

# Notch signaling mediates hypoxia-induced tumor cell migration and invasion

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Communicated by Tomas Hokfelt, Karolinska Institutet, Stockholm, Sweden, March 3, 2008 (received for review November 14, 2007)

**Tumor hypoxia is linked to increased metastatic potential, but the molecular mechanisms coupling hypoxia to metastasis are poorly understood. Here, we show that Notch signaling is required to convert the hypoxic stimulus into epithelial–mesenchymal transition (EMT), increased motility, and invasiveness. Inhibition of Notch signaling abrogated hypoxia-induced EMT and invasion, and, conversely, an activated form of Notch could substitute for hypoxia to induce these processes. Notch signaling deploys two distinct mechanisms that act in synergy to control the expression of *Snail-1*, a critical regulator of EMT. First, Notch directly up-regulated *Snail-1* expression by recruitment of the Notch intracellular domain to the *Snail-1* promoter, and second, Notch potentiated hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ) recruitment to the *lysyl oxidase* (*LOX*) promoter and elevated the hypoxia-induced up-regulation of *LOX*, which stabilizes the *Snail-1* protein. In sum, these data demonstrate a complex integration of the hypoxia and Notch signaling pathways in regulation of EMT and open up perspectives for pharmacological intervention with hypoxia induced EMT and cell invasiveness in tumors.**

E-cadherin | lysyl oxidase | Snail

Hypoxia (reduced oxygen) has received considerable attention as an inducer of tumor metastasis, and there is a strong correlation among tumor hypoxia, metastasis, and poor patient outcome (1, 2); for review, see refs. 3 and 4. It has been proposed that the initial steps in metastasis involve an epithelial–mesenchymal transition (EMT)-like process (5). EMT converts cells from an epithelial, nonmotile morphology to become migratory and prone to invade other tissues (5–7). EMT is accompanied by specific changes in gene expression, such as down-regulation of E-cadherin (6, 7). The *E-cadherin* promoter is repressed by several transcriptional repressors (8), including the zinc finger transcription factor *Snail-1* (9); for review, see ref. 10. In keeping with this, *Snail* factors are potent inducers of EMT. Up-regulation of *Snail-1* correlates with metastasis and poor prognosis, whereas silencing of *Snail-1* is critical for reducing tumor growth and invasiveness (11, 12); for review, see ref. 10.

When tumors outgrow the oxygen-supplying capacity of the vasculature, the low oxygen levels are sensed by a hypoxic response machinery in the cell. Low oxygen levels inactivate prolyl hydroxylases, which, in normoxia, hydroxylate hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ), making it a substrate for ubiquitylation by the von Hippel–Lindau (VHL) E3 ubiquitin ligase complex, followed by degradation by the proteasome (13, 14). This results in very low steady-state levels of HIF-1 $\alpha$  during normoxia and accumulation in hypoxia. When stabilized in hypoxia, HIF-1 $\alpha$  dimerizes with HIF-1 $\beta$  (ARNT) and activates downstream genes involved in the canonical hypoxic response via HRE elements in their promoters (15, 16). Mutations affecting *VHL* or growth factor-induced expression of HIF-1 $\alpha$  can lead to tumorigenesis (17).

The molecular mechanisms that relay the hypoxia signal into EMT and metastasis are still largely elusive, and it is an open issue whether the “canonical” hypoxic response, i.e., via HIF-1 $\alpha$  binding to hypoxia-response elements (HREs) in the promoter, is used or whether alternative molecular readouts cause EMT in

response to hypoxia. The Notch signaling pathway is an attractive candidate as a mediator for an alternative readout between hypoxia and EMT in tumor cells, because integration between Notch signaling and hypoxia was recently demonstrated in the control of stem cell differentiation (18). Furthermore, Notch can up-regulate *Snail-1* and induce EMT in normoxia during normal development in cardiac and kidney tubular cell differentiation (19, 20), although the detailed molecular mechanism for how Notch controls *Snail-1* expression remains to be established. Finally, deregulated Notch signaling correlates with cancer, for example in T cell lymphoblastic leukemia, where the majority of patients carry mutations in *NOTCH1* (21), and in breast cancer, where elevated expression of the activated form of Notch is observed (22, 23). The core Notch signaling pathway is composed of relatively few components (24). Ligand activation results in two proteolytic cleavage events in the transmembrane Notch receptor. This ultimately leads to liberation of the Notch intracellular domain (NICD), which constitutes the activated form of the receptor (24, 25). In the nucleus, NICD interacts with the DNA-binding protein CSL (RBP-J, Su(H), Rbbsuh, Lag-1) to regulate expression of downstream genes (24).

In this article, we provide evidence for a hypoxia/Notch/EMT axis in tumor cells, where Notch serves as a critical intermediate in conveying the hypoxic response into EMT. Hypoxia-induced increased motility and invasiveness of the tumor cells require Notch signaling, and activated Notch mimicked hypoxia in the induction of EMT. In this process, Notch signaling controls *Snail-1* expression by two distinct but synergistic mechanisms, involving both direct transcriptional activation of *Snail-1* and an indirect mechanism operating via lysyl oxidase (*LOX*), leading to elevated *Snail-1* protein levels.

## Results and Discussion

**Hypoxia Induces the Notch Downstream Response in Different Tumor Cell Types.** To address whether hypoxia potentiates the Notch downstream response in a tumor context, we examined the effect of hypoxia on the Notch signaling pathway in tumor cell lines of cervical (C-33A), colon (HCT-116), glioma (U-87MG), and ovarian (SKOV-3) origin. Coculture of these cell lines with Jagged 1-expressing cells resulted in a low level of activation of the Notch reporter construct 12XCSL-luc [see supporting information (SI) Fig. S1A for details] in normoxia and a substantially elevated response under hypoxic conditions (1% O<sub>2</sub>) (Fig. 1A). The increase in reporter gene expression was abrogated by the  $\gamma$ -secretase inhibitor (GSI) DAPT (Fig. 1A), which inhibits the final proteolytic cleavage of the Notch receptor. The

Author contributions: C.S., L.P., and U.L. designed research; C.S., M.V.G., and S.J. performed research; C.S., M.V.G., S.J., L.P., and U.L. analyzed data; and C.S., L.P., and U.L. wrote the paper.

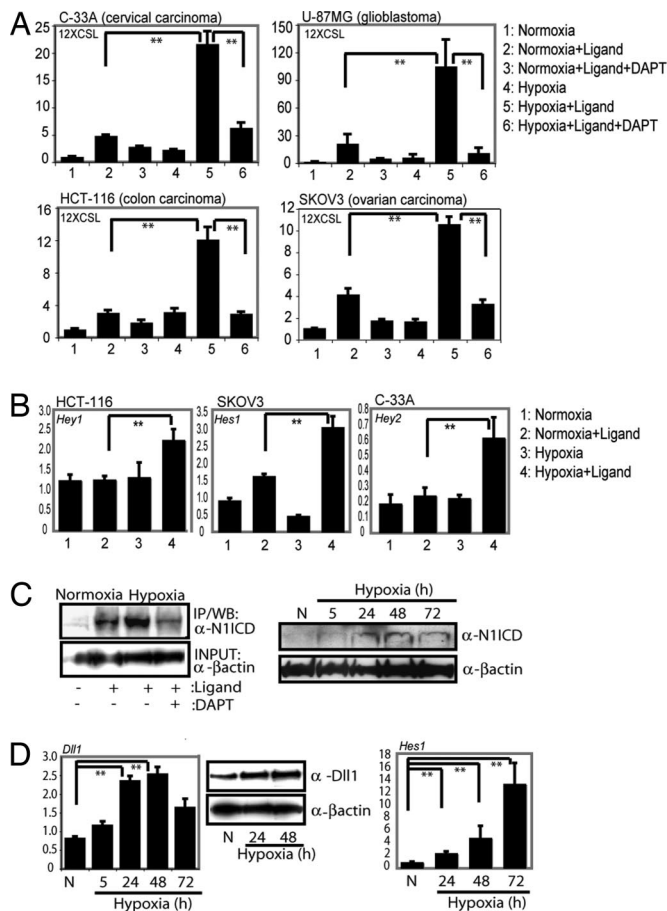
The authors declare no conflict of interest.

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This article contains supporting information online at [www.pnas.org/cgi/content/full/0802047105/DCSupplemental](http://www.pnas.org/cgi/content/full/0802047105/DCSupplemental).

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**Fig. 1.** Hypoxia potentiates Notch signaling in tumor cells. (A) C-33 A, HCT-116, U-87 MG, and SKOV-3 cells cocultured with 3T3-Babe (3T3-B) or 3T3-Jagged (ligand) cells kept at normoxia or hypoxia in the presence or absence of the  $\gamma$ -secretase inhibitor (DAPT), as indicated. Notch activity was measured by 12XCSL-luc activation. (B) Expression of Notch downstream genes (*Hey1*, *Hes1*, or *Hey2*, as indicated) measured by quantitative PCR in HCT-116, SKOV-3, and C-33 A cells activated by coculture as above, in normoxia or hypoxia. (C) (Left) SKOV-3 cells, immunoprecipitated for activated Notch 1 after coculture with 3T3-B or 3T3-Jagged (3T3-J) cells kept at normoxia or hypoxia in the absence or presence of GSI (Right). Western blot of N1ICD and  $\beta$ -actin expression in SKOV-3 cells subjected to normoxia (N) or hypoxia for 5, 24, 48, or 72 h. For the immunoprecipitation experiment (Left), the  $\beta$ -actin control is run on a separate gel. (D) (Left) Quantitative PCR analysis of *Delta-like 1* (*Dll1*) mRNA expression in SKOV-3 cells kept at normoxia or hypoxia for 5, 24, 48, or 72 h. (Center) Western blot showing expression of DLL1 in normoxia and hypoxia. (Right) Quantitative PCR analysis of *Hes1* mRNA expression in SKOV-3 cells kept at normoxia or hypoxia for 24, 48, or 72 h. Note that in this experiment, a small up-regulation of *Hes1* expression was observed already at 24 h, which is in contrast to the data in B, suggesting that *Hes1* up-regulation may be somewhat variable. Values are presented as mRNA expression relative to expression of  $\beta$ -actin mRNA. Values are significant at \*\*,  $P < 0.01$  and \*,  $P < 0.05$ , as indicated in the figure. Graphs represent average of three (A and B) or two (D) independent experiments.

hypoxia-potentiated Notch signaling was further corroborated by elevated expression of Notch downstream genes in hypoxia (Fig. 1B). In sum, these data show that Notch signaling is augmented by hypoxia in various tumor cell lines.

**Hypoxia Leads to Increased Levels of N1ICD and Up-Regulation of Notch Ligand Expression.** The elevated Notch response after hypoxia could result from effects at various levels in the Notch signaling pathway. Ligand-dependent activation of SKOV-3 cells resulted in increased levels of N1ICD, and the amount of

accumulated N1ICD was further elevated in hypoxia (Fig. 1C Left). The low level of N1ICD found in SKOV-3 cells in the absence of ligand stimulation (26) was also elevated by hypoxia, reaching a maximum at 24–48 h after the hypoxic switch (Fig. 1C Right). On the ligand side, up-regulation of *Delta-like 1* (*Dll1*) mRNA and protein was observed in SKOV-3 cells subjected to hypoxia (Fig. 1D). The increase in ligand expression and Notch ICD levels was paralleled by an increase in expression of the Notch target gene *Hes-1* in SKOV-3 cells (Fig. 1D). It is of note that elevated ligand levels and stabilization of Notch ICD are likely to act synergistically to increase the Notch signaling output. Furthermore, these data show that hypoxia not only can cooperate with preexisting Notch signaling but can also induce Notch signaling by increasing the level of Notch ligands.

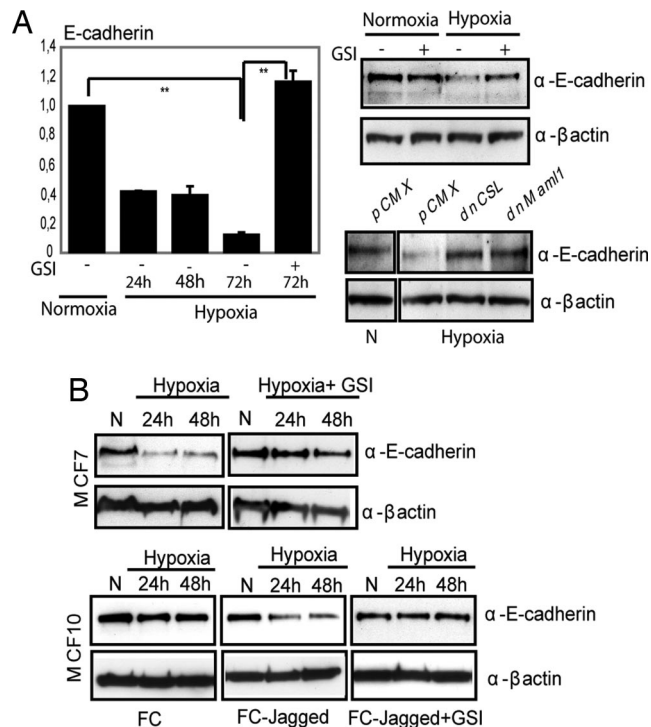
**Hypoxia-Induced EMT and E-Cadherin Down-Regulation Require Notch Signaling.** The ovarian carcinoma cell line SKOV-3 has been used to study the loss of epithelial features and gain of a mesenchymal phenotype in response to hypoxia (27). We cultured SKOV-3 cells under hypoxic conditions or exposed the cells to the hypoxia-mimicking compound  $\text{CoCl}_2$ . Both treatments induced morphological and molecular changes typical of EMT, i.e., cells converted from an epithelial to a more mesenchymal phenotype, down-regulated E-cadherin and  $\beta$ -catenin, and up-regulated vimentin, *N*-cadherin, and fibronectin (Fig. S1 B and D), in keeping with previous observations (27). Similar data were obtained for the breast cancer cell line MCF-7 (Fig. S1 C and D).

To assess the involvement of Notch in hypoxia-induced EMT in SKOV-3 cells, we inhibited Notch signaling by GSI or by expression of dominant-negative forms of CSL or Maml-1, key components in the Notch signaling cascade and present in the Notch ICD transcriptional complex (28). Inhibition of Notch signaling abrogated the down-regulation of *E-cadherin* mRNA observed during hypoxia (Fig. 2A), and the epithelial morphology was retained (Fig. S2A). Similarly, down-regulation of E-cadherin protein levels was observed after hypoxia and abrogated by GSI or transfection of dominant negative CSL or Maml1 (Fig. 2A). Furthermore, down-regulation of another cell-adhesion protein, occludin (data not shown), and reorganization of the actin cytoskeleton were substantially inhibited by GSI (Fig. S2A).

The GSI data would suggest that a certain endogenous level of Notch signaling is required for hypoxia to down-regulate E-cadherin expression. To test this, we analyzed E-cadherin regulation in two breast cancer cell lines with low (blocked because of high activity of Numb; MCF10) and high (MCF7) levels of Notch signaling (Fig. S2B). Hypoxia induced down-regulation of E-cadherin in MCF7 cells (Fig. 2B Upper) but not in the MCF10 cells (Fig. 2B Lower Left), and it was only when MCF10 cells were grown on immobilized Jagged1 to activate Notch signaling that hypoxia induced down-regulation of E-cadherin (Fig. 2B Lower Center). This ligand-induced down-regulation was blocked by GSI (Fig. 2B Lower Right). Taken together, these data indicate that hypoxia-induced EMT requires Notch signaling and that cells with blocked or too-low endogenous Notch signaling are refractory to hypoxia-induced EMT and E-cadherin down-regulation.

**Hypoxia-Induced EMT and E-Cadherin Down-Regulation Can Be Mimicked by N1ICD During Normoxia.** Because hypoxia-induced EMT required Notch signaling, we asked whether N1ICD could substitute for hypoxia in inducing EMT. Culturing of the SKOV-3 cells on immobilized Jagged-1 ligand under normoxic conditions induced morphological changes similar to what was observed in hypoxia, and the morphological change could be abrogated by GSI treatment (Fig. 3A). Expression of N1ICD led to a reduction in E-cadherin (Fig. 3B) and occludin (data not shown) levels during normoxia, and N1ICD was considerably



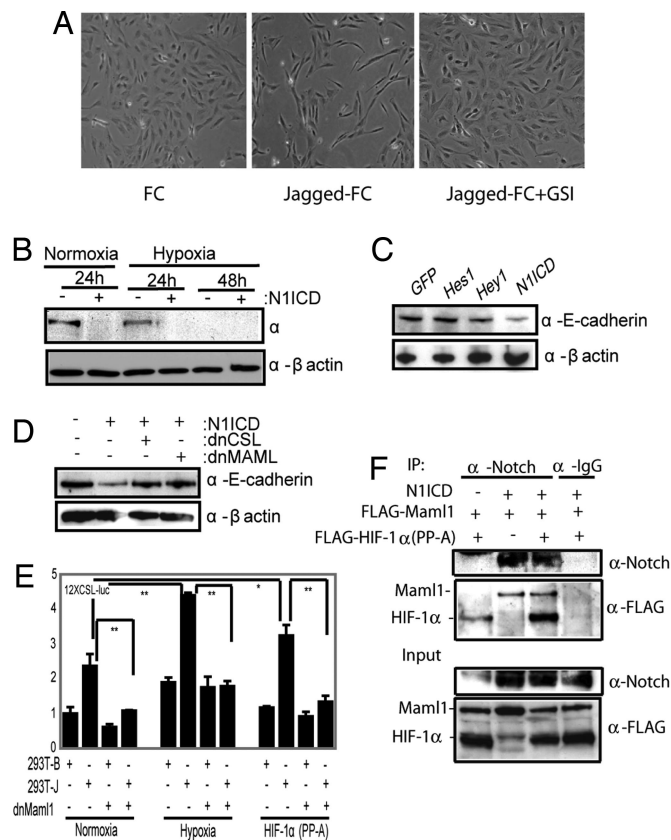


**Fig. 2.** Hypoxia-mediated EMT requires Notch. (A) Quantitative PCR analysis of *E-cadherin* mRNA expression in SKOV-3 cells kept at normoxia or treated with hypoxia for the indicated time (Left). Values represent average values of three independent experiments and are significant at \*\*,  $P < 0.01$  and \*,  $P < 0.05$  as indicated. Western blot analysis of E-cadherin protein levels in SKOV-3 cells cultured at normoxia or hypoxia in the absence or presence of GSI (Upper Right) or in SKOV-3 cells transfected with empty vector (PCMX), dnCSL, or dnMam11 (Lower Right). (B) E-cadherin protein expression in MCF7 cells in normoxia (N) or hypoxia (Upper). E-cadherin expression in MCF10 cells grown on recombinant FC domain or recombinant Jagged, to activate Notch signaling, in normoxia and hypoxia in the presence of absence of GSI as indicated (Lower).

more effective in down-regulating E-cadherin protein levels than Hes-1 and Hey-1 (Fig. 3C), indicating that E-cadherin down-regulation was mediated directly downstream of N1ICD rather than further down in the Notch signaling cascade.

N1ICD-induced repression of E-cadherin expression in SKOV-3 cells could be substantially abrogated when N1ICD was coexpressed with either dominant-negative CSL or Mam11 (Fig. 3D). Furthermore, dominant-negative Mam11 blocked ligand-mediated induction of 12XCSL-luciferase expression after hypoxia or introduction of a normoxically stabilized form of HIF-1 $\alpha$  (PP-A) in normoxia (Fig. 3E). These data suggest that HIF-1 $\alpha$  and Mam11 are present in the same transcriptional complex. In support of this notion, immunoprecipitation experiments revealed an interaction between N1ICD and both Mam11 and HIF-1 $\alpha$  (Fig. 3F). The small amount of HIF-1 $\alpha$  immunoprecipitated in cells not transfected with N1ICD is probably a consequence of a low level of endogenous Notch expression in these cells. In sum, these data show that N1ICD can substitute for hypoxia in controlling EMT and E-cadherin expression and that E-cadherin regulation requires CSL and Mam11 in addition to N1ICD.

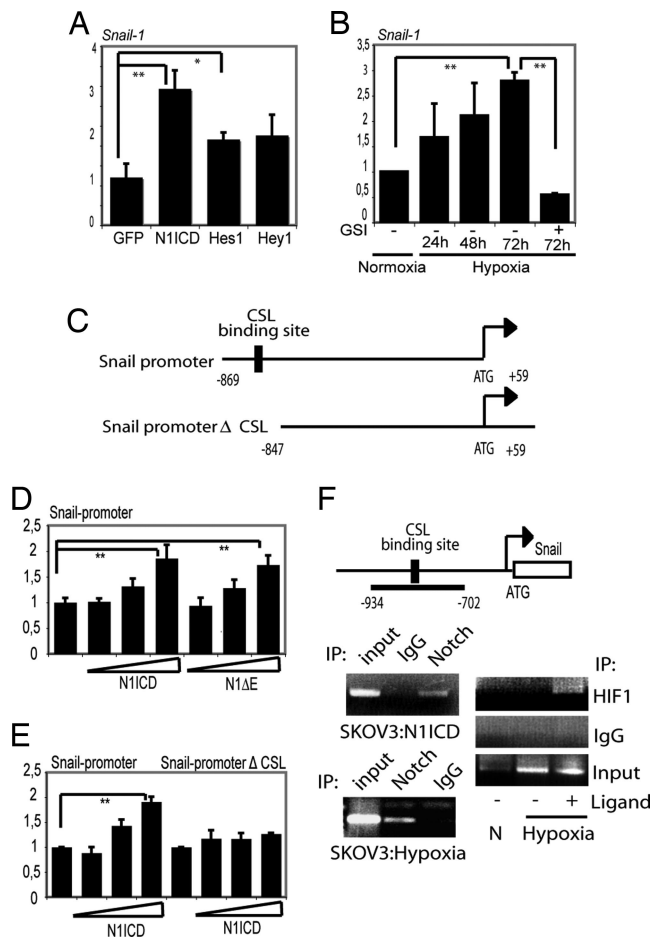
**Notch Directly Regulates Expression of *Snail-1*.** Because *Snail-1* is an important regulator of E-cadherin expression, we asked whether the effects of Notch on E-cadherin expression are mediated via N1ICD regulating *Snail-1* at the transcriptional level. *Snail-1* mRNA was up-regulated by N1ICD expressed from an adeno-



**Fig. 3.** Activation of Notch can mimic hypoxia-induced EMT. (A) Phase contrast images of SKOV-3 cells grown on recombinant FC or FC-Jagged in the presence or absence of GSI in normoxia. The same number of cells was seeded in each experiment. (B) Western blot analysis of endogenous E-cadherin protein levels in SKOV-3 cells kept at normoxia or hypoxia after transfection of N1ICD. (C) Western blot analysis of E-cadherin expression in SKOV-3 cells after infection with N1ICD, *Hes-1*, *Hey-1*, or *EGFP*. (D) E-cadherin protein levels after cotransfection of N1ICD with dnCSL or dnMam11 at normoxia. (E) 12XCSL-luciferase activation in 293T cells cocultured with wild-type (–) or Jagged1-expressing 293T 293T-J) cells transfected with dnMam11, treated with hypoxia or transfected with HIF-1 $\alpha$ (PP-A). (F) Coimmunoprecipitation analysis of HIF1 $\alpha$ -N1ICD-Mam11 interaction. Notch was immunoprecipitated from 293T cells transfected with N1ICD, FLAG-Mam11, and FLAG-HIF-1 $\alpha$ (PP-A) as indicated, and the presence of coimmunoprecipitated Mam11 and HIF-1 $\alpha$  was detected by Western blot analysis. The input control is shown below. Values are significant at \*\*,  $P < 0.01$  and \*,  $P < 0.05$  as indicated.

viral vector in the SKOV-3 cells compared with a control vector expressing EGFP or expression of Hes-1 or Hey-1 (Fig. 4A). Furthermore, hypoxia-induced up-regulation of *Snail-1* mRNA in SKOV-3 cells was abrogated by GSI treatment (Fig. 4B). In Fig. S3, data are presented indicating that Notch signaling alone was sufficient to induce *Snail-1* expression and that concomitant activation of the PI3-kinase/AKT, Ras/MAP kinase, TGF- $\beta$ , or Wnt pathways is not required.

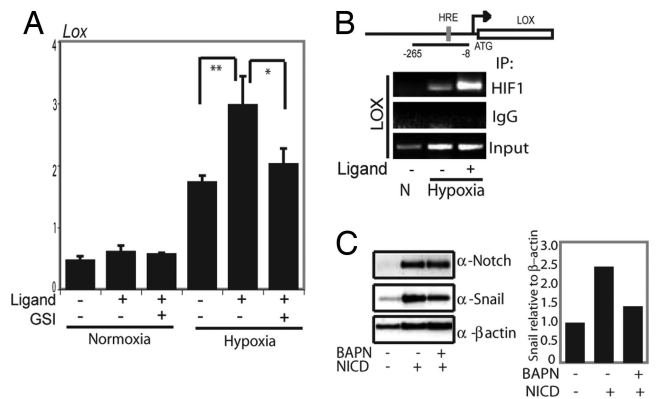
We identified a consensus CSL-binding motif (29) located at –847 to –839 bp upstream of the transcription start in the *Snail-1* promoter (Fig. 4C). A 0.9 kb *Snail-1* promoter-luciferase reporter, which encompasses the CSL-binding site (Fig. 4C), was activated in a dose-dependent manner by expression of N1ICD or Notch 1  $\Delta E$  [a membrane-tethered ligand-independent form of Notch (30)] (Fig. 4D). Deletion of the CSL-binding site (*Snail* promoter $\Delta$ CSL-luc, Fig. 4C) abolished Notch-induction (Fig. 4E). We next showed by ChIP assays that N1ICD in infected SKOV-3 cells was recruited to the region of the *Snail-1* promoter encompassing the –847/839 CSL-binding site (Fig. 4F). Recruit-



**Fig. 4.** *Snail-1* is a direct downstream target of Notch. (A and B) Quantitative PCR analysis of *Snail-1* expression in SKOV-3 cells after adenoviral infection of *N1ICD* (*N1CD*), *Hes-1*, *Hey-1*, or *EGFP* (A) or after exposure to hypoxia in the presence or absence of GSI (B). (C) Schematic depiction of the *Snail-1* promoter constructs used in the study: the promoter containing (Snail-promoter) or lacking (Snail-promoter $\Delta$ CSL) the CSL-binding site. (D) *Snail1-luciferase* activation by transfection of *N1ICD* or *N1*  $\Delta$ E. (E) Activation of the two promoter constructs shown in C by *N1ICD*. (F) Schematic representation of the *Snail1* promoter with the amplified promoter region and the position of the CSL-binding sites denoted. Below the schematic representation, ChIP analysis of recruitment of N1ICD to the *Snail-1* promoter after adenoviral infection of *N1ICD* into SKOV-3 cells (Upper Left) or of endogenous N1ICD after hypoxia in SKOV-3 cells (Lower Left) is shown. (Right) PCR amplification of the *Snail-1* promoter after immunoprecipitation for N1ICD. ChIP analysis of recruitment of HIF-1 $\alpha$  to the *Snail-1* promoter after coculture with 3T3-Jagged1 (3T3-J) or 3T3-Babe (3T3-B) cells under normoxia or hypoxia and PCR amplification of the *Snail-1* promoter after immunoprecipitation for HIF-1 $\alpha$  are shown. Values are significant at \*\*,  $P < 0.01$  and \*,  $P < 0.05$ , as indicated in A, B, D, and E. Values indicate the average of at least three independent experiments.

ment of N1ICD was also observed in hypoxic SKOV-3 cells at endogenous levels of Notch signaling (Fig. 4F Lower).

Given the interaction between N1ICD and HIF-1 $\alpha$  (Fig. 3F), we then asked whether HIF-1 $\alpha$  accompanied N1ICD to the *Snail-1* promoter. The data in Fig. 4F (Right) show that HIF-1 $\alpha$  was detected at the *Snail-1* promoter only under conditions of combined hypoxia and Notch-ligand stimulation. Further support for a role of HIF-1 $\alpha$  in controlling *Snail-1* expression comes from experiments in a renal carcinoma cell line (RCC), which is deficient in VHL and, consequently, has high HIF-1 $\alpha$  levels. Stable reintroduction of VHL into the RCC cell line (RCC-VHL) reduced *Snail-1* expression, whereas overexpression of N1ICD in the RCC-VHL cells increased *Snail-1* mRNA levels (Fig. S4). Treatment of the RCC



**Fig. 5.** Notch signaling enhances hypoxia-induced activation of *LOX* transcription. (A) Quantitative PCR analysis of *LOX* expression in SKOV-3 cells cocultured with 3T3-Babe (3T3-B) or 3T3-Jagged (3T3-J) cells kept at normoxia or treated with hypoxia for 16 h in the absence or presence of GSI. Values are significant at \*\*,  $P < 0.01$  and \*,  $P < 0.05$  as indicated. Values represent the average of three independent experiments. (B) Schematic depiction of the *LOX* promoter with the PCR-amplified promoter region and the location of the HRE sites denoted. For the ChIP experiments, SKOV-3 cells were cocultured with either 3T3-J cells or 3T3-B cells at normoxia or hypoxia, as indicated. PCR amplification of the *LOX* promoter after immunoprecipitation of HIF-1 $\alpha$ . (C) Western blot analysis of Snail-1 expressed from a heterologous promoter to analyze Snail-1 protein stability. SKOV-3 cells were transfected with wild-type *Snail-1* and *N1ICD* or an empty vector and subjected to hypoxia in the presence or absence of the *LOX* inhibitor BAPN (Left). Quantification of the Western blot (Snail-1/ $\beta$ -actin intensity) is shown (Right).

cells with GSI reduced the *Snail-1* mRNA levels (data not shown). These data are in good agreement with a recent report by Evans *et al.* (31). Collectively, these data demonstrate that N1ICD transcriptionally regulates *Snail-1*, in keeping with a previous report (19), and extend this study by demonstrating that *Snail-1* is a direct transcriptional target of Notch and that HIF-1 $\alpha$  is recruited to the *Snail-1* promoter only by the combination of hypoxia and active Notch signaling.

**Notch Signaling Indirectly Up-Regulates Expression of *LOX*, Leading to Increased Levels of the Snail-1 Protein in Hypoxia.** *LOX* was recently shown to link hypoxia to tumor invasion (32). Because *LOX* proteins also stabilize the Snail protein (33), we tested whether Notch would act in synergy with *LOX* to regulate Snail activity in hypoxia. As expected (32), hypoxia up-regulated the level of *LOX* mRNA in SKOV-3 cells (Fig. 5A). Interestingly, *LOX* expression was further increased by activation of Notch signaling by coculture with Notch ligand-expressing cells under hypoxic conditions, and Notch-mediated up-regulation was abrogated by GSI treatment (Fig. 5A). The Notch-induced elevation of *LOX* mRNA expression was observed only in hypoxia and not in normoxia (Fig. 5A). These data indicate that *LOX* is not a direct downstream gene of Notch, and in keeping with this notion, we could not identify any potential CSL-binding sites in a 2-kb proximal *LOX* promoter fragment, recently shown to respond to hypoxic stimulation (32). Furthermore, N1ICD did not bind to the *LOX* promoter at any detectable level, as assessed by ChIP analysis (data not shown). We therefore tested whether the recruitment of HIF-1 $\alpha$  to the *LOX* promoter was altered by Notch signaling in hypoxia. By ChIP analysis, we showed that HIF-1 $\alpha$  was recruited to the part of the *LOX* promoter containing a functional HRE (32). Recruitment of HIF-1 $\alpha$  was observed only in hypoxia, and the recruitment was augmented when the cells were cocultured with cells expressing Notch ligand (Fig. 5B).

The Notch-mediated elevation of *LOX* expression in hypoxia would be expected to lead to further stabilization, and thus higher levels, of the Snail-1 protein, because it is stabilized by *LOX* (34).

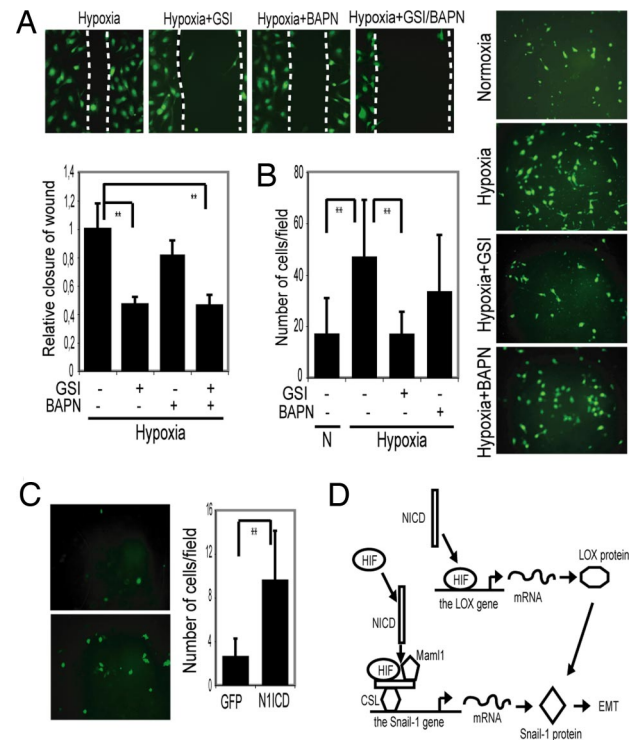
To test this, we expressed wild-type Snail-1 from a heterologous promoter (to avoid Notch-mediated transcriptional regulation) in SKOV-3 cells and analyzed the levels of Snail-1 protein in the presence or absence of N1ICD in hypoxia. Notch signaling increased Snail-1 protein levels, and the increase was reduced by treatment with the LOX inhibitor  $\beta$ -aminopropionitrile (BAPN) (Fig. 5C). Similarly, when ligand-induced Notch activation was combined with BAPN treatment, the Notch-mediated up-regulation of Snail-1 protein levels was reduced by BAPN (Fig. S5A and B). Furthermore, the observed increased stability of Snail-1 in ligand-activated cells was blocked by GSI (Fig. S5A and B). In contrast, *Snail-1* mRNA levels, which were augmented by hypoxia, were reduced by GSI but not by BAPN treatment (Fig. S5C). Collectively, these data show that hypoxia-induced transcription of Snail-1 depends on functional Notch signaling but not on LOX but that LOX exerts an effect on Snail-1 protein stability. This indicates that Notch signaling indirectly potentiates LOX expression in a hypoxia-dependent manner, which is manifested by elevated Snail-1 protein levels in response to combined hypoxia and Notch signaling.

**Hypoxia-Induced Cell Motility and Invasiveness Require Notch Signaling.** Increased cell motility and invasiveness are important consequences of hypoxia-induced EMT (5). To study cell motility, we used a scratch wound-healing assay, where the extent of migration of cells into the scratched area was measured. Migration of SKOV-3 cells was elevated during hypoxia but effectively blocked in the presence of GSI (Fig. 6A). To address the relationship between Notch and LOX in this context, we used BAPN and GSI to block LOX and Notch signaling, respectively. The addition of BAPN reduced migration, but less effectively than GSI, and the combined use of BAPN and GSI did not block migration to a higher extent than GSI treatment alone (Fig. 6A). Conversely, activation of Notch signaling by exposure to immobilized ligand increased motility in the wound scratch assay (Fig. S6A).

To study the invasive properties of the SKOV-3 cells, we used a Matrigel transmembrane invasion assay, where migration of cells across a membrane toward a source of serum attractant was monitored. Hypoxia induced an increase in invasiveness, which was substantially blocked by GSI (Fig. 6B) or by expression of dnMam1 (Fig. S6B). In contrast, inhibition of LOX by BAPN was less effective (Fig. 6B). In a converse experiment, we tested the effects on invasiveness of up-regulating Notch in normoxia. Transient expression of N1ICD increased migration and invasiveness during normoxia (Fig. 6C) and could thus substitute for hypoxia. This demonstrates that pharmacological intervention with Notch signaling effectively reduces hypoxia-induced migration and invasion capacity.

## Conclusions

This article provides evidence for a hypoxia/Notch/EMT axis in tumor cells, and in this process, Notch regulates Snail-1 levels in two different but synergistic ways. The regulation of *Snail-1* and *LOX* expression also sheds light on how the Notch and hypoxia signaling mechanisms are integrated and how Notch ICD and HIF-1 $\alpha$  cooperate but take on different roles on the two promoters, as schematically depicted in Fig. 6D. The role of Notch as a mediator of the hypoxic stimulus into enhanced cell migration and invasiveness opens perspectives for intervention. In keeping with this notion, a recent report shows that Notch controls expression of *Slug* (*Snail-2*) and that block of Notch signaling inhibits tumor growth and metastasis in an *in vivo* tumor model (35). Refinement of Notch inhibitors combined with more local containment, e.g., in epithelial



**Fig. 6.** Notch signaling is required for migration and invasiveness of SKOV-3 cells. (A) Scratch wound healing assay of fluorescently labeled SKOV-3 cells cultured at hypoxia and treated with GSI, BAPN, or GSI + BAPN. Quantification of relative closure of the scratch wound compared with the control. The graph shown below represents the results of three separate experiments each performed in triplicates, where the distance of the wound is measured at four to six reference points along the scratch wound. (B) Transmembrane invasion assay of fluorescently labeled SKOV-3 cells kept at normoxia or hypoxia in the absence or presence of GSI or BAPN. (C) Adenoviral infection of *N1ICD-GFP* or *GFP* into SKOV-3 cells kept at normoxia. Quantification of the number of invading cells per field. A minimum of six fields of three separate wells within each experiment were analyzed. The graphs represent data from three (B) and two (C) separate experiments. (D) Model for how Notch controls Snail-1 at two different levels. N1ICD is recruited to the *Snail-1* promoter, and we propose that HIF1 $\alpha$  is also part of the transcriptional complex (together with CSL and Mam1). For the *LOX* promoter, the binding of HIF1 $\alpha$  is potentiated by N1ICD, but N1ICD does not bind directly to the transcriptional complex. Notch is thus proposed to control Snail-1 in two different but synergistic ways, leading to elevated *Snail-1* transcription and stabilization of the resulting protein.

tumors at risk for metastasizing, may therefore be a productive way toward improving cancer therapy.

## Materials and Methods

Details for plasmid constructs, cell culture, Notch activity assay, RNA extraction and quantitative RT-PCR, immunocytochemistry and Western blot analysis, cell migration and invasion, activation of Notch signaling using immobilized recombinant Notch ligands, transfections and viral infections, and chromatin immunoprecipitation are given in *SI Experimental Procedures*.

**ACKNOWLEDGMENTS.** We are grateful to Drs. W. Pear (University of Pennsylvania, Philadelphia) and M. A. Nieto (Instituto de Neurociencias de Alicante, Elche, Spain) for the dominant-negative MAM1 and *Snail-1* promoter-reporter, respectively, and to Susanne Bergstedt for cell culture work. This work was supported by the Swedish Cancer Society, the Swedish Research Council, the Swedish Foundation for Strategic Research, the European Union project EuroStemCell (U.L.), and the Juselius Foundation and Academy of Finland (C.S.).

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