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Citation
Natsuizaka, M., K. A. Whelan, S. Kagawa, K. Tanaka, V. Giroux, P. M. Chandramouleeswaran, A. Long, et al. 2017. “Interplay between Notch1 and Notch3 promotes EMT and tumor initiation in squamous cell carcinoma.” Nature Communications 8 (1): 1758. doi:10.1038/s41467-017-01500-9. http://dx.doi.org/10.1038/s41467-017-01500-9.

Published Version
doi:10.1038/s41467-017-01500-9

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Interplay between Notch1 and Notch3 promotes EMT and tumor initiation in squamous cell carcinoma

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Notch1 transactivates Notch3 to drive terminal differentiation in stratified squamous epithelia. Notch1 and other Notch receptor paralogs cooperate to act as a tumor suppressor in squamous cell carcinomas (SCCs). However, Notch1 can be stochastically activated to promote carcinogenesis in murine models of SCC. Activated form of Notch1 promotes xenograft tumor growth when expressed ectopically. Here, we demonstrate that Notch1 activation and epithelial-mesenchymal transition (EMT) are coupled to promote SCC tumor initiation in concert with transforming growth factor (TGF)-β present in the tumor microenvironment. We find that TGFβ activates the transcription factor ZEB1 to repress Notch3, thereby limiting terminal differentiation. Concurrently, TGFβ drives Notch1-mediated EMT to generate tumor initiating cells characterized by high CD44 expression. Moreover, Notch1 is activated in a small subset of SCC cells at the invasive tumor front and predicts for poor prognosis of esophageal SCC, shedding light upon the tumor promoting oncogenic aspect of Notch1 in SCC.
Notch signaling regulates cell fate in a context-dependent manner. The ligand-activated intracellular domain of Notch (ICN) forms a transcriptional activation complex with the transcription factor CSL and the co-activator MAML. Notch1 drives terminal differentiation in stratified squamous epithelia in concert with other Notch receptor paralogs. Histopathology of squamous cell carcinomas (SCCs) features squamous-cell differentiation, a process normally regulated via direct transcriptional activation of Notch3 by ICN1, the activated form of Notch1, in esophageal epithelia. Loss-of-function Notch1 mutations are found in SCCs, suggesting a tumor suppressor role for Notch1. However, Notch1 can be stochastically activated or inactivated, with either scenario resulting in promotion of carcinogenesis in murine models of SCC. Many human SCC cell lines express ICN1 and ectopic ICN1 expression promotes xenograft tumor growth. While pharmacological modulation of Notch paralogs represents an attractive strategy for cancer therapy, a more detailed understanding of the functional role of the Notch pathway as it relates to tissue biology in the context of health and disease is necessary to guide such approaches.

In addition to squamous-cell differentiation, Notch1 regulates cell cycle, senescence, and epithelial–mesenchymal transition (EMT). Acquisition of mesenchymal properties facilitates malignant transformation by limiting oncogene-induced senescence. In human esophageal squamous cell carcinoma (ESCC), the deadliest form of all human SCCs, EMT is associated with chemoresistance and poor prognosis. EMT also regulates cancer stem cells (CSCs). CSCs defined by high CD44 expression (CD44H) have been identified in various tumor types, including SCCs. In transformed esophageal and oral keratinocytes, cells with low CD44 expression (CD44L) and...
epithelial properties are converted to CD44H cells with mesenchymal traits in response to transforming growth factor (TGF)-β, a potent EMT inducer present in the tumor microenvironment. During TGFβ-mediated EMT, expression of the Notch ligand JAG1 is induced via ZEB1, a transcription factor essential in TGFβ-induced EMT and microRNA-mediated regulation of Notch signaling. While emerging lines of evidence support Notch1 as a positive effector of EMT, Notch3 cooperate to drive squamous-cell differentiation, these Notch paralogs may play opposing roles in EMT and, potentially, regulation of CSC dynamics. The precise molecular mechanisms through which Notch signaling regulates distinct cell fates in a context-dependent manner have yet to fully elucidated.

Here, we aimed to define the functional role of Notch1 in SCC. We demonstrate that Notch1 activation and EMT are coupled to promote tumor initiation and intratumoral cancer cell heterogeneity in SCC. We find that the transcription factor ZEB1 represses NOTCH3, thereby limiting ICN1-induced differentiation while permitting ICN1-mediated EMT. Moreover, ICN1 expression in a small subset of SCC cells at the invasive tumor front predicts independently for poor prognosis of ESCC. These findings suggest an oncogenic role for Notch1 in SCC and identify the TGFβ–ZEB1–Notch1 axis as potential target for SCC therapy.

Results

EMT and Notch1 activation are features of carcinogen-driven ESCC in vivo. To study SCC initiation and progression in vivo, we treated mice with 4-Nitroquinoline 1-oxide (4NQO), a potent oral-esophageal carcinogen. We combined 4NQO treatment with a cell-lineage tracing experimental system in which murine oral and esophageal epithelial basal cells (keratinocytes) were marked permanently with tdTomato fluorescent protein following tamoxifen (TAM)-induced Cre-mediated recombination in K5CreERT2;R26tdTomatolsl/lsl mice (Fig. 1a), 4NQO-induced lesions showed tdTomato accumulation (Fig. 1b; Supplementary Fig. 1a, b), validating the basal keratinocyte origin of these tumors. Flow cytometry revealed the presence of cells displaying both negative and positive expression of EpCAM (EpCAMpos and EpCAMneg), an epithelial cell surface marker, within the tdTomato-positive (tdTomatopos) fractions of 4NQO-induced ESCC lesions (Supplementary Fig. 1c), suggesting a loss of epithelial characteristics in tumor cells originating from esophageal basal keratinocytes. In cell lineage tracing experiments, tdTomato expression assures that these EpCAMneg cells are not co-existing intratumoral stromal cells (e.g., fibroblasts) which are not labeled with tdTomato via K5CreERT2. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis coupled with fluorescence-activated cell sorting (FACS) demonstrated further upregulation of genes associated with mesenchymal cells (Fig. 1c) including Zeb1, in tdTomatopos,EpCAMpos cells originating from basal esophageal keratinocytes. Consistent with EMT as described in human ESCC, neoplastic lesions displayed E-cadherin loss or mislocalization as well as robust Zeb1 expression at the stromal interface (Fig. 1d). Single-cell-derived three-dimensional (3D) organoids generated from 4NQO-induced dysplastic mucosa and primary tumors recapitulated acquisition of mesenchymal properties as found in 4NQO-induced neoplastic lesions (Fig. 1e). We also included mice with conditional loss or expression of mutant p53 since p53 dysfunction promotes EMT and, potentially, regulation of CSC dynamics.

We next utilized this experimental platform to examine expression of ICN1 (ICN1Val1744), the activated form of Notch1, throughout the natural history of ESCC. In comparison to normal esophageal squamous epithelia, ICN1 expression was increased in dysplastic lesions and remained upregulated in primary and metastatic ESCC (Fig. 1f, g; Supplementary Fig. 1e). Moreover, ICN1 was detected in invasive ESCC cells displaying E-cadherin mislocalization and Zeb1 upregulation (Supplementary Fig. 1d). ICN1 was also expressed highly in neoplastic organoids displaying EMT (Fig. 1e), suggesting a potential role for ICN1 in the acquisition of mesenchymal properties by SCC cells. The tumor suppressor p53 protein can transcriptionally activate Notch1 in response to genotoxic stress. Since 4NQO activates p53 via DNA damage, ICN1 expression in 4NQO-induced early lesions may reflect p53 activation in dysplastic cells. Conversely, Notch1 downregulation may be accounted for by p53 inactivation during disease progression. Therefore, we evaluated the influence of p53 loss upon ICN1 expression in the esophageal epithelium. TAM-induced K5CreERT2-driven p53 loss did not affect ICN1 expression in mice without 4NQO treatment (P = 0.06 by Student’s t-test; n = 3 animals per group; two independent experimental replicates), a finding that recapitulates p53+/− murine epidermis. When p53 was deleted in oral-esophageal keratinocytes then mice were treated with 4NQO, p53 loss did not prevent ICN1 expression in neither normal esophageal epithelium nor 4NQO-induced neoplastic lesions (Fig. 1g; Supplementary Fig. 1e). Mice with p53 deletion did, however, display frequent metastases (Supplementary Fig. 1a). These findings suggest that p53 may be dispensable for Notch1 activation in...
4NQO-induced lesions, albeit essential for overall SCC progression.

**Notch1 promotes ESCC tumorigenicity and expansion of CD44H cells with mesenchymal properties.** To explore further the functional role of Notch1 in ESCC tumorigenicity, we first utilized the extensively characterized human ESCC cell lines TE11 and EN60. Both express ICN1 and form tumors upon xenograft transplantation in immunodeficient mice where doxycycline (DOX)-inducible ectopic ICN1 augmented tumor growth. To assess Notch activity in vivo, we used 8xCSL-GFP, a lentiviral green fluorescent protein (GFP) reporter driven by the minimal SV40 promoter fused to concatemeric CSL-binding sites (Fig. 2a). In TE11 and EN60 xenograft tumors, a subset of turboRFP (tRFP)-expressing tumor cells exhibited CSL-mediated trans-repression, indicating the Notch active population of tRFP-labeled ESCC cells without tRFP represent host-derived stromal cells.

**Representative flow cytometry scatter plots determining 8xCSL-mediated GFP reporter activation, indicating Notch active population of tRFP-labeled ESCC cells in TE11 and EN60 xenograft tumors.** On average, 43.5 ± 0.5% s.d. of EN60 and 11.2 ± 6.5% s.d. of TE11 cells comprised the live GFP	extsuperscript{pos}/tRFP	extsuperscript{pos} fraction across three independent tumors. Constitutively active UBC promoter drives tRFP expression concurrently. Cells without tRFP represent host-derived stromal cells.

**Tumor growth curves in immunodeficient mice carrying TE11 tumors of indicated genotypes.** Upon xenograft transplantation, mice were treated with DOX to induce ICN1 or NOTCH1-directed shRNA. Tumor growth was monitored for indicated time periods. In TE11-ICN1DoxOn, *, *P < 0.01 vs. zeo and DOX (−), **P < 0.05 vs. zeo and DOX (+), n = 6–8 per group. In EN60, *P < 0.05 vs. DOX (−); n = 6 per group. TE11 cells with CRISPR/Cas9-mediated NOTCH1 deletion. Immunoblotting confirms ICN1 loss in NOTCH1-deleted TE11 (N1Δ), but not non-targeted control TE11 (N1WT) cells. Bar graph shows tumor formation rate in immunodeficient mice 7 weeks after xenograft transplantation. *P < 0.05 vs. N1WT, n = 8 per group. Esophageal keratinocytes isolated from 4NQO-induced IEN (dysplasia) lesions or ESCC tumors of Notch1	extsuperscript{loxP/loxP} mice were utilized to generate 3D esophageal organoids in the presence or absence of ex vivo Cre-mediated recombination. In the absence of ex vivo Cre-mediated recombination, qRT-PCR analysis confirms inhibition of Notch1 gene expression upon ex vivo Cre-mediated recombination in ESCC organoids. *P < 0.000 vs. Cre (−), n = 3. Organoid formation rate was evaluated at day 14 post-plating and is represented as relative to Cre (−) for IEN and ESCC. *P < 0.000 vs. Cre (−) IEN, **P < 0.001 vs. Cre (−) ESCC. Line graphs represent mean ± s.e.m. in TE11. At least two independent replicates were performed for all experiments. Repeated measures ANOVA with Tukey’s post hoc test were used for multiple comparisons in TE11, Chi square with Fisher’s exact test was used for percentage comparisons in TE11. Student’s t-test was used for paired data comparisons in TE11.

**Tumor formation rate in immunodeficient mice carrying EN60 tumors of indicated genotypes.** Upon xenograft transplantation, mice were treated with DOX to induce ICN1 or NOTCH1-directed shRNA. Tumor growth was monitored for indicated time periods. In EN60-ICN1DoxOn, *, *P < 0.01 vs. zeo and DOX (−), **P < 0.05 vs. zeo and DOX (+), n = 6–8 per group. In EN60, *P < 0.05 vs. DOX (−); n = 6 per group. En60 cells with CRISPR/Cas9-mediated NOTCH1 deletion. Immunoblotting confirms ICN1 loss in NOTCH1-deleted EN60 (N1Δ), but not non-targeted control EN60 (N1WT) cells. Bar graph shows tumor formation rate in immunodeficient mice 7 weeks after xenograft transplantation. *P < 0.05 vs. N1WT, n = 8 per group. Esophageal keratinocytes isolated from 4NQO-induced IEN (dysplasia) lesions or ESCC tumors of Notch1	extsuperscript{loxP/loxP} mice were utilized to generate 3D esophageal organoids in the presence or absence of ex vivo Cre-mediated recombination. In the absence of ex vivo Cre-mediated recombination, qRT-PCR analysis confirms inhibition of Notch1 gene expression upon ex vivo Cre-mediated recombination in ESCC organoids. *P < 0.000 vs. Cre (−), n = 3. Organoid formation rate was evaluated at day 14 post-plating and is represented as relative to Cre (−) for IEN and ESCC. *P < 0.000 vs. Cre (−) IEN, **P < 0.001 vs. Cre (−) ESCC. Line graphs represent mean ± s.e.m. in EN60. At least two independent replicates were performed for all experiments. Repeated measures ANOVA with Tukey’s post hoc test were used for multiple comparisons in EN60, Chi square with Fisher’s exact test was used for percentage comparisons in EN60. Student’s t-test was used for paired data comparisons in EN60.

**Fig. 2 Notch1 promotes ESCC tumorigenesis.** a) Schematic of 8xCSL-GFP reporter. Notch activation permits GFP reporter expression via concatemeric CSL-binding sites. Constitutively active UBC promoter drives tRFP expression concurrently. Cells without tRFP represent host-derived stromal cells. b) Representative flow cytometry scatter plots determining 8xCSL-mediated GFP reporter activation, indicating Notch active population of tRFP-labeled ESCC cells in TE11 and EN60 xenograft tumors. On average, 43.5 ± 0.5% s.d. of EN60 and 11.2 ± 6.5% s.d. of TE11 cells comprised the live GFP	extsuperscript{pos}/tRFP	extsuperscript{pos} fraction across three independent tumors. c, d) Tumor growth curves in immunodeficient mice carrying TE11 tumors of indicated genotypes. Upon xenograft transplantation, mice were treated with DOX to induce ICN1 or NOTCH1-directed shRNA. Tumor growth was monitored for indicated time periods. In TE11-ICN1DoxOn, *, *P < 0.01 vs. zeo and DOX (−), **P < 0.05 vs. zeo and DOX (+), n = 6–8 per group. In EN60, *P < 0.05 vs. DOX (−); n = 6 per group. TE11 cells with CRISPR/Cas9-mediated NOTCH1 deletion. Immunoblotting confirms ICN1 loss in NOTCH1-deleted TE11 (N1Δ), but not non-targeted control TE11 (N1WT) cells. Bar graph shows tumor formation rate in immunodeficient mice 7 weeks after xenograft transplantation. *P < 0.05 vs. N1WT, n = 8 per group. f) Esophageal keratinocytes isolated from 4NQO-induced IEN (dysplasia) lesions or ESCC tumors of Notch1	extsuperscript{loxP/loxP} mice were utilized to generate 3D esophageal organoids in the presence or absence of ex vivo Cre-mediated recombination. In the absence of ex vivo Cre-mediated recombination, qRT-PCR analysis confirms inhibition of Notch1 gene expression upon ex vivo Cre-mediated recombination in ESCC organoids. *P < 0.000 vs. Cre (−), n = 3. Organoid formation rate was evaluated at day 14 post-plating and is represented as relative to Cre (−) for IEN and ESCC. *P < 0.000 vs. Cre (−) IEN, **P < 0.001 vs. Cre (−) ESCC. Line graphs represent mean ± s.e.m. in TE11. At least two independent replicates were performed for all experiments. Repeated measures ANOVA with Tukey’s post hoc test were used for multiple comparisons in TE11, Chi square with Fisher’s exact test was used for percentage comparisons in TE11. Student’s t-test was used for paired data comparisons in TE11.
EN60 cells are less tumorigenic than TE11 upon xenograft transplantation, displaying 12.5–25% tumor formation rates with 1–4 × 10^6 cells transplanted; however, DOX-induced ectopic ICN1 enhanced EN60 tumor formation rate to 100% (P < 0.01 by Fisher’s exact test; n = 12; two independent experimental replicates). Moreover, the ability of esophageal neoplastic cells to form single-cell-derived 3D organoids was attenuated upon Cre-mediated ex vivo Notch1 deletion in single-cell suspensions prepared from dysplastic lesions or ESCC tumors of 4NQO-treated Notch^loxP/loxP mice (Fig. 2f).

Human SCC tumors comprise CD44L and CD44H cells, the latter exhibiting augmented tumor-initiating capability. CD44H expression is associated with mesenchymal characteristics in transformed oral-esophageal cell lines. Following FACS-purification, early passage CD44H TE11 and EN60 cells maintained enhanced expression of the mesenchymal cell marker CD44H expression is associated with mesenchymal characteristics in transformed oral-esophageal cell lines. Following FACS-purification, early passage CD44H TE11 and EN60 cells maintained enhanced expression of the mesenchymal cell marker.

### Fig. 3

**Notch1 facilitates expansion of CD44H cells with mesenchymal properties.**

- **a** qRT-PCR analysis for indicated genes comparing EN60 intratumoral CD44L and CD44H cells. *P < 0.05 vs. CD44L; ^P < 0.0001 vs. CD44L; n = 3 per group.
- **b** Representative flow cytometry scatter plot determining CD44H cells in EN60 tumors grown for 4 weeks with or without DOX-induced ICN1 (EN60-ICN1^TetOn). Parental tumors were grown in mice without DOX treatment and dissociated for FACS-purification of CD44L and CD44H cells. Purified cells were serially transplanted (10^3 cells per injection site) into recipient mice to monitor tumor formation. Recipient mice were treated with or without DOX. TE11 carried DNMAML1 or zeo (empty vector control). *P < 0.05 for CD44L and DOX (+) vs. CD44L and DOX (–); n = 10-12 per group; ns not significant for CD44L and DOX (+) vs. CD44H (with or without DOX treatment), n = 10 per group; ^P < 0.05 vs. TE11-ICN1^TetOn^zeo CD44L and DOX (+), n = 10 per group.
- **c** Flow cytometry analysis for EpCAM-negative cells in TE11 with Cre-mediated Notch1 deletion (arrowheads). Box denotes area that is magnified in panel below. KC keratinized core of organoids. Scale bars, 20 μm. See Supplementary Table 2 for percentage comparisons in c.

**Fig. 4** Human esophageal cancer cell lines exhibit a mesenchymal plasticity phenotypic signature.

- **a** Relative mRNA level compared with N1WT and TGFβ (–).
- **b** Percent (%) of EpCAM^Neg cells (CD44L/CD44H) in TE11 with or without DOX treatment in the absence (−) or presence (+) of TGFβ. Data are presented as mean ± s.e.m. *P < 0.0001 vs. N1WT and TGFβ (–); #P < 0.0001 vs. N1WT and TGFβ (+); n = 3.
- **c** Multicolor IF for E-cadherin and Zeb1 in representative single-cell-derived organoids from 4NQO-induced ESCC tumors of Notch1^loxp/loxp mice with or without ex vivo Cre-mediated recombination. Zeb1 expression diminished in organoids upon Cre-mediated Notch1 deletion (arrowheads). Box denotes area that is magnified in panel below. KC keratinized core of organoids. Scale bars, 20 μm. See Supplementary Table 2 for percentage comparisons in c.
N-cadherin as compared to their CD44L counterparts in culture (Supplementary Fig. 2a). Additionally, CD44H cells isolated from TE11 and EN60 xenograft tumors exhibited upregulation of ZEB1 and CDH2 (N-cadherin) (Fig. 3a; Supplementary Fig. 2b). As ectopic ICN1 expression in EN60 tumors enhanced robustly intratumoral CD44H cell content (Fig. 3b), we hypothesized that Notch1 contributes to tumor initiation via generation of CD44H cells that have mesenchymal properties. To determine how Notch1 may influence tumor initiation by CD44L and CD44H cells, we performed serial transplantation experiments (Fig. 3c).

We first grew tumors without activating DOX-inducible ICN1. We then purified CD44L and CD44H cells by FACs from primary tumors and 1 × 10^5 cells were serially injected into recipient mice where DOX was given to induce ectopic ICN1 expression. CD44L cells purified from EN60 and TE11 tumors showed low (<30%) spontaneous tumor formation efficiency; however, ICN1 dramatically stimulated tumor initiation by CD44L cells to 80–90%. In TE11 CD44L cells, DNAML1 not only antagonized ICN1-mediated tumorigenicity, but also suppressed spontaneous tumor formation. When purified CD44H cells were highly tumorigenic (80–100%), neither ectopic ICN1 nor DNAML1 affected tumor initiation by CD44H cells, suggesting that established CD44H-mediated tumor initiation is independent of Notch1. To our knowledge, this is the first demonstration that Notch1 may facilitate tumor initiation by converting CD44L cells to highly tumorigenic CD44H cells with mesenchymal traits in vivo.

**TGFβ and Notch1 cooperate to drive EMT in the tumor microenvironment.** The functional role of Notch1 in EMT was suggested as NOTCH1 deletion in cultured TE11 cells attenuated sharply TGFβ-mediated EpCAM^{R8} cell induction (Fig. 3d). Cre-mediated ex vivo Notch1 deletion in 3D ESCC organoids generated from 4NQO-induced Notch1^{loxP/loxP} murine tumors resulted in a diminished expression of Zeb1 (Fig. 3e). Moreover, DOX-induced ectopic ICN1 augmented intratumoral EpCAM^{R8} cell content in TE11 xenograft tumors (Supplementary Fig. 2c). While these data support a role for Notch1 in promotion of ESCC tumor cells with attributes of EMT, EpCAM^{R8} cells were rare in cultured TE11 cells and ectopic ICN1 expression alone had no influence upon this cell population (Supplementary Fig. 2c), indicating that Notch1 activation alone may not be sufficient to drive EMT.

In the tumor microenvironment, Notch signaling may be modulated by other transcription factors such as HIF1α and...
SMAD345 via physical interactions with ICN1. We suspected that TGFβ influences Notch1-mediated tumor promotion and EMT since the TGFβ target gene PAI1 was upregulated in CD44H cells in tumors (Fig. 3a; Supplementary Fig. 2b). In agreement, an anti-TGFβ blocking therapeutic monoclonal antibody 1D1146 severely impaired growth of TE11 xenograft tumors as well as OCTT2 head and neck SCC patient-derived xenografts (PDXs) (Fig. 4a). In the context of 4NQO-mediated carcinogenesis, the percentage of esophageal mucosa occupied by neoplastic lesions classified as intraepithelial neoplasia (IEN) or ESCC was diminished upon treatment with 1D11 (26.0% ± 8.1 s.e.m.; n = 5) as compared to isotype control (56.3% ± 14.2 s.e.m.; n = 4; P = 0.09 by Student’s t-test; two independent experimental replicates). Administration of 1D11 to 4NQO-treated animals further...
attenuated ICN1 expression as well as morphological evidence of EMT in esophageal epithelium (Fig. 4b, c). Taken together, these data indicate that TGFβ functions as a critical positive effector of Notch1 and EMT in the context of the tumor microenvironment.

To dissect further the mechanistic role of Notch1 in the generation and maintenance of ESCC tumor cells with mesenchymal features, we utilized the genetically engineered transformed esophageal cell line EPC2T (EPC2-hTERT-EGFR-p53-R175H-cyclin D1), comprising discrete CD44L and CD44H subpopulations with epithelial and mesenchymal traits, respectively1 (Supplementary Fig. 3a). Under basal conditions, Notch activity, as measured using 8xCSL-GFP reporter (Fig. 2a), was highest within a subset of intermediate transitioning cells (designated as CD44T) as compared to CD44L or CD44H cells (Fig. 5a). In TGFβ-mediated EMT with a resulting increase in CD44T and CD44H cells, Notch activity was augmented further in the CD44T subpopulation (Fig. 5a), indicating that Notch activity may be augmented transiently during the CD44L-to-CD44H transition. Induction of CD44H cells may be accounted for by expansion of pre-existing CD44H cells as well as conversion from CD44L cells. Following FACS purification, CD44H cells failed to display CD44L repopulation even after extended passage (Supplementary Fig. 3b). CD44L cells that were cultured in the absence of TGFβ underwent expansion while permitting minimal CD44H cell repopulation within 5 weeks (four passages) (Supplementary Fig. 3b); however, purified CD44L cells robustly gave rise to CD44H cells upon TGFβ stimulation (Supplementary Fig. 3c; Fig. 5b). A requirement for TGFβ in CD44H cell induction was suggested as pharmacological inhibition of TGFβ receptor-mediated signaling suppressed spontaneous conversion of purified CD44L cells to CD44H cells (Supplementary Fig. 3d). A permissive role for Notch1 in CD44H cell generation via TGFβ-induced EMT was implicated further as a γ-secretase inhibitor (GSI), DNMAML1 or NOTCH1-directed shRNA each individually attenuated TGFβ-mediated CD44H cell expansion from purified CD44L cells (Supplementary Fig. 3c; Fig. 5b). Ectopic ICN1 augmented CD44H cell expansion from TGFβ-stimulated purified CD44L cells where ICN1 failed to influence spontaneous CD44H cell expansion in the absence of TGFβ (Fig. 5c). Taken together, these data indicate that Notch1 activation is required, but not sufficient, for EMT-mediated CD44H cell induction in EPC2T cells.

Transcriptional repression of Notch3 via Zeb1 permits Notch1-mediated EMT. As our findings indicate a role for Notch in EMT, we next sought to determine how Notch1 regulates cell fate in squamous epithelia. The 8xCSL-GFP reporter activity was unaffected in CD44T and CD44H cells upon calcium-mediated squamous-cell differentiation (Fig. 5a). In CD44L cells, which displayed differentiation in organotypic 3D culture (OTC) when compared to invasive CD44H cells (Fig. 5d), calcium treatment enhanced Notch activity in a subset of cells (Fig. 5a). Thus, local environmental cues may impact Notch signaling to direct cell fate determination. In agreement with this notion, pharmacological inhibition of TGFβ signaling in purified CD44H cells induced CD44L cells with concurrent downregulation of EMT markers and NOTCH3 upregulation (Fig. 5e, f). A trend toward increased Notch3 expression was also detected in peeled murine esophageal epithelia with 4NQO-induced neoplastic lesions following treatment with anti-TGFβ-blocking antibody ID11 (Supplementary Fig. 3e). Gene array analysis in EPC2T cells revealed further that ectopic ICN1 expression induced a gene expression pattern compatible with squamous-cell differentiation. However, TGFβ treatment in the context of ICN1-overexpression triggered a robust shift toward an EMT-associated gene expression signature (Fig. 5g; Supplementary Fig. 3f; GSE37994, GSE37993). qRT-PCR analysis confirmed that ICN1 and TGFβ cooperate to induce expression of EMT regulators, including ZEB1 and SNAIL, while TGFβ limited ICN1-mediated expression of NOTCH3 as well as IVL, the latter a marker of terminal differentiation (Fig. 5h).

Although the lack of discontinuous CD44L and CD44H cell populations in EN60 and TE11 prevented their isolation for long-term cell culture analyses, the role of Notch and TGFβ signaling in induction of CD44H cells and regulation of squamous-cell differentiation was recapitulated in TE11 cells (Supplementary Fig. 3g–i).

We investigated next the mechanistic role of Notch3 in EMT in the context of ESCC. TE11 serial transplantation experiments revealed that DOX-induced ectopic ICN3 suppressed tumor initiation by CD44L cells (Fig. 6a). In EPC2T cells, ectopic ICN3 induced IVL in the presence or absence of TGFβ stimulation (Supplementary Fig. 4a, b). Moreover, ectopic ICN3 abrogated TGFβ-mediated CD44H cell expansion in EPC2T cells (Supplementary Fig. 4c), while NOTCH3 knockdown was sufficient to promote CD44H cell expansion (Fig. 6b) coupled with decreased differentiation and increased EMT characteristics (Fig. 6c) in the absence of TGFβ stimulation. These findings indicate that Notch3, unlike Notch1, may limit EMT so as to permit squamous-cell differentiation. To define the mechanism through which Notch3 expression is suppressed during EMT, we analyzed the NOTCH3 locus by the ECR browser47. This analysis predicts two conserved ZEB-binding sites in the NOTCH3 second intron (N3Int2) adjacent to the CSL-binding sites (Fig. 6d) that is occupied by ICN1 during squamous-cell differentiation4. Hypothesizing that ZEB transcription factors repress Notch3, we evaluated the influence of ectopic ZEB1 or
**Fig. 6** ZEB1 represses NOTCH3, facilitating EMT and tumor initiation. a Serial transplantation experiments with DOX-inducible ICN3I-expressing TE11 (TE11-ICN3[Teo]). Tumor formation rates were determined as in Fig. 2e. *P < 0.05 vs. CD44L and DOX (−); n = 10. b Representative flow cytometry scatter plots of EPC2T cells with NOTCH3-targeted or non-silencing (NS) control shRNA. NOTCH3 shRNA increased CD44H cells (lower right quadrant) to 29.8 ± 0.4% s.d. as compared to 0.4 ± 0.1% s.d. in NS control (P = 0.0001 by Student’s t-test, n = 3). c qRT-PCR analysis for indicated genes comparing EPC2T cells with or without NOTCH3 knockdown. mRNA level for each gene in NS control cells was set to 1. *P < 0.05 vs. CD44L and IgG and TGFβ (−); #P < 0.0005 NS vs. TGFβ (−); n = 3. d) Schematic of NOTCH3 second intron (N3In2) region and ChIP PCR primers in f and Supplementary Fig. 4e. Primers amplify the region lacking ZEB1 or CSL-binding sites. e Transfection assays for pGL3-N3In2-luc reporter activity with or without ectopic ZEB1 or ZEB2 expression. bla empty vector control for ZEBs. *P < 0.05 vs. bla and pGL3-luc (empty reporter); #P < 0.05 ns vs. bla and pGL3-N3In2-luc; n = 4. f ChiP assays for ZEB1 binding to N3In2 region in purified CD44L and CD44H cells. CD44L cells were stimulated with TGFβ for 14 days to induce CD44H cells. *P < 0.0005 vs. CD44L and IgG and TGFβ (−); #P < 0.0005 vs. CD44H and IgG and TGFβ (−); *P < 0.05 vs. CD44L and anti-ZEB1 and TGFβ (−); ns not significant vs. IgG and TGFβ (−); n = 3. g) Representative flow cytometry scatter plots of EPC2T cells with ZEB1-targeted or NS control shRNA. *P < 0.05 vs. NS and TGFβ (−); #P < 0.0001 NS and TGFβ (−); ns not significant vs. NS and TGFβ (−); n = 3. In b, g, flow cytometry was done 7 days following lentivirus infection. All bar diagrams represent mean ± s.d. At least three independent replicates were performed for all experiments. Fisher’s exact test was used for percentage comparisons in a. Student’s t-test was used for paired data comparisons in b, c, f. ANOVA with Tukey’s post hoc test was used for multiple comparisons in e, g.

**Supplementary Fig. 4** a) Two introns and TGFβ (−) region in puri... (empty reporter); #P < 0.0005 vs. CD44H and IgG and TGFβ (−); ns not significant vs. IgG and TGFβ (−); n = 3. g) Representative flow cytometry scatter plots of EPC2T cells with ZEB1-targeted or NS control shRNA. *P < 0.05 vs. NS and TGFβ (−); #P < 0.0001 NS and TGFβ (−); ns not significant vs. NS and TGFβ (−); n = 3. In b, g, flow cytometry was done 7 days following lentivirus infection. All bar diagrams represent mean ± s.d. At least three independent replicates were performed for all experiments. Fisher’s exact test was used for percentage comparisons in a. Student’s t-test was used for paired data comparisons in b, c, f. ANOVA with Tukey’s post hoc test was used for multiple comparisons in e, g.

**Supplementary Fig. 5** a) Two introns and TGFβ (−) region in puri... (empty reporter); #P < 0.0005 vs. CD44H and IgG and TGFβ (−); ns not significant vs. IgG and TGFβ (−); n = 3. g) Representative flow cytometry scatter plots of EPC2T cells with ZEB1-targeted or NS control shRNA. *P < 0.05 vs. NS and TGFβ (−); #P < 0.0001 NS and TGFβ (−); ns not significant vs. NS and TGFβ (−); n = 3. In b, g, flow cytometry was done 7 days following lentivirus infection. All bar diagrams represent mean ± s.d. At least three independent replicates were performed for all experiments. Fisher’s exact test was used for percentage comparisons in a. Student’s t-test was used for paired data comparisons in b, c, f. ANOVA with Tukey’s post hoc test was used for multiple comparisons in e, g.

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ZEB2 expression upon N3Int2-modulated transcriptional activity. ZEB1 specifically suppressed basal pGL3-N3Int2-luc reporter activity and also abrogated reporter activation via DOX-induced ectopic ICN1 (Fig. 6e; Supplementary Fig. 4d). Chromatin immunoprecipitation (ChIP) analysis revealed enrichment of ZEB1 binding to the N3Int2 region in purified CD44L cells with TGFβ stimulation and purified CD44H cells without TGFβ stimulation (Fig. 6f). TGFβ did not prevent ICN1 from binding to the N3Int2 region (Supplementary Fig. 4e), suggesting that ZEB1 binding may predominate over ICN1 to repress NOTCH3 transcription. ZEB1-directed shRNA attenuated TGFβ-induced CD44H cell expansion, further implicating ZEB1 as a critical positive regulator in EMT-mediated generation of CD44H cells (Fig. 6g).

**Fig. 7** NOTCH1 activation and ZEB1 expression in invasive ESCC predicts poor prognosis. 

- **a**, **b** Representative IHC images for ICN1 and ZEB1 in ESCC cells in a deep invasive tumor nest and ESCC cells invading into lymphatic vessels. 
- **a** ESCC #55; **b** ESCC #62 in Supplementary Data 1. 
- **c** Representative IF image for ICN1 and CD44 in invasive ESCC cells. Scale bars, 50 μm in **a**–**c**. 
- **d** Survival curve for 185 post-surgical ESCC patients with or without ICN1 expression at the tumor invasive front. 
- **e** Model of the role of Notch1 in EMT in ESCC. Notch1 activation promotes tumorigenicity and heterogeneity in SCC via EMT. Notch1 drives squamous-cell differentiation by inducing Notch3 in normal squamous epithelia as well as SCC cells. Notch3 limits EMT. In response to TGFβ from the tumor microenvironment (e.g., cancer-associated fibroblasts and inflammatory cells), however, transcriptional repression of Notch3 by ZEB1 permits Notch1-mediated induction of CD44H cells via EMT. Notch activation may result in the generation and maintenance of CD44H cells possessing mesenchymal properties and enhanced malignant potential. EMT allows neoplastic cells to cope with stress during carcinogenesis and disease progression (e.g., genotoxic stress induced by 4NQO). CD44H cells produce pro-tumorigenic cytokines (e.g., IL-6) and tissue remodeling factors (e.g., MMP13, LOX, and POSTN) (Supplementary Fig. 3e).
Notch1 at SCC invasive fronts predicts poor patient prognosis.

To demonstrate further the importance of Notch1 activity in pathogenesis of human SCCs, we analyzed surgically procured tissue samples (Supplementary Data 1) by immunohistochemistry (IHC) with two independent NOTCH1 antibodies, both detecting nuclear NOTCH1 at a 95.24% concordance rate (κ = 0.664, 95% CI: 0.008−1.0, Kappa statistic, n = 21, Supplementary Fig. 5).

ICN1 (ICN1Val74) was readily detectable in normal esophageal squamous epithelia (n = 102) as described4 and was upregulated in superficial precancerous as well as early invasive ESCC lesions (Supplementary Fig. 6). Within tumors invading into submucosa or muscularis propria, most SCC cells (>80%) did not express ICN1 with or without NOTCH1 mutations47, 48; however, a small subset of SCC cells expressed ICN1 in the invasive tumor front (35.3% for HNSCC, n = 17; 37.0% for ESCC, n = 227) (Fig. 7a; Supplementary Fig. 7).

Accompanied by desmoplastic stroma, ICN1-positive invasive SCC often displayed spindle-shaped cell morphology and ZEB1 co-localization (17.1%, n = 174) (Fig. 7a; Supplementary Fig. 7) with ICN1 and ZEB1 expression being correlated (ρ = 0.31, P < 0.0001, Pearson correlation). When stratified by co-localization status, expression of ICN1 and ZEB1 was relatively uncorrelated for subjects lacking co-localization (ρ = −0.1, P = 0.2, Pearson correlation), while expression was correlated more strongly for those showing co-localization (ρ = 0.37, P < 0.05, Pearson correlation, n = 30). Additionally, these cells were often found invading into lymphatic vessels (Fig. 7b).

NOTCH1 expression was low, if not absent, in SCC cells with concurrent ICN1 and ZEB1 expression (Supplementary Fig. 7). Moreover, ICN1 was expressed in a subset of ESCC cells with elevated CD44 expression (Fig. 7c). Evaluation of ICN1 in relation to clinicopathological data revealed that ICN1 was significantly associated with increased lymph node and distant metastases and advanced disease stages (Supplementary Table 1).

Finally, analyses of clinical databases revealed that ICN1 expression at the invasive tumor front predicts independently for poor prognosis (Fig. 7d; Supplementary Table 2).

Discussion

The current study highlights an oncogenic role for Notch1 in SCC via EMT-mediated induction of CD44H cells with enhanced malignant potential (Fig. 7e). We demonstrate that Notch1 and Notch3 may have opposing functions to allow expansion of CD44H cells both in vitro and in vivo. Moreover, we find that TGFβ guides Notch1 to drive EMT via a previously undescribed robust shift in the spectrum of Notch1 target genes including Notch3, which is essential in squamous-cell differentiation and senescence12, 48. Given the microRNA-mediated crosstalk between Notch signaling and ZEB133, 49, our findings indicate that direct repression of NOTCH3 by ZEB1 is a novel mechanism through which ZEB1 may influence Notch1-mediated cell fate determination. Activated Notch1 (i.e., ICN1) interacts with TGFβ downstream effector SMAD345, ZEB1 also binds physically to SMAD3 to enhance TGFβ-mediated transcription44. Additionally, ZEB1 promotes tumorigenicity by repressing stemness-inhibiting microRNAs in pancreas cancer50, where ZEB1 induction and EMT have been implicated as the earliest event preceding tumor formation in cell-lineage tracing experiments47, 48.

Taken together, these preclinical studies suggest that Notch1 and ZEB1 may cooperate to promote carcinogenesis and SCC progression via TGFβ-mediated EMT and offer the TGFβ–ZEB1–Notch1 axis as potential therapeutic target in SCC.

Despite a well-established role for the Notch pathway in regulation of varied cell fates in a tissue-dependent and context-dependent manner, the molecular mechanisms governing Notch-mediated cell fate determination have yet to be fully elucidated. In non-transformed esophageal epithelium, Notch1-dependent transcriptional upregulation of Notch3 mediates squamous-cell differentiation as shown by us4. In the current study, we find Notch1 facilitates EMT-mediated expansion of CD44H cells with enhanced malignant potential. This raises the question of how Notch1 may act as a signal to induce both differentiation and dedifferentiation (i.e., EMT) in squamous epithelium. One potential explanation for this dichotomy is the influence of tissue microenvironment upon Notch-mediated cell fate determination. Indeed, microarray gene expression analysis in ICN1-overexpressing transformed esophageal keratinocytes indicates that TGFβ induces a dramatic shift in the spectrum of Notch1 target genes from a profile enriched for genes involved in squamous-cell differentiation to one consistent with EMT. Notch1 may also facilitate senescence in response to TGFβ in esophageal keratinocytes with intact cell cycle checkpoint functions45.

The complexity in the role of Notch signaling in tumor biology is echoed by that of TGFβ as intratumoral cancer cell heterogeneity represents cancer cells that can respond to TGFβ and those cannot, the latter emerging during disease progression32. Thus, like TGFβ, Notch1 may have differential roles in cancer initiation and development. Besides TGFβ, hypoxia and inflammation may activate Notch1 to promote EMT in the tumor microenvironment via transcriptional factors such as HIF1α17 and NF-κB33, respectively. Potential influence of these factors upon Notch1-mediated cell fates warrants further investigation in SCC as well as squamous epithelia under eosinophilic esophagitis where EMT and Notch3 downregulation are implicated (P.M.C. and H.N., unpublished observation).

While our own published findings and those of others have implicated Notch1 as a tumor promoter in SCCs12, 54–56, the current study is the first to demonstrate in vivo that Notch1 facilitates tumor initiation by converting CD44L cells to highly tumorigenic CD44H cells with mesenchymal traits, complementing the earlier studies implicating EMT and ZEB1 in the CD44L→CD44H transition60, 61. Tumor initiation by CD44H cells may be dispensable for their maintenance and tumor initiation by CD44H
cells. Such a premise is corroborated by ICN1 expression localized to tumor invasive fronts, but not in the majority of expanding ESCC cells in situ. Additionally, we have developed an esophageal 3D organoid platform that recapitulates esophageal tissue architecture ex vivo and can be utilized to study SCC and ESCC tumors display morphological characteristics of neoplastic epithelium, including nuclear atypia, perturbed squamous-cell differentiation and evidence of EMT, which are maintained upon organoid passing (K.A.W., P.M.C., and H.N., unpublished observation). We have also successfully performed ex vivo Cre-mediated recombination in 3D organoids generated from neoplastic esophageal keratinocytes of 4NQO-treated Notch1loxPloxP mice, demonstrating that Notch1 is required for organoid formation and EMT-like features in necrotic 3D organoids (Figs. 2f, 3e). Notch1 deletion in the murine skin promotes SCC development in a non-cell autonomous fashion due to inflammation associated with epidermal barrier defects, limiting the assessment of the cell-autonomous oncogenic role of Notch1 in vivo. The 3D organoid system provides insights about cell-autonomous oncogenic functions of Notch1 in the absence of inflammatory milieu ex vivo, complementing in vivo experiments. The 4NQO model requires a long-term 4NQO administration (16 weeks) where tumors arise 6–9 weeks after 4NQO withdrawal during the observation period; however, it is not precisely known when malignant transformation occurs in this model. The single-cell-derived 3D organoids have a potential to detect neoplastic changes in a more sensitive manner than conventional morphological tissue assessment. Such a study is underway. Once an appropriate window of 4NQO-induced malignant transformation is determined, our cell-lineage traceable mice can be utilized for genetic ablation of Notch1 following 4NQO administration to elucidate how Notch1 may exert its oncogenic role by promoting EMT and tumor initiation and/or progression in vivo. Alternatively, such mice may be treated with Notch1-specific antagonist antibody or anti-Notch3 agonistic antibody, the latter can be used for targeted activation of Notch3. While these findings identify 3D neoplastic organoids as sustainable resource for functional and mechanistic investigations into the biology of ESCC, it remains to be determined how faithfully organoid genetics and biology mimic that of the tissue from which they are derived upon extended ex vivo culture. Ongoing studies include procuring of a bank of human patient-derived ESCC 3D organoids that may be utilized as an experimental platform for discovery and validation of novel translational applications for prognosis and therapy in the setting of personalized medicine.

Taken together, the current study supports a tumor-promoting role of Notch1 in SCC while also providing novel mechanistic insight into how the local tissue microenvironment may influence Notch-mediated cell fate determination.

**Methods**

**Patients and tissue samples.** Surgically removed tissue samples (Supplementary Data 1) were described previously68 or newly procured at Kagoshima University Hospital in accordance with Institutional Review Board standards and guidelines. Informed consent was obtained from all human subjects. Most samples were available as tissue microarrays (TMA)s67,68 containing primary ESCC (n = 171), carcinoma in situ (CIS; n = 9), IEN (n = 7), and normal mucosa (n = 114). Samples with NOTCH1 mutations (n = 4; E753*, D409Y, R1279D, and D1457G) and wild-type NOTCH1 (n = 15) were identified by DNA sequencing (SRP072949). Given the limitation of TMA to assess intratumoral cancer cell heterogeneity, we examined whole paraffin blocks (n = 244) by Hematoxylin and Eosin (H&E) staining and IHC, as described below, following preliminary analysis with TMA. Survival analysis was done on IHC data for 185 ESCC patients who did not receive chemotherapy or radiation therapy prior to surgery.

**Esophageal epithelial cell-lineage traceable mice and 4NQO treatment.** The KSCERBT2 transgenic mouse strain was intercrossed with R26tdTomatofl/fl (Jackson Laboratory, Bar Harbor, ME) carrying the homozygous Rosa26 locus with knocked-in tdTomato fluorescent protein as a reporter under the loxP-stop-loxP sequence. The resulting KSCERBT2;R26tdTomatofl/fl mice were crossed with K5CreERT2;R26loxstoploxP mice to recombine the tdTomato gene and generate K5CreERT2;R26loxstoploxP;R26loxstoploxP mice, which were further crossed to generate K5CreERT2;R26loxstoploxP;p53fl/wt mice. The K5CreERT2;R26loxstoploxP;p53fl/wt mice were treated with 4NQO (0.20% in drinking water) for 10 weeks to induce esophageal tumors. The tumors were collected under the sternum after sacrifice and paraffin-embedded for morphological analyses. The other half of esophagi and tumors was dissociated as described below, following preliminary analysis with TMA. The L2Cre transgenic mouse strain was intercrossed with p53flR26loxR26lox mice to generate L2Cre;p53flR26loxR26lox mice. We administered TAM (Sigma–Aldrich, St. Louis, MO; 0.25 mg/kg body weight) via oral gavage to 3–4 month-old KSCERBT2;R26tdTomatofl/fl and KSCERBT2;R26loxstoploxP;R26loxstoploxP littermates 2 weeks before starting 4-Nitroquinoline N-oxide (4NQO) (Sigma–Aldrich) treatment. Mice received 100 µg/ml 4NQO in 2% propylene glycol (MP Biomedicals, Solon, OH) in drinking water for 16 weeks (or longer) and followed up for 8–10 weeks after 4NQO withdrawal as described73. To inhibit TGFβ1 receptor-mediated signaling, mice were treated for 2 weeks with either anti-TGFβ antibody 1D11 (intra-peritoneally), neutralizing all three isoforms of TGFβ (a gift of Dr. Singhal, University of Pennsylvania; 3 mg/kg, three times per week) or control IgG (intra-peritoneally) with injections initiated 4 weeks following 4NQO withdrawal at the time of sacrifice, one half of the dissected esophagus or a tumor (if visible macroscopically) from each mouse was fixed in 4% paraformaldehyde and paraffin-embedded for morphological analyses. The other half of esophagi and tumors was dissociated as described previously74 and cell suspensions were subjected to flow cytometry and FACs and organoid formation assays. All experiments were done under approved protocols from the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC). Sample size for groups were projected based on data from pilot studies. Animals were only excluded from analyses in event of death from procedure-related causes (e.g., death following oral gavage) that were unrelated to experimental differences between groups. Animals were randomized to treatment groups with consideration given to representation of both sexes. Investigators were informed of groups during treatment phase of experiments. Upon processing, tissues were given a unique identifier to blind investigators during outcomes and analysis assessments.

** Xenograft transplantation experiments.** Xenograft transplantation experiments were done as described11,12. In brief, 1–3 x 106 cells were suspended in 50% Matrigel and implanted subcutaneously into the dorsal of female athymic nu/nu mice (4–6 weeks old; Charles River Breeding Laboratories). PDX tumor transplantation was performed as previously described with fragments of passaged PDX tumors implanted under the dorsal skin of NOD/SCID/IL2 receptor γ-chain-deficient (NSG) mice13. Tumor growth was monitored and mice were sacrificed when tumors reached 10–15 mm diameter. After inoculation based on flow cytometry and histological analyses. One half of each tumor was fixed in formalin and embedded in paraffin blocks. The other half of each tumor was enzymatically dissociated and subjected to single-cell-derived 3D organoids as a reporter under the loxP-stop-loxP sequence. The resulting KSCERBT2;R26loxstoploxP;R26loxstoploxP mice were treated with 4NQO (0.20% in drinking water) for 10 weeks to induce esophageal tumors. The tumors were collected under the sternum after sacrifice and paraffin-embedded for morphological analyses. The other half of esophagi and tumors was dissociated as described below, following preliminary analysis with TMA. The L2Cre transgenic mouse strain was intercrossed with p53flR26loxR26lox mice to generate L2Cre;p53flR26loxR26lox mice. We administered TAM (Sigma–Aldrich, St. Louis, MO; 0.25 mg/kg body weight) via oral gavage to 3–4 month-old KSCERBT2;R26tdTomatofl/fl and KSCERBT2;R26loxstoploxP;R26loxstoploxP littermates 2 weeks before starting 4-Nitroquinoline N-oxide (4NQO) (Sigma–Aldrich) treatment. Mice received 100 µg/ml 4NQO in 2% propylene glycol (MP Biomedicals, Solon, OH) in drinking water for 16 weeks (or longer) and followed up for 8–10 weeks after 4NQO withdrawal as described73. To inhibit TGFβ1 receptor-mediated signaling, mice were treated for 2 weeks with either anti-TGFβ antibody 1D11 (intra-peritoneally), neutralizing all three isoforms of TGFβ (a gift of Dr. Singhal, University of Pennsylvania; 3 mg/kg, three times per week) or control IgG (intra-peritoneally) with injections initiated 4 weeks following 4NQO withdrawal at the time of sacrifice, one half of the dissected esophagus or a tumor (if visible macroscopically) from each mouse was fixed in 4% paraformaldehyde and paraffin-embedded for morphological analyses. The other half of esophagi and tumors was dissociated as described previously74 and cell suspensions were subjected to flow cytometry and FACs and organoid formation assays. All experiments were done under approved protocols from the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC). Sample size for groups were projected based on data from pilot studies. Animals were only excluded from analyses in event of death from procedure-related causes (e.g., death following oral gavage) that were unrelated to experimental differences between groups. Animals were randomized to treatment groups with consideration given to representation of both sexes. Investigators were informed of groups during treatment phase of experiments. Upon processing, tissues were given a unique identifier to blind investigators during outcomes and analysis assessments.

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Experiment. Animals were only excluded from analyses in event of death from procedure-related causes (e.g., sepsis) that were unrelated to experimental differences between groups. Animals were randomized by cage upon arrival. Investigators were informed of groups during treatment phase of experiments. Upon processing, tumors were given a unique identifier to blind investigators during analyses and outcome assessments.

DNA constructs. A lentiviral vector PTRIP expressing DOX-inducible shRNA directed against human NOTCH1 (Notch1-A; V2LHS_149557 and Notch1-B; V2LHS_671133) and PTRIP-Ns carrying a non-silencing scrambled sequence (RHS346) (GE Dharmaco, Lafayette, CO) were purchased. Other shRNA sequences in pGIPZ (GE Dharmaco) targeting NOTCH1 (Notch1-A; V2LHS_222974 and Notch1-B; V2LHS_93017), ZEB1 (ZEB1-A; V2LHS_116663 and ZEB1-B; V2LHS_116659), and a non-silencing scrambled sequence (RHS3448) were used as described previously11, 12, 19. To generate a lentiviral vector pTR-pRFP expressing constitutively turbo RFP (pRFP) under ubiquitin C (UBC) promoter, a DOX-inducible gene expression vector pTRIPZ-MCS12 was modified by removal of BamHI and XhoI sites and a subsequent insertion of new multiple cloning sites (MCS) comprising 5'-AgaCTGAAAGCTGAAGCTGATC-3' and 5'-AGGCGCGCCTGATCATGGC-3', respectively. We then amplified an open reading frame (ORF) for turbo RFP (pRFP) by PCR using pTRIPZ-NS (RHS4743, GE Dharmaco) as a template with primers 5'-AGCGCTAGCGTCCACCATGAGCGAGCTGATC-3' and 5'-TTATCTGTGCCAGTCCAGTTTGCTAGG-3'. Using subsequent analysis and outcome assessments.

ex vivo esophageal organoid 3D culture. Esophageal keratinocytes were isolated from vehicle-treated or 4NQO-treated KCSCreERT2:R26tdTomatoLox/Lox or Notch1CreERT2 (Jackson Laboratories) mice under an IACUC-approved protocol as described previously66, 84, 85. Using 24-well plates, 5000 cells were seeded per well in 50 µl Matrigel. After solidification, 500 µl of DMEM/F12 supplemented with 1x Glutamax, 1x HEPES, 1x N2 Supplement, 1x B27 Supplement, 0.1 mM N-acetyl-L-cysteine (Sigma-Aldrich), 50 ng/ml mouse recombinant EGF (R&D Systems), 2%/0% N-glycosylated conditioned media and 10 µM 2Y7632 (Tocris Biosciences, Bristol, UK) were added and replenished every other day. For ex vivo recombination, organoids were cultured in the presence of Adenovirus vector containing Cre recombinase and GFP (University of Iowa Gene Transfer Vector Core). Adenovirus vector containing GFP alone was used as a control. Adenovirus vectors were used at 1:500 at the time of organoid plating. Organoid formation rate was calculated as the percentage of the number of organoids formed at day 7 per total number of cells seeded at day 0. After 14 days organoids were recovered by digesting Matrigel with Dispase I (BD Biosciences, San Jose, CA; 1 U/ml) and fixed overnight in 40% paraformaldehyde. Specimens were embedded in 2% Bacto-Agar: 2.5% gelatin prior to paraffin embedding.

RNA isolation, cdNA synthesis, qRT-PCR, and microarray analyses. RNA isolation, cdNA synthesis, and qRT-PCR were done using StepOnePlus™ Real-Time PCR System (Applied Biosystems) with TaqMan® Gene Expression Assays (Assays-on-Demand™; Applied Biosystems) for NOTCH1 (Mm00435292_m1), BCL2L11 (Mm00433521_m1), NOTCH3 (Hs01126606_m1), CARKL (Mm00435270_m1), and SYBR® Green PCR for human ACTB (β-Actin) as well as 5'-TACAAGCGAGCTGCATCCATCTTGCCGTTGTC-3' and 5'-ATCCGGGAGTCTCAAGGGGTTG-3', hybridizing with 5'-GACTGCAGGCATTGCTGCAGACTG-3' and 5'-GACGCCGAGCCATTGCTGCAGACTG-3', respectively. We then amplified an open reading frame (ORF) for turbo RFP (pRFP) by PCR using pTRIPZ-NS (RHS4743, GE Dharmaco) as a template with primers 5'-AGCGCTAGCGTCCACCATGAGCGAGCTGATC-3' and 5'-TTATCTGTGCCAGTCCAGTTTGCTAGG-3'. Using subsequent analysis and outcome assessments.
Flow cytometry and FACS. Flow cytometry and FACS were performed as described previously. FACSCalibur or LSRII (BD Biosciences, San Jose, CA) and FlowJo (Tree Star, Ashland, OR) were used for flow cytometry. FACS Vantage SE and FACS Aria II (BD Biosciences) were used to sort purified CD44+CD24low–/– cells (CD44H) and CD44low–/–CD24hi CD44L from EP2C, TE1, and EN6 cells, and/or xenograft tumors. Cells were suspended in Hank’s balanced salt solution (Invitrogen) containing 1% BSA (Sigma-Aldrich) and stained with the following antibodies on ice for 30 min: human PE/Cy7-anti-CD24 clone G08 (1:50; 118.217, BioLegend), 4′-diamidino-2-phenylindole (DAPI; 2 μg/ml; Invitrogen) was used to assess viability. CD44T cells were defined as CD44hig, CD24low. To purify CD44L and CD44H cells from TE11 and EN6 xenograft tumors, cells were minced into 1 mm³ pieces and incubated in Dulbecco’s Modified Eagle Medium (DMEM; Invitrogen) containing 1 mg/ml collagenase I (Sigma-Aldrich) at 37 °C for 90 min. Following centrifugation, residual tissue pieces were digested in 0.05% trypsin-EDTA (Invitrogen) at 37 °C for 30 min and then with 1 U/ml Dispase (BD Biosciences) and 100 μg/ml DNase I (#1104159001, Roche) at 37 °C for 10 min. Dissociated tumor cells were filtered with a 40 μm cell strainer (BD Biosciences) and washed prior to incubation with a panel of antibody or isotype-paired control cells were distinguished via host-derived stromal cells by iRFP expression detected concurrently. In 8XGFP reporter assay, iRFP positivity was used to identify human cells while GFP flow cytometry was monitored to evaluate Notch activity. In tissue from iNQO-treated mice and controls, tdTomato expression was used to isolate epithelial cells that had undergone KSCreERT2-mediated recombinational regulation of TAD treatment. Following digestion, epithelial cells were prepared as described previously. For ZEB1, the following antibodies were used for detection: mouse anti-ICN1V1744 (2421; Cell Signaling; 1:100), both detecting cytoplasmic Notch1 protein, polyclonal anti-ICN1V1744 (ab27526 to detect nuclear Notch1 expression (Supplementary Fig.5). The ab27526 positivity was used to identify human cells while GFP expression was monitored to evaluate Notch activity. In tissue from 4NQO-treated mice and controls, tdTomato positivity was used to identify human cells while GFP expression was monitored to evaluate Notch activity. In tissue from 4NQO-treated mice and controls, tdTomato positivity was used to identify human cells while GFP expression was monitored to evaluate Notch activity.

**Chip assay.** 1 × 10⁶ cells were treated with 1% formaldehyde for 10 min at 37 °C and quenched with 0.125 M glycine for 5 min at room temperature. Cross-linked chromatin was sheared into ∼500 bp by DNA fragments with Branson Sonifier 250 (Branson, Danbury, CT) and subjected to immunoprecipitation with 2 μg/10⁶ cells with antibody for ZEB1 (sc10572, Santa Cruz), NOTCH1 (sc6014-R, Santa Cruz) or both goat IgG (sc208, Santa Cruz) or rabbit IgG (sc207, Santa Cruz) as negative controls. DNA was purified by QIAquick PCR purification kit (QIAGEN, Valencia, CA) and analyzed by real-time PCR using StepOnePlus™ Real-Time PCR System (Applied Biosystems, Carlsbad, CA). The following primers were used for real-time qPCR: 5′-CCCAACAGCCAACATCGGAGG-3′ and 5′-CCCGGCTCTGG AATAGCTGCG-3′ for ZEB1-binding sites at the 2nd intron of NOTCH3, 5′-GC TTGCTGGGTTCTTGGTATTG-3′ and 5′-GACC TCGACGGGTCTCTGTTCT-3′ for an off target control for NOTCH3, 5′-CCCTTCTGTGCAGTCCTCCT-3′ and 5′-GACC TTGCGTGTTCCTTCCAT-3′ and 5′-CCGCTGTATACGCCAGC-3′ for a CSL-binding site at the HES1 promoter86, 5′-TGGATCCATCCATTTCTGGC-3′ and 5′-CGGAGACT TGGAAATGTGT-3′ for an off target control for HES118. Data represent three independent experiments.

**Statistical analyses.** Data from experiments were presented as mean ± standard error (n = 3–6) in real-time RT-qPCR, luciferase assays, flow cytometry and IHC labeling index or mean ± standard deviation (n = 8–12) in xenograft transplanta- tion experiments. GraphPad Prism 7 (GraphPad Software, La Jolla, CA) or Stata Version 14 (StataCorp, College Station, TX) software were used for statistical analyses. The two-tailed Student’s t-test was used for paired comparisons. Fisher’s exact test was used for percentage comparisons in xenograft experiments. ANOVA with Tukey’s post hoc test was used for multiple pairwise comparisons. P < 0.05 was considered significant. The Krappa Statistic was used to evaluate concordance between NOTCH1-positive IHC staining as classified by independent antibodies. Survival curves were estimated using the Kaplan–Meier method and plotted. Candidate predictor variables were tested at P < 0.05 by univariate and multivariate Cox Regression using the z-score corresponding to the hazard ratio.

**Data availability.** Microarray data were deposited at the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) under accession number GSE37994. All data sets are available from the authors upon request.

Received: 28 July 2016 Accepted: 21 September 2017

Published online: 24 November 2017

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