HHS Public Access

Author manuscript

Oncogene. Author manuscript; available in PMC 2012 August 16.

Published in final edited form as:

Oncogene. 2012 February 16; 31(7): 907–917. doi:10.1038/onc.2011.279.

Modeling ductal carcinoma in situ: a HER2-Notch3 collaboration enables luminal filling

Chaluvally-Raghavan Pradeep[1,†], **Wolfgang J. Köstler**[1,*], **Mattia Lauriola**[1], **Roy Granit**[3], **Fan Zhang**[4], **Jasmine Jacob-Hirsch**[5], **Gideon Rechavi**[5], **Hareesh B. Nair**[6], **Bryan T. Hennessy**[4,9], **Ana M. Gonzalez-Angulo**[4,7], **Rajeshwar R. Tekmal**[8], **Ittai Ben-Porath**[3], **Gordon Mills**[4], **Eytan Domany**[2], and **Yosef Yarden**[1,*]

[1] Department of Biological Regulation, The Weizmann Institute of Science, Rehovot, Israel [2] Department of Physics of Complex Systems, The Weizmann Institute of Science, Rehovot, Israel [3] Institute for Medical Research – Israel-Canada, Hadassah School of Medicine, The Hebrew University of Jerusalem, Ein-Kerem, Jerusalem, Israel [4] Department of Systems Biology, University of Texas M.D. Anderson Cancer Center, Houston, Texas, USA [7] Department of Breast Medical Oncology, University of Texas M.D. Anderson Cancer Center, Houston, Texas, USA [5] Sheba Cancer Research Center, The Chaim Sheba Medical Center, Tel Hashomer, and Sackler School of Medicine, Tel Aviv University, Tel Aviv, Israel [6] Southwest National Primate Research Center, San Antonio, Texas, USA [8] Department of Obstetrics and Gynecology, Division of Reproductive Endocrinology and Infertility, University of Texas Health Science Center San Antonio, San Antonio, Texas, USA [9] Department of Medical Oncology, Beaumont Hospital, Dublin, Ireland

Abstract

A large fraction of ductal carcinoma in situ (DCIS), a non-invasive precursor lesion of invasive breast cancer, overexpresses the *HER2/neu* oncogene. The ducts of DCIS are abnormally filled with cells that evade apoptosis, but the underlying mechanisms remain incompletely understood. We overexpressed HER2 in mammary epithelial cells and observed growth factor-independent proliferation. When grown in extracellular matrix as 3- dimensional spheroids, control cells developed a hollow lumen, but HER2-overexpressing cells populated the lumen by evading apoptosis. We demonstrate that HER2 overexpression in this cellular model of DCIS drives transcriptional up-regulation of multiple components of the Notch survival pathway. Importantly, luminal filling required up-regulation of a signaling pathway comprising Notch3, its cleaved intracellular domain (NICD) and the transcriptional regulator HES1, resulting in elevated levels of c-MYC and Cyclin D1. In line with HER2- Notch3 collaboration, drugs intercepting either arm

[*]Corresponding author: Department of Biological Regulation, Candiotty Building (room 302), The Weizmann Institute of Science, 1 Hertzl Street, Rehovot 76100, Israel. Tel. 972-8- 9343974, FAX: 972-8-9342488, yosef.yarden@weizmann.ac.il.
[*]Present address: Clinical Division of Oncology, Department of Medicine 1, and Early Clinical Development Program, Comprehensive Cancer Center, Medical University of Vienna, Vienna A-1090, Austria
[†]Present address: Department of Systems Biology, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77054, USA

Supplementary information is available at Oncogene's website http://www.nature.com/onc/index.html

reverted the DCIS-like phenotype. In addition, we report up-regulation of Notch3 in hyperplastic lesions of HER2 transgenic animals, as well as an association between HER2 levels and expression levels of components of the Notch pathway in tumor specimens of breast cancer patients. Therefore, it is conceivable that the integration of the Notch and HER2 signaling pathways contributes to the pathophysiology of DCIS.

Keywords

breast cancer; DCIS; growth factor; spheroids; receptor tyrosine kinase; signal transduction

INTRODUCTION

The mammary gland grows rapidly at puberty to produce an elaborate tree-like structure composed of an inner layer of luminal cells, which are surrounded by an outer layer of myoepithelial cells. Later cycles of expansion and involution occur during each menstrual cycle and - even more dramatically - with each pregnancy (Howlin *et al.*, 2006). Mechanisms underlying formation of the lumen of mammary ducts include cell divisions with the metaphase plates organized perpendicular to the apical surface (Jechlinger *et al.*, 2009), and luminal apoptosis promoted by disengagement of inner cell layers from the basement membrane (Simpson *et al.*, 2008). However, the exact mechanisms that regulate duct renewal and apoptosis, as well as their relevance to malignant transformation, remain incompletely understood. In line with diverse mechanisms and cell type heterogeneity, human mammary tumors display marked morphological and molecular diversity (Perou *et al.*, 2000). One aggressive subtype, comprising 20–25% of all invasive ductal carcinomas, is characterized by amplification of the *HER2* gene, resulting in overexpression of the encoded HER2 oncoprotein (also known as ERBB-2/Neu) (Slamon *et al.*, 1987). Treatment with Trastuzumab, an antibody specific to HER2, has been shown to improve outcomes for women with high-risk, early stage or metastatic breast tumors that overexpress HER2 (Ignatiadis *et al.*, 2009; Slamon *et al.*, 2001).

In comparison to invasive lesions, HER2 is overexpressed at a higher frequency in pre-invasive lesions, including atypical ductal hyperplasia and DCIS. These lesions are associated with bypass of apoptosis, increased cellular proliferation and robust filling of the ductal lumen, without invasion through basement membranes (van de Vijver *et al.*, 1988). Importantly, HER2 overexpression is associated with an increased propensity of DCIS to recur after surgical tumor removal. Congruent with poor prognosis, the ligand-less HER2 receptor tyrosine kinase acts as the preferred heterodimerization partner of ligand-bound members of the ERBB family, namely the EGF-receptor (also called ERBB-1), ERBB-3 (HER3) and ERBB-4 (HER4) (Yarden and Sliwkowski, 2001). Because HER2-containing heterodimers are less susceptible to negative feedback regulation than homodimers devoid of HER2, biased heterodimer formation due to HER2 overexpression enhances and prolongs growth factor signaling. According to another model, HER2 overexpression results in ligand-independent formation of signaling-competent HER2-HER2 homodimers (Hudziak *et al.*, 1989; Lonardo *et al.*, 1990). Once activated by dimerization, HER2 can instigate a number of potent signaling pathways, including the mitogen activated protein kinase

(MAPK) pathway and the PI3-kinase (PI3K)/AKT pathway. It has previously been reported that HER2 induces luminal filling by promoting survival of luminal cells through the PI3K-AKT axis (Debnath *et al.*, 2002; Debnath *et al.*, 2003; Schafer *et al.*, 2009). In addition, HER2 disrupts the architecture of the glandular epithelium by interacting with the cell polarity machinery (Aranda *et al.*, 2006; Muthuswamy *et al.*, 2001). In contrast to the wealth of knowledge on biochemical pathways, the transcriptional programs launched by HER2 to enhance luminal filling at early stages of mammary tumorigenesis are less understood.

Another signal transduction pathway critical for breast cancer progression, comprises Notch family receptors and their membrane-bound ligands (Yin *et al.*, 2010). The family includes four conserved transmembrane receptors (Notch1 through Notch4) and five surfacelocalized ligands (Jagged1, Jagged2, Delta-like1 through Delta-like3), which play fundamental roles in self-renewal and proliferation of progenitor and adult stem cells of the mammary gland. For instance, Notch1 and Notch3 regulate expression of c-MYC and Cyclin D1 to promote cell proliferation (Cohen *et al.*, 2010; Palomero *et al.*, 2006). Notch signaling is activated through receptor-ligand interactions between neighboring cells, resulting in successive proteolytic cleavages of Notch proteins by the tumor necrosis factor converting enzyme (TACE; also called ADAM17) and the γ-secretase complex. This releases the Notch intracellular domain (NICD) from the plasma membrane, permitting its translocation into the nucleus and formation of a trimeric transcriptional activator complex with a DNA-binding protein, CSL (also termed CBF-1 and RBP-Jκ), and Mastermind. The complex induces transcription of the HERP and HES gene families, thereby regulating the expression of multiple genes involved in cell growth, differentiation and survival (Iso *et al.*, 2003).

The involvement of Notch signaling in mammary gland tumorigenesis is incompletely understood. The survival-promoting activity of the pathway likely underlay the observed ability of Notch family members to promote mammary tumors (Imatani and Callahan, 2000; Stylianou *et al.*, 2006). In humans, high co-expression of Notch1 and its ligand, JAG-1, associates with poor overall survival of breast cancer patients (Reedijk *et al.*, 2005), and an in vitro study implicated a Notch1-to-STAT3 pathway in mammary hyperproliferation (Mazzone *et al.*, 2010). Although no mutant forms of Notch or aberrant components of the downstream pathway have been reported in mammary tumors, according to one report frequent activation of the pathway occurs in tumors that have lost negative regulation of Notch by the cell fate determinant Numb (Pece *et al.*, 2004). Herein, we associate a Notchinduced pathway with overexpression of HER2. Because HER2 is frequently overexpressed in DCIS, and these lesions are characterized by the absence of cell death within their lumen, we characterized DCIS-like three-dimensional (3D) spheroids of mammary cells overexpressing HER2. Our results attribute luminal filling to the ability of an overexpressed HER2 to transcriptionally up-regulate several components of the Notch pathway. In line with in vivo relevance of the 3D model, we report up-regulation of Notch3 in pre-invasive lesions of a HER2-driven animal model of breast cancer. Moreover, we show association of expression of several Notch pathway components with HER2 expression in patients with invasive breast cancer. Our results imply that the uncovered HER2-Notch3 collaboration is required during early steps of mammary tumorigenesis.

RESULTS

Ectopic overexpression of HER2 confers autonomous growth to human mammary epithelial cells

Previous studies modeled DCIS by overexpressing fusion proteins, comprising HER2 (intracellular domain) and the ectodomain of the receptor for the nerve growth factor, in MCF10A immortalized human mammary cells (Debnath *et al.*, 2002; Muthuswamy *et al.*, 2001). To model the effects of wild type, full-length HER2 on DCIS, we constructed MCF10A cells ectopically overexpressing the oncoprotein (C-R. Pradeep et al., manuscript submitted). Briefly, cells were stably infected with retroviral particles encoding HER2 and IRES-GFP (hereafter denoted MCF10A-HER2 cells) or IRES-GFP alone (hereafter MCF10A; (Ueda et al, 2004)). Immunoblotting of cell lysates obtained before or after stimulation with growth factors (EGF or neuregulin; NRG1-beta), confirmed that HER2 overexpression results in increased autophosphorylation (Lonardo *et al.*, 1990), transphosphorylation of EGFR and ligand-independent activation of ERK. In addition, on growth factor stimulation we confirmed enhanced and prolonged activation of the ERK pathway in HER2-overexpressors (Figure 1A) (Pinkas-Kramarski et al, 1996; Worthylake et al., 1999). These effects on signaling kinetics translated to enhanced cellular proliferation: unlike MCF10A cells, whose proliferation rates depended on growth factors, the enhanced proliferation rates of MCF10A-HER2 cells were not affected by growth factors (Figure 1B). An independent bromodeoxyuridine (BrdU) incorporation assay detected an EGF-induced three-fold enhancement of BrdU signals in MCF10A cells, unlike MCF10A-HER2 cells that displayed high BrdU signals independent of EGF. Thus, when overexpressed in normal mammary cells, HER2 confers high phosphorylation signals and autonomous cell growth, independent of growth factors.

HER2 transcriptionally induces multiple components of the Notch pathway

To resolve molecular bases underlying the growth autonomy conferred by HER2, we employed a three-dimensional (3D) culture system (reviewed in (Debnath and Brugge, 2005)). When grown in a preparation of extracellular matrix (Matrigel™), MCF10A cells form hollow spheroids, which were reported to undergo luminal filling when an ectopically expressed chimeric HER2 was forced to form homodimers (Muthuswamy *et al.*, 2001). Our MCF10A-HER2 cells overexpressing wild type HER2 similarly exhibited luminal filling, even in the absence of further treatments. Notably the MCF10A-HER2 spheroids retained an intact outer structure without any evidence of invasion (data not shown and Figure 2D, left panels). To identify the gene expression programs that promote luminal filling, RNA was extracted from 3D structures and hybridized to oligonucleotide microarrays. As expected, analyses of mRNAs significantly altered in MCF10A-HER2 cells revealed up-regulation of cell proliferation modules (C-R. Pradeep et al., manuscript submitted). In addition, we noted persistent up-regulation of several components of the Notch pathway, including two receptors and three JAG/DLL ligands, as well as ADAM17 and Presenilin1, proteases that cleave and activate Notch (Figure 2A). Congruent with simultaneous, multi-site induction of the Notch pathway, two prototypic target genes of the pathway, HES1 and HES2, also displayed elevated expression. We confirmed transcriptional induction of several components by using quantitative real-time PCR (qRT-PCR; Figure 2B), and by employing

a mouse Notch3 promoter-reporter luciferase vector. The vector was co-transfected, along with HER2, into two different mammary epithelial cell lines, which were subsequently incubated in the presence or absence of Trastuzumab. The results show that ectopic expression of HER2 remarkably induced Notch3 promoter activity in both cell lines (Supplementary Figure S1). Moreover, a monoclonal antibody against HER2 (Trastuzumab) partially inhibited the HER2- induced activation of the Notch3 promoter. Next, by applying a MEK-specific inhibitor (U0126) we found that the MAPK-ERK pathway, the major downstream effector of HER2, contributes to the transcriptional induction of the Notch pathway in lumen-filled spheroids (Figure 2C).

On losing contact with their extracellular matrix, luminal mammary cells, as well as MCF10A cells grown in spheroids, undergo anoikis, resulting in lumen formation, unless oncogenes like HER2, which enhances proliferation and inhibits apoptosis, are activated (Debnath *et al.*, 2002; Simpson *et al.*, 2008). Consistent with the possibility that the bypass of anoikis is mediated by the Notch pathway, we found that MCF10A-HER2 cells strongly expressed Notch3, whereas the hollow spheroids formed by MCF10A cells exhibited relatively weak expression (Figure 2D, right panels). Western blotting of cell lysates from spheroids confirmed that Notch3 and its active cleavage product, NICD, were expressed at higher levels in MCF10A-HER2 cells compared to MCF10A cells (Figure 2E), and fractionation indicated that both forms were present in the cytoplasm, but only NICD partitioned with the nuclear fraction of MCF10A-HER2 cells (Supplementary Figure S2). Interestingly, treatment with Trastuzumab almost abolished the nuclear species. Immunostaining localized Notch3 to the cytoplasm of MCF10A cells, but specifically detected a fraction of Notch3 within nuclei of MCF10A-HER2 cells (Figure 2F). In conclusion, HER2 overexpression leads to induction of multiple components of the Notch survival pathway, and this associates with nuclear localization of Notch3, raising the possibility that Notch mediates the effects of HER2 on luminal filling.

Notch3 and DLL1 promote survival and proliferation of HER2-overexpressing cells

To test whether Notch3 is required for survival and proliferation, we stably reduced Notch3 expression by applying specific shRNAs (Figure 3A). Two out of four different shRNAs we tested effectively reduced Notch3 expression in MCF10A-HER2 cells (the results obtained with each shRNA are presented in Figure 3 and in Supplementary Figure S3). When analyzed in monolayers, Notch3 knockdown significantly decreased proliferation of MCF10A-HER2 cells, such that they displayed growth rates similar to MCF10A cells (Figure 3B). To analyze the effect of Notch3 knockdown in 3D cultures, we applied two distinct approaches. The first, a suspension culture in the polyHEMA polymer (Dontu *et al.*, 2003), revealed that neither shControl- nor shNotch3- expressing MCF10A cells formed spheroids. In agreement with the ability of HER2 to confer autonomous growth, MCF10A-HER2 cells formed large spheroids, with Notch3 knockdown significantly reducing both their number and size (Figure 3C). The second protocol, spheroids grown in Matrigel, indicated that unlike MCF10A cells, which developed hollow spheroids by day 8, HER2-overexpressing cells evolved lumen-filled spheroids. Notch3 knockdown largely reversed the HER2-induced phenotype (Figure 3D). Staining of spheroids at day 6 for the cleaved form of Caspase-3 revealed luminal activity of this apoptosis-executing protease in

MCF10A spheroids, as well as in Notch3 knocked-down MCF10A-HER2 spheroids, in line with the notion that the Notch pathway enables HER2- overexpressors to evade anoikis (Figure 3D). In conclusion, three different cellular approaches indicated that the Notch pathway underlies the effects of HER2 on proliferation and survival of mammary cells.

Along with Notch3 up-regulation, overexpression of HER2 up-regulates the ligand DLL1 (Figs. 2A and 2B). Low concentrations of a recombinant form of DLL1 enhanced cleavage of Notch3, and congruently increased proliferation of MCF10A-HER2 cells (Supplementary Fig. S4). However, no comparable mitogenesis was observed with the parental MCF10A cells, a difference that was reflected also in the ability to support spheroid formation. Conceivably, the observed HER2-induced up-regulation of several components of the Notch pathway sensitizes mammary cells to DLL1, as well as to other Notch agonists.

Notch3-induced c-MYC, Cyclin D1 and AKT activity underlie the growth-promoting effect of HER2

Previous studies implicated c-MYC and Cyclin D1 in Notch-induced growth and survival signals (Cohen *et al.*, 2010; Palomero *et al.*, 2006). Likewise, our analyses revealed much higher expression of both c-MYC and Cyclin D1 in MCF10A-HER2 spheroids, relative to MCF10A spheroids (Figure 4A), and immunoblotting confirmed these differences at the protein level (Figure 4B). Inhibition of either HER2 signaling (using Trastuzumab) or Notch signaling (using an inhibitor of γ-secretase; GSI) reduced c-MYC and Cyclin D1 protein levels, with maximal reduction occurring upon treatment with the combination of drugs (Figure 4B). In the same vein, immunoblot analysis confirmed that knockdown of Notch3 in MCF10A-HER2 cells decreased the expression of both Cyclin D1 and c-MYC (Figure 4C).

To substantiate the conclusion that transcriptional induction of Notch3 and its regulated proteolytic cleavage suffice to induce Cyclin D1 and c-MYC, we ectopically expressed NICD in two non-HER2 overexpressing mammary epithelial cell lines, MDAMB231 and MCF10A. As expected, this resulted in concomitant up-regulation of c-MYC and Cyclin D1 (Figure 4D) (Palomero *et al.*, 2006). Next, by using siRNA oligonucleotides, we silenced the expression of the transcriptional repressor HES1, a well-established target of NICD, in two HER2 overexpressing lines, BT474 and MCF10A-HER2. HES1 knockdown enhanced the expression of the lipid phosphatase PTEN, in line with previous reports (Palomero *et al.*, 2007; Whelan *et al.*, 2007), and accordingly diminished the activating phosphorylation of AKT on serine-473 (Figure 4E). As a complementary approach, we stably overexpressed AKT2 or c-MYC in MCF10A-HER2 cells, and treated the respective spheroids with GSI (Supplementary Figures S5A and S5B). Unlike parental MCF10A-HER2 cells, which displayed luminal apoptosis, both transfected lines evaded apoptosis. Taken together, these results implicate up-regulation of c-MYC and Cyclin D1, along with enhanced activation of AKT, in a HER2-Notch survival pathway of mammary cells.

To explore potential therapeutic implications, we treated MCF10A-HER2 spheroids with Trastuzumab and GSI. Whereas either drug alone enhanced apoptosis of luminal cells in MCF10A-HER2 spheroids, their combination almost completely abolished formation of filled lumina (Figure 4F). Similarly, when applied on MCF10A-HER2 spheroids, pathwayspecific inhibitors targeting MEK (U0126), c-MYC (10058-F4) or PI3K-AKT

(LY-294002) markedly enhanced Caspase-3 activation, resulting in significant inhibition of luminal filling (Supplementary Figures S5C and S5D). In conclusion, the HER2-to-Notch axis is linked to an apoptosis evasion mechanism that entails c-MYC and Cyclin D1, along with coupling of HER2 to AKT activation.

Notch3 expression correlates with HER2 levels in an animal model and in human breast cancer specimens

Studies using transgenic mice demonstrated that overexpression of an activated form of Notch1 or Notch3 in the mammary gland results in increased formation of mammary tumors (Hu et al, 2006). Our results using 2D and 3D models of HER2-overexpressing DCIS propose that HER2 activation harnesses the Notch pathway to accelerate cellular proliferation, and hence may support mammary tumors *in vivo*. To test this prediction, we stained for Notch3 mammary glands of transgenic mice carrying an activated form of the HER2/*neu* oncogene, under the control of the mouse mammary tumor virus (MMTV) long terminal repeat (Bouchard *et al.*, 1989; Tekmal *et al.*, 2007). Indeed, hyperplastic lesions, which frequently develop in the mammary glands of MMTV-HER2/neu transgenic mice, exhibited homogeneous weak to moderate immunohistochemical staining for Notch3, which was accentuated in cells facing the ductal lumen (Figure 5A). Conversely, normal mammary glands of non-HER2 transgenic mice from the same strain displayed a heterogeneous staining pattern, with Notch3 expression mostly confined to small ducts (Figure 5A), likely reflecting a role in the transition from small to mature ducts.

To determine the relevance of our findings to human breast cancer, we analyzed two clinical datasets (Desmedt *et al.*, 2007; Schmidt *et al.*, 2008), each derived from oligonucleotide microarray analyses of approximately 200 breast cancer patients, for possible associations between HER2 mRNA expression and presence of components of the Notch pathway. In line with our *in vitro* expression data (Figure 2B), Notch3 along with presenilin and HES1 presented highly significant correlations with HER2 expression (Table 1). Interestingly, our analyses found weak negative correlation between HER2 and Notch1, although co-expression of JAG-1 and Notch1 occurs in aggressive human breast tumors, which do not belong to the HER2 subtype (Reedijk *et al.*, 2005).

In order to confirm the association between HER2 and Notch3 at the protein level in clinical specimens, we used reverse-phase protein arrays (RPPA) (Hennessy *et al.*, 2010). Analyses of mammary tumors from two independent patient cohorts (approximately 100 patients per cohort) confirmed significant correlation between the phosphorylated, active form of HER2 (p1248HER2) and Notch3 (cohort 1: r=0.43, p=1.55E-05; cohort 2: r=0.23, p=2.58E-02; Figure 5B). Moreover, in both data sets Notch3 protein levels also significantly correlated with EGFR expression (r=0.37 or 0.28; p<1.00E-02 for both). Individual patientrelated data were available for the second cohort, for which subgroup analyses revealed correlation of Notch3 with levels of HER2 (r=0.31, p=3.16E-02) and p1248HER2 (r=0.34, p=1.80E-02) in 48 patients with poorly differentiated tumors. However, no such correlation was observed in moderately or well-differentiated tumors (HER2 r=0.03, p1248HER2 r=0.18, p>5.00E-02 for both). On the other hand, patient subgroups defined by age, menopausal status or expression of the estrogen receptor (ER) and/or the progesterone receptor (PR) did not

exhibit differences with respect to the correlation between Notch3 and either HER2 or p1248HER2 (data not shown).

In summary, our *in vitro* results, animal studies and clinical data lend collective support to an hypothesis arguing that the non-invasive cell proliferation associated with HER2-overexpressing mammary lesions, such as DCIS, is mediated, by the Notch pathway. Apparently, by activating proliferation and survival pathways comprising c-MYC, Cyclin D, and AKT, Notch signaling mediates filling of mammary ducts with HER2-overexpressing cells. Future studies will examine the ability of combination therapy targeting both HER2 and Notch to delay the putative transition from DCIS to infiltrating ductal carcinoma overexpressing the HER2 oncoprotein.

DISCUSSION

The evolutionary conserved Notch signaling pathway is considered a critical regulator of cell fate decisions in embryonic development, including hematopoiesis, neurogenesis and development of several organs, such as the mammary gland (Liu *et al.*, 2010). For example, proliferation and differentiation of mammary stem cells towards luminal and myoepithelial cell lineages are controlled in large part by the Notch pathway (Shackleton *et al.*, 2006; Stingl *et al.*, 2006). Thus, ectopic activation of Notch signaling commits mammary stem cells to the luminal lineage, as well as enhances proliferation of luminal cells, leading ultimately to their transformation (Bouras *et al.*, 2008). On the other hand, inhibition of Notch signaling enhances self-renewal, rather than differentiation, of mammary stem cells. It is, therefore, not surprising that the Notch pathway is amply employed by tumor cells to thrust their survival and growth. Whereas in small cell lung cancer, Notch may act as a tumor suppressive pathway (Sriuranpong *et al.*, 2001), gain-of-function mutations and a chromosomal translocation leading to constitutive activation of Notch1 were identified in human T-cell acute lymphoblastic leukemia (Ellisen *et al.*, 1991; Weng *et al.*, 2004), gene amplification of Notch3 was detected in ovarian cancer (Nakayama *et al.*, 2007), and relatively low levels of the Notch antagonist Numb were noted in breast tumors (Pece *et al.*, 2004). Our study unveils yet another mechanism that harnesses Notch signaling to promote malignant growth. Coordinated transcriptional induction of several Notch pathway components (summarized in Figure 5C) appears essential for HER2-induced enhancement of proliferation and survival of mammary epithelial cells. Importantly, the 3D experimental model we employed proposes that the HER2-to-Notch pathway, although robustly promoting growth factor-independent cell proliferation, is unable to induce basement membrane breakdown and subsequent invasive growth. Presumably, additional insults are needed to unleash the migratory potential of HER2-initiated cells. Interestingly, stimulation with EGF, which promotes formation of heterodimers of HER2 with the EGF-receptor, was reported to be sufficient for the emergence of an invasive phenotype of HER2-overexpressing spheroids (Zhan *et al.*, 2006).

Previous lines of evidence are consistent with our conclusion that HER2 overexpression in the mammary epithelium is functionally linked to the Notch pathway. For example, a recent study found that enhanced expression of Notch1 represents an early transforming event in both a murine model of DCIS and in human breast tumors (Zardawi *et al.*, 2010).

Interestingly, a positive feedback loop may escalate HER2 and Notch expression in tumors; on the one hand HER2's promoter contains a Notch-binding sequence (Chen *et al.*, 1997), and, on the other hand, overexpression of HER2 transcriptionally induces the Notch pathway, as we demonstrate in this study. Another emerging feature entails involvement of the HER2-Notch pathway in breast cancer stem cells. Notch-mediated up-regulation of HER2 enhances the tumor-initiating potential of mammary cells (Clemenz and Osipo, 2009), whereas Notch-driven HER2-overexpressing breast cancer cells show characteristics of tumor initiating cells that can be inhibited by Trastuzumab (Magnifico *et al.*, 2009). Interestingly, HER2 overexpression increases the proportion of stem/progenitor cells as demonstrated by the expression of the stem cell marker aldehyde dehydrogenase (ALDH) (Korkaya *et al.*, 2008). The effects of HER2 overexpression on breast cancer stem cells are blocked by Trastuzumab in sensitive, but not resistant, cell lines, an effect mediated by the PI3K-to-AKT pathway. It is notable that HER2 cannot directly recruit PI3K, hence it must engage a surrogate receptor, such as ERBB-3/HER3 (Prigent and Gullick, 1994; Wallasch *et al.*, 1995). The results presented herein delineate an alternative mechanism, analogous to the mode identified in leukemia (Palomero *et al.*, 2008): Notch activation reduces PTEN expression, and thereby elevates levels of 3′ phosphoinositides necessary for AKT stimulation.

Beyond the understanding that two oncogenic pathways, HER2 and Notch, jointly constitute a novel module that likely underlies the luminal filling characteristics of DCIS, our study bears potential clinical implications. Two implications are worth mentioning, especially in light of the current debate pertaining to relative risks and optimal treatment of this noninvasive neoplasm. For one, co-incidence of HER2 and active Notch may identify a group of DCIS patients who are at increased risk of relapse after surgery. Secondly, the ongoing interactions between HER2 and Notch in later stages of tumor development, as pointed out in our study, highlight the potential of treatment strategies that combine anti-HER2 antibodies with Notch antagonists (such as GSI) or with PI3K/AKT kinase inhibitors. Such combinations displayed effectiveness in our 3D model system, hence may prove useful in clinical settings.

MATERIALS AND METHODS

Reagents, cell lines, animals and breast tumor samples

The Notch3 antibody was purchased from Cell Signaling Technology (Beverly, MA). HRP–conjugated antibodies were from the Jackson Laboratories (Bar Harbor, Maine). Notch3-ICD-pCDNA3.1 was kindly provided by Dr. Isabella Screpanti (LaSapienza, Rome, Italy). HES1 siRNA was from Dharmacon (Lafayette, CO, USA) and DLL1 was purchased from R&D Systems. Cell growth was assayed by using a 3-(4,5-dimethylthiazol-z-yl)-2,5-diphenyl tetrazolium bromide (MTT) based kit. MCF10A cells were maintained as previously described (Katz *et al.*, 2007). Mammary fat pads of HER-2/neu transgenic or wild type FVB mice (Jackson Laboratories) were processed as previously described (Tekmal *et al.*, 2007). Breast tumor samples for RPPA were obtained from the Baylor College of Medicine Breast Centre Anonymized Tumor Bank (Cohort 1; (Speers et al, 2009)) and the M.D. Anderson Cancer Centre Tumor Bank (Cohort 2).

Retroviral infection

c-MYC-tagged HER2 cDNA cloned in a retroviral expression vector (pBMN-IRES-EGFP) was provided by Dr. Carlos L. Arteaga (Vanderbilt University School of Medicine, Nashville, TN). pBMN-HER2-IRES-EGFP or pBMN-IRES-EGFP (control) were co-transfected with a retroviral packaging plasmid, pSV-ψ-env-MLV (provided by Dr. Jane Burns, University of California, San Diego, CA) into 293T cells using FuGENE (Roche Applied Science, Indianapolis). Virus-containing medium was collected 48–72 hours later and passed through a 45-μm filter. MCF10A cells were transduced with control or HER2-encoding retroviral vectors and cells stably expressing GFP after 5 passages were selected by flow cytometry.

Immunofluorescence and confocal microscopy

Acinar structures were fixed on glass slides for 10 minutes in methanol-acetone (1:1; −20°C), and air-dried before blocking for 1 hour at room temperature in immunofluorescence buffer (130mM NaCl, 7mM Na_2HPO_4, 3.5mM NaH_2PO_4, 7.7mM NaN_3, 0.1% bovine serum albumin, 0.2% Triton X-100 and 0.05% Tween-20 and 10% goat serum). Secondary blocking was performed for 30 minutes in immunofluorescence buffer containing goat anti-mouse $F(ab')_2$ fragment (20 μg/ml). The primary antibody was incubated at 4°C for 15–18 hours. Secondary antibodies conjugated to fluorescent dyes and diluted in blocking buffer were subsequently incubated for 60 minutes at room temperature. Images presented are representative of three or more independent experiments.

Real-time quantitative PCR and oligonucleotide microarray hybridization

Total RNA was isolated using a Versagene kit (Gentra Systems, Minneapolis) and reverse transcribed with random hexamers (SuperScript II first-strand synthesis kit, InVitrogen, California). Real-time PCR analysis was performed using SYBR Green I (Applied Biosystems) in triplicates, and the results were normalized to beta-2 microglobulin. For oligonucleotide microarray hybridization, RNA (10μg) was labeled, fragmented and hybridized to Affymetrix HuGENE 1.0 ST arrays. After scanning of the arrays, we calculated gene expression values and normalized the results using the expression console of Affymetrix (RMA normalization). The GEO code for the data: GSE18938, and the link: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=vjgzdemksqmgqpi&acc=GSE18938

Cell cultures in polyHEMA

Cells were cultured in polyHEMA as previously described (Dontu *et al.*, 2003).

Reverse-phase protein arrays (RPPA)

RPPA analyses were performed as described previously (Hennessy *et al.*, 2010).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Amit Zeisel and Sara Lavi for help, Brenda Lilly for the Notch3 reporter, and Powel Brown, Corey Speers and the Kleberg Center for Molecular Markers at MD Anderson Cancer Center (CCSG grant NCI CA16672) for providing tumors for the RPPA analysis. We acknowledge research funding by the National Cancer Institute (CA072981, CA121994-01, and CA120248-01), the Israel Cancer Research Fund, Dr. Miriam and Sheldon G. Adelson Medical Research Foundation, Kekst Family Institute for Medical Genetics, Kirk Center for Childhood Cancer and Immunological Disorders, the Women's Health Research Center funded by Bennett- Pritzker Endowment Fund, Marvelle Koffler Program for Breast Cancer Research, Leir Charitable Foundation and the M.D. Moross Institute for Cancer Research, The Susan G. Komen Foundation (FAS0703849 to A.M.G., B.T.H. and G.B.M.), and a fellowship for Ph.D. track for specialist medical doctors by the Linda and Michael Jacobs Charitable Trust (W.J.K.). Y.Y. is the incumbent of the Harold and Zelda Goldenberg Professorial Chair and E.D. of the Henry J. Leir Professorial Chair.

References

Aranda V, Haire T, Nolan ME, Calarco JP, Rosenberg AZ, Fawcett JP, et al. Par6- aPKC uncouples ErbB2 induced disruption of polarized epithelial organization from proliferation control. Nat Cell Biol. 2006; 8:1235–45. [PubMed: 17060907]

Bouchard L, Lamarre L, Tremblay PJ, Jolicoeur P. Stochastic appearance of mammary tumors in transgenic mice carrying the MMTV/c-neu oncogene. Cell. 1989; 57:931–6. [PubMed: 2567634]

Bouras T, Pal B, Vaillant F, Harburg G, Asselin-Labat ML, Oakes SR, et al. Notch signaling regulates mammary stem cell function and luminal cell-fate commitment. Cell Stem Cell. 2008; 3:429–41. [PubMed: 18940734]

Chen Y, Fischer WH, Gill GN. Regulation of the ERBB-2 promoter by RBPJkappa and NOTCH. J Biol Chem. 1997; 272:14110–4. [PubMed: 9162037]

Clemenz AZ, Osipo C. Notch1 activates ErbB-2 through a PEA3-dependent mechanism. Cancer Research. 2009; 69:362s.

Cohen B, Shimizu M, Izrailit J, Ng NF, Buchman Y, Pan JG, et al. Cyclin D1 is a direct target of JAG1-mediated Notch signaling in breast cancer. Breast Cancer Res Treat. 2010; 123:113–24. [PubMed: 19915977]

Debnath J, Brugge JS. Modelling glandular epithelial cancers in three-dimensional cultures. Nat Rev Cancer. 2005; 5:675–88. [PubMed: 16148884]

Debnath J, Mills KR, Collins NL, Reginato MJ, Muthuswamy SK, Brugge JS. The role of apoptosis in creating and maintaining luminal space within normal and oncogeneexpressing mammary acini. Cell. 2002; 111:29–40. [PubMed: 12372298]

Debnath J, Walker SJ, Brugge JS. Akt activation disrupts mammary acinar architecture and enhances proliferation in an mTOR-dependent manner. J Cell Biol. 2003; 163:315–26. [PubMed: 14568991]

Desmedt C, Piette F, Loi S, Wang Y, Lallemand F, Haibe-Kains B, et al. Strong time dependence of the 76-gene prognostic signature for node-negative breast cancer patients in the TRANSBIG multicenter independent validation series. Clin Cancer Res. 2007; 13:3207–14. [PubMed: 17545524]

Dontu G, Abdallah WM, Foley JM, Jackson KW, Clarke MF, Kawamura MJ, et al. In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. Genes Dev. 2003; 17:1253–70. [PubMed: 12756227]

Ellisen LW, Bird J, West DC, Soreng AL, Reynolds TC, Smith SD, et al. TAN-1, the human homolog of the Drosophila notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. Cell. 1991; 66:649–61. [PubMed: 1831692]

Hennessy B, Lu Y, Gonzalez-Angulo AM, Myhre S, Carey M, Ju Z, et al. A technical assessment of the utility of reverse phase protein arrays for the study of the functional proteome in non-microdissected human breast cancers. Clinical Proteomics. 2010 in press.

Howlin J, McBryan J, Martin F. Pubertal mammary gland development: insights from mouse models. J Mammary Gland Biol Neoplasia. 2006; 11:283–97. [PubMed: 17089203]

Hudziak RM, Lewis GD, Winget M, Fendly BM, Shepard HM, Ullrich A. p185HER2 monoclonal antibody has antiproliferative effects in vitro and sensitizes human breast tumor cells to tumor necrosis factor. Mol Cell Biol. 1989; 9:1165–72. [PubMed: 2566907]

Ignatiadis M, Desmedt C, Sotiriou C, de Azambuja E, Piccart M. HER-2 as a target for breast cancer therapy. Clin Cancer Res. 2009; 15:1848–52. [PubMed: 19289395]

Imatani A, Callahan R. Identification of a novel NOTCH-4/INT-3 RNA species encoding an activated gene product in certain human tumor cell lines. Oncogene. 2000; 19:223–31. [PubMed: 10645000]

Iso T, Kedes L, Hamamori Y. HES and HERP families: multiple effectors of the Notch signaling pathway. J Cell Physiol. 2003; 194:237–55. [PubMed: 12548545]

Jechlinger M, Podsypanina K, Varmus H. Regulation of transgenes in threedimensional cultures of primary mouse mammary cells demonstrates oncogene dependence and identifies cells that survive deinduction. Genes Dev. 2009; 23:1677–88. [PubMed: 19605689]

Katz M, Amit I, Citri A, Shay T, Carvalho S, Lavi S, et al. A reciprocal tensin-3-cten switch mediates EGF-driven mammary cell migration. Nat Cell Biol. 2007; 9:961–9. [PubMed: 17643115]

Korkaya H, Paulson A, Iovino F, Wicha MS. HER2 regulates the mammary stem/progenitor cell population driving tumorigenesis and invasion. Oncogene. 2008; 27:6120–30. [PubMed: 18591932]

Liu J, Sato C, Cerletti M, Wagers A. Notch signaling in the regulation of stem cell self-renewal and differentiation. Curr Top Dev Biol. 2010; 92:367–409. [PubMed: 20816402]

Lonardo F, Di Marco E, King CR, Pierce JH, Segatto O, Aaronson SA, et al. The normal erbB-2 product is an atypical receptor-like tyrosine kinase with constitutive activity in the absence of ligand. New Biol. 1990; 2:992–1003. [PubMed: 1983208]

Magnifico A, Albano L, Campaner S, Delia D, Castiglioni F, Gasparini P, et al. Tumor-initiating cells of HER2-positive carcinoma cell lines express the highest oncoprotein levels and are sensitive to trastuzumab. Clin Cancer Res. 2009; 15:2010–21. [PubMed: 19276287]

Mazzone M, Selfors LM, Albeck J, Overholtzer M, Sale S, Carroll DL, et al. Dosedependent induction of distinct phenotypic responses to Notch pathway activation in mammary epithelial cells. Proc Natl Acad Sci U S A. 2010; 107:5012–7. [PubMed: 20194747]

Muthuswamy SK, Li D, Lelievre S, Bissell MJ, Brugge JS. ErbB2, but not ErbB1, reinitiates proliferation and induces luminal repopulation in epithelial acini. Nat Cell Biol. 2001; 3:785–92. [PubMed: 11533657]

Nakayama K, Nakayama N, Jinawath N, Salani R, Kurman RJ, Shih Ie M, et al. Amplicon profiles in ovarian serous carcinomas. Int J Cancer. 2007; 120:2613–7. [PubMed: 17351921]

Palomero T, Dominguez M, Ferrando AA. The role of the PTEN/AKT Pathway in NOTCH1-induced leukemia. Cell Cycle. 2008; 7:965–70. [PubMed: 18414037]

Palomero T, Lim WK, Odom DT, Sulis ML, Real PJ, Margolin A, et al. NOTCH1 directly regulates c-MYC and activates a feed-forward-loop transcriptional network promoting leukemic cell growth. Proc Natl Acad Sci U S A. 2006; 103:18261–6. [PubMed: 17114293]

Palomero T, Sulis ML, Cortina M, Real PJ, Barnes K, Ciofani M, et al. Mutational loss of PTEN induces resistance to NOTCH1 inhibition in T-cell leukemia. Nat Med. 2007; 13:1203–10. [PubMed: 17873882]

Pece S, Serresi M, Santolini E, Capra M, Hulleman E, Galimberti V, et al. Loss of negative regulation by Numb over Notch is relevant to human breast carcinogenesis. J Cell Biol. 2004; 167:215–21. [PubMed: 15492044]

Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, et al. Molecular portraits of human breast tumours. Nature. 2000; 406:747–52. [PubMed: 10963602]

Prigent SA, Gullick WJ. Identification of c-erbB-3 binding sites for phosphatidylinositol 3′-kinase and SHC using an EGF receptor/c-erbB-3 chimera. EMBO J. 1994; 13:2831–41. [PubMed: 8026468]

Reedijk M, Odorcic S, Chang L, Zhang H, Miller N, McCready DR, et al. High-level coexpression of JAG1 and NOTCH1 is observed in human breast cancer and is associated with poor overall survival. Cancer Res. 2005; 65:8530–7. [PubMed: 16166334]

Schafer ZT, Grassian AR, Song L, Jiang Z, Gerhart-Hines Z, Irie HY, et al. Antioxidant and oncogene rescue of metabolic defects caused by loss of matrix attachment. Nature. 2009; 461:109–13. [PubMed: 19693011]

Schmidt M, Bohm D, von Torne C, Steiner E, Puhl A, Pilch H, et al. The humoral immune system has a key prognostic impact in node-negative breast cancer. Cancer Res. 2008; 68:5405–13. [PubMed: 18593943]

Shackleton M, Vaillant F, Simpson KJ, Stingl J, Smyth GK, Asselin-Labat ML, et al. Generation of a functional mammary gland from a single stem cell. Nature. 2006; 439:84–8. [PubMed: 16397499]

Simpson CD, Anyiwe K, Schimmer AD. Anoikis resistance and tumor metastasis. Cancer Lett. 2008; 272:177–85. [PubMed: 18579285]

Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science. 1987; 235:177–82. [PubMed: 3798106]

Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Engl J Med. 2001; 344:783–92. [PubMed: 11248153]

Speers C, Tsimelzon A, Sexton K, Herrick AM, Gutierrez C, Culhane A, et al. Identification of novel kinase targets for the treatment of estrogen receptor-negative breast cancer. Clin Cancer Res. 2009; 15:6327–40. [PubMed: 19808870]

Sriuranpong V, Borges MW, Ravi RK, Arnold DR, Nelkin BD, Baylin SB, et al. Notch signaling induces cell cycle arrest in small cell lung cancer cells. Cancer Res. 2001; 61:3200–5. [PubMed: 11306509]

Stingl J, Eirew P, Ricketson I, Shackleton M, Vaillant F, Choi D, et al. Purification and unique properties of mammary epithelial stem cells. Nature. 2006; 439:993–7. [PubMed: 16395311]

Stylianou S, Clarke RB, Brennan K. Aberrant activation of notch signaling in human breast cancer. Cancer Res. 2006; 66:1517–25. [PubMed: 16452208]

Tekmal RR, Nair HB, Perla RP, Kirma N. HER-2/neu x aromatase double transgenic mice model: the effects of aromatase overexpression on mammary tumorigenesis. J Steroid Biochem Mol Biol. 2007; 106:111–8. [PubMed: 17604617]

van de Vijver MJ, Peterse JL, Mooi WJ, Wisman P, Lomans J, Dalesio O, et al. Neuprotein overexpression in breast cancer. Association with comedo-type ductal carcinoma in situ and limited prognostic value in stage II breast cancer. N Engl J Med. 1988; 319:1239–45. [PubMed: 2903446]

Wallasch C, Weiss FU, Niederfellner G, Jallal B, Issing W, Ullrich A. Heregulindependent regulation of HER2/neu oncogenic signaling by heterodimerization with HER3. EMBO J. 1995; 14:4267–75. [PubMed: 7556068]

Weng AP, Ferrando AA, Lee W, Morris JPt, Silverman LB, Sanchez-Irizarry C, et al. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. Science. 2004; 306:269–71. [PubMed: 15472075]

Whelan JT, Forbes SL, Bertrand FE. CBF-1 (RBP-J kappa) binds to the PTEN promoter and regulates PTEN gene expression. Cell Cycle. 2007; 6:80–4. [PubMed: 17245125]

Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. Nat Rev Mol Cell Biol. 2001; 2:127–37. [PubMed: 11252954]

Yin L, Velazquez OC, Liu ZJ. Notch signaling: emerging molecular targets for cancer therapy. Biochem Pharmacol. 2010; 80:690–701. [PubMed: 20361945]

Zardawi SJ, Zardawi I, McNeil CM, Millar EK, McLeod D, Morey AL, et al. High Notch1 protein expression is an early event in breast cancer development and is associated with the HER-2 molecular subtype. Histopathology. 2010; 56:286–96. [PubMed: 20459529]

Zhan L, Xiang B, Muthuswamy SK. Controlled activation of ErbB1/ErbB2 heterodimers promote invasion of three-dimensional organized epithelia in an ErbB1- dependent manner: implications for progression of ErbB2-overexpressing tumors. Cancer Res. 2006; 66:5201–8. [PubMed: 16707444]

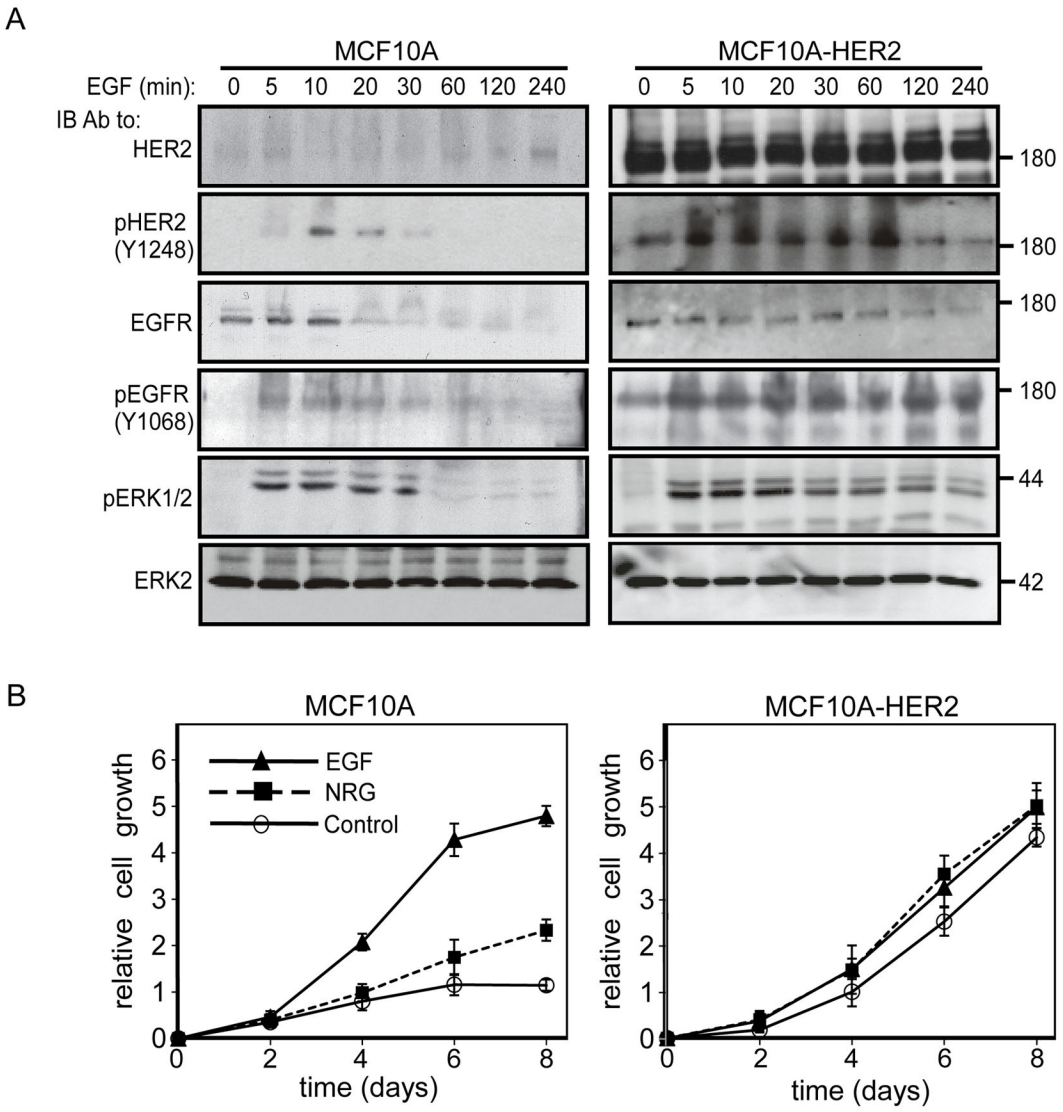

Figure 1. Ectopic overexpression of HER2 releases monolayers of mammary cells from growth saturation and from reliance on growth factors

A, Monolayers of MCF10A cells stably expressing the plasmid IRES-GFP (MCF10A) or HER2-IRES-EGFP (MCF10A-HER2) were starved for 24 hours and stimulated with EGF (20 ng/ml) for the indicated time intervals. Cell lysates were electrophoresed and immunoblotted (IB) with the indicated antibodies. **B**, MCF10A and MCF10A-HER2 cells were grown for up to 8 days in the presence or absence of EGF or NRG-1β (each at 20 ng/ml). Cell growth was monitored using the MTT assay. Data represent averages ± S.D. of triplicates. The experiment was repeated thrice.

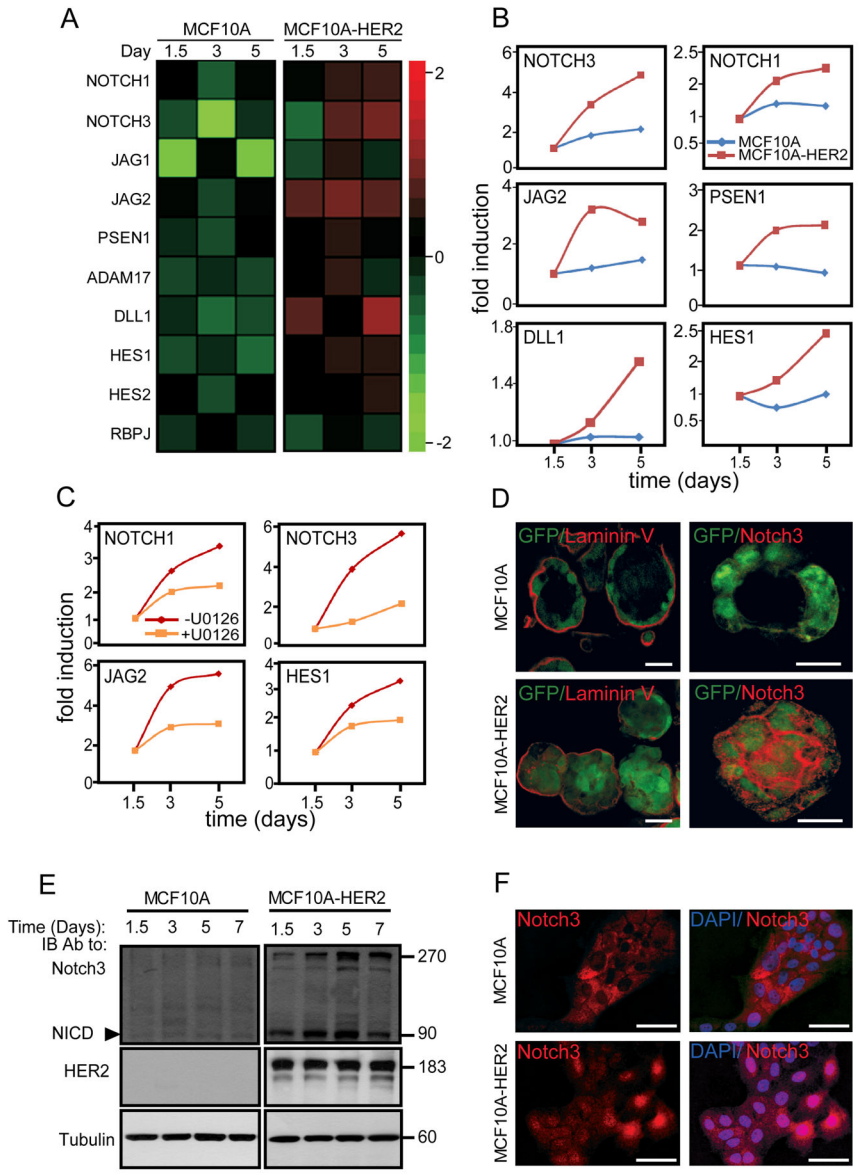

Figure 2. HER2 transcriptionally induces multiple components of the Notch pathway

A, Expression heatmaps of Notch pathway genes, whose expression levels, as determined using oligonucleotide microarrays, differ between spheroids of MCF10A and MCF10AHER2 cells seeded in Matrigel (day 0) and cultured for the indicated time intervals. The color bar depicts relative expression levels. **B,** Quantitative real-time PCR (qRT-PCR) was used for validation of microarray expression profiles of selected Notch pathways genes in MCF10A and MCF10A-HER2 spheroids seeded at day 0 and cultured in Matrigel for the indicated time intervals. **C,** qRT-PCR analyses of selected Notch pathway genes in MCF10A-HER2 spheroids incubated for up to five days in the absence or presence of the MEK inhibitor U0126 (1μM). **D,** Confocal photomicrographs showing GFP-expressing MCF10A and MCF10A-HER2 spheroids immunostained for Laminin V (left panels), or for Notch3 (right panels), eight days after seeding single cells in Matrigel™. Scale bars, 50μm. **E,** MCF10A and MCF10A-HER2 cells were grown on Matrigel for the

indicated intervals. The resulting spheroids were extracted and the lysates were immunoblotted with the indicated antibodies. NICD, Notch intracellular domain. **F,** Monolayers of MCF10A and MCF10A-HER2 cells were grown in serum-free medium, fixed, immunostained for Notch3 (red) and nuclei counterstained with DAPI (blue). Scale bar, 40μm.

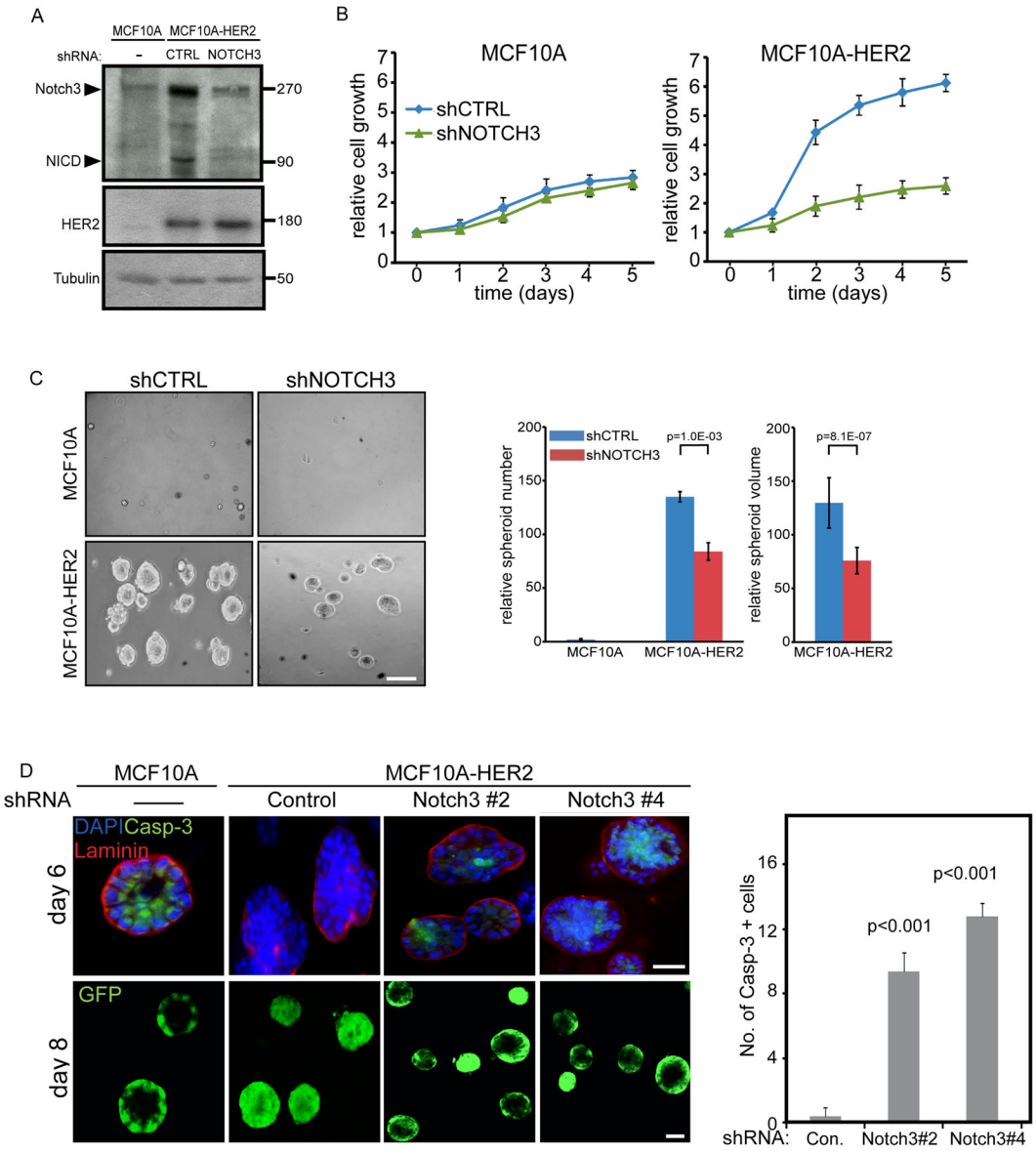

Figure 3. Enhanced survival and proliferation of HER2-overexpressing cells are enabled by Notch3

A, Extracts of monolayers of MCF10A cells and MCF10A-HER2 cells stably expressing control shRNA or shRNA targeting Notch3 were immunoblotted with the indicated antibodies. **B,** Proliferation of monolayer MCF10A and MCF10A-HER2 cells stably expressing the indicated shRNAs was determined using the MTT assay. Averages and standard deviation values (bars) of triplicates are presented**. C,** MCF10A and MCF10AHER2 cells stably expressing the indicated shRNAs were cultured for 8 days in polyHEMAcoated wells and photographed using a phase contrast microscope (upper part; scale bar, 100 μm). The number of spheroids per well was determined in triplicates and the average and standard deviations (bars) are presented (lower left panel). For MCF10A-HER2 cells, we estimated the volume of 120 spheroids per condition and presented the average volume and the standard errors (bars). **D,** Control MCF10A cells and MCF10-HER2 cells

stably expressing control shRNAs, or two different clones of cells expressing shRNAs targeting Notch3, were grown in Matrigel for the indicated time intervals and images captured by confocal microscopy. The upper row shows immunostaining for cleaved (active) Caspase-3 (green), laminin V (red) and DAPI (blue; scale bar, 25μm), whereas the lower panels present the anatomy of the GFP-expressing spheroids (scale bar, 50 μm). The bar graph presents the average fractions (± S.D., bars) of lumen-filled spheroids, as determined by analyzing 20 spheroids of each group.

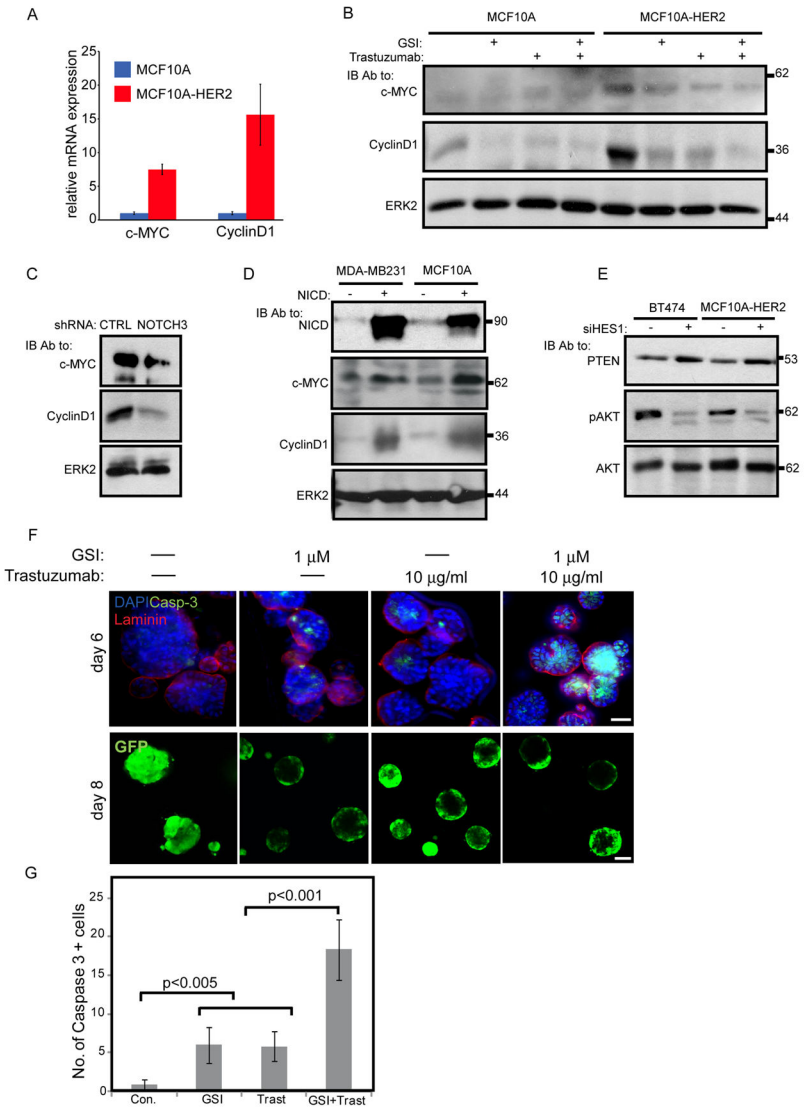

Figure 4. Notch3 promotes survival of HER2-overexpressing mammary cells

A, The relative expression levels of transcripts corresponding to c-MYC and Cyclin D1 (CCND1) were determined by applying quantitative real-time PCR to RNA samples from MCF10A and MCF10A-HER2 spheroids (in triplicates). **B,** MCF10A and MCF10A-HER2 cells were grown in Matrigel for 4 days and then the resulting spheroids were incubated in the presence of Trastuzumab (10 μg/ml) and/or a gamma-secretase inhibitor (GSI, 1μM). Two days later, cells were extracted and subjected to immunoblotting, as indicated. **C,** Monolayers of MCF10A-HER2 cells stably transduced with control or Notch3 shRNAs were lysed and immunoblotted for c-MYC and Cyclin D1. **D,** Monolayers of MDA-MB231 and MCF10A cells were transfected with pCDNA3-Notch3-NICD or with an empty plasmid, lysed 48 hours later and immunoblotted using the indicated antibodies. **E,** BT474 and MCF10A-HER2 cells were grown in monolayers and transfected with control or HES1-specific siRNA oligonucleotides, followed by lysis 48 hours later and immunoblotting with the indicated antibodies. **F** and **G,** MCF10A-HER2 spheroids were grown in Matrigel for 4

days and then incubated with Trastuzumab and/or GSI for up to 4 additional days. Confocal microphotographs show acinar morphology of GFP-expressing cells (lower panels), along with staining for the cleaved form of Caspase-3 (green), laminin V (red) and DAPI (blue) in the upper panels. Scale bars, 50 μm. The fraction of lumen-filled spheroids on day 8 was quantified by counting 20 spheroids in each treatment group. Data denote averages (±S.D.) of triplicates.

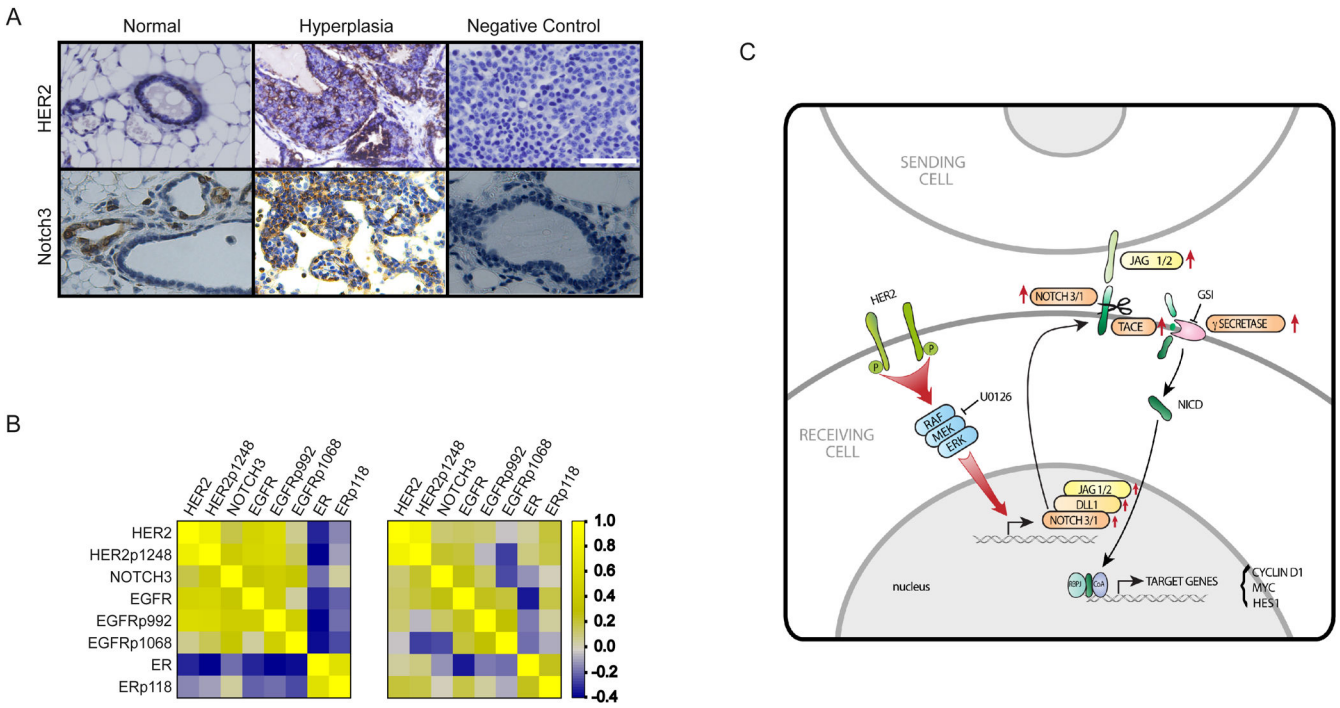

Figure 5. Notch3 expression correlates with HER2 levels in human mammary tumors and in an animal model overexpressing HER2

A, Immunohistochemical images of HER2 and Notch3 expression in mammary ducts of transgenic MMTV-HER2 mice. Both normal and hyperplastic ducts are shown, along with panels obtained with control antibodies. Scale bar, 200μm. **B,** Lysates of invasive breast cancer specimens were analyzed using reverse phase protein arrays (RPPA) for expression of Notch3, along with the levels of total and phosphorylated forms of EGFR, HER2 and ER. Two independent patient cohorts were employed: Cohort 1: left heatmap, n=102 patients (Speers *et al.*, 2009), and Cohort 2: right heatmap, n=95 patients. Heatmaps show correlation matrices of protein expression and the color scheme corresponds to Pearson correlation coefficients (r). Note high correlation between Notch3 and the phosphorylated form of HER2 (p1248) in both cohorts (r=0.43, p=1.55E-05 for the left cohort, and r=0.23, p=2.58E-02 for the right cohort). **C,** Schematic presentation of the effects of HER2 on the Notch pathway, specifically referring to components up-regulated (red vertical arrows) in HER2- overexpressing MCF10A cells. NICD, Notch intracellular domain.

Table 1

Correlation of mRNA expression of Notch pathway genes with HER2 mRNA expression

Two clinical datasets of gene expression microarrays of breast tumors were analyzed for correlation between HER2 expression and the indicated components of the Notch pathway. Correlation coefficients (r) and *p*-values are indicated.

	Dataset (number of patients)			
	Desmedt et al., 2007 (n=198)		**Schmidt et al., 2008 (n=200)**	
	Correlation coefficient (r)	***p*-value**	**Correlation coefficient (r)**	***p*-value**
NOTCH3	0.312	7.23E-06	0.257	2.36E-04
PSEN1	0.355	2.67E-07	0.425	3.44E-10
HES1	0.281	5.83E-05	0.309	6.30E-06
NOTCH1	−0.226	1.40E-03	−0.249	3.80E-04
NOTCH2	−0.100	1.61E-01	−0.306	1.03E-05