NOTCH1 and NOTCH3 Coordinate Esophageal Squamous Differentiation Through a CSL-Dependent Transcriptional Network

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BACKGROUND & AIDS: The Notch receptor family regulates cell fate through cell-cell communication. CSL (CBF-1/RBP-jκ, Su(H), Lag-1) drives canonical Notch-mediated gene transcription during cell lineage specification, differentiation, and proliferation in the hematopoietic system, the intestine, the pancreas, and the skin. However, the functional roles of Notch in esophageal squamous epithelial biology are unknown. METHODS: Normal esophageal keratinocytes were stimulated with calcium chloride to induce terminal differentiation. The squamous epithelium is renewed. Cytokeratins CK5 and CK14 are expressed in undifferentiated and proliferative keratinocytes in the basal cell layer. Cytokeratins CK4 and CK13 are specifically found in the suprabasal layer of the esophageal epithelium. The stratified squamous epithelia are regulated at an exquisite level. The esophageal epithelium consists of keratinocytes that migrate in an outward fashion toward the luminal surface. During migration, the cells undergo terminal differentiation in the suprabasal layer. Then, the cells are desquamated into the lumen and the epithelium is renewed. Cytokeratins CK5 and CK14 are expressed in undifferentiated and proliferative keratinocytes in the basa cell layer. Cytokeratins CK4 and CK13 are specifically found in the suprabasal layer of the esophagus and other noncornifying squamous epithelia. Involutrin (IVL) is also expressed in the suprabasal but not basal keratinocytes of the esophagus. Filaggrin (FLG) is a late differentiation marker localized to keratohyalin granules.

The Notch pathway regulates cell fate and differentiation through cell-cell communication. The Notch family comprises 4 structurally related single transmembrane receptor proteins Notch1–4 (N1–4). Ligand (Jagged and Delta-like) binding triggers a series of enzymatic cleavages of the receptor proteins mediated by metalloprotease and γ-secretase, thereby resulting in nuclear translocation of the intracellular domain of Notch (ICN). ICN forms a transcriptional activation complex containing a DNA binding transcription factor CSL (CBF-1/RBP-jκ, Su(H), Lag-1) and the coactivator Mastermind-like (MAML). Notch target genes include the hairy and enhancer of split (HES) and related (HEY) family of transcription factors. Notch signaling in the squamous epithelium has been investigated in the mouse epidermis. Prenatal CSL deletion impairs squamous differentiation with loss of early differentiation.
and late differentiation markers such as CK1/CK10 and FLG, causing barrier defects and death at birth due to severe dehydration. N3 has been proposed to have a central role in suppressing keratinocyte proliferation and promoting terminal differentiation. In fact, the N1-deficient epidermis displays progressive hyperplasia as mice age and becomes prone to chemical-induced carcinogenesis. Although N2 or N3 cannot compensate for N1 loss, compound loss of these Notch paralogues results in barrier defects and tumorigenicity even in the presence of N1, and that causes a more severe phenotype than N1 deficiency alone when all N1, N2, and N3 are knocked out, indicating the presence of interplay among the Notch family members in squamous epithelial homeostasis. N3 shows a relatively weak CSL-dependent transcriptional activity compared with the other Notch family members. Although N3 is expressed in the epidermis, no skin phenotype has been observed in N3 knockout mice. Thus, N3 regulation and its role in squamous epithelial biology remain to be elucidated.

In this study, we investigated how Notch signaling contributes to esophageal epithelial homeostasis in vitro and in vivo to find that N1 can trigger a robust induction and activation of N3 as a unique target for canonical CSL-dependent transcription at the onset of squamous differentiation and that a novel functional interplay between ICN1 and ICN3 has a critical role in transcriptional activation of cellular components essential in terminal differentiation. Notch inhibition not only impairs squamous differentiation but leads to basal cell hyperplasia and dysplasia in the mouse esophagus, providing novel insight into the role of Notch signaling in squamous epithelial biology.

Materials and Methods

Mice

DNMAML1VΔ mice carrying Lox-STOP-Lox dominant negative mastermind-like1 (DNMAML1) were described previously. DNAMAML1VΔ mice were intercrossed with KI4Cre transgenic mice, targeting DNAMAML1 into the basal cell layer of the esophageal epithelium. The KI4Cre:DNAMAML1 mice were compared with age-matched DNAMAML1VΔ and KI4Cre mice as controls. All experiments were performed under approved protocols from the University of Pennsylvania Institutional Animal Care and Use Committee and National Institutes of Health guidelines.

Human Esophageal Tissues

Normal human esophageal tissues (n = 7) used herein were described previously.

Cell Lines and Treatment

Primary and telomerase-immortalized nontransformed diploid human esophageal keratinocytes EPC2, EPC2-hTERT, and EPC1-hTERT are described elsewhere. Cells were kept undifferentiated in keratinocyte serum-free medium (Invitrogen, Carlsbad, CA) containing a low concentration (0.09 mmol/L) of CaCl2. Compound E (Calbiochem, La Jolla, CA), a γ-secretase inhibitor (GSI), was reconstituted in dimethyl sulfoxide.

Retrovirus- and Lentivirus-Mediated Gene Transfer and RNA Interference

Recombinant DNA work is described in the Supplementary Methods. Replication-incompetent retroviruses and lentiviruses were produced and transduced as described previously. Retroviral MigRI vector was used to express constitutively ICN1 as well as a green fluorescent protein (GFP)-DNMAML1 fusion gene. pTOF-DNMAML1 retroviral vector was created to express DNMAML1 in a tetracycline-regulatable (Tet-Off) manner. Lentiviral pGIPZ vector (Open Biosystems, Huntsville, AL) was used to express short hairpin RNA directed against N3 (N3-A, V2LHS_229748 and N3-B, V2LHS_93017) or a nonsilencing scramble control sequence (RHS4346). Cells transduced with MigRI or pGIPZ were selected, except for ICN1 transduction experiments, by flow sorting for the cells expressing GFP at the brightest level (top 20%). Cells transduced with pTOF-DNMAML1 were selected with 1 μg/mL of puromycin for 7 days. Small interfering RNA directed against N1 (N1-A, HSS181550 and N1-B, HSS107249) or a nonsilencing scramble control sequence (12935-300) (Invitrogen) was transfected transiently using the Lipofectamine RNAiMax reagent (Invitrogen), following the manufacturer’s instructions.

Luciferase Assays

Transient transfection and luciferase assays were performed as described previously. Briefly, 400 ng of 8xCBF1-luc (designated as 8xCSL-luc), a reporter plasmid containing 8 copies of the CSL DNA binding consensus sequence, or INV2.5-pGL3 containing a 2.5-kilobase IVL promoter (−2466 to +41) (IVL-luc) was transfected along with or without ICN expression plasmids or a control empty vector (p3XFLAG-CMV-7). Cells were incubated for 48 hours before cell lysis in ICN cotransfection experiments. Alternatively, CaCl2 was added to the final concentration of 0.6 mmol/L at 24 hours after transfection and for an additional 48 hours before cell lysis. The mean of firefly luciferase activity was normalized with the cotransfected Renilla luciferase activity.

Chromatin Immunoprecipitation Assays

Chromatin immunoprecipitation (ChIP) was performed as described previously. Briefly, chromatin samples were prepared from fixed 6 million cells and immunoprecipitated with rabbit immunoglobulin G (#sc-3888; Santa Cruz Biotechnology, Santa Cruz, CA), anti-N1, anti-CSL/RBP-jκ (#AB5790; Millipore, Billerica, MA), or anti-Histone H3 (#ab1791-100; Abcam, Cambridge, MA) anti-
body. Purified DNA was subjected to real-time polymerase chain reaction (PCR) with primers flanking potential CSL binding sites at −12 kilobases (TTCCCA) and −2.3 kilobase pairs (agCATGGGAGa) upstream the translation start and within intron 2 (nt TTCCCA/Cg-TTCCCA) of N3 (GenBank NG_009819). The N3 3′ end region was examined as an off-target control. The DNA quantity recovered from each ChIP sample is shown as the relative value to anti-Histone H3 ChIP samples. Supplementary Table 1 lists primer sequences. Data represent 3 independent experiments.

**RNA Isolation, Complementary DNA Synthesis, and Real-Time Reverse-Transcription PCR**

RNA isolation, complementary DNA synthesis, and real-time reverse-transcription (RT)-PCR were performed as described previously. Supplementary Table 2 lists used TaqMan Gene Expression Assays (Applied Biosystems, Carlsbad, CA). Relative gene expression was determined based on corresponding threshold cycle values and normalized to β-actin as an internal control.

**Western Blot Analysis**

Western blotting was performed as described previously. Supplementary Table 3 lists primary antibodies and the titers used for Western blotting.

**Immunofluorescence and Immunohistochemistry**

Immunofluorescence and immunohistochemistry were performed as described previously. Supplementary Table 4 summarizes antibodies, titers, and specific conditions. Stained objects were examined using a Nikon Microphot microscope (Nikon, Melville, KY) and images obtained with a digital camera. Ki67 labeling index was determined by counting at least 600 cells per group.

**Statistical Analyses**

Data from triplicate and hexaduplicate experiments in real-time RT-PCR, luciferase assays, and Ki67 labeling index are presented as mean ± SE and were analyzed by 2-tailed Student t test. Fisher exact test was performed to assess genotype-phenotype association in mice. P < .05 was considered statistically significant.

**Results**

**CSL-Mediated Canonical Notch Signaling Is Required at the Onset of Esophageal Squamous Differentiation**

Calcium (Ca²⁺) induces keratinocyte differentiation. Calcium (Ca²⁺) induces keratinocyte differentiation. Notch was activated in EPC2, EPC2-hTERT, and EPC1-hTERT cells upon Ca²⁺ stimulation, which promotes cell-cell contact (Supplementary Figure 1), CSL reporter activation, and induction of HES5 and early differentiation markers IVL and CK13 in a temporal and dose-dependent manner (Figure 1 and Supplementary Figure 2). These Ca²⁺-induced responses were blocked effectively at the transcriptional, messenger RNA (mRNA), and protein levels by a GSI as well as DNAMAML1, the latter antagonizing the MAML family of CSL-dependent transcriptional coactivator expressed in esophageal epithelial cells (Figure 1 and Supplementary Figure 3).

In the esophageal epithelium reconstituted in organotypic 3D culture, Notch inhibition suppressed sharply epithelial stratification as well as early (ie, IVL and CK13) and late (FLG) differentiation markers without affecting basal cell marker (ie, CK14) expression and proliferation (Figure 2 and Supplementary Figure 4). These data suggest there is canonical Notch signaling in the early stages of esophageal squamous epithelial differentiation.

The lack of squamous differentiation in the presence of Notch inhibitors can be accounted for by depletion of committed cells that are capable of undergoing differentiation in response to Ca²⁺ stimulation. When DNAMAML1 was repressed by doxycycline in the Tet-Off system, Notch functions and squamous differentiation were restored in both monolayer and 3D cultures (Figure 2D and E and Supplementary Figure 5), consistent with Notch-mediated direct transcriptional regulation of squamous differentiation.

**N1 and N3 Are Induced and Activated During Esophageal Squamous Differentiation**

We next explored whether the Notch receptor family members are involved in the regulation of squamous differentiation in the esophagus. N1 and N3 were expressed on the cell membrane of the suprabasal cells within the normal human esophageal epithelium, where nuclear localization of both N1 and N3 was also observed in the cells within the first 3 to 4 layers above the basal cell layer (Figure 3). Additionally, a subset of basal cells showed nuclear N1 and N3 expression (Figure 3).

We also determined the expression of N1–4 in primary and immortalized esophageal cells by real-time RT-PCR. Threshold cycle values implied relatively high basal levels of N1 and N2 mRNA and very low to undetectable levels of N3 and N4 mRNA (data not shown). Ca²⁺ increased N1 and N3 but not N2 and N4 mRNA (Figure 4A and Supplementary Figure 6), and that was reflected by the HES5, IVL, and CK13 mRNA levels (Figure 1 and Supplementary Figure 2). Constitutive N1 mRNA expression was corroborated by full-length (FL)-N1 protein detected by Western blotting in unstimulated EPC2-hTERT cells (Figure 4B, time 0). Upon Ca²⁺ stimulation, FL-N1 protein was down-regulated within 6 hours while ICN1 emerged in a reciprocal fashion (Figure 4B), indicating N1 activation. Both FL-N1 and ICN1 levels were increased thereafter (Figure 4B). Because ICN1 is generated by enzymatic FL-N1 cleavage triggered by ligand binding, ICN1 up-regulation (Figure 4B, 6h–48h) can be accounted for by an increase in the N1 mRNA level (Figure
NC1 before Ca\textsuperscript{2+} stimulation in EPC2-hTERT cells (Figure 4B). Consistent with a late N3 mRNA induction, both FL-N3 and ICN3 levels increased dramatically at 48 hours after Ca\textsuperscript{2+} stimulation (Figure 4A and B). Such a successive induction pattern of ICN3 preceded by ICN1 was documented in nuclear extracts (Figure 4C), implying sequential activation of N1 and N3.

Ligand-induced activation of N1 and N3 was suggested by the finding that the GSI blocked both ICN1 and ICN3 generation on Ca\textsuperscript{2+} stimulation (Figure 4D). Interestingly, GSI suppressed FL-N3 and N3 mRNA (Figure 4D and E), suggesting that N3 per se may be subjected to regulation by Notch signaling. Consistent with this premise, DNMAML1 prevented Ca\textsuperscript{2+} from inducing N3 mRNA and FL-N3 protein (Figure 4D and E). Importantly, ICN3 was found in the nuclear fraction by 48 hours after Ca\textsuperscript{2+} stimulation and that was suppressed by GSI or DNMAML1 (Figure 4C and Supplementary Figure 7A and B). Moreover, repression of DNMAML1 by doxycycline in the Tet-Off system augmented N3 mRNA and ICN3 induction (Supplementary Figure 7C and D), reinforcing the notion of direct CSL-mediated induction and nuclear translocation of the activated N3. N3 activation upon Ca\textsuperscript{2+}-mediated squamous differentiation was observed also in primary mouse esophageal keratinocytes and was antagonized by GSI (Supplementary Figure 8). Although GSI also suppressed N1 mRNA and ICN1 induction by Ca\textsuperscript{2+}, DNMAML1 augmented N1 mRNA and ICN1 expression on Ca\textsuperscript{2+} stimulation (Figure 4D and Supplementary Figure 9), suggesting that N1 mRNA induction may be CSL independent. These observations imply dynamic changes in expression and activation of N1 and N3 during esophageal squamous differentiation.

**N1 Induces N3 Through CSL-Dependent Transcription**

N1 activation preceded N3 induction (Figure 4A–C). This prompted us to investigate the possibility that N1 may regulate N3 directly. To this end, ICN1 was ectopically expressed in EPC2-hTERT cells. ICN1 not only activated the CSL reporter but also induced HES5, CK13, and IVL, whereas DNMAML1 blocked all these activities (Supplementary Figure 10). ICN1 induced N3 mRNA in the absence of DNMAML1 (Figure 5A), indicating that N1 may induce N3 through CSL-dependent transcription. Moreover, Western blotting showed that DNMAML1 blocked not only FL-N3 and ICN3 but also IVL proteins (Figure 5B).

We next addressed whether N1 is required for N3 induction by RNA interference experiments. N1 knockdown compromised Ca\textsuperscript{2+}-mediated induction and activation of N3 as well as Notch target genes (Figure 5C and D and Supplementary Figure 11), implying N1 in N3-mediated squamous differentiation.

Furthermore, ChIP assays documented Ca\textsuperscript{2+}-induced binding of ICN1 and CSL to the intron 2 of the N3 gene.
containing a putative CSL binding cis-element (Figure 5E and Supplementary Figure 12), providing evidence for direct transcriptional regulation of N3 by N1.

**N3 Cooperates With N1 for Esophageal Squamous Differentiation**

To elucidate the role of N3 in squamous differentiation, we established 2 independent lines of EPC2-hTERT cells of stable N3 knockdown. The RNA interference effect was validated by inhibition of Ca²⁺/H9262 or ICN1-mediated N3 mRNA and ICN3 protein induction (Figure 6A and B and Supplementary Figure 13A and B). N3 knockdown resulted in suppression of the IVL promoter activation as well as IVL mRNA and IVL protein induction (Figure 6B–D and Supplementary Figure 13B and C). Additionally, N3 knockdown suppressed HESS and CK13 induction (Supplementary Figure 13D and E), implying N3 is involved in their transcriptional regulation. Moreover, in organotypic 3D culture, N3 knockdown impaired squamous epithelial stratification and IVL expression without affecting basal cell proliferation (Figure 6E). Interestingly, ICN1 was induced on Ca²⁺ stimulation in N3 knockdown cells without circumventing the N3 knockdown effect on IVL expression (Figure 6B). Given the role of N3 in IVL transcriptional activation, we performed transient transfection of ICN1 and ICN3, either alone or in combination. ICN1, but not ICN3, activated the IVL promoter. However, ICN3 enhanced significantly the ICN1-mediated IVL promoter transactivation in EPC2-hTERT cells (Figure 6F). These findings indicate cooperation between N1 and N3 in squamous differentiation.

**Notch Inhibition in the Mouse Esophagus Leads to Deregulated Squamous Differentiation and Aberrant Basal Cell Proliferation With Notch3 Down-Regulation**

To determine the functional consequences of Notch inhibition in vivo, we targeted DNMAML1 in the
mouse esophagus. Born in the anticipated Mendelian ratio, the K14Cre;DNMAML1 mice developed normally, yet 4 of 10 mice exhibited weight loss by 3 months of age. Histologic examination revealed basal cell hyperplasia and/or dysplasia with disorganized nuclear polarity associated with an increase in Ki67-positive cells in the affected mouse esophagi. Neither the rest of K14Cre; DNMAML1 nor age-matched control mice presented histologic changes (Figure 7). Nevertheless, there was a statistically significant genotype-phenotype association in the affected K14Cre;DNMAML1 mouse esophagi compared with DNMAML1f/f or K14Cre controls (P = .023, n = 10). There was a marked reduction in the number and size of keratohyalin granules in the suprabasal stratum granulosum cell layer in the affected K14Cre; DNMAML1 esophagi, compared with unaffected K14Cre; DNMAML1 or control mouse esophagi, suggesting impaired terminal differentiation. This was corroborated by decreased Ivl and Flg expression (Figure 7). Finally, N3 expression was diminished greatly in the affected K14Cre; DNMAML1 esophagi (Figure 7), implicating CSL-dependent N3 expression in vivo. In aggregate, DNMAML1 perturbed Notch3 expression as well as the squamous differentiation program through inhibition of CSL-dependent transcription in the mouse esophagus.

Discussion

Our data indicate that the Notch pathway plays a critical role in esophageal squamous differentiation involving the canonical CSL-mediated transcriptional network (Figures 1–4). A novel functional interplay between N1 and N3 appears to be essential as N1 transcriptionally activates N3 to drive squamous differentiation (Figures 5 and 6). In the mouse, Notch inhibition not only impaired esophageal squamous differentiation but caused basal cell hyperplasia and dysplasia (Figure 7). These findings establish a novel model whereby cross talk between N1 and N3 regulates squamous differentiation (Supplementary Figure 14).

To our knowledge, this is the first demonstration of functionally active Notch signaling in normal esophageal epithelial cell biology. Both primary and immortalized human esophageal keratinocytes underwent terminal dif-
differentiation and formed stratified squamous epithelium in 3D culture in a CSL-dependent manner. Notch inhibition abrogated early and late differentiation in culture as well as in mice. GSI induces goblet cell metaplasia in the rat intestine without affecting normal esophageal squamous epithelium after 5 daily intraperitoneal injections. However, long-term GSI treatment may be required before GSI takes effect in the esophagus, given slower epithelial turnover (>14 days). DNMAML1 did not affect basal cell proliferation in 3D culture (Figure 2). Thus, esophageal hyperplasia and dysplasia in mice may be explained by non-cell autonomous effects of Notch inhibition.

Loss of Notch signaling in the skin leads to barrier defects and squamous cell carcinoma as well as atopic dermatitis-like disease. Thus, our mouse model may have implications in esophageal diseases such as cancer and eosinophilic esophagitis. Interestingly, focal inflammation was noted in a few K14Cre;DNMAML1 esophagi (Shinya Ohashi et al, unpublished data, June 2010). Only 40% of the K14Cre;DNMAML1 mice displayed esophageal phenotypes at 3 months after birth, although hair loss occurred with 100% penetrance rate, implying hair follicle dysfunction (John T. Seykora, manuscript in preparation).

**Figure 4.** Sequential induction and activation of N1 and N3 during Ca²⁺-induced squamous differentiation in esophageal keratinocytes. EPC2-hTERT cells were exposed to 0.6 mmol/L Ca²⁺ (A–C) for indicated time periods or (D and E) 72 hours and subjected to (A and E) real-time RT-PCR or (B–D) Western blotting. In D and E, Ca²⁺ stimulation was performed in the presence or absence of GSI or DNMAML1. Compound E (GSI) was used at indicated concentrations. β-actin served as an internal or loading control in A, B, D, and E. Histone H1 served as a loading control for nuclear extracts in C. (A) *P < .001 vs time 0 (n = 3). (E) *P < .001 vs 0.09 mmol/L Ca²⁺ + 0 μmol/L GSI or GFP; **P < .001 vs 0.6 mmol/L Ca²⁺ + 0 μmol/L GSI or GFP (n = 3). Arrowheads indicate FL-N3 and brackets indicate doublets of ICN3.

**Figure 5.** ICN1 directly induces and activates N3 through CSL-dependent transcription in esophageal keratinocytes. EPC2-hTERT cells expressing either DNMAML1 or GFP (control) were transduced with ICN1 to determine (A) N3 mRNA and (B) N3 protein levels 72 hours after retrovirus infection. In C and D, EPC2-hTERT cells were stimulated with Ca²⁺ for 72 hours following N1 small interfering RNA transfection to determine indicated molecules. Real-time RT-PCR and Western blotting determined mRNA and protein, respectively. β-actin served as an internal or loading control in A, B, D, and E. Histone H1 served as a loading control for nuclear extracts in C. (A) *P < .001 vs 0.09 mmol/L Ca²⁺ 0 μmol/L GSI or GFP (n = 3). (E) *P < .001 vs 0.6 mmol/L Ca²⁺ 0 μmol/L GSI or GFP (n = 3). Arrowheads indicate FL-N3 and brackets indicate doublets of ICN3.
Induction of CK13, an esophageal-specific cytokeratin, implies tissue-specific differentiation, whereas other markers such as IVL and FLG are shared with other squamous epithelia, including that in the epidermis. Lack of IVL and Flg expression in the K14Cre;DNMAML1 mouse is in concordance with impaired differentiation by embryonic K14Cre-mediated Csl loss in the mouse epidermis.7 Similar to CK13 expression in esophageal cells, Ca2+ induces skin-specific suprabasal cytokeratins CK1 and CK10 in a Csl-dependent fashion.7 However, there may be certain differences in the modes of Notch signaling during squamous differentiation between the esophagus and the skin, which includes hair follicle and interfollicular keratinocytes that are regulated differentially.

First, Ivl can be induced in a Csl-independent manner in mouse skin keratinocytes.29 By contrast, our data point to CSL-dependent IVL transcription in esophageal keratinocytes. Second, Hes1 is induced most predominantly among the HES/HEY family of transcription factors upon Notch activation in epidermal keratinocytes, where Hes1 regulates Hey1/Hey2 and other Notch downstream target molecules such as p21 and Wnt4.30 In human esophageal cells, Ca2+ induced HES5 most robustly (Figures 1 and Supplementary Figure 2) with HES1 induction only to a modest extent (data not shown). Functional redundancy of Hes1 and Hes5 has been implicated in neuronal differentiation.31 However, Hes5 determines T-cell fate depending on the ligand density, whereas Hes1 is indispensable for T-cell differentiation.32 Our data imply N3 serves an important functional role in HES5 induction.

ICN3, a weak transcriptional activator,10 failed to stimulate the IVL promoter by itself but enhanced ICN1-mediated IVL transactivation (Figure 6F). Our data suggest a novel modulatory role of N3 on N1-mediated IVL induction. Although ICN and CSL binding was not validated in this study, 4 potential CSL-binding cis elements exist within the 2.5-kilobase promoter sequence in IVL-luc construct. It is tempting to speculate that ICN1-ICN3 heterodimers may be formed on the IVL promoter to recruit histone acetyltransferases, chromatin remodeling factors, and other transcription factors as proposed previously.33 AP-1 and SP1 also regulate IVL.34 Thus, further study will be needed to identify factors cooperating with ICN1, ICN3, and CSL to regulate IVL.

N1-mediated CSL-dependent N3 transcription agrees with N3 mRNA induction by ICN1.35 Our ChIP assay data establish a direct link between ICN1 and N3. N3 down-regulation in the K14Cre;DNMAML1 mouse esophagus supports this notion. What is the biological role of N3 in the esophagus? Reminiscent of our K14Cre; DNMAML1 mouse phenotypes, esophageal hyperplasia has been noted when N3 was deleted in the NIN2 compound knockout mice, phenocopying the γ-secretase-deficient skin.11 We did not observe apoptosis upon N3 knockdown or DNMAML1 expression (data not shown).
Figure 7. DNMAML1 induce basal cell hyperplasia/dysplasia impairing squamous differentiation and N3 expression in the mouse esophageal epithelium. Representative K14Cre;DNMAML1 and control (DNMAML1f/f and K14Cre) mouse esophagi were subjected to H&E staining; immunohistochemistry for Ki67, Flg, and N3; or immunofluorescence for Ivl. Ki67 labeling index, 19.9% ± 5.0% for DNMAML1f/f, 21.8% ± 4.8% for K14Cre (not significant vs DNMAML1f/f); and 69.6% ± 5.5% for K14Cre;DNMAML1 (P < .001 vs DNMAML1f/f and K14Cre) (n = 6). Arrows denotes Ivl cytoplasmic staining, Flg localization to keratohyalin granules, and nuclear N3 staining in each panel. Scale bar = 50 μm.
However, N3 may inhibit apoptosis through AKT\textsuperscript{36} and the mitogen-activated protein kinase pathways.\textsuperscript{37} AKT activation by epidermal growth factor receptor leads to esophageal hyperplasia, suppressing late differentiation in organotypic 3D culture.\textsuperscript{38} Because epidermal growth factor receptor signaling negatively regulates N1,\textsuperscript{39} it will be of great interest to explore the role of N3 in this context.

In conclusion, our innovative approaches reveal novel functional cross talk between N1 and N3 during differentiation, providing mechanistic insights into the role of Notch signaling in esophageal squamous epithelial biology.

**Supplementary Material**

Note: To access the supplementary material accompanying this article, visit the online version of Gastroenterology at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2010.08.040.

**References**


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