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Notch signaling promotes mature T-cell lymphomagenesis

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Abstract

Peripheral T-cell lymphomas (PTCL) are agressive lymphomas that develop from mature T cells. The most common PTCLs are genetically, molecularly, and clinically diverse and are generally associated with dismal outcomes. While Notch signaling plays a critically important role in both the development of immature T cells and their malignant transformation, its role in PTCL is poorly understood, despite the increasingly appreciated function of Notch in regulating the proliferation and differentiation of mature T cells. Here, we demonstrate that Notch receptors and their Delta-like family ligands (DLL1/DLL4) play a pathogenic role in PTCL. Notch1 activation was observed in common PTCL subtypes, including PTCL-NOS. In a large cohort of PTCL-NOS biopsies, Notch1 activation was significantly associated with surrogate markers of proliferation. Complementary genetically-engineered mouse models and spontaneous PTCL models were

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utilized to functionally examine the role of Notch signaling, and Notch1/Notch2 blockade and pan-Notch blockade using dominant negative MAML significantly impaired the proliferation of malignant T cells and PTCL progression in these models. Treatment with DLL1/DLL4 blocking antibodies established that Notch signaling is ligand dependent. Together, these findings reveal a role for ligand-dependent Notch signaling in driving peripheral T-cell lymphomagenesis.

Keywords

Peripheral T-cell lymphoma; SWI/SNF; Notch1; Notch2; DLL1; DLL4

Introduction

The advent of modern immunochemotherapy regimens and improved understanding of B-cell lymphomagenesis have dramatically improved outcomes for patients afflicted with aggressive B-cell lymphomas. Consequently, aggressive lymphomas derived from mature (post-thymic) T-cells cause an increasingly disproportionate number of lymphoma-related deaths, as both primary refractory disease and acquired chemotherapy resistance are common, and complete or durable remissions with novel agents are rare. Despite improved understanding of peripheral T-cell lymphoma (PTCL) ontogeny (1–3), characterization of their genetic landscape (4), and increasing awareness of the tumor microenvironment's (TME) role in disease pathogenesis (5, 6), improved therapeutic strategies are needed.

Ligands and cytokines, provided by constituents of the TME, promote the growth and survival of malignant T cells upon binding their corresponding antigen-, costimulatory-, and cytokine-receptors. Many of these receptors or their downstream signaling intermediates, while recurrently subjected to gain-of-function genetic alterations in the PTCL, remain dependent upon the provision of exogenous ligands by the TME (6) (7).

Notch receptors are a highly conserved family of receptors with a well-described role in both early T-cell development in the thymus and in the pathogenesis of thymocyte-derived malignancies (8, 9). While the role of Notch ligand-receptor interactions in thymocyte biology has long been appreciated, their role in the activation, differentiation, and function of mature T cells has only been described more recently (10). Upon ligand binding, sequential juxtamembrane and intramembrane proteolytic reactions cleave Notch, releasing its intracellular domain (NICD), which transcriptionally regulates target gene expression upon nuclear entry. Intriguingly, while intranuclear NICD has been observed in selected PTCL and cutaneous T-cell lymphomas (CTCL) by immunohistochemistry (11, 12), its role in PTCL pathogenesis is largely unexplored. Herein, we demonstrate that Notch receptors and their Delta-like family ligands (DLL1/DLL4) play a pathogenic role in PTCL.

Materials and Methods

Genetically engineered mouse models and Notch pathway inhibition

Mouse studies were approved by the University Committee on Care and Use of Animals (UCUCA) and performed in accordance with guidelines established by the Unit for Laboratory Animal Medicine (ULAM) at University of Michigan (protocol number

PRO00009740). Mice were housed under specific pathogen-free conditions. Floxed (*SNF5*, p53) and CD4-Cre mice (RRID:MGI:3691126) were obtained from Jackson Laboratory and were crossed, and all F1 mice genotyped using tail DNA (Invitrogen Animal Tissue Direct PCR Kit). SNF5 mice were provided on a mixed background, but were backcrossed for at least 10 generations onto a B6 background. Mice generated during backcrossing were utilized for the experiments shown in supplementary figures 1–3, whereas the remaining work shown here was obtained using mice fully backcrossed on a B6 background. Offspring with the desired genotype for these studies, including $p53^{fl/fl\ or\ +/+}$, $SNF5^{fl/fl}$, CD4-Cre^{+} (lymphoma mice) and $p53^{+/+}$, $SNF5^{fl/+\ or\ +/+}$, CD4-Cre^{+} (littermate controls) were utilized. $ROSA^{DNMAML}$ mice (abbreviated DNMAML, expressing a Cre-inducible DNMAML-GFP pan-Notch inhibitor under control of the *ROSA26* promoter) have been previously described.(13) These mice were crossed with fully backcrossed *SNF5* floxed ($p53^{+/+}$) mice, to generate CD4-Cre^{+} $SNF5^{fl/fl}$ $ROSA^{DNMAML/DNMAML}$ or $ROSA^{DNMAML/+}$ ("Notch off"), and CD4-Cre^{+} $SNF5^{fl/fl}$ $ROSA^{+/+}$ ("Notch on"), offspring. Mice were genotyped using tail DNA (Invitrogen Animal Tissue Direct PCR Kit). Mice were monitored twice weekly for event-free survival (EFS), with an event defined as the development of massive hepatosplenomegaly, bulky lymphadenopathy (>5mm), or any other condition meeting euthanasia criteria. Necropsy was performed at the time an event was observed, and spleen and liver weights determined. Single cell suspensions were generated for flow cytometric analysis, and adoptive transfer experiments. Clonal (expressing a dominant TCR-Vβ, abbreviated TCR-$Vβ^{+}$) and non-clonal (not expressing a dominant TCR-Vβ, abbreviated TCR-$Vβ^{-}$) T cells from different genotypes were sorted for RNA sequencing and qRT-PCR. Tissue, including primary and secondary lymphoid organs, was formalin-fixed and paraffin-embedded for histologic examination. For adoptive transfer experiment, $5×10^{6}$ bulk splenocytes obtained from lymphoma-bearing donor mice were retro-orbitally injected into 12-16 week old recipient C57BL/6J mice (Jackson Laboratory; RRID:IMSR_JAX:000664). Where indicated, mice were treated with combined vincristine (0.5mg/kg, i.p) and cyclophosphamide (40mg/kg, i.p) weekly (two weeks after adoptive transfer) for 2-3 consecutive weeks. Tumor burden was evaluated by organ weights and the extent of CD3, clonal TCR-Vβ, and Ki67 expression by flow cytometry. For short-term Notch signaling blockade, mice were monitored after adoptive transfer of splenocytes from $SNF5^{fl/fl}$, CD4-Cre^{+} lymphoma-bearing donor mice, and upon lymphoma engraftment (demonstrated by palpable splenomegaly, usually within 2-4 weeks following adoptive transfer), isotype control (5mg/kg), anti-Notch1/2 or anti-DLL1/4 antibodies (5mg/kg) were administered intraperitoneally (for 2-3 doses, on day 0, 2 and 7), as previously described (14), and mice were euthanized 5-7 days after the last dose of treatment. For long-term anti-DLL1 experiment, $5×10^{6}$ bulk splenocytes obtained from $SNF5^{fl/fl}$, CD4-Cre^{+} lymphoma-bearing donor mice were retro-orbitally injected into 12-16 weeks old recipient C57BL/6 mice (Jackson Laboratory), then treated with either vehicle, chemotherapy (vincristine, 0.5mg/kg, cyclophosphamide, 40mg/kg), anti-DLL1 antibody (5mg/kg), or anti-DLL1 plus chemotherapy. Event-free survival was followed until they reached study endpoints (massive hepatosplenomegaly, bulky lymphadenopathy, or moribund). For short-term Notch blockade experiments, recipient C57BL/6J mice were monitored after adoptive transfer of splenocytes. Similarly designed experiments were performed utilizing a spontaneous PTCL that emerged in a Balb/c mouse (i.e. T8-28 model) utilizing Balb/c recipients, as previously

described (15). Upon lymphoma engraftment (demonstrated by palpable splenomegaly, usually within 2-4 weeks following adoptive transfer), anti-Notch1/2, anti-DLL1/4, or an irrelevant humanized IgG1 isotype control antibody were administered intraperitoneally for 2-3 doses (5 mg/kg, on days 0, 2 and 7), as previously described (14). Mice were euthanized 5-7 days after the last dose of treatment and lymphoma burden examined.

Human PTCL cases, immunohistochemistry, and multispectral imaging

Peripheral T-cell lymphoma (PTCL) cases were identified and cases with sufficient tissue and lymphoma content for construction of a tissue microarray selected with approval by each respective institution's Institutional Review Board. This study was conducted in accordance with US federal regulations and the Declaration of Helsinki. Cases were reviewed by at least two hematopathologists (C.M.Z., N.B., N.G.B., K.I., J.M., C.B., or L.T.) and classified in accordance with 2016 WHO criteria. Immunohistochemistry for the cleaved, intracellular domain of Notch1 (NICD1) was performed, as previously described (11), using anti-cleaved Notch1 antibody (titer 1:50 with overnight incubation, clone D3B8, specific for the Val1744 epitope in NICD1, Cell Signaling Technology). A nine-color Opal TSA multiplex immunofluorescence panel labeling CD3, CD20, CD68, CD163, CD8, PLK-1, Ki67, and the nuclear marker DAPI was optimized on reactive tonsil and independent lymphoma samples prior to staining on a Leica BOND RX autostainer according to the modified Opal 7-color (v5.2 plus) preset protocol. After satisfactory staining was achieved for individual targets, a multiplex run was performed on optimization samples, and signal localization and intensity values were compared to those in single stains. Finally, TMA slides were baked, deparaffinized on the BOND RX, and stained with the optimized panel. Multispectral images of each TMA core were acquired on an Akoya Polaris system using the previously optimized spectral profile for each target. Color channels were unmixed using inForm software (Akoya Biosciences). We trained two deep learning networks with HALO-AI (Indica Labs, Inc.) to identify tissue within each core and to segment all nuclei in each core image. Using the Highplex FL module in HALO (version 3.3), we phenotyped each cell based on its expression of lymphoma-informative (CD3, CD8, CD20) and proliferation (Ki67, PLK1) markers. A mean of 9,545 (IQR: 2,777-14,736) malignant T cells were analyzed in each case.

Statistics analysis

Data analyses were performed in GraphPad Prism 8.0 package (RRID:SCR_002798). Comparisons between groups were evaluated using two-tailed Student t-test or One-Way ANOVA and p-values<0.05 considered statistically significant. Event-free survival was summarized with Kaplan-Meier method, and comparisons made with log-rank test.

Data availability

The data generated in this study are publicly available in NCBI BioProject (RRID:SCR_004801) at PRJNA839400 and in Gene Expression Omnibus (GEO, RRID:SCR_005012) at GSE132550.

Additional methods are described more fully in the Supplementary Materials and Methods.

Results

Notch activation is associated with proliferation in human peripheral T-cell lymphoma, not otherwise specified (PTCL, NOS)

Given the importance of Notch signaling in both conventional T cells and T-cell acute lymphoblastic leukemia (T-ALL), and the observation that intranuclear, cleaved Notch1 has been observed in angioimmunoblastic T-cell lymphoma (AITL)(11), we sought to examine activated, cleaved Notch1 among the most prevalent PTCL subtypes. Expression and localization of the cleaved, intracellular domain of Notch1 (NICD1) was examined by immunohistochemistry among the most common PTCL subtypes (Fig. 1A, B), including AITL specimens, included as controls. Consistent with prior observations (11), NICD1 was observed in AITL. However, NICD1 detection was not confined to this subtype, as ≥20% of malignant T cells expressed NICD1 in the other PTCL subtypes examined, including PTCL, NOS (Fig. 1B). Notch-related transcripts were significantly enriched in an independent cohort of PTCL, NOS biopsies in comparison with benign lymph nodes (Fig. 1C), further implicating Notch signaling in PTCL.

Notch signaling promotes the proliferation of conventional T-cells and malignant T cells in adult T-cell leukemia/lymphoma (ATLL) harboring activating Notch mutations (16–18). Therefore, in order to examine the potential relationship between Notch1 activation, as demonstrated by intranuclear NICD1 expression, and proliferation in PTCL, NOS, multispectral imaging was performed in $CD4^+$ PTCL, NOS (Fig. 1D–F, Supplementary Figure 1A). Biopsy cores with >80% lymphoma content were obtained from PTCL, NOS diagnostic lymph node biopsies (n=50) and tissue microarrays constructed. The prevalence (Fig. 1E, F) and intensity (Supplementary Figure 1B, C) of staining for Ki67 (Fig. 1E, Supplementary Figure 1B) and PLK-1 (Fig. 1F, Supplementary Figure 1C) were examined in malignant T cells (defined as $CD3^+CD8^-$ $CD20^-CD68^-CD163^-$). Ki67 and PLK-1 were selected as complementary surrogate markers of cell proliferation. Multiple intrinsic and extrinsic factors drive the proliferation of malignant cells. Not surprisingly then, a subset of NICD1 negative cases were highly proliferative (Ki67 and/or PLK-1 “high”). Nonetheless, a positive correlation, albeit with varying degrees of significance, was observed between NICD1, Ki67 and PLK-1 (Fig. 1D–F, Supplementary Figure 1). As multiple intrinsic and extrinsic mechanism promote the proliferation of malignant T cells, the modest R^2 values may have been anticipated. Nonetheless, these findings suggest that Notch signaling promotes the proliferation of clonal T cells in PTCL, NOS. Unfortunately, clinical data were insufficient to determine the relationship between NICD1 and disease natural history in this cohort. Functional assessment of Notch signaling in primary specimens *ex vivo* is challenging, as malignant T cells undergo spontaneous apoptosis upon culture (5, 19), and the constituents of the TME that may present Notch ligands (e.g. fibroblastic reticular cells) are not readily available for *ex vivo* studies. Therefore, we next turned to genetically-engineered mouse (GEM) models for studies examining the functional role of Notch signaling in these lymphomas.

Clonal evolution and Notch receptor expression in *SNF5*-deficient T cells

In order to further examine the role of Notch receptor signaling in T-cell lymphomagenesis, we sought to identify a relevant GEM model. Many murine PTCL models harbor specific genetic derangements that are either relevant for a minority of PTCL or are not observed in human PTCL (Supplemental Table 1). Therefore, the phenotype observed in mice upon conditional deletion of the SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily b, member 1 (SMARCB1, also known as *SNF5*) in developing T cells is not only intriguing(20), but also relevant, as various subunits of the SWI/SNF complex are recurrently mutated or deleted in T-cell lymphomas (4, 21–25). For example, at least one ARID or SMARC family gene is deleted or mutated in >90% of Sezary syndrome genomes (21). SWI/SNF family members, in collaboration with members of the ARID family, are assembled into large, multisubunit complexes that promote chromatin remodeling and regulate the placement of Polycomb repressive complexes (26), which are now being therapeutically targeted in PTCL (27). Conditional deletion of *SNF5* in peripheral T cells in mice led to the preferential expansion of $CD8^+$ memory T cells (20).

Signs consistent with a lymphoproliferative disorder, most commonly significant hepatosplenomegaly and/or lymphadenopathy (Supplementary Figure 2A, B), were observed (n=25) with a median onset of 21 weeks (IQR: 18-26 weeks) upon *SNF5* deletion in mature T cells on a mixed (B6129SF1/J) background. Histomorphologic assessment of the bone marrow, secondary lymphoid organs, and liver revealed the expansion of morphologically atypical $CD3^+$ T cells (Supplementary Figure 2C). T-cell clonality was assessed using T-cell receptor Vβ specific antibodies. In contrast to littermate controls in which a polyclonal repertoire was appreciated, the preferential expansion of a dominant TCR Vβ-expressing T-cell population was observed in $SNF5^{fl/fl}$, CD4-Cre^+ mice exhibiting signs consistent with lymphoma (Supplementary Figure 3A, B, Supplementary Table 2). As observed in human PTCL, the preferential expansion of these $V\beta^+$ T cells was associated with a commensurate loss of TCR diversity. The expanded population of $V\beta^+$ PTCL observed were $CD3^+$, generally $CD8^{+/dim}$, but uniformly $CD4^-$, suggesting their derivation from $CD8^+$ T cells (Supplementary Figure 3C, Supplementary Table 2), as previously suggested (20). In addition, with few exceptions, these cells were $CD44^+CD62L^-CCR7^-$, suggesting derivation from antigen-experienced effector memory T cells (Supplementary Figure 3D, Supplementary Table 2). To further evaluate their malignant potential and sensitivity to conventional chemotherapeutic agents, bulk splenocytes from lymphoma-bearing mice were adoptively transferred into immunodeficient recipients, which were treated with cyclophosphamide and vincristine or vehicle control and followed for event-free survival (EFS). Lymphoma development was uniformly observed in sham-treated mice within 35 days (Supplementary Figure 4A; median EFS 20 days; 95% CI 15-27 days). While chemotherapy administration significantly prolonged EFS in recipient mice (median EFS 47 days, 95% CI 42-52 days, $p<0.0001$), these mice ultimately succumbed to disease progression.

After backcrossing onto a B6 background, bulk $CD3^+$ T-cell were sorted from both littermate control and young (non-lymphoma-bearing) *SNF5* cKO mice, as were $CD3^+$TCR-$V\beta^+$ T-cells from older, lymphoma-bearing mice. RNA-seq was performed, demonstrating

that 1459 transcripts were differentially expressed upon *SNF5* deletion prior to the emergence of a dominant clone, many of which were implicated in cell proliferation by GSEA (Fig. 2A; Supplementary Figure 5A, B; Supplementary Table 3). The emergence of a dominant clone in older (> 4 months old), lymphoma-bearing mice was associated with additional transcriptional changes, including the differential expression of 11,276 transcripts (Fig. 2A, B), many of which regulate cell cycle progression, are c-Myc dependent, and further associated with Notch signaling (Fig. 2C; Supplementary Table 3). Given the transcriptional reprogramming observed, the proliferation of clonal (TCR-Vβ^+) and non-clonal (TCR-Vβ^-) T cells was examined by Ki67 expression. In littermate controls, fewer than 10% of CD3$^+$ T cells expressed Ki67, but an incremental increase in Ki67 expression was noted upon *SNF5* loss, including an approximately ten-fold increase in Ki67 expression in clonal *SNF5*-deficient T cells (Fig. 2D, E). As both antigen- and costimulatory receptors have been implicated in PTCL pathogenesis and proliferation (5, 6), expression of both T-cell receptor inducible and costimulatory receptors was examined (Supplementary Table 2), many of which were highly expressed by clonal T cells (Supplementary Figure 4B). However, given the observed enrichment in Notch-related transcripts upon malignant transformation, we further examined the expression of cell surface Notch1 and Notch2 and intranuclear, cleaved Notch1 in malignant T cells. In contrast to either littermate controls or non-clonal T cells, malignant T cells highly expressed both cell surface Notch1 and Notch2 as assessed by flow cytometry (Fig. 3A, B), and cleaved intranuclear NICD1 by immunohistochemistry (Fig. 3C).

Notch signaling promotes the proliferation of malignant T cells

To examine the extent to which Notch signaling promotes the proliferation of malignant T cells in this model, the expansion and proliferation of clonal T cells was examined following short-term, combined Notch1/Notch2 blockade (Fig. 4A–D). As Notch1 and Notch2 are partially redundant (28), and in an effort to completely block Notch signaling, combined Notch1/Notch2 blockade was utilized in these experiments. Upon short-term (7-9 day) Notch blockade, a significant decrease in spleen and liver weights was observed (Fig. 4A, B), and a concordant reduction in total and Ki67$^+$ clonal T cells was similarly observed (Fig. 4C, D). Similar findings were obtained using a previously described (15), spontaneous murine PTCL model (Supplementary Figure 6A–D). As p53 deletions are recurrently observed in a high-risk subset of PTCL, NOS expressing the transcription factor GATA-3(4), we sought to investigate the role of Notch signaling in p53- and *SNF5* double-deficient mice. *SNF5* and p53 floxed mice were intercrossed, generating *SNF5*$^{fl/fl}$, p53$^{fl/fl}$, CD4-Cre mice that also developed PTCL, albeit with shorter latency, as the median time to PTCL development was 156 days in p53+ and 65 days in p53− mice ($p<0.0001$, Supplementary Figure 6E). While Notch1/Notch2 blockade appeared to reduce tumor burden irrespective of p53 status (Fig. 4A–D), we performed additional experiments exclusively in p53-deficient lymphomas. Also, non-canonical and ligand-independent Notch signaling has been described in conventional T cells (29, 30). Therefore, to further examine the role of Notch signaling in p53-deficient PTCL, and to examine whether Notch signaling was ligand dependent, similarly performed experiments were performed exclusively in p53-deficient lymphomas using antagonistic DLL1 and DLL4-specific antibodies. Similar findings to those obtained upon Notch1/Notch2 blockade were observed (Fig. 4E–H), as well as in

a spontaneous murine PTCL model (Supplementary Figure 7A–D). Collectively, these findings directly implicate DLL1/DLL4-dependent Notch signaling in PTCL progression, and in a “high-risk” genetic context.

Pan-Notch blockade significantly reduces PTCL prevalence and prolongs survival

In an effort to achieve T-cell specific, pan-Notch blockade, and to exclude any non-cell-autonomous effects of antibody-based Notch blockade, we introduced a GFP-tagged, dominant-negative MAML (DNMAML) in the *SNF5* model to inhibit Notch signaling downstream of all Notch receptors in mature T cells, as previously described (31). Impaired canonical Notch signaling in T cells obtained from DNMAML mice led to significant transcriptional changes, including significant decreases in cell cycle and proliferation-related transcripts (Fig. 5A, B; Supplementary Table 4). Downregulated expression of selected, well-established Notch target genes was observed (e.g *Dtx1, Hes1, IL2ra*). Concordant changes were observed in independently validated replicates and upon short-term Notch blockade for selected Notch targets (e.g Dtx1, Fig. 5C), but was variably observed for other genes (Fig. 5C, Supplementary Figure 8). GATA-3 has been recently implicated in PTCL pathogenesis (1, 2, 5), and may be a Notch target gene in certain contexts (32, 33). Thus, it is notable that pan-Notch blockade (in DNMAML mice) and antibody-based Notch1/Notch2 blockade were associated with relatively modest, and variable, alterations in GATA-3 expression, suggesting that alternative transcriptional regulators may promote its expression (Fig. 5C). We selected the top 50 differentially expressed transcripts that were downregulated upon pan-Notch blockade in DNMAML mice, and utilized these transcripts to generate a “Notch signaling” activity score. A significant enrichment in these Notch target genes was observed in PTCL, NOS specimens when compared to benign lymph nodes (Fig. 5D).

Importantly, the loss of Notch signaling in this GEM decreased the cumulative incidence of PTCL and prolonged survival in mice that eventually developed PTCL (Fig. 5E). Splenocytes from lymphoma-bearing wildtype (DNMAML negative) and DNMAML mice were adoptively transferred into B6 recipients in order to further examine the role of Notch signaling in regulating malignant T cell engraftment and progression. As anticipated, all recipient mice succumbed to PTCL progression within ≈50 days following adoptive transfer of splenocytes from wildtype (DNMAML negative) mice. In contrast, disease progression was significantly delayed upon adoptive transfer of DNMAML cells (Fig. 5F), and engraftment was not observed in ≈50% of these recipients with >150 days of follow up, further highlighting the importance of Notch signaling in PTCL progression.

Discussion

Gain-of-function Notch mutations, observed in most T-ALL (8), are rarely observed in most PTCL (34), with the exception of both anaplastic large cell lymphomas, where recurrent Notch1 mutations involving the extracellular domain have been observed in ≈18% of cases (35), and ATLL (16). Furthermore, and consistent with our results, loss of Notch expression in ALCL cell lines significantly impaired cell proliferation (35). Apart from ALCL, many

of which are primarily driven by oncogenic NPM-ALK signaling, Notch's role in PTCL pathogenesis more generally, or suitability as a therapeutic target, are largely unexplored.

In order to address these questions, we scrutinized the spontaneous PTCL that emerged in mice upon conditional deletion of *SNF5* (SMARCB1). Constituents of the SWI/SNF complex are commonly deleted or mutated in human PTCL, as deletions or mutations in at least one member of the SWI/SNF complex are observed in mature T-cell lymphomas (21, 22, 24, 36). Given the prevalence of p53 deletions in PTCL(4), we conditionally deleted p53 in this model to not only bolster its genetic relevance for human PTCL, but to also examine the importance of Notch signaling in an otherwise "high risk" genetic context. We observed upregulated Notch1 and Notch2 expression among clonal T cells that was further associated with increased transcription of selected Notch target genes upon *SNF5* loss. Therefore, we utilized complementary pharmacologic and genetic approaches to demonstrate that a Notch-dependent transcriptional program promotes PTCL proliferation and disease progression.

While Notch blockade led to anticipated changes in a subset of canonical Notch target genes previously identified in T-ALL or conventional T cells, similar changes were not observed in others, thus highlighting both the context-dependent nature of Notch driven transcriptional regulation and the likely heterogeneity of Notch-dependent transcription across the spectrum of conventional (non-malignant) T cells, thymocyte-derived malignancies, and mature (post-thymic) T-cell lymphomas (37). As most dynamically regulated Notch target genes are distally regulated upon Notch binding to distal enhancer elements (38), it seems quite likely that the variability in the Notch target genes identified may be attributed to distinct enhancer landscapes across the spectrum of T-cell derived malignancies. In addition, Notch may collaborate with recurrent epigenetic/genetic alterations observed in PTCL, including SWI/SNF loss (39). The SWI/SNF complex antagonizes epigenetic silencing mediated by members of the Polycomb group, including EZH1 and EZH2, the catalytic subunit of the PRC2 Polycomb repressor complex. Failure to displace Polycomb complexes and to reverse repressive histone modifications in SWI/SNF deficient cells leads to widespread H3K27 trimethylation, including at the Cdkn2a locus, which suppresses cell proliferation in normal cells, and is commonly deleted in PTCL (40). Loss of *SNF5* further promotes EZH2 expression, which is highly expressed in >90% of peripheral T-cell lymphomas (41), and was observed in our model (Supplemental Table 3). The extent to which EZH2 may collaborate with Notch, as observed in other malignancies (42), is unknown, but merits further study, as EZH1/EZH2 inhibitors are now being explored as therapeutic vulnerabilities in PTCL. Similarly, Notch signaling may also collaborate with alternative signaling pathways in the PTCL, including antigen- and cytokine-receptor dependent signaling.

Despite the context-dependent nature of Notch-dependent transcription and the need for future studies, DTX1 (Deltex1) has been identified as a Notch target gene in both conventional T cells, T-ALL and ALCL (10, 35, 38), and was the most significantly differentially expressed Notch-dependent gene we identified. Deltex1, an E3 ubiquitin ligase, negatively regulates Notch signaling in both a ubiquitin and non-ubiquitin-dependent manner (43, 44). While mutations in Deltex1 have been associated with poor outcomes in aggressive B-cell lymphomas (45), and while Deltex1 is not required for normal T-cell

development (46), Deltex1 promotes cell proliferation in other malignancies (47), and may confer resistance to corticosteroids in collaboration with Notch (48, 49). GATA-3 has also been reported to be a canonical Notch target gene in T cells in certain contexts. However, and in contrast to Deltex1, changes in GATA-3 transcripts were modest, and variably observed, upon Notch blockade in this model.

While our findings highlight a role for Notch in PTCL pathogenesis, and are consistent with both the increasingly appreciated role of Notch signaling in mature T cells generally (10), and the largely anecdotal evidence of cleaved, intranuclear Notch1 in PTCL cell lines and primary specimens (11, 50), our findings also have significant limitations. (19)For example, future studies will be needed to clarify the relative contribution of DLL1 and DLL4, and the cells that present these Notch ligands, within a PTCL context. Also, we cannot exclude a non-cell-autonomous role for Notch signaling in PTCL pathogenesis by promoting LAM survival and expansion, for example, as this has been suggested in alternative contexts (51), and LAM are relevant constituents of the TME that drive PTCL pathogenesis. While malignant T cells expressed DLL1 (Supplementary Figure 4B), we cannot exclude the contribution of alternative DLL1 (and/or DLL4)-expressing cells within the tumor microenvironment, including myeloid-derived cells or fibroblastic reticular cells (52). In addition, we did not examine the mechanism(s) driving increased Notch expression in clonal T cells, although STAT3-dependent transcription (2, 35), or copy number gains or gene amplification (4, 53), may be reasonably entertained in future studies.

In summary, the paucity of *bona fide* PTCL models with characteristics resembling the most common subtypes of human PTCL have hampered discovery in this field (54). Herein, using genetically engineered mouse models with genetic landscapes resembling aggressive and chemorefractory human PTCL, we identified ligand-dependent Notch signaling as a significant driver of PTCL pathogenesis. These findings have significant therapeutic implications for the use of antibody-based (or pharmacologic) Notch antagonists in a group of aggressive lymphomas for which novel therapeutic approaches are sorely needed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Significance:

This work demonstrates that ligand-dependent Notch activation promotes the growth and proliferation of mature T-cell lymphomas, providing new therapeutic strategies for this group of aggressive lymphomas.

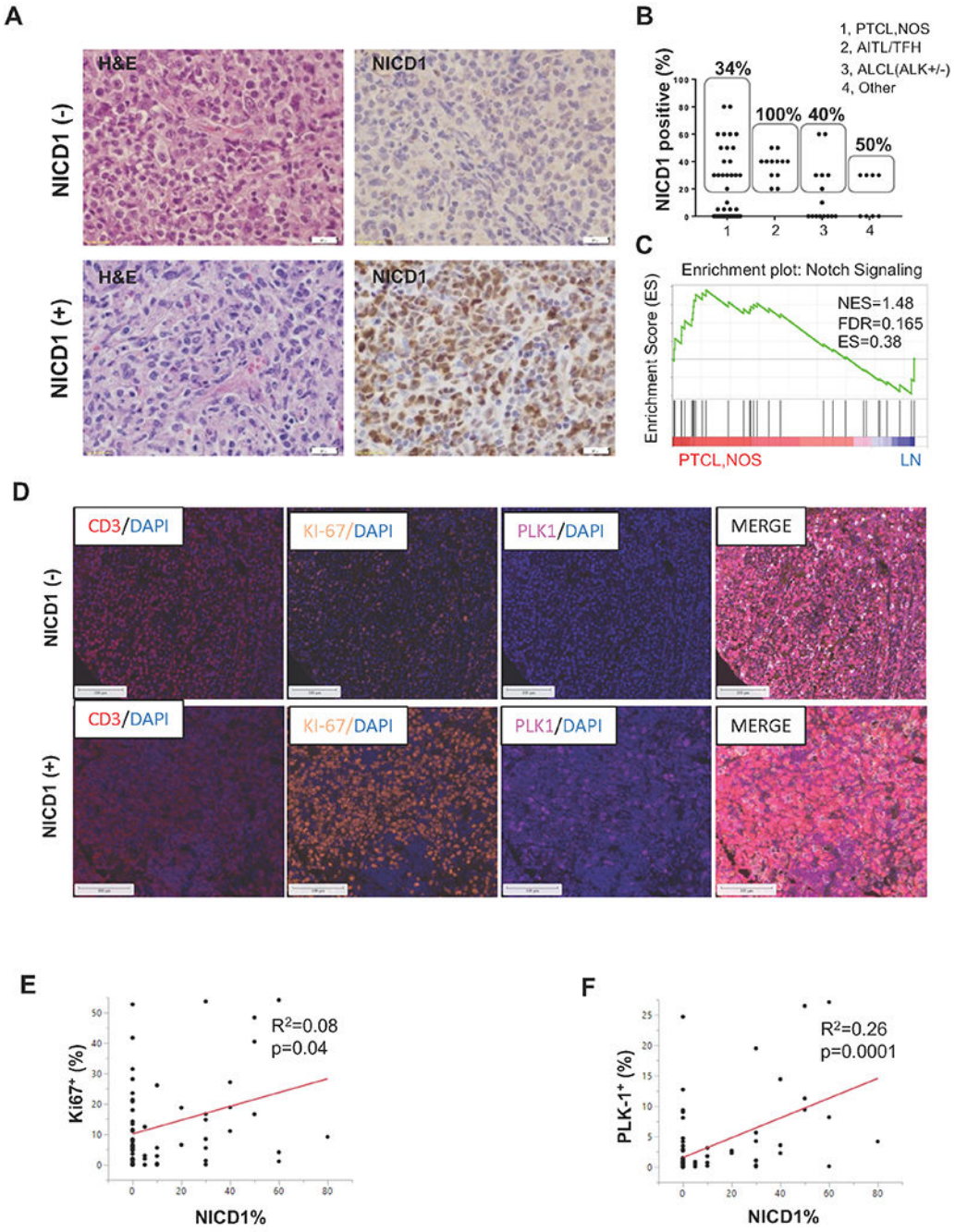

Figure 1.

NICD1 is expressed in PTCL, NOS and associated with proliferation. (A, B) Diagnostic biopsies from PTCL, NOS (n=56), AITL/TFH (n=12), ALCL (ALK+ and ALK−, n=15), and less common PTCL subtypes (n=8) were stained for NICD1. A representative NICD1 negative and NICD1 positive PTCL, NOS case are shown in (A) and the data summarized in (B). At least 20% of malignant T cells (as determined by morphologic and immunophenotypic assessment) were NICD1+ in 100% of AITL cases examined. NICD1 expression was similarly determined in the other subtypes indicated, and ≥20% positivity

utilized as a cut-off, and the percentage of NICD+ cases indicated (in B). (C) Notch pathway enrichment analysis was performed using microarray data obtained from PTCL, NOS and benign lymph nodes. (D) Multispectral imaging of CD3+CD8− PTCL, NOS cases (n=50), stratified by the extent of NICD1 expression, was performed using lymphoma (CD3, CD8, CD20)-, TME (CD68, CD163)-, and proliferation (Ki67, PLK1)-informative markers. CD3, Ki-67, and PLK1 stains in representative NICD1− and NICD1+ cases are shown. (E-F) As before, the extent of NICD1 expression as a percentage of PTCL cells was determined by immunohistochemistry. The proliferation-associated markers Ki67 (E) and PLK-1 (F), expressed as a percentage of $CD3^{+}CD8^{-}CD20^{-}$ PTCL cells (E, F) are shown and correlated with NICD1 expression.

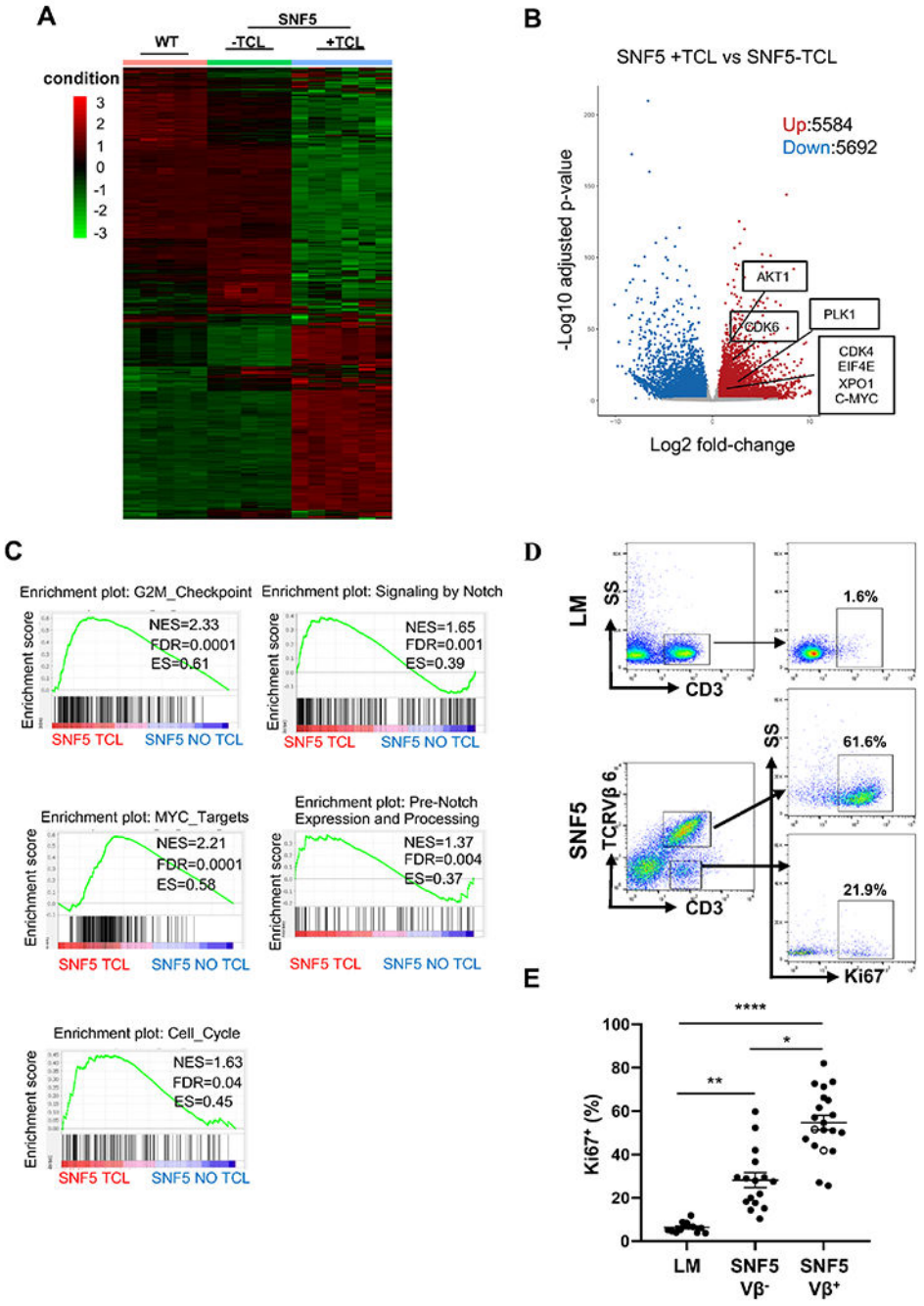

Figure 2.

PTCL progression in *SNF5*-deficient T cells is associated with transcriptional reprogramming. (A) Supervised hierarchical clustering of RNA-seq was performed in sorted CD3+ T cells from littermate control (WT, n=5), CD3+ T cells from young (<4 months of age) *SNF55*$^{fl/fl}$, CD4-cre mice without PTCL (*SNF5*-TCL, n=5), and clonal T cells (CD3^{+} TCR-Vβ^{+}) from older (> 4 months of age) *SNF5*$^{fl/fl}$, CD4-cre mice with PTCL (*SNF5* +TCL, n=6). Heat map showing differentially expressed genes is shown. (B) A volcano plot comparing gene expression between *SNF5*$^{fl/fl}$, CD4-cre mice without TCL

and clonal T cells from older *SNF5*$^{fl/fl}$, CD4-cre mice with TCL is shown. (C) Gene set enrichment analysis (GSEA) was performed and enrichment plots for selected pathways, including those that are Notch (shown at right) and c-myc or cell cycle related (shown at left), enriched in *SNF5*$^{fl/fl}$, CD4-cre mice with PTCL are shown. (D) Clonal (TCR-Vβ^{+}) and non-clonal (TCR-Vβ^{-}) T cells were identified by TCR-Vβ and CD3 expression in lymphoma-bearing mice (*SNF5*, n=20) and Ki67expression determined by flow cytometry. Open circles represent samples from *SNF5*$^{fl/fl}$, p53$^{fl/fl}$, CD4-Cre^{+} mice (n=2). (Non-clonal T cells were not observed, or were quantitatively insufficient for gating, in these mice.) Littermate control mice (LM, n=11) were stained with CD3 and Ki67. A representative example is shown in (D), and the data summarized in (E) (* P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001)

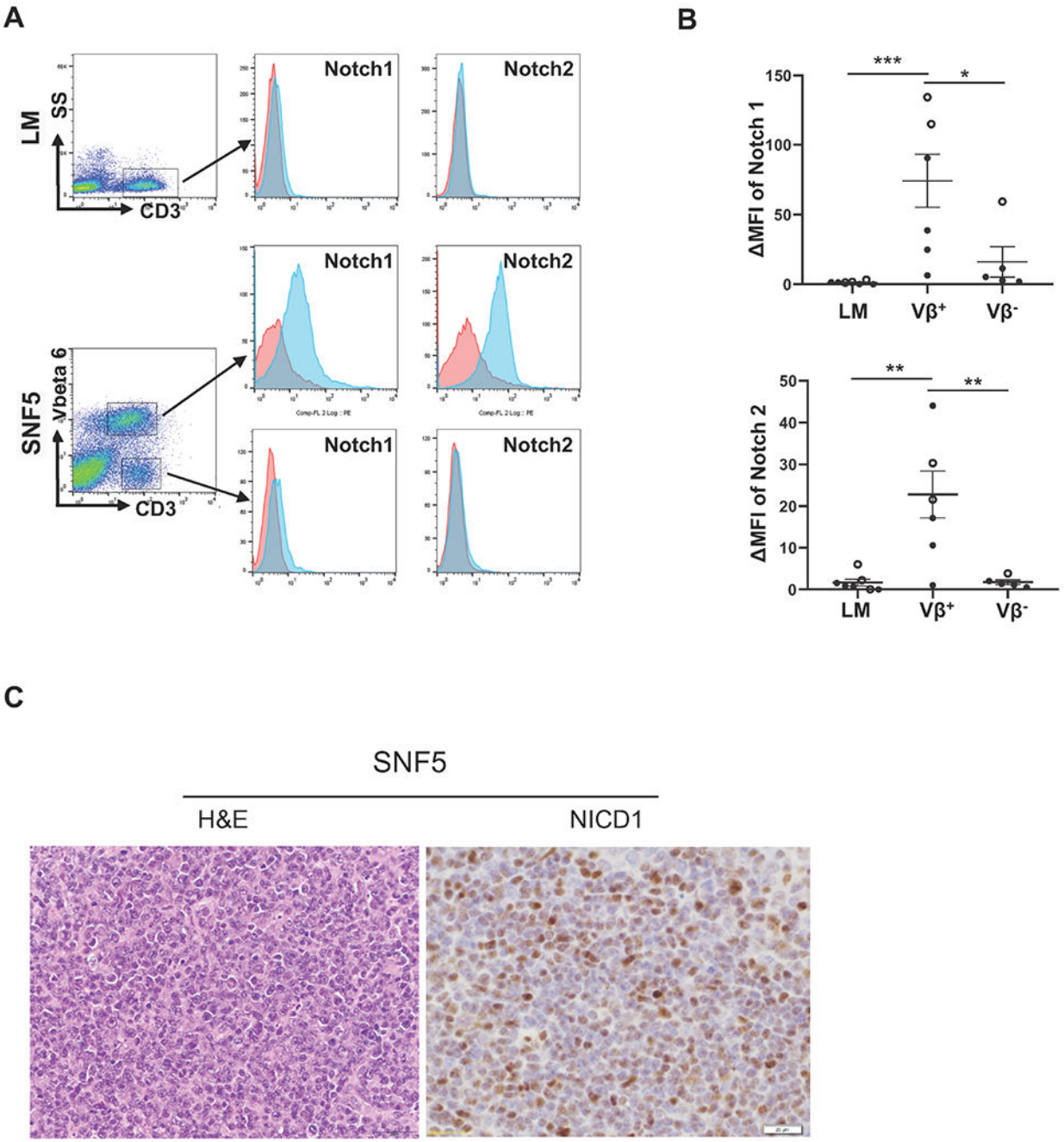

Figure 3.

Notch 1 and Notch 2 are highly expressed by PTCL cells. (A, B) The expression of Notch1 and Notch2 (isotype control, orange histogram; anti-Notch1/Notch2, blue histogram) were examined in littermate control (LM, n=5) and lymphoma-bearing *SNF5*$^{fl/fl}$ p53$^{+/+}$, CD4-Cre^{+} (*SNF5*, closed circles, n=4) and *SNF5*$^{fl/fl}$, p53$^{fl/fl}$, CD4-Cre^{+} (open circles, n=2) mice. A representative example is shown in (A). Notch1 and Notch2 expression, expressed as ΔMFI was examined on both clonal (Vβ^{+}) and non-clonal (Vβ^{-}) T cells, as summarized in (B). (*P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001). (C) Biopsies from LM and

SNF5 mice were stained for the Notch intracellular domain (NICD), a representative case is shown.

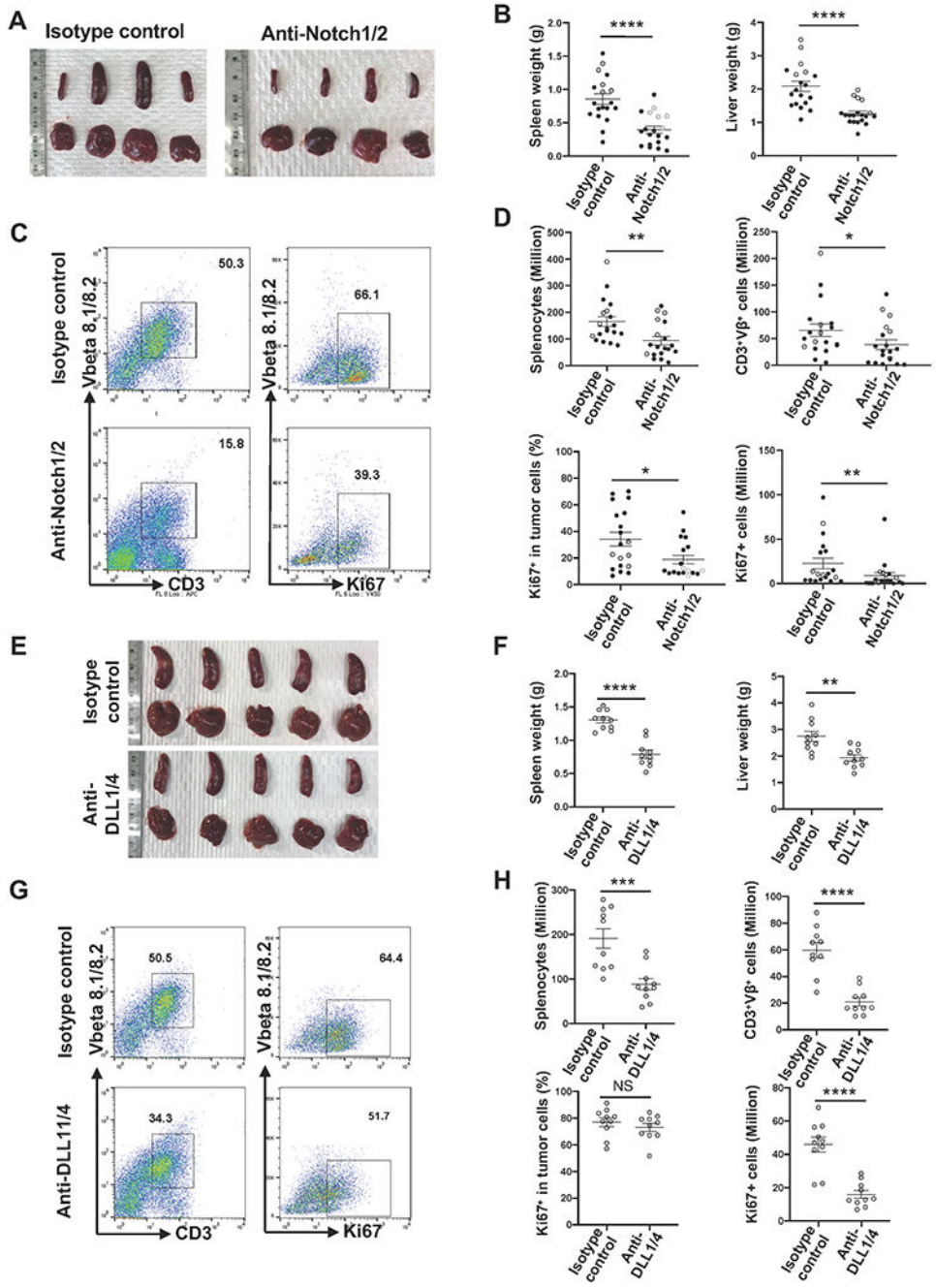

Figure 4.

Notch signaling promotes the proliferation of malignant T cells. (A-D), Splenocytes from lymphoma-bearing *SNF5*$^{fl/fl}$, p53$^{+/+}$, CD4-Cre^{+} (closed circles, •) or *SNF5*$^{fl/fl}$, p53$^{fl/fl}$, CD4-Cre^{+} (open circles, ○) mice were adoptively transferred into C57BL/6J recipient mice (n=4-5 recipients/experimental group for 4 biological replicates) and recipient mice were treated with isotype control or anti-Notch1/Notch2 antibodies on day 0 and 2 upon engraftment and euthanized 5-7 days after the last dose. Explanted spleens and livers were weighed, a representative example is shown in (A), summarized organ weight is shown

in (B). Clonal T cells ($CD3^+V\beta^+$) and Ki67 expression were quantified. A representative example is shown in (C), and summarized in (D). (E-H) Splenocytes from lymphoma-bearing *SNF5*$^{fl/fl}$, p53$^{fl/fl}$, CD4-Cre$^+$ (open circles) mice were adoptively transferred into C57BL/6J recipient mice (n=5 recipients/experimental group for 2 biological replicates) and recipient mice were treated with isotype control or anti-DLL1/DLL4 (Anti-DLL1/4) antibody at day 0, 2, and 7 upon engraftment and euthanized 5-7 days after last dose. Explanted spleens and livers were weighed, a representative example is shown in (E), and organ weight summarized in (F). Clonal (Vβ+) T cells and Ki67 expression was quantified. A representative example is shown in (G), and summarized in (H). (* P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001)

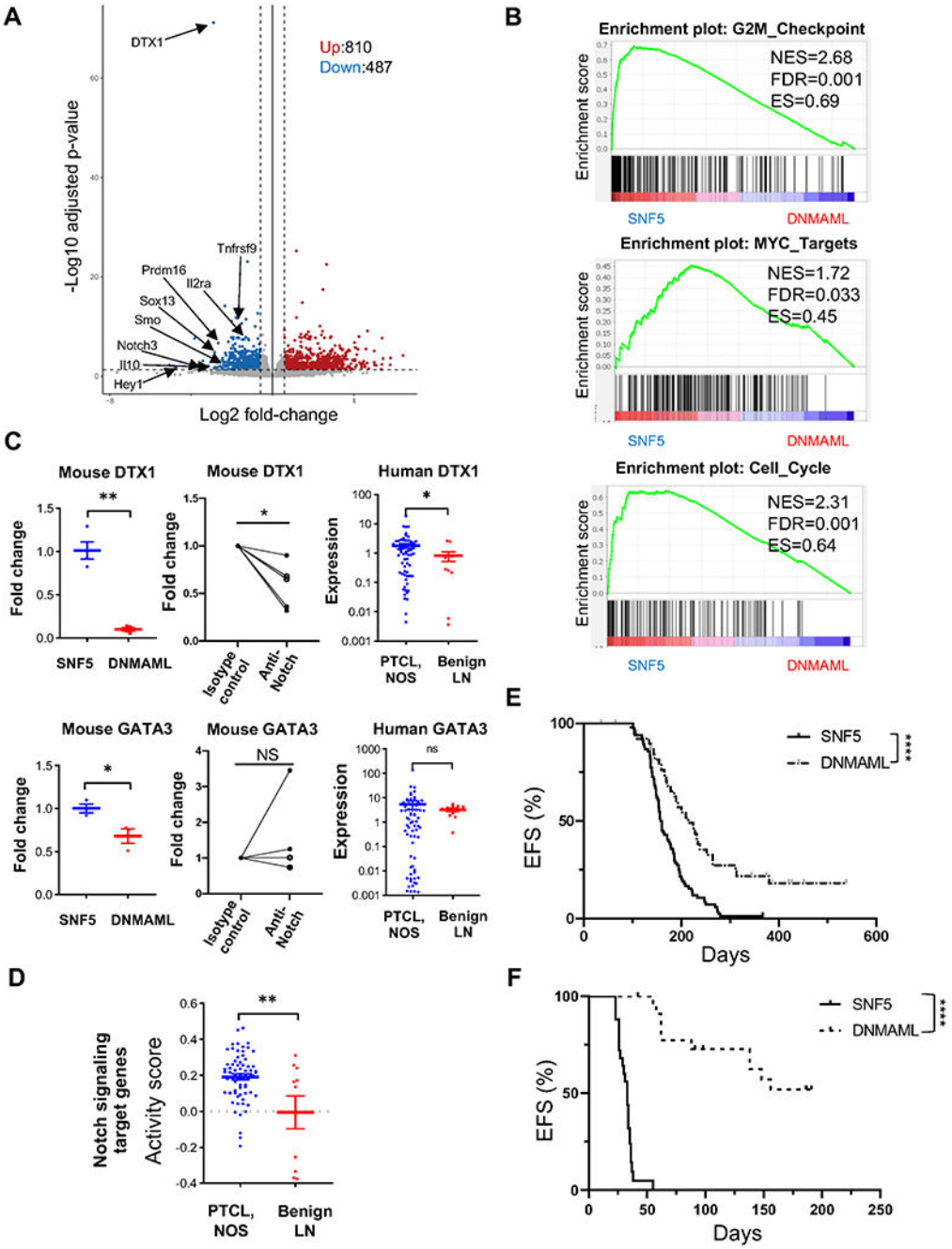

Figure 5.

Notch blockade in PTCL inhibits cell proliferation and impairs disease progression. (A) T cells were sorted from *SNF5*$^{fl/fl}$, DNMAML$^{+/+}$, CD4-Cre (n=5) and *SNF5*$^{fl/fl}$, DNMAML$^{fl/fl}$, CD4-Cre (n=3) mice and RNA-seq was performed. A volcano plot comparing gene expression is shown. (B) Gene set enrichment analysis (GSEA) was performed and enriched pathways are shown. (C) DTX1 and GATA-3 expression was similarly examined in T cells obtained from DNMAML negative ("Notch on") and DNMAML positive ("Notch off") T cells, (left). Clonal T cells (CD3^{+}Vβ^{+}) from short-term

isotype control, anti-Notch1/2 or anti-DLI1/4 antibody treated mice were sorted, mRNA was extracted and qRT-PCR was performed to examine the expression of selected Notch target genes. DTX1 and GATA3 expression in treatment group (anti-Notch1/2: closed circles; anti-DLL1/4: open circles) relative to control group is shown (middle). DTX1 and GATA-3 expression in human PTCL, NOS and benign lymph nodes was examined, as indicated (right). (D) Activity score of Notch signaling target genes for human PTCL, NOS (n=68) and benign (n=10) specimens is shown. (E) Event-free survival of *SNF5*$^{fl/fl}$, DNMAML$^{+/+}$, CD4-Cre^{+} (n=89) and *SNF5*$^{fl/fl}$ DNMAML$^{fl/fl\ or\ fl/+}$, CD4-Cre^{+} (n=38) mice is shown. (F) Splenocytes from lymphoma-bearing *SNF5*$^{fl/fl}$, DNMAML$^{+/+}$, CD4-Cre^{+} (n=5) and *SNF5*$^{fl/fl}$ DNMAML$^{fl/fl\ or\ fl/+}$, CD4-Cre^{+} (n=4) mice were adoptively transferred into B6 recipients (n=4-5/biologic replicate) and mice were followed for event-free survival (EFS). (* P<0.05, ** P<0.01, ***P<0.001, ****P<0.0001)