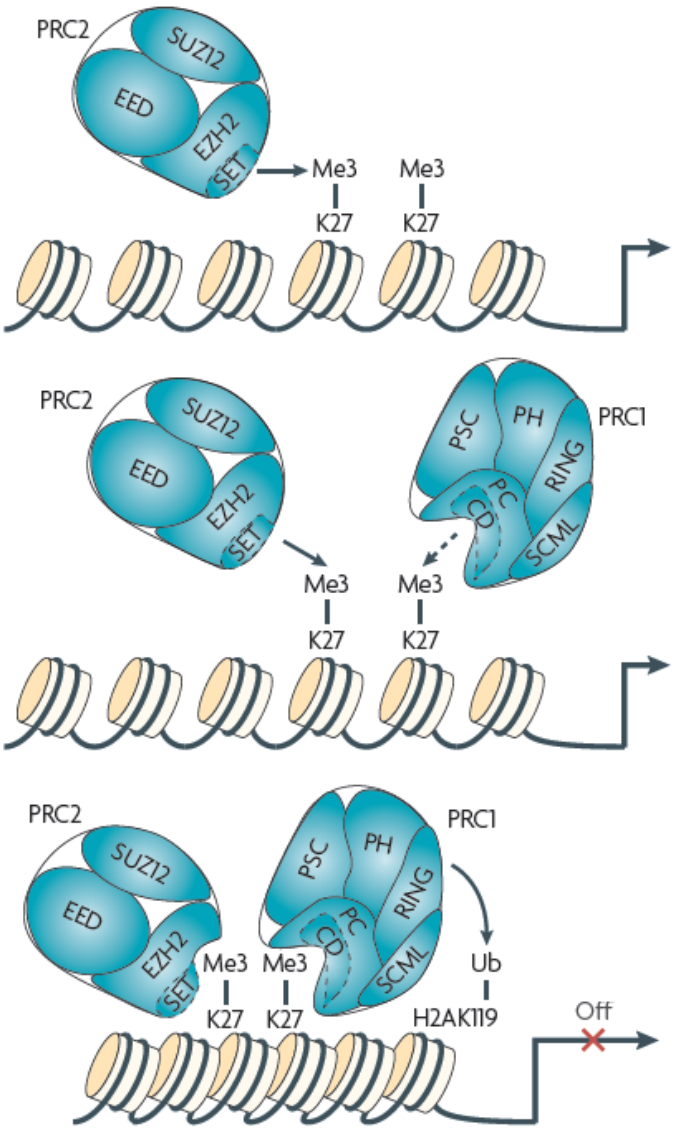

Figure 1-2 Polycomb Repressive Complexes 1 and 2 (PRC1 & PRC2) coordinately work together to repress gene expression.

(A) PcG proteins are mainly divided into two distinct groups dependent on their formation of the multimeric complexes PRC1 and PRC2. The methyltransferase EZH2 is the catalytic core member of PRC2, which trimethylates histone H3 at lysine 27 utilizing its SET domain (H3K27me3) in association with other members EED and SUZ12. The core composition of PRC1 is much more variable and contains one subunit of the CBX, RING1, SCML, PHC, and PCGF paralog protein groups. It is believed that PRC1 is recruited to the H3K27me3 mark through the affinity of chromodomains in CBX proteins. PRC1 recruitment leads to the monoubiquitination of histone H2A on lysine 119 by the RING1 proteins, which is thought to block the binding of transcription factors and inhibit transcription initiation by RNA Polymerase II resulting in transcriptional repression.

(From Bracken and Helin, Nature Reviews Cancer, November 2009, Volume 9)

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CHAPTER 2

EZH2 inhibition decreases p38 signaling and suppresses breast cancer motility and metastasis

The contents of following chapter have been published in Breast Cancer Research and Treatment (DOI: 10.1007/s10549-013-2498-x) under the above title by Heather M. Moore, Maria E. Gonzalez, Kathy A. Toy, Ashley Cimino-Mathews, Pedram Argani and Celina G. Kleer.

2-1. Abstract

EZH2 is a Polycomb group protein that exerts oncogenic functions in breast cancer, where its overexpression is associated with metastatic disease. While it reportedly acts a transcriptional repressor through trimethylation of histone H3 at lysine 27, EZH2 may exhibit context-dependent activating functions. Despite associations with worse outcome and metastasis in breast cancer, a functional role of EZH2 in breast cancer metastasis *in vivo* has not been demonstrated. Furthermore, whether EZH2 regulates cancer cell phenotype and motility are unknown. In this study, we discovered that knockdown of EZH2 induces a phenotypic reprograming from mesenchymal to epithelial, reduces motility, and blocks invasion in breast cancer cells. *In vivo*, EZH2 downregulation in MDA-MB-231 cells decreases spontaneous metastasis to the lungs. We uncover an unexpected role of EZH2 in inducing the p38 mitogen-activated protein kinase signaling pathway, an important regulator of breast cancer invasion and

metastasis. In breast cancer cells EZH2 binds to phosphorylated-p38 (p-p38) in association with other core members of the Polycomb Repressive Complex 2 (PRC2), EED and SUZ12, and EZH2 overexpression leads to increased levels of p-p38 and of activated, downstream pathway proteins. The effect on p-p38 was confirmed *in vivo* where it correlated with decreased spontaneous metastasis. In clinical specimens of matched primary and invasive breast carcinomas, we found that EZH2 expression was upregulated in 100% of the metastases, and that EZH2 and p-p38 were coexpressed in 63% of cases, consistent with the functional results. Together our findings reveal a new mechanism by which EZH2 functions in breast cancer, and provide direct evidence that EZH2 inhibition reduces breast cancer metastasis *in vivo*.

2-2. Introduction

Breast cancer is the 2nd most common cause of cancer-related deaths for women in the United States [1]. Despite advances in breast cancer detection and treatment strategies, metastatic breast cancer is essentially incurable and the 5 year survival rate for women diagnosed with distant metastatic disease is only 23% [2]. The degree of breast cancer cell differentiation directly impacts its metastatic ability; the more undifferentiated the primary invasive carcinoma, the greater likelihood to develop metastasis [3]. Thus, it is not surprising that dysregulation of cell type identity and differentiation programs directly impact breast cancer metastasis.

Polycomb group proteins are major regulators of cellular memory that function in multimeric complexes to regulate the expression of specific genes, mainly through transcriptional repression. Enhancer of Zeste Homolog 2 (EZH2) is the catalytic core member of the Polycomb Repressive Complex 2 (PRC2), which catalyzes the

trimethylation of histone H3 lysine27 (H3K27me3) [4-6]. Although primarily functioning in gene repression, EZH2 has been shown to exhibit gene activating functions, at times through mechanisms independent of its histone methyltransferase activity [7-13]. EZH2 is highly expressed in a wide range of human cancers and has been shown to mediate the expression of target genes involved in tumorigenesis, including cell cycle regulation and proliferation, stem cell maintenance, cell differentiation, and neoplastic cell transformation [14-16]. EZH2 protein is overexpressed in 55% of invasive breast carcinomas, and is significantly associated with poorly differentiated, estrogen receptor negative (ER^-) tumors. We have demonstrated that EZH2 is an independent prognostic biomarker in breast cancer as women with tumors expressing high EZH2 have worse disease free and overall survival than women with tumors expressing low EZH2 [17-21]. Despite these associations, direct demonstration that EZH2 downregulation decreases breast cancer metastasis is lacking.

The p38 mitogen-activated protein kinase (MAPK) signaling pathway plays a complex and key role in cancer progression by translating extracellular signals into cellular responses through phosphorylation of specific serine and threonine residues of downstream effector proteins, especially transcription factors and protein kinases. Four p38 isoforms have been identified, whose implications in tumorigenesis may depend on cell context and tumor type [22, 23]. Once activated, p38 has been associated with regulation of the epithelial-to-mesenchymal transition (EMT), invasion and motility of cancer cells, all cellular processes that are crucial to metastasis [22, 23]. Recently, elevated p38γ expression was shown to be associated with a lower overall survival of patients with breast cancer [24].

In this study, we demonstrate a previously undescribed function of EZH2: its role in cancer cell motility and cell phenotype. EZH2 knockdown in breast cancer cells induces a mesenchymal-to-epithelial transition (MET), decreases cancer cell motility and the speed of movement. We provide first evidence that EZH2 knockdown in breast cancer cells reduces lung metastasis *in vivo*. Mechanistically, EZH2 binds to phosphorylated p38 (p-p38) and upregulates p38 downstream signaling, while EZH2 inhibition in breast cancer cells decreases p-p38 binding, expression, and downstream signaling. The relevance of our *in vivo* and *in vitro* studies to human breast cancer is highlighted by the finding that human breast cancer distant metastases express high levels of EZH2 and p-p38. Taken together, this study identifies a novel function of EZH2 in controlling p-p38 activity, breast cancer cell motility, invasion and metastasis.

2-3. Materials and Methods

Cell culture

Breast cancer cell lines MDA-MB-231 and MCF7 and mammary epithelial cell line MCF10A were obtained from the American Type Culture Collection (Manassas, VA). All obtained cell lines were grown under recommended conditions. The SUM149 breast cancer cell line was obtained from the S. Ethier laboratory (Karmanos Cancer Institute, Detroit, MI) and cultured as previously reported [25].

The primary breast cancer cells (designated as Primary Tumor in text and figures) were derived from a donated tissue from a consenting 36 years old patient in accordance with a protocol approved by the Institutional Review Board at the University of Michigan (IRB#2002-227). The tumor was 2.4cm in size and classified as a Grade 3, triple negative, invasive ductal carcinoma. The tumor sample was obtained from the

operating room and immediately processed in the laboratory. A portion of the tumor was formalin fixed and paraffin embedded for staining with hematoxylin and eosin (H&E) and immunohistochemical staining with anti-EZH2 (Cell Signaling, #5246, 1:100); see the Immunohistochemistry section for a detailed staining protocol. Another portion was processed a to single cell suspension as previously described [26]. Briefly, the tumor specimen was manually minced and then dissociated in a collagenase-hyaluronidase solution (StemCell Technologies, #07912). Next, red blood cells were lysed with RBC Lysis Solution (Qiagen, #158902). For further purification, the tissue was treated with Trypsin-EDTA (GIBCO, #25200-056), then DNase1 (StemCell Technologies, #07900) and finally filtered through a 40µm cell strainer. Cells were then cultured in Mammary Epithelial Cell Medium (ScienCell, #7611) completed with Mammary Epithelial Cell Growth Supplement and penicillin-streptomycin (ScienCell, #7652 and #0503).

EZH2 knockdown using stable short-hairpin interfering RNA in lentivirus was completed as previously reported [20]. For targeting EZH2 (NM_152998, NCBI), the shRNA oligo ID# V2LHS_17507 was used and corresponded to #RHS4430-99139126 from Open Biosystems (Hunstville, AL); this oligo was cloned into a pLKO.1-Puro vector. The shRNA containing plasmid was packaged into lentiviral particles at the Vector Core at the University of Michigan. A lentivirus containing a plasmid encoding a scrambled shRNA oligo was used as a control. Cells were transduced and selected for antibiotic resistance with puromycin (Sigma-Aldrich, #P9620). EZH2 knockdown was also achieved using 3-Deazaneplanocin A (Cayman Chemical, #13828) at 1µM for 5 days treating every other day. As previously reported, transient EZH2 overexpression was achieved through infection with a wild type EZH2-encoding, myc-tagged pCMV for

48 hours, kind gift of A. Chinnaiyan [27, 28]. The p-p38 inhibitors, SB203580 (Cell Signaling, #5633) or SB202190 (abcam, #120638) were used at 10 or 20μM for 48 hours.

Western blotting and immunoprecipitations

Cells were lysed in RIPA lysis buffer with added protease and phosphatase inhibitors (Thermo Scientific, #89900, #78410 & #78420) and Western blot analyses were carried out using 50μg of whole cell extract. Samples were boiled in laemmli sample buffer, separated by SDS-PAGE gels and transferred onto PVDF membranes. Membranes were blocked and incubated with primary antibodies in 3% BSA (Sigma-Aldrich, #A3059) in TBS-T (Bio-Rad, #161-0372, with 0.05% Tween20) at 4°C overnight. Protein signals were visualized via chemiluminescence as described by manufacturer (Thermo Scientific, #32106). β-Actin-HRP (Santa Cruz, #47778) was used to confirm equal loading. The following antibodies from Cell Signaling antibodies: rabbit monoclonals anti-EZH2 (#5246), anti-E-cadherin (#3195), anti-SUZ12 (#3737), anti-p38β (#2339), anti-p38δ (#2308), anti-MAPKAPK-2 (#3042), anti-Snail1 (#3879); rabbit polyclonals anti-p38 (#9212), anti-p38α (#9218), anti-p38γ (#2307), anti-phospho-HSP27 (Ser82, #2401), anti-phospho-MAPKAPK-2 (#3007); mouse monoclonals anti-Snail1 (#3895) and anti-HSP27 (#2402). From Abcam: rabbit monoclonal anti-Cytokeratin-18 (#32118), rabbit polyclonal anti-EED (#4469) and mouse monoclonal anti-trimethyl-HistoneH3 (#6002). Additionally: rabbit polyclonal anti-ACTIVE®-p38 MAPK (pTGpY, Promega, #V1211) and rabbit monoclonal anti-Vimentin (Epitomics, #2707-1).

Immunoprecipitations (IPs) were conducted following protocol instructions (Sigma-Aldrich, #IP50). Protein was extracted from 70% confluent cells with provided IP buffer and added protease and phosphatase inhibitors (Thermo Scientific, #78410 & #78420). Whole protein extracts were precleared with provided Protein G agarose beads for 3 hours and then incubated with antibody (anti-normal mouse IgG [Santa Cruz, #2025], anti-p38 [Novus Biologicals, #NBP1-97545], anti-EED [abcam, #4469], anti-EZH2, anti-phospho-p38, or anti-SUZ12 [Cell Signaling, #5246, #9216, #3737, respectively]) overnight at 4°C. Next day, protein–antibody complexes were captured with Protein G agarose beads for 2 hours, washed in stringent conditions and eluted in laemmli sample buufer. Inputs and IPs were separated as by above described Western blot protocol. Immunoprecipitated EED was detected using Clean-Blot™ IP HRP (Thermo Scientific, #21230) to avoid interference from denatured IgG.

Proliferation Assays

Proliferation assays were conducted using the FluoReporter Blue Fluorometric dsDNA Quantitation Kit (Molecular Probes, #F-2962) following protocol instructions. SUM149, MDA-MB-231 and Primary Tumor breast cancer cells were plated at a density of 1000-2000 cells per well in a 96 well plate with at least 8 wells per condition. For the next seven days, Hoechst 33258 DNA staining was completed using kit reagents and fluorescence above blank background was measured at 460nm.

Invasion and motility assays

In vitro invasion was done using Matrigel Invasion Chambers (BD Biosciences, #354480) according to the manufacturer's instructions, in triplicate. Invasive cells located on the lower sides of chambers were crystal violet stained, air-dried and photographed. They were quantified using ImageJ to count colored pixels, or for colorimetric assays, inserts were treated with 10% acetic acid to remove dye and the absorbance was measured at 560nm using a spectrophotometer.

Random motion cell motility assays were completed as previously described [24]. Briefly, cells were plated on collagen-coated chambered coverslips at low density attaching overnight. Next day, cells were imaged every 10 minutes at 37ºC for 24 hours using the DeltaVision RT Live Cell Imaging System (Applied Precision, GE Healthcare) equipped with a UPlanAo 20X/0.7 NA lens at the University of Michigan Microscopy and Image analysis Laboratory. DIC images were acquired using SoftWoRx 3.5.1 software, and individual cell movements were quantified using MTrackJ /ImageJ software.

Real-time RT-PCR

Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) amplifications were carried out with 1µg of total RNA isolated from the indicated breast cells and conditions. Reactions were performed in triplicate using an Applied Biosystems StepOnePlus RT-PCR System available in the Michigan MicroArray Core with Qiagen primers and SYBR Green Master Mix (Applied Biosystems, #4309155). All primers were purchased from Qiagen: GAPDH #PPH00150E-200, MAPK1 (Total p38)

#PPH00715B-200, MAPK14 (p38α) #PPH00750B-200, MAPK11 (p38β) #PPH01778B-200, MAPK12 (p38γ) #PPH01779A-200, and MAPK13 (p38δ) #PPH00188B-200.

In vitro methylation

The *in vitro* methylation assay was completed as previously described [29]. Briefly, 1µg recombinant p38α-GST (BPS Bioscience, #40070) was incubated with 5µl methylation buffer (20mM Tris-HCl [pH 7.8], 5mM DTT, 0.5mM EDTA, 10% glycerol) in the presence of 10µM H^3-S-Adenosylmethionine, with or without the presence of 2µg purified PRC2 complex (total protein amount), at 30°C for 1 hr. Samples were then separated via SDS-PAGE, the gel was coomassie-stained to visualize proteins, and methylation was detected by autoradiograph at 21 days. We thank Yali Dou and Bo Zhou for assistance with this assay.

Spontaneous metastasis assay

Ten-week-old severe combined immunodeficiency mice (Jackson Laboratories) were used for examining tumorigenicity as previously reported [20]. Briefly, MDA-MB-231 cells expressing shEZH2 or scrambled control were orthotopically injected into the mammary fat pad at a concentration of 2×10^6 cells in 20 mice (n=10 per group). Tumor size was measured twice a week until tumors reached 2 cm^3 (tumor volume=(length x $width^2$)/2), at which time mice were sacrificed and primary tumor and lung tissues were collected. Tissues were formalin-fixed and paraffin-embedded for H&E staining and immunohistochemical staining with anti-EZH2 (Cell Signaling, #5246), anti-Cytokeratin-18 (abcam, #32118, 1:100), anti-Snail1 (Cell Signaling, #3895, 1:800), anti-Ki67 (Fisher,

#RM-9106, 1:2000), or anti-ACTIVE®-p38 MAPK (Promega, #V1211, 1:325); see the Immunohistochemistry section for a detailed staining protocol. Image analysis and quantification of only metastatic cells to determine the percentage of relative stained area was completed using FRIDA (FRamework for Image Dataset Analysis), a custom open source image analysis software package for the analysis of RGB color image datasets [30]. Additional information can be found at http://bui3.win.ad.jhu.edu/frida/. All procedures involving mice were approved by the University Committee on Use and Care of Animals at the University of Michigan and conform to their relevant regulatory standards.

Human breast tissue microarray

A high-density tissue microarray (TMA) containing 16 human primary invasive breast carcinomas with matched metastases was employed as previously reported [31, 32]. Immunohistochemistry on formalin-fixed, paraffin-embedded tissue blocks was performed using anti-EZH2 (Cell Signaling, #5246, 1:150) and anti-phospho-p38 MAPK (Cell Signaling, #9216, 1:3000); see the Immunohistochemistry section for a detailed staining protocol. EZH2 and p-p38 expression was evaluated as low or high based on intensity of staining and percentage of staining cells, following published literature [33]. The complete clinical and pathological information on these tumors is shown in Figure 1-11. We thank Dr. Ashley Cimino-Mathews and Dr. Pedram Argani at The Johns Hopkins Hospital for making the tissue microarray available to our laboratory for staining and analysis.

Immunohistochemistry

Formalin fixed, paraffin embedded tissue blocks were sectioned at 5u and placed on charged slides. Slides were deparaffinized in xylene and rehydrated through graded alcohols. Heat Induced Epitope Retrieval (HIER) was performed in the Decloaking Chamber (Biocare Medical) with Target Retrieval, pH 6.0 (DakoCytomation). Slides were incubated in 3% Hydrogen Peroxide for 5 minutes to quench endogenous peroxidases. Primary tumor tissue, mouse tissue and human TMA tissue slides were incubated for 1.5 hours at room temperature with previously indicated antibodies. Antibodies were detected with either anti-rabbit or anti-mouse Envision^{+} HRP Labelled Polymer (DakoCytomation) for 30 minutes at room temperature. HRP staining was visualized with the DAB^{+} Kit (DakoCytomation). Negative control slides were run. Slides were counterstained in hematoxylin, blued in running tap water, dehydrated through graded alcohols, cleared in xylene and then mounted with Permount.

2-4. Results

EZH2 knockdown induces a mesenchymal-to-epithelial transition and decreases the ability of breast cancer cells to move.

EZH2 overexpressing breast carcinomas have aggressive clinical behavior, high frequency of estrogen receptor negative status (ER^{-}) and are associated with a high propensity to metastasize [17]. However, direct evidence that EZH2 regulates cancer cell phenotype and motility are lacking. To test the hypothesis that EZH2 knockdown may reduce the ability of breast cancer cells to move and invade into the surrounding tissues, we employed breast cancer cell lines MDA-MB-231 and SUM149, both of which are ER^{-}, invasive, tumorigenic in vivo and express high levels of EZH2 protein in

comparison to nontumorigenic breast cell lines [20]. We utilized two independent and complementary methods to downregulate EZH2 protein levels in breast cancer cells: stable expression of a short hairpin RNA interference (shRNA) in a lentiviral vector and pharmacologic inhibition using 3-Deazaneplanocin A (DZNeP), a histone methyltransferase inhibitor which disrupts PRC2 (Fig. 2-1, A). As we previously reported that shRNA knockdown of EZH2 significantly reduced proliferation [20], we performed proliferation assays to confirm that DZNeP treatment also induced a decrease in proliferation (Fig. 2-1, B).

EZH2 knockdown through either shRNA or DZNeP treatment was sufficient to induce a morphologic and a molecular mesenchymal-to-epithelial transition (MET) of SUM149 and MDA-MB-231 cells when compared to scrambled shRNA or untreated controls, respectively (Fig. 2-2, A-D). The observed morphologic change was associated with a protein expression profile characteristic of MET: increased expression of the epithelial markers Cytokeratin-18 and E-cadherin and decreased expression of the mesenchymal markers Vimentin and Snail1 (Fig. 2-2, A-B). Knockdown of EZH2 with shRNA or DZNeP significantly reduced invasion in MDA-MB-231 and SUM149 cells when compared to corresponding controls (Fig. 2-2, E-F). Extending the relevance to human disease, EZH2 knockdown with DZNeP significantly decreased proliferation and invasion in primary breast cancer cells derived from a patient diagnosed with a triple negative, grade 3, invasive ductal carcinoma (Fig 2-3, A-D).

We next investigated the role of EZH2 on cell motility, a critical step in metastasis. Random cell motion was quantified using live cell imaging with time-lapse microscopy as previously described [24]. EZH2 downregulation by shRNA or DZNeP in

MDA-MB-231 cells significantly decreased the average cell velocity when compared to controls (Fig. 2-4, A-B). Furthermore, rescue of EZH2 expression using a myc-tagged EZH2 adenovirus partially reversed the decreased motility induced by EZH2 knockdown in MDA-MB-231 cells (Fig. 2-4, C). Collectively, these experiments show that EZH2 downregulation promotes a MET and reduces the motility and invasiveness of breast cancer cells.

EZH2 regulates the levels of phosphorylated p38 protein and signaling pathway.

p38 has emerged as an important regulator of cell migration and metastasis in breast cancer models [23, 24, 33]. Whether EZH2 influences the levels and function of p38 in human breast cancer is unknown. We found that EZH2 downregulation with shRNA or DZNeP reduced p-p38 protein and the phosphorylation of downstream targets MAPKAPK-2 (MK2) and Heat Shock Protein 27 (HSP27) in breast cancer cell lines and primary breast cancer cells when compared to controls (Fig. 2-5, A-B). No significant effect on the protein levels of total p38 was observed by EZH2 knockdown. Conversely, adenoviral overexpression of myc-tagged EZH2 in nontumorigenic MCF10A breast cells and in MCF7 breast cancer cells consistently led to upregulation of p-p38 protein levels when compared to controls (Fig. 2-5, C).

To further define the mechanistic link between EZH2 and p38, we tested whether p-p38 regulates the levels of EZH2 and other core components of PRC2. Treatment with SB203580 or SB202190, which inhibit the ability of activated p-p38 to phosphorylate downstream targets, such as HSP27, had no effect on EZH2, SUZ12, EED, or H3K27me3 protein levels in MDA-MB-231 cells (Fig. 2-6, A). These data

indicate that EZH2 levels in breast cancer cells are not affected by the p38 signaling pathway.

We next investigated whether EZH2 regulates a specific p38 isoform by testing the effect of EZH2 knockdown on the expression of phosphorylated p38α, p38β, p38γ and p38δ. As phospho-specific isoform antibodies are not available, total p-p38 was immunoprecipitated from whole cell extracts of MDA-MB-231 and SUM149 cells expressing scrambled or EZH2-targeted shRNA followed by Western blot analysis for the four isoforms. EZH2 knockdown decreased the phosphorylated levels of all isoforms when compared to controls, while total p38 isoform protein levels remained unaffected (Fig. 2-6, B). Further supporting a non-transcriptional role for EZH2 in the regulation of p-p38, quantitative real-time RT-PCR showed that neither knockdown nor overexpression of EZH2 affected the mRNA levels of total p38 or the individual p38 isoforms when compared to controls (Fig. 2-6, C). Collectively, these results show that EZH2 regulates the phosphorylated levels of all p38 isoforms in breast cancer cells and suggest either an indirect transcriptional or a post-transcriptional regulatory mechanism.

EZH2 protein binds with phosphorylated p38 protein.

To further understand the mechanism by which EZH2 regulates p-p38, we tested the hypothesis that EZH2 may bind to p-p38 in breast cancer cells. Immunoprecipitation and Western blot analyses revealed that endogenous EZH2 protein interacts with p-p38 in SUM149 and MDA-MB-231 breast cancer cells; and that the binding is reduced by EZH2 knockdown, thereby supporting the specificity of the interaction (Fig. 2-7, A-B). Furthermore, reciprocal immunoprecipitation experiments demonstrated that EZH2

protein binds to p38/p-p38 protein in association with EED and SUZ12 (Fig. 2-7, B). As PRC2 functions in protein methylation, we hypothesized that PRC2 may methylate p38. Even though all p-p38 isoforms were affected by EZH2 protein expression, we chose to analyze p38α because it is the most abundant and ubiquitously expressed [34]. An *in vitro* methylation assay shows that addition of PRC2 leads to p38α protein methylation (Fig. 2-7, C). Collectively, these data demonstrate that EZH2 binds to p-p38 in association with PRC2, and show that PRC2 can methylate p38α *in vitro*, which paves the way for future mechanistic investigations.

EZH2 knockdown is sufficient to reduce distant metastasis.

We previously demonstrated in a xenograft mouse model that stable EZH2 knockdown in MDA-MB-231 cells decreased the rate of breast tumor growth and the volume of tumors and improved survival, but the effect of EZH2 downregulation on the development of distant metastases in these mice was not determined [20]. Compared to the scrambled shRNA control, EZH2 knockdown reduced the ability of MDA-MB-231 cells to form spontaneous lung metastasis when injected into the mammary fat pads of NOD/SCID mice (Fig. 2-8, A-B). Histological analysis of lung tissues collected when primary tumors reached 2 cm^3, revealed that 8 of 10 (80%) MDA-MB-231/control mice developed metastases compared with 6 of 10 (60%) of MDA-MB-231/shEZH2 mice. Although no significant difference was observed in the number of mice developing lung metastases between conditions, the metastatic burden as determined by the number of lung metastases per mouse was significantly reduced in shEZH2 mice in comparison to control mice (Fig. 2-8, B). In addition, the metastases formed by shEZH2 cells were

smaller than controls; the average sizes of the largest lung metastasis per mouse were 304µm and 737µm from shEZH2 and control mice, respectively.

Consistent with our functional findings, pathological analyses revealed a change in the invasive pattern of the breast cancer cells at the metastatic site. The metastases formed by control cells exhibited irregular and infiltrative borders, and encased pre-existing normal structures such as bronchioles and blood vessels (Fig. 2-8, A). In contrast, metastases formed by shEZH2 cells were smaller and circumscribed, with round borders and minimal parenchymal infiltration (Fig. 2-8, A). Consistent with the *in vitro* data and the histopathological findings, metastases formed by shEZH2 cells exhibited increased Cytokeratin-18 and decreased Snail1 proteins compared to metastases formed by the controls, as demonstrated by double immunohistochemical analyses (Fig. 2-8, A); this effect was also observed in the primary xenografts (Fig. 2-9, A). EZH2 knockdown metastases had significantly reduced cell proliferation as measured by Ki67 immunohistochemical staining compared to controls, complementing the previous data which showed decreased mitotic activity in the primary breast cancers (Fig. 2-9, B). Supporting our *in vitro* observations and mechanistic studies, shEZH2 lung metastases had decreased p-p38 levels when compared to controls (Fig. 2-9, C).

Human breast cancer metastases exhibit high EZH2 and p-p38 protein expression.

The relevance of these novel findings to human breast cancer was validated by examining the expression of EZH2 and p-p38 proteins in a unique cohort of primary invasive carcinomas and their matched metastases from 16 patients, arrayed in tissue

microarrays [31, 32]. The patients in the cohort varied in age from 33 to 58 years old at diagnosis, and all had been initially diagnosed with invasive carcinoma. The interval to metastasis in the patients ranged from at time of initial diagnosis to seven years. Distant metastases were found in lung, brain, ovarian, pancreatic and bowel tissues. When present, both proteins predominantly localized to the nuclei of breast cancer cells (Fig. 2-10, A-B). EZH2 and p-p38 were scored as exhibiting low or high expression according to a previously validated schema [17, 33]. We found that EZH2 was significantly upregulated in all metastases when compared to primary carcinomas, and that EZH2 and p-p38 were co-expressed in 63% of the metastases (Fig. 2-10, C). The complete clinical and pathological information, including individual EZH2 and p-p38 scoring, on these tumors is shown in Figure 2-11.

2-5. Discussion

The data presented here reveal the previously undescribed findings that downregulation of EZH2 leads to MET and decreases motility in breast cancer cells. We show for the first time that EZH2 knockdown is sufficient to reduce distant metastasis in vivo. We uncovered a novel mechanism of EZH2 function by which EZH2 protein binds to p-p38 and leads to upregulated expression of p-p38 protein and its signaling pathway in breast cancer cells.

It has become increasingly evident that cancer cell plasticity influences the biologic behavior of breast cancer by allowing the conversion between epithelial and mesenchymal states [35]. EMT describes the reversible and dynamic process in which epithelial cells, characterized as organized and polarized cells closely attached by intercellular adhesion complexes, undergo a change into mesenchymal-like cells,

characterized by a lack of polarization and intercellular junctions. The process of epithelial cancer cells acquiring attributes of mesenchymal-like cells and being driven towards a motile state and metastasis is referred to as oncogenic EMT [35]. One of the hallmarks of EMT is the reduction of normal expression of the cell-cell junction protein E-cadherin [36]. We and other investigators have reported that EZH2 overexpression induces invasion in nontumorigenic breast and prostate cells, and decreases the expression of E-cadherin [27]. However, whether EZH2 can influence EMT and motility of breast cancer cells has not been previously considered. In this study, we show that EZH2 downregulation in breast cancer cell lines is sufficient to reprogram the phenotype of the cells from a spindle to an epithelial morphology with upregulation of E-cadherin and Cytokeratin-18, and downregulation of the mesenchymal protein Vimentin and the EMT transcription factor Snail1. The molecular and morphologic features indicative of EMT are tightly associated with the ability of cancer cells to migrate and invade, enabling metastasis. We found that EZH2 downregulation decreased invasion and motility in breast cancer cell lines. Time-lapse microscopy demonstrated that EZH2 downregulation decreased average cell velocity compared to controls.

p38 has been established as a regulator of transitions between epithelial and mesenchymal states as well as cancer cell migration [23, 24, 33]. Activated p-p38 regulates transcription factors responsible for E-cadherin repression including Snail1, Slug and Twist inducing a mesenchymal-like phenotype [37-40]. During TGF-β-induced EMT, p38 activation increases breast cancer lung metastasis [41]. p38α activity is required for the invasive capability of breast, pancreatic, hepatocellular, and head and neck squamous carcinoma cell lines, in part through regulation of matrix

metalloproteinases implicated in extracellular remodeling and degradation [33, 42-50]. Also, p38δ has been proposed to regulate the invasion of squamous cell carcinoma, while p38γ has been associated with Ras-induced invasion [43, 51]. Recently, down-regulation of p38γ markedly decreased the cell motility of breast cancer cells *in vitro* [24]. In human cancers, increased expression of p38α has been associated with cancer progression and poor prognosis in a number of malignancies, including breast carcinoma [43, 44, 52-55]. Despite the important role of p38 in motility, invasion and metastasis of human cancer, the mechanisms regulating its activation are still being defined. Through a combination of knockdown and overexpression strategies, EZH2 emerges as a novel regulator of p-p38 protein levels and the levels of its downstream signaling proteins in nontumorigenic breast cells and breast cancer cell lines.

The mechanisms implicated in the oncogenic role of EZH2 need further investigation. EZH2 has been considered largely a transcriptional repressor of tumor suppressor genes as part of PRC2, but recent evidence supports contextual, activating functions of EZH2 [7-13]. Here, we demonstrate that EZH2 regulates p-p38 via a non-transcriptional mechanism. We found that EZH2 had no effect on the mRNA levels of p38 isoforms using quantitative RT-PCR. Unexpectedly, in breast cancer cells, endogenous EZH2, EED and SUZ12 proteins bind to p-p38 protein, and that downregulation of EZH2 abrogates the binding of EZH2 and p-p38. Our *in vitro* methylation assay results suggest that PRC2 may methylate p-p38, and paves the way for future studies. Our data lead to the novel hypothesis that EZH2 in association with other PRC2 members may influence p-p38 activity, which is under investigation in our laboratory.

Our group has previously reported that high EZH2 protein expression is associated with the development of metastasis in breast cancer and worse clinical outcome [17]. Data presented here show for the first time that EZH2 knockdown reduces the number of distant breast cancer metastases in vivo. EZH2 knockdown in highly aggressive MDA-MB-231 cells decreased the metastatic burden and reduced the invasiveness of breast cancer cells at the metastatic site, as well as the expression of p-p38. In paired human samples of primary and metastatic carcinomas, EZH2 was significantly overexpressed at the metastatic site. Furthermore, co-expression of EZH2 and p-p38 were detected in 63% of the metastatic carcinomas.

In conclusion, our results demonstrate a previously unknown mechanism of EZH2 function in breast cancer metastasis. We have shown that EZH2 inhibition in breast cancer cell lines leads to a phenotypic change from mesenchymal to epithelial, with reduced motility, invasion and metastasis. We uncover a previously unknown molecular mechanism by which EZH2 binds to p-p38 and regulates the activation of the p38 signaling pathway. From a clinical perspective, the role of EZH2 in p38 signaling is of particular interest as activation of this pathway can be detectable and targetable in tumors to halt breast cancer metastasis.

2-6. Figures

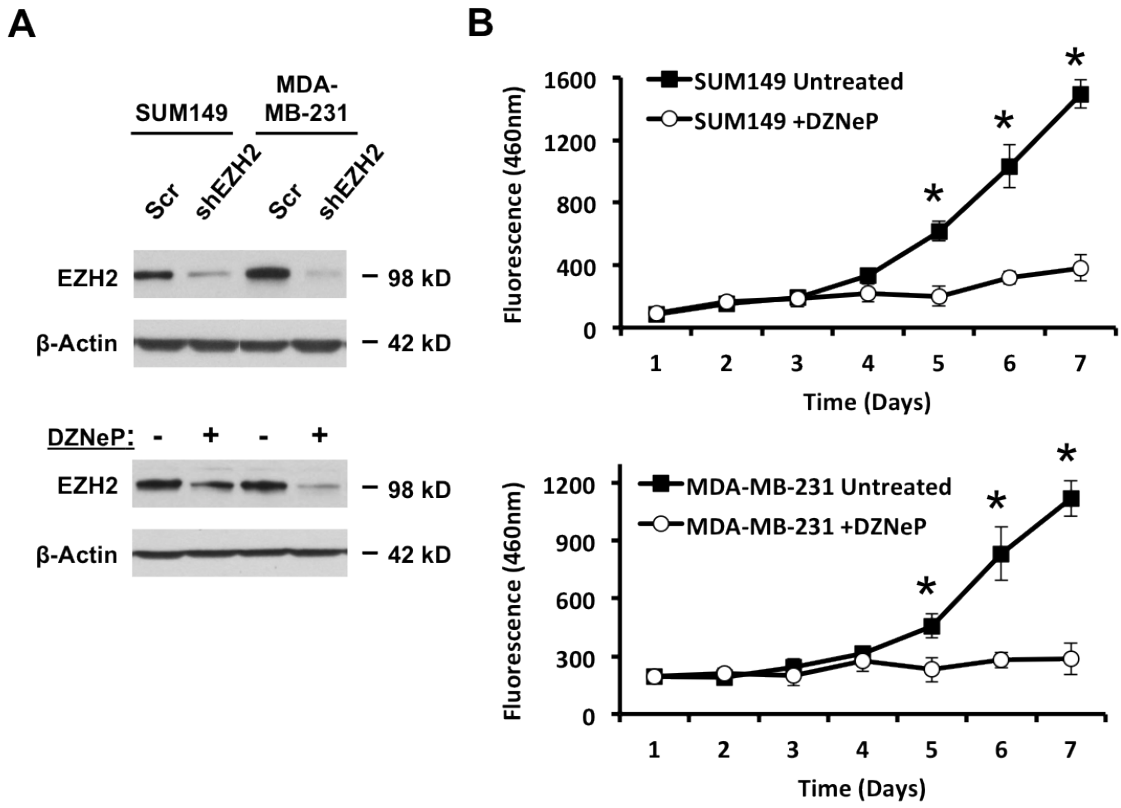

Figure 2-1 EZH2 knockdown in breast cancer cell lines can be achieved with targeted shRNA or DZNeP treatment and leads to decreased proliferation.

(A) Immunoblots of SUM149 and MDA-MB-231 breast cancer cells show that downregulation of EZH2 protein can be accomplished with either [top] EZH2-targeted shRNA [shEZH2] or [bottom] 1μM DZNeP treatment when compared to scrambled control shRNA [Scr] or untreated controls, respectively. **(B)** EZH2 downregulation with DZNeP reduces proliferation of SUM149 [top] and MDA-MB-231 [bottom] breast cancer cells as measured by Hoechst DNA staining and fluorescence emission at 460nm ± SD [Student's t-test, *$p<0.001$].

Figure 2-2 EZH2 knockdown induces a mesenchymal-to-epithelial transition and decreases invasion in breast cancer cells.

(A&B) Immunoblots of SUM149 and MDA-MB-231 breast cancer cells show that downregulation of EZH2 protein with either (A) EZH2-targeted shRNA [shEZH2] or (B) 1μM DZNeP treatment leads to a protein expression profile indicative of epithelial differentiation compared to scrambled control shRNA [Scr] or untreated controls, respectively. E-cadherin and Cytokeratin-18 [CK-18] represent epithelial marker proteins, and Vimentin and Snail1 represent mesenchymal marker proteins. (C&D) Representative phase contrast images show that EZH2 knockdown with either (C) shEZH2 or (D) DZNeP in SUM149 and MDA-MB-231 cells leads to a morphological change from mesenchymal-like to epithelial when compared to corresponding controls [200X magnification]. (E&F) EZH2 knockdown with either (E) shEZH2 or (F) DZNeP reduces invasion of SUM149 and MDA-MB-231 cells compared to corresponding controls using a reconstituted Boyden basement membrane invasion chamber assay. Left, representative images of either (E) entire chambers or (F) fields of chambers [200X magnification] which have been stained are shown; right, mean invaded area ± SD was calculated by either (E) quantifying stained image pixels using ImageJ [Student's t-test, *$p<0.0002$, **$p=0.03$] or (F) using colorimetry with absorbance at 560nm ±SD [Student's t-test, *$p=0.002$, **$p=0.01$].

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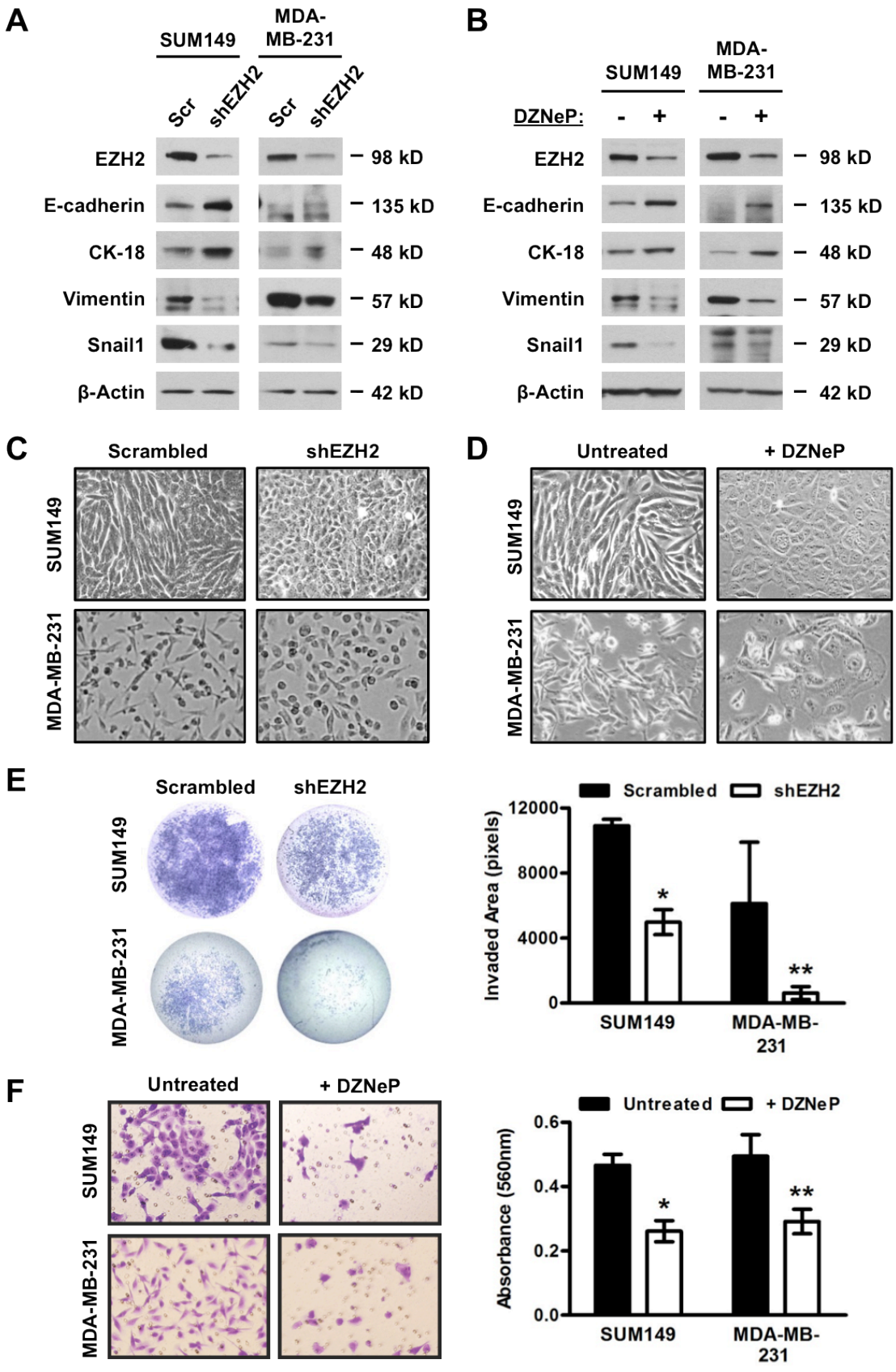
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SUM149
MDA-MB-231
Scr
shEZH2
Scr
shEZH2
EZH2
E-cadherin
CK-18
Vimentin
Snail1
β-Actin
98 kD
135 kD
48 kD
57 kD
29 kD
42 kD
B
SUM149
MDA-MB-231
DZNeP:
- + - +
EZH2
E-cadherin
CK-18
Vimentin
Snail1
β-Actin
98 kD
135 kD
48 kD
57 kD
29 kD
42 kD
C
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Scrambled
shEZH2
Invaded Area (pixels)
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F
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MDA-MB-231

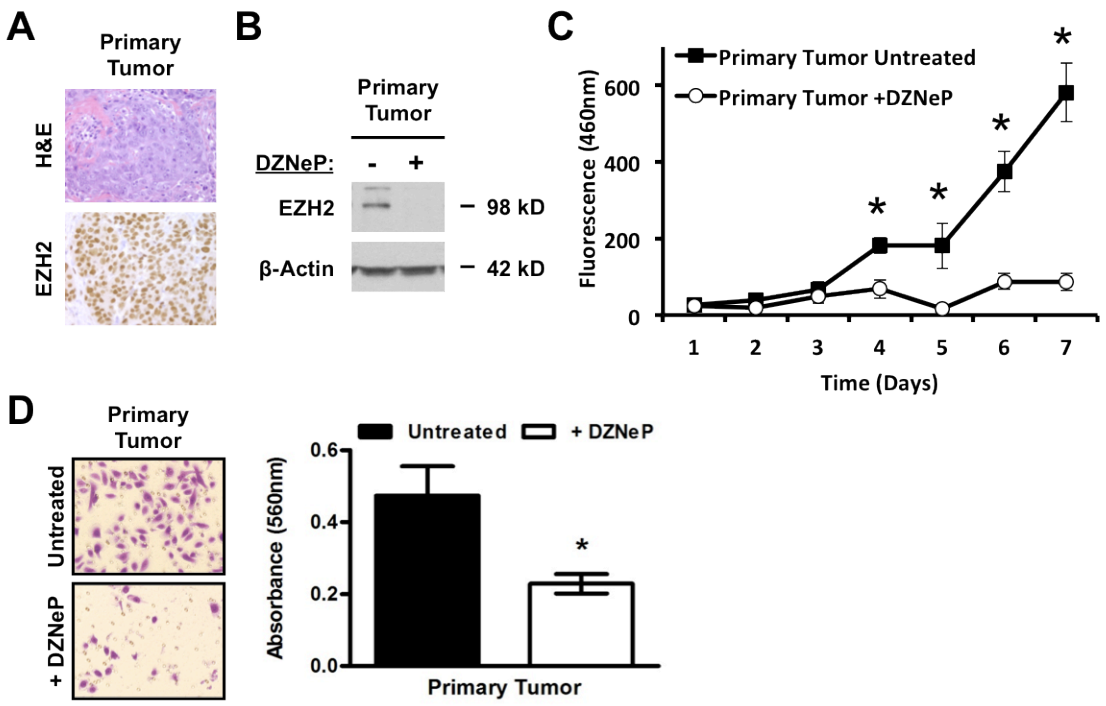


Figure 2-3 EZH2 downregulation decreases proliferation and invasion in primary patient breast cancer cells.

(A) Representative photomicrographs of hematoxylin and eosin [H&E] staining and EZH2 immunostaining of a patient tumor sample obtained from a primary invasive ductal breast carcinoma [Primary Tumor, 400X magnification]. **(B)** Immunoblots for EZH2 in primary cell cultures derived from the tumor sample described in **(A)** demonstrate EZH2 protein downregulation with 1μM DZNeP treatment compared to untreated controls. **(C)** EZH2 downregulation reduces proliferation of primary tumor cells as measured by Hoechst DNA staining and fluorescence emission at 460nm ± SD [Student's t-test, *p<0.001]. **(D)** EZH2 downregulation reduces invasion of primary tumor cells. Left, representative fields of invaded and stained Boyden chambers are shown [200X magnification]; right, invasion was quantified using colorimetry with absorbance at 560nm ± SD [Student's t-test, *p=0.008].

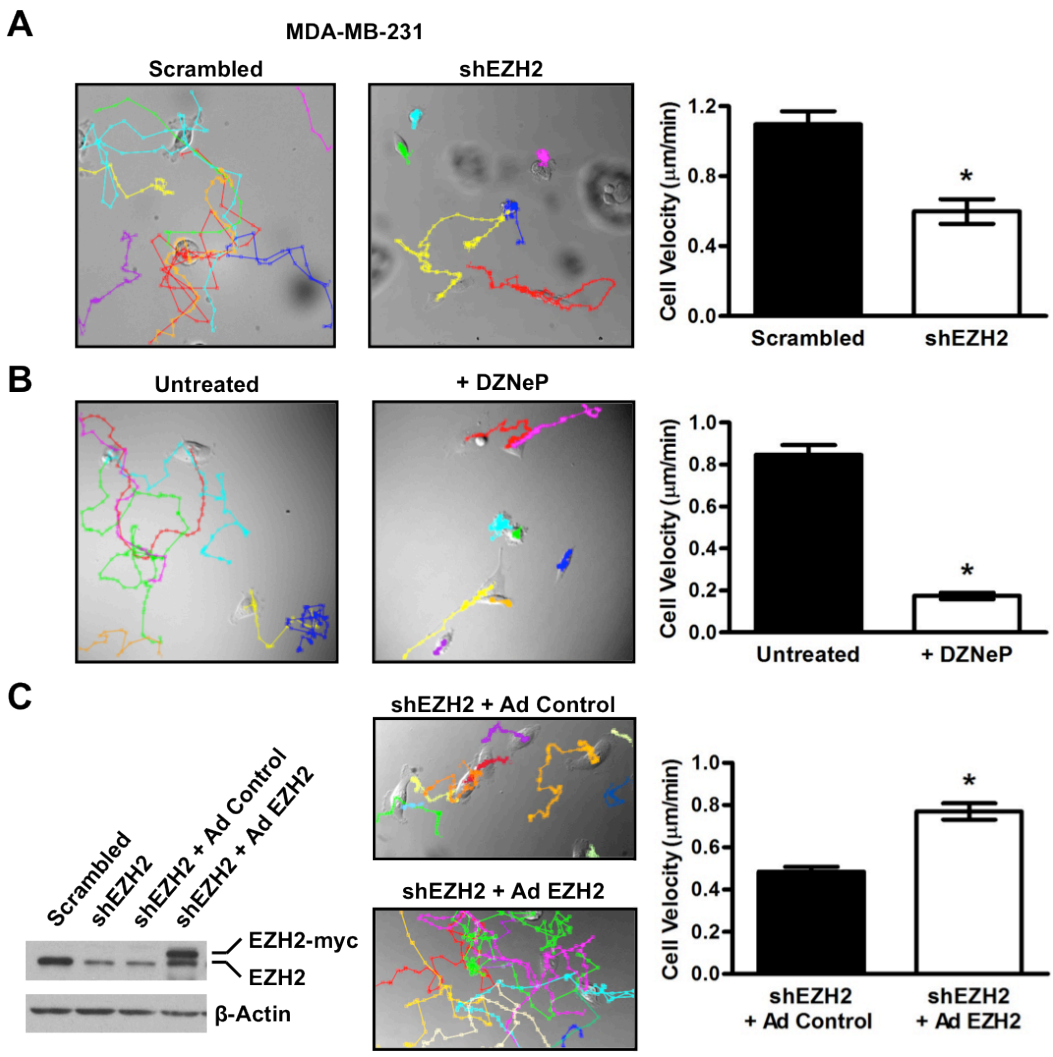


Figure 2-4 EZH2 knockdown decreases breast cancer cell motility.

(A & B) Left, representative images displaying MTrackJ individual MDA-MB-231 cell tracks, colored dots and connecting lines, from 24 hour time-lapse videos of **(A)** scrambled shRNA control and shEZH2 or **(B)** untreated and DZNeP treated cells [200X magnification]. Each dot represents a 10 minute time span and closely spaced dots indicate less movement over the elapsed time versus widely spaced dots. Right, bar graphs show that EZH2 KD cells are significantly slower than controls as demonstrated by the average cell velocity ± SEM [Student's t-test, *$p<1\times10^{-5}$, n ≥25 cells per condition]. **(C)** Transient rescue of EZH2 expression in MDA-MB-231 EZH2 KD cells using a myc-tagged EZH2-encoding adenovirus reverses the decreased motility of EZH2 KD cells. Representative images displaying cell tracks of shEZH2 cells infected with either control or EZH2 adenovirus [200X magnification]. The bar graph shows that shEZH2 cells with EZH2 adenoviral rescue are significantly faster than control adenoviral infected cells as demonstrated by the average cell velocity ± SEM [Student's t-test, *$p<9\times10^{-10}$, n ≥90 cells per condition].

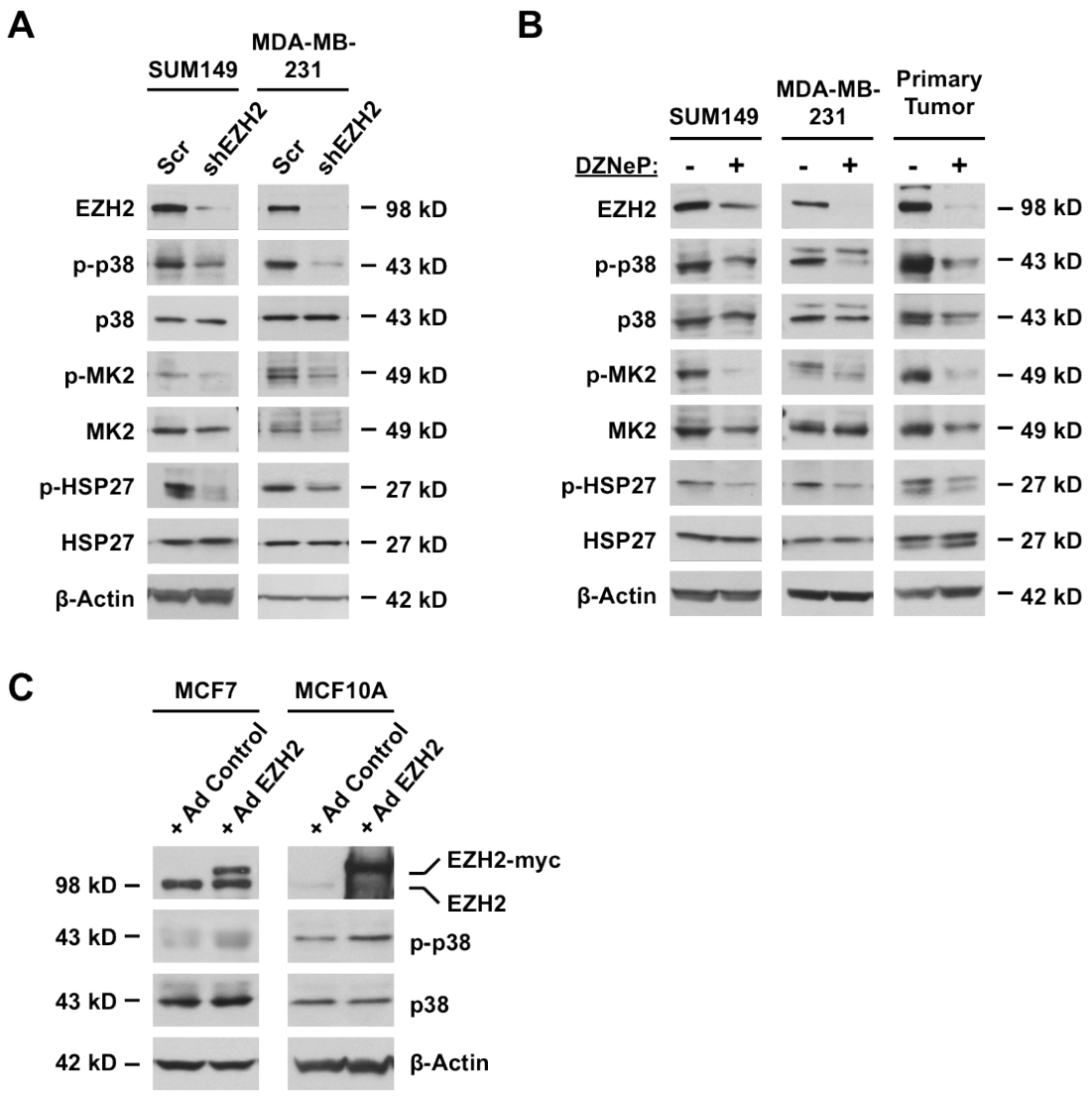
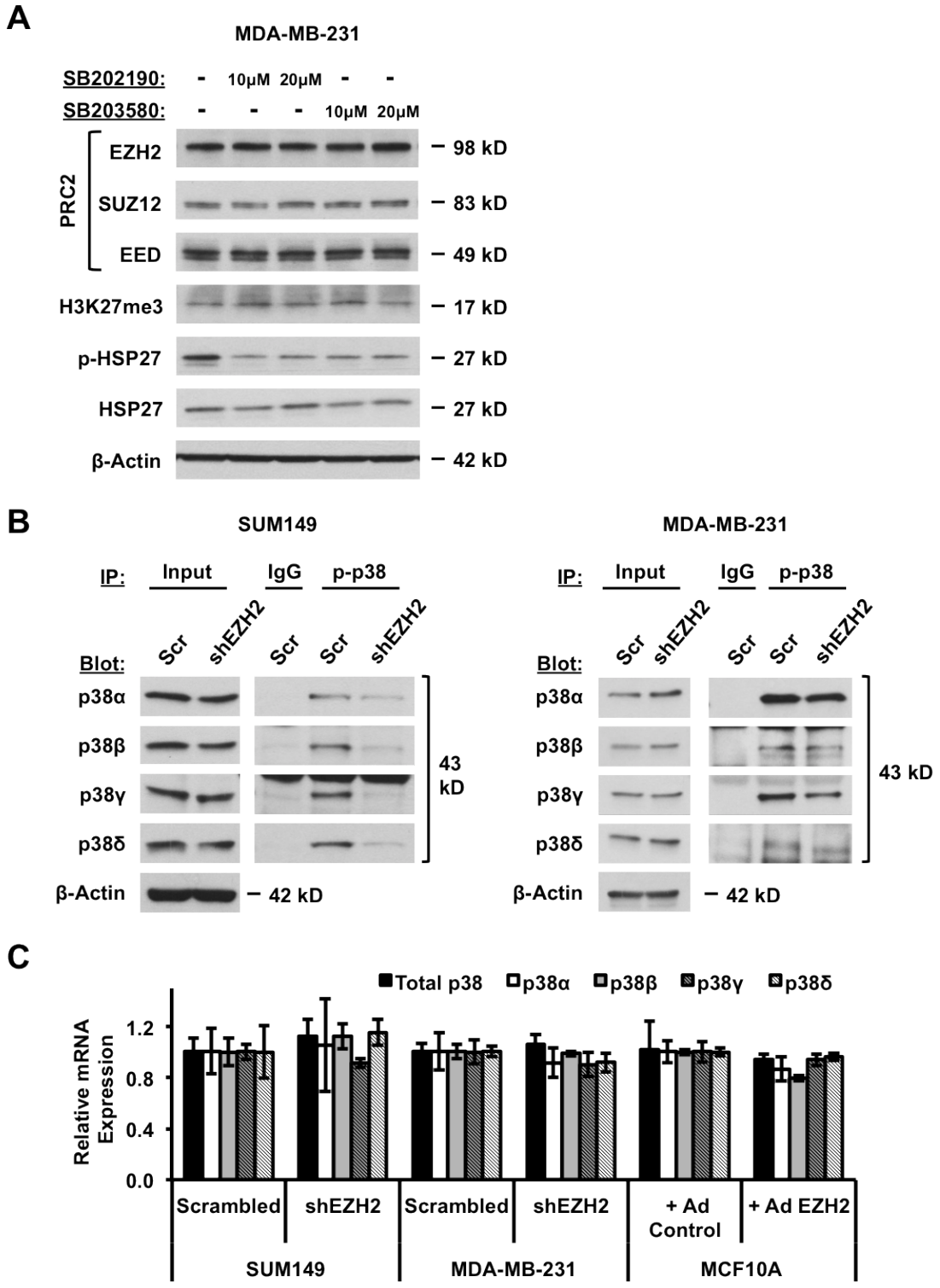


Figure 2-5 EZH2 regulates the activation of the p38 MAPK signaling pathway.

(A) Immunoblots of SUM149 and MDA-MB-231 breast cancer cells show downregulation of EZH2 protein with EZH2-targeted shRNA [shEZH2] have decreased levels of phosphorylated p38 [p-p38] and its activity as demonstrated by the phosphorylation of downstream signaling targets, MK2 and HSP27 when compared to scrambled shRNA control cells [Scr]. **(B)** Immunoblots of SUM149, MDA-MB-231 and Primary Tumor breast cancer cells show that EZH2 knockdown with DZNeP treatment decreases the levels of p-p38 and its downstream phosphorylating activity. **(C)** Immunoblots of MCF7 breast cancer cells and nontumorigenic MCF10A breast epithelial cells show that p-p38 levels are increased with adenoviral myc-tagged EZH2 over-expression when compared to adenoviral controls.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MDA-MB-231
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SB203580: - - - 10μM 20μM
PRC2
EZH2
SUZ12
EED
98 kD
83 kD
49 kD
H3K27me3
17 kD
p-HSP27
27 kD
HSP27
27 kD
β-Actin
42 kD
B
SUM149
MDA-MB-231
IP: Input IgG p-p38
Scr shEZH2 Scr Scr shEZH2
Blot:
p38α
p38β
p38γ
p38δ
43 kD
β-Actin
42 kD
C
Total p38
p38α
p38β
p38γ
p38δ
Relative mRNA Expression
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0.8
0.4
0.0
Scrambled
shEZH2
Scrambled
shEZH2
+ Ad Control
+ Ad EZH2
SUM149
MDA-MB-231
MCF10A

Figure 2-6 EZH2 regulates the activation of total and isoform specific protein levels of p38.

(A) Immunoblots show that inhibition of p-p38 phosphorylating activity in MDA-MB-231 cells with SB202190 or SB203580 at two different concentrations for 48 hours does not affect the levels of EZH2, SUZ12, EED or H3K27me3. **(B)** Activated, phosphorylated levels of all four p38 isoforms, but not total isoform protein levels, are decreased in SUM149 [left] and MDA-MB-231 [right] shEZH2 cells when compared to scrambled shRNA control cells [Scr]. Total p-p38 was immunoprecipitated from whole cell extracts followed by Western blot analysis for the four individual isoforms. **(C)** Quantitative real-time RT-PCR reveals that EZH2 knockdown in SUM149 and MDA-MB-231 breast cancer cells or transient adenoviral overexpression in nontumorigenic MCF10A breast cells has no significant effect on the mRNA levels of total p38 or of any of the four p38 isoforms when compared to controls. mRNA expression is shown relative to GAPDH mRNA levels ± SD.

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Figure 2-7 EZH2, along with the PRC2 complex, binds to phosphorylated p38 and can methylate p38α *in vitro*.

(A) Co-immunoprecipitations from whole cell extracts of SUM149 shEZH2 and scrambled shRNA control cells show that endogenous EZH2 immunoprecipitates with endogenous p-p38. Extracts were immunoprecipitated with EZH2, p-p38 or control IgG and bound proteins were revealed by Western blot via antibodies against EZH2 and p-p38. (B) Co-immunoprecipitations from whole cell extracts of SUM149 cells show that EZH2 binds p38/p-p38 in association with PRC2 members SUZ12 and EED. Extracts were immunoprecipitated with EZH2, p38, p-p38, EED, SUZ12 or control IgG and bound proteins were revealed by Western blot via antibodies against EZH2, p38, p-p38, EED and SUZ12. (C) *In vitro* methylation assay reveals that the addition of the PRC2 complex leads to the methylation of GST-p38α.

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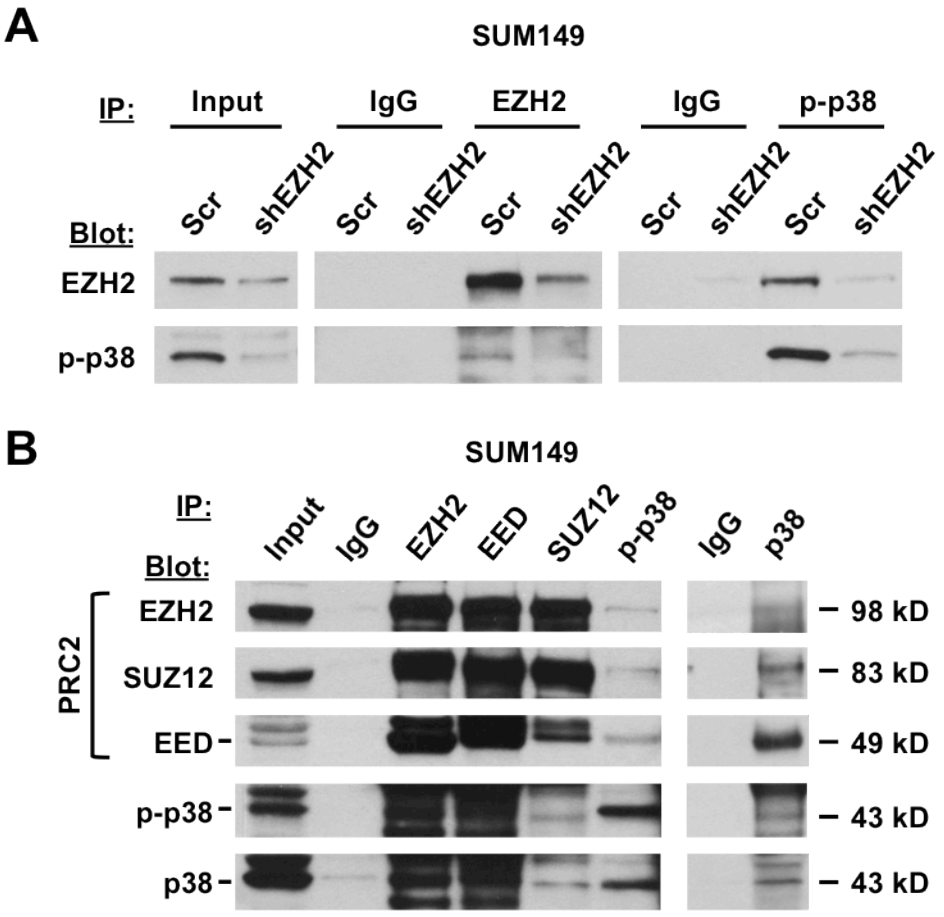
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SUM149
IP:
Input
IgG
EZH2
IgG
p-p38
Blot:
Scr
shEZH2
EZH2
p-p38
B
SUM149
IP:
Input
IgG
EZH2
EED
SUZ12
p-p38
IgG
p38
Blot:
PRC2
EZH2
SUZ12
EED
p-p38
p38
98 kD
83 kD
49 kD
43 kD
43 kD
C
Coomassie
Autoradio-graph
Protein Standard
p38α
p38α + PRC2
p38α
p38α + PRC2
kD
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100
75
50
37
25
20
PRC2 self-methylation
GST-p38α (67kD)

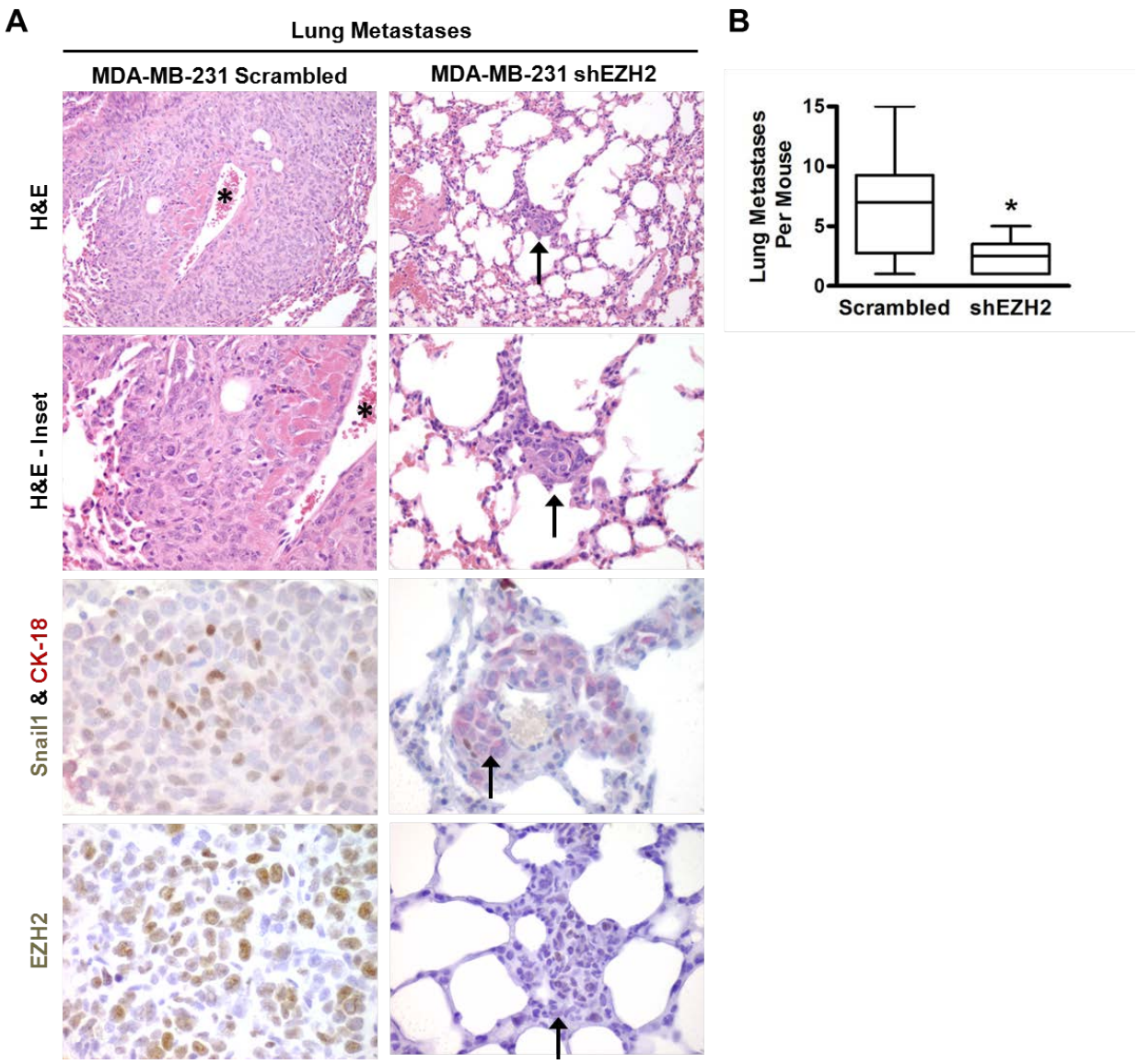
A
Lung Metastases
MDA-MB-231 Scrambled
MDA-MB-231 shEZH2
H&E
H&E - Inset
Snail1 & CK-18
EZH2
B
Lung Metastases Per Mouse
15
10
5
0
*
Scrambled
shEZH2

Figure 2-8 EZH2 knockdown in MDA-MB-231 cells is sufficient to reduce distant metastasis.

(A) Representative photomicrographs of mouse lung metastases of MDA-MB-231 scrambled shRNA control or EZH2-targeted shRNA [shEZH2] cells. EZH2 knockdown changed the tumor morphology from poorly circumscribed and highly invasive areas towards small, circumscribed foci. The asterisk shows a vessel encased by metastatic carcinoma. The arrows indicate metastases formed by MDA-MB-231 shEZH2 cells. Double immunostain with anti-Cytokeratin-18 [CK-18, red] and anti-Snail1 [brown] antibodies show that shEZH2 metastases exhibit upregulation of epithelial marker CK-18 and decreased expression of mesenchymal marker Snail1 in the nuclei of cancer cells compared to controls [H&E: 200X magnification; H&E-Inset: 400X magnification; CK-18/Snail1, EZH2: 600X magnification]. **(B)** EZH2 knockdown significantly reduced the number of lung metastases per mouse. Whiskers indicate the minimum and maximum number of lung metastases per mouse for each condition [Student's t-test, *p<0.05].

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Figure 2-9 Primary xenografts exhibit MET and lung metastases have significantly reduced expression of Ki67 and p-p38 with EZH2 knockdown in MDA-MB-231 cells.

(A) Representative photomicrographs of MDA-MB-231 scrambled control and shEZH2 primary xenografts with immunostaining for the epithelial marker CK-18 [red] and the mesenchymal marker Snail1 [brown, 400X magnification]. Arrow indicates the invasive edge of the tumor, on right, into the surrounding stroma, on left. **(B)** Left, representative photomicrographs of MDA-MB-231 control and shEZH2 lung metastases with immunostaining for the proliferative marker Ki67 [400X magnification]. Dotted line indicates boundaries of the shEZH2 lung metastasis. Right, bar graph shows Ki67 protein expression ± SEM in shEZH2 and control lung metastases quantified using FRIDA software [Student's t-test, *p=0.03]. **(C)** Left, photomicrographs of lung metastases of MDA-MB-231 control and shEZH2 cells exhibit decreased p-p38 protein [600X magnification]. Right, bar graph shows p-p38 protein expression ± SEM in shEZH2 and control lung metastases quantified using FRIDA software [Student's t-test, *p=0.01].

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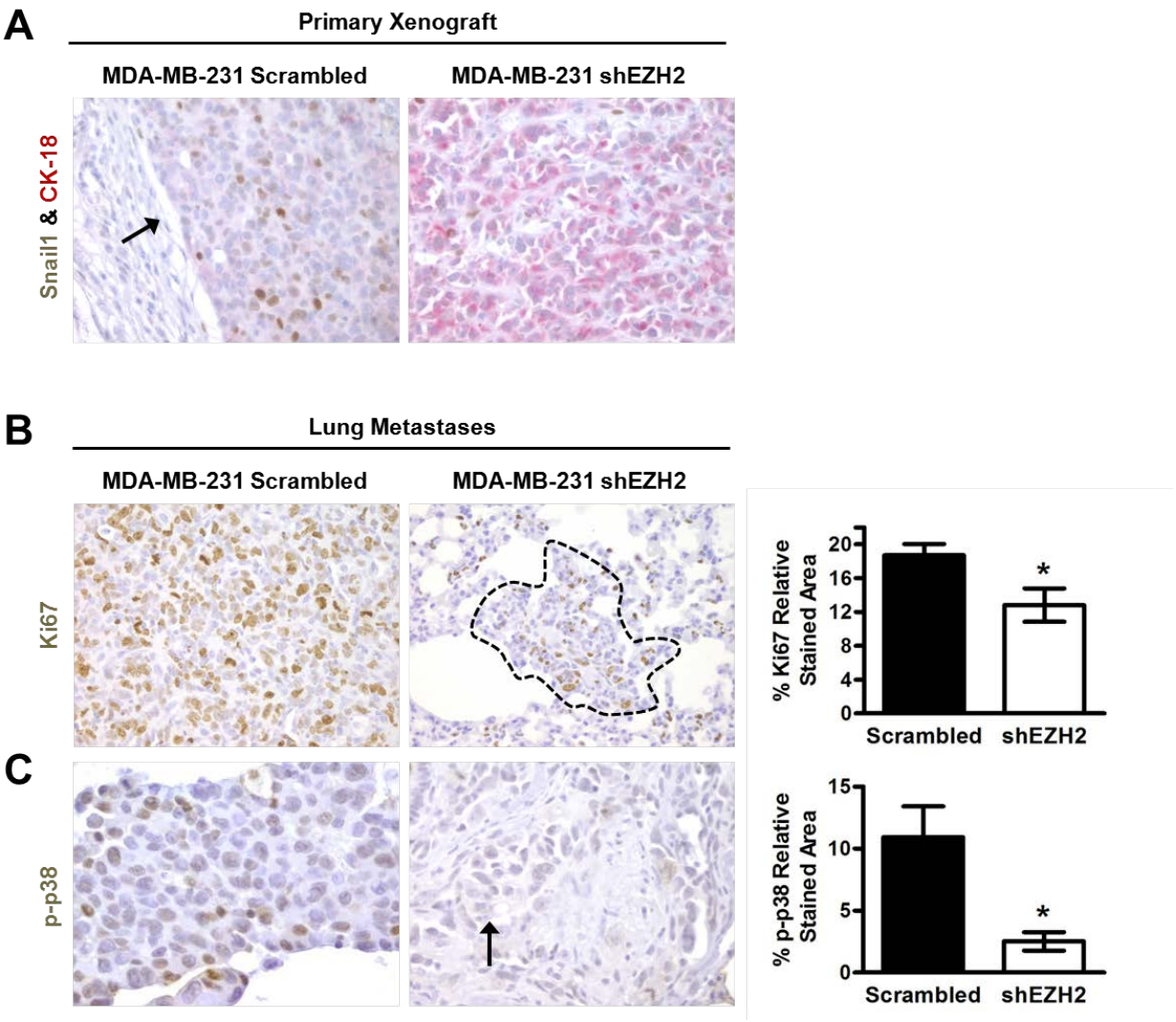
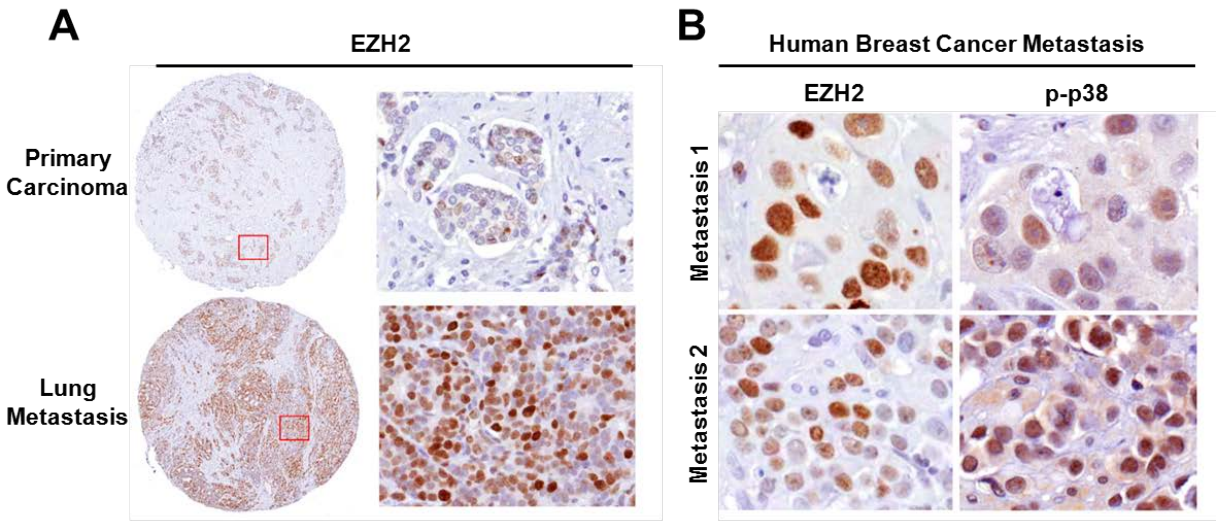

A
Primary Xenograft
MDA-MB-231 Scrambled
MDA-MB-231 shEZH2
Snail1 & CK-18
B
Lung Metastases
MDA-MB-231 Scrambled
MDA-MB-231 shEZH2
Ki67
C
p-p38
% Ki67 Relative Stained Area
20
16
12
8
4
0
*
Scrambled
shEZH2
% p-p38 Relative Stained Area
15
10
5
0
*
Scrambled
shEZH2



C

	EZH2 low	EZH2 high	Total	p-p38 low	p-p38 high	Total
Primary Carcinoma	7 (43.8%)	9 (56.3%)	16	11 (73.3%)	4 (26.7%)	15
Metastasis	0	16 (100%)	16	6 (37.5%)	10 (62.5%)	16
	Fisher's Exact Test, p<0.007			Fisher's Exact Test, p<0.05		

Figure 2-10 EZH2 and p-p38 are significantly upregulated in human breast cancer metastases when compared to matched primary tumors from the same patient.

(A) Representative images of matched primary human breast carcinomas and metastases (n=16 patients) immunostained for EZH2 [100X magnification, Inset: 400X magnification]. EZH2 is upregulated in the metastasis compared to the primary tumor. **(B)** Representative images of two metastases showing concordant high EZH2 and p-p38 expression [600X magnification] **(C)** The table shows the distribution of EZH2 and p-p38 protein expression in the 16 primary breast carcinomas and matched metastases; 62.5% of metastases exhibited high expression of both EZH2 and p-p38.

							Primary Carcinoma					EZH2 (low or high)		p-p38 (low or high)	
Case	Age at Diagnosis (years)	Pathologic Stage at Diagnosis	Primary Tumor Size (cm)	Nodal Status at Diagnosis	Tumor Type & Grade	Subtype	ER Status	PR Status	Her2 Status	Interval to Metastasis (years)	Metastasis Location	Primary Carcinoma	Metastasis	Primary Carcinoma	Metastasis
1	33	T3N1M1	6	5/24 positive	IDC, Grade 3	Her2	-	-	3+	At diagnosis	Lung	H	H	L	L
2	50	T2N1	2.3	1/19 positive	IDC, Grade 3	Luminal	+	+	0	6	Lung	L	H	L	L
3	34	T2N2M1	4.6	4/9 positive	IDC, Grade 3	TNC	-	-	0	At diagnosis	Brain	H	H	N/A	L
4	38	T1cN0	1.9	0	ILC, Grade 2	Luminal	+	+	1+	3	Ovary	L	H	L	H
5	45	T1aN1	< 0.5	1/3 positive	IDC, Grade 3	Luminal	+	+	0	7	Lung	L	H	L	L
6	36	yT1cN0	1.8	0	IDC, Grade 2	Her2	-	-	3+	4	Brain	L	H	L	H
7	39	T2N0	3	0	IDC, Grade 3	TNC	-	-	0	4	Brain	H	H	L	H
8	53	TXNX	radiologic size 7 cm	1/31 positive	IDC, Grade 3	TNC	-	-	0	1	Brain	L	H	L	L
9	38	T1cN0	1.4	0	IDC, Grade 3	TNC	-	-	0	5	Brain	H	H	H	L
10	53	T2N2	3.5	7/20 positive	IDC, Grade 3	Luminal	+	+	0	5	Brain	H	H	H	H
11	58	T2N0	3	0	ILC, Grade 2	Luminal	+	-	0	7	GI-Bowel	L	H	L	H
12	56	TXNXM1	diffuse radiologic abnormality	X	ILC, Grade 2	Luminal	+	+	0	At diagnosis	GI-Bowel	L	H	L	H
13	44	T2N0	2.6	0	IDC, Grade 3	TNC	-	-	0	1	Lung	H	H	L	H
14	33	yT2N1mi	3.7	micromet	IDC, Grade 2	Luminal	+	+	0	2	GI-Pancreas	H	H	H	H
15	57	T1N0	1.8	0	IDC, Grade 3	TNC	-	-	0	6	Lung	H	H	L	H
16	36	T1N0	multiple foci <2 cm	0	IDC, Grade 3	Luminal	+	+	0	2	Brain	H	H	H	H

Figure 2-11 Table of the complete clinical and pathological information, including EZH2 and p-p38 protein expression, for the tumor microarrays containing 16 human primary breast carcinomas with matched metastases.

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CHAPTER 3

EZH2 expands the stem cell population in benign and tumorigenic breast cells through regulation of Notch1 signaling

The following chapter represents a “manuscript in progress” and will be submitted for publication under the above title by Maria E. Gonzalez, Heather M. Moore, Xin Li, Kathy A. Toy, Kelley Kidwell, and Celina G. Kleer.

3-1. Abstract

Breast cancer is the second leading cause of cancer-related deaths in women, but the details of how it begins remain elusive. Increasing evidence supports the association of aggressive breast carcinomas with heightened expression of the Polycomb group transcriptional repressor EZH2 and increased numbers of tumor-initiating cells, often termed cancer stem cells (CSCs). However, mechanistic links between EZH2 and CSCs have remained unclear, and direct demonstration that EZH2 induces breast cancer development is lacking. We demonstrate *in vitro* that levels of EZH2 expression strongly correlate with stem cell numbers in nontumorigenic and tumorigenic breast cells. EZH2 overexpression in nontumorigenic breast cells leads to an increase in stem cells, whereas EZH2 downregulation in breast cancer cells leads to a decrease in CSCs. Mechanistically, we uncover a novel role of EZH2 in activating, rather than repressing, Notch1 signaling through binding to the *Notch1* promoter. EZH2

binding was found to be independent of its catalytic methyltransferase activity and the PRC2 complex, but corresponded instead to transcriptional activation marks. Inhibition of Notch1 activity prevented the EZH2-induced expansion of stem cell populations. We demonstrate *in vivo* that EZH2 overexpression promotes earlier breast cancer initiation and correlates with Notch1 expression in a transgenic breast cancer mouse model. In human clinical breast cancer tissues, we found that EZH2 and Notch1 were coexpressed in 44% of invasive breast carcinomas, while the expression of both proteins were absent in 35% of cases. In addition, EZH2, Notch1 and $CD44^{+}/CD24^{-/low}$ were coexpressed in 26% of tumor tissues, consistent with the *in vitro* functional results. These findings reveal a novel functional and mechanistic link between EZH2 expression, Notch1 signaling, and stem cell levels, and provide evidence that EZH2 enhances breast cancer initiation.

3-2. Introduction

It is thought that breast cancer arises through a continuum from epithelial cells in the terminal-ductal-lobular-unit, the functional unit of the breast. Normal cells may undergo hyperplasia and develop epithelial atypia, which may progress to ductal carcinoma *in situ*, and eventually to invasive carcinoma. Invasive carcinoma is characterized by abnormal proliferation and differentiation, and by the ability to invade normal tissues. The presence of cells lacking structural or functional differentiation, known as anaplasia, is considered a hallmark of cancer. Thus, it is not surprising that dysregulation of genes which govern cell type identify may lead to malignant transformation [1-4]. Members of the Polycomb protein family group play a major role in

maintaining cellular memory by transcriptionally repressing target genes involved in a wide array of cellular processes including differentiation, proliferation and stem cell maintenance [5-9]. Enhancer of zeste homolog 2 (EZH2) is the catalytic core member of the Polycomb Repressive Complex 2 (PRC2) where its most described function is in trimethylation of histone 3 lysine 27 (H3K27me3) leading to transcriptional repression [10]. EZH2, as a regulator of a number of critical cellular pathways, has been found to be upregulated in multiple malignancies of hematopoietic and solid organ origin, where its oncogenic activity is thought to be primarily mediated by silencing tumor suppressor genes [7-9, 11-13]. However, it has been recently demonstrated in several studies that EZH2 may exert transcriptional activating functions in context-dependent manners providing a contrasting role for EZH2 in cancer development [14-20].

EZH2 is overexpressed in clinically aggressive breast carcinomas where it independently predicts survival [3]. In benign breast tissues, elevated levels of EZH2 protein signal future development of breast cancer up to 12 years prior to diagnosis indicating that EZH2 upregulation precedes morphological changes of atypia or carcinoma [21, 22]. Recently, EZH2 has been shown to play a role in the self-renewal of cancer stem cells (CSCs) [23]. However, direct demonstration that EZH2 promotes breast cancer initiation is lacking and the responsible mechanisms remain to be elucidated.

The details of how human breast cancer initiates remain unknown. Studies have implicated CSCs in having enhanced tumor initiating capacity when compared to non-stem cells [24-26]. Breast CSCs are defined as a subpopulation of cells having an indefinite potential for self-renewal and the ability to recapitulate the cellular

heterogeneity of the primary tumor when transplanted into mice [27]. CSCs can be successfully isolated from primary tumor tissue and cultured cell lines. In human breast cancer, CSCs are enriched within cell subpopulations with a $CD44^{+}/CD24^{-/low}$ surface marker profile and by positive activity of the detoxifying enzyme aldehyde dehydrogenase-1 ($ALDH1^{+}$) [24, 25, 28-32]. Substantive data exist on the signaling dynamics and regulatory pathways that control breast CSCs, which include the Notch, Hedgehog, Wnt, PI3K, NF-κB, and JAK/STAT pathways [33-35]. In breast cancer, these pathways are dynamically regulated rather than activated via mutation [36]. However, the molecular mechanisms involved in the maintenance of the CSC pool and in breast cancer initiation remain poorly understood.

In this study, we demonstrate that EZH2 protein levels correlate strongly with the numbers of stem cells in nontumorigenic and malignant breast cells. The overexpression of EZH2 observed in breast cancer cell lines translates to an increase in stem cells, which can be reduced with EZH2 knockdown. We discover Notch1 expression, at both the mRNA and protein levels, to be regulated by the levels of EZH2 expression. Specifically, EZH2 is found to activate transcription of the *Notch1* promoter and control downstream signaling by interacting with the *Notch1* promoter through a mechanism independent of H3K27 trimethylation. Notch1 inhibition decreases the expansion of stem cells facilitated by EZH2 overexpression. We further show that EZH2 overexpression confers a distinct tumor initiation advantage to breast CSCs *in vivo.* In analysis of human breast cancers, we found a significant association between EZH2, Notch1 and $CD44^{+}/CD24^{-/low}$ protein expression. Together, these findings identify a novel role of EZH2 in regulating stem cell populations and Notch1 expression and

activity, and establish the first evidence that transgenic EZH2 overexpression accelerates breast cancer initiation *in vivo*.

3-3. Materials and Methods

Cell culture

The breast cancer cell line MDA-MB-231 and the immortalized human mammary epithelial cell line MCF10A were obtained from the American Type Culture Collection and grown under recommended conditions. The SUM149 breast cancer cell line was obtained from the S. Ethier laboratory (Karmanos Cancer Institute, Detroit, MI) and cultured as previously reported [37]. Human mammary epithelial cells were purchased from ScienCell Research Laboratories (#7610) and maintained following the provider's instructions. These cells were delivered frozen after being isolated from normal human breast tissue and being cryopreserved at first passage.

A fresh breast tumor tissue sample was obtained through the Tissue Procurement Service at the University of Michigan (IRB#2002-227). The donated sample was immediately processed in the laboratory. A portion was formalin fixed and paraffin embedded for staining with hematoxylin and eosin (H&E) and immunohistochemical staining with anti-EZH2 (Cell Signaling, #5246, 1:150); see the Immunohistochemistry section for a detailed staining protocol. Another portion was processed to a single cell suspension as previously described [38]. Briefly, the tumor specimen was manually minced and then dissociated in a collagenase-hyaluronidase solution (StemCell Technologies, #07912). Next, red blood cells were lysed with RBC Lysis Solution (Qiagen, #158902). For further purification, the tissue was treated with

Trypsin-EDTA (GIBCO, #25200-056), then DNase1 (StemCell Technologies, #07900) and finally filtered through a 40µm cell strainer. Cells were then cultured in Mammary Epithelial Cell Medium (ScienCell, #7611) completed with Mammary Epithelial Cell Growth Supplement and penicillin-streptomycin (ScienCell, #7652 and #0503).

Vectors/viral infections and inhibitors

EZH2 knockdown using stable short-hairpin interfering RNA in lentivirus was completed as previously reported [39]. For targeting *EZH2* (NM_152998 NCBI), the shRNA oligo ID# V2LHS_17507 was used and corresponded to #RHS4430-99139126 from Open Biosystems; this oligo was cloned into a pLKO.1-Puro vector and packaged into lentiviral particles at the University of Michigan Vector Core. A lentivirus containing a plasmid encoding a scrambled shRNA oligo was used for control. Cells were transduced and selected for antibiotic resistance with puromycin (Sigma-Aldrich, #P9620). Conditional overexpression of EZH2 in MCF10A cells (pLVX-EZH2) was achieved through a doxycycline-inducible system previously developed and reported in our laboratory [40]. Doxycycline treatments were done for 24 hours at a final concentration of 1µg/mL (Clontech, #631311). Transient EZH2 overexpression was also achieved through infection with myc-tagged pCMV encoding either wild type or specific deletion mutants of EZH2 for 48 hours, as previously reported [3, 41].

A pGreenFire lentiviral vector containing a GFP reporter gene driven by four transcriptional response elements of the *Notch1* gene promoter (GTGGGAACGGCATTGTAGCG) was purchased from System Bioscience (#TR020PA-1) and used for *Notch1* transcription reporter assays. Notch1 signaling inhibition was

completed by treating cells with γ-secretase inhibitor (GSI) (Calbiochem, #565750) for three days at final 17nM concentration in regular cell culture, and for seven days at a final 1.7nM concentration in mammosphere assays.

Western blot analyses

Western blot analyses were carried out with 100µg of whole cell extract derived as previously reported [40]. Membranes were blocked and incubated with primary antibodies in 4% milk (Sigma-Aldrich, #A3059) in TBS-T (Bio-Rad, #161-0372, with 0.05% Tween20) at 4°C overnight. Mouse monoclonals β-Actin-HRP (Santa Cruz, #47778), anti-GAPDH (abcam, #ab9484), anti-α-Tubulin (Sigma-Aldrich, #T9026) and rabbit polyclonal anti-Lamin B1 (abcam, #ab16048) were used to confirm equal loading. Primary antibodies from Cell Signaling included rabbit monoclonals anti-EZH2 (#5246), anti-CCND1 (#2978), anti-phospho-STAT3 (#9145), rabbit polyclonal anti-STAT3 (#9132), and mouse monoclonal anti-Myc-Tag (#2276). The mouse monoclonals anti-Notch1 from Santa Cruz (#SC-32745) and anti-Active-β-Catenin from Fisher (#05-665) and the rabbit polyclonal anti-Hes1 from abcam (#AB71559) were used.

Mammosphere assays

Single cell dissociation for mammosphere formation assays was performed following established protocols with SUM149 and MCF10A cells plated at density of $1x10^4$ cells/mL [25]. Mammospheres were cultured in MammoCult Human Basal Medium with added Proliferation Supplement (StemCell Technologies, #05621 & #05622) on Costar Ultra Low Attachment tissue culture plates (Corning, #3471). At the

end of seven days, for both primary and secondary generations, mammosphere sizes and numbers were determined using a Leica inverted microscope. Size was measured as the widest diameter with the scale bar.

Signaling microarrays

Pathway focused PCR arrays from SABiosciences were used to identify the gene expression profiles in the $ALDH1^+$ sorted, SUM149 scrambled and EZH2-targeted shRNA cells. The Notch Signaling Pathway PCR array (#PAHS-059Z) and the Stem Cell PCR array (#PAHS-0405Z) were performed in triplicate for each condition.

Real-time RT-PCR and ChIP assays

Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) amplifications were carried out with 1µg of total RNA isolated from the indicated breast cells and conditions. Reactions were performed in triplicate using an Applied Biosystems StepOnePlus RT-PCR System available in the Michigan MicroArray Core with Qiagen primers and SYBR Green Master Mix (Applied Biosystems, #4309155). All primers were purchased from Applied Biosystems: Actin (#Hs99999903_m1), GAPDH (#Hs99999905_m1), EZH2 (#Hs00544830_m1), and Notch1 (#Hs01062011_m1).

The ChIP-IT Express Enzymatic kit (Active Motif, #53009) was used following the manufacturer's instructions to perform ChIP assays. Antibodies were used at the manufacturer's recommended dilutions and included anti-EZH2, anti-Histone H3K27me2me3, anti-SUZ12, anti-H3K4me2 (Active Motif, #39875, #39535, #39877 & #39679) and anti-RNA Polymerase II (Fisher, #05-623). The following ChIP qPCR

promoter primers were purchased from SABiosciences: (Notch1) #GPH1027067(-)01A, #GPH1027067(-)02A, #GPH1027067(-)03A, #GPH1027067(-)04A, #GPH1027067(-)05A and (GAPDH) #GPH110001C(+)01A. The MYT1 promoter primers used as a positive control for EZH2 binding were made as previously reported [42]. ChIP real-time PCR was performed in triplicate with isolated DNA as described above.

Flow cytometry

Aldefluor assay was used for detection of the stem cell population using the ALDEFLUOR kit (StemCell Technologies, #01700) following the manufacturer's instructions. Stem cell populations were also measured by labeling $1x10^6$ cells with anti-CD44 conjugated to APC and anti-CD24 conjugated to PE (BD Biosciences, #559942 & #555428) at the manufacturer's recommended dilutions. For *Notch1* promoter reporter assays, $1x10^6$ cells transduced with the Notch1 reporter lentivirus (described above), were subjected to flow cytometry to determine the percentage of GFP^+ cells. All flow cytometry analyses were completed using the University of Michigan Flow Cytometry Core in triplicate.

Animal studies

All procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the University of Michigan. For tumorigenicity experiments, ten-week-old severe combined immunodeficiency mice (Jackson Laboratories) were used. SUM149 cells expressing EZH2-targeted or scrambled shRNA, sorted into $ALDH1^+$ and

ALDH1$^-$ groups, were orthotopically injected into the mammary fat pad at a concentration of 1x10^4 cells in 40 mice (*n*=10 per group). Tumor size was measured twice weekly until tumors reached 2 cm^3 (tumor volume=(length x width^2)/2), at which time mice were sacrificed. To generate EZH2$^+$/neu and EZH2^{wt}/neu transgenic mice, female MMTV-neu mice (Jackson Laboratories, #FVB/N-Tg(MMTVneu)202Mul/J) were bred with male heterozygous EZH2 transgenic mice using synchronized breeding methods [43]. Mammary glands were excised at 8 weeks, 16 weeks and when tumors reached 2cm^3. Mammary gland whole mounts were prepared by mounting the abdominal mammary fat pads on glass slides and processed following established protocols [43, 44]. In brief, glands were excised, dehydrated, stained with alum carmine, stored in methyl salicylate and mounted with Permount. Mammary gland tissue samples for histological analysis were fixed overnight in 10% neutral buffered formalin (Sigma-Aldrich, #HT501128), processed through graded alcohols, cleared in xylene and embedded in paraffin. Tissues were sectioned at 5u and placed on charged slides. Slides were H&E stained and immunohistochemically stained with anti-EZH2 (BD Biosciences, #612667, 1:250) and anti-Notch1/N-Terminus (Fisher, #07-1232, 1:800); see Immunohistochemistry section for a detailed staining protocol.

Human breast tissue microarrays

High-density tissue microarrays (TMA) containing human primary invasive breast carcinomas were employed. Formalin-fixed, paraffin-embedded tissue blocks underwent H&E staining and immunohistochemical staining with anti-EZH2 (BD Biosciences, #612667, 1:200-300), anti-CD24 (Biocare Medical, #CM323, 1:100), anti-CD44 (abcam,

#ab51037, 1:400) and anti-Notch1/ N-Terminus (Fisher, #07-1232, 1:800); see the Immunohistochemistry section for a detailed staining protocol. Expression for all stained proteins was evaluated as low or high based on intensity of staining and percentage of staining cells, following published literature [39, 45].

Immunohistochemistry

Formalin fixed, paraffin embedded tissue blocks were sectioned at 5u and placed on charged slides. Slides were deparaffinized in xylene and rehydrated through graded alcohols. Heat Induced Epitope Retrieval (HIER) was performed in the Decloaking Chamber (Biocare Medical) with Target Retrieval, pH 6.0 (DakoCytomation). Slides were incubated in 3% Hydrogen Peroxide for 5 minutes to quench endogenous peroxidases. Primary tumor tissue, mouse tissue and human TMA tissue slides were incubated for 1.5 hours at room temperature with previously indicated antibodies. Antibodies were detected with either anti-rabbit or anti-mouse Envision^{+} HRP Labelled Polymer (DakoCytomation) for 30 minutes at room temperature. HRP staining was visualized with the DAB^{+} Kit (DakoCytomation). Negative control slides were run. Slides were counterstained in hematoxylin, blued in running tap water, dehydrated through graded alcohols, cleared in xylene and then mounted with Permount.

3-4. Results

EZH2 levels regulate stem cell numbers in breast cancer cells.

To examine the effect of EZH2 on the stem cell populations of breast cancer cells, we utilized the established breast cancer cell lines SUM149 and MDA-MB-231, which express high levels of EZH2 protein in comparison to nontumorigenic breast cell

lines [39]. Downregulation of EZH2 protein was achieved through the stable expression of a lentiviral-mediated short hairpin RNA interference (shRNA), whereas re-expression of EZH2 protein in knockdown cells was accomplished through transient infection with a wild type, EZH2-encoding, myc-tagged adenovirus (Fig. 3-1, A). A sphere-forming assay based on the unique property of stem cells to survive in non-adherent, serum-free, tissue culture conditions was initially used to quantify stem cell activity and self-renewal [25]. EZH2 knockdown in SUM149 cells significantly reduced the number and size of mammospheres in primary and secondary generations when compared to scrambled shRNA controls (Fig. 3-1, B). The decrease in mammosphere numbers and size was subsequently rescued with re-expression of adenoviral EZH2, but not in knockdown cells infected with control adenovirus (Fig. 3-1, B).

In addition to mammosphere assays, we utilized the positive activity of ALDH1 ($ALDH1^+$) and the cell surface markers $CD44^+/CD24^{-/low}$ to test for CSC subpopulations. Intriguingly, in parental SUM149 cells, EZH2 mRNA expression was found to be higher in the sorted $ALDH1^+$ population versus the $ALDH^-$ population, signifying that EZH2 is preferentially expressed at higher rates in CSCs versus non-CSCs (Fig. 3-1, C). In support of the mammosphere results, EZH2 knockdown in SUM149 and MDA-MB-231 breast cancer cells led to a significant decrease in the $ALDH1^+$ and $CD44^+/CD24^-$ populations (Fig. 3-1, D-E & 3-2, A-C). To confirm and extend these observations to human breast cancer, we isolated primary cells from an, invasive human breast carcinoma exhibiting high endogenous EZH2 protein levels (Fig. 3-2, D). EZH2 knockdown in primary tumor cells using shEZH2 decreased the $CD44^+/CD24^-$ population (Fig. 3-2, E-F). Together, these data demonstrate that EZH2 regulates the

stem cell pool in breast cancer cells, that EZH2 expression is higher in CSCs versus non-CSCs, and that the proliferation of CSCs, as measured by mammopshere size, is reduced by EZH2 downregulation.

We next examined the biological consequences of decreased CSC populations due to EZH2 knockdown *in vivo*. SUM149 $ALDH1^+$ and $ALDH1^-$ cell populations transduced with either scrambled control or EZH2-targeted shRNA (shEZH2) were isolated using the Aldefuor assay. Immediately following sorting, $1x10^4$ cells were injected into the cleared mammary fat pads of female NOD/SCID mice, with 10 mice per condition, and mice were monitored for tumor growth. In mice injected with $ALDH1^+$ cells, EZH2 knockdown markedly decreased the time to tumor development and the growth rate of the breast tumors (Fig. 3-3, A-B). Furthermore, the tumors formed from $ALDH1^-$ cells significantly differed in time to tumor development and tumor volume from those formed by control $ALDH1^+$ cells as the time to tumor development and tumor volumes from these two groups resembled those of the $ALDH^+$ shEZH2 tumors (Fig. 3-3, A-B).

EZH2 knockdown reduces Notch1 levels and signaling in breast cancer.

In order to identify signaling pathways that might mediate the effect of EZH2 on breast CSCs numbers, we employed a real time RT-PCR microarray that profiles the expression of key genes involved in stem cell signaling. The array was completed in triplicate using mRNA prepared from sorted $ALDH1^+$ populations of SUM149 cells expressing either scrambled or EZH2-targeted shRNA. *Notch1* gene expression was found to be significantly downregulated, amongst other genes, in EZH2 knockdown cells

when compared to scrambled control cells (Fig. 3-4, A). Of the four Notch receptors, EZH2 downregulation primarily reduced *Notch1* expression and led to dysregulation of downstream signaling components as determined by an additional RT-PCR microarray profiling Notch signaling genes (Fig. 3-4, B). Supporting our data, the hitherto unknown correlation between *EZH2* and *Notch1* expression was also observed through examination of publicly available cDNA cancer microarray datasets on Oncomine. Correlation coefficients above 0.6 for *EZH2* and *Notch1* expression were obtained independently in four invasive breast carcinoma tissue datasets and two breast cancer cell lines datasets (Fig. 3-5).

The effect of EZH2 knockdown on *Notch1* gene expression was consistently translated to decreased protein levels of NICD, the activated and intracellular form of Notch1, with EZH2 knockdown in SUM149 and MDA-MB-231 breast cancer cell lines and in primary patient breast cancer cells (Fig. 3-4, C-D). Consistent with the mRNA data, the Notch1 downstream signaling targets Cyclin D1 and β-catenin were reduced at the protein level with EZH2 downregulation in SUM149 and MDA-MB-231 cells (Fig. 3-4, C). Likewise, the downstream targets Hes1 and phosphorylated-STAT3 were reduced in primary patient tumor cells with knockdown of EZH2 (Fig. 3-4, D). To determine the specificity of EZH2 in the regulation of Notch1 in breast cancer, EZH2 expression was transiently rescued in SUM149 and MDA-MB-231 shEZH2 cells using the EZH2-encoding adenovirus. Indeed, NICD protein expression was effectively rescued by the ectopic re-expression of wild type EZH2 in these cell lines (Fig. 3-4, E). In all, these data provide evidence that EZH2 depletion reduces the levels and the activity of Notch1 in breast cancer cells.

Notch1 signaling regulates EZH2-dependent stem cell expansion.

It has been shown that Notch1 inhibition reduces breast CSCs, but whether Notch1 is required for the effect of EZH2 overexpression in stem cell expansion has not been addressed [46-48]. In order to test this hypothesis, we utilized a conditional pLVX-TetOn system for ectopic EZH2 overexpression (pLVX-EZH2) in the nontumorigenic MCF10A breast epithelial cell line. Addition of doxycycline to control pLVX cells showed no change in EZH2 mRNA levels, however, a marked upregulation of EZH2 and Notch1 mRNA levels, principally in the $ALDH1^+$ population, was observed with doxycycline addition to pLVX-EZH2 cells (Fig. 3-6, A-B). At the protein level, a concomitant increase in EZH2 and NICD expression was seen in doxycycline treated pLVX-EZH2 cells when compared to untreated pLVX-EZH2 cells (Fig. 3-6, C). Complementing the previous assays conducted in SUM149 breast cancer cells, a significant increase in the number and size of primary and secondary mammospheres and in the $ALDH1^+$ and $CD44^+/CD24^-$ populations was induced by doxycycline treatment in pLVX-EZH2 cells, but not in control pLVX cells (Fig. 3-6, D-E & Fig. 3-7, A-B). In contrast, when doxycycline treated pLVX-EZH2 cells are treated with the γ-secretase inhibitor (GSI), preventing the release of NICD from the membrane and blocking Notch1 activity, the numbers of mammospheres, $ALDH1^+$ and $CD44^+/CD24^-$ cells are significantly reduced back to pLVX-EZH2 untreated levels (Fig. 3-6, C-E & Fig. 3-7, A-B) [49]. These results suggest that Notch1 signaling is required for the increased numbers in stem cell populations observed with EZH2 overexpression in a nontumorigenic breast cell line.

EZH2 regulates the *Notch1* promoter.

As ectopic EZH2 overexpression led to an increase in Notch1 mRNA levels (Fig. 3-6, B), we hypothesized that EZH2 may regulate Notch1 expression and function through interaction with the *Notch1* promoter. To test this hypothesis, we investigated the effect of conditional EZH2 overexpression in pLVX-EZH2 MCF10A cells in a transcription activation reporter assay. A pGreenFire lentiviral vector containing a GFP reporter gene driven by four transcriptional response elements of the *Notch1* gene promoter was utilized. We found that doxycycline treatment of pLVX-EZH2 cells revealed an increase in *Notch1* transcriptional activity compared to controls, as measured by the percentage of GFP-expressing cells (Fig. 3-8, A).

In order to determine which domains of EZH2 are necessary in the regulation of *Notch1* transcriptional activity, we generated several myc-tagged EZH2 deletion mutants in an adenoviral vector (Fig. 3-8, B). Mutants included deletions of the amino-terminal HI and HII homology domains, the carboxy-terminal SET domain which is necessary for methyltransferase activity, and the nuclear localization signal (NLS). The expression of the mutants, compared to wild-type EZH2 adenovirus, in parental MCF10A cells was confirmed via Western blot (Fig. 3-8, B). Interestingly, the increase in NICD protein levels mediated by ectopic wild type EZH2 expression was not observed with expression of any of the deletion mutants (Fig. 3-8, B). Additionally, all of the deletion mutants inhibited the *Notch1* transcriptional activity previously observed with wild type EZH2 upregulation (Fig. 3-8, C). While wild type EZH2 overexpression in parental MCF10A cells increased mammosphere numbers and sizes, we found that expression of all of the EZH2 deletion mutants in parental MCF10A cells prevented this

increase (Fig. 3-8, D). These findings signify that EZH2-mediated stem cell expansion and *Notch1* upregulation require the intact nuclear EZH2 protein.

EZH2 binds to the proximal *Notch1* promoter to activate *Notch1* transcription.

EZH2 has been characterized as a transcriptional repressor but there is recent evidence suggesting it can contextually activate gene transcription [14-17]. We hypothesized that the ability of EZH2 to increase *Notch1* expression may be linked directly to its ability to bind to the *Notch1* promoter. To test this hypothesis, we performed chromatin immunoprecipitation (ChIP) assays on primary, nontumorigenic breast epithelial cells transduced with adenoviral vectors containing wild-type EZH2 or EZH2 mutants, ΔSET and ΔHII. These mutants were selected because the SET domain is required for the histone methylating function of EZH2 and the HII domain has been reported to promote gene activation [4, 6, 7, 13, 14, 18-20]. Walking primers encompassing the *Notch1* promoter regions from -532 to -4510 base pairs of the transcription start site were used (Fig. 3-9, A). Primers flanking the *GAPDH* promoter region were used as a negative control; and primers flanking the *MYT1* promoter region, a known direct transcriptional repression target of EZH2 through trimethylation of histone 3 lysine 27 (H3K27me3), were used as a positive control [50, 51]. Upon wild-type EZH2 overexpression in nontumorigenic primary breast cells we observed a significant increase in EZH2 binding at the -1.2 kb *Notch1* site, which was blocked by expression of the ΔHII mutant (Fig. 3-9, B). EZH2 binding to the *Notch1* promoter was not associated with H3K27me3 or SUZ12 binding, but coincided with increased methylation of H3K4 and RNA Polymerase II binding, known transcriptional activation

marks (Fig. 3-9, B). These intriguing results strongly suggest that EZH2 binds to the *Notch1* promoter independent of the Polycomb Repressive Complex 2 (PRC2) and of its repressive trimethylating activities, and may instead be leading to transcriptional activation. These results further demonstrate that the amino-terminal HII domain is indispensible for the binding.

We next investigated whether the binding of EZH2 protein to the *Notch1* promoter occurs in breast cancer cells with high endogenous levels of EZH2. Indeed, ChIP assays demonstrated enrichment for endogenous EZH2 at the *Notch1* promoter in MDA-MB-231 cells and in primary patient breast cancer cells (Fig. 3-9, C-D). Consistently, EZH2 binding was associated with enhanced H3K4 methylation and occurred independent of SUZ12 and H3K27me3 binding. Stable knockdown of EZH2 in MDA-MB-231 cells decreased the binding of EZH2 to the *Notch1* promoter, validating the specificity of the interaction (Fig. 3-9, C). Taken together, these data identify a previously undescribed function of EZH2 that involves binding to the *Notch1* promoter to activate transcription.

Transgenic EZH2 overexpression upregulates Notch1 and accelerates tumor initiation in MMTV-neu mice.

We were interested to determine whether the observed *in vitro* effects of EZH2 on breast stem cells translated to an *in vivo* model. Towards this end, we genetically overexpressed EZH2 in the MMTV-neu transgenic breast cancer mouse model by crossing MMTV-neu mice with the MMTV-EZH2 transgenic mice developed previously in our laboratory rendering $EZH2^{+}$/neu and control $EZH2^{wt}$/neu mice [43, 52]**.** Virgin $EZH2^{+}$/neu mice exhibited ductal hyper-branching with increased numbers of tertiary

branches compared to the EZH2^{wt}/neu mice as early as 8 weeks of age, which persisted at 16 weeks of age (Fig. 3-10, A). Providing *in vivo* validation to our mechanistic studies, EZH2^{+}/neu mice developed mammary glands with atypical intraductal hyperplasia similar to the human counterpart, had increased levels of NICD and phospho-STAT3 proteins, and increased cell proliferation as measured by using Ki-67 immunostaining, when compared to EZH2^{wt}/neu mice (Fig. 3-10, B)**.** Furthermore, EZH2^{+}/neu mice formed invasive mammary carcinomas significantly earlier and had increased levels of NICD in the tumors compared to EZH2^{wt}/neu mice (Fig. 3-11, A-B). Collectively, these findings provide direct *in vivo* evidence that precancerous EZH2 upregulation promotes epithelial hyperplasia with increased Notch1 expression and pathway activation. Our data show that EZH2 overexpression is sufficient to accelerate the initiation of mammary carcinomas in MMTV-neu mice.

EZH2 expression is associated with NICD and CD44^{+}/CD24^{-} expression in human invasive breast cancer tissues.

The relevance of these novel findings to human breast cancer was validated by examining the expression of EZH2, NICD, and CD44/CD24 proteins in tissue microarrays containing 107 cases of invasive breast carcinomas. Immunostained proteins were scored as exhibiting low or high expression according to a previously validated schema [39, 45]. We found a significant correlation between EZH2 and NICD expression, as both proteins were highly co-expressed in 55 cases (44%) and lowly co-expressed, or not expressed at all, in 37 cases (35%) (Fisher's exact test, $p<0.0001$)(Figure 3-12, A-B). Of the 107 cases, 70 were available for double

immunostaining with CD44 and CD24. We observed that 26% of tumor tissues were positive for stem cells ($CD44^+/CD24^-$), EZH2 and NICD whereas 34% were negative for stem cells ($CD44^-/CD24^-$), EZH2 and NICD (Fisher's exact test, p=0.0004) (Figure 3-12, A&C). These associations in human breast cancer underscore our *in vitro* and animal experiments and further highlight the role of EZH2 in regulating *Notch1* expression and stem cell numbers.

3-5. Discussion

It was estimated that over 200,000 new breast cancer diagnoses and nearly 40,000 breast cancer-related deaths were anticipated in the United States in 2011 alone [53]. Despite major advances in the diagnosis and treatment of breast cancer, there is a considerable gap in our understanding of the mechanisms by which breast cancer originates and progresses. In this study, we have identified that EZH2 has a direct causal role in breast cancer progression and that it is a crucial modulator of Notch1 signaling in breast cancer. Although EZH2 has been reported to exert oncogenic functions in the breast, those studies were based on *in vitro* and xenograft models. We demonstrate for the first time that genetic overexpression of EZH2 accelerates breast cancer initiation in a transgenic mouse model of mammary tumorigenesis.

By employing breast cancer cell lines and primary breast cancer cells derived from a patient tumor, we found that EZH2 expression regulates the abundance of cancer stem cells *in vitro*. Ectopic EZH2 overexpression increased the stem cell pool in nontumorigenic breast cells, while EZH2 downregulation reduced the breast cancer stem cell population in breast cancer cell lines. In accordance with our finding is an earlier report showing that EZH2 can promote breast cancer stem cell expansion [23].

However, the consequences of these functions on breast cancer initiation *in vivo* have not been investigated. Our data show that genetic overexpression of EZH2 in MMTV-neu mice decreases the latency to breast cancer initiation. The role of EZH2 in promoting breast stem cell expansion and breast cancer development is intriguing in light of our previous study showing that EZH2 protein is increased in histologically normal breast tissues from women up to 12 years *before* they develop breast cancer compared to controls [21, 22]. From a clinical perspective, blocking EZH2 may prevent breast cancer initiation in women with EZH2 overexpression in their breast epithelium.

We show that EZH2 is a novel modulator of Notch1 expression and signaling, regulating Notch1-dependent expansion of breast cancer stem cells. Inhibition of Notch1 activity by γ-secretase inhibitors prevented EZH2-mediated induction of breast stem cells. By utilizing domain specific mutants of EZH2, we demonstrate that intact nuclear EZH2 protein is required for *Notch1* gene upregulation and function on the stem cell pool. The association and mechanistic link between EZH2 and Notch1 was validated *in vitro, in vivo*, and in human breast cancer tissue samples.

Substantive data show that canonical EZH2 function is exerted through transcriptional repression of tumor suppressor genes through its methyltransferase activity on H3K27. Intriguingly, recent studies have reported that EZH2 can exert transcriptional activating functions in breast cancer, but the underlying mechanisms warrant further investigation [14-17, 54-56]. Our study defines a new role for EZH2 in breast cancer, by which EZH2 binds to the *Notch1* promoter in a region which coincides with RNA Polymerase II binding and enrichment for the H3K4 activating mark, leading to transcriptional activation. This occurs in the vicinity of a previously reported

recruitment site for the transcription factor PEA3, an ETS family member who activates *Notch1* transcription [57]. Further supporting our findings, a recent study demonstrated that EZH2 physically interacts with RelA and RelB proteins to promote the expression of NF-κB target genes in basal-like breast cancer cells, independent of the SET domain [16]. Another recent study demonstrated in prostate cancer cells that EZH2 binding to transcriptionally active gene sites occurred independent of PRC2 complex members and H3K27me [18]. Although independent of its canonical trimethylating mark on H3K27, the authors found the SET domain to be required for gene activation. These two studies highlight interesting discoveries within our own work. In this study, we found that an intact EZH2 protein, including the SET domain, was necessary for *Notch1* promoter activity, but that the SET domain was not necessary for *Notch1* promoter binding by EZH2. We demonstrate that the N-terminal HII domain of EZH2 is responsible and necessary for *Notch1* promoter binding. Our data are in agreement with a previous study, which showed that the EZH2 N-terminal homology domains are involved in enhancing gene transactivation [14].

In conclusion, our study advances the current understanding of the mechanisms of EZH2 function in breast cancer, and lends support to the emerging transcriptional activating role of EZH2. By providing first direct evidence that EZH2 overexpression accelerates breast cancer initiation *in vivo,* our work paves the way to targeting EZH2 to halt breast cancer progression.

3-6. Figures

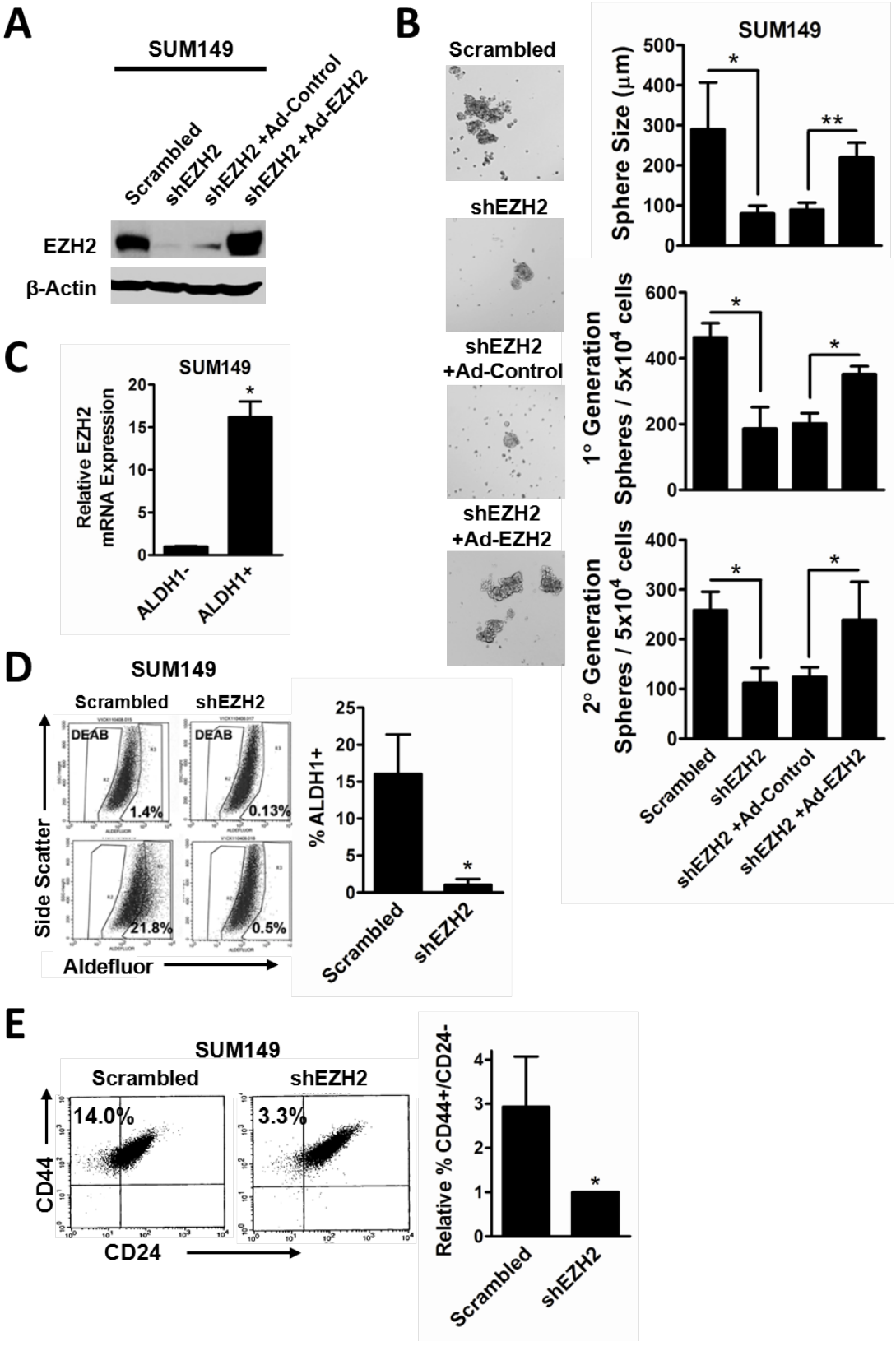
A
SUM149
Scrambled
shEZH2
shEZH2 +Ad-Control
shEZH2 +Ad-EZH2
EZH2
β-Actin
B
Scrambled
shEZH2
shEZH2 +Ad-Control
shEZH2 +Ad-EZH2
SUM149
Sphere Size (μm)
1° Generation Spheres / 5x10^4 cells
2° Generation Spheres / 5x10^4 cells
Scrambled
shEZH2
shEZH2 +Ad-Control
shEZH2 +Ad-EZH2
C
SUM149
Relative EZH2 mRNA Expression
ALDH1-
ALDH1+
D
SUM149
Scrambled
shEZH2
DEAB
1.4%
0.13%
21.8%
0.5%
Side Scatter
Aldefluor
% ALDH1+
Scrambled
shEZH2
E
SUM149
Scrambled
shEZH2
14.0%
3.3%
CD44
CD24
Relative % CD44+/CD24-
Scrambled
shEZH2

Figure 3-1 EZH2 levels regulate stem cell numbers in SUM149 breast cancer cells.

(A) Immunoblots of SUM149 breast cancer cells show that downregulation of EZH2 protein is accomplished with EZH2-targeted shRNA [shEZH2] compared to scrambled shRNA controls. Transient rescue of EZH2 expression in shEZH2 cells was completed using a myc-tagged, EZH2-encoding adenovirus [+Ad-EZH2], but rescue did not occur in shEZH2 cells expressing control adenovirus [+Ad-Control]. **(B)** EZH2 knockdown in SUM149 cells significantly reduces the size and numbers of mammospheres in primary and secondary sphere generations [7 days/generation], while re-expression of EZH2 in knockdown cells rescues the phenotype. Left, representative images of mammospheres formed after 7 days in culture [200X magnification]. Right-top, average mammosphere sizes ± SD in the primary generation [Student's t-test, *p=0.04, **p=0.005]. Right-middle and right-bottom, average number of mammospheres ± SD per $5x10^4$ plated cells in the primary and secondary generations, respectively [Student's t-test, 1º Gen. *p<0.0001, 2º Gen. *p≤0.0004]. **(C)** EZH2 mRNA expression is significantly higher in the parental SUM149 $ALDH1^+$ flow cytometry-sorted population versus the $ALDH1^-$ sorted population. EZH2 mRNA expression presented as relative to β-Actin mRNA expression ± SD [Student's t-test, *p=0.0001]. **(D)** shEZH2 significantly decreased the percentage of SUM149 $ALDH1^+$ cells ± SD compared to scrambled shRNA controls [Student's t-test, *p=0.002]. **(E)** shEZH2 significantly decreased the relative percentage of SUM149 $CD44^+/CD24^{-/low}$ cells ± SD compared to scrambled shRNA controls [Student's t-test, *p=0.04].

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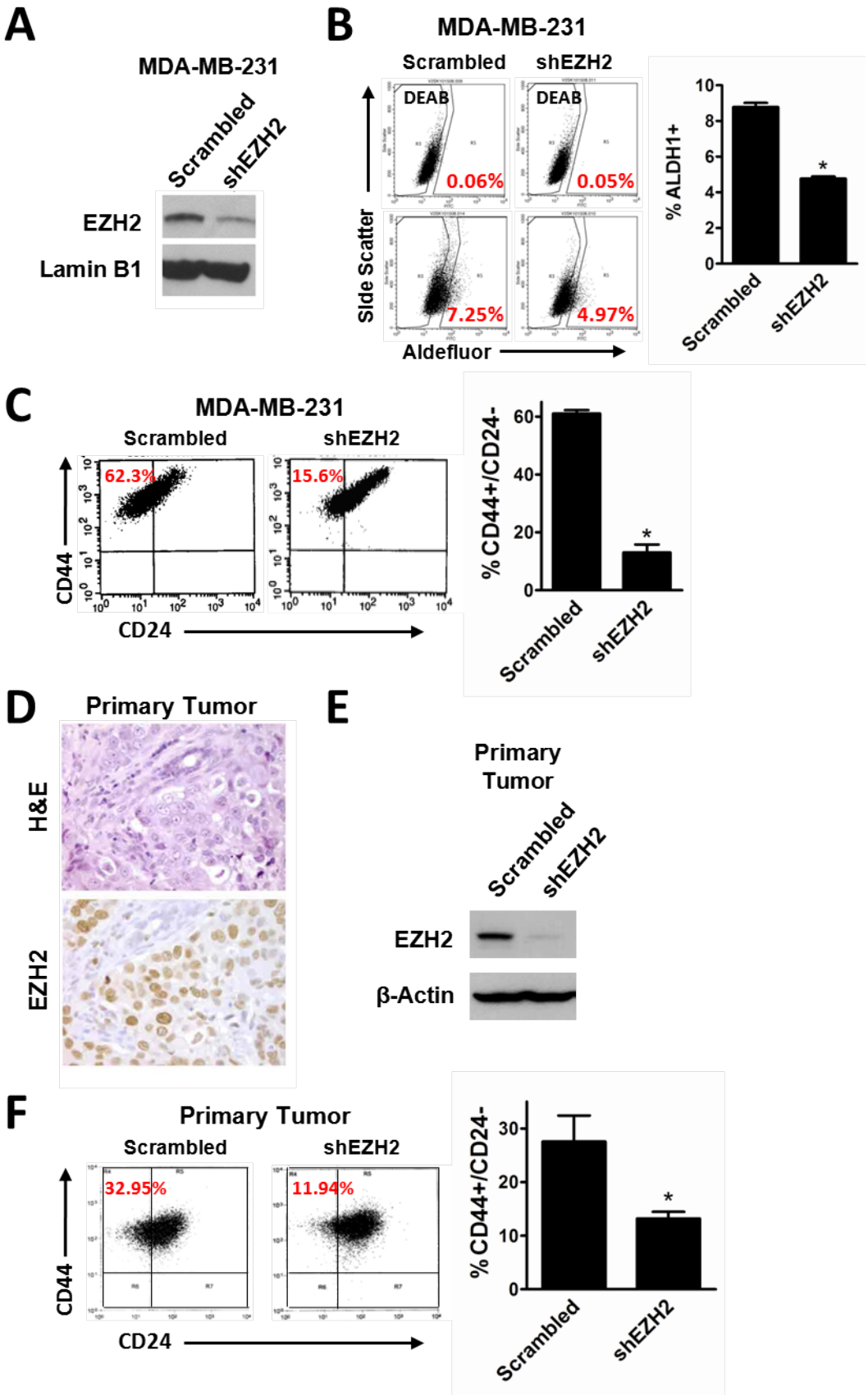
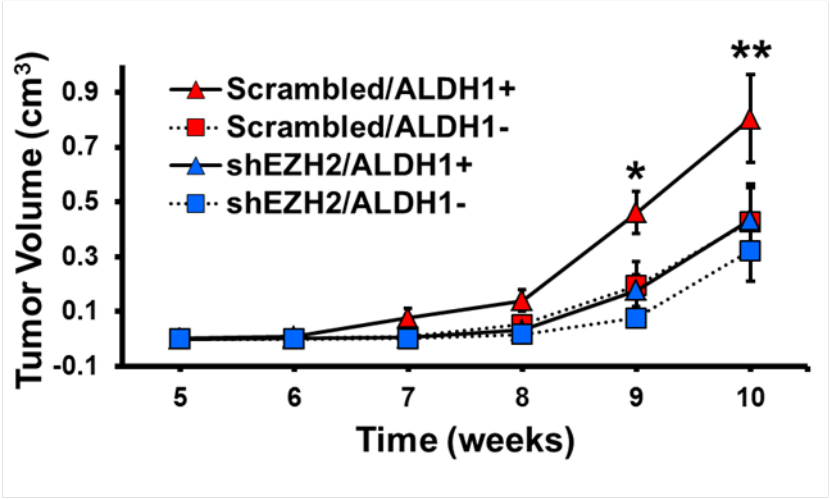
Figure 3-2 EZH2 levels regulate stem cell numbers in MDA-MB-231 breast cancer cells and in patient primary breast cancer cells.

(A) Immunoblots of MDA-MB-231 breast cancer cells show that downregulation of EZH2 protein is accomplished with EZH2-targeted shRNA [shEZH2] compared to scrambled shRNA controls. **(B)** shEZH2 significantly decreased the percentage of MDA-MB-231 ALDH1+ cells ± SD compared to scrambled shRNA controls [Student's t-test, *p=0.04]. **(C)** shEZH2 significantly decreased the relative percentage of MDA-MB-231 $CD44^+/CD24^{-/low}$ cells ± SD compared to scrambled shRNA controls [Student's t-test, *p<0.0001] **(D)** Representative photomicrographs of hematoxylin and eosin [H&E] staining and EZH2 immunostaining of a patient tumor sample obtained from a primary invasive ductal breast carcinoma [Primary Tumor, 400X magnification]. The invasive carcinoma cells exhibited strong nuclear expression of EZH2. **(E)** Immunoblots of patient primary breast cancer cells derived from the tumor in (C) show that downregulation of EZH2 protein is accomplished with shEZH2 compared to scrambled shRNA controls. **(F)** In patient primary breast cancer cells, shEZH2 significantly decreased the percentage of $CD44^+/CD24^{-/low}$ cells ± SD compared to scrambled shRNA controls [Student's t-test, *p=0.008].

(Figure on following page)


A
MDA-MB-231
Scrambled
shEZH2
EZH2
Lamin B1
B
MDA-MB-231
Scrambled
shEZH2
DEAB
DEAB
0.06%
0.05%
Side Scatter
7.25%
4.97%
Aldefluor
% ALDH1+
10
8
6
4
2
0
*
Scrambled
shEZH2
C
MDA-MB-231
Scrambled
shEZH2
62.3%
15.6%
CD44
CD24
% CD44+/CD24-
60
40
20
0
*
Scrambled
shEZH2
D
Primary Tumor
H&E
EZH2
E
Primary Tumor
Scrambled
shEZH2
EZH2
β-Actin
F
Primary Tumor
Scrambled
shEZH2
32.95%
11.94%
CD44
CD24
% CD44+/CD24-
30
20
10
0
*
Scrambled
shEZH2

A



B

SUM149 10,000 Cells		No. of mice with tumors (No. of total mice)					
		Week 5	Week 6	Week 7	Week 8	Week 9	Week 10
ALDH1-	Scrambled	0(9) 0%	2(9) 22%	5(9) 56%	7(9) 78%	9(9) 100%	9(9) 100%
	shEZH2	0(8) 0%	0(8) 0%	2(8) 25%	5(8) 63%	6(8) 75%	6(8) 75%
ALDH1+	Scrambled	2(9) 22%	5(9) 56%	9(9) 100%	9(9) 100%	9(9) 100%	9(9) 100%
	shEZH2	0(10) 0%	2(10) 20%	5(10) 50%	7(10) 70%	7(10) 70%	8(10) 80%

Chi squared, p<0.0001

Figure 3-3 EZH2 knockdown in SUM149 ALDH1$^+$ cells decreased the growth rate and time to tumor initiation of *in vivo* breast tumors.

(A) EZH2 knockdown significantly decreased the tumorigenicity of ALDH1$^+$ SUM149 cells compared to controls [mixed effects regression model, $p<0.05$]. Tumorigenicty assay using ALDH1$^+$ and ALDH1$^-$ sorted populations of SUM149 cells expressing either scrambled shRNA or shEZH2. After flow cytometry sorting, 10,000 cells were injected into the cleared mammary fat pad of female NOD/SCID mice [*n*=10 mice per condition] and mice were monitored for tumor growth. Average tumor volume ± SEM for weeks 5-10 post-injection for the four conditions is graphed [Student's t-test, *p=0.004, **p=0.04]. **(B)** EZH2 knockdown significantly increased the time to tumor initiation in SUM149 ALDH1$^+$ cells compared to controls [Chi-squared, $p<0.0001$]. The table shows the number of mice with tumors for weeks 5-10 post-injection.

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Figure 3-4 EZH2 downregulation reduces Notch1 and downstream signaling in breast cancer cells.

(A) Heatmap of quantitative RT-PCR microarray (SABiosciences Stem Cell PCR Array) performed using mRNA isolated from sorted ALDH1$^+$ SUM149 cells expressing scrambled shRNA or shEZH2. Upon EZH2 knockdown, *Notch1* gene expression was one of the most significantly reduced [Student's t-test, $p<0.0001$]. **(B)** mRNA from cells described in (A) were used in further quantitative RT-PCR assays analyzing expression of genes within the Notch signaling family. Bar graph shows that EZH2 knockdown in stem cells leads to deregulation of genes involved in Notch signaling. **(C)** Immunoblots of MDA-MB-231 and SUM149 breast cancer cells show that EZH2 downregulation reduces the expression of the intracellular, activated form of Notch1 [NICD] and NOTHC1 downstream targets CCND1 and CTNNB compared to controls. **(D)** Immunoblots of patient primary breast cancer cells show that EZH2 downregulation reduces the expression of NICD and Notch1 targets Hes1 and phosphorylated-STAT3 [p-STAT3]. **(E)** Immunoblots of MDA-MB-231 and SUM149 cells show that transient adenoviral re-expression of EZH2 in shEZH2 cells [+Ad-EZH2] rescues NICD protein levels compared to adenoviral control cells [+Ad-Control].

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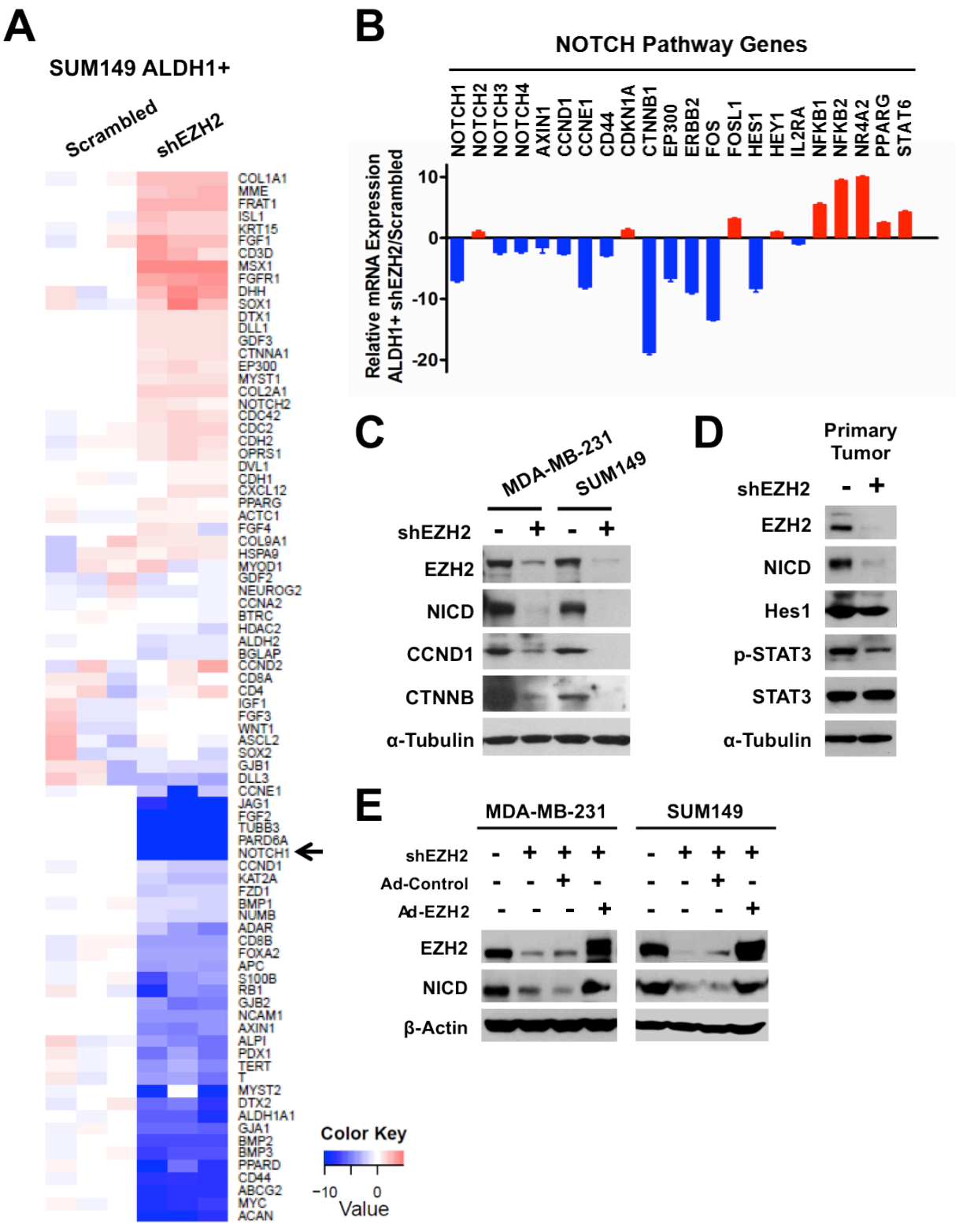
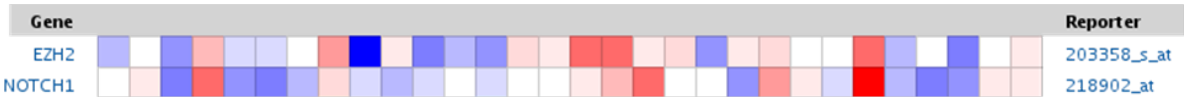

A
SUM149 ALDH1+
Scrambled
shEZH2
COL1A1
MME
FRAT1
ISL1
KRT15
FGF1
CD3D
MSX1
FGFR1
DHH
SOX1
DTX1
DLL1
GDF3
CTNNA1
EP300
MYST1
COL2A1
NOTCH2
CDC42
CDC2
CDH2
OPRS1
DVL1
CDH1
CXCL12
PPARG
ACTC1
FGF4
COL9A1
HSPA9
MYOD1
GDF2
NEUROG2
CCNA2
BTRC
HDAC2
ALDH2
BGLAP
CCND2
CD8A
CD4
IGF1
FGF3
WNT1
ASCL2
SOX2
GJB1
DLL3
CCNE1
JAG1
FGF2
TUBB3
PARD6A
NOTCH1
CCND1
KAT2A
FZD1
BMP1
NUMB
ADAR
CD8B
FOXA2
APC
S100B
RB1
GJB2
NCAM1
AXIN1
ALPI
PDX1
TERT
T
MYST2
DTX2
ALDH1A1
GJA1
BMP2
BMP3
PPARD
CD44
ABCG2
MYC
ACAN
Color Key
−10
0
Value
B
NOTCH Pathway Genes
NOTCH1
NOTCH2
NOTCH3
NOTCH4
AXIN1
CCND1
CCNE1
CD44
CDKN1A
CTNNB1
EP300
ERBB2
FOS
FOSL1
HES1
HEY1
IL2RA
NFKB1
NFKB2
NR4A2
PPARG
STAT6
Relative mRNA Expression
ALDH1+ shEZH2/Scrambled
10
0
-10
-20
C
MDA-MB-231
SUM149
shEZH2 - + - +
EZH2
NICD
CCND1
CTNNB
α-Tubulin
D
Primary Tumor
shEZH2 - +
EZH2
NICD
Hes1
p-STAT3
STAT3
α-Tubulin
E
MDA-MB-231
SUM149
shEZH2 - + + + - + + +
Ad-Control - - + - - - + -
Ad-EZH2 - - - + - - - +
EZH2
NICD
β-Actin

Comparison of EZH2 and NOTCH1 mRNA expressions in publicly available breast cancer datasets on Oncomine

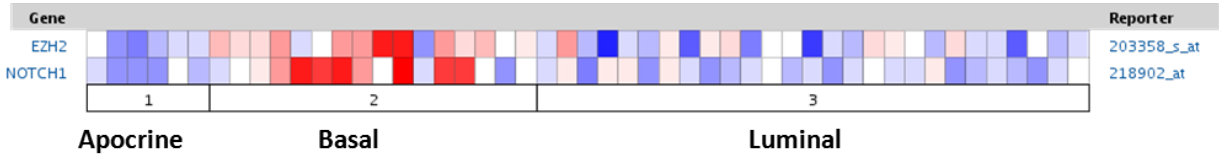
BREAST CANCER TISSUE SAMPLES

Hoeflich et al, Correlation Coefficient 0.76



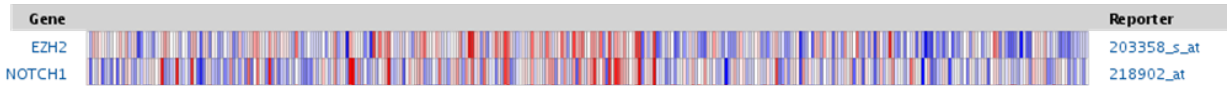
Clin Cancer Res 2009/07/15	30 samples
mRNA	19,574 measured genes
Human Genome U133 Plus 2.0 Array	

Farmer et al, Correlation Coefficient 0.61



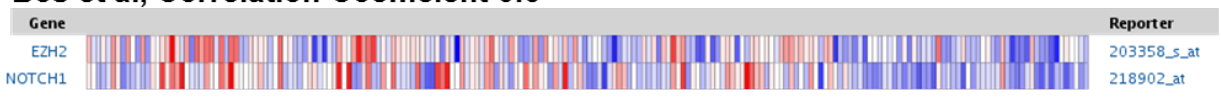
Oncogene 2005/07/07	49 samples
mRNA	12,624 measured genes
Human Genome U133A Array	

Bittner database, Correlation Coefficient 0.6



Unpublished 2005/01/15	336 samples
mRNA	19,574 measured genes
Human Genome U133 Plus 2.0 Array	

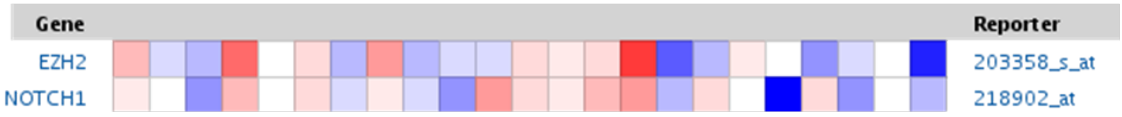
Bos et al, Correlation Coefficient 0.6



Nature 2009/06/18	204 samples
mRNA	19,574 measured genes
Human Genome U133 Plus 2.0 Array	

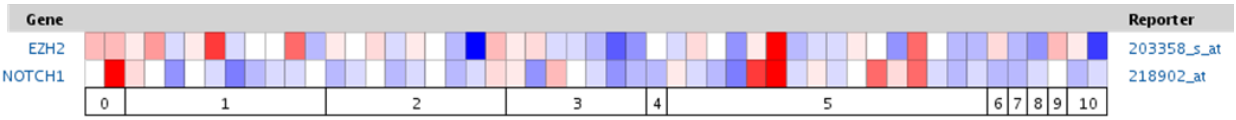
BREAST CANCER CELL LINES

Huang et al, Correlation Coefficient 0.69



Cancer Res 2007/03/01	23 samples
mRNA	12,624 measured genes
Human Genome U133A Array	

Neve et al, Correlation Coefficient 0.6



Cancer Cell 2006/12/01	51 samples
mRNA	12,624 measured genes
Human Genome U133A Array	

Figure 3-5 *EZH2* expression is associated with *Notch1* expression in independent datasets of human invasive breast carcinomas.

Significant associations [correlation coefficient > 0.6] were identified between EZH2 and Notch1 mRNA levels in publicly available datasets of human breast cancer tissues, first page, and human breast cancer cell lines, second page, utilizing Oncomine.

(Figure includes previous page)

Figure 3-6 Notch1 is required for EZH2-induced expansion of MCF10A breast stem cells, part 1.

(A) Real-time RT-PCR of MCF10A pLVX [control] and pLVX-EZH2 [conditional EZH2 TetOn system] cells either untreated or treated with doxycycline [DOX] for 24 hours at 1μg/ml and sorted into $ALDH1^-$ and $ALDH1^+$ populations; mRNA expression is relative to GAPDH mRNA levels ± SD. Upon DOX treatment, a significant increase in EZH2 mRNA expression is seen in both $ALDH1^-$ and $ALDH1^+$ pLVX-EZH2 cells. Between these two groups specifically, EZH2 mRNA is significantly increased in the $ALDH1^+$ population compared to the $ALDH1^-$ population [Student's t-test, *p<0.0007]. **(B)** Real-time RT-PCR of MCF10A pLVX-EZH2 cells either untreated or treated with DOX for 24 hours and sorted into $ALDH1^-$ and $ALDH1^+$ populations; mRNA expression is relative to GAPDH mRNA levels ± SD. Upon DOX treatment and EZH2 overexpression, Notch1 mRNA levels significantly increase in the $ALDH1^+$ population, but not in the $ALDH1^-$ population [Student's t-test, *p=0.0001]. **(C)** Immunoblots of MCF10A pLVX-EZH2 cells show that DOX treatment results in an increase in EZH2 and NICD protein levels. Upon Notch1 inhibition with the γ-secretase inhibitor [GSI] for 3 days at 17nM in EZH2 overexpressing cells, NICD protein levels are reduced. **(D)** Representative images of MCF10A pLVX and pLVX-EZH2 mammospheres formed after 7 days in culture [200X magnification]. **(E)** DOX-induced overexpression of EZH2 in MCF10A pLVX-EZH2 cells leads to a significant increase in mammosphere sizes and numbers compared to controls, which is blocked with Notch1 inhibition. For mammosphere assays, GSI treatment was done for each 7-day generation at 1.7nM. Top, average mammosphere sizes ± SD in the primary generation [Student's t-test, *p<0.002]. Middle and Bottom, average number of mammospheres ± SD per $5x10^4$ plated cells in the primary and secondary generations, respectively [Student's t-test, 1º & 2º Gen. *p<0.0001].

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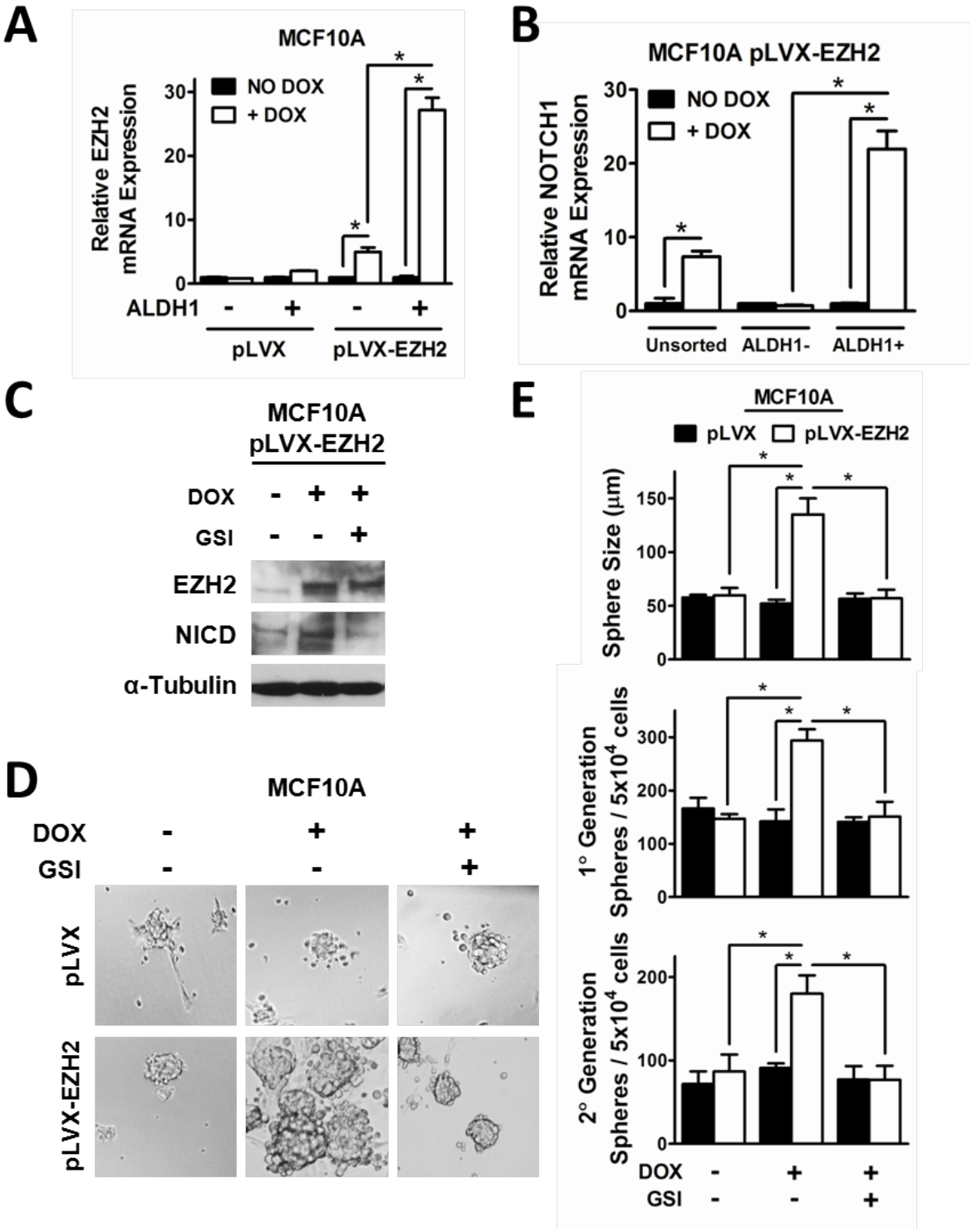
A
MCF10A
Relative EZH2 mRNA Expression
NO DOX
+ DOX
ALDH1
pLVX
pLVX-EZH2
B
MCF10A pLVX-EZH2
Relative NOTCH1 mRNA Expression
NO DOX
+ DOX
Unsorted
ALDH1-
ALDH1+
C
MCF10A pLVX-EZH2
DOX
GSI
EZH2
NICD
α-Tubulin
D
MCF10A
DOX
GSI
pLVX
pLVX-EZH2
E
MCF10A
pLVX
pLVX-EZH2
Sphere Size (μm)
1° Generation Spheres / 5x10^4 cells
2° Generation Spheres / 5x10^4 cells
DOX
GSI

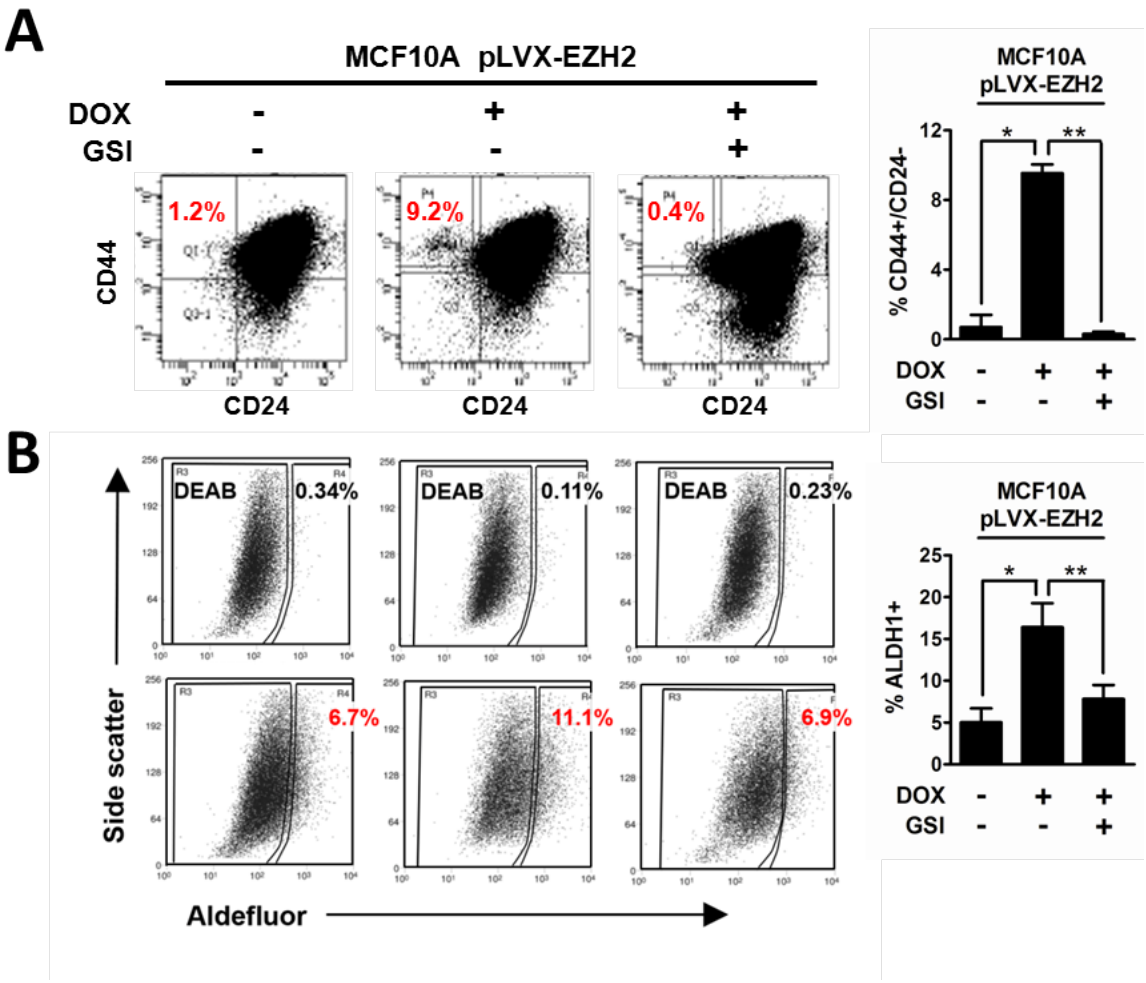

Figure 3-7 Notch1 is required for EZH2-induced expansion of MCF10A breast stem cells, part 2.

(A&B) DOX-induced overexpression of EZH2 in MCF10A pLVX-EZH2 cells leads to a significant increase in the percentage of $ALDH1^{+}$ **(A)** and $CD44^{+}/CD24^{-/low}$ **(B)** cells compared to controls, which is blocked with Notch1 inhibition. GSI was added for 3 days at 17nM; percentages are expressed ± SD [Student's t-test, (A): *p=0.005, **p=0.002; (B): *p=0.008, **p=0.02].

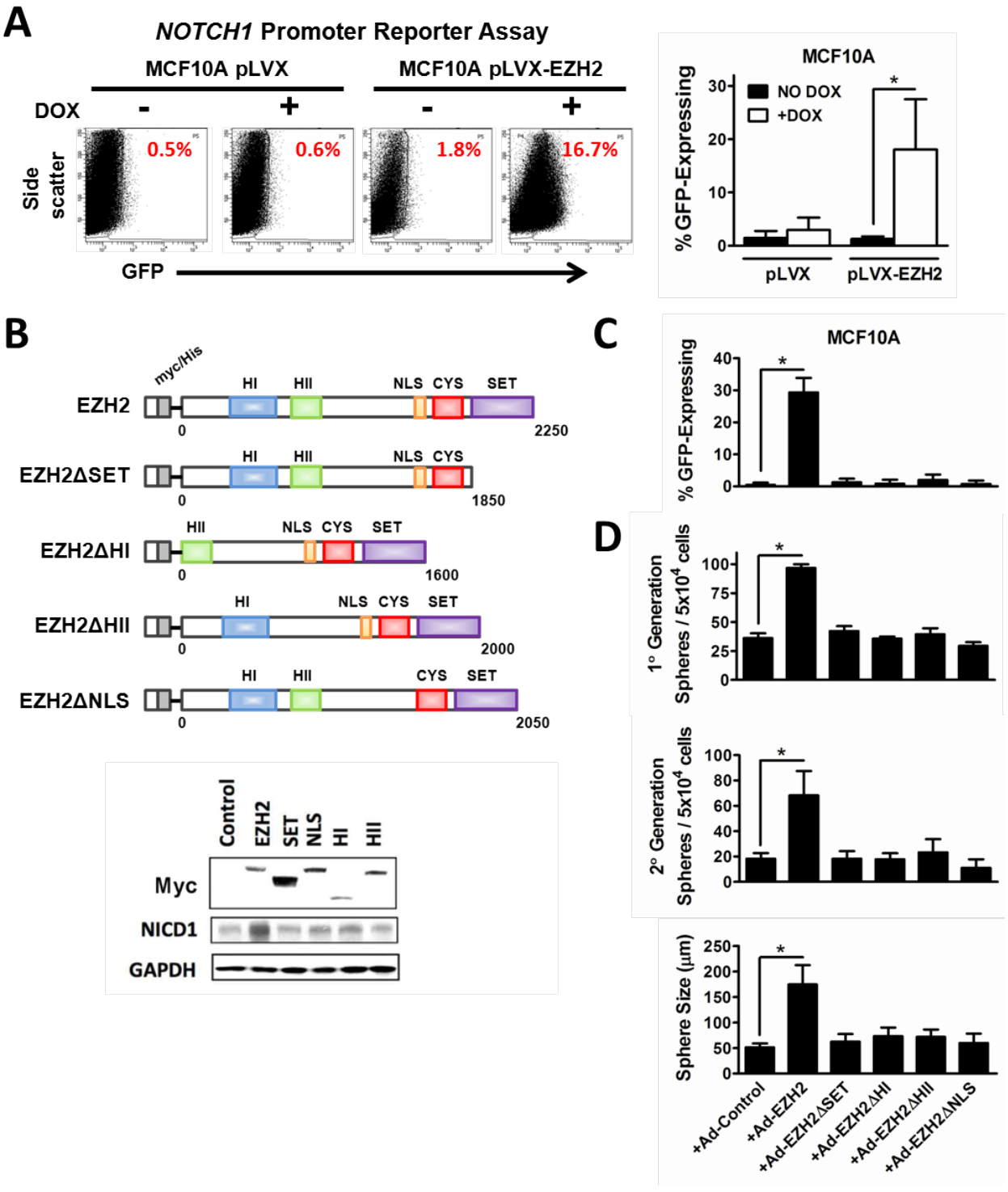
A
NOTCH1 Promoter Reporter Assay
MCF10A pLVX
MCF10A pLVX-EZH2
DOX
-
+
-
+
Side scatter
0.5%
0.6%
1.8%
16.7%
GFP
MCF10A
NO DOX
+DOX
% GFP-Expressing
pLVX
pLVX-EZH2
B
myc/His
HI
HII
NLS
CYS
SET
EZH2
0
2250
EZH2ΔSET
1850
EZH2ΔHI
1600
EZH2ΔHII
2000
EZH2ΔNLS
2050
Control
EZH2
SET
NLS
HI
HII
Myc
NICD1
GAPDH
C
MCF10A
% GFP-Expressing
D
1° Generation Spheres / 5x10^4 cells
2° Generation Spheres / 5x10^4 cells
Sphere Size (μm)
+Ad-Control
+Ad-EZH2
+Ad-EZH2ΔSET
+Ad-EZH2ΔHI
+Ad-EZH2ΔHII
+Ad-EZH2ΔNLS

Figure 3-8. Full length EZH2 is required for *Notch1* promoter activation and expansion of mammosphere sizes and numbers.

(A) EZH2 overexpression in MCF10A pLVX-EZH2 cells leads to a significant increase in *Notch1* promoter transcriptional activation. MCF10A pLVX and pLVX-EZH2 cells with and without DOX treatment were transduced with a pGreenFire lentivirus containing a GFP reporter gene driven by the *Notch1* promoter. Transcriptional activation was measured as the percentage of GFP-expressing cells ± SD by flow cytometry [Student's t-test, *p=0.02]. **(B)** Top, diagram of adenoviral, myc-tagged, EZH2 deletion mutants: ΔSET, ΔHI (Homology Domain I), ΔHII (Homology Domain II), and ΔNLS (Nuclear Localization Signal). Below, immunoblot demonstrates ectopic expression of full length and mutant EZH2 proteins in parental MCF10A cells and their effect on NICD protein levels. **(C)** Full length EZH2, but not any of the deletion mutants, is required for *Notch1* promoter transcriptional activation as measured by the percentage of GFP-expressing cells ± SD [Student's t-test, p=0.0004]. **(D)** Full length EZH2, but not any of the deletion mutants, is required for the EZH2-induced expansion in mammosphere sizes and numbers. Top and Middle, average number of mammospheres ± SD per $5x10^4$ plated cells in the primary and secondary generations [Student's t-test, 1º & 2º Gen., *p<0.0001]. Bottom, average mammosphere sizes ± SD in the primary generation [Student's t-test, *p=0.005].

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Figure 3-9 EZH2 binds to the *Notch1* gene promoter in benign breast cells, breast cancer cell lines and in patient primary breast cancer cells.

(A) Diagram of the *Notch1* promoter regions analyzed for EZH2 binding in chromatin immunoprecipitation [ChIP] assays. EZH2 was found to bind region "B" at -1.2kb; only ChIP real-time PCR data using primers designed for site "B" are shown. **(B)** ChIP assays were performed using nontumorigenic primary breast cells expressing control, full length EZH2, EZH2ΔSET or EZH2ΔHII. Primers flanking the *GAPDH* promoter were used as negative binding controls, and primers flanking the *MYT1* promoter, a known H3K27me3-repression target of EZH2, were used as positive binding controls. EZH2 binds the *Notch1* promoter independent of H3K27me3 and SUZ12, but coincides with RNA Polymerase II binding and enrichment for the transcriptional activating mark H3K4me2. The N-terminal HII domain is required for EZH2 binding whereas the C-terminal SET domain is dispensable. **(C)** ChIP assays as described in (B) were perfomed using MDA-MB-231 breast cancer cells expressing scrambled control shRNA or shEZH2. Endogenous EZH2 binds the *Notch1* promoter with similar associations as observed in (B), and binding is blocked with shEZH2. **(D)** ChIP assays as described in (B) were performed using patient primary breast cancer cells. Endogenous EZH2 binds to the *Notch1* promoter with similar associations as observed in (B). [Student's t-test, *p <0.05}

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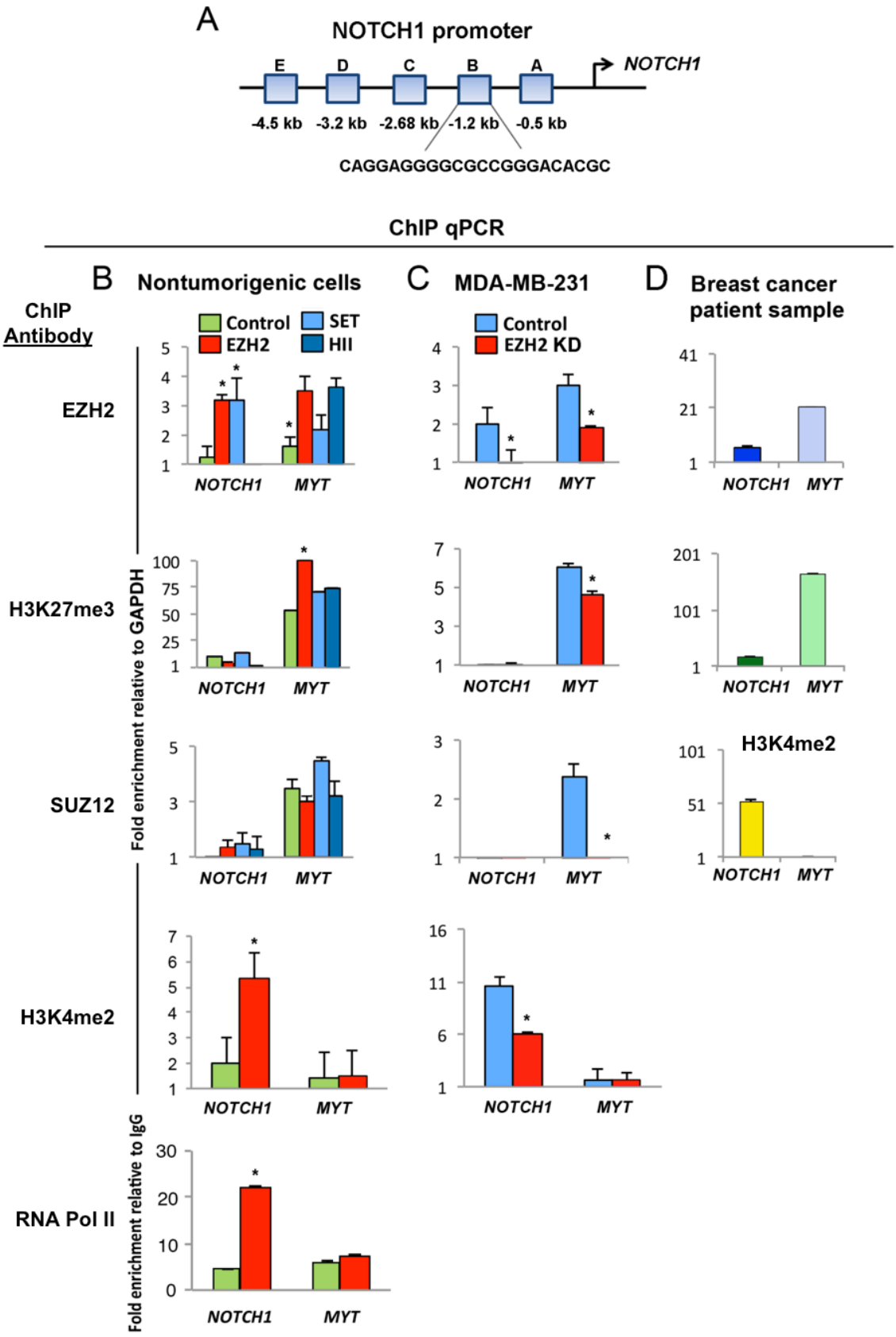
A
NOTCH1 promoter
E
D
C
B
A
NOTCH1
-4.5 kb
-3.2 kb
-2.68 kb
-1.2 kb
-0.5 kb
CAGGAGGGGCGCCGGGACACGC
ChIP qPCR
B
Nontumorigenic cells
C
MDA-MB-231
D
Breast cancer patient sample
ChIP Antibody
Control
SET
EZH2
HII
Control
EZH2 KD
EZH2
H3K27me3
SUZ12
H3K4me2
RNA Pol II
Fold enrichment relative to GAPDH
Fold enrichment relative to IgG
NOTCH1
MYT
H3K4me2

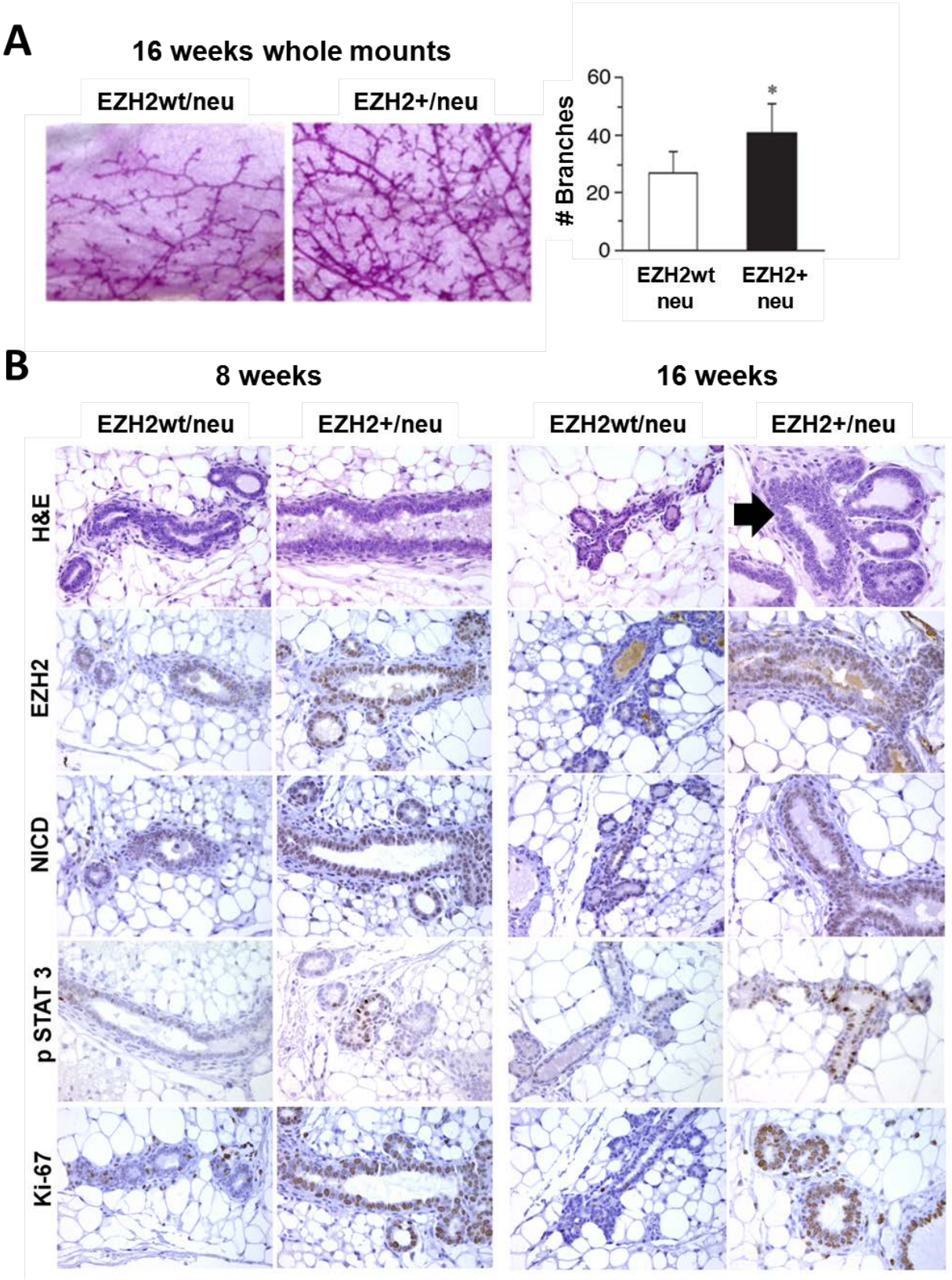
A
16 weeks whole mounts
EZH2wt/neu
EZH2+/neu
Branches
60
40
20
0
*
EZH2wt neu
EZH2+ neu
B
8 weeks
16 weeks
EZH2wt/neu
EZH2+/neu
EZH2wt/neu
EZH2+/neu
H&E
EZH2
NICD
p STAT 3
Ki-67

Figure 3-10 Characterization of EZH2^{+}/neu and EZH2^{wt}/neu transgenic mice.

(A) Left, representative images of whole mounts of mammary glands from 16 weeks old virgin female mice show that EZH2^{+}/neu glands exhibit ductal hyperbranching compared to EZH2^{wt}/neu glands controls. [100X magnification]. Right, graph shows the average number of tertiary branches ± SD in whole mounts preparations described in (A) [Student's t-test, *$p<0.05$]. **(B)** Representative histological sections at 8 and 16 weeks of age [400X magnification]; EZH2^{+}/neu mammary glands show upregulation of EZH2 protein in the nuclei of breast epithelial cells. At 8 weeks, EZH2^{+}/neu mice exhibit ductal hyperplasia, and at 16 weeks atypical intraductal hyperplasia becomes evident (arrow); changes were not seen in controls for the indicated timepoints. EZH2^{+}/neu mammary glands also show increased expression of NICD, phosphorylated-STAT3 and Ki67 compared to EZH2^{wt}/neu mammary glands.

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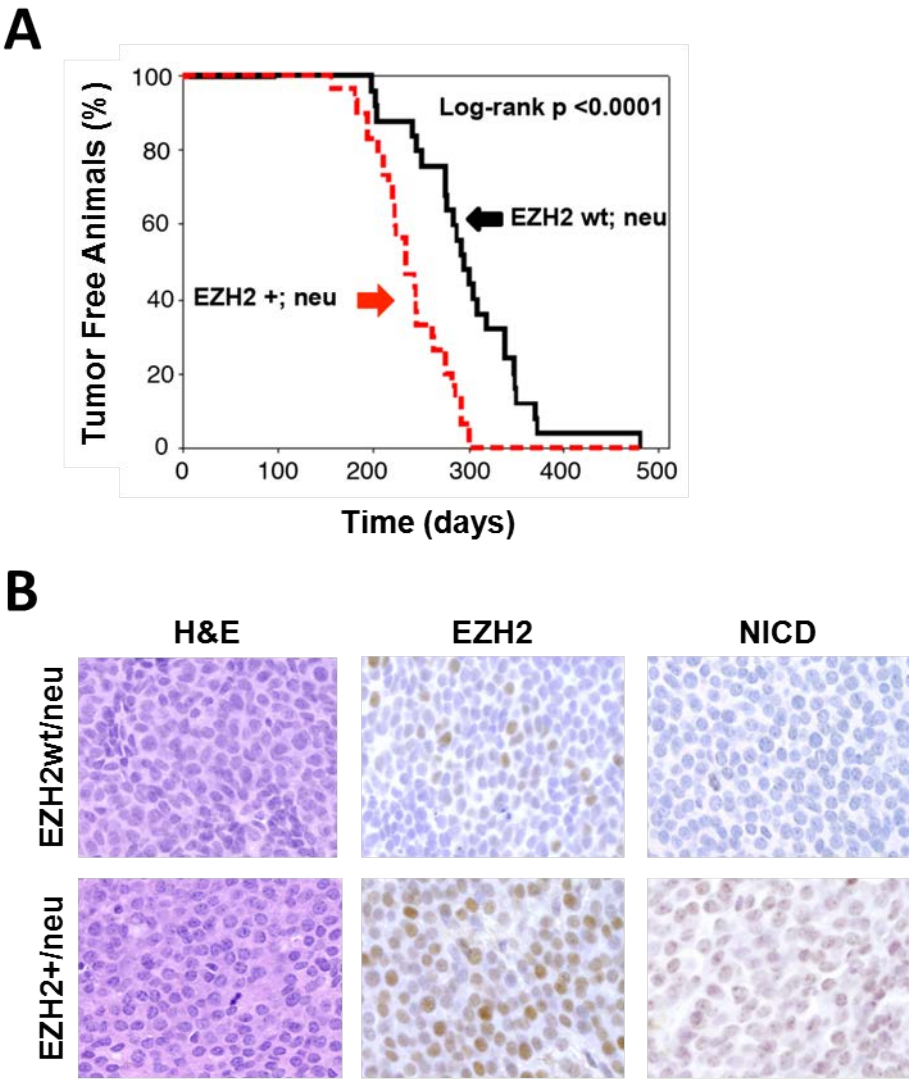


Figure 3-11 Transgenic EZH2 overexpression accelerates tumor initiation and upregulates Notch1 in MMTV-neu mice.

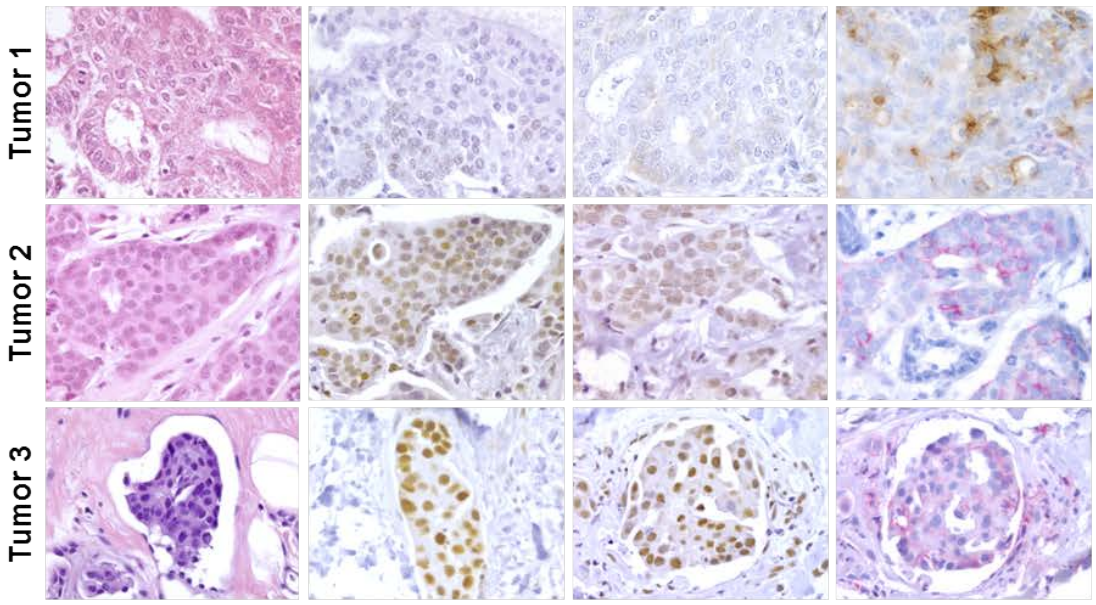
(A) Kaplan Meier curve shows that $EZH2^{+}$/neu mice formed mammary carcinomas significantly earlier than $EZH2^{wt}$/neu mice [Log-rank test, $p<0.0001$]. **(B)** Representative photomicrographs of breast carcinomas from $EZH2^{+}$/neu and control $EZH2^{wt}$/neu mice stained for H&E and immunostained for EZH2 and NICD [400X magnification].

Figure 3-12 EZH2 expression is associated with NICD and $CD44^+/CD24^-$ expression in human invasive breast cancer tissues.

(A) Representative images of three primary human invasive breast carcinomas (*n*=107 patients) stained for H&E and immunostained for EZH2, NICD and CD44/CD24 [400X magnification] **(B)** The table shows the distribution of EZH2 and NICD protein expression in 107 primary invasive breast carcinomas scored from immunostained tissue microarrays; 43.9% of tumors exhibited high expression of both EZH2 and NICD [Fisher's Exact Test, $p<0.0001$, 2-tailed]. **(C)** The table shows the distribution of EZH2, NICD and CD44/CD24 protein expression in 70 primary invasive breast carcinomas; 25.7% of tumors exhibited high expression of EZH2, NICD and $CD44^+/CD24^-$ [Fisher's Exact Test, $p=0.0004$, 2-tailed].

(Figure on following page)

A



B

	EZH2 low	EZH2 high	Total
NICD low	37 (34.6%)	8 (7.5%)	45
NICD high	15 (14.0%)	47 (43.9%)	62
Total	52	55	107

Fisher's Exact Test, p<0.0001

C

	EZH2 low		EZH2 high		Total
	NICD low	NICD high	NICD low	NICD high	
CD44+/CD24-	2 (2.9%)	3 (4.3%)	1 (1.4%)	18 (25.7%)	24
CD44-/CD24-	24 (34.3%)	3 (4.3%)	4 (5.7%)	15 (21.4%)	46
Total	26	6	5	33	70

Fisher's Exact Test, p=0.0004

3-7. References

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CHAPTER 4

Conclusions

4-1. Discussion and Future Directions

Breast cancer development and metastasis are complex processes, but through research, our understanding of these processes has evolved. The aim of this thesis has been to delve into the mechanisms underlying the importance of EZH2 overexpression in breast malignancies. Before our studies, EZH2 was highly associated with aggressive breast cancer and metastasis, but the work presented here has directly demonstrated that downregulation of EZH2 in breast cancer cells leads to a decrease in metastatic burden. In all, our findings have revealed important and novel functional links between EZH2, stem cells and breast cancer migration and invasion, and the underlying mechanisms involving EZH2-mediated regulation of p38 and Notch1 signaling pathways. This work establishes EZH2 as regulator of breast cancer progression and metastasis. However, as in all research, answers to certain questions inevitably lead to the development of new questions and research goals. Here, many of the new questions generated by the reported data will be addressed with proposed future directions.

The observed interaction between EZH2 and p38 proteins through immunoprecipitation (IP) experiments in Chapter 2 has raised several interesting and important questions. First, does EZH2 have preferential binding affinity for the unphosphorylated or the phosphorylated form of p38, or does it bind both equally?

Another related question might be where does this interaction occur, in the cytoplasm or the nucleus, or both? Although IPs were conducted using pan p38 and phosphorylated-p38 (p-p38) antibodies, the question of preferential affinity could not be answered in these experiments. The pan p38 antibodies that were used recognize both p38 and p-p38 species, so even though probing with a p-p38 antibody in a Western blot shows binding, an interaction between unphosphorylated p38 cannot be excluded.

As EZH2 predominantly localizes to the nucleus, it may seem more likely that EZH2 preferentially binds p-p38 since it is usually accumulates in the nucleus after activation [1, 2]. However, unphosphorylated p38 can be found in the cytoplasm and the nucleus, and it has been reported that phosphorylated p38 can be trafficked out of the nucleus into the cytoplasm by its substrate MK2 [3, 4]. Additionally, in a study where an activating function for EZH2 in actin polymerization was defined, it was also revealed that EZH2 can exist in the cytoplasm [5]. This cytosolic EZH2 retained methyltransferase capabilities as it was found to complex with the other core PRC2 members SUZ12 and EED in the cytoplasm, so our data demonstrating that SUZ12 and EED immunoprecipitate with p38 does not aid in answering the question of where these interactions occur. A cytosolic role for EZH2 has been implicated in research conducted in our laboratory as well. For instance, immunohistochemistry for EZH2 in mouse and human breast tumors does show a primary localization of EZH2 to the nuclei of cancer cells, but a lighter staining in the cytoplasm can also be seen (Figures 1-9, 1-10, 2-12). Also, in Western blot analyses, EZH2 has been seen in our laboratory to be present in both cytoplasmic and nuclear fractionations, although much lower in cytoplasmic fractions. Therefore, although IP experiments employing lysates from cytoplasmic and

nuclear fractionations may answer the important localization question, the question of possible preferential affinity cannot be resolved in this manner.

In order to determine if EZH2 has a preference for unphosphorylated or p-p38, a couple of different experiments could be conducted. First, immunoprecipitates collected using an EZH2 antibody could be run out via 2-dimensional gel electrophoresis, which allows for the separation of proteins by size and isoelectric point. As phosphorylated proteins are more acidic than their unphosphorylated counterparts, this assay should make them distinguishable if present. Second, lysates could be treated *in vitro* with Lambda Phosphatase before conducting IPs. If the phosphorylation mark is required for EZH2 binding, the loss of phosphorylation on p38 caused by phosphatase treatment should inhibit binding when compared to untreated controls.

Another significant question that the interaction between EZH2 and p38 generates is if there is a functional relevance to the binding. For example, does the binding promote the activation of p38 or ensure the stability of p-p38, or does it just coincide with the correlation between EZH2 and p-p38 protein levels in breast cancer cells with no functional relevance? In muscle stem cells, it has been recently demonstrated that EZH2 binds to p38α in nuclear extracts in association with the p38 upstream activator MKK6 [6]. EZH2 and p38α were detected on the promoter region of *Pax7*, a typical marker of stem cells, only after MKK6-mediated activation of p38. This study supports our findings of an interaction between EZH2 and p38, and suggests that the binding these two proteins may be functional as it was necessary for repression of *Pax7* in another system. In breast cancer cells, it would be of interest to see if MKK3 or MKK6, the protein kinases thought to be majorly responsible for p38 activation, also

complex with EZH2 and p38 through IPs [3]. These experiments could be expanded with the use of adenoviral constructs that express constitutively active forms of MKK3 or MKK6. Would the expression of these constructs, which would lead to p38 activation, lead to enhanced binding of p38 with EZH2? Additionally, does p38 co-occupy with EZH2 at PRC2 target genes and, if so, is its activation necessary for this recruitment?

It would also be of interest to determine what domains of EZH2 are necessary for binding with p38. Expression of the EZH2 mutants described in Chapter 3 in association with IP experiments could help to answer this question. Functionally, if a certain domain is deemed required for binding, the expression of its corresponding mutant could be used to determine if the activity of p-p38 is dependent on binding, as measured by the phosphorylation of downstream targets. It must also be kept in mind, however, that the binding between EZH2 and p38 may not be direct as another cofactor may be necessary for the interaction. The identification of other binding partners could be accomplished with IPs followed by mass spectrometry.

On a more basic level, experiments could be performed to see if EZH2 binding to p-p38 leads to stabilization. The use of proteasomal inhibitors, such as epoxomicin and lactacystin, could be used in scrambled control and shEZH2 breast cancer cells. If high EZH2 levels lead to increased p-p38 protein stability, the use of such inhibitors in the EZH2 knockdown cells should allow for a greater accumulation of p-p38 than what would be observed in treated control cells. Additionally, it must be considered that the increase in p-p38 levels seen with high EZH2 expression may be the result of PRC2-mediated repression of a phosphatase that normally targets p-p38. Interrogation of the

mRNA and protein levels of known MAPK-targeted phosphatases in control and EZH2 knockdown cells would easily address this issue.

The last set of questions generated from the interaction between EZH2 and p38 relate to the normal catalytic functions of these proteins. For instance, does the association lead to the methylation of p38 by EZH2? The post-translational methylation of several non-histone proteins has been reported in recent years, and in many cases, the methylation leads to enhanced stability or activity of these proteins [7]. We have demonstrated in preliminary experiments that PRC2 can methylate p38α *in vitro*. As knockdown of EZH2 was found to decrease levels of all p-p38 isoforms, *in vitro* experiments should be extended to include the other three isoforms. Moreover, *in vivo* experiments should be performed to see if the methylation occurs in live cells using metabolic labeling and antibodies that recognize methylated residues. Analysis of the p38 amino acid sequence using PMeS (Prediction of protein Methylation Sites), did not suggest any lysine residues as potential sites for methylation, but two arginine residues located at 189 and 237 were proposed with a threshold value above 0.5. If *in vivo* experiments show promising results, mass spectrometry of methylated p38 proteins should be performed in order to identify potential methylation sites. Most importantly, though, if methylation is confirmed *in vivo*, it should be determined if the methylation regulates the activation and activity of p-p38.

Alternately, does the interaction between EZH2 and p38 lead to the phosphorylation of EZH2 by p-p38? It has been published in recent years that EZH2 can be phosphorylated by several kinases affecting its activity. Most importantly, Palacios and colleagues have found in muscle stem cells that p38α is capable of

phosphorylating EZH2 on threonine 372, which enhances its repression of *Pax7* [6]. Additionally, phosphorylation of EZH2 by AKT on serine 21 decreases H3K27me3 levels, leads to derepression of silenced genes and contributes to tumor progression [8]. Several other studies have demonstrated that cyclin dependent kinases phosphorylate EZH2 at threonines 350 and 492 [9-12]. Phosphorylation of these two residues appears to have contrasting roles in EZH2 activity as phosphorylation of threonine 350 and of threonine 492 positively and negatively impact PRC2 functions, respectively. These studies in EZH2 phosphorylation emphasize the need for further interrogation of the interaction between EZH2 and p38. Numerous serine and threonine phosphorylation sites are predicted on EZH2, some specifically for MAPKs, utilizing several phosphorylation prediction software programs implicating a need for further research. If a phosphorylation event does occur by p38 on EZH2, it would be intriguing to see if an effect on EZH2 activity is rendered. We have demonstrated that inhibition of p-p38 activity does not affect EZH2 or H3K27me3 protein levels, so perhaps phosphorylation of EZH2 may influence the recruitment of EZH2 and PRC2 to target genes.

Our findings in Chapter 3 related to the EZH2-mediated regulation of stem cell numbers and Notch1 signaling raise several questions as well. First, a fusion of the ideas presented in Chapter 2 with those in Chapter 3 can be imagined. Two independent studies have found that induction of EMT in human mammary epithelial cells generates cells with stem-like properties [13, 14]. We have shown separately that high EZH2 expression in breast cancer cells promotes EMT and an expansion in CSCs. Experiments should be extended to determine the levels of epithelial or mesenchymal marker proteins in control and shEZH2 breast cancer cells sorted into stem and non-

stem populations based on expression of $ALDH1^+$ and $CD44^+/CD24^{-/low}$. Additionally, control and shEZH2 breast cancer cells grown using the mammosphere assay should be assayed for the expression of EMT proteins and compared to cells grown under normal tissue culture conditions. Based on the recent studies, it is hypothesized that down-regulation of EZH2 in CSCs will lead to an epithelial-like protein expression pattern compared to controls.

Through the use of established cell surface marker proteins and the mammosphere assay, we were able to interrogate the effects of EZH2 up- and downregulation on CSC numbers. It has been demonstrated by other research groups using the breast cancer cell lines MDA-MB-231 and SUM149 that cells isolated through the expression of these cell surface markers and through mammosphere assays are indeed bona fide CSCs with the required properties discussed in Chapter 1 [15-17]. Although previously shown, it would be beneficial if we were to personally demonstrate tumor initiating capacity in our isolated CSC populations. To do so, limiting dilution xenograft transplantations should be conducted in immunocompromised mice with isolated “potential” CSCs. As we have shown that knockdown of EZH2 decreases the number of CSCs *in vitro*, we would expect that shEZH2 CSCs would have less tumor initiating capacity over several transplantations. Moreover, cells deemed non-CSCs after sorting, should show low to no tumor initiating capacity over several transplantations.

Our findings that $EZH2^+$/neu transgenic mice have increased Notch1 expression and earlier tumor initiation create further research questions. For instance, even though EZH2 overexpression leads to increased Notch1 expression in tissues, we cannot

explicitly say that this is due to an effect of EZH2 regulating stem cells in the murine mammary gland. In order to show a direct correlation between EZH2 and stem cells/CSCs in these mouse models, primary cells should be collected from early non-tumorigenic and late malignant mammary glands. Establishing the percentage of stem-like cells obtained from these glands could be accomplished using the aforementioned cell surface marker proteins and the mammosphere assay. Additionally, immunohistochemistry for CD44/CD24 in both early and late mammary glands would provide valuable information. As we observed a concomitant increase in stem cell numbers with EZH2 overexpression *in vitro*, it is expected that $EZH2^{+}$/neu mice will contain more stem cells and CSCs in nonmalignant and tumorigenic breast tissues, respectively, when compared to $EZH2^{wt}$/neu mice. Furthermore, it can be asked if treatment of these mice with a γ-secretase inhibitor (GSI) will have an effect on tumor initiation. We found that $EZH2^{+}$/neu mice formed breast tumors significantly earlier than $EZH2^{wt}$/neu mice, so it can be hypothesized that treatment of these mice with GSI will lead to later development of breast tumors. It would also be interesting to determine the number of CSCs in the mammary glands after these mice develop tumors and are treated with GSI. Will GSI reduce the number of CSCs present in the tumors? Additionally, the same GSI treatment could be used in xenograft models. We found that control SUM149 $ALDH1^{+}$ cells had earlier tumor initiation and a faster tumor growth rate compared to shEZH2 $ALDH1^{+}$ and all $ALDH1^{-}$ cells. Would GSI treatment of mice injected with SUM149 $ALDH1^{+}$ cells prevent this earlier tumor initiation and increased tumor volume?

Lastly, the data demonstrating that EZH2 localizes to the *Notch1* promoter in a manner independent of H3K27me3 and PRC2 and instead correlates with transcriptional activation marks raises the question of cofactors. Are other proteins localizing with EZH2 to this gene promoter, and, if so, is their presence required for EZH2 recruitment and subsequent gene activation? IPs using antibodies targeted against EZH2 followed by mass spectrometry may allow for identification of potential cofactors. Additionally, conducting IPs in sorted stem and non-stem cell populations would be of particular interest. Would it be found that EZH2 binds the *Notch1* promoter in both populations or only preferentially in stem cell-like populations?

4-2. Clinical and Therapeutic Implications

It has been established that EZH2 plays a crucial role in stem cell maintenance and in many types of tumor development. However, there are no therapies currently available that target histone methylation or EZH2. In the laboratory setting, DZNeP has emerged as the most promising PRC2 inhibitor and it has been widely used to decrease EZH2 protein expression. But, the effect of DZNeP on histone methylation is considered to be global versus EZH2-specific, so there is warranted concern in its potential therapeutic use as it may affect many processes requiring histone methylation [18-20]. Nevertheless, DZNeP has been found to have anti-tumor activity in numerous cancer cells, including breast, making it a valuable research tool [21]. A recent study from researchers at GlaxoSmithKline has introduced the small-molecule inhibitor GSK126, which they have characterized as a potent and highly selective inhibitor of EZH2 methyltransferase activity [22]. They demonstrated in lymphoma cells that GSK126 was capable of decreasing proliferation and inhibited growth of xenograft tumors. Of note,

these tumors expressed the Y641 EZH2 mutant, which has enhanced H3K27me3 due to altered substrate preferences. Hopefully, more work utilizing this inhibitor in cancers that overexpress wild type EZH2 will emerge, implicating a wider potential application for this drug. In regards to therapeutically targeting EZH2 in human cancers, however, caution should be taken in certain malignancies. Mutations of EZH2 in myeloid cancers have been shown to lead to inactivation and loss of gene repression, thus it is vital to determine the activation status of EZH2 in a tumor before initiating treatment strategies aimed at inhibiting EZH2 [23].

As interactions between PcG gene regulation and other forms of epigenetic modifications have emerged, it may be promising to use therapies targeted against DNMTs and HDACs. Many inhibitors for these proteins are available with clinical trials currently underway [18, 24]. Interestingly, it has been revealed that simultaneous treatment with DNMT and HDAC inhibitors in cell and animal models has anti-tumorigenic effects [25]. Moreover, combinatorial treatment of human leukemia cells *in vitro* with DZNeP and an HDAC inhibitor led to a synergistic apoptotic effect [26].These studies emphasize the complex nature of the interactions between different epigenetic marks.

The link between EZH2 and CSCs implies that therapies targeting pathways involved in CSCs may be beneficial in patients with tumors expressing high EZH2 protein levels. Indeed, it has been postulated that CSCs within a tumor are resistant to chemotherapy and radiation, and are likely to cause relapse in patients [27, 28]. This may due to the fact that conventional therapeutics efficiently target actively proliferating cells, but have little effect on quiescent or slowly proliferating cells, which may include

the CSC population [29, 30]. It has been demonstrated in a mouse xenografts that treatment with oncolytic adenoviruses was effective in killing the $CD44^{+}/CD24^{-/low}$ population and preventing tumor formation [31]. Another study has shown that treatment of mouse xenografts with the CSC active compound 8-quinolinol in combination with the mitotic inhibitor paclitaxel shows anti-tumor activity and prevents relapse [32]. But, as we have found the expression of Notch1 in CSCs to be regulated by EZH2, therapies targeted against Notch signaling in patients with high EZH2 expression may be promising in eliminating resilient CSCs. Studies using monoclonal antibodies targeted against Notch receptors and ligands have revealed a decrease in tumorigenic activities and in the frequency of CSCs [33, 34]. Likewise, the use of GSI in mouse xenograft leukemia models and in transgenic ERBB2-breast cancer models demonstrates promising anti-tumor effects [35, 36]. Specifically, in another study researchers discovered that treatment of mice injected with breast cancer cells with the Notch1 GSI DAPT led to a significant decrease in brain metastases [37]. Although all of the research into inhibiting CSCs *in vivo* is vital to a progression in breast cancer treatment, it is yet to be seen how such methods will affect human breast tumors. Additionally, the effect of these inhibitory methods on the normal stem cell populations within the mammary epithelium must be taken into account along with possible side effects. Taken together, these studies show encouraging results for a number of methods in targeting CSCs that may evolve into potent therapeutics for human breast cancer patients in the near future.

4-3. References

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