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Down-regulation of Enhancer of Zeste-2 decreases growth of estrogen receptor negative invasive breast carcinoma and requires BRCA1

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Abstract

Increased levels of EZH2, a critical regulator of cellular memory, are associated with negative estrogen receptor (ER) expression and disease progression in breast cancer. High levels of EZH2 signal the presence of metastasis and poor outcome in breast cancer patients. To test the hypothesis that deregulation of EZH2 contributes to ER negative breast cancer progression, EZH2 expression was inhibited in ER negative breast cancer cells MDA-MB-231 and CAL51 using a lentivirus system. EZH2 knockdown decreased proliferation and delayed the G2/M cell cycle transition, while not affecting apoptosis. *In vivo*, EZH2 down-regulation significantly decreased breast xenograft growth and improved survival. EZH2 knockdown up regulated BRCA1 protein. Of note, BRCA1 knockdown was sufficient to rescue the effects of EZH2 down-regulation in proliferation, G2/M arrest, and on the levels of hyperphosphorlated mitotic Cdc25C and Cyclin B1 proteins, crucial for entry into mitosis. Invasive ER negative breast carcinomas show significant overexpression of EZH2 and down-regulation of BRCA1 proteins. Taken together, we show that EZH2 plays a role in ER negative breast cancer progression *in vivo* and *in vitro*, and that BRCA1 is required for the proliferative effects of EZH2. Blockade of EZH2 may provide a prime target to prevent and/or halt ER negative breast cancer progression.

Keywords

EZH2; enhancer of zeste; breast cancer; basal cell phenotype; BRCA1; proliferation; G2/M

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Introduction

Breast cancer is the second most common cause of death in women in the Western world, with over 44,000 deaths per year in the US alone (American Cancer Society, 2008). Despite advances in breast cancer treatment at present there is no cure once metastasis develop (Ellis et al., 2000). The metastatic ability of breast cancer is inversely related to its degree of differentiation, evidenced pathologically by nuclear pleomorphism and the formation of glandular structures (Hayes et al., 2001). The more undifferentiated the invasive carcinoma, the greater likelihood to develop metastasis.

Our laboratory has shown that EZH2, a protein that controls cell differentiation, is elevated in aggressive and metastatic breast cancer (Kleer et al., 2003). EZH2 is an independent predictor of survival in women with breast cancer (Kleer et al., 2003). Invasive carcinomas with high EZH2 expression are significantly larger and more likely to metastasize than tumors with low EZH2. Studies have shown that EZH2 expression correlates with markers of the basal-cell breast cancer subtype characterized by infrequent expression of estrogen (ER) and progesterone receptors(PR), and rare Her-2/neu overexpression, but robust expression of markers of myoepithelial differentiation (Bachmann et al., 2006; Collett et al., 2006; Kleer et al., 2003).

EZH2 is a Polycomb group (PcG) protein homologous to *Drosophila* Enhancer of Zeste and involved in the regulation of cellular memory (Laible et al., 1997; Ringrose and Paro, 2004; Satijn and Otte, 1999). PcG together with the Trithorax group (TrxG) proteins function forming multimeric complexes that activate and repress transcription. While PcG proteins act mainly by repressing gene expression, TrxG proteins promote gene expression (Laible et al., 1997; Ringrose and Paro, 2004; Satijn and Otte, 1999). Deregulation of the PcG proteins can lead to cancer (Jacobs et al., 1999a; Jacobs et al., 1999b; Laible et al., 1997). Studies have shown that EZH2 specifically trimethylates lysine 27 of histone H3 ($H2K27me^3$) which recruits other members of the PcG to specific genetic loci (Cao et al., 2002; Kirmizis et al., 2004; Kuzmichev et al., 2002). Of note, recent studies show that EZH2 was able to activate gene transcription through mechanisms that do not involve histone methylation (Shi et al., 2007). EZH2 was reported to function by forming transcriptional complexes independent of its methyltrasnferase activity (Tonini et al., 2004). In addition, mechanisms that do not involve gene transcription but affect protein activation have been proposed (Su et al., 2005). The mechanism by which EZH2 is associated with breast cancer progression, especially in the context of ER negative breast cancer remains to be elucidated.

The tumor suppressor BRCA1 regulates DNA repair, activation of cell-cycle checkpoints, and maintenance of chromosome stability (Venkitaraman, 2002). Heterozygous germ-line mutations in the BRCA1 gene predispose women to breast and ovarian cancer with a lifetime risk of breast cancer of up to 80% (Narod and Foulkes, 2004). Although somatic mutations of BRCA1 are not common, expression of its messenger RNA and protein are reduced in approximately 40 % of sporadic breast carcinomas (Turner et al., 2007; Wilson et al., 1999; Yoshikawa et al., 2000). The vast majority of breast tumors in these patients display a basal-like phenotype, and BRCA1 dysfunction, by down-regulation, mutation, or other mechanisms, has been suggested to play an etiological role in the development of this

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aggressive breast cancer subtype (Foulkes et al., 2003; Lakhani et al., 2005; Turner et al., 2007).

To evaluate the oncogenic function of EZH2 in the breast, we generated a lentivirus-mediated short hairpin RNA to stably knockdown EZH2 in breast cancer cells. Our *in vivo* and *in vitro* studies show that EZH2 plays a critical role in breast cancer growth. We provide the first evidence that EZH2 knockdown decreased the rate of breast tumor growth, the volume of tumors, and improved survival of mice. EZH2 down-regulation decreased proliferation of ER negative breast cancer cells and caused a delay in the G2/M transition of the cell cycle. Our data revealed that EZH2 modulates BRCA protein levels and the levels of BRCA1 phosphorylated at serine 1423, which is important in the regulation of the G2/M transition. Of note, we show that BRCA1 is required for the proliferative and G2/M effects of EZH2. The relevance of our *in vitro* and animal model findings to human breast cancer is highlighted by demonstrating that ER negative invasive carcinomas have high levels of EZH2 and concomitant low levels of BRCA1 protein.

Results

EZH2 protein is elevated in breast cancer cells

We investigated the levels of EZH2 protein expression in a panel of breast cells including the non-tumorigenic line MCF10A and breast cancer cells, MDA-MB-231, SUM149, CAL51 and MCF-7. EZH2 protein is elevated in breast cancer cells compared to benign cells (Figure 1A).

The expression of EZH2 in benign non-mammary diploid fibroblasts has been shown to be cell cycle regulated (Bracken et al., 2003). To determine the pattern of EZH2 expression in the cell cycle during growth, benign and cancerous breast cells were subjected to double thymidine block and subsequently re-stimulated by their medium to enter the cell cycle. As shown in Figure 1B left, EZH2 protein is cell growth regulated and accumulates at the G1/S transition in benign MCF10A cells. This pattern of expression is similar to that reported in fibroblasts (Bracken et al., 2003). However, this regulation is lost in cancer cells, as the four different breast cancer cells studied had deregulated and high levels of EZH2, that were not regulated through the cell cycle (Figure 1B right). The deregulation of EZH2 in CAL51 breast cancer cells during cell cycle progression is likely not due to activating *EZH2* mutations, as no mutations in the open reading frame of *EZH2* of CAL51 cells were detected by direct sequencing (data not shown).

Lentivirus-mediated knockdown of EZH2 decreases cell proliferation

Guided by our previous data showing that EZH2 overexpression in invasive carcinomas is significantly associated with negative estrogen receptor (ER) status (Kleer et al., 2003), we concentrated our studies on the ER negative cell lines CAL51 and MDA-MB-231 cells, both of which have high levels of EZH2 protein (Figure 1A) and intact BRCA1 (Elstrodt et al., 2006; Yuli et al., 2007). To directly investigate the contribution of EZH2 to breast tumorigenesis we used short hairpin RNA interference (shRNA) in a lentivirus vector, which effectively downregulated EZH2 expression in breast cancer cells (Figure 2A).

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EZH2 knockdown in MDA-MB-231 and CAL51 cells significantly decreased proliferation (Figure 2B). Figure 2C shows that the doubling time of both breast cancer cell lines was increased after EZH2 knockdown when compared to empty vector transfected cells (36 h for CAL51 shEZH2 vs. 24 h for CAL51/vector, and 33 h for MDA-MB-231 shEZH2 vs. 24 h for MDA-MB-231/vector, ANOVA $p<0.05$ for both cell lines). Of note, EZH2 knockdown did not induce apoptosis, as assessed by annexin V staining of nuclei and flow cytometry (6.11 % shEZH2 vs 6 % control).

EZH2 knockdown induces a delay in the G2 phase of the cell cycle and decreases the mitotic activity of breast cancer cells

We wished to understand the observed variability of EZH2 expression in active cell growth. For this, we analyzed the number and distribution of cells in the phases of the cell cycle by flow cytometry. As shown in Figure 3A, EZH2 knockdown significantly increased the number of cells at the G2/M transition. In asyncronized CAL51 cell cultures, EZH2 knockdown resulted in 30 % increase in the number of cells in G2/M when compared to empty vector transfected cells, normalized to total cell count. We next investigated the effect of EZH2 on crucial G2/M checkpoint proteins. For this, nuclear extracted fractions of CAL51 shEZH2 and vector controls were anayzed for the level of hyperphosphorylated mitotic Cdc25C, Cdc2 and Cdc2-Tyr15 phosphorylation, whose activities are essential for entry into mitosis (Bonnet et al., 2008; Hoffmann et al., 1993; Peng et al., 1997; Strausfeld et al., 1994), as well as for total Cdc25C protein. As shown in Figure 3B, EZH2 knockdown resulted in decreased hyperphosphorylated mitotic Cdc25C and increased Cdc2 and Cdc2-Tyr15 phosphorylation, further supporting the observed G2 delay.

To directly determine the effect of EZH2 on the mitotic activity of breast cells, we monitored the mitotic fractions of cultured cells using the mitotic marker phosphorylated histone H3 using both immunofluorescence and flow cytometry. EZH2 knockdown caused over 50 % reduction in the percentage of breast cancer cells undergoing mitosis (Figure 3C). Consistent with this result, overexpression of EZH2 in MCF10A cells resulted in a four-fold increase in the mitotic activity (Figure 3C, right). The overexpression of EZH2 was confirmed by immunoblot analysis (Figure 3C, inset). Collectively, these data show that EZH2 plays a role in the regulation of the G2/M transition and the number of cells undergoing mitosis, which have a major influence in tumor growth.

EZH2 knockdown decreases *in vivo* proliferation and tumorigenicity, and improves survival of mice

We analyzed the effect of EZH2 knockdown on MDA-MB-231 xenograft tumor growth and survival of immunocompromised mice. Two groups of mice were injected in the mammary fat pads with MDA-MB-231 shEZH2 (10 mice), and MDA-MB-231 sh-control (10 mice). Quantitative real time RT-PCR and immunohistochemistry confirmed the stable down-regulation of EZH2 in the MDA-MB-231 shEZH2 tumors compared to controls (Supplementary Figure 1).

MDA-MB-231 cells with shRNA knockdown of EZH2 exhibited a slower tumor growth rate and smaller tumor volumes than controls ($p = 0.014$ based on mixed effects regression

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model for the serial tumor volume measurements, Figure 4A). Importantly, mice bearing MDA-MB-231 shEZH2 tumors had a significantly better survival when compared to control mice (log rank p = 0.016).

Histopathologic examination of the tumors resulting from the different experimental groups did not reveal significant morphological differences. We observed a striking difference in the mitotic activity of the tumors. Xenografts formed by MDA-MB-231 cells transfected with the empty vector exhibited high mitotic activity (mean 7.8 mitoses per high power field, 400x). In contrast, tumors derived from MDA-MB-231 shEZH2 cells had markedly decreased number of mitoses (mean 3.5 mitoses per high power field) (Figure 4B). Taken together, these data show for the first time that EZH2 down-regulation decreases *in vivo* mitotic activity and breast tumor growth, and set the foundation for further studies to explore EZH2 as a therapeutic target for breast cancer.

The EZH2 effects on cell proliferation and G2/M transition require BRCA1

Studies including our own have demonstrated that EZH2 overexpression in human breast carcinomas is associated with the ER negative basal-like phenotype, characterized by low BRCA1 protein expression (Bachmann et al., 2006; Kleer et al., 2003; Turner et al., 2007). We next tested the hypothesis that EZH2 may promote breast tumorigenesis by regulating BRCA1 in this subtype of breast cancer. As shown in Figure 5A, EZH2 down-regulation increased the protein levels of total BRCA1 and BRCA1 phosphorylated at serine 1423 (pBRCA1 s1423) in the nuclei of MDA-MB-231 and CAL51 breast cancer cells. Consistently, ectopic expression of EZH2 in non-tumorigenic MCF10A breast cells resulted in 75 % decrease in nuclear BRCA1 protein and in 67 % decrease in nuclear pBRCA1 s1423 (Figure 5B). We further confirmed the *in vivo* upregulation of BRCA1 protein by EZH2 knockdown (Figure 5C).

To investigate whether the effects of EZH2 down-regulation on cell proliferation and transition from the G2 phase to mitosis necessitate BRCA1 protein, we employed shRNA to knockdown BRCA1 in CAL51 cells with EZH2 down-regulation and controls. As expected, BRCA1 shRNA resulted in almost complete inhibition of nuclear BRCA1 protein compared with cells transfected with the empty vector. In CAL51 shEZH2 cells, BRCA1 shRNA effectively reduced BRCA1 protein to levels similar to the empty vector transfected cells, and therefore, specifically abrogated the increase in BRCA1 caused by EZH2 knockdown (Figure 6A).

To ascertain the role of BRCA1 in EZH2 mediated cell proliferation, we subjected these breast cancer cells to proliferation assays and flow cytometry. Figures 6B and 6C show that BRCA1 knockdown in shEZH2 CAL51 cells was sufficient to completely rescue the reduction in cell proliferation and the G2/M arrest caused by EZH2 down-regulation. Of note, inhibition of BRCA1 in CAL51 cells had no effect in their proliferative activity. This result is not surprising given previous studies showing that BRCA1 knockdown in breast and prostate cancer cells does not affect their proliferation kinetics (Bae et al., 2005).

Entry into mitosis is tightly regulated by the Cdc2/Cyclin B1 complex (Smits and Medema, 2001). Cdc25C is the key enzyme that triggers the activation of CyclinB1/Cdc2 by

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dephosphorylating Cdc2 at Thr14 and Tyr15 (Dunphy, 1994). Our data show that BRCA1 knockdown abolished the effect of EZH2 inhibition on the levels of Cdc25C mitotic protein (Figure 6D) as well as the levels of total and phosphorylated Cyclin B1 (Figure 6E). Taken together, these data provide strong evidence supporting the hypothesis that the decrease in cell proliferation and prolongation of G2 caused by EZH2 knockdown require BRCA1.

ER negative invasive breast carcinomas exhibit high EZH2 and low pBRCA1 proteins

To investigate the relevance of our *in vitro* and *in vivo* xenograft studies to human breast cancer, we investigated the expression of pBRCA1 s1423 in tissue samples that we have previously characterized for ER and EZH2 proteins (Kleer et al., 2003). These tissues consisted of 136 consecutive invasive breast carcinomas. Immunohistochemical analysis showed that pBRCA1 s1423 protein was localized exclusively to the nuclei of cancer cells, and was scored as positive or negative (Figure 7A). We found a novel significant association between pBRCA1 s1423 expression and ER status. Forty three of 86 (50 %) ER positive tumors were negative for pBRCA1 s1423, while 38 of 50 (76 %) ER negative tumors were negative for pBRCA1 s1423 (Fisher's exact test $p=0.015$). Importantly, concomitant with elevated EZH2 protein expression, ER negative invasive breast carcinomas had significantly reduced pBRCA1 s1423 expression (Chi square test, $p<0.0001$, Table 1).

Discussion

EZH2 is an independent marker of recurrence and metastasis in women with breast cancer (Kleer et al., 2003). EZH2 overexpression occurs mainly in the basal-type tumors, characterized by ER, PR, and Her-2/neu negative status as well as low levels of BRCA1 protein (Bachmann et al., 2006; Collett et al., 2006). A major novel finding presented in our study that EZH2 down-regulation in aggressive ER negative breast cancer cells greatly decreases their proliferative capacity and rate of progression through the cell cycle. Specifically, we found that EZH2 knockdown prolonged the doubling time of ER negative breast cancer cell lines, and caused an arrest at the G2/M transition of the cell cycle, with corresponding changes in mitotic Cdc25C, Cdc2 and Cdc2-Tyr15 phosphorylation. Consequently, EZH2 knockdown led to a decreased number of breast cancer cells undergoing mitosis. In support of these data, ectopic expression of EZH2 in the non-tumorigenic MCF10A cells caused a striking increase in the mitotic fraction. Work from our and other laboratories support these findings. Braken et al demonstrated that ectopic overexpression of EZH2 promotes cell proliferation in human diploid fibroblasts, and that EZH2 siRNA inhibits BrdU incorporation suggesting a role for EZH2 for progression through the cell cycle (Bracken et al., 2003). Varambally et al reported that EZH2 siRNA induces a G2/M arrest in prostate cell lines (Varambally et al., 2002). Using tissue samples, it has been shown that EZH2 overexpression is significantly associated with increased proliferative activity determined by detection of the Ki-67 antigen in ductal carcinoma *in situ* and invasive carcinomas of the breast (Bachmann et al., 2006; Ding et al., 2006).

There have been intensive studies concerning the oncogenic mechanism underlying EZH2 overexpression. Both repression and activation of genes that regulate fundamental processes have been implicated, and new mechanisms that do not involve gene transcription have been

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proposed (Shi et al., 2007; Su et al., 2005; Tonini et al., 2004). However, direct demonstration of EZH2's function in *in vivo* breast tumorigenesis is currently lacking. In the present study, we show that EZH2 modulates breast cell proliferation and tumor growth *in vivo*. Targeted down-regulation of EZH2 in aggressive ER negative breast cancer cells resulted in a significant reduction of mammary tumor size as well as better survival. Although the histological features of the EZH2 inhibited and control tumors were similar, we found that EZH2 knockdown decreased the mitotic activity of breast cancer cells. Our xenograft mouse models thus suggest that EZH2 inhibiting compounds may have potential utility in treating ER negative breast cancer patients, and provide the foundation for the design of pre-clinical models.

In addition to its role in hereditary breast cancer, BRCA1 expression is reduced in up to 40 % of sporadic breast carcinomas (Turner et al., 2004; Turner et al., 2007; Wilson et al., 1999; Yoshikawa et al., 2000). Although BRCA1 promoter methylation is responsible for BRCA1 reduction in 10–15 % of sporadic breast carcinomas, it does not explain BRCA1 deficiency in the remainder of the tumors and it has been suggested that a group of ER basal-like breast carcinomas are characterized by BRCA1 deficiency (Turner et al., 2004; Turner et al., 2007). Discovery of novel strategies to restore BRCA1 expression and function is highly desirable to develop therapies for women carrying BRCA1-deficient tumors.

It has been proposed that the function BRCA1 in cell cycle regulation complements its role in DNA damage response, by allowing time for DNA repair to occur more effectively (Mullan et al., 2006; Venkitaraman, 2002). We demonstrate that EZH2 knockdown in ER negative breast cancer cell lines causes up-regulation of BRCA1 protein levels with a concomitant increase in pBRCA1 s1423; the total amount of the latter being crucial for G2/M arrest (Cortez et al., 1999; Xu et al., 2001). Consistently, ectopic overexpression of EZH2 in benign breast cells decreased nuclear BRCA1 and pBRCA1 s1423 protein levels. The *in vivo* relevance of these findings is underscored by our xenograft mouse models showing that EZH2 knockdown increased BRCA1 and pBRCA1 s1423 proteins in the nuclei of breast cancer cells *in situ*. Of note, our data demonstrate that the observed effects of EZH2 down-regulation on breast cancer cell proliferation and G2/M transition require BRCA1, as BRCA1 inhibition was sufficient to completely rescue the decrease in cell proliferation and the delay in G2 caused by EZH2 down-regulation.

Ectopic overexpression of BRCA1 in breast cancer cells has been shown to cause G2/M arrest by regulating the levels and activity of Cdc2/Cyclin B1 complex and the dual phosphatase Cdc25C, both of which are essential for G2/M checkpoint control (MacLachlan et al., 2000; Yan et al., 2005). At the G2/M transition, hyperphosphorylation of Cdc25C on several sites within its regulatory N-terminal domain coincides with mitotic activation of Cdc25C (Bonnet et al., 2008; Bulavin et al., 2003; Roshak et al., 2000; Strausfeld et al., 1994). Once activated, Cdc25C is able to dephosphorylate Ccd2-Tyr15, which increases the activity of the Cdc2/Cyclin B1 complex and results in entry into mitosis (Dunphy, 1994). Providing strong evidence in support of EZH2 participation in BRCA1-mediated G2/M checkpoint control, EZH2 knockdown decreased nuclear mitotic Cdc25C, increased Cdc2 and Cdc2-Tyr15 phosphorylation, and decreased total and phosphorylated Cyclin B1.

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Importantly, BRCA1 knockdown reverted the effects of EZH2 down-regulation on the levels of both mitotic Cdc25C, and total and phosphorylated Cyclin B1.

Our experiments show that EZH2 knockdown is sufficient to increase BRCA1 levels in breast cancer cells and trigger similar effects in G2/M as those reported after ectopic expression of BRCA1 using an adenovirus vector in the absence of ionizing irradiation (MacLachlan et al., 2000; Yan et al., 2005). We propose that EZH2 knockdown in breast cancer cells reduces their growth by enhancing the cell cycle regulatory effects of BRCA1, slowing the transition from G2 to M phases, and allowing more time for DNA repair to occur. This hypothesis is supported by our experiments showing that breast cancer cells with EZH2 knockdown have increased levels of pBRCA1 s1423 protein, which is crucial for G2/M arrest and is activated during the DNA damage response (Xu et al., 2001). Even though the precise mechanism by which EZH2 modulates BRCA1 protein levels will require further studies, unpublished preliminary data from our laboratory suggest that it does not involve BRCA1 gene transcription since EZH2 knockdown had no effect on BRCA1 messenger RNA. This finding is not surprising in light of studies showing that EZH2 regulates the activity of Cdc42 without directly affecting its transcription (Su et al., 2005). Furthermore, we have no evidence by co-immunoprecipitation of a direct interaction between EZH2 and BRCA1 proteins (data not shown). A functional connection of EZH2 and BRCA1 proteins is currently under investigation.

The relevance of the association between EZH2 and BRCA1 proteins to human breast cancer is highlighted by the finding that 76 % of ER negative invasive carcinomas overexpress EZH2 and are negative for pBRCA1 s1423 protein. Previous studies have demonstrated that ER negative breast carcinomas have significantly lower BRCA1 protein than ER positive tumors (Turner et al., 2007). However, the expression of pBRCA1 s1423 has not been reported. Our study shows for the first time that pBRCA1 s1423 levels are significantly associated with the ER status of invasive breast carcinomas as invasive carcinomas with negative ER exhibit significantly higher levels of pBRCA1 s1423 protein.

In conclusion, our results demonstrate a previously undescribed function of EZH2 during ER negative breast cancer progression by showing that EZH2 knockdown decreases tumor proliferation and growth *in vivo* and *in vitro*, and influences the transition from G2 phase to mitosis. We provide the first link between EZH2 and BRCA1 proteins and show that EZH2 knockdown depends on BRCA1 up-regulation to decrease breast cancer proliferation and progression through G2 phase (Figure 7B). Our data raise a novel hypothesis that restoration of BRCA1 function by EZH2 knockdown may effectively decrease tumor progression enabled by BRCA1 deficiency, and provide a new lead toward future developments of specific strategies to restore BRCA1 levels and function, and for possible prevention of ER negative tumors.

Methods

Cell lines

SUM149 cell line was developed and provided by S.P. Ethier (Karmanos Cancer Institute, Detroit, MI). Additional breast cancer cell lines and immortalized human mammary

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epithelial cell lines (MCF10A) were obtained from the American Type Culture Collection (AATC, Manassas, VA) and grown under recommended conditions.

Western immunoblots

Immunoblot analysis was performed as previously described (Kleer et al., 2003) using 100 μg of nuclear enriched fractions extracted with NE-Per kit, (Pierce, Rockford, IL), or whole cell extract as indicated in the legends. The following antibodies were used: mouse anti-β-actin (1:10,000), mouse anti-α-tubulin (1:1000), goat anti-rabbit:HRP secondary antibody (1:10,000), and goat anti-mouse HRP also (1:10000). These antibodies were purchased from Sigma (St Louis, MO). In addition, we used anti-EZH2 (1:1000, BD Biosciences, San Jose, CA), anti-BRCA1 (D-9) (1:200, Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-BRCA1 (Ser1423) (1:1000, ABCAM, Cambridge, MA), anti-cdc25C (5H9), anti-cdc2, anti-phosphor-cdc2-tyr15, used at 1:1000 dilution, from Cell Signaling Technology, Danvers, MA, anti-Cyclin B1 (1:1000 dilution, Calbiochem, EMD Chemicals, La Jolla, CA) and anti-phospho-Cyclin B1 (Ser126) (1:1000 dilution, Novus Biologicals, Littleton, CO). Control immunoblots using preimmune IgG confirmed the specificity of the antibodies. Semi-quantitative protein expression levels were determined by densitometry using IMAGE J 1.38x software.

Cell synchronization

Cells were synchronized with a double thymidine block as described previously (Fan et al., 2000). Briefly, cells were incubated in medium containing 2 mM thymidine for 12 hours, released into their normal medium for 8–10 hours, and then incubated for 12 hours in medium containing 2 mM thymidine (See Supplemental Methods for details).

Knockdown of shEZH2 and shBRCA1 in breast cancer cells

To generate stable hairpin short interfering RNA-EZH2 and RNA-BRCA1 in MDA-MB-231 and/or CAL51 breast cancer cells, cells were transduced with lentivirus and selected for antibiotic resistance in ATCC recommended media with puromycin (100 μg/ml, Sigma), at 37°C under 10% CO_2. Lentivirus was purchased from the Vector Core, University of Michigan. Background vector control was Lenti-PuroEMPTY-VSVG. For targeting *EZH2* (NM_152998 NCBI) and *BRCA1* (NM_009764 NCBI) the shRNA oligos ID used were as follows: V2LHS_17507 targeting EZH2 and V2LHS_254648 targeting BRCA1, corresponding to these catalog numbers RHS4430-99139126 and RHS4430-99157192 respectively from Open Biosystems, Huntsville, AL.

EZH2 overexpression in non-tumorigenic mammary epithelial cells

To generate MCF10A cells stably overexpressing EZH2, MCF10A cells were transduced with a lentivirus construct containing EZH2 or empty vector, and cultured in growth media supplemented with puromycin (100ug/ml). Lentivirus bearing the control vector: pLentiLoxRSV-puro was purchased from the Vector Core at the University of Michigan. *EZH2* gene was isolated from pCDNA3-myc EZH2 plasmid (gift of Dr. Chinnaiyan).

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Lentiviral Transduction

Briefly, cell transductions were performed in 6-well plates using short hairpin RNA in a lentivirus vector targeting *EZH2* and *BRCA1* or lentivirus overexpressing EZH2 for 48 h and selecting for two weeks for puromycin resistance colonies.

Cell Proliferation

Cells were plated at the same density and cultured for 24 hours in a 96-well microplate. WST-1 reagent was added and absorbance at 450 nm was measured after 3 hours of incubation, following the manufacturer's instructions (Roche Molecular Systems, Pleasanton, CA).

Mitotic Index

CAL51-shRNA (control, EZH2, and BRCA1) cells were grown in 100-mm plates and fixed at 70 % (v/v) ethanol at −20 °C for 2 h, then incubated with a rabbit antibody to pH3 (anti-phospho histone H3, Ser10 mitotic maker, Upstate Biotechnology, Lake Placid, NY). Cells were then stained with Alexa Fluor-633 goat anti-rabbit IgG (H+L) (Invitrogen Molecular Probes, Carlsbad, CA). Stained cells were treated with RNase A, incubated with propidium iodide and then analyzed by flow cytometry.

Breast Tumor Xenografts

Ten week-old SCID mice (Jackson Laboratories, Bar Harbor, Maine) were used for examining tumorigenicity. To evaluate the role of EZH2 down-regulation in tumor 20 formation, shEZH2 MDA-MB-231 cells or vector control cells were orthotopically injected into the mammary fat pad at a concentration of 2×10^6 cells in 20 mice (n = 10 mice per group). Additional information can be found in the Supplemental Methods.

Real-time Quantitative PCR

Total RNA was isolated from following the manufacturer's instructions with the RNeasy kit, (QIAGEN Inc., Valencia, CA). cDNA samples from breast cancer cells were amplified in triplicate from the same starting material of total RNA following the manufacturer's instructions (High-Capacity cDNA reverse Transcription kit, Applied Biosystems, Foster City, CA). Samples were amplified using TaqMan MGB FAM dyelabeled probes from Applied Biosystems (Foster City, CA) in an ABI7900HT model Real-Time PCR machine. The following probes were used: Hs99999903_m1 *(ACTIN)*, Hs00173233_m1 (*BRCA1*) and Hs00544830_m1 *(EZH2)*.

Human Tissue Specimens and immunohistochemical analysis

A high-density tissue microarray (TMA) containing 136 consecutive invasive carcinomas of the breast, previously analyzed for ER and EZH2 (Kleer et al., 2003) was employed. A 5 μthick section was immunostained using a rabbit polyclonal antipBRCA1 s1423 antibody at 1:700 (Abcam, catalog number AB2838, Cambridge, MA) following standard biotin-avidin complex technique (Kleer et al., 2003). Details are in the Supplemental Methods.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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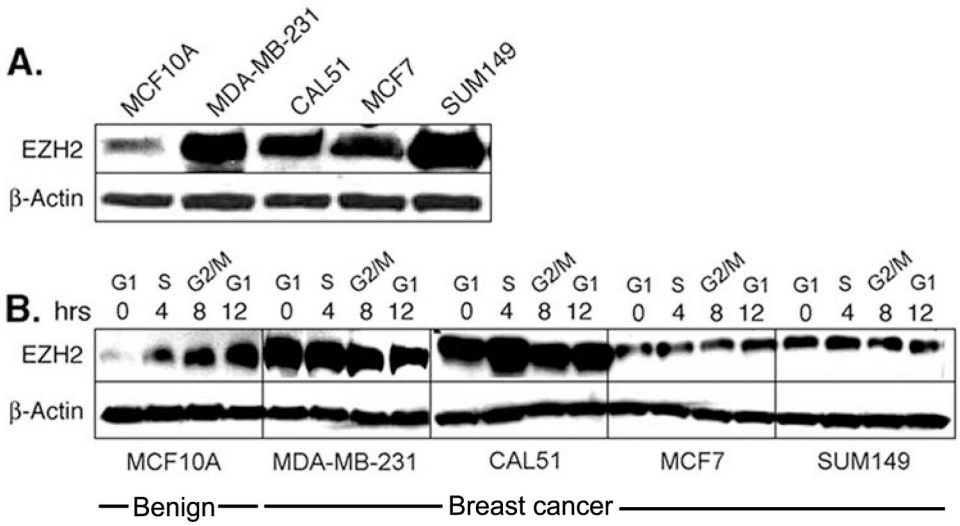

Figure 1. EZH2 is upregulated and deregulated in breast cancer cells

A. Immunoblot for EZH2 in a panel of breast cells shows that EZH2 protein is elevated in breast cancer cells when compared to the non-tumorigenic MCF10A cell line. **B.** EZH2 is deregulated in breast cancer cell lines compared to MCF10A cells. Cells were synchronized using a double thymidine block that induces a cell block in G1 phase and were released into their culture medium to progress to S phase. Western immunoblots were performed concomitantly with flow cytometry analysis (not shown). Note that while EZH2 protein in benign MCF10A cells is cell growth regulated and accumulate at the G1/S transition as has been previously reported (Bracken et al., 2003), this regulation is lost in breast cancer cell lines.

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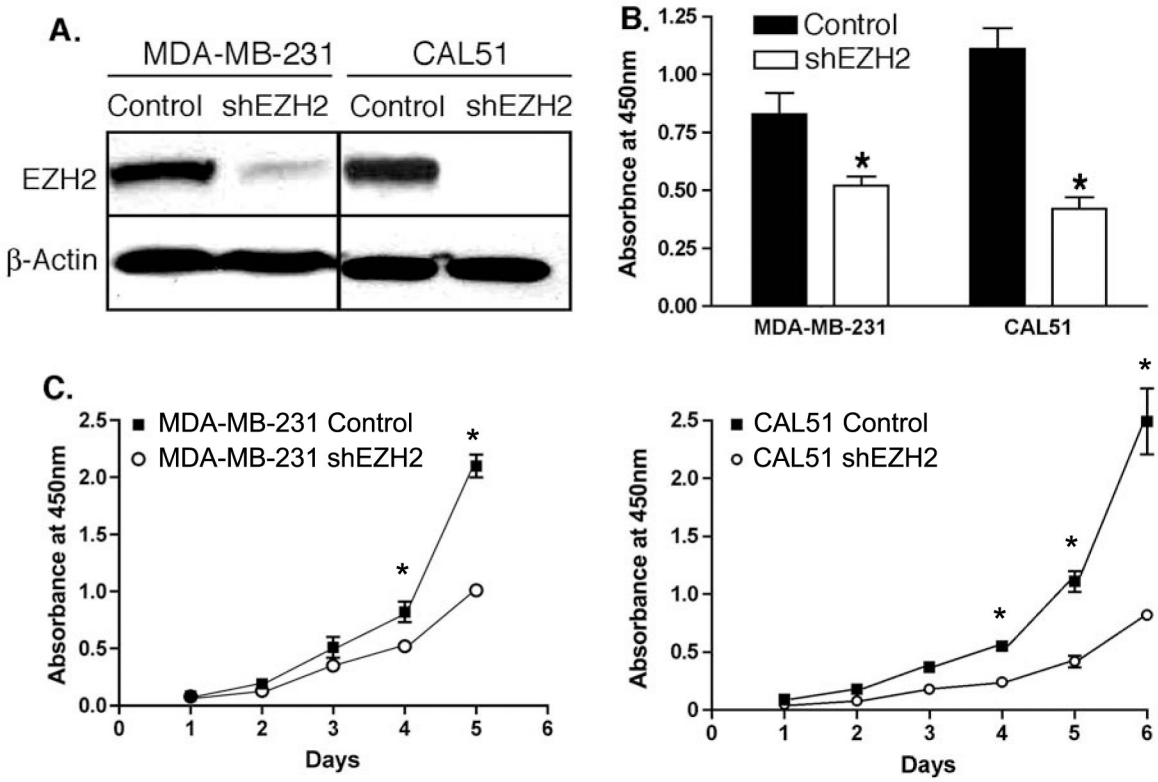

Figure 2. EZH2 inhibition decreases proliferation of breast cancer cells

A. Immunoblot analysis of EZH2 in stable vector and EZH2 shRNA breast cancer cells. MDA-MB-231 and CAL51 cells were stably transfected with EZH2 shRNA in a lentivirus vector or empty vector control. **B.** EZH2 inhibition in MDA-MB-231 and CAL51 breast cancer cells decreases proliferation, as measured with the Wst-1 assay at 6 days after plating the cells. Significant inhibition of proliferation is seen in cells transfected with EZH2 shRNA relative to those transfected with the empty vector (Student's *t*-test, $P < 0.001$ for both cell lines). **C.** Time course of proliferation determined using the Wst-1 assay. shEZH2 in CAL51 and MDA-MB-231 cells significantly decreases their doubling time when compared to vector control transfected cells (Student's *t*-test, $P < 0.001$ for both cell lines). Data are from at least three independent experiments carried out in triplicate.

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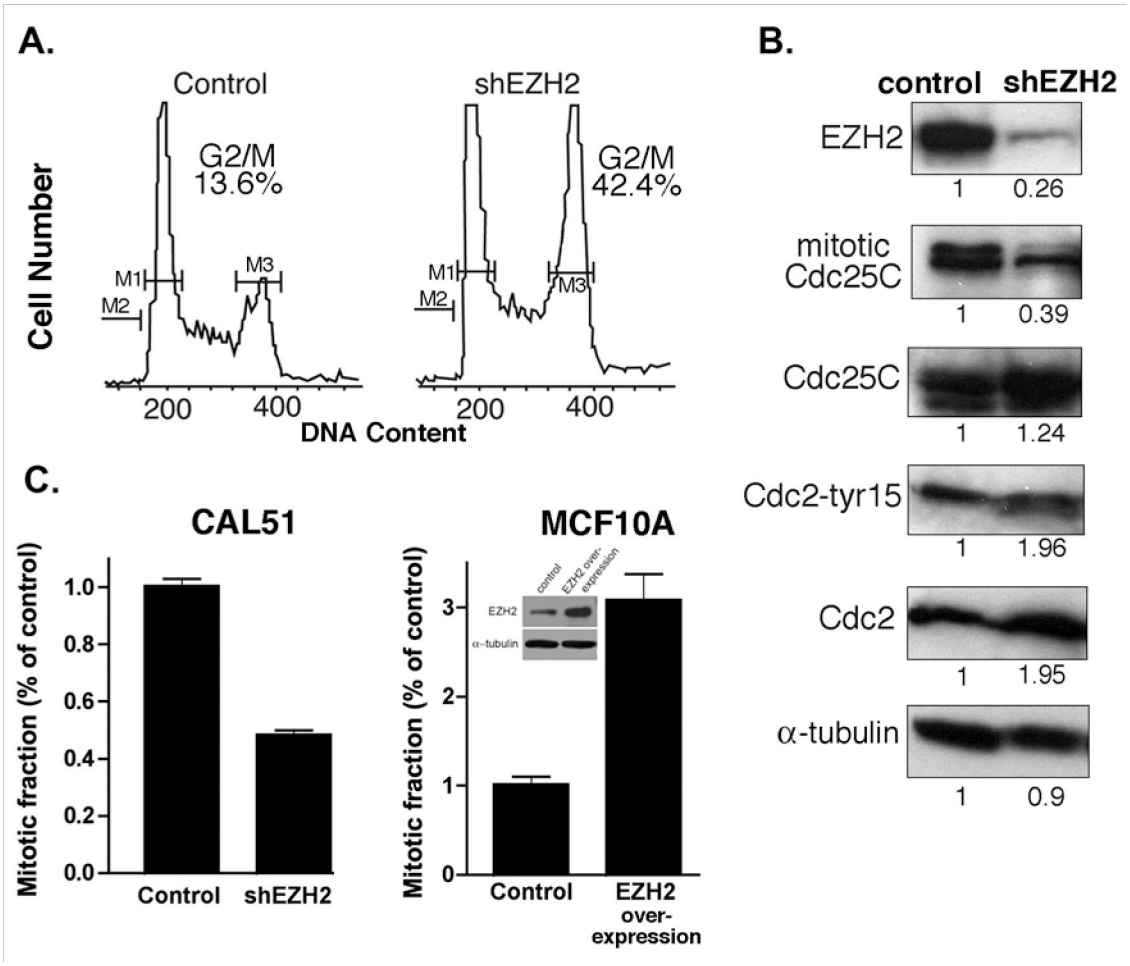

Figure 3. EZH2 knockdown induces G2/M cell cycle arrest in breast cancer cells

A. DNA content analysis by flow cytometry. Samples were fixed and stained with PI. PI-stained cells were gated on the basis of their DNA content. The percentage of cells with 4N-DNA content, indicative of G2/M phase of the cell cycle, are indicated. EZH2 knockdown increases the number of cells at G2/M. **B.** Immunoblot analyses show that EZH2 knockdown in CAL51 cells affects the levels of proteins crucial in G2/M transition including phosphorylated mitotic Cdc25C, total Cdc2 and Cdc2 phosphorylated at Tyr15 in the nuclear fractions of the cells. No effect was noted on total Cdc25C protein. In immunoblots, the numbers underneath each band indicate the fold change in intensity of the corresponding band relative to the control. **C.** Left, EZH2 knockdown decreases the number of cells undergoing mitosis as determined by flow cytometry using phosphorylated histone 3 antibody. Right, EZH2 overexpression in MCF10A cells increases the number of cells in mitosis. Inset shows an immunoblot using anti-EZH2 antibody demonstrating EZH2 overexpression in MCF10A cells.

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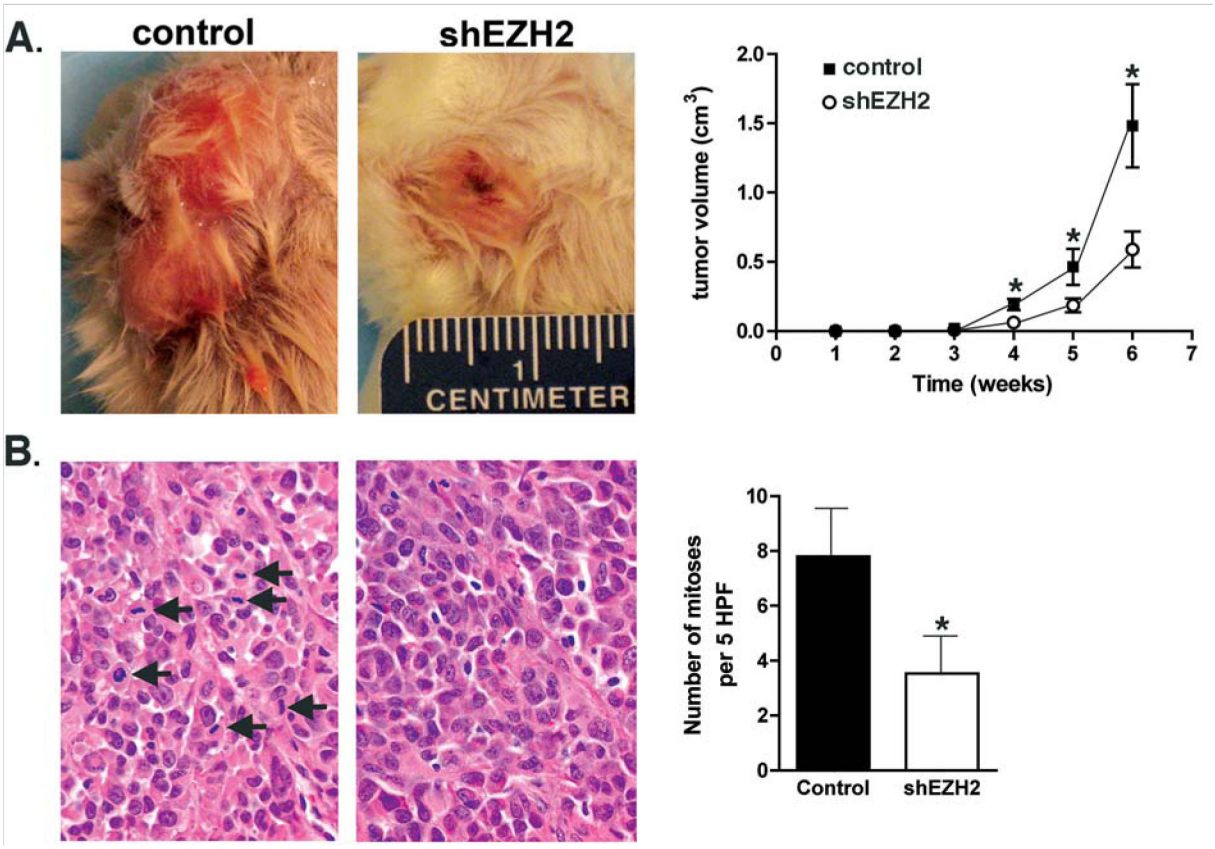

Figure 4. EZH2 down-regulation inhibits breast tumor growth *in vivo*

A. Stable MDA-MB-231- shEZH2 cells have reduced tumor growth in a xenograft mouse model. Tumor growth rates significantly different between the control and EZH2 knockdown cells, mixed effects regression model (p=0.014). **B.** Histopathological study of the tumors shows no significant morphological differences. Both tumors are poorly differentiated and growing in sheets of malignant cells. Note that tumors formed by EZH2 shRNA cells had decreased mitotic activity than tumors derived from empty vector transfected cells. The arrows show mitotic figures (Hematoxylin and eosin stain, light microscopy, x400). The bar graph shows the quantification of the mitotic activity in the xenografts. The number of mitosis per high power field was quantified by light microscopy in at least 5 high power fields per tumor in all xenografts (n = 10 per group, two groups). Error bars: n = 10, mean ± SD Student's t test $p < 0.001$.

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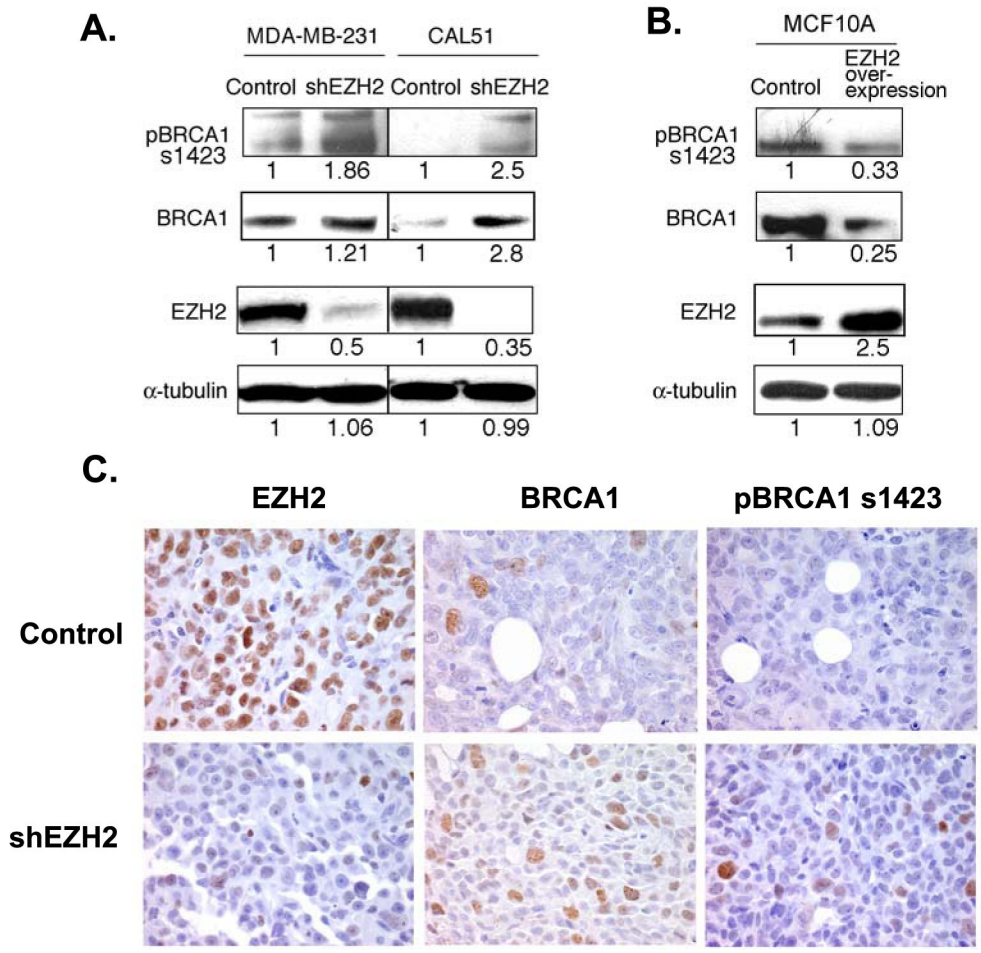

Figure 5. EZH2 knockdown up-regulates BRCA1 protein levels in breast cancer *in vivo* and *in vitro*

A and B. Immunoblot analyses of EZH2, BRCA1, BRCA1 phosphorylated at serine 1423, and α-tubulin proteins in breast cancer cells MDA-MB-231 and CAL51 (A), and in non-tumorigenic spontaneously immortalized breast cells MCF10A following stable lentiviral-mediated EZH2 overexpression or transfected with the empty vector (B). **C.** *In vivo* association of EZH2 and BRCA1 protein expression. Representative immunostaining of EZH2, BRCA1, and BRCA1 phosphorylated on serine 1423 (pBRCA1-s1423) in tumor xenografts derived from MDA-MB-231 shEZH2 cells and controls. Magnification 600x.

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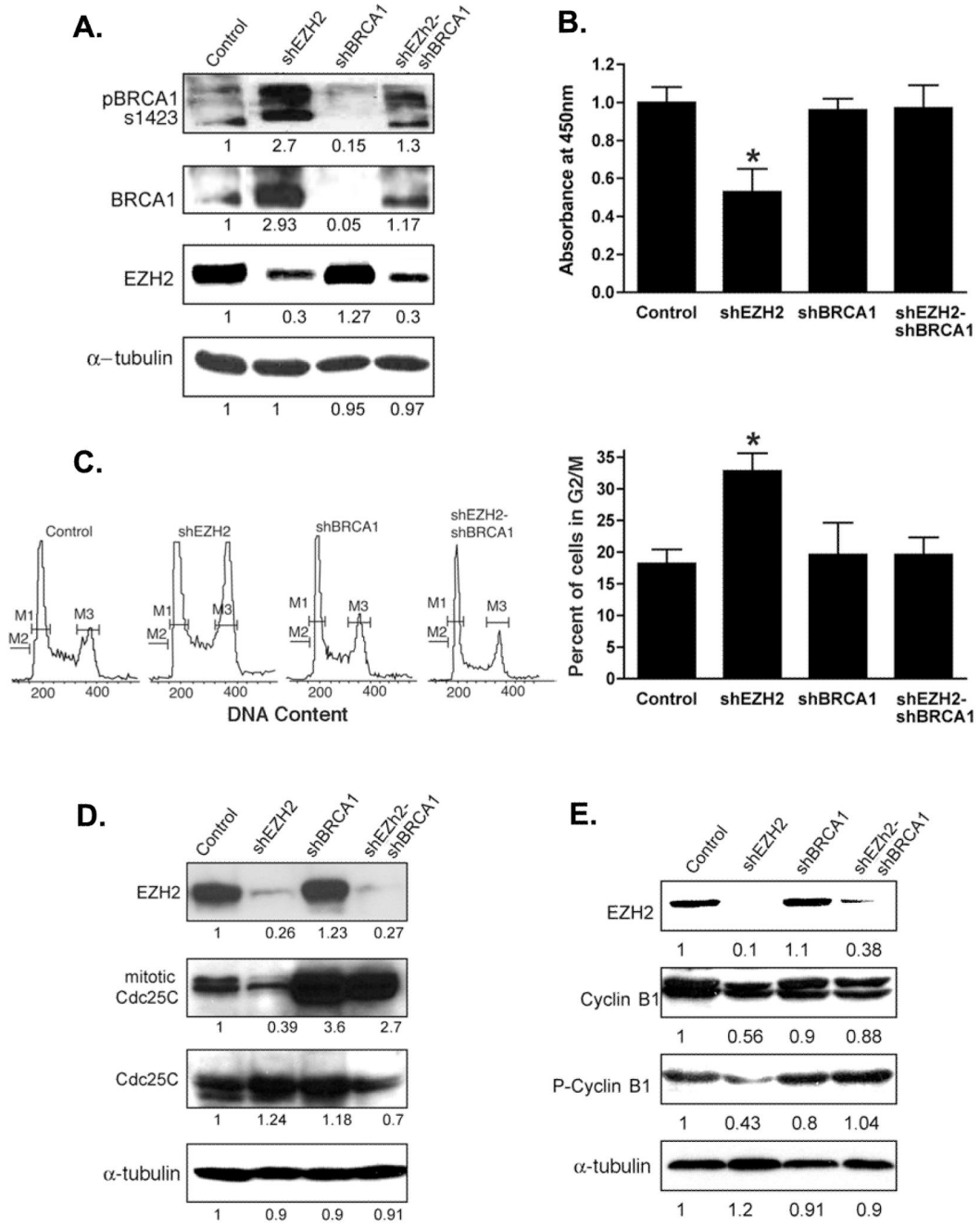

Figure 6. The effects of EZH2 on cell proliferation and G2/M transition require BRCA1

A. CAL51 cells were transfected with vector or EZH2 shRNA alone or in combination with BRCA1 shRNA. Immunoblots show strong up-regulation of BRCA1 in EZH2 shRNA CAL51 cells when compared to CAL51 cells transfected with the empty vector. BRCA1 knockdown in the setting of EZH2 down-regulation abrogates the increase in BRCA1 protein to levels similar to vector transfected cells. **B.** BRCA1 knockdown is sufficient to rescue the effect of EZH2 inhibition on cell proliferation. Inhibition of BRCA1 in CAL51 cells does not affect cell proliferation, as has been previously reported by Rosen and

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coworkers (Bae et al., 2005). **C.** BRCA1 knockdown rescues the effect of EZH2 inhibition on G2/M cell cycle arrest by DNA content analysis by flow cytometry. **D and E.** Consistently, BRCA1 knockdown reverts the effect of EZH2 down-regulation on crucial proteins that regulate G2/M transition: phosphorylated Cdc25C and total and phosphorylated Cyclin B1 in nuclear fractions of CAL51 cells.

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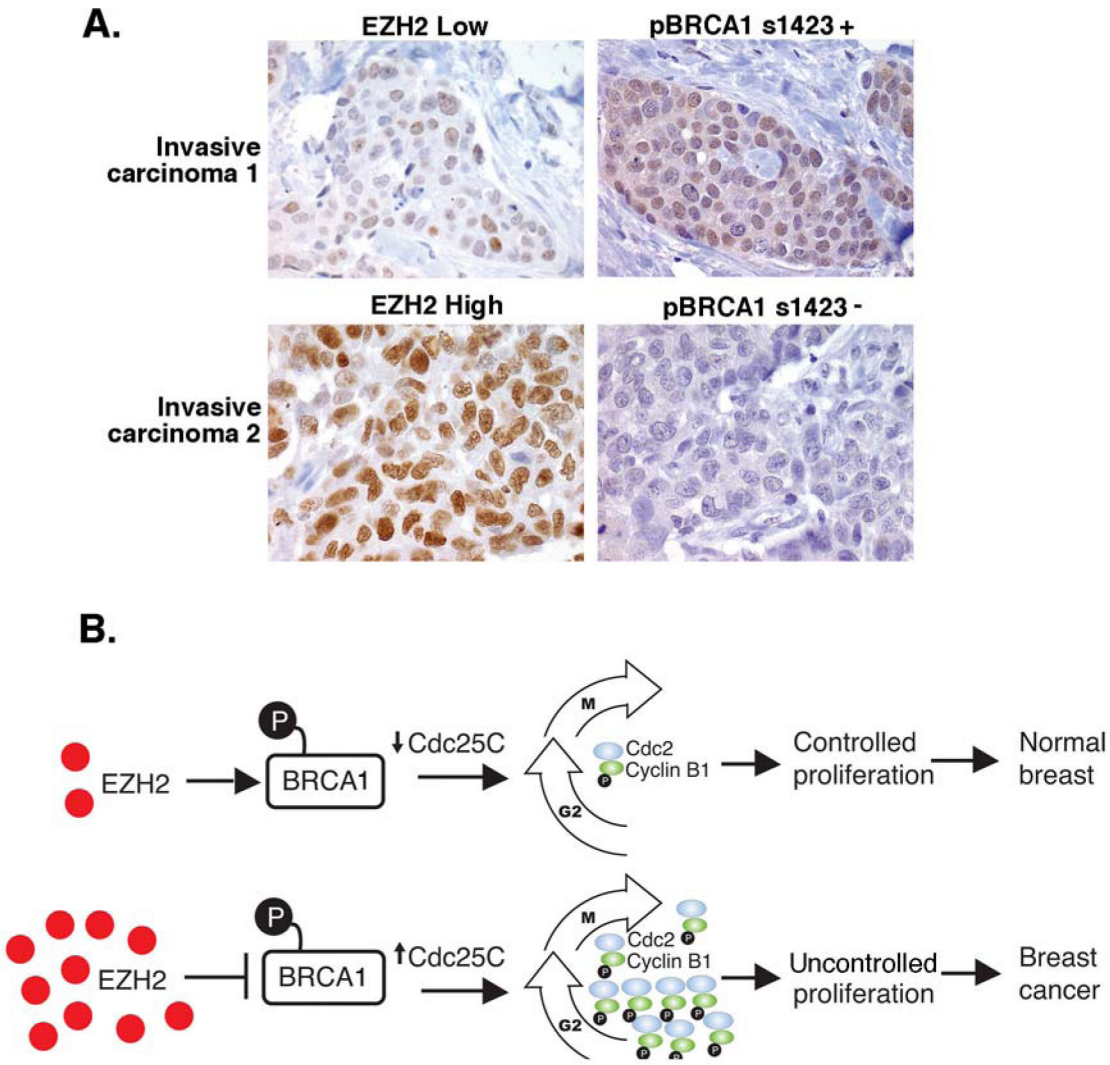

Figure 7. EZH2 expression is associated with phosphorylated BRCA1 in ER negative human breast carcinoma tissue samples

A. Human breast cancer tissue samples (n=136) immunostained for EZH2 and pBRCA1 s1423. Representative ER negative invasive carcinomas with low EZH2 expression and positive p-BRCA1 s1423 levels (*TOP*), and another tumor with high expression of EZH2 and negative p-BRCA1 s1423 proteins (*BOTTOM*). **B.** Working model for the role of EZH2 in cell proliferation and breast tumor development. Based on our data, we propose that EZH2 regulates the levels of BRCA1 protein and its serine 1423 phosphorylation in the breast epithelium. Low levels of EZH2, as occurs in normal breast epithelial cells (Ding et al., 2006; Kleer et al., 2003), regulate BRCA1 and control cell proliferation in the normal breast epithelium by regulating the levels of Cdc25C and the Cdc2-CyclinB1 complex. EZH2 overexpression results in decreased BRCA1 and pBRCA1 s1423 with 30 high levels of Cdc2-CyclinB1 complex and uncontrolled proliferation and mitosis, which contributes to breast carcinogenesis.

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Table 1

Association between EZH2 and pBRCA1 s1423 proteins in invasive carcinomas of the breast according to the ER status

Parameter	EZH2 high pBRCA1 s1423−	EZH2 high pBRCA1 s1423+	EZH2 low pBRCA1 s1423−	EZH2 Low pBRCA1 s1423+	*P**
	N %	N %	N %	N %	
ER negative	29 (76.3)	9 (75)	9 (23.7)	3 (25)	<0.0001
ER positive	16 (50)	16 (50)	27 (50)	27 (50)	NS

* Chi square test