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Histone Methyltransferase EZH2 Induces Akt-Dependent Genomic Instability and BRCA1 Inhibition in Breast Cancer

Maria E. Gonzalez^{1,2}, Matthew L. DuPrie^{1,2}, Heather Krueger^{1,2}, Sofia D. Merajver^{2,3}, Alejandra C. Ventura^{2,3}, Kathy A. Toy^{1,2}, and Celina G. Kleer^{1,2}

Abstract

Increased levels of EZH2, a critical regulator of cellular memory, signal the presence of metastasis and poor outcome in breast cancer patients. High levels of EZH2 are associated with nuclear pleomorphism, lack of estrogen receptor expression, and decreased nuclear levels of BRCA1 tumor suppressor protein in invasive breast carcinomas. The mechanism by which EZH2 overexpression promotes the growth of poorly differentiated invasive carcinomas remains to be defined. Here, we show that EZH2 controls the intracellular localization of BRCA1 protein. Conditional doxycycline-induced upregulation of EZH2 in benign mammary epithelial cells results in nuclear export of BRCA1 protein, aberrant mitoses with extra centrosomes, and genomic instability. EZH2 inhibition in CAL51 breast cancer cells induces BRCA1 nuclear localization and rescues defects in ploidy and mitosis. Mechanistically, EZH2 overexpression is sufficient for activation of the phosphoinositide 3-kinase/Akt (PI3K/Akt) pathway specifically through activation of Akt isoform 1. EZH2-induced BRCA1 nuclear export, aneuploidy, and mitotic defects were prevented by treatment with the PI3K inhibitors LY294002 or wortmannin. Targeted inhibition of Akt-1, Akt-2, and Akt-3 isoforms revealed that the EZH2-induced phenotype requires specific activation of Akt-1. The relevance of our studies to human breast cancer is highlighted by the finding that high EZH2 protein levels are associated with upregulated expression of phospho-Akt-1 (Ser473) and decreased nuclear expression of phospho-BRCA1 (Ser1423) in 39% of invasive breast carcinomas. These results enable us to pinpoint one mechanism by which EZH2 regulates BRCA1 expression and genomic stability mediated by the PI3K/Akt-1 pathway. *Cancer Res*; 71(6); 2360–70. ©2011 AACR.

Introduction

EZH2 is a Polycomb group protein involved in the regulation of cellular memory with roles in tumorigenesis including cancer cell proliferation, stem cell maintenance, cell differentiation, and neoplastic cell transformation (1–7). In breast cancer, EZH2 protein is elevated in aggressive and metastatic tumors and is an independent predictor of survival (8). Immunohistochemical studies of human breast tissue samples have shown that while EZH2 expression is low in normal epithelium, EZH2 is overexpressed in 54% of invasive carcinomas, especially in estrogen receptor (ER)-negative tumors with low BRCA1 nuclear expression (8–11).

The tumor suppressor BRCA1 regulates DNA repair and activation of cell-cycle checkpoints and has a central role in the maintenance of chromosomal stability (12). 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% (13). Although somatic mutations of *BRCA1* are not common, expression of its messenger RNA and protein is reduced in approximately 40% of sporadic (nonhereditary) breast carcinomas (14–16). Independent of the mechanism underlying the decrease in nuclear BRCA1 protein, the vast majority of breast carcinomas with reduced nuclear BRCA1 are poorly differentiated, aneuploid, and lack expression of ER (16–18).

BRCA1 protein exerts its tumor suppressor functions in the nucleus and can shuttle between the nucleus and the cytoplasm (19). Recent studies have provided information on the subcellular localization of BRCA1 protein during the cell cycle in normal and cancerous breast cells (20–22). BRCA1 protein is exported from the nucleus transiently during the initial part of S phase. By late S phase, BRCA1 resumes being a predominantly nuclear protein (20–22). Activation of the protein kinase B (Akt) has been implicated in the nuclear/cytoplasmic shuttling of BRCA1 protein in breast cells (23–26).

EZH2 has been proposed to participate in cell growth and invasion in breast cancer and has been studied to modulate BRCA1-mediated proliferation (8, 11, 27). However, no studies

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have been carried out to investigate the mechanism by which EZH2 influences BRCA1 protein and the link between EZH2 and genomic stability in breast cancer. Here, we show that EZH2 regulates the intracellular localization of BRCA1 protein in benign and malignant breast cells. Conditional doxycycline (Dox)-induced EZH2 overexpression in MCF10A cells leads to nuclear export of BRCA1 protein and is sufficient to trigger aberrant mitoses and numerical chromosomal alterations. EZH2 inhibition in ER-negative CAL51 breast cancer cells induces BRCA1 nuclear localization and rescues their ploidy and mitotic defects. Mechanistically, our data show that EZH2-induced BRCA1 nuclear export, mitotic, and ploidy abnormalities require activation of the phosphoinositide 3-kinase (PI3K)/Akt-1 signaling pathway.

Materials and Methods

Cell lines and breast tissues

CAL51 breast cancer cell line was purchased from German Collection of Microorganism and Cell Cultures (DSMZ GmbH; Cat. No. DSMZ ACC 302) and grown under recommended conditions. Immortalized human mammary epithelial cells MCF10A were obtained from American Type Culture Collection and grown under recommended conditions.

Lentiviral transductions

To conditionally overexpress EZH2 in MCF10A cells, a Dox-inducible system was employed. *EZH2* gene was isolated from pCDNA3-myc EZH2 plasmid (gift of Dr. Chinnaiyan) and cloned into the pLVX-Tight-Puro on the Lenti-X Tet-On advance inducible expression system (Clontech). Briefly, the Lenti-X Tet-On system is based on cells that express the *E. coli* Tet repressor protein (TetR), which negatively regulates the tetracycline operon on the Tn 10 transposon (vector: pLVX-Tet-On) together with the *tetO* (tet operator sequences; vector: pLVX-Tight-Puro). In the presence of tetracycline or Dox, TetR dissociates from *tetO*, and transcription of the resistance-mediating genes begins. Lentivirus-bearing EZH2 conditional system and vector control were used to transduce MCF10A cells. Cells were cultured in complete media supplemented with puromycin (10 µg/mL). EZH2 expression was transiently induced with Dox (500 ng/mL) by following the manufacturer's instructions.

Short hairpin RNA (shRNA) targeting human *EZH2* (NM_152998 NCBI; V2LHS_17507; Open Biosystems; Cat. No. RHS4430-99139126) was cloned into a pLKO.1-Puro vector. The shRNA-containing plasmid was packaged into lentiviral particles at the Vector Core (University of Michigan, Ann Arbor, MI). Background control was Lenti-PuroEMPTY-VSVG. To generate stable CAL51 breast cancer cells with EZH2 knockdown, 1×10^6 cells per 100-mm plate were transduced with the corresponding lentivirus-containing supernatant diluted 1:1 with fresh serum-free medium for 48 hours. Stable clones were selected for antibiotic resistance with 10 µg/mL puromycin (Sigma) at 37°C under 10% CO₂ for 3 weeks (11).

Human Akt-1 siRNA (sense: 5'-CCAAGCACCGCGUGAC-CAU-3'; antisense: 5'-AUGGUCACGCGGUGCUUGG-3'), Akt-2

siRNA (sense, 5'-CAGAAUGCCAGCUGAUGAA-3'; antisense, 5'-UUCAUCAGCUGGCAUUCUG-3'), Akt-3 siRNA (sense, 5'-GAAAGAUUGUGUACCGUGA-3'; antisense, 5'-UCACG-GUACACAAUCUUUC-3'), and human siRNA negative control oligonucleotides were purchased from Sigma (28). Cells were split into complete medium for 24 hours before subconfluence. siRNA oligos were transfected into subconfluent cells with Oligofectamine (Invitrogen) in accordance with the manufacturer's instructions.

Western blot analysis

Nuclear enriched fractions were separated by utilizing the NE-Per kit (Pierce). Western blot analyses were carried out with 100 µg of whole cell extract, nuclear, or cytoplasmic enriched fractions as indicated in the corresponding figure. Samples were boiled in $1 \times$ SDS loading buffer, separated by SDS-PAGE gels, and transferred onto a nitrocellulose (NC) membrane. NC membranes were blocked with 5% nonfat dry milk and were incubated with corresponding primary antibodies at 4°C overnight. Immunoblot signals were visualized by a chemiluminescence system as described by the manufacturer (Amersham Bioscience). Blots were reprobbed with α -tubulin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to confirm the equal loading of samples and with laminin B1 to confirm the nuclear enrichment of the fractionated samples.

Primary antibodies, including anti-EZH2 and anti-phospho-Polo-like kinase 1 (Plk1) Thr210 (BD Biosciences), anti-BRCA1 (EMD Chemicals), anti-phospho-BRCA1 (p-BRCA1; Ser1423), anti-laminin B1 (Abcam), anti-Akt, anti-Akt-1, anti-phospho-Akt (anti-p-Akt; Ser473), anti-Akt-3, anti-p-Akt-3 (Ser472), anti-Aurora A, anti-Aurora B, anti-phospho-Aurora A (anti-p-Aurora A; Thr288; Cell Signaling Technology), anti-p-Akt-1 (Ser473) (Upstate Biotechnology), anti-Akt-2, anti-p-Akt-2 (Ser474), and anti-Plk1 (Santa Cruz Biotechnology), anti- β -actin, anti- α -tubulin (Sigma), anti-p-Aurora B (Thr232; Biologend), were used at the manufacturers' recommended dilutions. The PI3K/Akt inhibitors LY294002 and wortmannin (Invitrogen) were employed to investigate the contribution of the PI3K/Akt pathway on EZH2 function by following previous procedures (29).

Analysis of mitosis and mitotic index

Cells were grown in slides, fixed with paraformaldehyde, rotated for 45 minutes at room temperature, and incubated with anti-phospho-histone H3 (anti-p-H3) antibody (Ser10 mitotic maker)-FITC-conjugated (Millipore). The *mitotic index* is the percentage of cells staining for p-H3. The presence of abnormal mitosis was studied by immunofluorescence with antibodies against p-H3 (Ser10), α -tubulin, and Aurora A. 4',6-Diamidino-2-phenylindole (DAPI) identified the nuclei. Slides were visualized by a confocal microscope. The number of cells with abnormal mitosis and greater than 2 Aurora A foci was recorded. A total of 300 cells were counted in triplicate. Details on cell synchronization, nocodazole treatment, Aurora A activity assay, flow cytometry, immunofluorescence, and human tissue samples are found in the Supplementary Methods.

Metaphase analysis

MCF10A cells were grown to 70% confluency and treated with Dox (500 ng/mL) for 24 hours at 3 and 5 days. Untreated cells served as controls. Cells were treated with 50 ng/mL colcemid (Invitrogen) for 24 hours and then collected and resuspended in a hypotonic solution of 2% KCl and 2% Na₃C₆H₅O₇ for 7 minutes at 37°C. Metaphase spreads were prepared and stained with Giemsa as described (30). Slides were examined with an ImagingZ1 microscope (Carl Zeiss) equipped with the ISIS image processing software (Meta-Systems). One hundred metaphases were counted in triplicate for each sample. Tetraploidy was defined as chromosome numbers of 81 to 100 by following established criteria (31–33).

Results

EZH2 regulates the nuclear/cytoplasmic shuttling of BRCA1 in benign and breast cancer cells

To determine the oncogenic phenotype of EZH2 overexpression in nontumorigenic human breast epithelial cells, we generated a Dox-regulated system to overexpress EZH2 in MCF10A cells. The empty vector served as negative control (pLVX). EZH2 was detected in whole cell lysates of Dox-induced MCF10A cells transduced with EZH2-containing plasmid (pLVX-EZH2) but not in the lysates of cells transduced with the empty vector (pLVX; Fig. 1A, top). We also generated CAL51 breast cancer cells with stable downregulation of EZH2 by using previously validated shRNAs (11). CAL51 breast cancer cells were chosen for EZH2 downregulation because they overexpress EZH2, are human cells, ER negative, and lack *BRCA1* mutations (11, 34).

Western blot analyses showed that Dox treatment of MCF10A-pLVX-EZH2 cells decreased nuclear BRCA1 protein and increased BRCA1 in the cytoplasm (Fig. 1A, middle and bottom). To investigate the effect of EZH2 on the kinetics of BRCA1 shuttling between the nucleus and cytoplasm during the cell cycle, MCF10A-pLVX-EZH2 cells with or without Dox treatment were synchronized at G₁/S by using double thymidine block and released and analyzed at the specified time points of early S phase. By immunofluorescence, BRCA1 localized to the nucleus of untreated MCF10A-pLVX-EZH2 cells. In contrast, Dox-induced EZH2 upregulation led to cytoplasmic localization of BRCA1 (Fig. 1B). Fluorescence signals of individual cells in the nucleus and cytoplasm were quantified using the ImageJ NIH software (Fig. 1B). On confirming the specificity of these results, no effect on BRCA1 intracellular localization was observed when MCF10A-pLVX cells (empty vector) were treated with Dox (Supplementary Fig. S1).

EZH2 KD on CAL51 breast cancer cells increased BRCA1 protein in the nuclear enriched fraction immediately after release from cell-cycle block at G₁/S (Fig. 1C). While CAL51 controls exhibited predominantly cytoplasmic and perinuclear BRCA1 protein as previously reported (20), EZH2 KD cells accumulated BRCA1 in the nucleus (Fig. 1C and D). We conclude that EZH2 influences the intracellular localization

of BRCA1 protein in nontumorigenic breast cells and breast cancer cells.

Overexpression of EZH2 protein induces extra centrosomes and abnormal mitosis

Immunofluorescence studies showed that Dox-induced EZH2 overexpression led to mitotic defects, including multiple mitotic spindles which contrasted with the absence of mitotic defects in untreated controls (Fig. 2A, left). To determine the effect of EZH2 overexpression on centrosome number, we detected Aurora A by immunofluorescence. Early in mitosis, Aurora A localizes to the centrosomes to mediate their maturation, separation, and spindle formation (35). As expected, Aurora A localized to the centrosomes during metaphase of untreated MCF10A-pLVX-EZH2 cells as evidenced by the 2 distinct foci that colocalized to the spindle poles (Fig. 2A, right). Dox-induced EZH2 overexpression led to a 6-fold increase in the percentage of mitotic cells with more than 2 Aurora A foci (Fig. 2A, right).

Because CAL51 cells contain a tetraploid population with centrosome amplification and multiple mitotic spindles, they constitute a good model to test the effect of EZH2 KD on centrosome number, mitotic spindle, and mitotic defects (34). EZH2 KD on CAL51 cancer cells significantly reduced the number of aberrant mitosis and the number of cells with more than 2 Aurora A foci (Fig. 2B).

We found that EZH2 expression in MCF10A and CAL51 cells regulates the levels and activity of Aurora A and B kinases, essential for mitotic entry and progression. Corresponding with the increase in Aurora A and B proteins observed in asynchronous cultures (Fig. 2C, left), EZH2 overexpression increased their enzymatic activity in nocodazole-treated samples. EZH2 overexpression induced phosphorylation of Aurora A on Thr288, Aurora B on Thr232, Aurora A interacting protein Plk1 on Thr210, and Aurora kinase substrate p-H3 Ser10, as well as Aurora A *in vitro* kinase activity (Fig. 2C, right; Supplementary Fig. S2A; ref. 36). EZH2 KD in CAL51 cells had the opposite effect (Fig. 2D). Further strengthening these data, EZH2 protein regulated Aurora A and B protein levels during cell-cycle progression and their messenger RNA levels (Supplementary Fig. S2C–D). Collectively, these data implicate EZH2 in mitosis and show a novel regulatory role for EZH2 on expression and activity of Aurora A and B kinases and on centrosome number in benign and breast cancer cells.

EZH2 regulates genomic stability

Errors in mitosis can lead to genomic instability. In contrast to the diploid chromosome number of untreated MCF10A cells, EZH2 overexpression resulted in 16.8% and 26.8% polyploidy (≥ 51 chromosomes) after 72 and 120 hours of Dox treatment, respectively. Chromosome counting indicated that 57% of cells within the polyploid population were near-tetraploid (51–100 chromosomes) at 5 days of Dox treatment (Fig. 3A; refs. 31–33). In contrast, EZH2 KD decreased the percentage of tetraploid CAL51 cells from 23.2% to 9.2% (Fig. 3B). These data reveal that besides its ability to regulate the number of centrosomes, EZH2 plays a role in the maintenance of genomic stability.

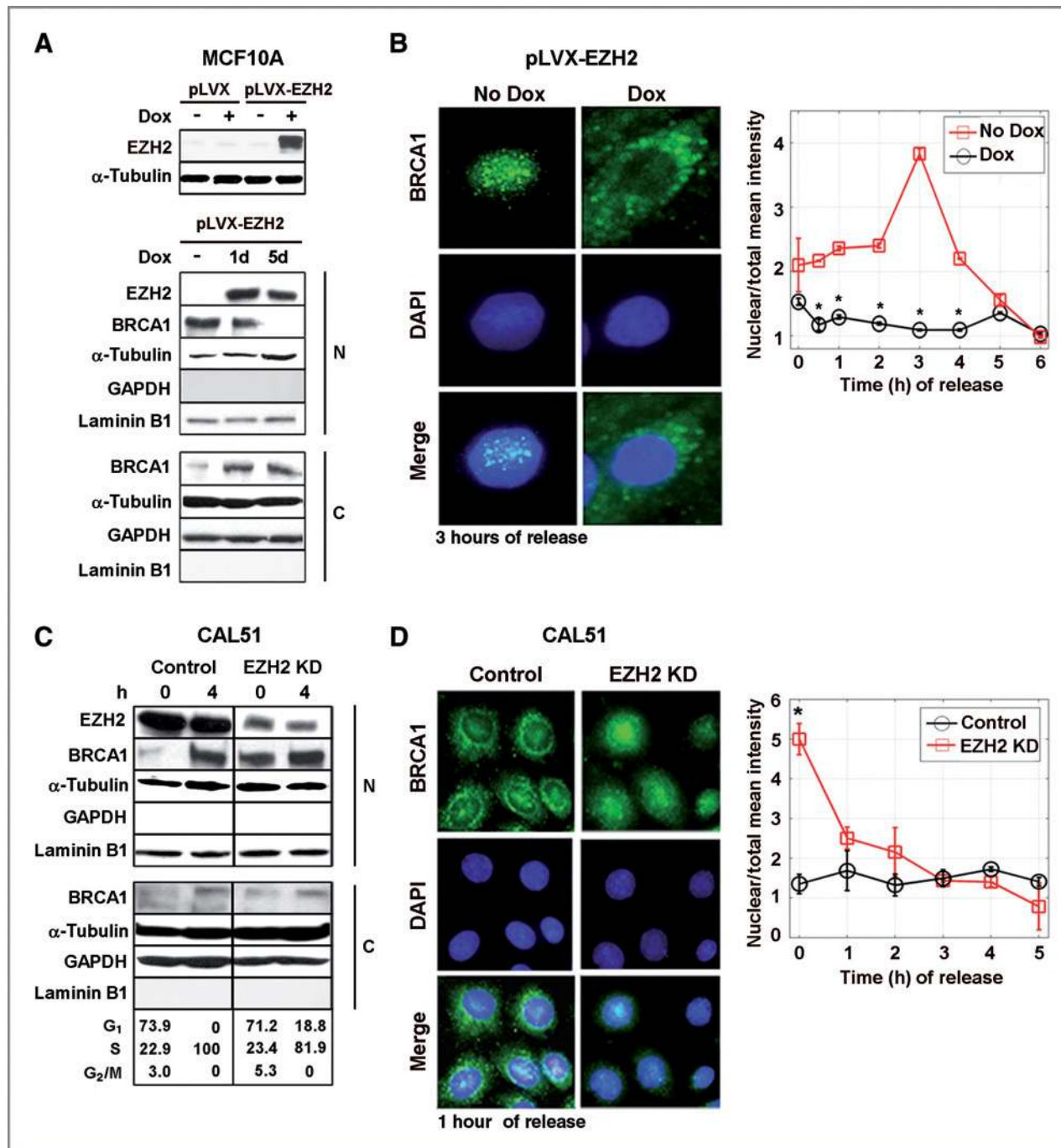


Figure 1. EZH2-dependent regulation of BRCA1 intracellular localization. A, inducible synthesis of EZH2 in MCF10A cells and its effect on BRCA1 protein. Western blot analysis of MCF10A cells transduced with the empty vector (pLVX) and EZH2-containing vector (pLVX-EZH2). Cells were untreated or treated with Dox (500 ng/mL) to induce EZH2 expression. Underneath is a Western blot of EZH2 and BRCA1 proteins in nuclear and cytoplasmic enriched fractions of pLVX-EZH2 cells untreated or treated with Dox. Laminin B1 and GAPDH confirm nuclear and cytoplasmic enrichment of the fractionated samples, respectively. B, effect of EZH2 overexpression on the intracellular localization of BRCA1 protein. Representative immunofluorescence images of BRCA1 protein at 3 hours after release of G₁/S cell-cycle block (double thymidine block). The graph shows the mean intensity of BRCA1 protein expression in the nucleus normalized to the total intensity at different times after release from cell-cycle block. The percentage of cells in each cell-cycle phase after release of cell-cycle block for untreated and Dox treated cells was at 0 hours: G₁, 94.84%; S, 5.15%; G₂/M, 0%; and G₁, 94.09%; S, 4.97%; G₂/M, 0.94%, respectively. At 4 hours: G₁, 78.52%; S, 21.02%; G₂/M, 0.46%; and G₁, 74.27%; S, 25.7%; G₂/M, 0.03%, respectively. C, Western blot for EZH2 and BRCA1 proteins in CAL51/EZH2 KD and controls at 0 and 4 hours after release of cell-cycle block. The number of cells in G₁, S, and M cell-cycle phases was obtained by flow cytometry. D, representative immunofluorescence images of BRCA1 protein at 1 hour after release of cell-cycle block and quantification of results, as described earlier. The experiment was repeated for 3 independent times. Error bars, SD; *, *P* < 0.05.

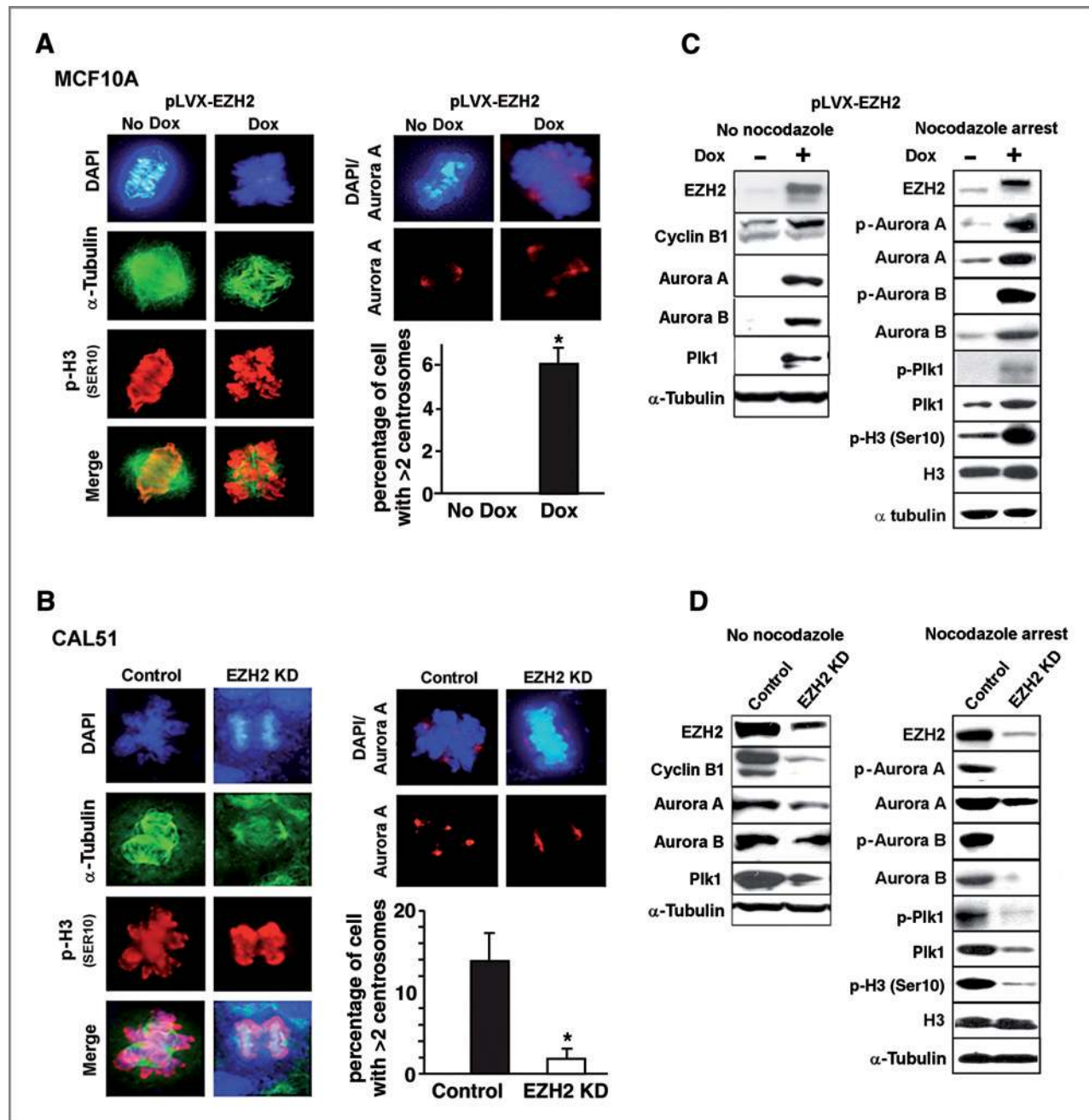


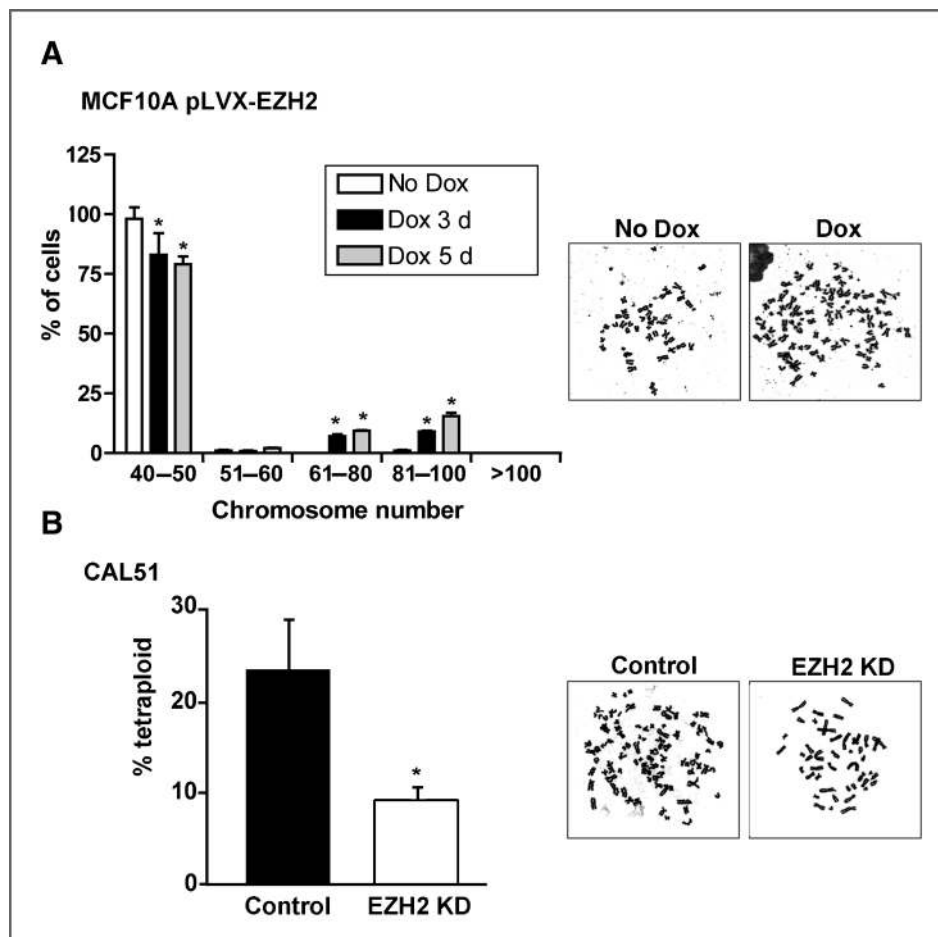
Figure 2. EZH2-mediated induction of abnormal mitosis and Aurora kinase activity. **A** and **B**, immunofluorescence analysis of mitotic MCF10A-pLVX-EZH2 cells untreated or treated with Dox for 3 days (**A**) and in CAL51/EZH2 KD and controls (**B**). p-H3 marks mitotic DNA; Aurora A marks the centrosomes. Bar graphs, the mean percentage of cells with more than 2 centrosomes \pm SD. The number of mitotic cells per 300 cells was 59 (19%) and 78 (26%) for untreated and Dox-treated cells, respectively. The number of mitotic cells per 300 cells was 198 (66%) and 78 (26%) for control and EZH2 KD cells, respectively. **C**, Western blot analysis of MCF10A-pLVX-EZH2 cells untreated or treated with nocodazole (50 ng/mL for 20 hours at 80% confluence) to induce mitotic arrest. EZH2 overexpression induces levels of Aurora A and B in asynchronous cultures, and upregulates p-Aurora A and B, p-Plk1, and the Aurora kinase substrate p-H3 Ser10 in nocodazole-treated cells. **D**, Western blotting of CAL51 cells untreated or treated with nocodazole. EZH2 KD in CAL51 cells shows the opposite effects to that of EZH2 overexpression.

EZH2-induced BRCA1 nuclear export, mitotic, and ploidy defects require activation of PI3K/Akt-1

We found that Dox treatment of MCF10A-pLVX-EZH2 cells increased the levels of Akt phosphorylated at Ser473, required

to promote its maximal activation (Fig. 4A, left). As expected, Dox treatment of MCF10A-pLVX cells did not alter p-Akt expression (Supplementary Fig. S3). To pinpoint which Akt isoform is necessary for the EZH2-induced phenotype, we

Figure 3. EZH2 regulates genomic stability in benign and breast cancer cells. A, MCF10A-pLVX-EZH2 cells untreated or treated with Dox for 3 and 5 days were subjected to Giemsa-stained metaphase spread analysis. While untreated cells are diploid, EZH2 overexpression induced polyploidy (≥ 51 chromosomes) and increased near-tetraploid cells (81–100 chromosomes). Bar graph, the mean percentage of cells \pm SD. B, metaphase counts in CAL51 control and EZH2 KD cells. For both experiments, 100 metaphases per sample were counted and the assay was done in triplicate. *, $P < 0.05$.



investigated the effect of EZH2 on the expression and phosphorylation of Akt isoforms 1, 2, and 3 in benign and breast cancer cells. EZH2 overexpression in MCF10A cells increased Akt-1 protein but did not influence Akt-2 or Akt-3 expression or phosphorylation, compared with controls (Fig. 4A, left). Consistently, CAL51/EZH2 KD cells exhibited decreased Akt-1 phosphorylation at Ser473 compared with scrambled controls (Fig. 4A, right). Reciprocal coimmunoprecipitation showed that EZH2 could directly interact with Akt-1 in MCF10A cells (Supplementary Fig. S4). These data led us to hypothesize that Akt-1 may mediate the observed EZH2-induced phenotype.

We next investigated the specific contribution of each Akt isoform to EZH2-induced functions by independent siRNA knockdown of Akt-1, Akt-2, and Akt-3 followed by Dox treatment to induce EZH2 overexpression (Fig. 4B). Specific inhibition of Akt-1 decreased EZH2-induced BRCA1 nuclear export. In contrast, knockdown of Akt-2 or Akt-3 had no effect (Fig. 4C). Akt-1 was required for EZH2-induced genomic instability and abnormal mitosis. siRNA inhibition of Akt-1 completely prevented EZH2-induced polyploidy and mitotic defects (Fig. 5A and B). Akt-2 and Akt-3 proteins were dispensable for EZH2-induced polyploidy (Fig. 5A). Similarly, Akt-3 expression was not required for EZH2 effect on abnor-

mal mitosis (Fig. 5B). Interestingly, Akt-2 KD blunted mitosis in MCF10A cells independent of EZH2 expression (mitotic index for siRNA control/no Dox = 18.3% and for siRNA control/Dox = 27%; compared with mitotic index for Akt-2 siRNA/no Dox = 5.6% and for Akt-2 siRNA/Dox = 5%). Further supporting the role of Akt pathway on BRCA1 localization and genomic instability, pharmacologic inhibition of PI3K/Akt, using LY294002 or wortmannin, prevented the EZH2-induced phenotype (Supplementary Fig. S5; Supplementary Table S1). Altogether, these results directly show that activation of PI3K/Akt-1 pathway is essential for EZH2-induced BRCA1 nuclear export, aneuploidy, and mitotic defects in benign breast cells.

EZH2 overexpression is associated with increased Akt-1 phosphorylation and decreased phospho-BRCA1 nuclear localization in human invasive breast carcinomas

To examine whether this regulation also exists in tumor tissues, we compared the levels of EZH2 and p-Akt-1 and the expression and localization of phospho-BRCA1 (p-BRCA1) in 138 tumors by immunostaining. Consistent with our observations in cell cultures, upregulation of EZH2 was significantly

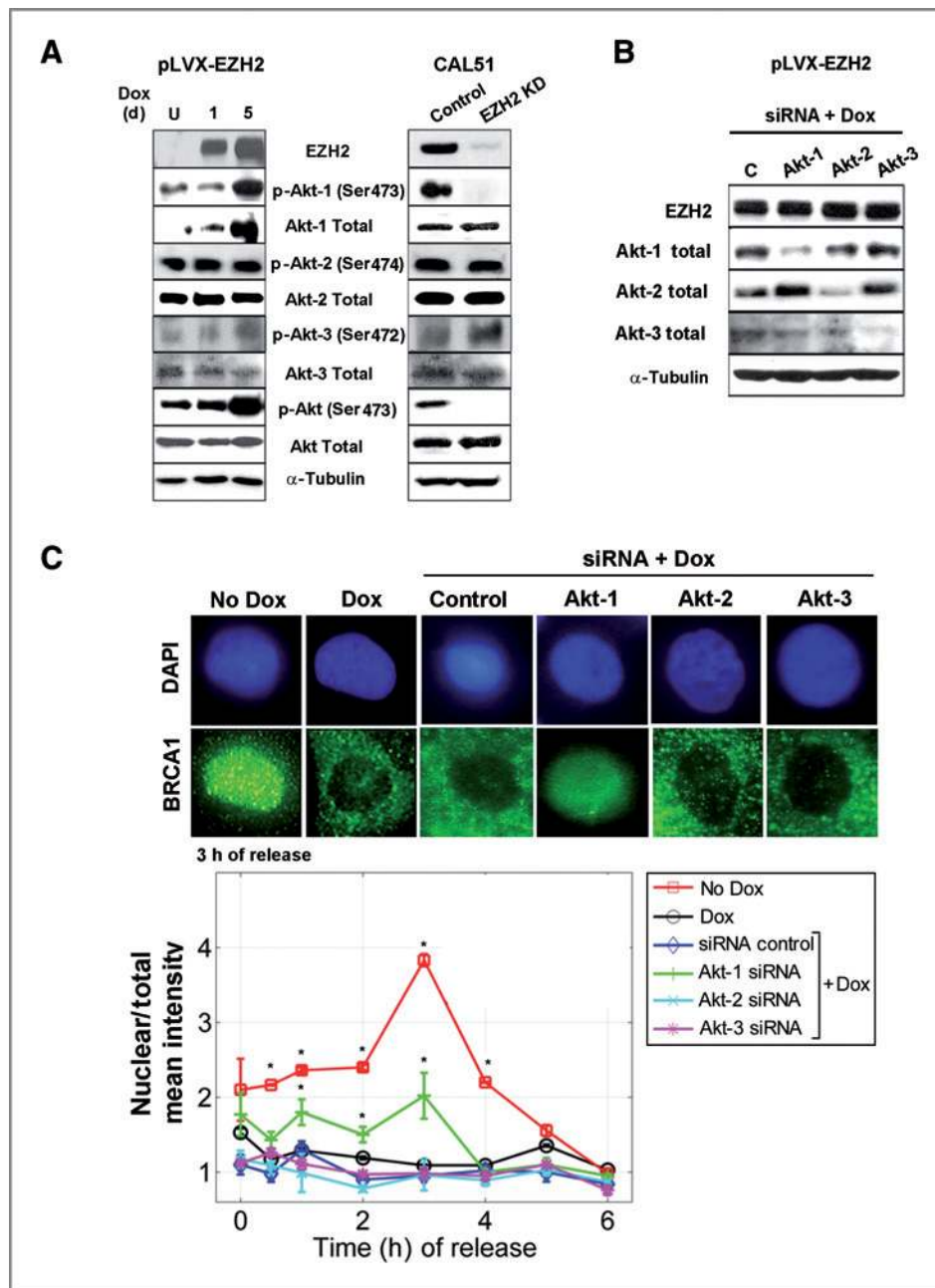


Figure 4. EZH2 requires Akt-1 to regulate the intracellular localization of BRCA1. A, immunoblots of MCF10A and CAL51 cells show that EZH2 expression regulates Akt-1 protein, whereas it has no effect on Akt-2 or Akt-3 protein levels. B, Western blotting of MCF10A-pLVX-EZH2 cells treated with siRNA control or Akt-1 siRNA, Akt-2 siRNA, or Akt-3 siRNA and then subjected to Dox for 3 days to induce EZH2 overexpression. Immunoblot shows specific inhibition of Akt isoforms. C, cells (described in B) and pLVX-EZH2 untreated or treated with Dox were subjected to immunofluorescence analyses of BRCA1 protein at the indicated time points after release from cell-cycle block at G₁/S. Representative confocal images at 3 hours after release from cell-cycle block. More than 500 cells were counted per slide in triplicate. Mean intensity of BRCA1 protein in the nucleus was normalized to the total intensity by utilizing the ImageJ NIH program. Error bars, SD. *, $P < 0.05$.

associated with upregulation of p-Akt-1 and decreased nuclear levels of p-BRCA1 protein (Fig. 6; Table 1). Of the 138 tumors, 86 (62.3%) exhibited reciprocal expression of EZH2 and p-BRCA1 proteins [49 (35.5%) had high EZH2 and low nuclear p-BRCA1, and 37 (26.8%) had low EZH2 and high nuclear p-BRCA1 (Fisher's exact test, $P < 0.005$; Table 1]. Invasive breast carcinomas with high EZH2 and high p-Akt-1 significantly showed low nuclear p-BRCA1 expression, whereas those tumors with low EZH2 and low p-Akt-1 exhibited high p-BRCA1 expression (Fisher's exact test, $P = 0.03$;

Table 1). Concomitant high EZH2/high p-Akt-1/low nuclear p-BRCA1 is associated with ER-negative status and high histologic grade compared with low EZH2/low p-Akt-1/high nuclear p-BRCA1 (Fisher's exact test, $P = 0.005$; Supplementary Table S2).

Discussion

A salient feature of EZH2-overexpressing human invasive breast carcinomas is their high histologic grade and poorly

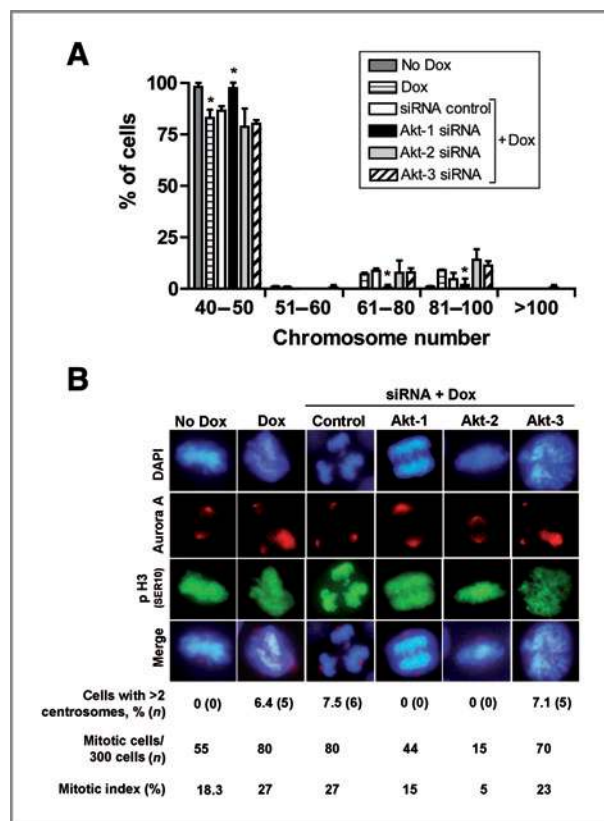


Figure 5. Akt-1 is required for EZH2-dependent regulation of mitosis and genomic stability. **A**, MCF10A-pLVX-EZH2 cells treated with siRNA control or Akt-1 siRNA, Akt-2 siRNA, or Akt-3 siRNA were subjected to Dox for 3 days to induce EZH2 overexpression. Giemsa-stained metaphase spread analyses show that Akt-1 siRNA is sufficient to prevent EZH2-induced polyploidy (≥ 51 chromosomes). In contrast, EZH2 overexpression increased numerical chromosomal alterations in cells treated with siRNA control, Akt-2 siRNA, or Akt-3 siRNA. Bar graph, the mean percentage of cells \pm SD. **B**, immunofluorescence analysis of mitotic MCF10A-pLVX-EZH2 cells treated with siRNA control or Akt-1 siRNA, Akt-2 siRNA, or Akt-3 siRNA and then subjected to Dox for 3 days. p-H3 (Ser10) marks mitotic DNA; Aurora A marks the centrosomes; DAPI marks the nuclei. Note that Akt-1 siRNA prevented EZH2-induced mitotic aberrations and the number of cells with extra centrosomes. Akt-2 siRNA caused a 3-fold decrease in the number of mitotic cells independent of EZH2 overexpression (mitotic index for siRNA control/no Dox = 18.3% and for siRNA control/Dox = 27% compared with mitotic index for Akt-2 siRNA/no Dox = 5.6% and Akt-2 siRNA/Dox = 5%). Akt-3 KD could not prevent EZH2-induced chromosomal alterations.

differentiated cells with pleomorphic nuclei (8–10). EZH2-overexpressing invasive carcinomas are largely ER negative and exhibit BRCA1 downregulation (8–11). We discovered that EZH2 regulates the intracellular distribution of BRCA1 protein in benign breast cells and ER-negative breast cancer cells. To draw these conclusions, we investigated the effect of EZH2 on the intracellular localization of BRCA1 protein by utilizing independent and complementary gain- and loss-of-function approaches. By measuring the nuclear and cytoplasmic expression of BRCA1 protein at different time points after release from G_1/S cell-cycle block, it was concluded that EZH2

overexpression in MCF10A induced nuclear export with cytoplasmic retention of BRCA1 protein. Consistent with this observation, while BRCA1 was mainly localized to the cytoplasm of CAL51 breast cancer cells, it was translocated to the nucleus upon lentiviral-mediated EZH2 KD.

The mechanisms governing the nuclear/cytoplasmic shuttling of BRCA1 protein are not fully elucidated, but recent studies implicate the membrane serine/threonine protein kinase B/Akt (23–26). A tumorigenic mechanism of Akt upon its phosphorylation is the induction of cytoplasmic localization of tumor suppressor proteins including p21 Cip1/WAF1 and FOXO3a (37, 38). The functional relationship between Akt and BRCA1 is complex and contextual (23). The PI3K/Akt pathway promoted nuclear translocation of BRCA1, and reciprocally, BRCA1 deficiency could activate the PI3K/Akt signaling (26, 39). Akt-1 activation was shown to induce cytoplasmic retention of BRCA1 protein in breast cancer cells (24). By using pharmacologic pathway inhibition and transient, specific siRNA interference of Akt isoforms, we provide direct evidence that the effect of EZH2 on BRCA1 intracellular localization necessitates the activation of Akt-1, whereas Akt-2 and Akt-3 are dispensable for this function. Immunostaining of surgical samples highlights the relevance of our mechanistic studies to human breast cancer, as EZH2-overexpression is significantly associated with increased p-Akt-1 and decreased p-BRCA1 nuclear protein.

The stepwise progression from an atypical lesion to full-blown malignancy with metastatic capacity is associated with increases in genomic instability (40). BRCA1 deficiency can cause tetraploidy and aneuploidy (41). However, whether EZH2 regulates genomic stability is not known. Conditional EZH2 upregulation induced numerical chromosomal alterations in MCF10A cells as early as 72 hours after the addition of Dox. Of note, more than 50% of polyploid cells were near-tetraploid. These results are intriguing, as several lines of evidence show that tetraploidy can be an initiator of chromosomal instability and tumorigenesis *in vivo* and has been detected in human tissues before aneuploidy occurs (42–44). Our data on CAL51 breast cancer cells support the possible therapeutic role of EZH2 blockade in breast cancer, as EZH2 KD was sufficient to significantly decrease the percentage of tetraploid breast cancer cells. Thus, preventing or reverting tetraploidization through EZH2 inhibition may halt breast cancer development.

Although multiple mechanisms can lead to aneuploidy (40), alterations in mitosis play an important role. Overexpression of Aurora kinases A and B are required for centrosome maturation, bipolar spindle assembly, and mitotic entry, and their overexpression in human cells results in abnormal mitosis and aneuploidy (40, 45, 46). We show that transient EZH2 overexpression in benign breast cells was sufficient to induce aberrant mitosis with extra centrosomes. The effect of EZH2 on mitosis was also evident in CAL51 breast cancer cells. While CAL51 controls exhibited aberrant mitosis with supernumerary centrosomes and multiple mitotic spindles, EZH2 KD abrogated these abnormalities. Mechanistically, EZH2 overexpression increased the messenger RNA and protein levels of Aurora kinases A and B and enhanced their kinase

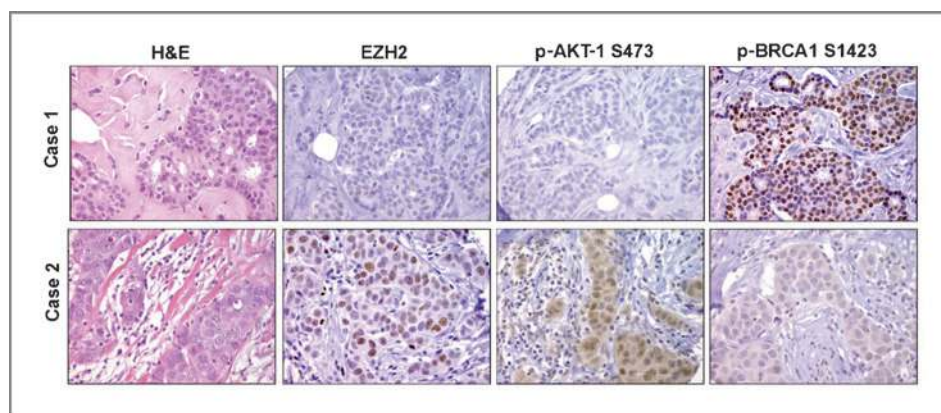


Figure 6. EZH2 overexpression is associated with upregulation of p-Akt-1 and decreased nuclear BRCA1 expression. Human breast cancer tissue samples ($n = 138$) were immunostained for EZH2, p-Akt-1, and p-BRCA1. Representative images of invasive ductal carcinomas. H&E, hematoxylin and eosin.

activity. These data implicate EZH2 in mitosis and the regulation of Aurora kinase function in benign and breast cancer cells.

Although Akt has been reported to play a role in mitosis and aneuploidy, the specific mechanisms have not been fully defined. Similarly, the specific role of each Akt isoform in the maintenance of genomic stability is unknown. Akt was shown to mediate abnormal checkpoint control and aneuploidy in PTEN-deficient cells by impairing CHK1 through phosphorylation, ubiquitination, and reduced nuclear localization (47). Especially intriguing in light of our data are results from a recent study showing that Akt-1 activation induced supernumerary centrosomes and genomic instability through cytoplasmic retention of BRCA1 in a hamster ovary cell line (25).

Here, we show that the effects of EZH2 overexpression on mitosis and genomic instability require specific activation of Akt-1. Interestingly, our data suggest a novel role for Akt-2 during mitosis unrelated to EZH2 expression. We observed that Akt-2 siRNA inhibition caused a 3-fold decrease in the number of cells undergoing mitosis in an EZH2-independent manner. On the basis of our data, we hypothesize that the blunting of mitoses may explain the absence of mitotic defects in Akt-2 KD cells after induction of EZH2 overexpression, as

was observed with Akt-3 KD. The function of Akt-2 in mitosis warrants further study.

In conclusion, these data show a novel function of EZH2 in breast tumorigenesis: its ability to promote the nuclear export of BRCA1 and induce aberrant mitosis and genomic instability. Our results enable us to pinpoint one mechanism by which EZH2 controls BRCA1 intracellular localization and genomic stability by activating Akt-1. In breast cancer cells, EZH2 downregulation induces nuclear localization of BRCA1, decreases mitotic aberrations, and reverses tetraploidy. We propose that modulation of EZH2 expression may be a valid strategy to prevent or halt neoplastic progression in the breast.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Table 1. Relationships between EZH2 expression and p-Akt-1 and p-BRCA1 in human invasive carcinomas of the breast

	EZH2 expression			EZH2 low/p-Akt-1 low	EZH2 high/p-Akt-1 high	P
	Low	High	P			
p-BRCA1						
Low nuclear	31	49		19	30	
High nuclear	37	21	<0.005	18	9	<0.03
p-Akt-1						
Low	37	8				
High	14	46	<0.0001			

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