SUPPLEMENTAL MATERIALS AND METHODS:

Immunohistochemistry: Five μm sections were cut, deparaffinized and stained with appropriate antibodies and controls. Estrogen receptor (clone 6F11, Ventana, Tucson, AZ), progesterone receptor (clone PGP-1A6, Ventana), p53 (clone Bp53-11, Ventana), Her2/Neu (monoclonal TAB250, Zymed, San Francisco CA) and Ki67 (monoclonal 7B11, Zymed, San Francisco, CA) immunostaining was conducted using standard operating procedures for diagnostic pathology. Her2/neu was evaluated in a semiquantitatively fashion and was assigned a 0, 1+, 2+, and 3+ score (negative, weak, moderate or strong) according to surgical pathology criteria. Notch receptors and ligands were stained using commercial goat polyclonal antibodies from Santa Cruz Biotechnology (Notch-1 C-20, Notch-4 C-19, Delta-1 C-20, and Jagged-1, C-20). An automated Leica Histostainer Ig was used. Serial sections were simultaneously stained with goat non-specific IgG as negative controls. Incubation with primary antibody was performed for 3 h at room temperature. Detection was performed using the Mouse ABC staining kit (Vectastain, Vector Laboratories, Burlingame, CA), which includes biotinylated secondary antibodies and macromolecular avidin-biotinylated horseradish peroxidase. Counterstaining was avoided in most cases to prevent hiding weak nuclear Notch signal. Slides were dehydrated and permanently mounted in non aqueous mounting medium. Staining intensity compared to simultaneously run negative controls was determined by two independent observers based on an arbitrary scale from 0 to 3+. Observers concurred in most cases. Rare discrepancies were resolved by jointly re-examining sections. For the second group of 31 tumors, the same Notch-1 antibody (C-20) was used. However, the Notch-4 antibody was replaced because the new batches of

C-19 were of lower quality. After screening a panel of Notch-4 antibodies on cell lines, we selected antibody N-17 (Santacruz, Santa Cruz, CA). The screening involved Western blotting on T47D:C42 cells. This antibody at the same concentration used for immunohistochemistry recognized 2 bands, of molecular masses consistent with full-length and N^{EC} Notch-4, with very little background. These bands were specifically suppressed by Notch-4 siRNA, while Notch-1 bands in the same cell lysates were not.

Cell lines and constructs: HMEC were cultured in mammary epithelial cell basal medium (MEBM) with 52 μg/ml Bovine Pituitary Extract (BPE), 10 μg/ml human recombinant Epidermal Growth Factor (hEGF), 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, 50 μg/ml gentamicin and 50 μg/ml amphotericin-B. MCF-7 were cultured in MEM medium with 10% FBS, 100 μM non-essential amino acid, 1 mM sodium pyruvate and 10 μg/ml insulin. MDA-MB231 were cultured in DMEM medium with 10% FBS. T47D:C42, T47D:A18 and SKBR3 were propagated in RPMI 1640 with 10% FBS, 100 μM non-essential amino acid and 6 ng /ml insulin (1). For experiment involving the use of hormones, T47D:A18, T47D:C42 cells and MCF-7 were cultured in phenol red-free RPMI 1640 with 10% charcoal-stripped serum, 100 μM non-essential amino acid and 6 ng/ml insulin and then treated with the hormones. Duration of charcoal stripped serum treatment and hormone treatment was determined for each cell line and for each assay by pilot experiments.

RNA Interference: Sequence specificity for siRNAs was determined by BLAST searches which determined the uniqueness of our sequences. The siRNA sequences we designed were validated for specificity in CaSki cells and human primary keratinocytes, examining all four Notches and 2 housekeeping proteins. In these cells, each affects only

the specific Notch homologue to which it is targeted (Song et al., submitted). The Notch-1 siRNA has been used successfully in other models (2, 3). Transfection of siRNAs was performed using Oligofectamine (Invitrogen). The same siRNA sequences were also cloned in pSuper (OligoEngine, Seattle, WA) which directs intracellular synthesis of siRNA-like transcripts. Pilot experiments showed that the knockdown effectiveness and specificity of these constructs was identical to those of RNA oligos. These constructs were used instead of dsRNA oligonucleotides for experiments shown in Supplemental Figure 7. The negative control in these experiments was pSuper vector containing the same scrambled sequence used for oligos. The constructs were transfected using either Fugene (Roche, Indianapolis, IN), Lipofectamine 2000 (Invitrogen, Carlsbad, CA) or electroporation using GenePulser II, Biorad, Hercules, CA) depending on the cell type.

Western blotting: Lysates were sonicated 4 X 5 seconds, incubated on ice 30 min and centrifuged at 10,000 *g* 20 min at 4º C. Supernatants were used as total cell lysates. Nuclear extracts were prepared as described (4). Protein concentrations were estimated with the BCA protein assay (Pierce, Rockford, IL). Supernatants were boiled in reducing SDS sample buffer (Invitrogen, Carlsbad, CA). Twenty µg protein per lane were run on a 3-8% Tris acetate gel (Invitrogen, Carlsbad, CA) in Tris Acetate Reducing Running Buffer (Invitrogen, Carlsbad, CA). Protein bands were transferred onto PVDF membranes (BioRad, Hercules, CA) using transfer buffer (Invitrogen, Carlsbad, CA). Membranes were then blocked overnight at 4º C in 2% blocking solution in TBS (Roche, Indianapolis IN). Primary antibodies were diluted in 2% blocking solution. Membranes were incubated one h at room temperature, then washed in TBS. Membranes were

incubated in secondary antibody and bands were detected with chemiluminescent reagent (Roche, Indianapolis, IN).

Chemoinvasion assays: 300 µl of cell suspension (10^6 cells/mL in serum-free medium) were added to invasion inserts (and incubated 22 h at 37ºC, 5% CO_2. Cells were stained with 0.9% crystal violet in 10% ethanol for 20 min. Inserts were dipped in water several times and air-dried. After removal of non-invading cells from inserts interiors, stained invading cells were lysed in 10% acetic acid and OD_{595} was determined. Pilot experiments determined that this method was more reproducible than and gave comparable results to counting individual cells. Isobolograms were constructed using TableCurve (SPSS).

Real-time RT-PCR: 1 µg of RNA was reverse transcribed in 20 µl using 15 units of AMV reverse transcriptase (Invitrogen, Carlsbad, CA) and 500 ng of oligo$(dT)_{20}$. Reaction conditions were as suggested by manufacturer. 2.5 µl of the cDNA mixture were used for Real-time PCR experiments to measure the amount of Notch-1 transcript. GAPDH mRNA was used for normalization of RNA amount. Real-time PCR reactions were conducted on a ABI PRISM 7700 Sequence Detector using Platinum Quantitative PCR SuperMix-UDG (Invitrogen, Carlsbad, CA) with 5.5 mM $MgCl_2$ in a final volume of 25 µl. The following primers and probes were used for Notch-1: forward 5'CGG GTC CAC CAG TTT GAA TG3', reverse GTT GTA TTG GTT CGG CAC CAT, probe 6 FAM-CCG CTC TGC AGC CGG GAC AG-TAMRA. Primers and probe for GAPDH mRNA (Applied Biosystem, Foster City, CA) were used for normalization of RNA amount. For the detection of HEY1 mRNA 1 µg of RNA was reverse transcribed in 20 µl reaction using a kit from Fermentas Inc. (Hanover MD) according to the manufacturer's

protocol. 25 pmoles of the specific primers were used for cDNA synthesis. 2 µl of the cDNA mixture were used for Real-time PCR to measure the amount of HEY1 transcript. HPRT mRNA was used for normalization of RNA amount. Real-time PCR reactions were conducted on a Biorad Sequence Detector using BioRad master mix containing SYBR green (BioRad Hercules CA) in a final volume of 25 µl. The following primers were used at 500 nM for HEY1: HEY-Forward 5'-TGGATCACCTGAAAATGCTG-3' and HEY-Reverse 5'TTGTTGAGATGCGAAACCAG'-3.

Biotinylation experiment: T47D:A18 cells were grown in charcoal stripped serum for 48 h with or without 100 nM 17β-estradiol. Cells were then rinsed three times with PBS and 2 mM biotinylation reagent (EZ-Link Sulfo-NHS-Biotin Reagent, (Pierce, Rockford IL) was added to the cells. Cells were shaken for 15 min at 4ºC and then the same volume of growth medium without serum was added. After 15 min cells were scraped, rinsed twice in PBS and then lysed in RIPA buffer containing proteases inhibitors. One mg of total protein lysate was immunoprecipitated in 500 µl of RIPA buffer using 30 µl of Immobilized Neutravidin Protein (Pierce, Rockford, IL). The beads were washed 4 times in PBS and the protein eluted in 2X SDS Laemmli buffer. 15 µl of the immunoprecipitated protein were analyzed by Western Blot C-20 Notch-1 antibody (Santacruz, Santa Cruz, CA). To check the total levels of Notch, 30 µg of total cell lysate were analyzed by Western blot using the same antibody. In order to distinguish between effects on cellular localization and effects on total protein amount, for these experiments we used shorter exposure times to both charcoal-stripped serum and estrogen compared to Figure 1C, D. Pilot experiments identified conditions that would affect Notch localization before any effects on Notch total amount were observed.

Xenografts studies: MDA-MB231 xenografts were monitored for tumor growth and randomized in 2 treatment groups consisting of 5 mice: Control (0.5% DMSO), and GSI 1.2 mg/kg. Injections were done peri-tumorally. Tumor volume was determined weekly by Vernier caliper and calculated using the formula $4/3\pi r^3$. Treatment was begun as soon as tumors became palpable (the average tumor volume at day 0 was 0.031 ± 0.006 cm^3). In T47D:A18 xenografts, estradiol was admistered via silastic capsules (1.0 cm long containing 25 mg of estradiol) implanted s.c. between the scapulas. Tumor volume was determined as above. Relative tumor volume was plotted against time in weeks to monitor tumor growth. Treatment was initiated when the tumors were well established (at week 4). This was designated as time 0. Mice were randomized into 4 treatment groups consisting of 10 mice/group: Control (1% DMSO), TAM (0.5 mg tamoxifen in 100 µl of carboxymethylcellulose suspension), GSI (1.2 mg/kg in 100 µl 1% DMSO), and TAM plus GSI. The average tumor volume at day 0 was 0.231 ± 0.05 cm^3. Tamoxifen was administered every day (5 days ON, 2 days OFF) by gavage whereas GSI was administered every other day by peri-tumoral injections. The last treatment was performed 8 h before sacrificing the mice. Tumors were excised and snap frozen in OCT to perform immunohistochemical analyses. Frozen blocks were cut into 10 µm sections and stained with hematoxylin/eosin.

Statistical Analyses, clinical studies: To evaluate the correlation between Notch expression and known prognostic factors, we analyzed various clinical variables on 31 breast cancer patients treated at Loyola Medical Center. The measured and/or recorded variables included age, menopausal status, pathological diagnosis, ER status, HER2/Neu status, Ki67 fraction, histological grade, node status and tumor size. Paraffin sections

were stained for Notch-1 and Notch-4. The sample set was validated by determining the association between tumor size and other clinical variables. Subsequently, we investigated the association between Notch-1 and Notch-4 with clinical variables. Associations involving two-class variables (age, nodal status, menopausal status, ER status, and HER status) with tumor size were performed using a t-test. Associations involving tumor size with variables with multiple measurements levels (Notch-1, Notch-4, tumor grade), were performed using analysis of variance (ANOVA). In our validation analysis we found that node status was significantly associated with tumor size ($P<0.01$). The mean for tumor size in the node-negative group ($N =15$) was 1.36 ± 0.87 cm. The corresponding values for the node-positive group ($N=15$) were 2.38 ± 1.33 cm. Other variables showed no statistical significant association, presumably due to small sample size.

SUPPLEMENTAL RESULTS

Immunohistochemical studies of clinical specimens: Only one out of 4 normal adolescent breast biopsies showed weakly detectable Notch-1 in mammary ducts. Notch-4 was undetectable. Interestingly, normal ducts in pathological specimens from older women did show both Notch-1 and Notch-4 signal (not shown). Whether this represents an age-related phenomenon or reflects the presence of nearby neoplastic lesions remains unclear. Hyperplastic lesions and areas of apocrine metaplasia were intensely positive for Notch-1. Also, apparently normal ducts near DCIS lesions were intensely positive for Notch-1, even when the nearby DCIS was Notch-1 negative. DCIS lesions showed heterogeneous patterns (Supplemental Table 1). Eleven/27 were intensely positive for

Notch-1 alone. Five showed only Notch-4, 7 co-expression of Notch-1 and Notch-4 and 4 were negative for both Notch-1 and -4. DCIS of histological grades I, II or III showed Notch receptor expression. In grade I non-comedo DCIS 4/7 tumors were Notch-1 positive, 2 were Notch-4 positive and 1 was double-positive for Notch-1 and -4. In grade II, 8/15 non-comedo DCIS expressed Notch-4, either alone (2) or with Notch-1 (6). Three grade II DCIS were Notch-1 positive without Notch-4. Conversely, comedocarcinomas (grade III) did not include samples co-expressing Notch-1 and -4. The most common pattern (4/6) was strong Notch-1 expression, with one tumor showing Notch-4 without Notch-1. Notch-4 expression in DCIS was more common in patients older than 50 (11/21 versus 1/5 in patients younger than 50). There was no statistically significant correlation between Notch expression and immunostaining for either ER or p53. However, 5/6 p53-positive tumors were positive for Notch-1 and 2 were also positive for Notch-4. Among 41 infiltrating carcinomas, (27 ductal, 14 lobular), staining patterns different between Notch-1 and Notch-4. For Notch-1, some tumors (17/27) showed variable expression, i.e., different levels of Notch-1 staining in different cells within the same lesion. Others (10/27) showed uniform staining intensity throughout individual lesions. Notch-4 staining was consistently uniform and varied in intensity. Notch-1 and -4 staining was primarily cytoplasmic but individual cells showed punctate nuclear signal.

Validation of antibody specificity in Western blots. The specificity of bands observed in Western blots was verified by transfecting authentic intracellular Notch-1 and Notch-4 into primary keratinocytes, which are spontaneously positive for both Notch-1 and 4 (5) and staining Western blots with the same antibodies used for breast cell lines (Supplemental Figure 1).

Validation and dose-response of GSI. In MDA-MB231 cells, GSI caused a clear, dose-dependent decrease in N^{IC} relative to N^{TM}, which was virtually complete at 2 μM (Supplemental Figure 4A). GSI inhibited the proliferation of MDA-MB231 cells at 1 μM and caused time-dependent cytotoxicity at concentrations ≥ 2 μM (Supplemental Figure 4B). Its IC_{50} for Notch cleavage inhibition in the quantitative γ-secretase assay (6) was determined to be 1.23 μM (Supplemental Figure 4C). Consistent with this observation, GSI's IC_{50} for growth inhibition in MDA-MB231 cells was 1.3 μM (Supplemental Figure 4D). We determined that at these concentrations GSI did not have significant proteasome inhibitory activity in our intact cells (up to 10 μM, not shown), while purified 20S proteasome was inhibited (not shown). Importantly, the effects of GSI on MDA-MB231 cells below 1.5 μM were significantly rescued by transient transfection of Notch-1 N^{IC} (Supplemental Figure 4E). This is consistent with what we observed in BJ fibroblasts (7), and indicates that the growth inhibitory effects of GSI, at least at concentrations below 2 μM, are in large part mediated by Notch inhibition. At higher concentrations, off-target effects cannot be ruled out.

Supplemental Figure Legends:

Supplemental Figure 1: Validation of Notch-1 and Notch-4 antibodies. The antibodies to Notch-1 and Notch-4 were validated by Western blotting in normal human keratinocytes transfected with pLZRS constructs encoding Notch-1 or Notch-4 N^{IC}. Normal human keratinocytes were chosen because they spontaneously express all 4 Notch receptors, thus allowing a comparison between the endogenous bands and overexpressed proteins.

Supplemental Figure 2: Notch-1 and Notch-4 are required for Matrigel invasion in MDA-MB231 cells and stimulate proliferation and Matrigel invasion in MCF-7 cells. A, silencing of Notch-1 (Notch-1i) or Notch-4 (Notch-4i) significantly inhibited migration of MDA-MB231 cells through Matrigel-coated filters; B, Western blots showing that transient transfection of MCF-7 cells with two different constructs encoding Notch-1 N^{IC} (amino acids 1759-end or 1770-end) induced Notch-4 expression; C, MCF-7 cells expressing Notch-1 proliferated significantly faster than cells transfected with empty vector; D, MCF-7 cells expressing Notch-1 invaded through Matrigel-coated filters significantly more effectively than cells transfected with empty vector. Error bars are standard deviations. Each experiment was repeated at least 3 times.

Supplemental Figure 3: inhibition of Notch signaling in MDA-MB231 cells causes G2 accumulation and increased E2F-1 activation, followed by death. A, RNAi silencing of Notch-1 (top) or Notch-4 (bottom) in MDA-MB231 cells caused G2/M accumulation. Data representative of 3 independent experiments each performed in triplicate ($p = 0.000026$ for Notch-4 and 0.01 for Notch-1 respectively); B, Western blotting for phosphorylated histone H3, showing that Notch-1 silencing virtually abolished H3 phosphorylation, an indicator of entry into mitosis; C, Western blots showing that Notch-1 siRNA silencing in MDA-MB231 cells causes accumulation of E2F-1 at 48 h; D, representative raw data from flow cytometry experiments, showing the effects of RNAi silencing of Notch-1 and -4 on cell cycle distribution of MDA-MB231 cells. Notice the relative accumulation of cells in G2/M at 24 h followed by appearance of prominent "sub-G1" peaks at 48 h, indicative of cell death. Numbers above the graphs indicate percentages of cells in subG1, G1, S, and G2/M respectively. Sub-G1 induction

was statistically significant ($p = 0.01$) compared to controls transfected with an inactive siRNA.

Supplemental Figure 4: Inhibition of γ-secretase inhibits proliferation of MDA-MB231 cells in a Notch-1-dependent way. A, Western blots showing dose-dependent reduction of N^{IC} levels in the presence of γ-secretase inhibitor cbz-LLNle-CHO (GSI) for 24 h; B, time and concentration-dependent growth inhibition tests, showing that GSI had apparent cytostatic effects at 1 μM and cytotoxic effects above 2 μM, becoming evident at 48 h and beyond; C, γ-secretase cleavage assay showing dose-dependent inhibition of γ-secretase activity by GSI with $IC_{50} = 1.23$ μM; D, dose-response curve showing that the IC_{50} for cell growth inhibition at 48 h is 1.30 μM, very close to the value for Notch cleavage inhibition; E, transient transfection of intracellular Notch-1 (N^{IC}) rescued cell killing by GSI compared to empty vector. Since transfection efficiency in these experiments is never 100%, and 100% of the cells are exposed to GSI, the extent of rescue, though nearly complete below 1.5 μM, is underestimated by these experiments.

Supplemental Figure 5: Effects of GSI/4-hydroxytamoxifen combinations on cyclins A and B expression: Western blots of T47D:A18 cells treated for 48 h, showing synergism of combinations of 0.4 μM GSI and 1, 2 or 5 μM 4-hydroxytamoxifen in inhibition of expression of cyclins A and B1.

Supplemental Figure 6: Notch-4 expression in clinical specimens correlates with Ki67 status. Immunostaining of human biopsies for Notch-4 and Ki67 (400X original magnification). Note the higher Ki67-stained fraction in the Notch-4-high cancer (10 cells/hpf vs. 2 cells/hpf). This picture is representative of 31 patients.

Supplemental Figure 7: Notch 1 downregulation by siRNA or by GSI induces a decrease in the levels of c-Myc after 48 h in T47D:A18 cells. Treatment with GSI does not affect the levels of c-Myc inMDA-MD231.

References

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