Crucial role of the Rap G protein signal in Notch activation and leukemogenicity of T-cell acute lymphoblastic leukemia

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The Rap G protein signal regulates Notch activation in early thymic progenitor cells, and deregulated Rap activation (Raphigh) results in the development of Notch-dependent T-cell acute lymphoblastic leukemia (T-ALL). We demonstrate that the Rap signal is required for the proliferation and leukemogenesis of established Notch-dependent T-ALL cell lines. Attenuation of the Rap signal by the expression of a dominant-negative Rap1A17 or Rap1GAP, Sipa1, in a T-ALL cell line resulted in the reduced Notch processing at site 2 due to impaired maturation of Adam10. Inhibition of the Rap1 prenylation with a geranylgeranyl transferase inhibitor abrogated its membrane-anchoring to Golgi-network and caused reduced proprotein convertase activity required for Adam10 maturation. Exogenous expression of a mature form of Adam10 overcome the Sipa1-induced inhibition of T-ALL cell proliferation. T-ALL cell lines expressed Notch ligands in a Notch-signal dependent manner, which contributed to the cell-autonomous Notch activation. Although the initial thymic blast cells barely expressed Notch ligands during the T-ALL development from Raphigh hematopoietic progenitors in vivo, the ligands were clearly expressed in the T-ALL cells invading extrathymic vital organs. These results reveal a crucial role of the Rap signal in the Notch-dependent T-ALL development and the progression.
Results

The Rap signal is required for Notch activation in T-ALL cell lines. FL0 cell line derived from T-ALL by BMT of Rap1high HPCs expressed intact Notch receptors with no detectable Notch1 mutation and showed Notch-dependent proliferation (Figure S1). Retroviral transduction of dominant-negative Rap1 (Rap1A17) in FL0 cells causing a decrease of the Rap1-GTP resulted in a reduced expression of NICD and its target Hes1 (Figure 1a). Accordingly, the expression of p27Kip1, a target of Hes1-mediated repression \(^{14}\) was increased, and the proliferation was significantly reduced (Figure 1a). The FL0/Rap1A17 cells showed significantly compromised leukaemic activity in scid mice, and 20% of the recipients remained free of leukemia, whereas all recipients of control FL0/vect cells died within 25 days (Figure 1b, left). Moreover, the leukemia cells developed in the recipients of FL0/Rap1A17 cells revealed significantly reduced expression of the retrovirus-driven NGFR (Figure 1b, right). Such an effect was not observed in the recipients of FL0/vect cells, suggesting a counter selection against FL0/Rap1A17high cells in vivo. We then transduced Rap1-specific GAP, Sipa1, in FL0 cells with a doxycycline (Dox)-inducible system. Induction of Sipa1 expression also resulted in the decreased NICD expression and cell proliferation in concordance with reduced Rap1-GTP levels in a Dox-dose dependent manner (Figure 1c). Furthermore, treatment of the FL0 cells with a geranylgeranyl transferase inhibitor (GGTI)\(^ {15}\), which inhibited the Rap prenylation required for membrane anchoring, also suppressed the NICD generation and cell proliferation at a dose-range that did not affect the proliferation of irrelevant leukemia cells (Figure 1d). Other T-ALL cell lines of mice and humans similarly showed significantly higher susceptibility to GGTI than leukemia cells of non-T-ALL types (Figure S2). The results suggest that the Rap signal plays an important role in sustaining Notch activation and proliferation of established T-ALL cell lines.

The Rap signal controls Notch S2 processing by regulating intracellular Adam10 maturation. The conditional expression of Sipa1 in FL0 cells did not affect the cell surface expression of Notch1 in FL0 cells that did not affect the surface expression of Notch1 (Figure 2a). However, analysis with Notch1-immunoprecipitation followed by immunoblotting with S2 (V1711)-specific antibody revealed that the Notch S2 product precedent to the NICD generation was also decreased by Sipa1 expression (Figure 2a). In T-ALL cells, Notch cleavage at S2 site is mediated by Adam10\(^ {16}\), which maturates intracellularly via prodomain-cleavage of the immature form\(^ {17}\). Sipa1 expression in FL0 cells caused a decrease of the mature form of Adam10 (m-Adam10), which maturates intracellularly via prodomain-cleavage of the immature form. Sipa1 expression in FL0 cells caused a decrease of the mature form of Adam10 (m-Adam10), which maturates intracellularly via prodomain-cleavage of the immature form. Sipa1 expression in FL0 cells caused a decrease of the mature form of Adam10 (m-Adam10), which maturates intracellularly via prodomain-cleavage of the immature form. Sipa1 expression in FL0 cells caused a decrease of the mature form of Adam10 (m-Adam10), which maturates intracellularly via prodomain-cleavage of the immature form.
(85 kDa) with a concomitant decrease of the cleaved transmembrane and intracellular (TMIC) form (29 kDa); FACS analysis confirmed a slight yet clear increase in the cell surface Dll1 expression (Figure 2c).

We next examined the proprotein convertase activity required for the Adam10 maturation19, with the use of a fluorescence-labeled synthetic substrate (Pyr-Arg-Thr-Lys-Arg-AMC)20. Although proprotein convertase activity requires Ca2+, early-phase cleavage activity in the cell lysate tended to be resistant to EDTA and was probably attributed to other trypsin-like proteases. Sipa1 expression in FL0 cells rather preferentially inhibited the EDTA-sensitive late-phase activity (Figure 2d). The results suggest that the Rap signal regulates the activation of proprotein convertase activity required for the maturation of Adam10 mediating Notch S2 processing.

Prenylation-mediated anchoring of the Rap1 at the Golgi-network is crucial for proprotein convertase activation. We then investigated the intracellular localization of the Rap1 and Furin, a main proprotein convertase. Because the analysis was rather difficult in small T-ALL cells with minimal cytoplasm, we made use of an epithelial Eph4 cell line to this end, whose Sipa1 expression could be conditionally induced with a Rheo-switch system (Eph4/Rheo-Sipa1) (Figure S3a). It was confirmed that the induction of Sipa1 expression in Eph4/Rheo-Sipa1 cells resulted in the decrease of late-phase, EDTA-sensitive proprotein convertase activity (Figure 3a). Treatment of Eph4/Rheo-Sipa1 cells with GGTT inhibiting the prenylation of Rap1 also caused a reduction of the proprotein convertase activity dose-dependently (Figure 3b, Figure S3b). In agreement with previous reports21,22, Furin was detected in the cytosol as small clusters enriched at the perinuclear region corresponding to the Golgi-network, and the Rap1 was distributed at the same regions to Furin, with additional localization in the nuclei in some cells (Figure 3c, left). After GGTT treatment, however, unprenylated Rap1 was barely detected in the cytosol any more and was localized mostly in the nuclei, whereas Furin localization was hardly affected (Figure 3c, right). The results suggest that the Rap1 anchoring at the Golgi-network membrane is crucial for the activation of proprotein convertases.

Exogenous expression of mature Adam10 overcomes the Sipa1-induced growth inhibition of T-ALL cells. To confirm the role of Rap signal in Adam10 maturation, we expressed a mature form of Adam10 in FL0/rtTA-Sipa1 cells using pMSCV-hNGFR (MIN) retroviral vector (Figure 4a). The FL0/rtTA-Sipa1/m-Adam10 cells showed increased NICD and Hes1 expression compared with control FL0/rtTA-Sipa1/vect cells as anticipated (Figure 4a). We then cultured the cells in the absence or presence of Dox. The FL0/rtTA-Sipa1/m-Adam10 cells showed no or significantly less inhibition of the proliferation in the presence of Dox than FL0/rtTA-Sipa1/vect cells (Figure 4b). The results are consistent with

Figure 2 | The Rap signal is involved in Notch S2 processing by sustaining Adam10 maturation via proprotein convertase activity. (a) FL0/rtTA-Sipa1 cells were cultured in the absence (fine line) or presence (solid line) of Dox (1 µg/mL), and the surface expression of Notch1 was analyzed with flow cytometry. These cells were cultured with or without varying doses of Dox for 3 days, and the lysates were immunoprecipitated with anti-Notch1 antibody followed by immunoblotting with S2-specific (V1711) antibody. The mean signal intensities of Notch S2 in 3 independent experiments are shown. *; p < 0.01. (b) FL0/rtTA-Sipa1 cells were cultured in the absence or presence of Dox for 3 days, and the lysates were immunoblotted with anti-Adam10 antibody. Adam10 transcripts were assessed with qRT-PCR in the aliquots of cells. The mean signal intensities of mature (m)-Adam10 and the relative transcripts in 3 independent experiments are shown. *; p < 0.01. NS; not significant. (c) The cells in (b) were immunoblotted and FACS analyzed with anti-Dll1 antibody. (d) FL0/rtTA-Sipa1 cells were cultured in the absence or presence of Dox for 3 days, and proprotein convertase activity of the cell lysates was assessed with the use of a fluorescence-labeled synthetic substrate in the presence or absence of EDTA. In (a), (b), and (c), relevant parts of immunoblot images were cropped from full-length blots shown in Figure S8.
Figure 3 | Prenylation-mediated anchoring of Rap1 to Golgi-network is required for the proprotein convertase activation. (a, b) Eph4/Rheo-Sipa1 cells were cultured in the absence or presence of RSL1 (a) or GGTI (b) for 3 days, and the intracellular proprotein convertase activity was assessed with or without EDTA. (c) Eph/Rheo-Sipa1 cells were cultured in the absence (left) or presence (right) of 4 µM GGTI for 24 h and multi-color immunostained with the indicated antibodies.

Figure 4 | Exogenous expression of mature Adam10 overcomes Sipa1-induced growth inhibition of T-ALL cells. (a) FL0/rtTA-Sipa1 cells were transduced with a MIN vector containing Flag-tagged m-Adam10 cDNA as illustrated or empty vector. Arrow indicates a cleavage site. The cells were immunoblotted with indicated antibodies. (b) The FL0/rtTA-Sipa1/m-Adam10 (solid circles) and control (open circles) cells were cultured in the absence or presence of varying concentrations of Dox at 10^5 cells/mL for 3 days, and the viable cell numbers were assessed with Cell Titer Glo assay. The means and SEs of triplicate culture are indicated. *; p < 0.005, **; p < 0.001. The experiments were repeated three times with essentially similar results. In (a), relevant parts of immunoblot images were cropped from full-length blots shown in Figure S9.
the notion that the Rap signal is required for the Notch-dependent proliferation of T-ALL cells by promoting endogenous Adam10 maturation.

Expression of Notch ligands and their involvement in the cell-autonomous Notch activation of T-ALL cell lines. Because Notch1 receptor in FL0 cell line showed no mutation, the initiation of Notch processing might be expected to depend on the ligand engagement. We therefore examined the expression of Notch ligands on T-ALL cell lines. FACS analysis revealed that Notch-dependent T-ALL cell lines including FL0 variably expressed Jagged1, Jagged2 and Dll1, but rarely Dll4, although normal thymocytes expressed none of them (Figure 5a). Moreover, co-culture of these T-ALL cell lines with Notch ligand–responsive C2C12 myoblast cells induced significant expression of Notch1 and Jagged1 in the C2C12 cells (Figure 5b), indicating that the ligands were functional. The expression of Jagged1 was abrogated in the presence of a \( \gamma \)-secretase inhibitor (DAPT) and was suggested to be dependent on the Notch signal (Figure 5c). To examine the possible involvement of ligands in cell-autonomous Notch activation, we cultured the T-ALL cell lines in the presence of the mixture of monoclonal antibodies against Jagged1, Jagged2 and Dll1 (60 \( \mu \)g/mL each) for 1 day and immunoblotted with the indicated antibodies. Relative intensities of NICD to actin are indicated. Aliquots of the cells were cultured for 5 days, and the viable cell numbers were determined in triplicate culture. (e) FL0 and T-lymphoma (EL4, RLm1) cell lines were cultured in methylcellulose medium (100 cells/dish) in triplicate for 7 days, and the colony numbers were counted. Images of typical colonies of RLm1 and FL0 cells are shown. In (a), relevant parts of immunoblot images were cropped from full-length blots shown in Figure S10.

Notch ligand expression in primary T-ALL cells correlates with the systemic leukemia invasion in vivo. To validate the significance of Notch-dependent ligand expression during T-ALL development in vivo, we performed BMT of Rap\(^{sh} \) HPCs. As reported previously\(^\text{13} \)\(^\text{13} \), the Rap\(^{sh} \) HPCs caused highly aggressive T-ALL eventually involving most vital organs in the BMT recipients. The thymi of Rap\(^{sh} \) HPC-recipients were remarkably enlarged in 3 weeks after BMT, mostly consisting of blastic immature (CD3\(^- \)CD4\(^- \)CD8\(^- \)) T cells, whereas control HPCs repopulated the thymi with normal T cell differentiation (Figure 6a). The thymic Rap\(^{sh} \) blast cells showed no detectable expression of any Notch ligand on the surface, although...
these cells exhibited markedly enhanced expression of Notch1 and Notch3 compared with control repopulating thymocytes (Figure 6a). In contrast, the CD3^+^CD4^+^CD8^+^ blast cells that invaded the spleen significantly expressed cell-surface Notch ligands in addition to the enhanced Notch1/3, whereas the T cells derived from control HPCs barely did so (Figure 6b). Moreover, the T-ALL cells that invaded other vital organs such as BM and liver showed even more Jagged1 transcripts than those in the spleen, although the increase in Notch1/3 transcripts was comparable (Figure 6c). These results suggest that the expression of Notch ligands in the T-ALL cells coincides with their extrathymic spreading and invasion to peripheral vital organs in vivo.

**Discussion**

In humans, meta-analyses of gene expression (NCBI Gene Expression Omnibus, http://lifesciencedb.jp/geo/) show that the negative Rap regulator SIPA1 is one of the most prominently under-expressed genes in T-ALL (Figure S5). Chromosomal translocation causing a fusion of NUP98 and RAP1GDS1 encoding Rap1 guanine nucleotide dissociation factor was also reported in a case of human T-ALL. In mice, we reported that BMT of Sipa1^−/−^C3G-F^1^ (Raphigh) HPCs invariably resulted in the development of Notch-dependent T-ALL in a cell-autonomous manner. Although these results suggest the involvement of the Rap signal in T-ALL, the mechanistic basis remained unknown.

Our current study indicated that specific attenuation of the Rap signal in a Notch-dependent T-ALL cell line by the expression of Rap1A17 or Sipa1 caused significantly compromised Notch activation and proliferation. Current results revealed that the Rap signal attenuation resulted in the decrease of an intracellular mature form of Adam10 and accordingly the reduced Notch S2 processing precedent to the NICD generation. Adam10 has multiple targets in various cell types, and Adam10-mediated extracellular cleavage of Dll1 was also reduced by Sipa1 expression in T-ALL cells. Maturation of Adam10 is achieved by proprotein convertases such as Furin that proteolytically remove a prodomain of Adam10 in the Golgi-network before a mature form is transported to the plasma membrane, and artificial inhibition of the proprotein convertase activity may result in the surface expression of the immature form of Adam10.

The majority of Rap1 is localized at the perinuclear Golgi-network, and our current results indicated that the inhibition of the Rap1 prenylation with GGTI caused a dislodgement of the Rap1 from the Golgi-network to the nuclei. Nuclear Rap1 sporadically detected in untreated Eph4 cells may represent unprenylated Rap1. The Rap1 dislodgement with GGTI was associated with the significant decrease of proprotein convertase activity. The convertase activity is activated by auto-cleavage of a prodomain and is regulated by various factors such as H^+^ and Ca^2+^ concentrations in the Golgi-network. Thus, it is suggested that the Rap signal at the Golgi-network membrane regulates the activation of proprotein convertases inside the Golgi-network. Although Furin also mediates intracellular Notch S1 processing, cell surface Notch expression was scarcely affected by the Rap signal inhibition, probably due to the expression of unprocessed Notch. The effects of GGTI can be broad in various cell types, however Notch-dependent T-ALL cell lines of humans and mice commonly showed much higher susceptibility to GGTI than other types of leukemia cells, suggesting that the Rap signal-mediated regulation of Adam10 maturation is crucially important in T-ALL cells. To support the notion, the exogenous expression of a mature form of Adam10 significantly overcame the Sipa-1-induced proliferation inhibition of FLO T-ALL cells. It is also noted that T-cell conditional deletion of Adam10 causes...
arrested thymic T-cell development, similar to T-cell conditional Sipa1 overexpression12,23.

Although deregulated Rap activation in normal ETPs induces remarkably enhanced Notch-mediated proliferation, the effect is dependent on the Notch ligands provided by stroma cells, and thus the enhanced Rap signal alone is incapable of bypassing the ligand requirement11. How Notch activation occurs cell-autonomously in T-ALL cells without other ligand-donor cells has been an open question, and several mechanisms have been reported. Activating Notch1 mutations, particularly at an HD region, may result in an intrinsic increase of spontaneous S2 cleavage28. It was also reported that unique Notch isoforms with spontaneous S2 cleavage are generated via cryptic Notch1 promoters in murine T-ALL models29. Our current results indicated that T-ALL cell lines express functional Notch ligands, and that the Notch activation and proliferation of those expressing intact Notch1 are significantly inhibited by the mixture of antibodies against the ligands. As expected, those of a T-ALL cell line with Notch1 receptor lacking the ligand-binding region were unaffected by the antibodies despite the ligand expression. The expression of Jagged1 in the T-ALL cells was dependent on the Notch signal, apparently forming an auto-amplification circuit as reported in human macrophages26. The results provide another mechanism for cell-autonomous Notch activation in T-ALL cells, in which Notch receptor engagement is achieved via a paracrine manner among the T-ALL cells, being reminiscent of “lateral” Notch activation30. Requirement of the intact cell–cell contacts for the optimal proliferation of T-ALL cells is consistent with the notion.

Using a T-ALL model by the BMT of Rap1high HPCs13, we also investigated the Notch ligand expression in the primary T-ALL cells in vivo. The results indicated that the initial intrathymic blast cells showed remarkably enhanced Notch expression but barely expressed Notch ligands. In contrast, the leukemic cells that spread and invaded into peripheral organs such as spleen, BM and liver significantly expressed the ligands on the cell surface with a remarkable increase of the transcripts. The possible mechanisms leading to the ligand expression under sustained Notch signaling in T-ALL cells remain to be investigated. A recent report indicates that a Toll-like receptor signal induces Notch ligand expression in collaboration with the Notch signal in macrophages28. We reported that, unlike Sipa1–/– C3G–F– HPCs, WT C3G–F– HPCs caused a remarkable increase in the oligoclonal thymic blast cells with little systemic leukemia, implying that the Rap signal strength might influence the leukemic spread of blast cells23. In any case, it may be suggested that the subclones of thymic blast cells expressing Notch ligands have an apparent advantage for the survival and proliferation outside the thymic tissues, owing to the liberation from the requirement of other ligand-donor cells. We thus propose that the Notch ligand expression may represent one of the early steps toward systemic T-ALL progression (Figure S6). It remains to be seen when and where the characteristic Notch mutations take place in the T-ALL-genic process, however Notch mutations leading to the Notch activation bypassing the ligand requirement28 as well as other secondary genetic changes bypassing the Notch signaling per se29 may further aggravate the disease (Figure S6).

Our current results disclose a mechanistic link between the Rap signal and Notch activation in T-ALL cells and may provide a novel strategic clue for therapeutic control of human T-ALL.

**Methods**

**Mice.** C57BL/6 (B6) and scid mice were purchased from Japan SLC (Shizuoka, Japan) and CLEA Japan (Tokyo, Japan), respectively; Sipa1–/– mice were described previously24. All mice were maintained under specific pathogen–free conditions at the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University, Tokyo, Japan, according to the University’s guidelines for the treatment of animals. All protocols were approved by the committee on the ethics of animal experiments of Kyoto University ( Permit Number: MediKyotol4049). All efforts were made to minimize suffering.

**Cell lines.** Murine T-ALL cell lines were reported previously15,16, and human T-ALL cell lines were obtained from RIKEN BRC, Saitama, Japan. FL0/rTAA Sipa1 and FL0/RapA17 cells were established by the transduction of FL0 cells with pRetroX-Tight-Sipa1 plus pRetroX-Tet-On Advansat vector (Clontech, Palo Alto, CA) and pMSCV-hNGFR (MIN)-RapA17, respectively. Because the addition of tag to RapA17 cDNA abrogated the dominant negative effect, untagged RapA17 cDNA was used. Mature (M) and immature (IM) fractions of the 7-aminoactinomycin D-stained (7-AAD) events/Disintegrin domain was cloned from FL0 cells with Flag-tagged at the 5′-terminus, and was integrated into a MIN retrovirus vector. FL0/rTAA-Sipa1 cells were infected with the MIN/m-Adam10. The mammary epithelial Eph4 cell line was transduced with pNBEKX1-Hygro-Sia-125 and pNBEKX-R1 (New England Bio Labs Inc., Beverly, MA) (Eph/Eph-Rho-Sipa1). The myeloblastic cell line (C2C12) was provided by Dr. R. Kageyama, Institute for Virus Research, Kyoto University, Kyoto, Japan. Cell viability was assessed with Cell Titer Glo assay (Promega, Madison, WI).

**Reagents.** The γ-secretase inhibitor (GSI) (DAPT, Calbiochem, San Diego, CA), the geranylgeranyl transferase inhibitor (GGT1) (GTGT298, Sigma-Aldrich, Poole, UK), and the Rho-switch ligand (RSL1, New England Biolabs Inc., Beverly, MA) were obtained commercially.

**Flow cytometry.** Multicolor flow cytometry analysis was performed with FACSCalibur flow cytometers (Becton Dickinson, Sunnyvale, CA). Antibodies included biotin-conjugated anti-Notch1, anti-Notch2, anti-Notch3, anti-Notch4, anti-Jagged1, anti-Jagged2, anti-Dll1, anti-Dll4, PE-conjugated anti-mouse Adam10 (BD Biosciences, Franklin Lakes, NJ), and anti-Notch1, biotin-conjugated anti-human nerve growth factor receptor (NGFR) antibodies (BD Bioscience, San Jose, CA). PE/Cy7-conjugated and APC-conjugated streptavidin (BioLegend, San Diego, CA) served as second reagents.

**Immunoblotting, immunoprecipitation, and pull-down assay.** Cells were lysed with lysis buffer (150 mM NaCl, 50 mM Tris [pH 7.6], 0.5% Triton X-100, protease and phosphatase inhibitors) and subjected to immunoblotting. Antibodies included anti-Notch1 (C-20, anti-actin (I-19), anti-unprenylated Rap1 (C-17), anti-Dll1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Hes1, anti-p27Kip1 (BD Transduction Laboratories, San Diego, CA), anti-Sipa1–/–, anti-Adam10 (ab39180) (Abcam, Cambridge, MA), and anti-Notch1 (Val7144) (DB3) (Cell Signaling Technology, Danvers, MA) antibodies. Antibodies for detection of the S2 product of Notch1, the lysate was immunoprecipitated with anti-Notch1 (C-20) and protein A–conjugated beads and then immunoblotted with S2-cleavage (V1711) specific antibody as reported previously30. Rap1GTP was assessed by a pull-down assay.

**Immunostaining.** Cells grown on cover glasses were fixed with chilled 100% methanol, blocked in PBS containing 1% BSA (w/v), and then incubated with primary antibodies, followed by fluorophore-conjugated secondary antibodies. The primary antibodies used were mouse anti-mouse Furin (Enzo Life Science, Farmingdale, NY) and rabbit anti-Rap1A (Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies were Alexa Fluor–488– or Cy3–conjugated antibodies. Nuclei were stained with 4, 6-diamidino-2-phenylindole (DAPI). Cover glasses were mounted on slides and examined by Axiosvert 200M inverted fluorescence microscope (Carl Zeiss, New York, NY).

**Notch ligand assay.** Notch ligand activity was assessed as reported by Luo et al.31. Briefly, normal thymocytes or T-ALL cells were cultured with C2C12 cell monolayers. Two days later, C2C12 cells were recovered by depleting CD45+ cells with rat anti-CD45 magnetic beads (Daynabeads, Life Technologies, Oslo, Norway), and Notch1 and Jagged1 transcripts were assessed by qRT-PCR.

**Proprotein convertase assay.** Cells were lysed with reaction buffer (500 mM HEPES, pH 7.0–2.5% Triton X-100, 5 mM CaCl2, 5 mM β-mercaptoethanol), and the lysates were incubated in black opaque 96-well plates (Perkin Elmer, Wellesley, MA) containing 0.1 mM Furin fluorogenic substrate (Calbiochem, La Jolla, CA) in the absence or presence of 5 mM EDTA. Fluorogenic intensity was measured with excitation at 355 nm and emission at 450 nm, 1 sec in every 1.5 min.

**Quantitative real-time PCR.** Total RNAs were isolated with TRIzol Reagent and treated with DNase I (Invitrogen, Carlsbad, CA), and cDNAs were synthesized with SuperScript III (Invitrogen, Carlsbad, CA). Quantitative real-time PCR (qRT-PCR) was performed with LightCycler 480 SYBR Green I Master Kit (Roche, Basel, Switzerland) on a LightCycler 480 instrument (Roche, Basel, Switzerland). The relative transcript levels were normalized to those of Gapdh. Primers were as follows:

Notch1, sense; gcagatgctcagggtgtctt, antisense; agttgtgccatcatgcattc,

Notch3, sense; cttgccatactctatttctc, antisense; ggtgtgccagtcagttc,

Jagged1, sense; gcagatgctcagggtgtctt, antisense; ggtgtgccagtcagttc,

Jagged2, sense; gcagatgctcagggtgtctt, antisense; ggtgtgccagtcagttc,

Propagin convertase assay.** Cells were lysed with reaction buffer (500 mM HEPES, pH 7.0–2.5% Triton X-100, 5 mM CaCl2, 5 mM β-mercaptoethanol), and the lysates were incubated in black opaque 96-well plates (Perkin Elmer, Wellesley, MA) containing 0.1 mM Furin fluorogenic substrate (Calbiochem, La Jolla, CA) in the absence or presence of 5 mM EDTA. Fluorogenic intensity was measured with excitation at 355 nm and emission at 450 nm, 1 sec in every 1.5 min.

**Retroviral infection and BM.** Isolation of BM Lin– HPCs and retroviral expression of C3G-F were performed as described before11. GFP+ cells were sorted with a FACSAria II (Becton Dickinson, San Jose, CA) and injected into 8.5 Gy γ-ray-irradiated B6 mice together with normal rescue BM cells.
Statistical analysis. Statistical analysis was performed using the Student’s t-test.

1. Zuniga-Pflucker, J. C. T-cell development made simple. Nature Rev. Immunol. 4, 67–72 (2004).
2. Maillard, I., Fang, T. & Pear, W. S. Regulation of lymphoid development, differentiation, and function by the Notch pathway. Annu. Rev. Immunol. 23, 945–974 (2005).
3. Logeat, F. et al. The Notch1 receptor is cleaved constitutively by a furin-like convertase. Proc. Natl Acad. Sci. 95, 8108–8112 (1998).
4. Kopan, R. & Ilagan, M. X. The canonical Notch signaling pathway: unfolding the activation mechanism. Cell 137, 216–233 (2009).
5. Hozumi, K. et al. Delta-like 4 is indispensable in thymic environment specific for T cell development. J. Exp. Med. 205, 2507–2513 (2008).
6. Van Vlierberghe, P. & Ferrando, A. The molecular basis of T cell acute lymphoblastic leukemia. J. Clin. Invest. 122, 3398–3406 (2012).
7. Weng, A. P. et al. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. Science 306, 269–271 (2004).
8. Chiang, M. Y. et al. Leukemia-associated NOTCH1 alleles are weak tumor initiators but accelerate K-ras-initiated leukemia. J. Clin. Invest. 118, 3181–3194 (2008).
9. Koch, U. & Radtke, F. Notch in T-ALL: new players in a complex disease. Trends Immunol. 32, 434–442 (2011).
10. Lobry, C., Oh, P. & Aifantis, I. Oncogenic and tumor suppressor functions of Notch in cancer: it’s NOTCH what you think. J. Exp. Med. 208, 1931–1935 (2011).
11. Kometani, K. et al. Essential role of Rap signal in pre-TCR-mediated beta-selection checkpoint in alphabeta T-cell development. Blood 112, 4565–4573 (2008).
12. Minato, N. & Hattori, M. Spa-1 (Sip1) and Rap signaling in leukemia and cancer metastasis. Cancer Sci. 100, 17–29 (2009).
13. Wang, S. E. et al. Development of Notch-dependent T-cell leukemia by deregulated Rap1 signaling. Blood 111, 2878–2886 (2008).
14. Murata, K. et al. Hes1 directly controls cell proliferation through the transcriptional repression of p27Kip1. Mol. Cell. Biol. 25, 4262–4271 (2005).
15. Berzat, A. C., Brady, D. C., Fiordalisi, J. J. & Cox, A. D. Using inhibitors of prenylation to block localization and transforming activity. Methods Enzym. 407, 575–597 (2006).
16. van Tetering, G. et al. Metalloprotease ADAM10 is required for Notch1 site 2 cleavage. J. Biol. Chem. 284, 31018–31027 (2009).
17. Anders, A., Gilbert, S., Garten, W., Postina, R. & Fahrenholz, F. Regulation of the alpha-secretase ADAM10 by its prodomain and proprotein convertases. FASEB J. 15, 1837–1839 (2001).
18. Six, E. et al. The Notch ligand Delta1 is sequentially cleaved by an ADAM protease and gamma-secretase. Proc. Natl Acad. Sci. 100, 7638–7643 (2003).
19. Edwards, D. R., Handsley, M. M. & Pennington, C. J. The ADAM metalloproteinases. Mol. Aspects Med. 29, 258–289 (2008).
20. Bourne, G. L. & Grainger, D. J. Development and characterisation of an assay for furin activation. J. Immunol. Meth. 364, 101–108 (2011).
21. Thomas, G. Furin at the cutting edge: from protein traffic to embryogenesis and disease. Nat. Rev. Mol. Cell Biol. 3, 753–766 (2002).
22. Anderson, E. D., VanSluyk, J. K., Thulin, C. D., Jean, F. & Thomas, G. Activation of the furin endoprotease is a multiple-step process: requirements for acidification and internal propeptide cleavage. EMBO J. 16, 1508–1518 (1997).
23. Hussey, D. J., Nicola, M., Moore, S., Peters, G. B. & Dobrovic, A. The (4;11) (q21;p15) translocation fuses the NUP98 and RAP1GDS1 genes and is recurrent in T-cell acute lymphoblastic leukemia. Blood 94, 2072–2079 (1999).
24. Carey, R. M., Bluvstein, J. K. & Slack, B. E. Surface expression and limited proteolysis of ADAM10 are increased by a dominant negative inhibitor of dynamin. BMC Cell Biol. 12, 20 (2011).
25. Nomura, K., Kenemura, H., Satoh, T. & Kataoka, T. Identification of a novel domain of Ras and Rap1 that directs their differential subcellular localizations. J. Biol. Chem. 279, 22664–22673 (2004).
26. Nichols, J. T. et al. DSL ligand endocytosis physically dissociates Notch1 heterodimers before activating proteolysis can occur. J. Cell Biol. 176, 445–458 (2007).
27. Tian, L. et al. ADAM10 is essential for proteolytic activation of Notch during thymocyte development. Int. Immunol. 20, 1181–1187 (2008).
28. Malecki, M. J. et al. Leukemia-associated mutations within the NOTCH1 heterodimerization domain fall into at least two distinct mechanistic classes. Mol. Cell. Biol. 26, 4642–4651 (2006).
29. Gomez-del Arco, P. et al. Alternative promoter usage at the Notch1 locus supports ligand-independent signaling in T cell development and leukemogenesis. Immunity 33, 685–698 (2010).
30. Folds, J. et al. Autamplification of Notch signaling in macrophages by TLR-induced and RBP-J-dependent induction of Jagged1. J. Immunol. 185, 5023–5031 (2010).
31. Artavanis-Takonas, S., Matsuno, K. & Fortini, M. E. Notch signaling. Science 268, 225–232 (1995).
32. Palomer, T. et al. Mutational loss of PTEN induces resistance to NOTCH1 inhibition in T-cell leukemia. Nature Med. 13, 1203–1210 (2007).
33. Ishida, D. et al. Myeloproliferative stem cell disorders by deregulated Rap1 activation in SPA-1-deficient mice. Cancer cell 4, 55–65 (2003).
34. Lee, J. S., Ishimoto, A., Honjo, T. & Yanagawa, S. Murine leukemia provirus-mediated activation of the Notch1 gene leads to induction of HES-1 in a mouse T lymphoma cell line, DL-3. FEBS Lett. 455, 276–280 (1999).
35. Koyanagi, A., Sekine, C. & Yagita, H. Expression of Notch receptors and ligands on immature and mature T cells. Biochem. Biophys. Res. Commun. 418, 799–805 (2012).
36. Luo, B., Aster, J. C., Hasserjian, R. P., Kuo, F. & Sklar, J. Isolation and functional analysis of a cDNA for human Jagged2, a gene encoding a ligand for the Notch1 receptor. Mol. Cell. Biol. 17, 6057–6067 (1997).

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Author contributions

K.D., T.I., C.K. and J.I. performed experiments and collected data; H.Y. and M.V. developed and provided antibodies; Y.A. and Y.H. helped gene construction and immunostaining, respectively; and N.M. designed the research and wrote the paper.

Additional information

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