NEOPLASIA
www.neoplasia.com

Volume 16 Number 12 December 2014 pp. 1007–1017 1007

Notch3/Jagged1 Circuitry Reinforces Notch Signaling and Sustains T-ALL[1,2]

Maria Pelullo*, Roberta Quaranta*, Claudio Talora*, Saula Checquolo[†], Samantha Cialfi*, Maria Pia Felli[‡], Geertruy te Kronnie[§], Chiara Borga[§], Zein Mersini Besharat*, Rocco Palermo[||], Lucia Di Marcotullio*, Anthony J. Capobianco[¶,#], Alberto Gulino*,[||],, Isabella Screpanti*,[||],[††] and Diana Bellavia***

*Department of Molecular Medicine, Sapienza University, Rome, Italy; [†]Department of Medico-Surgical Sciences and Biotechnologies, Sapienza University, Latina, Italy; [‡]Department of Experimental Medicine, Sapienza University, Rome, Italy; [§]Department of Women's and Children's Health, University of Padova, Padova, Italy; [||]Center for Life Nano Science@Sapienza, Istituto Italiano di Tecnologia, Rome, Italy; [¶]Molecular Oncology Program, DeWitt Daughtry Family, Department of Surgery; Miami, FL, USA; [#]Department of Biochemistry and Molecular Biology, Sylvester Comprehensive Cancer Center, University of Miami Miller School of Medicine, Miami, FL, USA; **Neuromed Institute, Pozzilli, Italy; [††]Institute Pasteur-Foundation Cenci Bolognetti, Sapienza University, Rome, Italy

Abstract

Deregulated Notch signaling has been extensively linked to T-cell acute lymphoblastic leukemia (T-ALL). Here, we show a direct relationship between Notch3 receptor and Jagged1 ligand in human cell lines and in a mouse model of T-ALL. We provide evidence that Notch-specific ligand *Jagged1* is a new Notch3 signaling target gene. This essential event justifies an aberrant Notch3/Jagged1 cis-expression inside the same cell. Moreover, we demonstrate in Notch3-IC–overexpressing T lymphoma cells that Jagged1 undergoes a raft-associated constitutive processing. The proteolytic cleavage allows the Jagged1 intracellular domain to empower Notch signaling activity and to increase the transcriptional activation of Jagged1 itself (autocrine effect). On the other hand, the release of the soluble Jagged1 extracellular domain has a positive impact on activating Notch signaling in adjacent cells (paracrine effect), finally giving rise to a Notch3/Jagged1 auto-sustaining loop that supports the survival, proliferation, and invasion of lymphoma cells and contributes to the development and progression of Notch-dependent T-ALL. These observations are also supported by a study conducted on a cohort of patients in which Jagged1 expression is associated to adverse prognosis.

Neoplasia (2014) 16, 1007–1017

Introduction

Notch receptors and their specific ligands play a key role in the early development of multicellular organisms and deregulated Notch signaling can cause human diseases, including cancer [1,2]. The mammalian Notch signaling network consists of four transmembrane

Address all correspondence to: Diana Bellavia, PhD and Isabella Screpanti, MD, Laboratory of Molecular Pathology, Department of Molecular Medicine, Sapienza University of Rome, Viale Regina Elena 291, 00161 Rome, Italy.
E-mail: diana.bellavia@uniroma1.it; isabella.screpanti@uniroma1.it

[1] This article refers to supplementary materials, which are designated by Figures S1 to S3 and are available online at www.neoplasia.com.

[2] This study was supported by the Italian Association for Cancer Research (AIRC; IG13314), the Italian Ministry of University and Research (MIUR), FIRB-RBAP11WCRZ and PRIN Programs (2010MCLPLB), the European Union (FP7-MC-ITN 215761-NotchIT), and Fondazione Roma. No potential conflicts of interest were disclosed.
Received 18 June 2014; Revised 1 October 2014; Accepted 6 October 2014

1476-5586/14

http://dx.doi.org/10.1016/j.neo.2014.10.004

receptors (Notch 1, 2, 3, and 4), which can interact with five transmembrane Notch ligands (Jagged1 and 2 and Delta-like 1, 3, and 4) [3,4]. Under physiological conditions, the activation of Notch signaling requires the binding "in trans" between Notch receptors, expressed on the surface of signal-receiving cells, with Notch ligands, expressed on the surface of adjacent signal-sending cells. Such an interaction renders Notch susceptible to proteolytic processing that ends in the release of its intracellular domain (Notch-IC) [5,6]. Notch-IC moves into the nucleus, where it directly interacts with the Recombination signal Binding Protein for immunoglobulin kappa J region (RBP-Jκ) transcription factor [7,8] and recruits co-activators to transcriptionally activate several downstream effectors, such as the ubiquitous hairy and enhancer of split [8] and the T cell precursor–specific pTalpha (pTα) [9].

Like Notch, Jagged1, after binding, becomes a substrate for proteolysis by a member of A Disintegrin And Metalloprotease (ADAM) family. The ADAM17 activity allows the shedding of the ectodomain fragment (sJag1-ECD) and generates a membrane-tethered intracellular domain (Jag1-TMICD) [10–12]. Then, the Jag1-TMICD fragment undergoes an intramembrane cleavage mediated by presenilin/γ-secretase complex activity that releases a soluble intracellular fragment (Jag1-ICD) [11], which moves into the nucleus [13]. Intriguingly, it has been suggested that Jag1-ICD plays a role in controlling the expression of both Jagged1 itself and Notch3 mRNAs and contribute to transformation of E1A-immortalized baby rat kidney (RKE) cells, although through an unknown molecular mechanism [13]. Consistent with this original observation, it has been reported that the overexpression of Jagged1 protein was associated with a poor prognosis in several human tumors [14–16]. Moreover, it has been suggested that Jagged1/Notch3 co-expression could be important for the malignant transformation [17–21].

In this report, we show the existence of a direct relationship between Notch3 receptor and Jagged1 ligand in T cell acute lymphoblastic leukemia (T-ALL) context. Our data first suggest that *Jagged1* is a novel Notch3 target gene and the enforced expression of Notch3-IC is able to determine a cell membrane lipid raft–associated constitutive processing of Jagged1. Second, we show a Notch3/Jagged1 cis-interaction within the same cell, which results in the autocrine reinforcement of Notch3 signaling. Finally, the shedding of sJag1-ECD trigged by the ADAM17 activity ends in the paracrine amplification of Notch signaling in adjacent cells. These findings suggest that the dysregulated expression and processing of Jagged1 protein, sustained by Notch3, takes part in a multistep oncogenic process, playing a role in controlling cell growth, apoptosis, and migration, favoring tumor aggressiveness and progression.

Materials and Methods

Mice

The generation and typing of Notch3-IC transgenic (N3-ICtg) mouse have been described elsewhere [22]. The studies involving animals have been conducted following the Italian National Guidelines for Animal Care, established in DL No. 26, 2014 and in accord with Directive 2010/63/UE.

Cell Culture and Treatments

HEK-293T, preT 2017 [23], SCB29 [24], N3-232T [22], KE37, Molt3, Dnd4.1, Cem, Jurkat, P12-Ichikawa, SIL-ALL, and Kopkt [25,26] were maintained as described elsewhere. An opportune amount of cells was treated with different compounds: 10 mM methyl-β-cyclodextrin (MβCD; Sigma-Aldrich, St. Louis, MO, USA) for 30 minutes at 37°C before harvesting, 10 μM TAPI-2 (Peptides International, Louisville, KY, USA), or 5 μM γ-secretase inhibitor I (GSI-I; Calbiochem, Darmstadt, Germany), for the times indicated in the figures. The SCB29 cells were grown in N3-232T cell culture-conditioned medium (CCM) in the presence or absence of both 10 μM GSI-I for 12 hours before processing and 10 μg/ml anti-CD339 Jagged1 neutralizing antibody (eBioscience, San Diego, CA, USA).

Plasmids

The two putative *Jagged1* promoter regions were amplified by polymerase chain reaction (PCR) from N3-232T genomic DNA (ENSMUST00000028735), using specific primers containing *Mlu*I and *Bgl*II restriction sites. The following primers were used to amplify murine pJ1pro$^{-3794/-2156}$ and murine pJ1pro$^{-1351/-237}$, respectively: 5′-GCGCACGCGTACCATCTCCTTCTCCCATCTCCT-3′ and 5′- GCGCAGATCT ACCGGACTTCTT ACCTGCA TCTC-3′; 5′- GCGCACGCGT ACCA TCACCT ATCTCAGGTGGTT AA AA TG-3′ and 5′-GCGCAGATCTACCGATCAGTGTCCCGGG GAG-3′. Briefly, both DNA fragments were amplified for 30 cycles and then purified by means of a Qiagen quick spin column. The DNA fragments amplified were digested using *Bgl*II (New England BioLabs, Ipswich, MA, USA) and *Mlu*I (New England BioLabs) enzymes and ligated into pGL3 Basic Vector (Promega, Madison, WI). Cloned PCR products were verified by sequencing. The following plasmids were kindly provided by A.J. Capobianco: pBABE-Ser^{TMIC} (Jagged1-TMICD) and pBABE-Jagged1 WT (Jagged1-FL). Furthermore, the expression vectors for CMV-Notch3-IC, CMV-Notch1-IC, RBP-Jκ, Flag-MAML1, and pTα-luc were previously described [22,27].

Cell Transfection and Luciferase Assay

Transient transfection of preT 2017 and HEK-293T cell lines with pTα-luc, pJ1pro$^{-3794/-2156}$, or pJ1pro$^{-1351/-237}$ reporter plasmids with different combinations of the expression vectors, described above, was performed by using the Lipofectamine 2000 kit (Life Technologies, Carlsbad, CA, USA, Invitrogen), according to the manufacturer's instructions. pcDNA3 has been used as a control empty vector. Renilla luciferase reporter vector, pTK-Renilla-Luc, was also incorporated into each transfection for normalization (Promega). For luciferase assay, Firefly- and pRL-TK–derived Renilla luciferase activities were measured in each sample with the Dual Luciferase Assay System (Promega) on GloMax (Promega).

RNA Interference Analysis

The target sequences of small interfering RNA (siRNA) oligonucleotide probes for human Notch3 were purchased from Dharmacon, GE Healthcare, Lafayette, CO, USA, Thermo Fisher Scientific, Waltham, MA USA (L-011093-00-0005). Human Molt3, P12-Ichikawa, SIL-ALL, and Kopkt cell lines at subconfluence were transfected with the indicated siRNA using the Neon Transfection System (Invitrogen).

Protein Extract Preparation, Immunoprecipitation, and Immunoblot Analysis

Protein extract preparation, immunoprecipitation assay, and sucrose gradient for raft isolation were performed as described elsewhere [28]. For immunoblot analysis, protein extracts were separated by NuPage 4% to 12% Bis-Tris gels (Life Technologies, Invitrogen), transferred to a nitrocellulose membrane, and probed with the following primary

antibodies: anti–α-tubulin (Santa Cruz Biotechnology), anti-p56^{Lck} (Santa Cruz Biotechnology), anti-Notch3 M20 (Santa Cruz Biotechnology), anti–lamin B (Santa Cruz Biotechnology), anti-HA (Santa Cruz Biotechnology, Dallas, TX, USA), anti-Jagged1 C20 (Santa Cruz Biotechnology), anti–β-actin (Sigma-Aldrich), anti–c-Myc (Sigma-Aldrich) and anti-Flag (Sigma-Aldrich), anti-ADAM17 (Abcam, Cambridge, MA, USA), anti-Jagged1 N-terminal (Abcam), and anti–RBP-Jκ (Cell Signaling Technology, Beverly, MA, USA). Bound antibodies were detected with enhanced chemiluminescence (ECL kit, Amersham, GE Healthcare, Lafayette, CO, USA).

Extracellularly Shed Protein Preparation

To detect the extracellular soluble Jagged1 (sJag1-ECD) protein into the mice serum, an appropriate amount of whole blood was collected in a Vacutainer covered test tube (Becton Dickinson, BD, San Jose, CA, USA). After collection, the blood is allowed to clot by leaving it at room temperature for 30 minutes. Then, the blood was centrifuged at 300*g* for 15 minutes and the resulting supernatant fraction was transferred in a clean polypropylene tube using a Pasteur pipette. Conversely, to identify sJag1-ECD in N3-232T CCM, an amount of CCM was collected in a polypropylene tube and centrifuged at 200*g* for 10 minutes. For immunoblot analysis, soluble proteins were separated by NuPage 4% to 12% Bis-Tris gels (Life Technologies, Invitrogen), transferred to a nitrocellulose membrane, and probed with anti-Jagged1 N-terminal (Abcam) antibody.

RNA Analysis, Reverse Transcription–PCR, and Quantitative Real-Time Reverse Transcription–PCR

Total RNA was extracted from unfractioned T lymphocytes (both from thymi and splenocytes), from untransfected preT 2017 [23] and N3-232T cell lines [22] using MiniKit RNA Extraction (Qiagen, Germantown, MD, USA) according to the manufacturer's instructions. A 1-μg portion of RNA was processed for reverse transcription (RT)–PCR as previously described [27]. Quantitative real-time RT-PCR (qRT-PCR) analysis of pTα, Jagged1, and HPRT mRNA was performed on cDNA using TaqMan gene expression assays according to the manufacturer's instructions (Applied Biosystems, Life Technologies Brand, Carlsbad, CA, USA) and using the ABI Prism 7900HT (Applied Biosystems). mRNA quantification was expressed, in arbitrary units, as the ratio of the sample quantity to the calibrator or to the mean values of control samples.

Immunofluorescence Staining

To detect Jagged1 and Notch3, N3-232T cells were plated onto polylysine-coated Lab-Tek chamber slides. Briefly, cells were fixed with 4% paraformaldehyde for 20 minutes at room temperature, incubated first with 1 M glycine for 15 minutes and then with 0.2% Triton X-100 and successively in blocking solution (1% BSA). Cells were stained with primary antibodies diluted in blocking solution for 1 hour at room temperature; after three washes, the cells were incubated with secondary antibodies. Primary antibodies were goat anti-Jagged1 C20 (Santa Cruz Biotechnology) and rabbit anti-Notch3 M-134 (Santa Cruz Biotechnology). 594- and 488-conjugated anti-rabbit and anti-goat secondary antibodies were purchased from Molecular Probes (Life Technologies, Invitrogen). Nuclei were counterstained with Hoechst reagent. Images were acquired with Carl Zeiss microscope (Axio Observer Z1) using Apotome technology and AxioVision Digital Image Processing Software.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed as described earlier [27]. Briefly, protein complexes were cross-linked to DNA in living nuclei of N3-232T cells. In total, 5 μg of antibody (anti–RBP-Jκ sc-28713X or rabbit IgG sc-2027; Santa Cruz Biotechnology) was added to each aliquot of chromatin. Immunoprecipitated DNA was eluted and analyzed by PCR with primer set 5′GTTAGGTTCTTTTCAGGCCG-3′ and 5′CTGCTGGGAACTTTTGAAAAAC-3.

Cell Growth, Apoptosis, and Invasion Assay

After treatment with TAPI-2 or vehicle, N3-232T and Molt3 cells were harvested and plated in an opportune amount for each assay. To analyze the cell growth rate, 5000 cells per well were plated onto a 96-well plate. The MTT solution (Sigma-Aldrich) was added to each well according to the manufacturer's instructions and dissolved by adding DMSO. Spectrophotometric absorbance at a wavelength of 570 nm was determined by the plate reader GloMax-Multi Detection System (Promega).

To check apoptosis, N3-232T cells were resuspended in 1 × binding buffer (BD Pharmigen) at a final concentration of 10^6 cells/ml and Allophycocyanin (APC)–Annexin V (BD Pharmigen) was added at the opportune concentration, following the manufacturer's instructions. The analysis was performed by using a FACS-Calibur cytometer (BD Biosciences, San Jose, CA), within 1 hour. To test the invasion ability of the Jurkat cell line after TAPI-2 treatment, 300,000 cells per well were plated in pre-warmed Matrigel Invasion Chamber (Corning, Tewksbury, MA, USA) and allowed to move into the lower layer characterized by the presence of a chemoattractant such as 5% FBS, in tissue culture medium. After 22 hours, the %invasion was measured as the percentage of the rate between the mean of cells invading through the Matrigel insert membrane and the mean of cells migrating through the control insert membrane, following the manufacturer's instruction.

Statistical Analysis

Results were reported as the mean ± SD. A Student's *t* test for paired samples was used to assess differences among groups. For multiple comparisons of groups, one-way analysis of variance was used. A P value < .05 was considered statistically significant ($^*P < .05$, $^{**}P < .01$, and $^{***}P < .001$.

Results

Jagged1 Constitutive Processing Occurs in Notch3-IC–Overexpressing Lymphoma Cells

To study the putative relationship between Notch3 and Jagged1 (Jag1) in T-ALL, we first investigated Jag1 expression in N3-232T lymphoma cells, a cell line established from a lymphoma-bearing Notch3-IC transgenic (N3-ICtg) mouse, which we previously demonstrated to represent a mouse model of human T-ALL [22,29]. We show that N3-232T cells display a significantly high expression of Jag1 full-length protein (Jag1-FL), when compared to the preT 2017 cell line [23], T-lymphoma cells phenotypically comparable to N3-232T cells, but expressing only Notch3 full-length and not the Notch3-IC and which does not express at all Jag1 (Figure 1*A*).

Notably, preT 2017 cells express instead the constitutively active Notch1-IC, as revealed by immunoreactivity to anti-Notch1 Val1744 (N1-Val1744) antibody (Figure 1*A*). Moreover, double immunofluorescence staining shows that in N3-232T cells the expression of Jag1 and Notch3 takes place within the same cell, a phenomenon defined

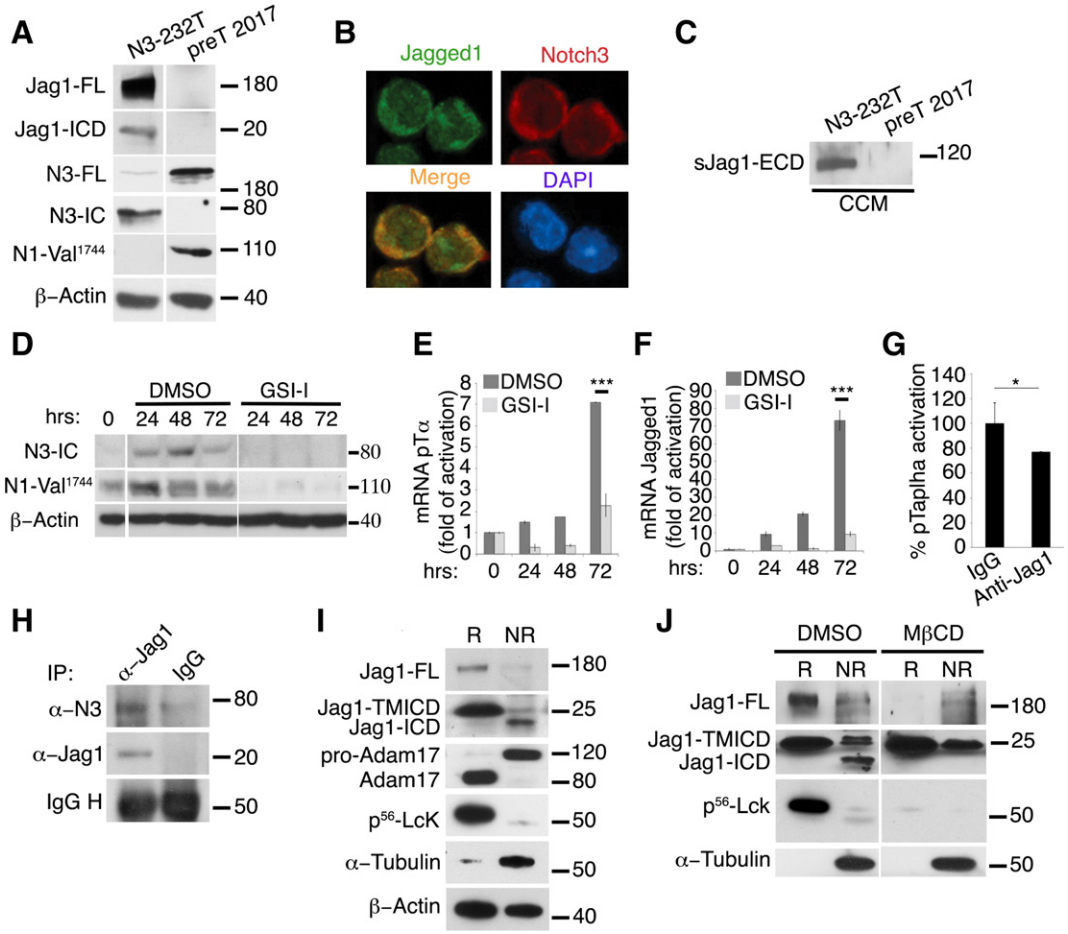

Figure 1. Cis-expression and interaction of Jagged1 and Notch3 in N3-232T lymphoma cells. (A). Jagged1, Notch3, and Notch1 expression was determined in WCEs by immunoblot analysis using specific antibodies raised against the C-terminal region of the analyzed proteins and able to detect their full-length and/or the processed forms. To reveal the activated form of Notch1, an anti-Notch1 Val1744 antibody was used. Anti–β-actin was used as a loading control (B) Jagged1 (green) and Notch3 (red) cis-expression (merge) was analyzed by double immunofluorescence staining. Magnification: ×40. (C) sJag1-ECD sheds in N3-232T CCM revealed by immunoblot analysis. (D–F) sJag1-ECD triggers Notch signaling on neighboring cells. (D) SCB29 cells were cultured with N3-232T CCM for the indicated time, in the presence of vehicle alone (DMSO) or GSI-I. The expression of cleaved Notch3-IC (N3-IC) and activated Notch1 (N1-Val^{1744}) was analyzed by Western blot. (E and F) qRT-PCR analysis of pTα and Jag1 target genes in the presence of DMSO or GSI-I in SCB29 cells grown in N3-232T CCM. The data are represented as fold of activation with respect to starting time. The graph bars display the means ± SD of three independent experiments, made in triplicate. ***$P < .001$. (G) Expression levels of pTα mRNA evaluated by qRT-PCR in SCB29 cultured in N3-232T CCM in the presence of control IgG or JAG1 neutralizing antibody (anti-Jag1). The level of pTα mRNA from SCB29 cells cultured in CCM *plus* IgG was set as 100%. Mean values ± SD are shown and they were obtained from three independent experiments, each in triplicate. *$P < .05$. (H) N3-232T WCEs were subjected to immunoprecipitation with anti-Jagged1 antibody and revealed in Western blot (WB) with anti-Notch3. Heavy chain IgGs (IgG H) are used as a loading control. (I) Raft (R) and non-raft (NR) fractions from N3-232T cells were analyzed in immunoblot assays to detect the localization of Jag1-FL, Jag1-TMICD, Jag1-ICD, pro-ADAM17, and mature ADAM17. (J) R and NR fractions derived from MβCD-treated and untreated (DMSO) N3-232T cells were used for immunoblot assay with anti-Jagged1 antibody. Anti-616 p56^{lck} and anti–α-tubulin were used as fraction markers; anti–β-actin was used as a loading control. All data are representative of at least three independent experiments, each in triplicate.

as "cis-expression" (Figure 1*B*). Notably, N3-232T cells show also the products of Jag1 processing, represented by the soluble intracellular fragment (Jag1-ICD; Figure 1*A*) and by the extracellular fragment (sJag1-ECD), shed into the CCM (Figure 1*C*). Interestingly, whole-cell extracts (WCEs) from N3-ICtg thymocytes and splenocytes not only show a high expression of Jag1-FL (Figure SS1*A*, *upper panel*) but also a Jag1 constitutive processing, represented by the presence of the soluble Jag1-ICD (Figure SS1*A*, *middle panel*) and the appearance of sJag1-ECD in the blood serum of mice (Figure SS1*B*). Furthermore, consistently with the notion that Jag1 is a substrate of the catalytic activity of ADAM17, Figure SS1*C* shows that N3-ICtg thymocytes display the expression of the ADAM17 active form, while wild-type thymocytes only express the pro-ADAM immature form.

Interestingly, we observed that sJag1-ECD is able to trigger Notch signaling in adjacent cells. Indeed, when we cultured SCB29 lymphoid cells [24], we previously demonstrated to bear a functional Notch3 receptor [28] and which constitutively express activated Notch1 (Figure 1*D*), in the presence of the CCM of N3-232T cells, we observed a progressive increase of both cleaved Notch3-IC and activated Notch1 (Figure 1*D*). The addition of GSI-I completely abrogates the expression of Notch3-IC and activated Notch1 (Figure 1*D*), further validating the hypothesis that sJag1-ECD, present in N3-232T CCM, is able to

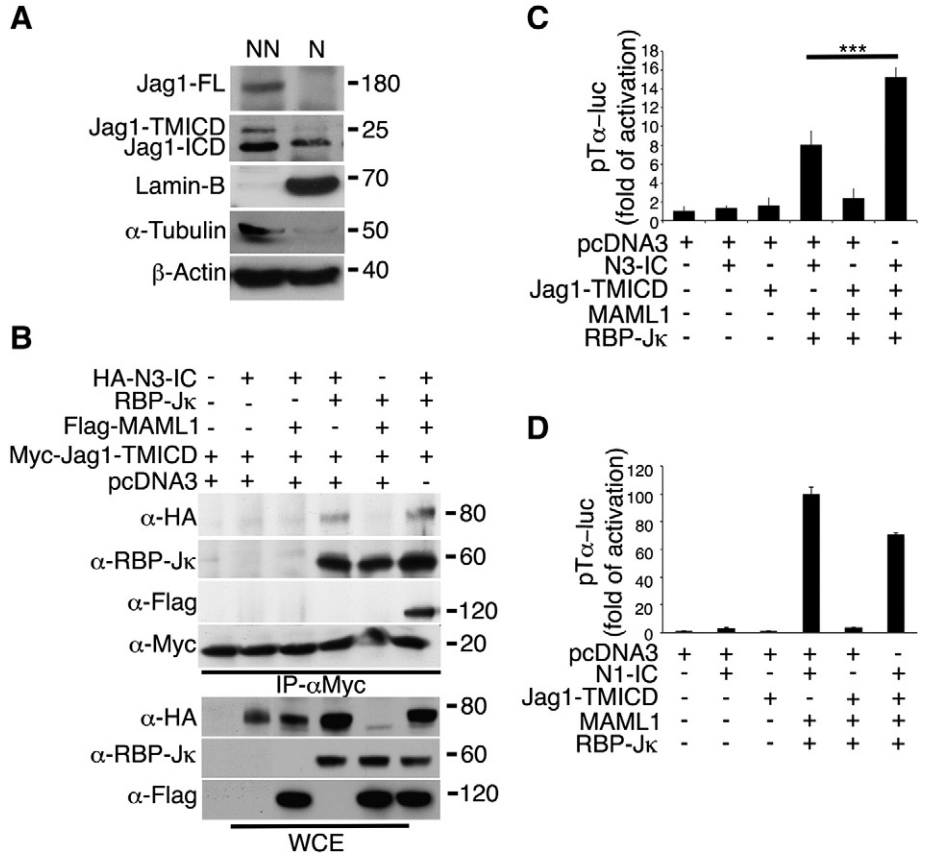

Figure 2. Jag1-ICD interacts with and empowers the Notch3-driven transcriptional complex. (A) Extracts from non-nuclear (NN) and nuclear (N) fractions were probed with anti-Jag1 antibody. Anti–lamin B and anti–α-tubulin were used as fraction controls. (B) WCEs from HEK-293T cells co-transfected with different combinations of Notch3-IC-HA, RBP-Jκ, MAML1-Flag, and Jag1-TMICD-Myc expression vectors were immunoprecipitated with anti–c-Myc and immunoblotted with antibodies as labeled. (C and D) Luciferase assay on preT 2017 cells co-transfected with pTα-luc and several combinations of plasmids encoding for MAML1, RBP-Jκ, Jag1-TMICD, and Notch3-IC or Notch1-IC. All luciferase data are graphed as fold of activation with respect to pcDNA3 negative control. The bars represent the mean of three independent experiments ± SD, all made in triplicate; ***$P < .001$.

trigger the activation of Notch signaling in neighboring cells. As a readout of such activation, we observed a significant increase of mRNA expression levels of endogenous pTα (Figure 1*E*), which is rescued upon addition of GSI-I (Figure 1*E*). To further confirm the specific role of sJag1-ECD shed into CCM, we used a Jag1 neutralizing antibody (anti-Jag1) that is able to counteract the effects of the sJagged1-ECD ligand. Figure 1*G* shows that the addition of anti-Jag1 in N3-232T CCM is able to induce a 25% decrease of pTα transcript levels with respect to the control, suggesting that the positive effects on Notch signaling are mediated by the Jagged1 ligand. Notably, we also observed that sJag1-ECD–dependent activation of Notch signaling results in increased expression of Jagged1 mRNA, which is rescued by GSI treatment (Figure 1*F*).

To determine whether Notch3 and Jag1 could also interact with each other, within the same cell, we performed co-immunoprecipitation experiments using specific antibodies recognizing carboxyl-terminal (C-terminal) region of Notch3 and Jag1 proteins. Figure 1*H* shows that in WCE the C-terminal regions of the endogenous Jag1 and Notch3-IC interact with each other.

Jagged1 Constitutive Processing Is a Lipid Raft–Associated Event

We have recently shown that Notch3 is constitutively recruited to lipid raft in N3-232T lymphoma cells suggesting a possible role of Notch3 in triggering and/or sustaining thymocyte activation [28]. Given the observations above, we hypothesized that Jag1 may also be recruited to lipid rafts in N3-232T cells. Figure 1*I* shows that Jag1-FL and Jag1 transmembrane domain (Jag1-TMICD) are constitutively recruited to lipid raft microdomains, whereas Jag1-ICD is detected only in the non-raft fraction. Membrane raft microdomain integrity depends on the presence of cholesterol and MβCD, which removes cholesterol from the plasma membrane, disrupting the rafts. Figure 1*J* shows that after MβCD treatment we cannot appreciate anymore the release of the Jag1-ICD signaling fragment in the non-raft fraction, suggesting a possible blockade of Jag1-FL processing. Anti-p56^{LCK} and anti–α-tubulin immunoblot analyses were, respectively, used as controls for Raft (R) and non-Raft (NR) compartments (Figure 1, *I–J*). ADAM17 metalloprotease is mainly responsible for the cleavage of Jag1 that allows the release of sJag1-ECD and that represents an obligatory step before the cleavage by the presenilin/γ-secretase complex [11]. It is known that ADAM proteins are synthesized as inactive zymogens that are proteolytically processed to the catalytically active forms [30]. We thus analyzed the expression of ADAM17 in N3-232T lipid rafts and we observed a significant recruitment of its 80-kDa active form in such microdomains (Figure 1*I*).

Together, these observations strongly suggest that lipid raft platform plays a functional role in triggering the ADAM17-mediated Jag1 processing in N3-232T lymphoma cells.

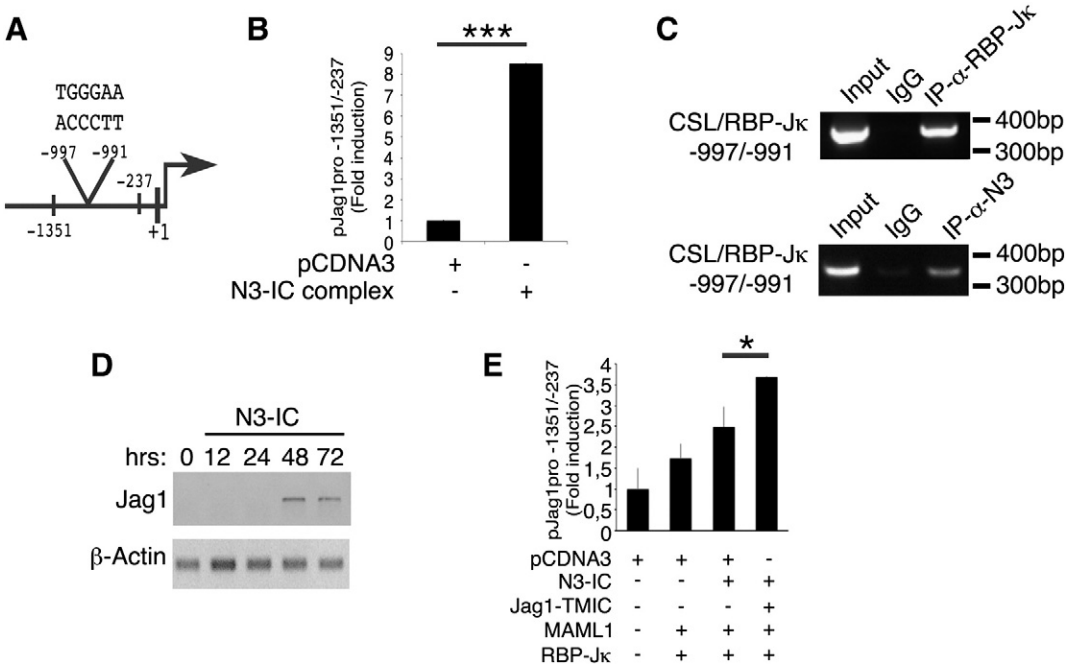

Figure 3. *Jagged1* is a Notch3-signaling target gene. (A) Schematic representation of murine *Jagged1* putative promoter sequence. The genomic DNA region of *Jagged1* promoter ranging between −1351 and −237 bp upstream the start site (pJ1pro$^{-1351/-237}$) was cloned into pGL3 Basic luciferase vectors as described in the Materials and Methods section. (B) Activation of the *Jagged1* promoter constructs by Notch3 signaling. HEK-293T cells were co-transfected with a luciferase reporter construct containing pJ1pro$^{-1351/-237}$ and Notch3 transcriptional activator complex (Notch3-IC + RBP-Jκ + MAML1). (C) ChIP assay in N3-232T cells using anti–RBP-Jκ or anti-Notch3 antibodies or IgG as control. Immunoprecipitated DNA was analyzed by PCR using a primer set that amplifies the CSL/RBP-Jκ consensus binding site spanning between −997 and −991 bp on pJ1pro$^{-1351/-237}$ promoter. (D) preT cells were transfected with Notch3-IC expression vector and Jag1 mRNA was analysed by RT-PCR. Anti–β-actin was used as a loading control. (E) preT 2017 cells were transfected with pJ1pro$^{-1351/-237}$-luc and Notch3-IC, MAML1, and RBP-Jκ, with or without Jag1-TMICD expression vectors. All data presented were collected from three independent experiments, each made in triplicate. The luciferase graph bars show the means ± SD and analyzed as fold of activation with respect to pcDNA3 empty control. ***$P < .001$; *$P < .05$.

Jag1-ICD Takes Part in and Positively Regulates Notch3-IC–Driven Transcriptional Complex

To address the function of different Jag1 fragments in lymphoma cells, we have first studied their respective subcellular localization. We observed that Jag1-FL and Jag1-TMICD are specifically located in the non-nuclear fraction, while Jag1-ICD is able to move into the nucleus (Figure 2*A*). Furthermore, co-immunoprecipitation assays using HEK-293T cells, co-transfected with Notch3-IC–, RBP-Jκ–, MAML1-, and Jag1-TMICD–expressing vectors, show that Jag1 is able to interact with the transcription factor RBP-Jκ and to take part in the Notch3-IC transcriptional complex (Figure 2*B*). To investigate whether the released Jag1-ICD was able to influence Notch3-dependent transcription, we first monitored the ability of Jag1-ICD to modulate the transcription of the pre-TCRα chain (pTα), which we have previously shown to be directly regulated by Notch3 [9] and to sustain the leukemogenesis process [29]. A luciferase assay performed in preT 2017 cells with the pTα promoter as a luciferase reporter (pTα-luc) shows that the addition of Jag1-TMICD to the Notch3-IC–driven transcriptional complex gives rise to a two-fold increase in transcriptional activity of the pTα promoter (Figure 2*C*).

Together, these results suggest that Jag1-ICD, by binding to RBP-Jk, positively cooperate with the Notch3-driven transcriptional complex, empowering its transcriptional activity. Intriguingly, it has been previously shown that Jag1-ICD is able to directly interact with Notch1 and such an interaction prevents the formation of a Notch1-driven transcriptional complex, enhancing the degradation of Notch1-IC, by the FBXW7 ligase [31]. In keeping with this, we observed that the addition of Jag1-ICD significantly reduces Notch1 transcriptional activity in the pTα-luc (Figure 2*D*).

Jagged1 Is a Transcriptional Target of Notch3 and Contributes to Its Own Transcriptional Activation

The observations reported above allowed us to speculate that the increased expression and processing activation of Jag1 could be the result of the constitutive activation of Notch3. To this purpose, we investigated the ability of Notch3 to induce the activation of the *Jagged1 promoter*. We identified through the Genomatix Software two different genomic DNA fragments putatively matching the promoter sequence of Jagged1: pJ1pro$^{-3794/-2156}$ and pJ1pro$^{-1351/-237}$, respectively, characterized by the presence of two RBP-Jκ binding sites (Figure SS2*A*) or one RBP-Jκ binding site (Figure 3*A*).

Luciferase assays in HEK cells demonstrated that the enforced expression of the Notch3-IC–driven transcriptional complex results in an eight-fold induction of the pJ1pro$^{-1351/-237}$ luciferase reporter construct (pJ1pro$^{-1351/-237}$-luc; Figure 3*B*), while it was not able to increase the transcriptional activity of the pJ1pro$^{-3794/-2156}$ (Figure

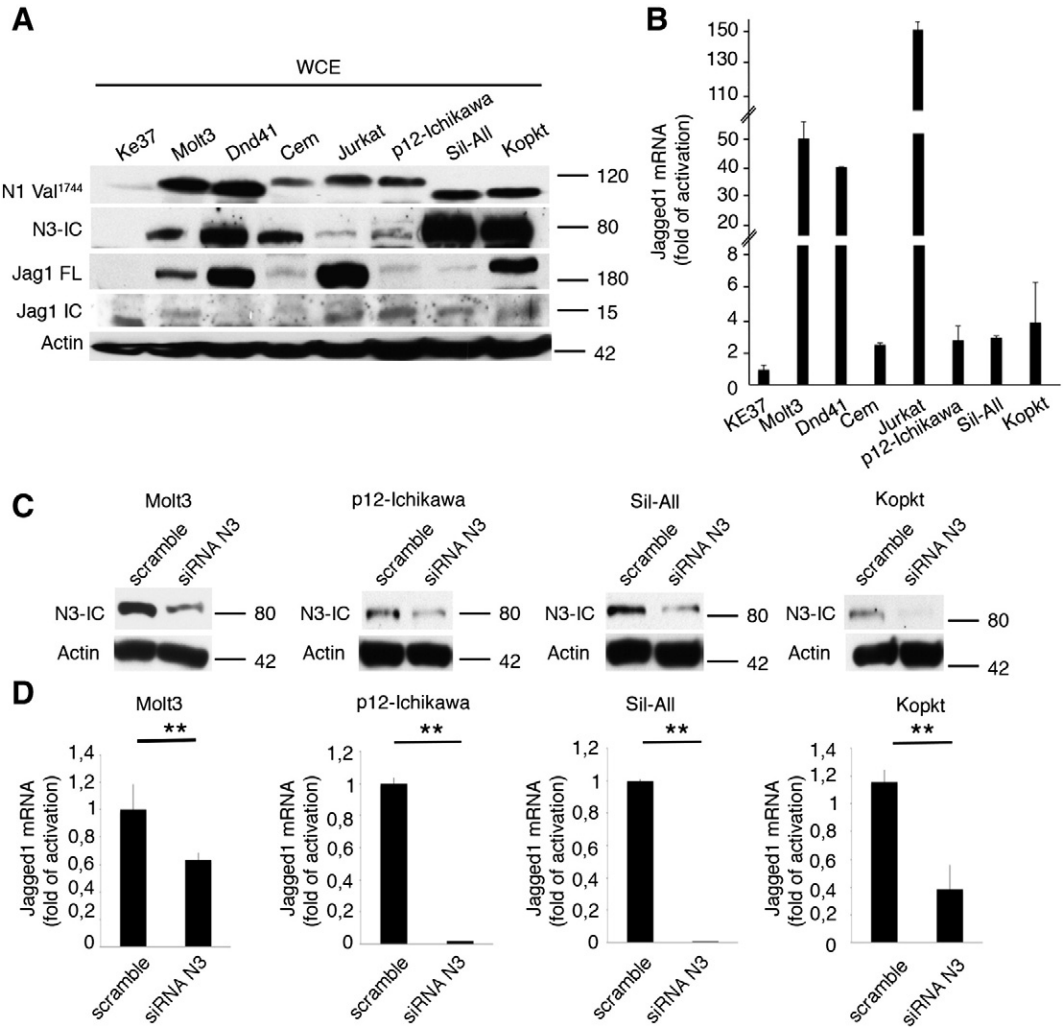

Figure 4. Relationship between Notch3 and Jag1 in human T-ALL cell lines. (A) WCEs from KE37, Molt3, Dnd4.1, Cem, Jurkat, P12-Ichikawa, SIL-ALL, and Kopkt cell lines were analyzed by immunoblot analysis with anti-Notch3, anti-Notch1-Val^{1744}, and anti-Jagged1 antibodies, as labeled. Anti–β-actin was used as a loading control. (B) mRNA derived from the same human T-ALL cell lines was analyzed by qRT-PCR using a primer set specific for Jagged1. The data are analyzed as fold of activation with respect to KE37 negative control. The graph bars display the means ± SD of three similar experiments, each in triplicate. (C) Immunoblot WCE from Molt3, P12-Ichikawa, SIL-ALL, and Kopkt cells after silencing of Notch3 (Notch3 siRNA) compared to the control (CTR siRNA). Anti–β-actin was used as a loading control. (D) qRT-PCR was used to determine the effects of Notch3 knockdown on Jagged1 target gene expression in Notch3-silenced cells after 48 hours. The graph bars display the means ± SD of at least three independent experiments, analyzed as fold of activation with respect to scramble control. $^{**}P < .01$.

SS2*B*). Moreover, a ChIP assay in N3-232T cells shows that both RBP-Jκ and Notch3-IC constitutively bind the canonical RBP-Jk binding site, spanning between −997 and −991 bp in the *Jagged1 promoter*, indicating that *Jag1* is a Notch3 target gene (Figure 3*C*). Moreover, Figure 3*D* shows that Notch3-IC transient transfection is able to induce the increased expression of endogenous Jag1 in preT 2017 cells.

It has been previously suggested that Jag1 is able to transcriptionally activate itself [13]. To see whether Jag1-ICD was also able to cooperate with Notch3-IC to drive the activation of its own promoter, we co-transfected preT 2017 cells with the pJ1pro$^{-1351/-237}$-luc and Notch3-IC, RBP-Jκ, and MAML1 expression plasmids in the presence or absence of Jag1-TMICD. Figure 3*E* confirms that the Notch3-IC–driven transcriptional complex increases the luciferase activity of *Jagged1 promoter*, and as we hypothesized, the addition of Jag1-TMICD further increases the activation of its own promoter. Altogether our data demonstrate, for the first time, the ability of Notch3 to regulate the transcription of its own ligand Jagged1 in a T cell precursor context and that Jag1-ICD, in turn, is able to strengthen the activity of Notch3-driven transcriptional complex in triggering its own transcription.

Jagged1 Expression Is Regulated by Notch3 Activity in Human T-ALL Cell Lines and Its Processing Regulates T-ALL Cell Survival, Proliferation, and Invasion

To assess the possible relevance of Notch3 and Jagged1 relationship in human T-ALL, we first analyzed the expression of Jagged1 and Notch3-IC in several human T-ALL cell lines. Figure 4*A* shows that all of the T-ALL cell lines analyzed express not only high levels of activated Notch1-IC (N1-Val^{1744}), reflecting an aberrant activation of the Notch pathway, but also the cleaved form of Notch3 (except for KE37). Intriguingly, the presence of a cleaved Notch3-IC correlates with the expression of Jag1-FL protein at different levels in all of the human T-ALL cell lines analyzed, while most of them (Molt3,

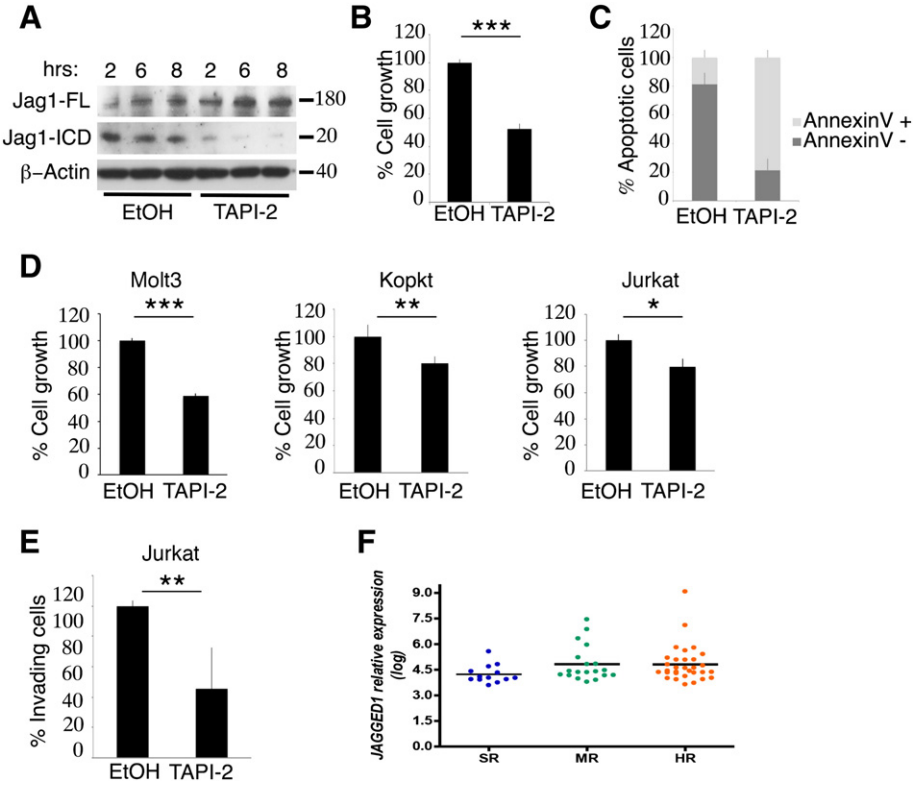

Figure 5. Inhibition of Jag1 processing impairs mouse and human lymphoma cell proliferation, apoptosis, and invasiveness. (A) WCEs of N3-232T cells treated with TAPI-2 or vehicle alone (EtOH) were immunoblotted with anti-Jag1 antibody. Anti–β-actin was used as a loading control. (B and C) The TAPI-2 effect on growth was tested by the MTT assay, whereas apoptosis was measured by the analysis of Annexin V staining (Annexin V+, gray bars; Annexin V−, dark bars). (D) Molt3, Kopkt, and Jurkat cell lines were treated with TAPI-2 or EtOH and the effect on cell growth was tested by the MTT assay. (E) The Jurkat cell line treated with TAPI-2 or EtOH was used in invasion Matrigel assay. All data presented were collected from three independent experiments. The graph bars display the means ± SD, analyzed as fold of activation with respect to EtOH negative control. ***$P < .001$; **$P < .01$; *$P < .05$. (F) The graph represents gene expression profiling (GEP) data for the expression of probe set 216268_s_at (U133 2.0 plus array; Affymetrix) representing the *Jagged1* gene in a cohort of 76 pediatric T-ALL patients. Patients were stratified in three final risk groups based on Minimal Residual Disease (MRD) levels, response to the first week of steroids, and resistance to induction therapy. Each dot corresponds to one patient and the expression value of Jagged1 is given in $\log_2$ scale after normalizing GEP data with justRMA algorithm normalization. JustRMA is an algorithm fulfilling two steps, namely, background adjustment of all the probe sets present on the GeneChip and quantile normalization to make the values of all the GeneChips comparable. The *X*-axis represents patient stratification according to final risk groups, and the *Y*-axis represents *Jagged1* gene expression level.

Jurkat, P12-Ichikawa, SIL-ALL, and Kopkt) display a constitutive release of Jag1-IC (Figure 4*A*). The expression levels of Jagged1 mRNA in different cell lines, assayed by qRT-PCR, are represented in Figure 4*B*. Altogether, these observations allow us to assume a direct correlation between Notch signaling activation and Jagged1 ligand expression in human T-ALL cell lines. Moreover, we observed that Notch3 silencing in Molt3, P12-Ichikawa, SIL-ALL, and Kopkt cells, confirmed by Western blot analysis (Figure 4*C*), results in the significant decrease of Jagged1 transcript expression (Figure 4*D*), suggesting a possible direct correlation between Notch3 and Jagged1. Then, to finally ascertain whether Jag1 cleavage may have an impact on lymphoma cell survival, we used the TAPI-2 compound to inhibit ADAM17 activity [27]. We observed that TAPI-2 treatment abrogates the cleavage of endogenous Jag1 in N3-232T cells (Figure 5*A*) and is able to induce a 50% decrease of cell growth, as determined by the MTT assay (Figure 5*B*). Moreover, Figure 5*C* shows a sharp increase (from 20% up to 80%) of apoptosis in TAPI-2–treated N3-232T cells. In addition, even Molt-3, Koptk1, and Jurkat cells that display a constitutive Jagged1 cleavage (Figure 4*A*), when treated with the TAPI-2 compound, disclose a reduction of cell growth, respectively, of 40% for Molt3 and 20% for Jurkat and Koptk1 by MTT analysis, with respect to vehicle-treated cells (Figure 5*D*). Interestingly, TAPI-2 treatment was not able to induce the reduction of cell growth in KE37 T-ALL cells, which do not express Jagged1 (Figure WS3). Notably, the block of Jagged1 cleavage obtained by TAPI-2 treatment strongly reduced Jurkat cell migration (55%), measured in Matrigel invasion assay (Figure 5*E*). Together, these data indicate that a deregulated expression and an uncontrolled processing of Jagged1 are involved in lymphoma cell proliferation and migration/invasion, suggesting a potential role of Jagged1 in malignant tumor progression in T-ALL. Analyses of gene expression in a cohort of 76 pediatric T-ALL patients revealed a general moderate expression of Jagged1, except for six cases. Intriguingly, these six patients with a higher Jagged1 expression were stratified in the medium and high final risk groups.

Discussion

The data presented here indicate that a direct relationship exists between Notch3 and its ligand Jagged1, and such a relationship could be a new puzzle's piece to unravel the molecular mechanisms that sustain the development and progression of T-ALL.

We first demonstrate that *Jagged1* is a Notch3 target gene in lymphoma cells from a mouse model of T-ALL and in human T-ALL

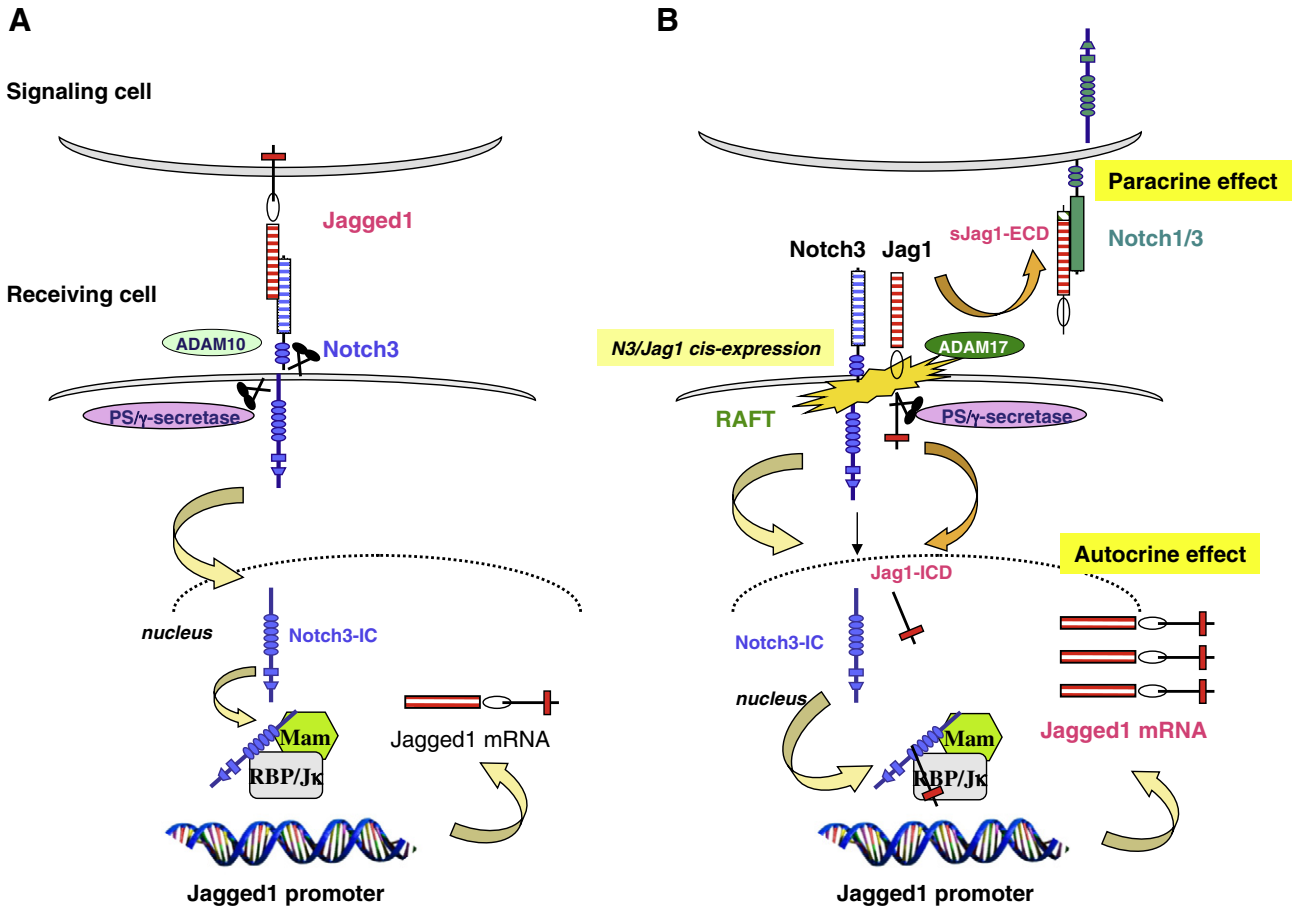

Figure 6. Schematic representation of the reciprocal auto-sustaining loop between Jagged1 ligand and Notch3 receptor in T-ALL. (A) The Jagged-1–triggered transactivation induces cleavage of Notch, by sequential activation of ADAM10/TACE and γ-secretase, to generate Notch-IC. In a T ALL cell context, a Notch3-IC–driven transcriptional complex activates the *Jagged1* target gene, by constitutively binding the canonical RBP-Jκ binding site spanning between −997 and −991 bp in the *Jagged1 promoter*. (B) The constitutive expression of Notch3 intracellular domain in thymocytes and lymphoma cells from N3-ICtg mice determines a Notch3/Jagged1 cis-expression within the same cell and a constitutive raft-dependent Jagged1 processing, whose result is the empowering of Notch3 signaling. The Jagged1 intracellular domain is able to translocate into the nucleus where it is recruited specifically by the Notch3 transcriptional complex (Notch3-IC + MAML1 + RBP-Jκ), acting as a co-activator able to sustain the activation of specific target genes, such as *pTα* and *Jagged1* itself (autocrine effect). On the other hand, the shedding of soluble extracellular domain of Jagged1 (sJag1-ECD) has a positive impact on triggering Notch signaling pathway in neighboring cells (paracrine effect).

cell lines. In this regard, we have identified the specific genomic region corresponding to *Jagged1* promoter (pJag1pro$^{-1351/-237}$) that contains a canonical RBP-Jκ binding site, which is bound by RBP-Jκ and Notch3. Our data also indicate that in both Notch3-IC–overexpressing T lymphoma cells and in human T-ALL cell lines, expressing a cleaved Notch3-IC, Jagged1 undergoes a constitutive processing, revealed by the expression of the released Jag1-ICD that is able to translocate into the nucleus and to empower Notch transcriptional activity. We have also observed that Notch3 silencing results in the significant decrease of Jagged1 transcript expression, suggesting that the expression of Jagged1 can be Notch signaling mediated in a dysregulated cell context.

Interestingly, we show that Jagged1 and the active form of ADAM17 segregate in lipid rafts of Notch3-IC overexpressing lymphoma cells, suggesting that Jagged1 constitutive processing is a lipid raft microdomain–associated event. Consistently, the disruption of the rafts, by using MβCD, inhibits the release of the Jag1-ICD in a non-raft fraction. We hypothesize that such a subcellular distribution would permit a higher degree of interaction between Jag1 and ADAM17, which may favor the sJag1-ECD shedding. Notably, it has been previously reported that ADAM17-mediated cleavage of Jag1 is a lipid raft–independent event in a non–T cell context [32], suggesting that our observation may be due to a specific effect of the Notch3 constitutive recruitment to lipid rafts in N3-232T lymphoma cells, which we previously reported [28]. Moreover, we present compelling evidence that the sJag1-ECD shed in the N3-232T cell CCM is able to activate Notch signaling in Notch receptor–bearing neighboring cells, as revealed by the increased expression of specific Notch target genes, supporting literature data concerning the positive effects of sJag1-ECD [33].

Noteworthy, we show that once Jag1-ICD moves to the nucleus it can behave as a co-activator of the Notch3-IC–driven transcriptional complex. In particular, we demonstrate that Jag1-ICD binds to the transcription factor RBP-Jκ and is able to empower the

transcriptional effect of Notch3-IC on target genes, such as *pTα* and *Jagged1* itself. Such a functional cooperation between Notch3 and Jag1-ICD seems to be specific, since Kim et al. demonstrated that the presence of Jag1-ICD accelerates the protein degradation of Notch1-IC through FBW7 [31]. The proline, glutamic acid, serine, threonine-rich (PEST) domain of Notch intracellular domain (NICD) is known to regulate protein stability and is a hot spot for mutations in primary human and mouse T-ALLs [34,35] and FBW7 binding to Notch is dependent on an intact PEST [31,36]. Thus, we may assume that a deregulated expression of Notch ligand Jagged1, Notch3-mediated, could synergize with PEST domain–mutated Notch1 to potently activate the pathway. These observations allow us to assume that a multistep oncogenic process that involve both mutant Notch1 and unmutated Notch3 could be necessary to regulate the expression of Jagged1 *in cis*, that, in turn, once cleaved, is able to synergize with Notch3-driven transcriptional complex in the same cell and to empower Notch signaling *in trans* in adjacent cells.

Jagged1 up-regulation has been recently observed in several Notch-overexpressing human tumors, such as ovarian cancer [17], lung cancer [18], and renal cell carcinoma [20]. Moreover, it has been suggested that Jagged1 is able to play a role in progression and metastasization of prostate cancer and may be a useful marker to distinguish indolent and aggressive cancers and to predict poor outcome in renal cell carcinoma [14,20,37]. In addition, it has been demonstrated that the down-regulation of Jagged1 induces S phase arrest and cell growth inhibition, regulating CDK2, cyclin A, and p27 in prostate cancer cell [38]. Finally, a role for Jagged1 in controlling cell growth and invasion has been identified in colorectal cancer, renal cell carcinoma, and colon cancer [20,39]. In keeping with these data, we observe that the pharmacological inhibition of Jagged1 processing can significantly reduce lymphoma cell proliferation and migration and induces apoptosis of both mouse and human T-ALL cell lines. Intriguingly, for the first time, we show that in a cohort of 76 pediatric T-ALL patients, a higher Jagged1 expression is observed in the medium and high final risk groups.

Taken together, these data suggest that a Notch-dependent dysregulation of Jagged1 protein takes part in a multistep oncogenic process, playing a role in controlling not only cell growth and apoptosis but also lymphoma cell migration, finally sustaining tumor aggressiveness and progression.

Collectively, our results strongly suggest a new molecular mechanism, which supports the hypothesis that a dysregulated Notch3 activation is able to increase the transcription of Jagged1 ligand inside the same cell (Figure 6*A*) and the deregulated expression of Jagged1 is able, in turn, to empower the transcriptional activation of Notch target genes (e.g., *pTα*), involved in the onset and progression of T-ALL, and to elicit a Jagged1 auto-sustaining loop, mediated by the transcriptional activation of Jagged1 itself (autocrine effect). On the other hand, our data demonstrate that the constitutive release of the sJag1-ECD has a positive impact on activating Notch signaling in adjacent cells (paracrine effect), as suggested in the model depicted in Figure 6*B*.

Finally, the significant reduction of the proliferation and the increased apoptosis of mouse and human T-ALL cell lines, obtained with the pharmacological block of the Jagged1 cleavage, suggest that Jagged1 processing inhibition may be exploited, as an additional target therapy for T-ALL.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.neo.2014.10.004.

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