Supporting Information

Landor et al. 10.1073/pnas.1104943108

SI Materials and Methods

Cell Culture. Human breast cancer cell lines MCF7 (including stable and inducible cell lines) and MDA-MB-231 were grown in DMEM (Sigma D6171) supplemented with 10% FCS (PromoCell C-37360), 1 mmol L-glutamine (Sigma G7513), 25,000 units penicillin/25 mg streptomycin (Sigma P0781), nonessential amino acids (Sigma M7145), and sodium pyruvate (Sigma S8636). Stable cell lines were selected with puromycin (1 μg/mL). Notch activity was blocked by addition of 5 μg/mL γ-secretase inhibitor *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester (DAPT; Calbiochem). Inducible Tet-on cell lines designated MCF7-EGFP and MCF7-N1ΔEGFP were induced by addition of 1 μg/mL doxycyclin. Phosphatidylinositol 3-kinase (PI3K) and AKT serine/threonine kinase were inhibited using LY294002 and AKT inhibitor XI, respectively (Calbiochem).

Western Blot Analysis. For Western blot analysis, cells were lysed in Laemmli serum buffer [30% (vol/vol) glycerol, 3% SDS, 0.1875 M Tris·HCl (pH 6.8), 0.015% bromophenol blue, and 3% β-mercaptoethanol]. The lysates were heated at 100 °C for 10 min and then stored at −20 °C. The protein separations were done on 10–20% SDS/PAGE gels and the proteins were transferred onto PVDF membranes (Millipore Immobilon-P). The membranes were blocked for 1 h in 3% milk/0.3% PBS-Tween. Primary antibodies used were mouse anti-p53 (Santa Cruz), mouse anti–β-actin (Sigma), rabbit anti-AKT, anti–phospho-AKT (Ser473), phosphatase and tensin homolog (PTEN), PI3K class III, PI3K subunit p110β (Cell Signaling), mouse anti-cytochrome *c* oxidase 2 (Santa Cruz), MitoProfile Total OXPHOS Detection Kit (MitoSciences MS601: mouse), and rat anti-HSC70 (Stressgen). Secondary antibodies used were HRP-conjugated sheep anti-mouse IgG (GE Healthcare) and goat anti-rat IgG (Cell Signaling) diluted 1:20,000. Luminescence was achieved using Western Lightning Chemiluminescence Reagent Plus (Perkin-Elmer).

Flow Cytometry Experiments. All flow cytometry experiments were performed using BD FACSCalibur running BD CellQuest PRO v.5.1.1.

For measuring of the mitochondrial membrane potential, cells were incubated in tetramethylrhodamine, methyl ester (Invitrogen) containing medium (10 nM) for 20 min. The cells were then washed in PBS, trypsinized, and pelleted by centrifugation at 450 × *g* for 5 min. The pellets were resuspended in PBS, and the mitochondrial potential analyzed.

Determination of apoptotic populations after glucose deprivation was done by propidium iodide (Sigma P4170) staining. Cell medium and trypsinized cells were pooled and pelleted by centrifugation at 450 × *g* for 5 min and then incubated with propidium iodide containing sodium citrate buffer (40 mM sodium citrate, 0.3% Triton X-100, 50 μg/mL propidium iodide) for 10 min at room temperature. Samples were immediately analyzed on flow cytometry. Inhibition of electron transport chain complexes was performed using 50-μM concentrations of oligomycin (Cell Signaling) and rotenone (Acros).

Glucose uptake was measured using 2-NBDG and 6-NBDG[(2/6-(*N*-(7-nitrobenz-2-oxa-1,3- diazol-4-yl)amino)-2-deoxyglucose)] fluorescent probes. (Invitrogen). Cells were incubated in medium containing 100-μM probe for 20 min. Cells were then washed twice in PBS, trypsinized, pelleted, and resuspended in PBS followed by flow cytometry analysis.

Histological Analysis and Immunohistochemistry. Paraformaldehyde-fixed tumors were cut into 10-μm sections and stained with Harris' H&E. Immunohistochemistry was carried out with standard techniques, using commercial monoclonal rat anti-mouse K_i-67 (1/200) (DakoCytomation). Briefly, formalin-fixed, paraffin-embedded tumors were sectioned (2.5 μm thick), dewaxed in xylene 2 × 5 min, dehydrated in an alcohol row, and subjected to antigen retrieval in a microwave 2 × 7 min in 10 mM sodium citrate buffer (pH 6.0) (Dako). After cooling for 20 min, tumor sections were washed three times in PBS (pH 7.4). Endogenous peroxidase activity was blocked by immersion in 3% hydrogen peroxide for 10 min and then washed in PBS 2 × 5 min. Sections were incubated overnight with primary antibodies diluted in PBS. After washing with PBS, sections were incubated with secondary antibody (Dako Cytomation EnVision System Labeled Polymer-HRP Anti-Mouse) for 30 min and then washed in PBS 2 × 5 min. The reaction products were visualized applying DAB solution (Dako Cytomation Liquid DAB Substrate Chromogen System) for 10 min. Sections were then counterstained with Mayer's acidic hematoxylin and washed in an alcohol multiple row (70–100%); after xylene treatment, they were covered. Negative controls were obtained by omission of the primary antibody.

cDNA Synthesis and Quantitative RT-PCR. Cells were lysed directly in wells and RNA was prepared using the QIAgen RNeasy Mini Kit according to the manufacturer's instructions. cDNA was synthesized from 1.5 μg total RNA using SuperScript III Reverse Transcriptase, oligo(dT)12–18 primers and RNaseOUT (Invitrogen) according to the protocol. RT-PCR was carried out on a 7500 Fast Real-Time PCR system with Fast SYBR Green Master Mix (Applied Biosystems) according to the manufacturer's recommendations. The primers were synthesized by Eurofin MWG Operon and were as follows, β-actin: 5′CCAGAGGCGTACAGGGATAG3′ and 5′CCAACCGCGAGAAGATGA3′; p53: 5′CACTGCCCAACAACACCAGCTCCT3′ and 5′GTCTGAGTCAGGCCCTTCTGTCTTG3′; hexokinase 2: 5′GGCTGTGGATGAGCTTTCACTC3′ and 5′TCCACGCTTGGTGAAATCG3′; glucose transporter 1 (GLUT1): 5′CTGTGTGGTCCCTACGTCTTCA3′ and 5′AAGCGATCTCATCGAAGGTCC3′; puryvate dehydrogenase kinase 2 (PDK2): 5′GGA CTC ATG GCT TTC CAC AGT3′ and 5′CAA ACA GCC GAT TCA CAT GGT3′; aldolase A (ALDOA): 5′GTC CCT TCC CCC AAG TTA TCA A3′ and 5′GGT GGT AGT CTC GCC ATT TGT C3′. Experiments were repeated at least three times.

Small-Animal PET/CT. Small-animal PET/CT scans were obtained using the Inveon Multimodality PET/CT scanner (Siemens Medical Solutions). ^{18}F-fluorodeoxyglucose (FDG) production and quality control were performed by the Turku PET Centre radiopharmaceutical chemistry laboratory. Mice were fasted for at least 8 h before ^{18}F-FDG injection with free access to water. For ^{18}F-FDG injection and imaging, mice were anesthetized by inhalation of 2% isoflurane in oxygen. Following a 10-min CT scan that also was used for attenuation correction of the PET data, an emission scan was acquired in list mode between 60 and 80 min after the injection. Images were reconstructed using a 2D-filtered back-projection with a 0.5-mm ramp filter. Levels of blood glucose were measured in all mice after scan by using Accu-Chek (Roche). Three groups of mice bearing Notch^{high}-MCF7, Notch^{medium}-MCF7, and Notch^{low}-MCF7 tumors (n = 5) were imaged three times: 1 wk after orthotopic xenotransplantation and 1 and 2 mo after the first scan.

Image Analysis. Images from the last scan (9 wk after orthotopic xenotransplantation) were analyzed using Inveon Research Workplace software (Siemens). Regions of interest were drawn manually around the anatomical lesions (palpable and visual tumors or fibrotic loci, as in the Notch^{low}-MCF7 group). The intensity of ^{18}F-FDG uptake was calculated using the mean standardized uptake value, which is defined as the tissue concentration of the tracer divided by the activity injected divided by body weight.

Cell Invasion. In vitro cell invasion was analyzed by Boyden chamber experiment using 8-μm 24-multiwell insert plates with FluoroBlok membrane (BD BioCoat Tumor Invasion System 354165). Before seeding, cells were starved in serum-free medium overnight and dyed with DilC12(3) (BD 354218) the following morning. A total of 200,000 cells were then seeded in each of the chambers with 0.1% FCS. Medium containing 15% FCS was placed outside the chamber. Invasion was determined by fluorescent plate reader at 549/565 nm (absorbance/emission).

3D Growth Assay. In vitro 3D cultures were obtained by seeding cells in Cultrex reduced growth factor basement membrane extract (Trevigen). Colonies were allowed to form for 10 d.

Proliferation Assay. Cell proliferation in vitro was assayed by seeding 30,000 cells in each well of a 12-well cell cluster. Cells were then counted each day for 3 d using a Cedex cell counter (Innovatis).

Activation of Notch by Immobilized Recombinant Notch Ligands. Activation of Notch by recombinant ligands was performed as previously described (1).

Activity of Electron Transport Chain Complexes and ATP Synthase. Mitochondria were isolated from cultured cells using MitoProfile Benchtop Mitochondria Isolation Kit (MitoSciences). Activity of complexes I and IV were measured using enzyme activity dipstick assay kits (MitoSciences MS130 and MS430, respectively) according to the manufacturer's protocol. ATP synthase activity was measured using an enzyme activity microplate assay kit (MitoSciences MS541).

Tumor Xenograft Growth in Vivo. All animal experiments were conducted in accordance with the institutional animal care policies of the University of Turku and Åbo Akademi University. Athymic nude *Foxn1nu* 5- to 6-wk-old female mice (Harlan) were housed in Scantainer cages (Tecniplast), five per cage, and supplied with mouse chow and tap water ad libitum in controlled conditions of light and temperature. After 1 wk of acclimatization, mice underwent implantation of 17β-estradiol pellets (60-d release; Innovative Research of America). Four days later, animals were randomly assigned for three groups (n = 5): Notch^{high}-MCF7, Notch^{medium}-MCF7, and Notch^{low}-MCF7. To initiate tumors, mice were anesthetized with isoflurane 2–3% and injected bilaterally into upper inguinal mammary gland fat pads (orthotopic xenotransplantation) with 1×10^6 cells mixed with Matrigel (BD Biosciences) 1:1; total volume 100 μL per injection. Tumor diameter was determined by vernier caliper every third day, and tumor volume was calculated assuming the shape as sphere. At the end of the experiment, animals were killed, and tumors were removed and fixed in 4% paraformaldehyde for H&E staining and immunohistochemistry for Ki67.

Oxygen Consumption. Oxygen consumption was monitored using the Oxygraph together with the Oxygraph software (Hansatech) in a sealed chamber at 37 °C. A total of 500,000 cells were resuspended in a total volume of 1 mL 37 °C warm cell culture medium and added to the Oxygraph chamber with magnetic stirring. For inhibition of ATP synthase, oligomycin A (Sigma) was used at a final concentration of 10 μM. Experiments were repeated at least three times.

Quantification of ATP. Cells grown in 12-well plates were harvested in 150 μL Tropix Lysis Solution (Applied Biosystems). Lysates were heated for 2 min at 98 °C in a heating block to extract ATP and spun down at 150,000 × g for 10 min. Measurements were carried out using 50-μL supernatant with the FLAA ATP Bioluminescent Assay Kit (Sigma) and the GloMax 96 Microplate Luminometer (Promega) according to the protocol. For normalization, total protein was quantified with the BCA Protein Assay kit (Thermo Scientific). DAPT experiments were performed with naïve MCF7 cells incubated with 2.5 μM DAPT or DMSO control 3 h before measurement. Experiments were repeated at least three times.

Glucose Consumption and Lactate Production. The concentration of glucose and lactate was determined in medium collected at different time points (0, 24, and 48 h). The concentration of glucose was measured using Accu-Chek (Roche), and the concentration of lactate was measured at the Turku University Hospital; concentrations were adjusted to the number of cells. For glucose consumption, the amount of glucose used during 24 or 48 h was given as related to the number of cells.

1. Sahlgren C, Gustafsson MV, Jin S, Poellinger L, Lendahl U (2008) Notch signaling mediates hypoxia-induced tumor cell migration and invasion. *Proc Natl Acad Sci USA* 105:6392–6397.

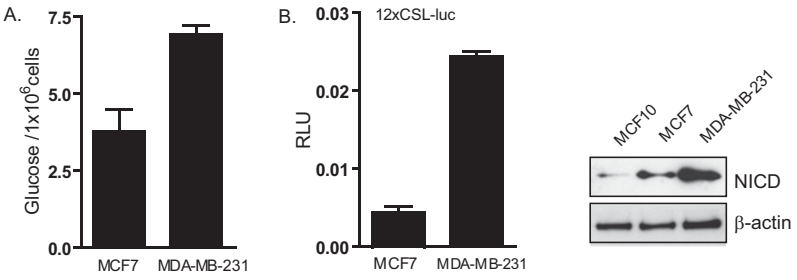

Fig. S1. Notch activity corresponds to the glycolytic phenotype. (*A*) Glucose uptake in MCF7 and MDA-MB-231 breast cancer cell lines demonstrates the glycolytic phenotype of MDA-MB-231 cells. (*B*) Notch signaling activity in MCF7 and MDA-MB-231 cells as measured by the 12xCSL-luciferase reporter and by Western blot using an antibody against the active cleaved form of Notch, Notch intracellular domain (NICD). MCF10 is a nontumorigenic breast epithelial cell line.

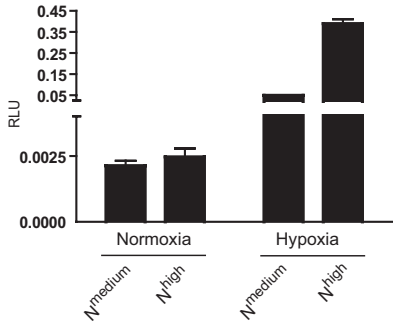

Fig. S2. Notch enhances signaling by hypoxia-inducible factor 1 in hypoxia but not in normoxia. Hypoxic signaling activity was measured by the hypoxia response element–luciferase reporter in N^{high} and N^{medium} cells cultured under normoxic or hypoxic conditions.

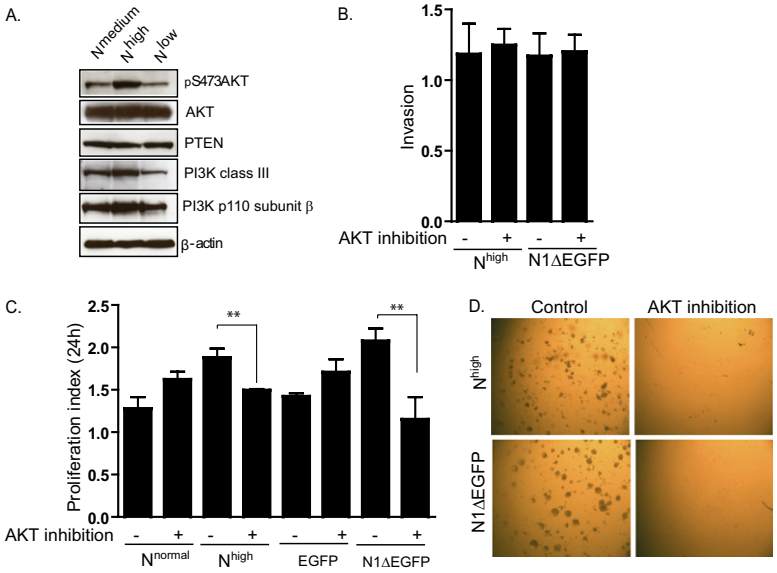

Fig. S3. Notch and PI3K/AKT cross-talk influence cell proliferation and 3D growth. (*A*) Immunoblotting of N^{high}, N^{medium}, and N^{low} cells with antibodies for phosphorylated (pS473) AKT, total AKT, PTEN, PI3K class III, and PI3K p110 subunit β and β–actin. (*B*) AKT inhibition does not affect invasion in N^{high} cells, as measured by a transmembrane invasion assay. (*C*) AKT inhibition reduces proliferation in cells with high Notch activity. (*D*) AKT is required for 3D growth and colony formation.

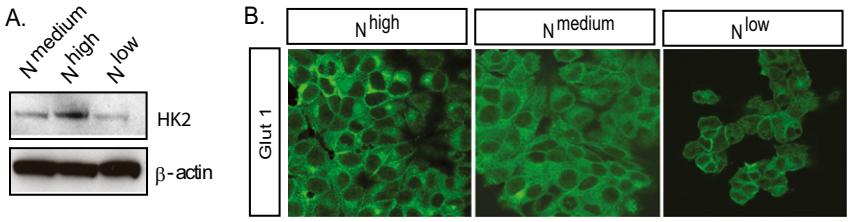

Fig. S4. Notch increases HK2 protein levels and promotes membrane localization of GLUT1. (*A*) Immunoblot showing hexokinase 2 (HK2) levels in N^{high}, N^{medium}, and N^{low} cells. (*B*) Fluorescence microscopy images showing GLUT1 N^{high}, N^{medium}, and N^{low} cells. Note the enhanced membrane localization of GLUT1 in N^{high} and N^{low} cells.

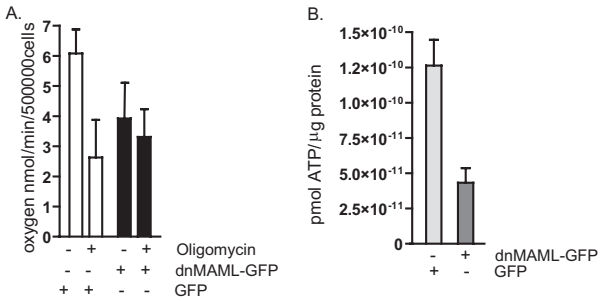

Fig. S5. Oxygen consumption and ATP production are reduced in cells with blocked Notch signaling. (*A*) Oxygen consumption in untreated or oligomycin-treated MCF7 stably expressing GFP or dominant-negative Mastermind-like (dnMAML)–GFP. Average of 5–7 different measurements. (*B*) ATP production in MCF7 stably expressing GFP or dnMAML–GFP. Average of three different experiments.

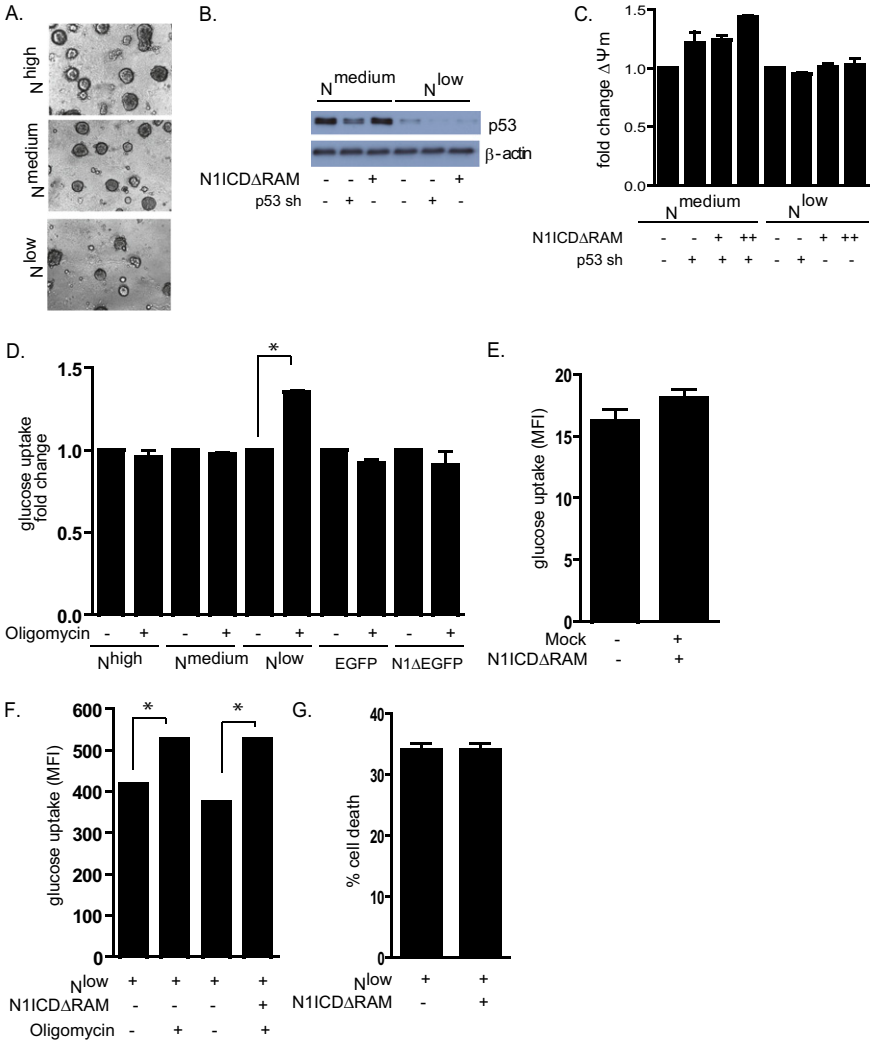

Fig. S6. Canonical vs. noncanonical Notch signaling in tumor progression and cancer cell metabolism. (*A*) Anchorage-independent growth N^{high}, N^{medium}, and N^{low} cells. (*B*) Noncanonical Notch signaling does not affect p53 levels in N^{medium} and N^{low} cells. Immunoblot shows p53 in N^{medium} and N^{low} cells transiently transfected with Notch1 ICDΔRAM (N1ICDΔRAM), a construct that activates Notch in a noncanonical manner. (*C*) p53 knockdown increases the mitochondrial membrane potential (MMP) in N^{medium} cells. N1ICDΔRAM does not affect the MMP in N^{low} cells. The graph shows fold change in MMP compared with untransfected cells, which were given the value 1 (original values ~152 for N^{medium} and ~454 for N^{low}). (*D*) N^{low} cells are dependent on glucose for mitochondrial ATP production and respond to oligomycin treatment by increasing glucose uptake. Glucose uptake in N^{high}, N^{medium}, and N^{low} cells and MCF7 cells expressing EGFP or N1ΔEGFP in response to oligomycin treatment as measured by flow cytometry (FACS) using a fluorescent probe 6-NBDG (Molecular Probes). (*E*) Noncanonical Notch signaling does not affect glucose uptake. Glucose uptake analyzed by FACS in naïve MCF7 cells transfected with an empty vector or N1ICDΔRAM. (*F* and *G*) Noncanonical Notch signaling does not rescue the metabolic phenotype of N^{low} cells. (*F*) Glucose uptake in N^{low} control cells and N^{low} cells transfected with N1ICDΔRAM in response to oligomycin treatment. (*G*) Cell death in N^{low} control cells and N^{low} cells transfected with N1ICDΔRAM upon glucose deprivation.