EZH2 expands breast stem cells through activation of NOTCH1 signaling

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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 triple-negative (TN) breast cancer with heightened expression of the Polycomb group protein Enhancer of Zeste Homolog 2 (EZH2) and increased tumor-initiating cells (TICs). However, mechanistic links between EZH2 and TICs are unclear, and direct demonstration of a tumorigenic function of EZH2 in vivo is lacking. Here, we identify an unrecognized EZH2/NOTCH1 axis that controls breast TICs in TN breast carcinomas. EZH2 overexpression increases NOTCH1 expression and signaling, and inhibition of NOTCH1 activity prevents EZH2-mediated stem cell expansion in nontumorigenic breast cells. We uncover a unique role of EZH2 in activating, rather than repressing, NOTCH1 signaling through binding to the NOTCH1 promoter in TN breast cancer cells. EZH2 binding is independent of its catalytic histone H3 lysine 27 methyltransferase activity and of the Polycomb Repressive Complex 2 but corresponds instead to transcriptional activation marks. In vivo, EZH2 knockdown decreases the onset and volume of xenografts derived from TN breast TICs. Conversely, transgenic EZH2 overexpression accelerates mammary tumor initiation and increases NOTCH1 activation in mouse mammary tumor virus-neu mice. Consistent with these findings, in clinical samples, high levels of EZH2 are significantly associated with activated NOTCH1 protein and increased TICs in TN invasive carcinomas. These data reveal a functional and mechanistic link between EZH2 levels, NOTCH1 signaling activation, and TICs, and provide previously unidentified evidence that EZH2 enhances breast cancer initiation.

EZH2 up-regulates in clinically aggressive breast carcinomas, where it independently predicts survival (11). EZH2 overexpression is significantly associated with triple-negative (TN) carcinomas, a biologically aggressive group of breast cancer characterized by lack of estrogen and progesterone receptor expression and absence of HER-2/neu overexpression (11). In benign breast tissues, elevated levels of EZH2 protein signal future development of breast cancer up to 12 y before diagnosis, indicating that EZH2 up-regulation precedes morphological atypia or carcinoma (12). Recently, EZH2 has been shown to play a role in self-renewal of breast tumor-initiating cells (TICs) (13). However, direct demonstration that EZH2 promotes breast cancer initiation is lacking, and the responsible mechanisms need further investigation. Our data identify a unique molecular mechanism by which EZH2 promotes breast cancer development and provide support for targeting the gene activating function of EZH2 in TN invasive breast cancer.

Results

EZH2 Knockdown Reduces TICs and Inhibits the NOTCH1 Pathway in Breast Cancer. To examine the effect of EZH2 on the TIC populations of TN breast cancer, we used primary human breast cancer cells and SUM149 and MDA-MB-231 cell lines. As is the case for clinical samples of TN breast cancer, these cells exhibit high endogenous levels of EZH2 in comparison with benign breast cells (14). EZH2 knockdown (KD) was achieved through stable lentiviral-mediated short hairpin RNA interference (shRNA) previously developed in our laboratory (14). To reconstitute EZH2 expression in KD cells, we used a wild-type EZH2-encoding, myc-tagged adenovirus (11) (Fig. 14). To understand the role of EZH2 in the regulation of breast TICs, we performed mammosphere assays, based on the property of TICs to survive in nonadherent, serum-free culture conditions (15). EZH2 KD in SUM149 and MDA-MB-231 cells reduced sphere numbers compared with controls (Fig. L4 and Table 1). EZH2 knockdown decreases both the number and size of mammospheres. In vivo, EZH2 knockdown decreases the onset and volume of xenografts derived from TN breast TICs. Conversely, transgenic EZH2 overexpression accelerates mammary tumor initiation and increases NOTCH1 activation in mouse mammary tumor virus-neu mice. Consistent with these findings, in clinical samples, high levels of EZH2 are significantly associated with activated NOTCH1 protein and increased TICs in TN invasive carcinomas. These data reveal a functional and mechanistic link between EZH2 levels, NOTCH1 signaling activation, and TICs, and provide previously unidentified evidence that EZH2 enhances breast cancer initiation.

Invasive breast carcinoma arises in the terminal-duct lobular unit and progresses through phases of increasing proliferation and altered differentiation to atypical ductal hyperplasia and carcinoma in situ. Anaplasia, which describes cells lacking differentiation, is a hallmark of cancer. Disregulation of genes governing cell type identity may lead to malignant transformation (1). The transcriptional memory of cells is tightly regulated through epigenetic mechanisms largely by Polycomb and Trithorax group proteins (2). Enhancer of Zeste Homolog 2 (EZH2) is the catalytic subunit of Polycomb Repressive Complex 2 (PRC2), which silences gene transcription through trimethylation of histone H3 on lysine 27 (H3K27me3) (3). EZH2 protein is up-regulated in multiple malignant states of breast tissue (14). EZH2 protein is up-regulated in multiple malignancies (4, 5), where its oncogenic activity is thought to be primarily mediated by silencing tumor suppressor genes (6). Recent evidence implicates EZH2 in transcriptional activation (7–10), but the mechanisms are not well-defined.

EZH2 is up-regulated in clinically aggressive breast carcinomas, where it independently predicts survival (11). EZH2 overexpression is significantly associated with triple-negative (TN) carcinomas, a biologically aggressive group of breast cancer characterized by lack of estrogen and progesterone receptor expression and absence of HER-2/neu overexpression (11). In benign breast tissues, elevated levels of EZH2 protein signal future development of breast cancer up to 12 y before diagnosis, indicating that EZH2 up-regulation precedes morphological atypia or carcinoma (12). Recently, EZH2 has been shown to play a role in self-renewal of breast tumor-initiating cells (TICs) (13).

Significance

Triple-negative breast cancers comprise 10% of invasive breast carcinomas but are responsible for a disproportionate number of deaths and remain poorly understood. Unfortunately, current therapies are only weakly effective, and the median disease-free survival is 4 y among young women. Clinical studies support the relevance of Enhancer of Zeste Homolog 2 (EZH2) overexpression to the progression of triple-negative breast carcinomas. Our study shows that EZH2 acts as an activator of the NOTCH1 promoter and signaling to expand the stem cell pool, leading to accelerated breast cancer initiation and growth. We discovered that this function is independent of EZH2 histone methyltransferase activity and of its Polycomb Repressive Complex 2-binding partners, paving the way for novel therapeutic strategies.


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In SUM149 cells, mRNA and ALDH1 cells compared with A and family genes. EZH2 KD significantly decreases and ALDH1 populations in the ALDH1+ population compared with the ALDH1− cells (9,687 fold vs. 18 fold, respectively; Student t test P < 0.0001; Fig. S2A and B). Real-time RT-PCR of NOTCH signaling pathway genes validated these results and showed that EZH2 KD most significantly reduces the mRNA levels of NOTCH1 compared with the other NOTCH receptors and deregulates NOTCH signaling pathway components in the ALDH1+ population (Fig. 1D).

To search for critical genes and pathways mediating the effect of EZH2 on TICs, we used a stem cell signaling focused PCR array comparing the ALDH1+ and ALDH1− populations of SUM149 EZH2 KD and control cells. NOTCH1 was one of the most significantly down-regulated genes by EZH2 KD in the ALDH1+ population compared with the ALDH1− cells (Fig. S1C and Table S1).

The in vivo consequences of decreased TICs attributable to EZH2 KD were investigated by injecting ALDH1+ and ALDH1− populations of NOD/SCID mice. EZH2 KD in SUM149 cells significantly delayed tumor onset and decreased the tumor volume of ALDH1+ cells compared with controls, whereas it had no significant effect on the ALDH1− populations (Kaplan–Meier, log-rank P = 0.0019; and mixed-regression model, P < 0.05; Fig. 1C, Fig. S1C, and Table S1). Extending these observations to human breast cancer, EZH2 KD ALDH1+ cells exhibited decreased NOTCH1 signaling proteins compared with controls (Fig. S2C). Interrogation of publicly available human breast cancer cDNA array datasets using Oncomine confirmed the significant and hitherto unknown association between EZH2 and NOTCH1 mRNA expression in breast carcinomas from six independent datasets (Fig. S3).

EZH2 KD in MDA-MB-231, SUM149, and patient-derived breast cancer cells down-regulated NOTCH1 intracellular domain (NICD1) protein, the activated intracellular form of NOTCH1, and reduced the expression of NOTCH pathway proteins, consistent with the mRNA data (Fig. S1 D, F, and G). Ectopic expression of EZH2 was sufficient to rescue NICD1 protein expression in SUM149 and MDA-MB-231 EZH2 KD cells (Fig. S1E). Collectively, we provide evidence that the TIC-enriched population in TN breast cancer manifests increased NOTCH1 signaling in an EZH2-dependent manner and exhibits gene expression signatures of stemness.

An EZH2/NOTCH1 Axis Regulates Stem Cells. Endogenous EZH2 mRNA levels in primary epithelial cells derived from mammo- plasities are higher in the mammosphere forming population compared with adherent cell cultures (Fig. S4A). We used a conditional doxycycline (DOX)-mediated system to overexpress EZH2 in MCF10A cells (18). Whereas DOX treatment of MCF10A-pLVX-EZH2 cells significantly induced EZH2 overexpression in ALDH1+ and ALDH1− cells, EZH2 mRNA levels were significantly higher in ALDH1+ cells (Fig. S4B). Consistently, DOX-induced EZH2 overexpression increased NOTCH1 mRNA levels in ALDH1+ MCF10A cells (Fig. S4C). DOX-mediated EZH2 overexpression in MCF10A-pLVX-EZH2 cells increased NICD1 protein, mammospheres, and the percentage of CD44+/CD24− and ALDH1+ cells compared with untreated controls (Fig. 2 A–C and Fig. S4D). Reduction of NOTCH activation by pretreatment with a γ-secretase inhibitor (GSI) or NOTCH1 siRNA was sufficient to prevent these effects (Fig. 2 A–C and Fig. S4D). Demonstrating the importance of this pathway to human breast cancer, expression of constitutively active NICD1 (19) in patient-derived breast cancer cells effectively rescued the decreased sphere formation due to EZH2 KD (Fig. S1H).

EZH2 Regulates NOTCH Transcriptional Activity and Expands Stem Cells in a Histone Methyltransferase Activity-Independent Manner. To investigate the effect of EZH2 on NOTCH signaling, we used a lentiviral NOTCH reporter vector that drives the expression of GFP under the minimal essential CMV promoter downstream of NOTCH transcriptional response elements (20).
EZH2 binds to the Proximal NOTCH1 Promoter to Activate Transcription. EZH2 has been reported to function as a transcriptional repressor, but there is recent evidence supporting an activating role by yet unclear mechanisms (7–10). In TN breast cancer, EZH2 has been shown to form a complex with RelA and RelB to activate transcription (9). We hypothesized that EZH2-induced NOTCH1 up-regulation may be linked directly to an ability to bind to the NOTCH1 promoter. We performed chromatin immunoprecipitation (ChIP) assays on primary nontumorigenic breast epithelial cells transduced with adenoviral vectors containing wild-type EZH2 or the ΔSET and ΔHII mutants. These mutants were selected because the SET domain is required for HMT activity and the HII domain has been reported to promote gene activation (5, 7). We used primers targeting the NOTCH1 promoter region from −532 to −4510 base pairs upstream of the transcription start site (Fig. 4A). Primers flanking the GAPDH promoter and the MYT1 promoter, a known direct transcriptional repression target of EZH2 through H3K27me3 (22), were used as negative and positive binding controls, respectively. Upon overexpression of wild-type EZH2, we observed a significant increase in EZH2 binding to the NOTCH1 promoter (Fig. 4C).

We generated myc-tagged EZH2 deletion mutants involving the amino-terminal homology domains I and II (ΔHI and ΔHII), the carboxyl-terminal SET domain (ΔSET), and the nuclear localization signal (ΔNLS) in adenoviral vectors and expressed them in MCF10A cells (Fig. 3A and Fig. S4E). Functionally, all deletion mutants blocked the ability of EZH2 to enhance NOTCH transcriptional activity and to increase spheres and the percentage of CD44+/CD24− cells (Fig. 3B and Fig. S4F and G). To elucidate whether the observed effects of ΔSET are attributable to its enzymatic function, we overexpressed the EZH2-H689A mutant, which has reduced histone methyltransferase (HMT) activity in MCF10A cells (21). Our data show that EZH2-H689A increased NOTCH1 signaling and sphere numbers to levels similar to wild-type EZH2, suggesting that the HMT activity may not be required for these functions (Fig. 3C).

Fig. 2. NOTCH1 pathway activation is required for EZH2-mediated breast stem cell expansion. (A, Left) Immunoblots of MCF10A pLVX-EZH2 cells DOX-induced and controls probed with anti-EZH2 and anti-NICD1. GSI (17 nM for 3 d) was added 24 h before DOX. Representative images of mammospheres after 7 d in culture. (Magnification: 200×) (A, Right) mammosphere assay of MCF10A pLVX and pLVX-EZH2 cells DOX-induced and controls, with or without GSI Average sphere number ± SD per 5 × 10⁶ plated cells in the secondary generation (*P < 0.0001). (B) Flow cytometric assays to detect CD44+/CD24− populations in MCF10A pLVX-EZH2 DOX-induced and controls. GSI was added (1.7 nM for 7 d) 24 h before DOX. Percentages are expressed ± SD (*P ≤ 0.0005). (C) Immunoblots and mammosphere assays of MCF10A pLVX and pLVX-EZH2 cells DOX-induced and controls, treated with NOTCH1 siRNA or scrambled controls 24 h before DOX. Average sphere number ± SD per 5 × 10⁶ plated cells in the secondary generation (*P < 0.0005).

Fig. 3. EZH2 regulates NOTCH1 transcriptional activity and expands stem cells in an HMT activity-independent manner. (A) Schematic diagram of myc-tagged, EZH2 deletion mutants: ΔSET, ΔHI (homology domain I), ΔHII (homology domain II), and ΔNLS (nuclear localization signal). (B, Top) GFP-NOTCH promoter reporter assay of MCF10A cells overexpressing full-length EZH2, EZH2 deletion mutants, or controls. Percentages of GFP-expressing cells ± SD (*P = 0.0004). (B, Middle and Bottom) Mammosphere assays and representative images after 7 d. (Magnification: 200×.) Average number of mammospheres ± SD (*P < 0.0001). (C, Upper) Immunoblot of MCF10A cells transduced with EZH2 and EZH2-H689A mutant probed with anti-NICD1, anti-HES1, anti-H3K27me3, and anti-histone H3. (C, Lower) Mammosphere assay. Average sphere numbers per 5 × 10⁶ plated cells in the second generation ± SD (*P ≤ 0.0001).
Transgenic mammary-specific EZH2 overexpression up-regulates NOTCH1 promoter activity and accelerates tumor initiation in MMTV-neu mice. (A) Whole mounts of mammary glands from 16-wk-old virgin female EZH2wt;neu and EZH2+;neu mice (n = 10 mice per group). (Magnification: 200×). Average number of tertiary branches ± SD at 16 wk quantified using FRIDA software (*P < 0.0001). (B) Kaplan–Meier curve shows that EZH2+;neu mice (n = 30) formed mammary carcinomas significantly earlier than EZH2wt;neu mice (n = 25) (median time to tumor initiation: 243 ± 295 d, respectively; log-rank P < 0.0001). (C) Representative pictures of mammary tumors from EZH2+;neu and EZH2wt;neu mice stained for H&E and immunostained for NOTCH1 and NICD1. (Magnification: 400×). Percentage of relative expression ± SD was quantified using FRIDA software (*P < 0.003, **P = 0.009).

Fig. 4. EZH2 binds to the NOTCH1 promoter in benign and in breast cancer cells. (A) Diagram of the NOTCH1 promoter regions analyzed for EZH2 binding in ChIP assays. EZH2 binds to region “B” at −1.2 kb; subsequent experiments were performed in this area. (B) ChIP assays using qRT-PCR in nontumorigenic primary breast cells overexpressing full-length EZH2, ASET, ΔHII, or control adenovirus. Primers flanking the GAPDH promoter region are negative binding controls. Primers flanking MYT1, a known direct EZH2 transcriptional repression target through H3K27me3, are positive binding controls. (C) ChIP assays (as in B) in MDA-MB-231 cells show that endogenous EZH2 protein binds the NOTCH1 promoter. EZH2 KD reduces EZH2 binding. (D) ChIP assays (as in B) in patient-derived breast cancer cells reveal that endogenous EZH2 protein binds the NOTCH1 promoter. *P < 0.05 (B and C). All ChIP assays are representative of three independent experiments.

Fig. 5. Transgenic mammary-specific EZH2 overexpression up-regulates NOTCH1 and accelerates tumor initiation in MMTV-neu mice. (A) Whole mounts of mammary glands from 16-wk-old virgin female EZH2wt;neu and EZH2+;neu mice (n = 10 mice per group). (Magnification: 200×). Average number of tertiary branches ± SD (*P = 0.01). (B) Representative H&E-stained histological sections of mammary glands of EZH2wt;neu and EZH2+;neu mice at 8 and 16 wk of age. Arrow shows atypical intra ductal hyperplasia in EZH2+;neu mice. Immunohistochemical detection of EZH2, NICD1, phosphorylated-STAT3 (p-STAT3), and Ki-67 proteins. (Magnification: 400×). Bar graphs show the percentages of relative expression ± SD at 16 wk quantified using FRIDA software (*P < 0.0004). (C) Kaplan–Meier curve shows that EZH2+;neu mice (n = 30) formed mammary carcinomas significantly earlier than EZH2wt;neu mice (n = 25) (median time to tumor initiation: 243 ± 295 d, respectively; log-rank P < 0.0001). (D) Representative pictures of mammary tumors from EZH2wt;neu and EZH2+;neu mice stained for H&E and immunostained for EZH2 and NICD1. (Magnification: 400×). Percentage of relative expression ± SD was quantified using FRIDA software (*P < 0.003, **P = 0.009).
Transgenic EZH2 Overexpression Up-Regulates NOTCH1, Increases Stem Cells, and Accelerates Tumor Initiation. Because of tumor latency, the mouse mammary tumor virus (MMTV)-neu mouse model is well-suited to test the effect of EZH2 overexpression on accelerating tumor initiation (25). Mammary-specific EZH2 transgenic mice developed in our laboratory (26) were crossed with MMTV-neu mice (27). Female 8-wk-old virgin EZH2-neu mice exhibited ductal hyperbranching compared with EZH2wt-neu mice (Fig. 5A). Virgin EZH2wt-neu mice developed atypical intraductal hyperplasia similar to human disease, had up-regulation of NOTCH1 signaling proteins, and increased cell proliferation, compared with EZH2wt-neu mice (Fig. 5B). EZH2wt; neu mice formed invasive mammary carcinomas significantly earlier than EZH2wt; neu mice (Kaplan–Meier, log-rank P < 0.0001; Fig. 5C). Although no notable histopathological differences were apparent, EZH2wt; neu tumors exhibited increased EZH2 and NICD1 (Fig. 5D). Collectively, these data provide direct in vivo evidence that precancerous EZH2 up-regulation promotes atypical epithelial hyperplasia with heightened NOTCH1 pathway activation and that EZH2 overexpression is sufficient to accelerate tumor initiation in MMTV-neu mice.

Flow cytometric analyses in the lineage negative (Lin−) population using ESA and CD49f, shown to delineate cellular subsets in MMTV-neu mice and in the human breast (27, 28), demonstrated that EZH2wt; neu glands had increased stem cells (Lin− ESAmed CD49fhigh) and progenitors (Lin− ESAhigh CD49fmed) compared with EZH2wt; neu glands (Fig. S6A). The transition from preneoplasia to tumor formation in MMTV-neu mice is characterized by an increase in ESA−, CD49f−, and CD61−expressing cells detected by flow cytometry (28). Using dual immunohistochemistry, we detected areas of increased numbers of tumor cells expressing these markers in EZH2wt; ne tumours compared with EZH2wt; neu tumours (Fig. S6D).

Transplantation experiments (29) showed that stem cells, but not progenitor cells, isolated from preneoplastic EZH2wt; neu and EZH2wt; neu mammary glands exhibited in vivo gland-reconstituting activity. Of note, stem cells from EZH2wt; neu glands formed hyperplastic outgrowths with increased number and size of terminal end buds, higher NICD1 and Ki-67 expression, and elevated percentages of stem cells compared with EZH2wt; neu outgrowths (Fig. S6B and C).

EZH2/NICD1 Axis in TN Breast Cancer Tissues. To examine whether EZH2-mediated regulation of NOTCH1 and TICs exists in tumor tissues, tissue microarrays from 143 primary invasive breast carcinoma patients (67 luminal, 9 HER-2/neu over-expressing, 58 TN, and 9 unknown subtype) were interrogated for EZH2 in non-neoplastic breast tissue samples immunostained for EZH2 and NICD1 and coimmunostained with CD44 (red) and CD24 (brown). Case 1 is a luminal-type invasive carcinoma with low EZH2 expression, negative NICD1, and CD44+/CD24−. Case 2 is a TN invasive carcinoma with high EZH2 expression and positive NICD1 and contains CD44+/CD24− cancer cells. Case 3 is a representative picture of a tumor em-

Discussion

EZH2 is an independent marker of recurrence and metastasis in women with breast cancer, where EZH2 overexpression occurs mainly in TN compared with luminal tumors (11). Despite major advances in diagnosis and treatment, there is a considerable gap in our understanding of the mechanisms that induce breast cancer development and progression. In this study, we identify a previously undescribed role for EZH2 in regulating NOTCH1-dependent breast TIC expansion and show that EZH2 has a direct role in breast cancer progression.

We found that EZH2 expression regulates the abundance of TICs in vitro and in vivo. Ectopic EZH2 expression increased the stem cell pool in nonmutumorigenic breast cells, whereas EZH2 down-regulation reduced the breast TIC population in vitro and in xenograft studies. Our data strengthen those from an earlier study showing that EZH2 can promote breast TIC expansion (13) and further demonstrate the consequences of EZH2 levels on breast cancer initiation. EZH2 down-regulation in TN breast cancer cells retarded breast cancer initiation. Providing previously unidentified in vivo evidence for a role of EZH2 in breast cancer.
cancer initiation in transgenic models, overexpression of EZH2 in MMTV-neu mice decreased the latency to breast cancer onset. The role of EZH2 in promoting breast stem cell expansion and cancer initiation sheds light on our previous study showing that EZH2 is increased in histologically normal breast tissues from women up to 12 y before they develop breast cancer (12). From a clinical perspective, blocking EZH2 may prevent or ameliorate cancer initiation in women with high EZH2 protein expression levels in their breast epithelium.

Despite interest in the association between EZH2 functions, breast TICs, and TN breast cancer (13), the molecular mechanisms underlying the tumorigenic function of EZH2 in this cancer subtype and the relationship to NOTCH1 signaling have not yet been considered. Furthermore, whereas a role for NOTCH1 in breast tumorigenesis has been established in vivo (30), the factors regulating increased NOTCH1 expression and signaling in breast cancer cells are largely unknown (31, 32). We show that EZH2 is a regulator of NOTCH1 expression and pathway activation in TN breast cancer and that NOTCH1 signaling activation is required for EZH2-dependent stem cell expansion. The association and mechanistic link between EZH2 and NOTCH1 was validated in vitro, in vivo, and in human breast cancer samples. The relevance of our findings is further supported by a research study, which, by using the NOTCH-GFP reporter assay used here, demonstrated that NOTCH activity identifies the cancer stem cell population in the cancer-stem (20).

Substantial studies show that the canonical function of EZH2 is exerted via transcriptional repression through its HMT activity on H3K27 (4, 11, 13, 22). Most studies have focused on PRC2-mediated repression of tumor suppressor genes as the main oncogenic mechanism of EZH2 (6). More recently, EZH2 was shown to activate transcription via non-PRC2-mediated mechanisms, including interaction with RelA/RelB to activate NF-κB targets (9). Our study defines a unique role and mechanism for EZH2 in TN breast cancer, whereby EZH2 binds to the NOTCH1 promoter and induces epigenetic activation. We demonstrate that the amino-terminal HII domain of EZH2 mediates binding to the NOTCH1 promoter and that the HMT activity of EZH2 is not required for NOTCH1 transcriptional activation. Our results are consistent with a recent study documenting the importance of the amino-terminal domain of EZH2 in enhancing gene transactivation through an HMT-independent mechanism in breast cancer (7). Together, our data strengthen the emerging notion that overexpressed EZH2 may function through non-canoncal mechanisms leading to activation of target genes in an HMT-independent manner.

In clinical invasive breast cancer samples, high EZH2 and NCD1 are significantly coexpressed in TN compared with luminal tumors, supporting the contention that the EZH2/NCD1 axis is operative in vivo and in humans. Furthermore, EZH2hi/NCD1+ tumors are more frequently poorly differentiated and exhibit high numbers of TICs compared with tumors without this phenotype. In conclusion, our findings establish a previously unrecognized link between EZH2, NOTCH1 signaling activation, and TICs in TN invasive carcinomas. These data advance the current understanding of the mechanisms of EZH2 in breast cancer and lend support to the emerging transcriptional activating role of EZH2. By providing previously unidentified direct evidence that EZH2 overexpression accelerates breast cancer initiation in vivo our work paves the way to targeting EZH2 to halt breast cancer progression.

Materials and Methods

Detailed protocols regarding cell culture, vectors, pharmacologic treatments, Western blot analyses, antibodies, microarrays, mammomassary assays, flow cytometry, ChIP analyses, immunohistochemistry, and animal studies are described in SI Materials and Methods. The breast cancer patient cohort has been described previously (see SI Materials and Methods). Original Western blots are shown in Fig. S7.

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References

6. Bracken AP, et al. (2003) EZH2 is downstream of the pRB-E2F pathway, essential for tumorigenesis and that NOTCH signaling activation is required for EZH2-dependent stem cell expansion. The association and mechanistic link between EZH2 and NOTCH1 was validated in vitro, in vivo, and in human breast cancer samples. The relevance of our findings is further supported by a research study, which, by using the NOTCH-GFP reporter assay used here, demonstrated that NOTCH activity identifies the cancer stem cell population in the cancer-stem (20).

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