Supporting Information

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Experimental Procedures

Plasmid Constructs. The 12XCSL luciferase reporter (1, 2), dnMAML (3), dnCSL (4), the Snail-1 promoter luciferase construct (−69/+59) (5), and the FLAG-HIF-1α (PP-A)(P402A/P563A) (6) constructs have been described.

Cell Culture. Human ovarian carcinoma SKOV-3, human colon carcinoma HCT-116, breast cancer cell lines MCF7 and MDA-231, breast epithelial cells MCF10, and the human glioblastoma U-87 MG cell lines were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% FBS and nonessential amino acids (MCF10). Cervical carcinoma C-33 A cells were grown in DMEM Earl's Salt supplemented with 10% FBS. For hypoxia, cells were grown for 1–3 days in 1% oxygen. Notch activity was blocked by a 2.5 μM concentration of the γ-secretase inhibitor DAPT (Calbiochem), added fresh at each day of culturing.

Notch Activity Assay. Notch activity in cells expressing the 12XCSL luciferase reporter construct was measured by a luciferase assay as described (7).

RNA Extraction and Quantitative (Q) RT-PCR. RNA was extracted and RT-PCR performed as described (8). The following primers were used for Q-PCR:

Hes-1: 5′-AGGCGGACATTCTGGAAATG-3′ (forward), 5′-TCGTTCATGCACTCGCTGA-3′ (reverse); *Hey-2*: 5′-AGATGCTTCAGGCAACAGGG-3′ (forward), 5′-CAAGAGCGTGTGCGTCAAAG-3′ (reverse); *E-cadherin*: 5′-GGDCTGAAGTGACTCGTAACGA-3′ (forward), 5′-CAGCCGCTTTCAGATTTTCATC-3′ (reverse); *Snail-1*: 5′-CATCCTTCTCACTGCCATGGA-3′ (forward), 5′-AGGCAGAGGACACAGAACCAGA-3′ (reverse); *Lox*: 5′-TGCTTGATGCCAACACCCA-3′ (forward), 5′-ATGCAAATCGCCTGTGGTAGC-3′ (reverse); *Loxl2*: 5′-TGTGCAGCGACAAAAGGATTC-3′ (forward), 5′-GTAGGTTGAGAGGATGGCTCGA-3′ (reverse); and β-actin: 5′-AAGAGATGGCCACGGCTG-3′ (forward), 5′-GAACCGCTCATTGCCAATG-3′ (reverse).

Immunocytochemistry and Western Blot Analysis. Immunocytochemistry was performed as described (7). The antibodies used for immunocytochemistry are described below. Immunostaining was visualized by using a Zeiss LSM510 META confocal microscope, adjusted for brightness and contrast in Photoshop and assembled in Illustrator (Adobe Systems). For Western blot analysis, cells were lysed in RIPA [0.15 M NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.05 M Tris·HCl (pH 8), supplemented with protease inhibitors (Roche), and DTT 10 μM/ml]. Lysates were cleared by centrifugation for 15 min at 15,000 $\times$ *g*. Primary antibodies used for immunocytochemistry were rabbit anti-E-cadherin (Cell Signaling Technology); goat anti-Occludin (Santa Cruz Biotechnology); and goat anti-vimentin (Santa Cruz Biotechnology), all diluted 1:250. Secondary antibodies used were goat anti-mouse, donkey anti-goat, or goat anti-rabbit Alexa Fluor 488 (Molecular Probes), diluted as suggested by the manufacturer. Actin filaments on paraformaldehyde-fixed samples were visualized by Alexa Fluor 633-conjugated phalloidin (Molecular Probes), used according to the manufacturer's instructions. Antibodies used for Western blot analysis were goat anti-vimentin (Santa Cruz Biotechnology), rabbin anti-cleaved Notch 1 (Cell Signaling Technology), rabbit anti-E-cadherin (Cell Signaling Technology), and goat anti-Occludin (Santa Cruz Biotechnology), all used at dilutions suggested by the manufacturers.

Cell Migration and Invasion. Cell migration was analyzed by a scratch wound assay. Cells were grown to confluency, and a scratch wound in the monolayer was performed by dragging a pipette tip across the layer. Detached cells were washed away with cell culture medium. The remaining cells were treated as described, and the closure of the wound was followed by microscopy at 12, 24, and 48 h after inflicting the wound. For better visualization of the cell layer, the cells were prelabeled by Cell Tracker Green CMFDA (Molecular Probes) according to the manufacturer's instructions. Cell invasion was analyzed by using the BD BioCoat tumor invasion system according to the manufacturers' instructions. Briefly, Cell Tracker-labeled cells were serum starved for 24 h and plated in the upper insert at 50,000 cells per ml for a 24-well chamber in serum-free medium or medium supplemented with 1% serum. Medium containing 10% serum as a chemoattractant was added to the well. The cells were placed in hypoxia (1% 0_2) or normoxia for 24 and 48 h. Noninvading cells were removed from the upper surface by scrubbing with a cotton swab, after which the membrane was fixed with 4% formaldehyde for 10 min at room temperature (RT), and the number of invading cells was analyzed by fluorescence microscopy. For treatment with 2 μM γ-secretase inhibitor DAPT or 200 μM LOX inhibitor BAPN (Sigma), the cells were pretreated for 2–4 h and the treatment continued during the invasion experiment.

Activation of Notch Signaling by Using Immobilized Recombinant Notch Ligands. Cell culture plates were coated with Protein G (Zymed) 50 μg/ml in PBS at RT overnight. After coating, the plates were washed three times with PBS and further blocked with 10 mg/ml BSA in PBS for 2 h at RT. The blocked plates were washed three times with PBS and incubated with recombinant Jagged1-FC chimera (R & D Systems) or only CromPure IgG, Fc fragment (Jackson ImmunoResearch) at concentrations of 2 μg/ml in 0.1% BSA/PBS for 2–4 h at RT. After washing three times with PBS, cells were immediately plated on the coated plates.

Transfections and Viral Infection. The 3T3 and 293T cells were transfected by using FuGene6 transfection reagent (Roche) according to the manufacturer's instructions. SKOV-3 and MCF7 cells were transfected by using Lipofectamine Plus (Life Technologies) or MirusTransIT transfection reagents were used according to manufacturers' instructions, yielding transfection efficiencies of 35–50% for MCF7 and SKOV-3 as estimated by the number of cells expressing GFP-tagged proteins. Alternatively, SKOV-3 cells were infected with adenoviral vectors for N1ICD, Hes1, or Hey1 (8).

Chromatin Immunoprecipitation Experiments. Cells were cross-linked by incubation with 1% formaldehyde for 10 min at room temperature. After two washes with ice-cold PBS, the cells were collected, and a chromatin immunoprecipitation experiment was performed with an Upstate Biotechnology kit according to the manufacturer's description. Antibodies (rabbit-anti-N1ICD (C-20) (Santa Cruz Biotechnology) diluted 1:200; rabbit-anti-HIF-1α (2185) (Abcam) diluted 1:300, or rabbit anti-mouse IgG (DAKO) diluted 1:2,000) were added and incubated overnight at 4°C with rotation. The mixture was then incubated with 40 μl of Sepharose

beads and eluted according to the protocol from Upstate Biotechnology. The eluates were combined, and the cross-linking was reversed by heating at 65°C overnight. DNA fragments were then purified by using a QIAquick PCR purification kit (Qiagen), followed by PCR analysis to amplify the *Snail-1* promoter region (from −934 to −702) spanning the CSL-binding site. For the LOX promoter region, PCR analysis amplified a region (from −265 to −8) spanning the HRE site. The PCR products were analyzed on a 1.5% agarose gel. The system for activation of Notch signaling by coculture (7) and chromatin immunoprecipitation for Notch 1 ICD (8) have been described (7). The sequences of the primers used in the PCRs are described below:

Snail-1 promoter: 5′-ATCCCTGGAAGCTGCTCTCT-3′ (forward), 5′-TCTGGTCCAGTGAGGGAG-3′ (reverse); *LOX* promoter: 5′-GCCCGCTCTCCGGGACTGCC-3′ (forward, 5′-TTGDDAGATTGACCCCGCTCG-3′ (reverse).

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6. Masson N, William C, Maxwell PH, Pugh CW, Ratcliffe PJ (2001) Independent function of two destruction domains in hypoxia-inducible factor-alpha chains activated by prolyl hydroxylation. *EMBO J* 20:5197–5206.
7. Gustafsson MV, *et al*. (2005) Hypoxia requires Notch signaling to maintain the undifferentiated cell state. *Dev Cell* 9:617–628.
8. Chapman G, Liu L, Sahlgren C, Dahlqvist C, Lendahl U (2006) High levels of Notch signaling down-regulate Numb and Numblike. *J Cell Biol* 175:535–540.

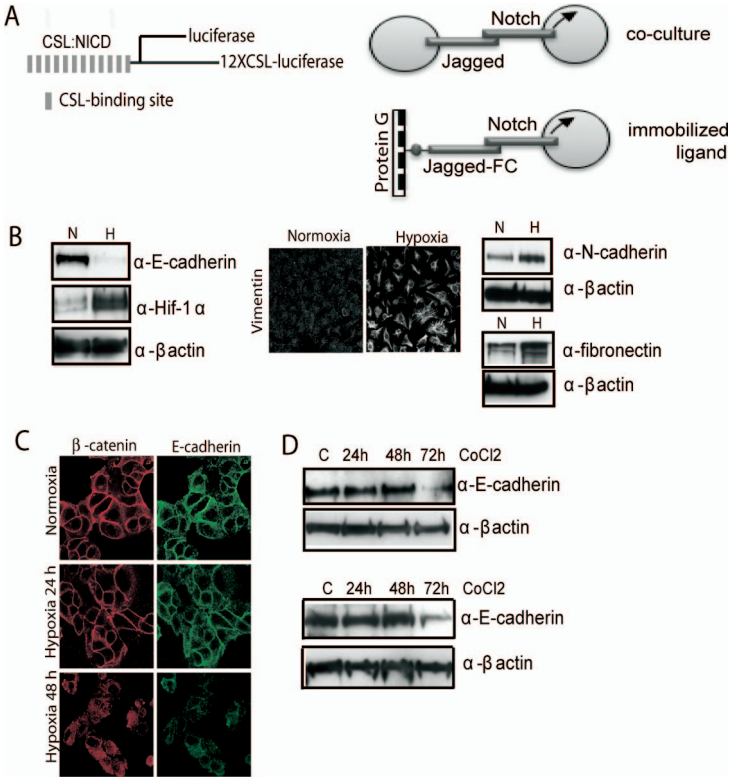

Fig. S1. Analysis of hypoxia-induced EMT in ovarian and breast epithelial cancer cells. (*A*) (*Left*) To measure activity immediately downstream of the Notch receptor, i.e., at the CSL/Notch ICD level, we used a Notch reporter construct containing multimerized CSL-binding sites linked to the luciferase gene (12XCSL-luciferase). (*Right*) To activate Notch signaling, cells were cocultured with cells expressing the Notch ligand Jagged1 or cultured on recombinant Jagged-Fc proteins immobilized by protein G on cell culture plates. (*B–D*) Hypoxia induces EMT in ovarian and breast epithelial cancer cells. (*B*) Hypoxia (72 h), as identified by elevated HIF-1α levels, induces down-regulation of E-cadherin (*Left*) in SKOV-3 cells. Expression of the mesenchymal markers vimentin (*Center*), *N*-cadherin and fibronectin (*Right*) increases. (*C*) Immunofluorescence images of MCF7 cells show loss of membrane-associated E-cadherin and β-catenin in response to hypoxia. (*D*) The hypoxia-mimicking compound $CoCl_2$ down-regulates E-cadherin in SKOV-3 (*Upper*) and MCF7 (*Lower*) cells as demonstrated by Western blot analysis.

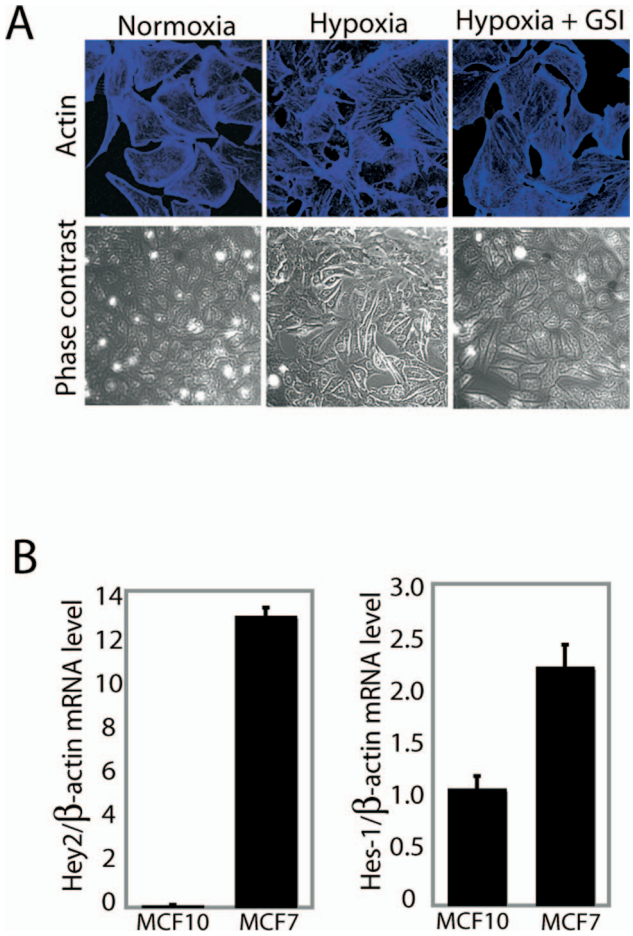

Fig. S2. Actin filament organization in response to Notch and hypoxia. (*A*) (*Upper*) Immunofluorescence images showing actin filament organization in SKOV-3 cells subjected to hypoxia for 72 h in the absence or presence of GSI. (*Lower*) Phase-contrast images of SKOV-3 cells subjected to hypoxia for 72 h in the absence or presence of GSI. (*B*) Different levels of Notch signaling in a breast epithelial (MCF10) and a breast epithelial cancer (MCF7) cell line as demonstrated by expression of the Notch target genes *Hey2* and *Hes1*.

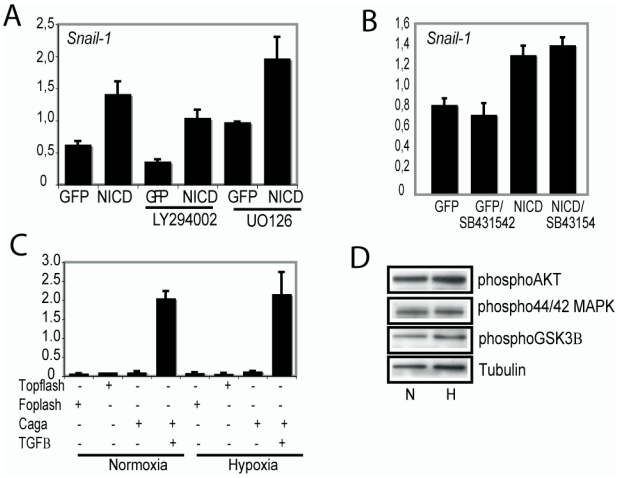

Fig. S3. Notch-induced expression of *Snail-1* does not require the PI3-kinase/AKT pathway or the Ras/MAP kinase pathway. (*A*) Pharmacological blocking of the PI3-kinase/AKT and the Ras/MAP kinase pathways by LY294002 or U0126, respectively, did not reduce *Snail-1* up-regulation in SKOV-3 cells induced by Notch 1 ICD. (*B*) TGFβ is not required for Notch-induced *Snail-1* expression. TGFβ signaling was blocked by the TGFβ receptor inhibitor SB431542. (*C*) Hypoxia does not alter the activity of TGFβ and WNT signaling pathways in SKOV-3 cells. The graph shows results from luciferase reporter assays with reporters for TGFβ (caga) and WNT signaling (Topflash and negative control reporter Fopflash). (*D*) Hypoxia did not induce activation of the PI3-kinase/AKT pathway or the MAP/kinase pathway or an increase in the inhibitory phosphorylation of GSK3β in SKOV-3 cells.

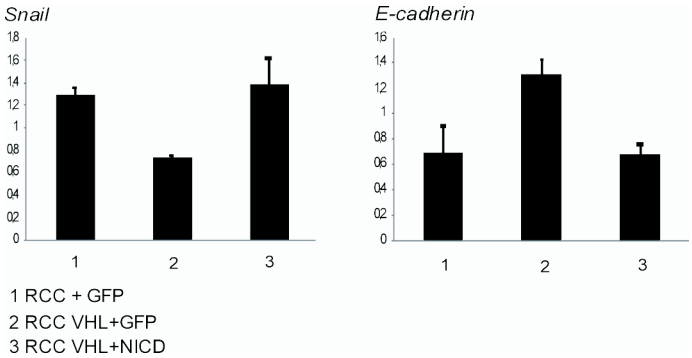

Fig. S4. Snail-1 and E-cadherin mRNA expression as measured by real-time Q-PCR in renal cell carcinoma cells (RCC) (1) and in RCC cells where VHL has been reintroduced (RCC VHL) (2), and in RCC VHL cells overexpressing Notch 1 ICD (RCC VHL+NICD) (3).

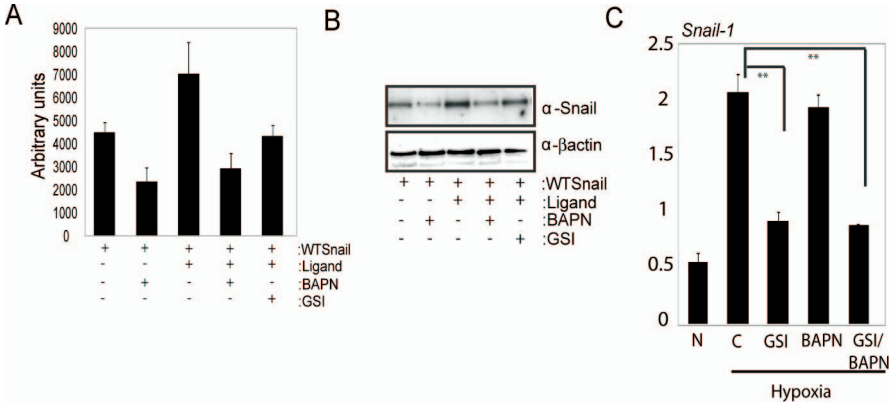

Fig. S5. Control of Snail-1 expression by Notch and LOX. (*A*) Quantification of Snail-1 protein levels in hypoxic SKOV-3 cells transfected with wild-type Snail-1 (WT Snail) with or without ligand-activated Notch signaling treated with vehicle, DAPT, or BAPN in different combinations. The graph represents data from three separate experiments. The analysis was done by Scionimage GelPlot2 software. (*B*) Snail-1 protein levels in hypoxic SKOV-3 cells transfected with WT Snail alone or together with Notch 1 ΔE (NΔE) treated with vehicle, DAPT, or BAPN in different combinations. (*C*) *Snail-1* mRNA expression in normoxic (N) or hypoxic SKOV-3 cells treated with DAPT or BAPN either separately or in combination.

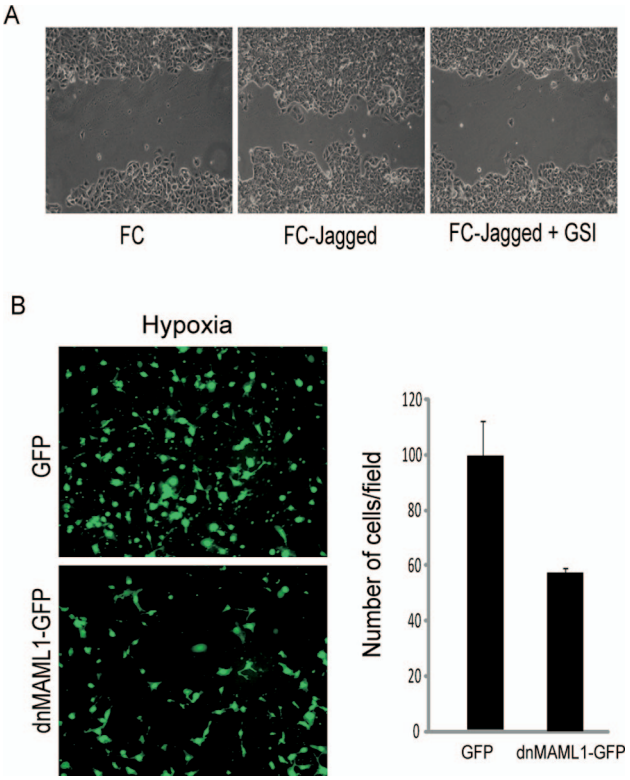

Fig. S6. Notch activation increases motility in normoxia. (*A*) Phase-contrast images demonstrate elevated motility in response to Notch activity as analyzed by a scratch assay of cells with low Notch activity (FC), high Notch activity (FC-Jagged), and GSI-inhibited high Notch activity (FC-Jagged+GSI). (*B*) Transmembrane invasion assay of SKOV-3 cells expressing GFP or dnMAML1-GFP in hypoxia. GFP- or dnMAML1-GFP-expressing cells were sorted by FACS, and positive cells were added to Matrigel invasion chambers and incubated for 48 h in hypoxia.