Breast cancer mortality is principally due to recurrent tumors that arise from a reservoir of residual tumor cells that survive therapy. Remarkably, breast cancers can recur after extended periods of clinical remission, implying that at least some residual tumor cells pass through a dormant phase prior to relapse. Nevertheless, the mechanisms that contribute to breast cancer recurrence are poorly understood. Using a mouse model of recurrent mammary tumorigenesis in combination with bioinformatics analyses of breast cancer patients, we have identified a role for Notch signaling in mammary tumor dormancy and recurrence. Specifically, we found that Notch signaling is acutely upregulated in tumor cells following HER2/neu pathway inhibition, that Notch signaling remains activated in a subset of dormant residual tumor cells that persist following HER2/neu downregulation, that activation of Notch signaling accelerates tumor recurrence, and that inhibition of Notch signaling by either genetic or pharmacological approaches impairs recurrence in mice. Consistent with these findings, meta-analysis of microarray data from over 4,000 breast cancer patients revealed that elevated Notch pathway activity is independently associated with an increased rate of recurrence. Together, these results implicate Notch signaling in tumor recurrence from dormant residual tumor cells and provide evidence that dormancy is a targetable stage of breast cancer progression.
Notch promotes recurrence of dormant tumor cells following HER2/neu-targeted therapy

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Introduction

Breast cancer mortality is principally due to recurrent tumors that arise from a reservoir of residual tumor cells that survive therapy. Remarkably, breast cancers can recur after extended periods of clinical remission, implying that at least some residual tumor cells pass through a dormant phase prior to relapse. Nevertheless, the mechanisms that contribute to breast cancer recurrence are poorly understood. Using a mouse model of recurrent mammary tumorigenesis in combination with bioinformatics analyses of breast cancer patients, we have identified a role for Notch signaling in mammary tumor dormancy and recurrence. Specifically, we found that Notch signaling is acutely upregulated in tumor cells following HER2/neu pathway inhibition, that Notch signaling remains activated in a subset of dormant residual tumor cells that persist following HER2/neu downregulation, that activation of Notch signaling accelerates tumor recurrence, and that inhibition of Notch signaling by either genetic or pharmacological approaches impairs recurrence in mice. Consistent with these findings, meta-analysis of microarray data from over 4,000 breast cancer patients revealed that elevated Notch pathway activity is independently associated with an increased rate of recurrence. Together, these results implicate Notch signaling in tumor recurrence from dormant residual tumor cells and provide evidence that dormancy is a targetable stage of breast cancer progression.

While recurrence is perhaps the most important determinant of clinical outcome, the cellular and molecular mechanisms underlying this stage of cancer progression remain poorly defined. In particular, little is known about the signaling pathways that permit residual neoplastic cells to survive in a dormant state and eventually resume growth. Unfortunately, detailed examination of the molecular and cellular mechanisms that contribute to cellular dormancy and recurrence in breast cancer has been limited by difficulties in obtaining clinical samples from patients with MRD and recurrent cancers. There is also the challenge of accurately modeling these complex biological processes in vivo. To overcome these limitations, our laboratory has developed genetically engineered mouse models that recapitulate key features of breast cancer progression, including spontaneous recurrence arising from dormant MRD that persists following therapy (11–16).

In MMTV-rtTA;TetO-HER2/neu (MTB/TAN) mice, treatment with doxycycline induces HER2/neu signaling in the mammary epithelium, which in turn drives the formation of invasive adenocarcinomas. Upon doxycycline withdrawal and resultant oncogene downregulation, primary tumors regress to a nonpalpable state as a consequence of oncogene addiction. However, a small population of surviving tumor cells persists. Following a variable period of cellular dormancy, residual tumor cells re-enter the cell cycle and give rise to recurrent tumors in a stochastic manner. In line with clinical observations that HER2/neu-positive primary tumors can give rise to HER2/neu-negative residual disease (17, 18) and recurrences (19) following therapy, recurrence in this preclinical mouse model occurs in the absence of HER2/neu expression. This behavior parallels clinical observations in which resistance to HER2/neu targeted therapies can occur through activation of compensatory pathways (20, 21).
Accumulating data support a role for the Notch pathway in the pathogenesis and progression of human breast cancer. Recurrent gene rearrangements resulting in constitutive NOTCH1 activation have been identified in patients with estrogen receptor–negative (ER-negative) adenocarcinomas of the breast (22), and constitutive activation of Notch signaling in transgenic mice results in the formation of mammary tumors (23–27). Recent data also implicate Notch signaling in resistance to chemotherapy, hormone therapy and targeted therapy in breast cancer cell lines (28). Additionally, positive staining for the intracellular domain of NOTCH1 (NICD1) has been reported to be associated with an increased risk of recurrence in a series of 50 patients with ductal carcinoma in situ (29). Furthermore, elevated expression of NOTCH1 and JAG1 mRNAs in primary breast cancers has been associated with poor overall survival in a cohort of 184 patients, with coexpression of NOTCH1 and JAG1 mRNAs associated with the highest risk of relapse (30). However, whether Notch plays a functional role in breast cancer recurrence has not been addressed.

In the present study, we employed computational interrogation of breast cancer patient datasets and genetically engineered mouse models for HER2/neu-targeted therapy to elucidate a role for Notch signaling in breast cancer recurrence. Our observations that Notch signaling is associated with a decrease in recurrence-free survival in breast cancer patients, that Notch signaling is upregulated following HER2/neu downregulation, and that inhibition of Notch signaling impairs recurrence of dormant tumor cells implicate Notch signaling as a potential mechanism through which tumor cells evade therapy and recur in breast cancer patients. Our findings identify dormancy as a distinct, rate-limiting, and targetable stage of tumor progression and suggest a new therapeutic approach to the clinical problem of MRD and recurrence.

Results

Notch pathway activity is associated with an increased risk of recurrence in women with breast cancer. To investigate the potential role of Notch in breast cancer recurrence, we first developed a gene-expression signature reflecting Notch activation in mammary epithelial cells. We generated MMTV-rTst3;TetO-NICD1 (MTB/TICNX) transgenic mice that conditionally express NICD1 in the mammary epithelium upon doxycycline administration, and we performed gene expression profiling following 96 hours of doxycycline treatment. As anticipated, canonical Notch targets such as Hey1 and Hey2 were acutely upregulated following NICD1 induction (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI74883DS1). In parallel, we compared expression profiles of human breast cancer cell lines (31) with and without activating NOTCH1 gene rearrangements (22). A 72-gene signature was derived from the intersection of the differentially expressed gene lists generated from these 2 data sets (Supplemental Figure 1B and Supplemental Table 1).

Using a previously described scoring method for estimating pathway activity from gene expression data (32), we validated the performance of this Notch signature on independent gene-expression datasets. The signature accurately identified Notch activation in an independent cohort of MTB/TICNX mice induced for 48 hours (Supplemental Figure 1C), increases in Notch activity in MCF-10A cells transduced with increasingly potent NOTCH1 alleles (ref. 32 and Supplemental Figure 1D), activation of Notch signaling in human T-ALL cell lines bearing Notch pathway-activating mutations (ref. 30 and Supplemental Figure 1E), and inhibition of Notch signaling in human T-ALL cell lines treated with a γ-secretase inhibitor (GSI; refs. 33–36 and Supplemental Figure 1F).

We next used this Notch pathway signature to classify human breast cancers according to their predicted levels of Notch activity and asked whether Notch signaling was associated with recurrence-free survival in women with breast cancer. Meta-analysis of data from 17 studies, including 4,463 patients, revealed a robust association between elevated Notch activity and reduced recurrence-free survival (Figure 1; P < 5 × 10^{-8}). Furthermore, while estimated Notch activity was higher in subsets of breast cancers associated with poor clinical outcomes — including basal-like tumors, ER-negative tumors, and high-grade tumors — its association with recurrence-free survival was independent of these prognostic factors in multivariate analyses, and it also remained predictive of recurrence within these high-risk subgroups (Supplemental Figures 2 and 3). These findings suggest that elevated Notch pathway activity is associated with an increased risk of tumor recurrence in breast cancer patients.

Notch signaling is upregulated acutely following HER2/neu downregulation. In light of the strong association between Notch path-
way activity and relapse-free survival in breast cancer patients, we hypothesized that Notch signaling might play a functional role in mammary tumor recurrence. It has been suggested that resistance of human breast cancer cell lines to HER2/neu inhibition can be mediated, at least in part, by activation of Notch signaling in vitro (37) and in xenograft models (38). However, the mechanisms underlying this proposed association are unknown.

First, we used the above Notch pathway signature to address whether HER2/neu blockade alters Notch signaling. Applying our bioinformatics approach to published microarray data from SKBR3 cells, which are ER/PR/HER2− (39), we found that Notch pathway activity increased with elevating doses of lapatinib (Supplemental Figure 4A). Next, to determine whether Notch activation following HER2/neu inhibition is evolutionarily conserved, we downregulated HER2/neu in vitro in oncogene-dependent primary tumor cells derived from Mtb/Tan mice by removing doxycycline from the media. This revealed that HER2/neu downregulation was accompanied by acute upregulation of NICD1, Hey1, and Hey2 in a GSI-sensitive manner, confirming that HER2/neu downregulation results in acute Notch pathway activation (Figure 2, A and B). While levels of NOTCH1 and DLL1 also increased, these changes were not GSI sensitive, suggesting that they occurred upstream of Notch activation. Importantly, parallel experiments in SKBR3 cells revealed a similar pattern of alterations in HEY1, HEY2, and DLL1 levels, suggesting that the mechanisms of crosstalk between HER2/neu and Notch signaling identified in HER2/neu-driven mouse tumor cells may also be operative in HER2-amplified human breast cancer cells (Supplemental Figure 4B).

To assess the contribution of signaling pathways downstream of HER2/neu, we performed analogous experiments, in which the effects of HER2/neu downregulation were compared with those resulting from treatment with inhibitors of RAS/MAPK or AKT signaling. While AKT inhibition had little effect on Hey1, Notch1, or DLL1 levels, MEK inhibition closely paralleled the effects of HER2/neu downregulation on these Notch signaling components in Mtb/Tan primary tumor cells (Figure 2, C–E, and Supplemental Figure 5, A and B). Consistent with this, treatment with a selective ERK1/2 inhibitor also increased NICD levels (Supplemental Figure 5C). In contrast, increased Notch signaling was not observed in SKBR3 cells upon MEK inhibition, suggesting that more than one signaling pathway downstream of HER2/neu may be capable of mediating crosstalk with Notch signaling (data not shown).

**HER2/neu represses Notch signaling through HES1 and NRARP.** Unexpectedly, HES1 and NRARP expression decreased following HER2/neu downregulation or MEK inhibition (Figure 2, A, C, and D). Although both are canonical Notch targets — and behaved as such in our experiments — evidence exists suggesting that HES1 may also be upregulated through activation of receptor tyrosine kinases, including signaling downstream of FGF and ErbB families (40–43). Indeed, we found that HES1 and NRARP were each downregulated within 6 hours of doxycycline withdrawal and rapidly upregulated by re-addition of doxycycline, suggesting that these genes may be directly regulated by HER2/neu in addition to Notch signaling (Figure 3A).

Of note, HES1 and NRARP have each been reported to inhibit Notch signaling, by either repressing DLL1 expression (44) or inhibiting NICD-mediated activation of target genes (45), respectively. Therefore, we determined the impact of enforced NRARP on Notch signaling in Mtb/Tan tumor cells (Figure 3, B and C, and Supplemental Figure 6, A and B). While HER2/neu deinduction led to downregulation of HES1 and upregulation of DLL1 and Hey1 in control cells, increases in DLL1 and Hey1 expression were attenuated by enforced HES1 expression. Enforced NRARP expression also decreased Hey1 upregulation upon HER2/neu downregulation. These observations suggest that HES1 and NRARP negatively regulate Notch signaling.

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**Figure 2. HER2/neu downregulation activates Notch signaling.** (A–E) Western blot and qRT-PCR analyses of Notch signaling components in HER2/neu-dependent primary tumor cells derived from MTB/TAN mice: (A and B) 48 hours after doxycycline withdrawal with and without GSI treatment. (C–E) Treatment for 4 hours (C) or 48 hours (D and E) with MEKi (PD0325901), AKTi (MK 2206), or doxycycline withdrawal. Data in B and D are shown as the mean ± SD. *P < 0.05, **P < 0.01, and ***P < 0.001 by 1-way ANOVA followed by the Bonferroni (C) or Dunnett (D) multiple comparisons tests; n = 3. Western blot results are representative of 3 different experiments.
and D). In control experiments in which HER2/neu expression was maintained by inclusion of doxycycline in the medium, activation or inhibition of Notch signaling altered colony size but did not affect the number of colonies that formed. In contrast, in the absence of HER2/neu signaling, Notch pathway activation rescued colony formation and, conversely, Notch pathway inhibition impaired colony formation (Figure 4).

Next, to determine the impact of Notch activation on the recurrence of HER2/neu-induced mammary tumors in vivo, we used MTB/TAN tumor cells transduced with NICD1 in a doxycycline-inducible orthotopic mouse model (16). Primary tumor cells expressing control or Notch1 gain-of-function constructs were injected into the mammary fat pads of recipient nu/nu mice maintained on doxycycline. Following primary tumor formation in the presence of HER2/neu signaling, HER2/neu downregulation induced by doxycycline withdrawal was used to model HER2/neu-targeted therapy. All tumors regressed to a nonpalpable state, irrespective of NICD1 expression (data not shown). However, the rate of recurrence of
primary tumors that overexpressed NICD1 was dramatically accelerated compared with control tumors (Figure 5A; hazard ratio [HR] 21.8, 95% confidence interval [CI] 8.26–57.7; \( P < 0.0001 \)). This demonstrates that Notch signaling is sufficient to promote mammary tumor recurrence following HER2/neu downregulation.

We then adapted this model to permit Notch pathway inhibition by excision of floxed alleles of the canonical Notch effector Rbpj (48, 49). Individual MTB/TAN primary tumors were digested to yield single-cell suspensions, infected with Cre or GFP adenovirus (AdCre and AdGFP), and orthotopically injected into recipient mice without intervening culture. Expression of Cre in control tumor cells isolated from mice with WT Rbpj alleles did not alter primary orthotopic tumor growth, time to regression, or time to recurrence; these findings supported the feasibility of this experimental approach (Supplemental Figure 7, A–C).

As anticipated, AdCre infection of primary tumor cells derived from MTB/TAN/Rbpj\(^{\text{fl/fl}}\) mice resulted in knockdown of Rbpj expression in primary orthotopic tumors (Supplemental Figure 7, D and E). Although Rbpj knockdown did not alter primary tumor growth (Supplemental Figure 7F), it markedly inhibited tumor recurrence (Figure 5B; HR 0.30, 95% CI 0.10–0.91; \( P = 0.03 \)).

While knockdown of Rbpj impaired recurrence, a subset of tumors in this group recurred despite treatment with AdCre. To evaluate the mechanism by which these tumors recurred, we interrogated the RBPJ status of the resulting recurrent tumors. We found that expression of the excised Rbpj\(^{\text{fl/fl}}\) allele (Rbpj\(^{\Delta}\)) was lost in recurrent tumors arising from AdCre-infected tumor cells (Supplementary Figure 8A). Consistent with this, IHC for RBPJ revealed many fewer RBPJ-expressing cells in primary tumors treated with AdCre than in tumors treated with AdGFP, but little difference in the frequency of RBPJ-expressing cells in recurrent tumors treated with AdCre versus treated with AdGFP (Supplemental Figure 8B). These data suggest that strong selection pressure exists for maintaining Notch signaling during the process of recurrence but does not exclude the possibility of escape through alternative signaling pathways. In aggregate, our experiments demonstrate that activation of Notch signaling is a rate-limiting step in tumor recurrence in vivo following HER2/neu downregulation.

GSI treatment prevents recurrence of dormant MRD by blocking Notch signaling. Having established that constitutive activation of Notch signaling promotes recurrence, and that constitutive inhibition of Notch signaling delays recurrence, we used a GSI in the MTB/TAN mouse model of autochthonous HER2/neu driven tumor formation to dissect the role of Notch signaling during each phase of tumor progression (Figure 6A). To assess a role for Notch signaling in primary tumor formation or growth, GSI treatment was initiated either concurrently with HER2/neu activation induced by doxycycline or after primary tumors had arisen in the presence of HER2/neu signaling. Consistent with our results above regarding the lack of effects of Rbpj deletion on primary tumor formation, GSI treatment did not alter primary tumor latency or growth (Supplemental Figure 9).

The kinetics of tumor recurrence in women with breast cancer (2–5), along with studies of DTCs (7, 8), have suggested that...
Figure 5. Notch signaling promotes tumor recurrence following HER2/neu downregulation. (A) Kaplan-Meier survival curves showing recurrence-free survival for mice harboring MTB/TAN orthotopic tumors expressing NICD1 (n = 18) or MigR1 (n = 21) constructs: HR = 21.8, 95% CI 8.26–57.7; P < 0.0001 by the Mantel-Haenszel method. (B) Kaplan-Meier survival curves showing recurrence-free survival for mice harboring orthotopic tumors generated from uncultured primary MTB/TAN/Rbpjfl/fl tumor cells infected with AdGFP (n = 13) or AdCre (n = 15): HR 0.30, 95% CI 0.10–0.91; P = 0.03 by the Mantel-Haenszel method.

GSI on recurrence were Notch-dependent or Notch independent. This was accomplished by performing a rescue experiment in which we determined the impact of GSI treatment on the recurrence of tumors expressing a GSI-insensitive allele of NICD1.

As above, primary HER2/neu-dependent tumor cells expressing control or NICD1 constructs were orthotopically injected into nu/nu mice maintained on doxycycline. Following primary tumor formation, weekly treatment with 300 mg/kg GSI or vehicle control was initiated at the time of doxycycline withdrawal to induce tumor regression. Consistent with our previous results (Figure 5A), recurrent breast cancers in patients may arise from a dormant population of residual tumor cells. In an analogous manner, we have found that MTB/TAN mice in which tumors have regressed to a nonpalpable state following HER2/neu downregulation harbor a residual population of dormant, Ki-67-negative, and BrdU-resistant tumor cells (refs. 13, 16, and our unpublished observations). Therefore, to address the role of Notch signaling in dormant tumor cells, we initiated GSI treatment in a cohort of mice bearing dormant residual disease generated from primary tumors that had fully regressed to a nonpalpable state following doxycycline withdrawal and HER2/neu downregulation. GSI treatment of mice bearing dormant residual tumor cells resulted in a dose-dependent inhibition of recurrence (Figure 6B; HR 0.37, 95% CI 0.15–0.94, P = 0.037 for 150 mg/kg GSI; HR 0.28, 95% CI 0.10–0.78, P = 0.015 for 300 mg/kg GSI) of a magnitude similar to that observed after constitutive knockdown of RBPj expression throughout tumor progression.

To address the possibility that the suppressive effect of GSI treatment on tumor recurrence was due to inhibition of recurrent tumor cells that had already re-entered the cell cycle, rather than to an effect of GSI treatment on dormant residual tumor cells, control experiments were performed in which GSI treatment was not initiated until recurrent tumors had been detected. GSI treatment of existing recurrent tumors did not alter recurrent tumor growth rates (Supplemental Figure 10 and data not shown).

As γ-secretase is necessary for ligand-dependent Notch signaling, these findings suggest that while endogenous Notch signaling may not be required for the growth of primary or recurrent tumors, it does affect a rate-limiting step in tumor recurrence from dormant residual tumor cells. Consistent with a role for Notch signaling in dormant tumor cells, analysis of residual neoplastic lesions in mice bearing fully regressed tumors 28 days following HER2/neu downregulation revealed the presence of a subset of residual tumor cells with positive nuclear staining for NICD1 that was abrogated by GSI treatment (Figure 6C). This indicates that Notch signaling remains active within a subpopulation of residual tumor cells that survive HER2/neu downregulation for extended periods of time.

Since Notch receptors are not the only known substrates of γ-secretase, we wished to address whether the inhibitory effects of GSI on recurrence were Notch-dependent or Notch independent. This was accomplished by performing a rescue experiment in which we determined the impact of GSI treatment on the recurrence of tumors expressing a GSI-insensitive allele of NICD1.

As above, primary HER2/neu-dependent tumor cells expressing control or NICD1 constructs were orthotopically injected into nu/nu mice maintained on doxycycline. Following primary tumor formation, weekly treatment with 300 mg/kg GSI or vehicle control was initiated at the time of doxycycline withdrawal to induce tumor regression. Consistent with our previous results (Figure 5A),
weeks. Longitudinal luciferase imaging revealed that, while residual disease remained stable in vehicle-treated mice, GSI treatment of mice bearing MRD resulted in a reduction in luciferase signal (Figure 7, B–D). Since prior studies have revealed that residual tumor cells do not proliferate during this time frame, this finding implies that GSI treatment results in a decrease in the burden of dormant residual tumor cells. We cannot exclude the possibility that the decrease in luciferase signal was due to a factor other than a decreased number of residual tumor cells, such as a change in the size or distribution of tumor cells; however, in light of our findings that GSI treatment inhibits tumor recurrence only when administered during the dormant phase of tumor progression, these data are consistent with the hypothesis that Notch signaling contributes to the maintenance of MRD.

Discussion
Since recurrent breast cancer is typically incurable, the propensity of breast cancers to recur following surgery, chemotherapy, and hormonal therapy is the most important determinant of clinical outcome. However, while tumor dormancy and recurrence are responsible for the majority of breast cancer deaths, the mechanisms underlying these critical stages of cancer progression are largely unknown. Here, we demonstrate that Notch signaling plays an important role in tumor recurrence following HER2/neu inhibition. Specifically, we found that Notch activity is positively associated with breast cancer recurrence in patients and that Notch activation is sufficient to promote the recurrence of HER2/neu-driven mammary tumors in genetically engineered mice. Furthermore, we demonstrated that endogenous Notch signaling is upregulated in response to HER2/neu downregulation and is rate-limiting for tumor recurrence. Consistent with this, pharmacologic inhibition of Notch signaling suppressed tumor recurrence when administered to mice bearing dormant MRD and reduced residual disease burden following HER2/neu blockade in a manner suggestive of a reduction in the number of residual tumor cells.

Recent large-scale sequencing and screening approaches have identified recurrent Notch gene rearrangements (22), implicated Notch signaling in therapeutic resistance (28), and reinvigorated

Figure 6. GSI treatment blocks recurrence of dormant residual tumor cells. (A) Schematic of MTB/TAN tumor progression and treatment paradigms. (B) Kaplan-Meier survival curves showing recurrence-free survival of MTB/TAN mice after treatment with vehicle (n = 12), 150 mg/kg (n = 13) or 300 mg/kg GSI (n = 11) initiated during tumor dormancy: HR 0.37, 95% CI 0.15–0.94, P = 0.037 for 150 mg/kg; HR 0.28, 95% CI 0.10–0.78, P = 0.015 for 300 mg/kg. (C) Representative immunofluorescence analysis of NICD1 in GFP+ dormant residual tumor cells in mice, 28 days following HER2/neu downregulation and 24 hours after treatment with GSI or vehicle (×40 original magnification; n = 3). (D) Kaplan-Meier survival curves showing recurrence-free survival for mice harboring MTB/TAN orthotopic tumors expressing NICD1 or MigR1 constructs treated with GSI or vehicle initiated with doxycycline withdrawal: MigR1+GSI (n = 16) vs. MigR1 + vehicle (n = 14): HR 0.23, 95% CI 0.08–0.68, P = 0.008; NICD1+GSI (n = 14) vs. NICD1 + vehicle (n = 16): P = NS; NICD1 + vehicle vs. MigR1 + vehicle: HR 10.0, 95% CI 3.64–27.39, P < 0.0001; NICD1+GSI vs. MigR1+GSI: HR = 25.47, 95% CI 7.94–81.69, P < 0.0001. P values calculated by the Mantel-Haenszel method and log-rank test for trend.
While it is currently unclear whether drugs targeting the Notch pathway will impair the growth of established primary or recurrent metastatic tumors, their use as adjuvant agents in the setting of dormant MRD — or in the neoadjuvant setting in combination with HER2/neu-targeted therapies — could enable the elimination of dormant residual cancer cells and, consequently, could enable the prevention of recurrence.

**Methods**

**Human breast cancer microarray data.** Publicly available microarray data for 4,463 patients contained within 17 human primary breast cancer data sets (54–69), along with the corresponding clinical annotations, were downloaded from NCBI GEO or authors’ websites. Microarray data were converted to base 2 logarithmic scale where necessary. Affymetrix microarray data were renormalized using Robust Multi-array Average (RMA; ref. 70) when CEL files were available.

**RNA isolation and gene-expression profiling.** RNA was isolated from tumors and cells using Trizol (Ambion) or RLT (QIAGEN) followed by RNeasy columns (QIAGEN). For qRT-PCR, 1 or 2 μg of RNA was reversed transcribed using high-capacity cDNA synthesis reagents (Applied Biosystems). qRT-PCR was performed on Applied Biosystems 7900 HT Fast and ViIA 7 Real-Time PCR systems using the following 6-carboxyfluorescein–labeled TaqMan probes: Dll1 (Mm00432841_m1), Hey1 (Mm00468865_m1), Hey2 (Mm00469280_m1), ErbB2 (Rn00566561_m1), Notch1 (Mm00435245_m1), NOTCH1 (Hs01062014_m1), Nrarp (Mm00482529_SL), Tbp (Mm00446973_m1), and custom NRARP cDNA (Forward: CCGGAGGGCCAGACA; Reverse: GCTTCAC-CAGCTCCAGGTT; Probe: ACACACGTCGTCATCG).

For microarray profiling, all samples were processed by the University of Pennsylvania Molecular Profiling Core. RNA integrity was...
analyzed using the 2100 Bioanalyzer (Agilent Technologies). Samples were reverse transcribed and labeled using the GeneChip 3′ IVT Express Kit (Affymetrix). The resulting cRNA was hybridized to Affymetrix Mouse genome 430 2.0 arrays. Raw microarray data were normalized by RMA using the Bioconductor affy package in R version 2.15.1. MA55 detection calls were generated using the same package. Probe sets with absent detection calls in all samples or with a dynamic range of less than 1.2-fold across all samples were removed from downstream analysis. Probe set-to-gene mapping was performed in R using the Bioconductor annotation packages. For multiple probe sets mapping to the same gene, only the probe set with the highest percentage-present call was retained, with ties broken by choosing the probe set with the highest median expression across samples. Normalized gene expression data are deposited in NCBI Gene Expression Omnibus (GEO) under the accession number GSE51628.

**Tissue culture and colony formation assays.** MTB/TAN primary tumor cells were derived and grown at 37°C in 5% CO2 as described (16) and were cultured in DMEM with 10% super calf serum, 1% Penicillin/Streptomycin, and 1% L-glutamine supplemented with 10 μg/ml EGF, 5 μg/ml insulin, 1 μg/ml hydrocortisone, 5 μg/ml prolactin, 1 μM progesterone, and 2 μg/ml doxycycline to maintain HER2/neu expression. SKBR3 cells were purchased from ATCC and cultured as recommended. Measurements of cell viability and cell number were performed by staining with trypan blue and counting on a ViCell cell counter (BD Biosciences). For colony formation assays, 1,000 cells were plated on a 10-cm dish in complete growth medium. The next day, doxycycline was withdrawn to induce HER2/neu downregulation. Colonies were allowed to form for 2–3 weeks, after which they were fixed and stained with crystal violet for visualization and manual quantification.

**Drug treatments.** MRK-003 GSI was provided by Merck & Co. Inc. and was dissolved in DMSO for in vitro studies. For in vivo studies, MRK-003 was resuspended in 0.5% methycellulose vehicle and administered by oral gavage once per week. Lapatinib (B-Bridge International Inc.), MK-2206 AKTi-1/2 (Selleck Chemicals), PD0325901 MEKi-1/2 (Sigma-Aldrich), and SCH772984 ERKi-1/2 (Selleck Chemicals) were dissolved in DMSO for in vitro studies.

**Western blotting.** Western blotting was performed as described (16) using the following antibodies (obtained from Cell Signaling Technology, unless otherwise noted): NICD1 (1:1000, D3B8), NOTCH1 NTM (1:2000, D6P2U), RBPJ (1:1,000, D10A4), pERK1/2 (1:2,000, D13.14.4E), pAKT (1:2,000, D9E), GAPDH (1:1,000, 14C10), and β-tubulin (1:2,000, BioGenex, MU122-UC). Secondary antibodies conjugated to Alexa fluor 680 (1:10,000, Invitrogen) or IRDye 800 (1:5,000, LI-COR Inc.) were detected and quantified with the Odyssey CLx Infrared Imaging System and Image Studio software (LI-COR Inc.). Secondary antibodies conjugated to HRP (Jackson ImmunoResearch Laboratories Inc.) were developed with Luminata Classico Western HRP substrate (Millipore) and exposed to film (Amersham or Kodak).

**Plasmids and retrovirus production.** MigR1, MigR1-NICD, and MigR1-dnMAML retroviral constructs were provided by Warren Pear. HES1 and NRARP cDNAs encoding the full-length mouse proteins were amplified by RT-PCR from primary MTB/TAN tumor cells and cloned into the pCRII-TOPO vector (Invitrogen) using the following primers: Hes1 Forward: ATGCCAGCTGATAATGGAGA; Hes1 Reverse: TCAAGTTCCGCAGCGGTT; NRARP Forward ATGAGCCAAGCAGAGCTGTCCACCT; and NRARP Reverse: TCACCGGCGCGTGGCCGCAGTGTA. Retroviral expression constructs were generated by subcloning Hes1 and Nrarp into pKl. For luciferase expression, Renilla luciferase (RLuc) was subcloned from pRL-CMV (Promega) into MigR1.

Retrovirus was produced by transfecting the packaging line plat-E (71) with retroviral constructs using Lipofectamine 2000 (Invitrogen). Retroviral supernatant was collected 48 hours after transfection, was filtered, and was used to transduce cells in the presence of 4 μg/ml polybrene (Sigma-Aldrich). Cells were selected using puromycin or fluorescence-activated cell sorting.

**Animals and recurrence assays.** Animal care and experiments were performed with the approval of, and in accordance with, guidelines of the University of Pennsylvania IACUC. Mice were housed under barrier conditions with 12-hours light/12-hours dark cycles and access to food and water ad libitum. TICNX mice were engineered by subcloning an NICD1 construct (gift from Warren Pear) downstream of the tet operator sequences in the TMILA plasmid (14). Founder lines were generated by injecting the linearized construct into fertilized oocytes harvested from super-ovulated FVB/N mice and crossed with MTB mice (12). Mice were generated, induced with doxycycline, and sacrificed as described (12, 13).

Tumor recurrence assays were performed as described (13, 16). Briefly, for orthotopic experiments, 1 × 10⁶ cells were injected into the inguinal mammary fat pads of female nu/nu mice maintained on 2 mg/ml doxycycline in their drinking water. Mice were monitored for tumor formation twice weekly. Once primary tumor endpoints were reached, doxycycline was removed to initiate oncogene downregulation and tumor regression. Mice were palpated twice weekly to monitor for tumor recurrence.

For Rbpj knockdown experiments, Rbpjfl/fl mice were obtained from RIKEN BRC, backcrossed onto an FVB background, and then interbred with MTB/TAN mice to generate MTB/TAN/Rbpjfl/fl. Primary tumor formation was induced by doxycycline treatment. To generate a single-cell suspension, tumors were manually minced and then digested in MEGM (Lonza), 1X B-27 (Invitrogen), 20 ng/ml bFGF (Sigma-Aldrich), 4 μg/ml heparin (StemCell Technologies Inc.), 5% Super Calf Serum (Gemini Bio-Products), and 1X Collagenase/Hyaluronidase (StemCell Technologies Inc.) for 1 hour at 37°C. Red blood cells were removed by suspension in red blood cell lysis buffer for 5 minutes. Cells were counted, resuspended in media with 2% serum, and infected with Ad5CMV-eGFP or Ad5CMV-Cre (University of Iowa Gene Transfer Vector Core) at an MOI of 100 for 2 hours at 37°C. Following infection, cells were washed and used for orthotopic injections.

For GSI treatment experiments, bitransgenic female MTB/TAN mice were generated, induced with doxycycline, monitored for tumor development, and sacrificed as described (12, 13). Mice were randomly assigned to 4 different experimental cohorts in which treatment was administered once per week, initiated at the time of doxycycline induction, primary tumor detection, full tumor regression, or recurrent tumor detection; treatment continued until humane tumor endpoints were reached. Each cohort contained 3 different treatment arms: 150 mg/kg GSI, 300 mg/kg GSI, or vehicle control. Mice were randomized between treatment cohorts by cage.

**NICD immunofluorescence.** GFP-labeled orthotopic tumors were generated from MTB/TAN cells expressing H2B-eGFP. Following primary tumor formation, doxycycline was withdrawn to induce tumor regression. Mice were treated with a single dose of vehicle or GSI 28 days after deinduction and sacrificed 24 hours later. Mammary tumors
were fixed in 4% PFA overnight, dehydrated, and embedded in paraffin blocks following standard protocols.

Paraffin tissue sections, which were 8 μm thick, were prepared using a standard xylene-based dewaxing procedure. Sections were subjected to antigen retrieval in a 2100 Retriever using Buffer A (Electron Microscopy Sciences). Slides were blocked in 5% BSA and 10% normal goat serum for 1 hour before overnight incubation at 4°C with primary antibodies. After washing, slides were incubated with secondary Alexa-Fluor-conjugated antibodies for 1 hour at 1:1000 (Invitrogen) followed by Hoechst to visualize nuclei. Anticleaved Notch1 (Cell Signaling Technology, D3B8) was used at 1:200, and Anti-GFP (Novus Biologicals, NB100-1770) was used at 1:1000.

*Rbpj* PCR and IHC. AdGFP- and AdCre-treated primary and recurrent orthotopic tumors were generated from *MTB/TAN/Rbpj* mice as described, fixed in 4% PFA overnight, dehydrated, and embedded in paraffin blocks following standard protocols. For PCR, genomic DNA was purified from paraffin sections using the QIAamp DNA FFPE Tissue Kit (Qiagen). *Rbpj* was amplified with the following primers using standard PCR protocols: *Rbpj Forward: CCTTGGTTGTGGTGTGGTT and Rbpj Reverse: GTTGCTCTCAACTCCCAATCGT.*

IHC was performed using the VECTASTAIN ABC System (Vector Laboratories). Briefly, 8-μm thick paraffin tissue sections were prepared using a standard xylene-based dewaxing procedure. Sections were subjected to antigen retrieval in a 2100 Retriever using Buffer A (Electron Microscopy Sciences). Slides were treated with H2O2 and blocked in 10% normal goat serum for 30 minutes before overnight incubation at 4°C with an anti-RBPJ primary antibody (D10A4, Cell Signaling Technology) at 1:1000. After washing, slides were incubated with a secondary biotinylated goat anti-rabbit antibody (BA-1000, Vector Laboratories) at 1:300 for 1 hour and developed following the kit protocol. Slides were counterstained with hematoxylin following standard protocols.

*In vivo luciferase imaging.* Luciferase-labeled orthotopic tumors were generated from *MTB/TAN primary tumor cells transduced with MigR1-RLuc. Following primary tumor formation, doxycycline was withdrawn to induce tumor regression. Mice were imaged once before deinduction and then twice per week thereafter. After 28 days of deinduction, once-weekly treatment with vehicle or GSI was initiated. Mice were randomized between treatment cohorts by cage. For imaging, mice were anesthetized using isoflurane and administered 100 μl CTZ-SOL (2.5 μg/μl), a water-soluble formulation of native Coe-lenterazine (NanoLight Technologies) by i.v. injection. Bioluminescence images were taken immediately after substrate injection using the IVIS Spectrum (PerkinElmer). Peak signal intensity was quantified using Living Image 4.3 software (PerkinElmer).

**Statistics.** Two-tailed Student’s *t* tests (72) were used to assess differences between 2 groups, substituting the Mann-Whitney *U* test (73) when data did not follow a normal distribution, as determined by the Shapiro-Wilk test (74). For multiple comparisons, 1-way or 2-way ANOVA was followed with Dunnett’s multiple comparisons test (75), the Bonferroni multiple comparisons test (76), or the post hoc test for linear trend. The repeated-measures ANOVA was used for paired analyses, and the Geisser-Greenhouse method (77) was used to correct for violations of the sphericity assumption. Survival curves were created using the Kaplan-Meier method (78), with *P* values and hazard ratios calculated by the Mantel-Haenszel method (79) and log-rank test for trend. All tests were performed using Prism software (GraphPad Software). *P* < 0.05 was considered indicative of statistical significance. In vitro analyses are representative of at least 3 independent experiments.

To estimate relative Notch pathway activity in human breast cancer samples, we generated a Notch signature containing 72 genes concordantly regulated in 2 gene-expression microarray data sets: mammary glands from *MTB/TICNX* mice induced with doxycycline for 96 hours (data are deposited in NCBI GEO under the accession number GSE51628) and human breast cancer cell lines with and without activating NOTCH1 gene rearrangements (22) profiled in the Cancer Cell Line Encyclopedia (CCLE; GSE36133, ref. 31). Signature genes were selected from genes common to the 2 platforms used in the above data sets with cross-species gene mapping performed using data from NCBI HomoloGene (https://www.ncbi.nlm.nih.gov/homologene).

Differentially regulated genes in the *MTB/TICNX* experiment were determined using Cypher-T (80) with a false discovery rate (81) of less than 0.01 and an absolute fold change of greater than 1.5 between the *MTB/TICNX* mice and the *TICNX* controls. In CCLE breast cancer cell lines, expression of each gene was used to rank breast cancer cell lines and then to calculate a rank-sum statistic defined as the sum of the ranks for positive cell lines with activating NOTCH1 gene rearrangements. The significance of each rank sum was determined by its location in the distribution of rank sums from 10,000 random permutations of the ranks. Genes with rank sums in the top or bottom 2.5% of the distribution were considered as being significantly associated with NOTCH1 activation. Among the 58 breast cancer cell lines, HCC1599 and HCC2218 were considered as having activating NOTCH1 gene rearrangements (22); 9 cell lines (DU4475, HCC1187, HS578T, MDAMB468, MCF7, ZR751, CAL51, MDAMB231, and SKBR3) were excluded from the analysis due to ambiguous NOTCH1 activation status; and the remaining 46 cell lines were considered as lacking activating NOTCH1 gene rearrangements.

We validated the 72-gene Notch signature in one in-house data set (GSE51628) and 3 publicly available microarray data sets — GSE20285 (33), the T-ALL subset in GSE36133 (31), and GSE5716 (36) — using a previously described scoring method for estimating pathway activity (32). Microarray data for the validation sets were RMA normalized when CEL files were available, or taken directly from GEO and converted to base 2 logarithmic scale when CEL files were not available. Signature generation and validation were performed in R version 2.15.1.

Within each publicly available human breast cancer microarray data set, the effect size of the association between estimated Notch pathway activity and 5-year relapse-free survival was estimated using hazard ratio from Cox proportional hazards regression, in which Notch pathway activity was modeled as a continuous variable. Each type of effect-size estimate was combined across data sets by meta-analysis using the inverse-variance weighting method (82). Between-study heterogeneity of survival association was tested using the inverse-variance weighting method (82). Between-study heterogeneity of survival association was tested using the inverse-variance weighting method (82).
The association between estimated Notch activity and categorical prognostic variables in human breast cancers—including ER status, HER2/neu status, lymph node status, tumor grade, and intrinsic molecular subtype—was assessed by ANOVA in pooled microarray data sets. For each categorical prognostic variable, estimated Notch activity scores were normalized against the mean scores of the same baseline group in each data set and pooled across all data sets for which the prognostic variable was available. Baseline groups used for ER status, HER2/neu status, lymph node status, tumor grade, and intrinsic molecular subtype were ER-positive, HER2/neu-negative, lymph node–positive, grade I, and the normal-like, respectively. Baseline normalization was performed by subtracting mean Notch pathway score in the baseline group from the score for each sample. For each prognostic variable significantly associated with estimated Notch activity, we assessed the association between estimated Notch activity and relapse-free survival after adjusting for the prognostic variable in multivariate Cox proportional hazards models, and we aggregated the adjusted effect sizes using meta-analysis as described above.

Since HER2/neu IHC status was not available for many of the data sets, HER2/neu status was approximated by ERBB2 mRNA expression, as measured by microarray in a similar fashion as the Cancer Outlier Profile Analysis (86). In each data set, HER2/neu-positive and HER2/neu-negative samples were defined as being above and below a cutoff of 1.5 median absolute deviations above the median, which resulted in average specificity of 98% and sensitivity of 78% in 5 validation data sets (56, 58, 59, 66, 87). Due to the nonrandom association between ER and HER2/neu status, approximation of HER2/neu status was not attempted in data sets consisting entirely of hormone-positive or hormone-negative cancers. Assignments of intrinsic subtype were done using the PAM50 (88) classifier after expression data were median-centered for each gene.

Study approval. Animal care and experiments were performed with the approval of, and in accordance with, guidelines of the University of Pennsylvania IACUC.

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