NOTCH and EZH2 collaborate to repress PTEN expression in breast cancer

Kyrie Pappas1,2,3,6, Tiphaine C. Martin1,2, Andrew L. Wolfe1,2,7, Christie B. Nguyen1,2, Tao Su4, Jian Jin1,2,5, Hanina Hibshoosh4 & Ramon Parsons1,2,7

Downregulation of the PTEN tumor suppressor transcript is frequent in breast cancer and associates with poor prognosis and triple-negative breast cancer (TNBC) when comparing breast cancers to one another. Here we show that in almost all cases, when comparing breast tumors to adjacent normal ducts, PTEN expression is decreased and the PRC2-associated methyltransferase EZH2 is increased. We further find that when comparing breast cancer cases in large cohorts, EZH2 inversely correlates with PTEN expression. Within the highest EZH2 expressing group, NOTCH alterations are frequent, and also associate with decreased PTEN expression. We show that repression of PTEN occurs through the combined action of NOTCH (NOTCH1 or NOTCH2) and EZH2 alterations in a subset of breast cancers. In fact, in cases harboring NOTCH1 mutation or a NOTCH2 fusion gene, NOTCH drives EZH2, HES-1, and HEY-1 expression to repress PTEN transcription at the promoter, which may contribute to poor prognosis in this subgroup. Restoration of PTEN expression can be achieved with an EZH2 inhibitor (UNC1999), a γ-secretase inhibitor (Compound E), or knockdown of EZH2 or NOTCH. These findings elucidate a mechanism of transcriptional repression of PTEN induced by NOTCH1 or NOTCH2 alterations, and identifies actionable signaling pathways responsible for driving a large subset of poor-prognosis breast cancers.
Phosphatase and tensin homolog deleted on chromosome ten (PTEN) is a haploinsufficient, dosage-sensitive tumor suppressor that is commonly inactivated or downregulated in cancer. Although genetic mutation of PTEN is frequent across many cancer types\(^1\)\(^{-}\)\(^5\), the loss of PTEN activity in cancer more often occurs in the absence of mutation through complex mechanisms including epigenetic transcriptional repression, microRNAs, noncoding RNAs, and posttranslational modifications, among others\(^6\)\(^{-}\)\(^8\). In fact, just a 20% decrease in PTEN levels is sufficient to develop breast tumors, and the progressive reduction of PTEN levels is associated with increasingly aggressive tumor phenotypes\(^9\)\(^{-}\)\(^10\).

Transcription of PTEN can be both positively and negatively regulated by a wide variety of transcription factors and chromatin modifying complexes. PTEN has been documented to be transcriptionally activated by peroxisome proliferation-activated receptor γ (PPARγ), early growth-regulated transcription factor-1 (ERG1), p53, and activating transcription factor 2 (ATF2)\(^11\)\(^{-}\)\(^15\). Conversely, PTEN has been shown to be transcriptionally repressed by c-Jun and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB)\(^16\)\(^{-}\)\(^17\). Two transcription factors, Snail and inhibitor of DNA binding (ID1), can compete for binding with p53 on the PTEN promoter to repress PTEN transcription\(^18\)\(^{-}\)\(^19\). Interestingly, NOTCH can regulate the expression of PTEN through opposing mechanisms depending on the context. Constitutively active NOTCH1 can induce expression of PTEN by the MYC and CBF-1 transcription factors in embryonic kidney cells\(^20\)\(^,\)\(^21\) and through direct binding of the Notch intracellular domain to the PTEN promoter in endothelial cells\(^22\), and can repress PTEN through binding of the HES-1 transcription factor to the PTEN promoter in T cells\(^23\). The polycomb repressive complex 2 (PRC2) binds chromatin and represses PTEN transcription in nasopharyngeal epithelial cells and leukemia through enhancer of zeste homolog 2 (EZH2)-mediated trimethylation of histone 3 lysine 27 (H3K27Me3) at the PTEN promoter\(^24\)\(^,\)\(^25\), and is reported to be guided to the site of action at the PTEN locus by long noncoding RNA (lncRNA) originating from the PTENP1 pseudogene locus in certain contexts\(^26\). Furthermore, histone deacetylases (HDACs) can also restrain PTEN expression\(^27\).

In breast cancer, downregulation of PTEN occurs frequently, especially in poor-prognosis triple-negative breast cancer (TNBC) without any evidence of genetic alteration of the PTEN locus in most cases\(^28\)\(^,\)\(^29\). In fact, PTEN expression is diminished in 19% of all breast cancers, and in over 50% of TNBCs (RNA-seq z-score cutoff for downregulation < -1)\(^30\)\(^,\)\(^31\), where PTEN transcript level rather than mutation or posttranslational modification is the primary determinant of PTEN protein expression\(^28\). Baseline expression of p53 also controls PTEN expression in breast cancer, where p53 mutation is associated with decreased expression\(^15\). NOTCH1 and NOTCH2 mutations occurring in breast cancer are required for tumor viability but the signaling pathways through which they maintain tumor growth are similarly unclear\(^32\).

To clarify the mechanisms responsible for the silencing of PTEN expression, we carried out an analysis of primary breast cancer samples, adjacent normal epithelial tissue, and existing breast cancer datasets for genes that could be responsible for PTEN loss of expression and determined that NOTCH1 or NOTCH2 alterations (mutation, fusions, or overexpression) occur in a large proportion of TNBC cases exhibiting PTEN downregulation. Using breast cancer cell lines, we found that mutant forms of NOTCH1 or NOTCH2 collaborate with EZH2 to mediate the transcriptional repression of PTEN in these poor-prognosis breast cancers. PTEN expression could be restored by interfering with NOTCH or EZH2 function, thus highlighting a therapeutic strategy for these patients.

**Results**

The PTEN locus is transcriptionally repressed in breast tumors relative to normal breast tissue. Many previous measurements of PTEN mRNA have been based on a comparison among tumors without regard to the normal level of expression. However, PTEN mRNA measurements correlate well with protein levels measured by immunohistochemistry (IHC)\(^32\), which are scored relative to normal epithelium in the same section. To better understand the relationship between normal mammary epithelial tissue and breast tumor tissue expression, and to potentially identify cell culture models for studying PTEN downregulation, we measured expression of PTEN in epithelial cells isolated from normal breast and a large series of breast cancer cell lines that were genetically wild-type for PTEN\(^33\). PTEN transcript was downregulated in breast cancer cell lines compared to normal mammary epithelial cells in breast cancers of all subtypes, and the same was true regarding the neighboring gene ATAD1, that shares an enhancer with PTEN, and is part of the PTEN-loss signature (Fig. 1a, Supplementary Table 1)\(^28\). mRNA levels of ATAD1 are highly correlated with PTEN mRNA levels in a large cohort of breast cancers of all subtypes (P < 0.0001, Supplementary Fig. 1a)\(^30\)\(^,\)\(^31\). These data suggest that breast tumor cell lines could be potential models for studying the downregulation of PTEN by epigenetic regulation of the neighborhood of chromatin including PTEN, and also suggest that PTEN is more frequently downregulated than previously realized.

To determine if the PTEN mRNA downregulation in tumors relative to normal was also present in patient samples, we used Nanostring digital barcoding technology to measure PTEN expression in normal and tumor breast biopsy samples (four normal samples, four normal–tumor pairs) using multiple different probes, and determined that PTEN levels are decreased in tumor compared to normal samples (Supplementary Fig. 1b, Supplementary Data 1, Supplementary Table 2). Furthermore, PTEN-downregulated samples were associated with changes in the expression of selected previously published PTEN-loss signature genes including ATAD1 (Supplementary Fig. 1c and Supplementary Data 1), indicating agreement between the breast cell lines and patient samples.

PTEN expression inversely correlates with EZH2 and NOTCH1. To explore the cause of loss of PTEN expression in breast cancer, we treated breast cancer cell lines with the epigenetic inhibitors Trichostatin A (TSA), which inhibits Class I and II histone deacetylases (HDACs), and 5-aza-2'-deoxycytidine (AZA) which is a cytidine analog that blocks DNA methylation (Fig. 1b, c). We saw substantial restoration of PTEN transcript levels in multiple cell lines following TSA treatment, with the exception of MDA-MB-231 (Fig. 1b). This restoration was not as apparent with AZA treatment, where only HCC-1187 and BT-20 cells showed an increase in PTEN transcript upon treatment (Fig. 1c). We assessed potential regulators of PTEN for their association with PTEN mRNA in a large cohort of breast cancer cases from The Cancer Genome Atlas (TCGA)\(^30\)\(^,\)\(^31\)\(^,\)\(^34\). Interestingly, EZH2, the histone lysine methyltransferase component of the PRC2 complex, had the largest inverse correlation with PTEN expression in a large cohort of breast cancer cases, and the transcriptional regulator NOTCH1 was also inversely correlated with PTEN expression (Fig. 1d, Supplementary Data 2 for all genes). Furthermore, the expression of HDACs (HDAC1 and HDAC2) that interact with the PRC2 complex was also negatively correlated with PTEN expression (Fig. 1d, Supplementary Data 2), consistent with the result of TSA treatment in breast cancer cell lines. The expression of the ATAD1 and KLLN genes that are in genomic proximity to the PTEN locus were strongly
positively correlated with PTEN expression, as previously reported\textsuperscript{28}, again suggesting a common epigenetic mechanism of regulation controlling the region (Fig. 1d, Supplementary Data 2).

To characterize a subset of seven cell lines further, we performed immunoblot measurements of PTEN from protein lysates and confirmed that PTEN protein levels reflect the change in mRNA expression (Fig. 1e, Supplementary Fig. 1d). In a larger cohort of 841 breast tumors, PTEN transcript and protein levels are highly correlated ($P < 0.0001$, Supplementary Fig. 1e)\textsuperscript{30,31}. Furthermore, we show that EZH2 protein levels are increased in breast cancer cell lines compared to normal breast epithelial cells (Fig. 1e, Supplementary Fig. 1d). We measured PTEN and EZH2
protein expression by IHC in breast cancer cases, and we observed the same inverse correlation (Fig. 1f, g, Supplementary Data 3). Generally, PTEN staining decreases in tumor versus adjacent normal, and EZH2 staining increases, though the magnitude of the changes between tumor and normal varies between cases (Fig. 1f). These results strengthen the observation that PTEN downregulation is exceptionally frequent in all subtypes of breast cancer (Supplementary Data 3). Adjacent normal ducts typically have low/no EZH2 staining and robust PTEN protein levels (Fig. 1g). We also observed that EZH2 levels in tumor (compared to normal) tend to be higher in TNBC cases (Supplementary Data 3). In fact, in a larger cohort of breast cancer cases\(^{34}\), we found that EZH2 mRNA expression is increased in more aggressive subtypes of breast cancer including TNBC (Fig. 1h)\(^{30,31,34}\). Thus, we decided to investigate a putative PR2-mediated mechanism by which PTEN may be transcriptionally downregulated in breast cancer cell lines and primary tumors.

The PTEN promoter contains repressive domains that are prominent in some breast cancer cell lines. We sought to determine which regions of the PTEN promoter are the most important for PTEN transcriptional repression in breast cancer. We chose a panel of PTEN wild-type cell lines comprised of the non-tumorigenic mammary epithelial cell line MCF10A, and breast cancer cell lines that have low PTEN transcript levels, including HCC-1187, MDA-MB-157, SUM-159, BT-20, and HS758T. We performed a luciferase reporter assay using previously defined sections of the PTEN promoter/ regulatory region that contain transcriptionally active chromatin elements in various non-tumorigenic breast cell lines and tissue (separated by compartment) as well as in breast cancer cell lines (Supplementary Fig. 2) fused to a luciferase reporter gene (Fig. 2a)\(^{23}\). Furthermore, this region contains hotspot mutations in breast cancer\(^{35}\), suggesting its importance in transcriptional regulation of PTEN (Supplementary Fig. 2). The strength of the luciferase signal corresponds to the transcriptional activity of that section of the PTEN promoter. We found that compared to the longest PTEN promoter/regulatory region reporter, multiple truncated portions caused increased transcriptional reporter activity in all of the cancer cell lines with a particularly notable increase for HCC-1187, consistent with the full-length PTEN promoter containing repressive elements (Fig. 2b). On the other hand, for the non-tumorigenic line MCF10A, only one truncated region (Pgl3-2) led to a relatively modest increase in PTEN transcription, indicating that less active repressive mechanisms may also be present in non-tumorigenic cells (Fig. 2b).

**Fig. 1 Repression of the PTEN locus occurs in breast cancer, and associates with increased expression of EZH2 and other known transcriptional regulators.** a PTEN (red) and ATAD1 (blue) transcript levels were measured using qRT-PCR in breast cancer cell lines compared to normal mammary epithelial cells. Error bars are mean ± s.d., triplicate measurements. Significance from normal mammary epithelial cells derived from normal mammary speci...m: two-way ANOVA, Dunnett’s correction. b Trichostatin A (TSA) and (c) 5-aza-2'-deoxycytidine (AZA) treatment was performed at the indicated doses and transcript levels of PTEN were measured by qRT-PCR in breast cancer cell lines. Error bars: mean ± s.d., triplicate measurements. Significance from Ctrl: one-way ANOVA, Sidak’s correction. d Analysis of co-expression with PTEN including Pearson correlation coefficients and P values in normalized RNA-seq data for previously reported PTEN-repressing genes and genes in the PTEN genomic locus (ATAD1 and KLLN). Cohort includes 818 breast cancer cases\(^{34}\). Significance: two-tailed t test, Pearson correlation (n = 818), black dot indicates a statistically significant result. E Protein levels of EZH2 and PTEN were measured by immunoblotting in the panel of indicated cell lines. Vinculin was loading control. Quantification is shown below each blot (signal normalized to Vinculin, calculated as a fold change compared to normal breast epithelium isolated from normal mammary speci...m: two-way ANOVA, Dunnett’s correction. (****P < 0.0001; ***P < 0.001; **P < 0.01; *P < 0.05).

**EZH2 binds to the PTEN genomic locus at regions important for transcriptional repression containing H3K27 trimethylation.** We then further investigated epigenetic landscape of the PTEN promoter/ regulatory region that are important for transcriptional repression in cancer. To do this, we performed chromatin immunoprecipitation (ChIP) coupled with qPCR for H3K27Me3, the mark of PRC2-mediated transcriptional repression, in the HCC-1187 TNBC cell line, which we selected because it is PTEN wild-type, exhibited marked co-downregulation of PTEN and ATAD1 (Fig. 1a), and showed the greatest amount of reporter activation when portions of the PTEN promoter were deleted (Fig. 2b). Examination of HCC-1187 at the PTEN promoter/ regulatory region revealed extensive H3K27Me3 (Fig. 2c). Notably, the regions containing interaction with H3K27Me3 overlapped with those that were important for transcriptional repression in the luciferase reporter assay (Fig. 2b). To determine if the PRC2 member EZH2 could also be present in this region, we performed ChIP for EZH2 and observed binding of EZH2 to the PTEN promoter/ regulatory region in the same regions of chromatin (Fig. 2d). This result suggested that the PRC2 complex acts at the PTEN locus and may contribute to the transcriptional repression of PTEN observed in breast cancer.

**Depletion of EZH2 restores PTEN expression in cases harboring NOTCH1 or NOTCH2 mutations.** We next wanted to investigate a possible role for EZH2 in the repression of PTEN, which we examined in HCC-1187 and two additional cell lines that exhibited low levels of PTEN (in the absence of mutation), HCC-1954, and MDA-MB-231. We performed a stable knockdown of EZH2 in HCC-1187 cells and observed that PTEN transcript and protein levels were restored following knockdown (Fig. 3a, d, respectively, Supplementary Fig. 2b). We saw a similar increase in PTEN transcript and protein levels following EZH2 knockdown in HCC-1954 cells (Fig. 3b, e, respectively) but not in MDA-MB-231 cells (Fig. 3c, f, Supplementary Fig. 2b). Furthermore, transcriptional activity at the PTEN promoter was increased upon EZH2 knockdown in both HCC-1187 and HCC-1954 cells (Fig. 3g, h). It has been previously demonstrated that subtle variations in PTEN dose can also influence tumorigenic properties such cell proliferation\(^9\). The effect of EZH2 knockdown on proliferation was evaluated in HCC-1187 and MDA-MB-231, and EZH2 knockdown decreased proliferation in HCC-1187 cells (Fig. 3i) but did not change proliferation in MDA-MB-231 cells (Fig. 3j), suggesting that the decrease in proliferation in HCC-1187 cells may at least in part be due to the increase in PTEN expression.
As S-adenosyl-L-methionine (SAM)-competitive EZH2 inhibitors are highly selective and are currently showing promise in clinical trials for lymphoma and malignant rhabdoid tumors, we decided to test whether the preclinical EZH2 inhibitor UNC1999 could restore expression of PTEN in breast cancer cell lines. UNC1999 restored PTEN expression considerably in the HCC-1187, HCC-1954, and HCC-2218 cell lines, restored it weakly in MDA-MB-175-VII, but did not in the MDA-MB-231 or HCC-1143 cell lines (Fig. 4a–f), and these results were consistent with our EZH2 stable knockdown experiments. Given the reported association between NOTCH and EZH2 in invasive breast cancer, we examined the NOTCH status of these cell lines and found that the three cell lines that restored PTEN expression considerably in response to EZH2 inhibition or knockdown all harbor mutations or fusions in NOTCH1 or NOTCH2, whereas the other cell lines harbor wild-type NOTCH1 and NOTCH2 (Fig. 4g). To determine the relevance of our findings in tissue culture to the downregulation of PTEN that is observed in human breast tumor surgical samples, we examined the large TCGA data cohort containing mRNA and protein expression data and found that cases expressing high levels of EZH2 (RNA-seq z-score >1, about 15% of all breast cancers and luminal A tumors are highly selective and are currently showing promise in clinical trials for lymphoma and malignant rhabdoid tumors) tended to have increased expression of NOTCH1 and mutations in breast cancer can increase activity or create a truncated form of NOTCH resembling cleaved NOTCH that enters the nucleus to regulate transcription of target genes.

Interestingly, the HCC-1187 cell line, where we detected evidence of EZH2-mediated repression of PTEN, harbors a transforming SEC22-NOTCH2 translocation. To examine the role of NOTCH2 in the repression of PTEN in HCC-1187 cells, we performed a stable knockdown of NOTCH2 and observed a restoration of PTEN transcription and protein levels (Fig. 5a, b, Supplementary Fig. 3d). Concomitant with the stable restoration of PTEN transcript and protein levels (Fig. 5a, b, Supplementary Fig. 3d), we observed a decrease in the NOTCH target genes HEY-1 and HES-1, as well as a decrease in EZH2 transcript and protein levels (Fig. 5a, b, Supplementary Fig. 3d). These results show that depletion of EZH2 activity may be effective at restoring repressed PTEN in cases harboring NOTCH alterations.

Mutant NOTCH drives both increased expression of EZH2 and transcriptional repression of PTEN in breast cancer. We demonstrated that a subset of breast cancer cell lines exhibit reduced expression of PTEN mediated by the PRC2 complex, but we wanted to investigate the possible upstream signaling changes that cause increased PRC2 activity at the PTEN promoter. EZH2 can activate NOTCH signaling in breast cancer, and we have observed a correlation between increased NOTCH1 expression and the presence of a PRC2 repressive mark at the PTEN promoter. However, we have also observed that NOTCH1 can lead to repression of PTEN through the HES-1 transcription factor in T cells, and NOTCH1/2 translocations and mutations in breast cancer can increase activity or create a truncated form of NOTCH resembling cleaved NOTCH that enters the nucleus to regulate transcription of target genes.

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Fig. 3 Depletion of EZH2 restores PTEN expression and reduces proliferation in a subset of cell lines. Stable shRNA knockdown of EZH2 was performed. EZH2 and PTEN transcript levels were measured by qRT-PCR (a–c) and protein levels were measured by immunoblotting in (d–f) HCC-1187 cells, HCC-1954 cells, and MDA-MB-231 cells, respectively. Vinculin was loading control. Quantification is shown below each blot (signal normalized to Vinculin, calculated as a fold change compared to Empty Vector). Error bars: mean ± s.d. (triplicate measurements). Significance from Ctrl: two-way ANOVA, Tukey’s correction. Luciferase activity at the PTEN promoter was measured (Pgl-1 from Fig. 2a) following stable knockdown of EZH2 in (g) HCC-1187 and (h) HCC-1954 cells. Error bars: mean ± s.e.m., n = 3 experiments. Proliferation was measured following EZH2 knockdown, the percentage of confluence over time (days) is displayed in (i) HCC-1187 cells and (j) MDA-MB-231 cells. Readings taken every 6 h. Error bars: mean ± s.d., triplicate measurements. Corresponding representative photos from indicated timepoints (red arrow) shown, Scale bar (red): 800 µm. Significance from Ctrl: two-way ANOVA, Tukey’s correction. (**P < 0.001; ***P < 0.0001; ****P < 0.0001).
same sites on the PTEN promoter (Fig. 5c). Furthermore, HES-1 and EZH2 binding to the PTEN promoter was diminished by NOTCH2 knockdown (Fig. 5c).

The HCC-1954 and HCC-2218 cell lines harbor NOTCH1 mutations (missense and translocation, respectively)\(^{32,40}\). To investigate the role of NOTCH1 in the transcriptional repression of PTEN in these cell lines, we inhibited downstream NOTCH signaling with the γ-secretase inhibitor Compound E (CompE). In HCC-1954 cells, treatment with CompE caused a decrease in both HES-1 and HEY-1 transcript levels, whereas in HCC-2218 cells, the treatment only inhibited HEY-1 transcript levels (Fig. 5d). This result is consistent with previous research showing that the NOTCH1 translocation present in HCC-2218 cells signals primarily through HEY-1\(^{32}\). Furthermore, treatment with CompE resulted in an increase in PTEN transcript levels in both HCC-1954 and HCC-2218 cell lines (Fig. 5d), and an increase in PTEN protein levels in HCC-1954 cells but not MDA-MB-231 cells (Fig. 5c, Supplementary Fig. 3d), further supporting this conclusion.

NOTCH1 and NOTCH2 alterations are correlated with reduced PTEN expression in breast cancer biopsies and represent a poor-prognosis subset of TNBC. To determine if NOTCH1 and NOTCH2 alterations could be regulating PTEN in tumor biopsies, we examined the same TCGA breast cancer cohort that we analyzed for EZH2\(^{30,31,34}\), and found that the presence of NOTCH1 or NOTCH2 mutations in breast cancer (about 3% of cases) correlated with reduced PTEN expression\(^{30,31}\) (Fig. 5f). Furthermore, within TNBC, a subtype of breast cancer harboring high expression of EZH2, combined NOTCH1 and NOTCH2 mutation or overexpression (occurring in about 30% of TNBC cases) is associated with decreased overall survival and decreased disease-free survival\(^{30,31,34}\) (Fig. 5g), which likely depends on multiple NOTCH outputs including PTEN. These results combined with our cell line findings suggest that alteration of NOTCH1 or NOTCH2 (mutation or overexpression) could contribute to increased PRC2 complex activity at the PTEN promoter in these breast cancers, and may account for a large proportion of the

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**Fig. 4** Mutation of NOTCH1/2 enhances restoration of PTEN expression in response to EZH2 inhibition. Inhibition of EZH2 by UNC1999 was performed for 7 days and 11 days and transcript levels of PTEN were measured by qRT-PCR in (a) HCC-1187, (b) HCC-1954, (c) HCC-2218, (d) MDA-MB-231, (e) HCC-1143, and (f) MDA-MB-175-VII. Error bars: mean ± s.d., triplicate measurements. Significance from Ctrl: one-way ANOVA, Sidak’s correction.

**Table** shows the NOTCH1/NOTCH2 mutation status of breast cancer cell lines\(^{32,40}\).

**Boxplots of NOTCH1 and PTEN RNA and protein levels in EZH2-high (RNA-seq z-score > 1) and EZH2-low (RNA-seq z-score < 1) breast cancer cases, measured by RNA-seq and RPPA, respectively\(^{34}\). Number of cases indicated for each group (total n = 816). (**P < 0.0001; ***P < 0.001; **P < 0.01; *P < 0.05).
PTEN-downregulated cases in TNBC. The HES-1 and HEY-1 transcription factors could recruit the PRC2 complex to the PTEN cis-regulatory elements such as the PTEN promoter. Moreover, these results suggest that this signaling activity could be responsible for the poor prognosis for this subset of patients.

Discussion

Here, we discover that dysregulation of both PTEN and EZH2 occurs in almost all breast cancers when compared to adjacent normal ducts, regardless of subtype. However, the degree of downregulation of PTEN and upregulation of EZH2 is most
Increased expression of NOTCH1 and EZH2 individually associate with poor prognosis in breast cancer, and their expression levels are positively correlated with one another. Although mutation of NOTCH1 or NOTCH2 only occurs in a small proportion of breast cancer cases, inclusion of cases harboring both mutation and overexpression accounts for 30% of poor-prognosis TNBC cases. It has been reported that MDA-MB-231 cells harbor a slight elevation in NOTCH1 protein and thus, are subtly susceptible to a γ-secretase inhibitor (PZ0187), which is cytostatic to subcutaneous MDA-MB-231 xenografts, and has a slight restorative effect on PTEN protein levels. We did not see the same effect on PTEN with a different γ-secretase inhibitor (CompE). These studies combined with our findings support the idea that NOTCH1 or NOTCH2 alterations could generate a broadly relevant positively reinforcing feedback loop between the NOTCH and EZH2 pathways that may have important roles in driving a large proportion of the poor-prognosis cancers that display downregulation of PTEN (Fig. 6). Importantly, the action of NOTCH is highly tissue specific; therefore, the insight that NOTCH plays this potentially oncogenic role in the repression of PTEN in poor-prognosis breast cancer is of interest to the field. It is possible that cells in the tumor microenvironment can activate NOTCH signaling and downregulate PTEN in a cell non-autonomous manner through juxtacrine signaling to tumor epithelial cells, as has been demonstrated with macrophages and other components of the breast cancer stroma. Additionally, ER-negative breast cancer stem cells rely on NOTCH-dependent paracrine signaling from ER-positive cells in the mammary epithelium.

Our findings also suggest that NOTCH1 and NOTCH2-driven breast cancers may represent a distinct biological form of breast cancer that is driven in part through the silencing of the PTEN tumor suppressor gene, in addition to the activation of oncogene targets such as MYC. Interestingly, the repression of tumor suppressors by polycomb group (PcG) proteins including PRC2 could be a more broadly relevant mechanism of tumor suppressor repression in cancer. Collaboration between PcG proteins and the NOTCH pathway contributes to malignancy in Drosophila through silencing of the retinoblastoma (Rb1) tumor suppressor. In fact, in breast cancer tumor samples, RB1 expression levels are correlated with PTEN expression, and inversely correlated with EZH2, HDAC1, HDAC2, and NOTCH1 expression (Supplementary Data 4), which suggests that a similar mechanism of repression may be at play for RB1. However, correlation does not necessarily imply a mechanistic linkage, and this hypothesis warrants further experimental testing.

A large body of research has demonstrated that PTEN is a haploinsufficient tumor suppressor that is extremely dosage sensitive. Thus, the strong transcriptional downregulation of PTEN observed in many types of cancer, including TNBC, could contribute to tumorigenic phenotypes in many cases. Taken together, our results suggest that NOTCH and EZH2, working together in a feed forward loop, could control tumorigenic phenotypes in a subset of breast cancer cases through repression of PTEN expression. EZH2 is a promising therapeutic target for many different types of cancer, and our results show that EZH2 inhibitors have the potential to restore PTEN expression, which may present therapeutic benefit in breast cancer patients with NOTCH alterations. The development of compounds to target EZH2 in cancer remains to be an area of active interest, and EZH2-targeting compounds have been published displaying increased specificity and potency, including novel EZH2 degraders. Further preclinical studies should include these improved inhibitors and degraders, as the effects on tumor cell viability in vitro and in vivo may be improved. Even though EZH2 can act as a tumor suppressor in certain tissues, EZH2 appears to act exclusively as an oncogene in breast cancer. Our results and others show that γ-secretase inhibitors could also be a viable

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**Fig. 6 Model of transcriptional repression of PTEN.** The NOTCH and PRC2/EZH2 pathways form a regulatory loop where both can feed into the transcriptional repression of PTEN. Mutation or translocation of NOTCH1 or NOTCH2 leads to upregulation of HES-1/HEY-1 and PRC2/EZH2; however, NOTCH mutation is not required for EZH2 activity or transcriptional repression. EZH2 signaling can also upregulate NOTCH38. PRC2/EZH2 adds the H3K27Me3 mark of transcriptional repression (purple dots), and binds with the HES-1 transcription factor on the PTEN promoter leading to repression. Numbers 1 and 2 in black boxes represent exons of PTEN.
approach to restore PTEN expression in a subset of NOTCH1/2-altered patients that still harbor the γ-secretase cleavage site, either alone or in combination with EZH2 inhibitors.

Importantly, therapies that specifically aim to restore PTEN expression represent a largely unexplored strategy to boost tumor suppressor signaling. The strategies used in this study to restore PTEN expression could be relevant to other tumor suppressors in breast cancer, such as RB1.

Methods

Cell culture. Cell lines were purchased from ATCC. ATCC authenticates cell lines using several methods, including DNA fingerprinting. Cell lines were further authenticated in 2015 by LabCorp using a short tandem repeat method. Cell lines were tested quarterly for mycoplasma, and tested negative throughout the period of this study as determined by the Lonza Kit (LT07-418). MDA-MB-435S was used in this study as determined by the Lonza Kit (LT07-418). CDNA was synthesized using the SuperScript Reverse Transcriptase II kit (Thermo 18064-014). The Applied Biosystems 7500 Fast Real-Time PCR System was used according to the manufacturer’s instructions.

Compound E (CompE) was prepared using the QiaShredder (79654) followed by the QIAquick PCR purification kit (QIAGEN). CDNA was synthesized using the SuperScript Reverse Transcriptase II kit (Thermo 18064-014). The Applied Biosystems 7500 Fast Real-Time PCR System was used according to the manufacturer’s instructions.

Luciferase reporter assay. Cells were seeded at 2 × 10^5 cells/well of Falcon six-well dishes. The transfections were carried out the following day using Lipofectamine (18324-020) and Plus (11514-015) reagents according to the manufacturer’s instructions. The cells were harvested 24 h later using reagents supplied by the Dual-Luciferase® Reporter Assay System (Promega E1910). Luciferase expression is normalized to Renilla activity, and was calculated as a fold change from the pg3-1 plasmid. The luciferase assays were performed as specified by the manufacturer’s instructions and were quantitated using a TD-20e Luminometer (Turner).

Luciferase plasmid. The pGL3 basic reporter vector was used (as described above). See below for the sections of the PTEN promoter that were cloned into the pGL3 vector, including the restriction sites that flank each section. These constructs were made by S. Nagase in the Parsons Laboratory and were sequenced before performing this experiment.

tQR-PCR. RNA was prepared using the QiaShredder (79654) followed by the Qiagen RNeasy Kit (74104). CDNA was synthesized using the SuperScript Reverse Transcriptase II kit (Thermo 18064-014). The Applied Biosystems 7500 Fast Real-Time PCR System was used according to the manufacturer’s instructions.

Table 1 qRT-PCR primers.

| Gene target | Sequence |
|-------------|----------|
| PTEN-For    | CCAGTCGTCGTAACACATC |
| PTEN-Rev    | CTTCTTCTCAGGATTTAGGG |
| ADAT1-For   | AGTTGCCGAGAAACACTGATG |
| ADAT1-Rev   | GTTGAACAGGGCGGATTTCA |
| EZH2-For    | TGGTTGGCGGAAGGCCGTAATTC |
| EZH2-Rev    | ACCCTTCTGAGTTGCAAGCC |
| NOTCH2-For  | GCCCTGGCAGACACACTGAGC |
| NOTCH2-Rev  | ACCCTTCTGAGTTGCAAGCC |
| NOTCH2-SEC22B-For | GGGTAAACTGTTGCGGCG |
| NOTCH2-SEC22B-Rev | GAGTGAAACCTTCATGAAATG |
| HES-1-For   | CTGGAATACGACGTGAAGGACCT |
| HES-1-Rev   | ATGGATCGTGGTGATCGG |
| HEY-1-For   | TGGTGACGTGGATCGG |
| HEY-1-Rev   | CCGGAAACCTGTTGCGGCG |
| GAPDH-For   | TACCCGAGGTGCTTTTAC |
| GAPDH-Rev   | AATAGGAAGGGGCTATGGG |

Antibodies. EZH2 (Roche-SP219), PTEN (CST-138G6).

Human tissue samples. De-identified breast tissue samples used for Nanosting were distributed by the Tumor Bank in the Herbert Irving Comprehensive Cancer Center Molecular Pathology Shared Resource. De-identified breast tissue samples used for IHC, as well as H&E stains were distributed by the Biorepository and Pathology core at Icahn School of Medicine at Mount Sinai. All samples were considered non-human subject research by the IRBs of each institution.

Immunohistochemistry. IHC was performed on formalin-fixed paraffin-embedded blocks from breast cancer cases from MSSM. Staining for EZH2 was performed and validated at the Molecular Cytology Core at MSKCC. Staining for PTEN was performed at Mount Sinai using the Leica-BOND automated IHC stainer. H&E slides for each case were provided with the blanks by MSSM. QuPath software was used for the analysis of IHC stain intensity quantification for PTEN and EZH2.

qRT-PCR. RNA was prepared using the QiaShredder (79654) followed by the Qiagen RNeasy Kit (74104). CDNA was synthesized using the SuperScript Reverse Transcriptase II kit (Thermo 18064-014). The Applied Biosystems 7500 Fast Real-Time PCR System was used according to the manufacturer’s instructions.

Table 1 qRT-PCR primers.

| Gene target | Sequence |
|-------------|----------|
| PTEN-For    | CCAGTCGTCGTAACACATC |
| PTEN-Rev    | CTTCTTCTCAGGATTTAGGG |
| ADAT1-For   | AGTTGCCGAGAAACACTGATG |
| ADAT1-Rev   | GTTGAACAGGGCGGATTTCA |
| EZH2-For    | TGGTTGGCGGAAGGCCGTAATTC |
| EZH2-Rev    | ACCCTTCTGAGTTGCAAGCC |
| NOTCH2-For  | GCCCTGGCAGACACACTGAGC |
| NOTCH2-Rev  | ACCCTTCTGAGTTGCAAGCC |
| NOTCH2-SEC22B-For | GGGTAAACTGTTGCGGCG |
| NOTCH2-SEC22B-Rev | GAGTGAAACCTTCATGAAATG |
| HES-1-For   | CTGGAATACGACGTGAAGGACCT |
| HES-1-Rev   | ATGGATCGTGGTGATCGG |
| HEY-1-For   | TGGTGACGTGGATCGG |
| HEY-1-Rev   | CCGGAAACCTGTTGCGGCG |
| GAPDH-For   | TACCCGAGGTGCTTTTAC |
| GAPDH-Rev   | AATAGGAAGGGGCTATGGG |
Table 2 ChIP-qPCR primers.

| Name          | Sequence                | Hg19 Loc. (Chr10) |
|---------------|-------------------------|-------------------|
| Site 1-For    | GAGAGATTTGGGACATGGGA    | 89618828          |
| Site 1-Rev    | GCCGTAAGTGTCGGCTACCA    | 8961913           |
| Site 2-For    | GCCACCCGTTGGTCACTGCCT   | 89620304          |
| Site 2-Rev    | CCCGGAGGACCTCTACCATG    | 8962093           |
| Site 3-For    | GCTCAGGGGGATGGACTGGA    | 8962149           |
| Site 3-Rev    | TTGAGGGTATCTCTCTGCTC    | 89621567          |
| Site 4-For    | GCCGGCTCAAAATGTTAGTGG   | 89625500          |
| Site 4-Rev    | CCCCATCCTAATCAAAACC     | 89625583          |
| Site 5-For    | GTGATGGGCGGGGACTCTTAT   | 89623313          |
| Site 5-Rev    | CTCTCCTCCCTGCGTCGAG     | 89623472          |

Primary antibodies. Vinculin (Sigma V9131, 1:10,000), β-actin (Sigma A3316, 1:10,000), PTEN (138G6, CST 9559 1:1000), EZH2 (Active Motif 39901, 1:1000), and HES-1 (H-140, SC-25392, 1:300).

Secondary antibodies. Mouse (Thermo 31432, 1:5000), Rabbit (Thermo 31460, 1:5000).

Chromatin immunoprecipitation (ChIP-qPCR). ChIP assays were performed as previously described61. In summary, cells were cross-linked in 1% formaldehyde (J. T. Baker 2106-01) for 5 min on ice. After quenching with glycine, the cells were harvested in 1× PBS containing 1× protease inhibitor cocktail (Sigma P8340) and pelleted. For ChIP-qPCR, cells were sonicated for 20 min (30 s on, 30 s off) on the Diagenode Bioruptor Twin (UCD-400) sonicator at 4 °C. Lysates were precleared for 1 h with Protein A Agarose/Salmon Sperm DNA beads (Emdmillipore 16-157). Precleared lysates were then incubated with 7 μg of antibody overnight at 4 °C. Samples were then incubated with beads (same as preclear) for at least 2 h at 4 °C and beads were repeatedly washed. The Protein-DNA complexes were eluted, crosslinks were reversed, and DNA was purified using phenol/chloroform extraction followed by sodium acetate/ethanol precipitation. % input was calculated and normalized as a fold change from IgG. Antibodies: IgG (sc-2025), EZH2 (Active Motif 39901), HES-1 (H-140, SC-25392), H3K27Me3 (Millipore 07-449). ChIP-qPCR primers are presented in Table 2.

Proliferation assay. Cells (Empty Vector and EZH2 shRNA#1 and EZH2 shRNA#2) were plated at 8000 cells/well (HCC-1187) or 2000 cells/well (MDA-MB-231) in 96-well tissue culture plates (Corning 3595) full media. Cells were allowed to grow for the indicated number of days. The Essen BioScience IncuCyte® ZOOM Live-Cell Analysis System took phase-contrast images in triplicate wells every 6 h. The IncuCyte® software package was used to estimate confluence at each time point.

Data from the cancer genome atlas (TCGA). TCGA data shown is from the invasive breast cancer dataset (818 cases)34 including pre-analyzed mutation, RNA-seq, and reverse phase protein array data was downloaded from the cBioPortal30,31,34 except for the data contained in Supplementary Fig. 1a, e, which are from the breast invasive carcinoma TCGA Firehose Legacy cohort (1108 cases). All complete tumors were used for any given analysis, and the number of cases used is indicated for each analysis.

Co-expression analysis. Genes that were co-expressed with PTEN in an invasive breast cancer dataset published by TCGA were analyzed using the co-expression tool in cBioPortal30,31,34. Pearson (r) scores were provided by cBioPortal and P values were calculated using a two-tailed t-test (n=818). P values were adjusted for multiple comparisons using the Benjamini and Yekutieli method62. We established a significance cutoff defined by a P value (P < 1.0 x 10^-7).

Gene set enrichment analysis (GSEA). Enrichment of the list of invasive breast cancer cases34 (pre-ranked from high to low EZH2 expression by RNA-seq z-score) for cases with mutations in the TP53 gene was quantified using the GSEA package63.

Analysis of chromatin landscape of PTEN promoter. Supplementary Fig. 2 was generated using R. Details and code can be accessed at the following link, https://github.com/TiphaineCMartin/Regulation_PTN_KyriePappas2018. Various previously published and publicly available datasets were used for this analysis from COSMIC (Release v84, February 14, 2018, cancer.sanger.ac.uk), ENCODE (ChromHMM IEMEC GEO sample accession: GSM356684, DNA-seq hMRC sample accession: GSM366364, MCP7 sample accession: GSM376588), ENSEMBL (Release 91 of GRCh37.13), and others15,55,64,67.

Statistics and reproducibility. No statistical methods were used to determine sample size, and experiments were not randomized. The experimenters were not blinded. Replicates and sample sizes were determined for each experiment based on feasibility within method being used. Aside from traditional Mann–Whitney, Pearson correlation test, and student t tests to compare data sets, statistical methods were used in order to make appropriate multiple comparisons of data (following one-way or two-way ANOVA as indicated in figure legends). Graphpad Prism 6 was used to make these simple predetermined statistical comparisons. When multiple cell lines were used for an experiment, corrections for multiple comparisons were performed on the combined data.

Dunnett’s multiple comparisons correction. Used for comparing all samples to a control sample, but not for controlling the non-control samples to one another.

Sidak’s multiple comparisons correction. Used when specific multiple comparisons are pre-selected.

Tukey’s multiple comparisons correction. Used when all pairwise comparisons are performed.

Benjamini and Yekutieli correction62. Used for Pearson correlation test.

We also use the Logrank nonparametric test to determine the statistical difference between Kaplan–Meier survival distributions.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. Data analyzed in Supplementary Fig. 2 can be found at https://github.com/TiphaineCMartin/Regulation_PTN_KyriePappas2018. Source data for all figures can be found in Supplementary Data 5. All other data generated or analyzed during this study are included in this published article (and its Supplementary Information files).

Code availability. All custom scripts have been made available at https://github.com/TiphaineCMartin/Regulation_PTN_KyriePappas2018.
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**Author contributions**

K.P. and R.P. designed the study and interpreted results. K.P. performed and analyzed most experiments. T.M. performed the analysis of the detailed transcriptional activity/state of the PTEN promoter and helped with preparation and editing of the paper. A.W. performed and analyzed the Nanostring experiments. C.N. helped perform qRT-PCRs and western blots in EZH2 knockdown and UNC1999 experiments. T.S. and H.H. helped to procure the breast tissue samples from CUMC. J.J. provided reagents and input on UNC1999 experiments and analysis. H.H. provided guidance on scoring and analysis of tissue specimens, and also provided extensive guidance and pathology perspective on the content and editing of the paper. R.P. supervised experiments. K.P. and R.P. wrote the paper with input from all authors.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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**Correspondence** and requests for materials should be addressed to R.P.

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