Cross talk between EBV and telomerase: the role of TERT and NOTCH2 in the switch of latent/lytic cycle of the virus

S Giunco1, A Celeghin1, K Giansesi1, R Dolcetti2, S Indraccolo3 and A De Rossi1,3

Epstein–Barr virus (EBV)-associated malignancies, as well as lymphoblastoid cell lines (LCLs), obtained in vitro by EBV infection of B cells, express latent viral proteins and maintain their ability to grow indefinitely through inappropriate activation of telomere-specific reverse transcriptase (TERT), the catalytic component of telomerase. Our previous studies demonstrated that high levels of TERT expression in LCLs prevent the activation of EBV lytic cycle, which is instead triggered by TERT silencing. As lytic infection promotes the death of EBV-positive tumor cells, understanding the mechanism(s) by which TERT affects the latent/lytic status of EBV may be important for setting new therapeutic strategies. BATF, a transcription factor activated by NOTCH2, the major NOTCH family member in B cells, negatively affects the expression of BZLF1, the master regulator of viral lytic cycle. We therefore analyzed the interplay between TERT, NOTCH and BATF in LCLs and found that high levels of endogenous TERT are associated with high NOTCH2 and BATF expression levels. In addition, ectopic expression of TERT in LCLs with low levels of endogenous telomerase was associated with upregulation of NOTCH2 and BATF at both mRNA and protein levels. By contrast, infection of LCLs with retroviral vectors expressing functional NOTCH2 did not alter TERT transcript levels. Luciferase reporter assays, demonstrated that TERT significantly activated NOTCH2 promoter in a dose-dependent manner. We also found that NF-κB pathway is involved in TERT-induced NOTCH2 activation. Lastly, pharmacologic inhibition of NOTCH signaling triggers the EBV lytic cycle, leading to the death of EBV-infected cells. Overall, these results indicate that TERT contributes to preserve EBV latency in B cells mainly through the NOTCH2/BATF pathway, and suggest that NOTCH2 inhibition may represent an appealing therapeutic strategy against EBV-associated malignancies.

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viral lytic cycle. BATF is a transcription factor mainly expressed in hematopoietic tissues and in B cells infected with EBV. Interestingly, BATF is a target gene of NOTCH signaling in B cells. The NOTCH gene family encodes transmembrane receptors that modulate differentiation, proliferation and apoptotic programs in response to extracellular stimuli. NOTCH signaling is activated by the interaction of the extracellular domain of NOTCH with one of its ligands, belonging to the delta-like and jagged families. This interaction induces a conformational change in NOTCH, resulting in two proteolytic cleavages mediated by ADAM protease and gamma-secretase, and cytoplasmic release of the NOTCH intracellular domain (NOTCH-ICD), allowing its translocation to the nucleus, where it participates in transcriptional regulation of target genes.

In particular, NOTCH2 has an important role in the development of marginal zone B cells, and NOTCH2 gene mutations or overexpression can be detected in B-cell malignancies. These observations, together with the demonstration that NOTCH2 can induce the expression of BATF, prompted us to examine the possible involvement of NOTCH2 in the mechanisms underlying the regulation of EBV latent/lytic status affected by TERT in LCLs. As viral lytic replication is associated with the death of infected cells, discovering the pathways involved in the mechanisms by which TERT regulates the balance between EBV latency and lytic replication may be useful in designing new strategies to treat EBV-driven malignancies.

Results

BATF and NOTCH2 are expressed at high levels in TERT-positive LCLs. We first examined the expression of BATF and NOTCH2 in LCLs expressing various levels of endogenous TERT. LCLs greatly differed in their timing of TERT expression and telomerase activation; in fact, they exhibit telomerase activity (TA) since their early culture passages after EBV infection or else become telomerase positive during their establishment in culture. Figures 1a and b show seven representative LCLs with low (4134/Early, 4810/Early and 4193) or high (4134/Late, 4810/Late, 4815 and 4141) levels of endogenous TERT and TA; LCLs with low/undetectable TERT levels and TA had significantly lower BATF mRNA levels ($P=0.0215$; Figure 1c). TERT-positive LCLs also had more than threefold higher NOTCH2 transcript levels than TERT-negative LCLs ($P=0.0174$; Figure 1d). Western blot analysis showed higher levels of NOTCH2 protein in TERT-positive LCLs, thus supporting results of the transcript analysis (Figure 1e). Overall, these findings suggest a possible interplay between TERT, BATF and NOTCH2 levels in LCL cells.

Ectopic expression of TERT is followed by increased expression of functional NOTCH2 protein. To investigate whether TERT could modulate NOTCH2 expression, we examined 4134/Early cells transfected with a retroviral vector containing TERT (4134/TERT+) or with the control vector (4134/BABE); 4134/TERT+ cells expressing ectopic TERT showed much higher levels of TERT transcripts than parental or control 4134/BABE cells (Figure 2a). Increased TERT mRNA levels were paralleled by a concomitant increase in TA (Figure 2b). Cells expressing ectopic TERT showed increased levels of both NOTCH2 and BATF transcripts and relative proteins (Figures 2c and d). Interestingly, forced TERT expression was accompanied by a significant increase in transcripts and protein expression of jagged 1 (JAG1), one of the NOTCH ligands (Figures 2d and e). The expression of NOTCH2 and JAG1 in 4134/TERT+ cells was also associated with increased NOTCH signaling, as indicated by upregulation of transcripts of hes family bHLH transcription factor 1 (HES1), a canonical target gene activated by the NOTCH pathway (Figure 2e). NOTCH signaling in our LCL model is probably activated by interactions between cells expressing both JAG1 and NOTCH2, which may occur in the tight clumps typically formed in B cells after EBV infection. Upregulation of JAG1 and HES1 expression was also detected in cells expressing high levels of endogenous TERT transcripts (data not shown).

NOTCH2-ICD does not induce TERT expression. To ascertain whether NOTCH2 could also induce TERT expression, 4134/Early cells were infected with a retroviral vector expressing the functional NOTCH2-ICD (4134/MigRI-ICN2 or 4134/MSCVpuro-ICN2) or with a control vector (4134/BABE). NOTCH2 overexpression in infected cells was confirmed by PCR amplification (Figure 3a) and, as expected, was followed by increased expression of the NOTCH target gene HES1 (Figure 3b). As shown in Figure 3c, ectopic expression of NOTCH2-ICD did not significantly modify the level of TERT transcripts. Since NOTCH2 participates directly in transcriptional regulation of nuclear genes, we performed additional experiments to confirm that NOTCH2 did not activate TERT expression. TERT-negative U2OS cells were co-transfected with pHtERTpromoterLuc, expressing luciferase under the control of the TERT promoter, and a plasmid expressing NOTCH2-ICD (pMigRI-ICN2 or pMSCVpuro-ICN2), or a plasmid expressing v-myc avian myelocytomatosis viral oncogene homolog (MYC; pMT2TMyc), a well-known activator of TERT promoter. Luciferase analysis showed that, unlike MYC, NOTCH2-ICD does not activate the TERT promoter (Figure 3d).

TERT transactivates the NOTCH2 promoter via NF-$\kappa$B signaling. The finding that TERT overexpression increased NOTCH2 mRNA levels prompted us to ascertain whether the NOTCH2 promoter could be activated by TERT. HCT116 cells were co-transfected with the pGL3N2PR-2327/-99 plasmid, carrying the luciferase gene under the control of NOTCH2 promoter and with pEGFP-+hTERT, a plasmid encoding a enhanced green fluorescent protein (EGFP)–TERT fusion protein, or the pEGFP-C1 vector as a control. Luciferase analysis disclosed that the NOTCH2 promoter was significantly activated by ectopic TERT expression in a dose-dependent manner (Figure 4a).

Recent studies have indicated a telomere-independent role for TERT as a transcriptional modulator of the Wnt and NF-$\kappa$B signaling pathways. Therefore, in order to identify the mechanism exploited by TERT to induce NOTCH2 expression, we examined the effects of Wnt and NF-$\kappa$B inhibitors on
the regulation of the NOTCH2 promoter by TERT. HCT116 cells were co-transfected with pGL3N2PR-2327/-99 and pEGFP–hTERT or with pGL3N2PR-2327/-99 and pEGFP-C1 as control, and cultured in serial dilutions of the NF-κB signaling inhibitor NF-κB activation inhibitor (N-AI) or dimethyl-sulfoxide (DMSO). Luciferase reporter assay performed 24 h after co-transfection showed that N-AI treatment reduced NOTCH2 promoter activation by TERT in a dose-dependent manner compared with DMSO-treated cells (Figure 4b). To ascertain the specificity of the N-AI effect, we performed parallel experiments by co-transfecting HCT116 cells with pGL3N2PR-2327/-99 and pCGN-HA-S33Y-β-catenin, a plasmid encoding an S33Y-mutated β-catenin protein known to activate the NOTCH2 promoter through the Wnt pathway.33 Results showed that N-AI treatment did not modify the ability of S33Y-β-catenin to activate the NOTCH2 promoter compared with DMSO-treated cells (Figure 4c). Conversely, experiments with the Wnt signaling inhibitor XAV-939 showed that XAV-939 did not counteract NOTCH2 promoter induction by TERT compared with DMSO-treated cells (data not shown). These data suggest that NF-κB, but not Wnt signaling, is involved in the TERT-mediated activation of NOTCH2 expression.

To further investigate the involvement of NF-κB signaling in the regulation of the NOTCH2 promoter by TERT, we performed the luciferase reporter assay with a smaller NOTCH2 luciferase promoter reporter construct (pGL3N2PR-110), covering the most proximal LEF-1/TCF-site but lacking two putative NF-κB binding motifs.33,38 In agreement with a previous study,33 we observed that luciferase activity was similarly increased by co-transfection with pCGN-HA-S33Y-β-catenin with either pGL3N2PR-2327/-99 or pGL3N2PR-110 (7.5-fold increase for pGL3N2PR-2327/-99 and 6.6-fold increase for pGL3N2PR-110 compared with co-transfection with the control pcDNA3 plasmid; Figure 4d). In contrast, pEGFP–hTERT activated pGL3N2PR-110 significantly less efficiently than pGL3N2PR-2327/-99 (P<0.001), (3.4-fold

Figure 1  High levels of TERT are associated with high levels of BATF and NOTCH2 expression in LCLs. TERT levels, telomerase activity (TA), BATF and NOTCH2 expression in several LCLs. (a) TERT transcripts were quantified by real-time PCR. Values are means and S.D. (bar) of three replicates. (b) TA was analyzed by TRAP assay. TL, telomerase ladder; ITAS, internal telomerase assay standard. Panels are representative of three separate analyses. (c) BATF and (d) NOTCH2 transcripts were quantified by real-time PCR. Relative units were calculated according to 2−ΔΔCt formula, with HPRT1 as housekeeping gene. Values are means and S.D. (bar) of three replicates. (e) Expression of NOTCH2 and housekeeping α-tubulin proteins was assessed by western blot. Panels are representative of three separate analyses. Presence (+) or absence (−) of TA in cell cultures is reported below.
increase for pGL3N2PR-110 and 6.8-fold increase for pGL3N2PR-2327/-99 compared with co-transfection with the control pEGFP-C1 plasmid; Figure 4d). Taken together, these results demonstrate that TERT activates the NOTCH2 promoter and that this effect is mostly mediated by the NF-κB signaling pathway.

Inhibition of NOTCH signaling triggers EBV lytic cycle. We have previously demonstrated that, in the LCL system, the viral latent membrane protein 1 (LMP1) activates TERT at transcriptional level, whereas TERT silencing triggers the viral lytic cycle. This effect is associated with downregulation of BATF, a transcription factor able to modulate BZLF1 expression. According to these previous findings, we hypothesized that TERT can affect EBV latent/lytic status by modulating NOTCH2 expression which, in turn, may influence BATF expression. Thus, inhibition of NOTCH2 signaling was predicted to induce activation of the EBV lytic cycle. To test this hypothesis, we treated 4134/TERT+ cells with gamma-secretase inhibitors (GSIs), including compound E (CompE) and dibenzazepine (DBZ). GSIs are compounds that block the final cleavage of the precursor form of NOTCH, thus preventing generation of ICD and inhibiting NOTCH signaling. GSIs treatment of 4134/TERT+ cells reduced both
the canonical NOTCH target gene, HES1 and BATF expression in a time- and dose-dependent manner (Figures 5a and b). Western blot analysis confirmed that the cleavage of NOTCH2 was inhibited by treatment with GSIs (Figure 5c).

Inhibition of NOTCH signaling at 5 days also resulted in the expression of lytic viral BZLF1 and E-AD genes (Figures 5c and d). GSI treatment consistently resulted in a remarkable increase in the number of positive cells expressing late viral lytic gp350 (from <1% in control cell cultures to ~35% in cell cultures treated with 5μM CompE or 0.5 μM DBZ; Figure 5e). B95.8 cells were employed as positive control for EBV lytic protein expression (Supplementary Figure 1).

The complete induction of the viral lytic cycle was confirmed by the release of EBV virions, as shown by increased EBV DNA levels in DNase-treated culture supernatants (Figure 5f) after 5 days of GSI treatment compared with supernatant from untreated control cells.

As the EBV lytic cycle promotes the death of infected cells, we studied the possible pro-apoptotic effect of GSI treatment on EBV-infected cells. Treatment of 4134/TERT+ cells for 5 days with 5μM of CompE or 0.5μM of DBZ induced an increase in the number of apoptotic cells (by >30%) compared with DMSO-treated cells (Figure 6a). The number of cells undergoing apoptosis after GSI treatment was
consistent with the percentage of cells positive for late lytic gp350 viral protein. In agreement with published data, treatment with GSIs (5 μM of CompE or 0.5 μM of DBZ), only slightly increased the percentage of apoptotic cells (Supplementary Figure 2). In EBV- and TERT-negative U2OS cells, treatment with 10 μM of CompE or with 1 μM of DBZ did not alter cell viability compared with untreated control cells (data not shown). Furthermore, in view of the emerging interest in approaches combining lytic cycle inducers with antiviral drugs to treat EBV-driven tumors, we studied the apoptotic effect of combined treatment with GSI and ganciclovir (GCV), an antiviral pro-drug that is activated by viral lytic protein kinase. 41–43 As shown in Figure 6a, the combined treatment of 4134/TERT+ cells with GSI and GCV

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**Figure 4** Transcriptional activation of NOTCH2 promoter by TERT. (a) HCT116 cells were co-transfected with a plasmid expressing luciferase under control of NOTCH2 promoter (pGL3N2PR-2327/99) and with 1 or 2 μg of plasmid expressing TERT (pEGFP–hTERT) or control vector (pEGFP–C1). (b) HCT116 cells were co-transfected with NOTCH2 reporter plasmid pGL3N2PR-2327/99 and with pEGFP–hTERT or control pEGFP–C1. Three hours before co-transfection, cells were treated with indicated concentrations of N-AI or with DMSO as control. (c) HCT116 cells were co-transfected with NOTCH2 reporter plasmid pGL3N2PR-2327/99 and with a plasmid encoding an S33Y-mutated β-catenin protein (pCGN-HA-S33Y–β-catenin) or a control plasmid (pDNA3). Three hours before co-transfection, cells were treated with indicated concentrations of N-AI or with DMSO as control. (d) HCT116 cells were co-transfected with vectors allowing the expression of S33Y-mutated β-catenin (pCGN-HA-S33Y–β-catenin) or TERT (pEGFP–hTERT) or with control vectors (pDNA3 and pEGFP–C1) and with a plasmid expressing luciferase under control of larger NOTCH2 promoter (pGL3N2PR-2327/99) or smaller NOTCH2 promoter (pGL3N2PR-110). A plasmid expressing bacterial β-Gal (pCMV–β-Gal) gene was also co-transfected in each experiment as internal control for transfection efficiency. Luciferase assay was performed 24 h post transfection. Values are means and S.D. (bar) of four separate experiments. NS, not significant; *P < 0.05
further increased the rates of apoptotic cells compared with cells treated with GSI alone (Figure 6a). However, treatment with GCV alone did not affect cell viability. The increased rate of apoptotic cells in cultures treated with a combination of GCV and GSI compared with GSI treatment alone suggests that in some GSI-treated cells the EBV lytic reactivation is abortive, but sufficient to produce the early lytic EBV protein kinase able to activate the pro-drug GCV. Lastly, activation of the EBV lytic cycle after GSI treatment was also observed in EBV-positive cells of BL cell line BL41/B95.8 (data not shown). As observed in 4134/TERT+, also in BL41/B95.8 cells GSI treatment induced a pro-apoptotic effect enhanced by combined treatment, GSI with GCV (Figure 6b), indicating that this is a general phenomenon for EBV-carrying B lymphocytes.

Discussion

Despite their pathogenic importance, the mechanisms underlying EBV reactivation in vivo are poorly understood. Available data obtained from in vitro models indicate that EBV lytic cycle can be elicited by treatment of latently infected cells with a
variety of reagents including 12-O-tetradecanoyl-phorbol-1-acetate, calcium ionophores, sodium butyrate, anti-immunoglobulin antibodies and TGF-β. The effects of all these reagents converge on the upregulation of two EBV immediate–early genes, BZLF1 and BRLF1, which orchestrate the activation of viral lytic replication. Nevertheless, in all experimental conditions investigated so far, only a fraction of cells treated with these reagents enter the lytic cycle, the remainder of the population being refractory to lytic replication. Therefore, identification of cellular factors that regulate the balance between latency and lytic replication of EBV is critical for better understanding of the complex interplay between virus and infected cells.

It is well established that TERT activation is a prerequisite for an efficient EBV-driven B-cell immortalization. Accumulating evidence indicates that TERT may have additional functions,
beyond its role in preserving telomere homeostasis. Our previous studies have demonstrated that high levels of TERT expression in LCLs prevent the induction of the EBV lytic cycle, which is instead triggered by TERT silencing. However, the fine mechanisms by which TERT levels affect EBV lytic/latent status have not yet been elucidated. In the present study, we demonstrate that high expression of TERT in LCLs induces an increase in levels of NOTCH2 and its target genes, including BATF, an inhibitor of the expression of BZLF1, the main EBV lytic cycle inducer. Therefore, our findings show that TERT contributes to preserve EBV latency in B cells mainly through NOTCH2-dependent BATF activation.

BATF is a member of the AP-1/ATF superfamily of basic leucine zipper transcription factors, able to form heterodimers with Jun proteins to bind to AP-1 consensus sites preferably. Available data indicate that BATF expression may antagonize the B-cell growth and inhibit pro-apoptotic gene expression in these cells. EBV nuclear antigen 2 (EBNA2) has been shown to induce BATF immediately after infection of primary B cells. Notably, EBNA2 could be regarded as a functional homolog of an active NOTCH receptor, due to its ability to be tethered to promoter regions by interaction with the DNA-binding protein RBPU. EBNA2 and NOTCH2 appear to be partially interchangeable as regards to their ability to activate target genes and modulate signaling pathways in B-cell lines. In the absence of EBNA2, NOTCH may transcriptionally upregulate the expression of EBV LMP 2A, which in turn activates the NOTCH pathway in a positive feedback loop. On these grounds, our results indicate that, in B cells, TERT activates the NOTCH/BATF cascade, a cellular pathway that is functionally hijacked by EBV for critical regulation of the balance between latency and lytic replication, and induction of immortalization.

The role of NOTCH signaling in B-cell lymphomagenesis is not yet clear, and only limited data are available, particularly for EBV-driven lymphomas; nevertheless, dysfunctions of this pathway may be involved in neoplastic development. In particular, deregulation of NOTCH2 signaling and consequent CD23 upregulation have been observed in B-cell malignancies, such as B-cell chronic lymphocytic leukemia. The involvement of the NF-κB pathway in lymphomas is involved in NOTCH2 promoter induction by TERT. These results were observed with both an NF-κB signaling inhibitor and a NOTCH2 luciferase reporter construct lacking two putative NF-κB binding motifs. The involvement of the NF-κB pathway in NOTCH2 promoter activation was also recently demonstrated by Wang et al., who reported that inflammatory cytokine-dependent induction of NOTCH2 in nucleus pulposus cells requires direct interaction of the NF-κB/p65 protein with the NOTCH2 promoter. Overall, the results of the present study provide new insights into how cellular genes coordinately control EBV latency. In particular, we demonstrate that TERT contributes to preserve EBV latency through the NOTCH2 cellular pathway; as virus latency is required for EBV-driven cell transformation, this study suggests that NOTCH2 has a significant role in LCL immortalization. Our finding that NOTCH2 inhibition triggers the EBV lytic cycle and cell apoptosis is of particular importance in the light of increasing interest in developing strategies to reactivate EBV lytic gene expression in latently infected tumor cells to treat overt EBV-associated lymphomas. Several chemotherapeutic drugs are known to trigger EBV replication, and the combination of antivirals with lytic cycle inducers is emerging as a highly promising strategy for the treatment of EBV-driven tumors. On these grounds, our results also demonstrate that the antiviral drug GCV can enhance the apoptotic effect induced by GSI treatment in both LCL and EBV-positive BL. Our findings therefore suggest that GSIs can be combined with other drugs in therapeutic schedules aimed at inducing EBV lytic reactivation against EBV-associated lymphomas. In this respect, it will be of interest in future studies to assess whether TERT inhibitors can synergize with GSIs.

**Materials and Methods**

**Plasmids.** Plasmids were provided as follows: a plasmid expressing luciferase under the control of NOTCH2 promoter (pGL3N2PR-2327/99), a smaller NOTCH2
reporter plasmid lacking two putative NF-κB binding motifs (pGL3N2PR-110) and a plasmid expressing an S33Y-mutated β-catenin protein (pCGN-HA-S33Y-βcatenin)33 obtained from Jonas Ungerber (Linkops University, Linkoping, Sweden); human TERT linked to EGFP in pEGFP (pEGFP-HTERT) and the control empty vector pEGFP-C1 was obtained from Chantall Axenti (Lady Davis Institute for Medical Research, Jewish General Hospital, Montreal, Quebec, Canada); plasmids expressing the intracellular domain of NOTCH2 (pMigRI-ICN2 and pMSCVpuro-ICN2) from Adolfo Ferrando (Columbia University, New York, NY, USA); plasmids containing 800-bp fragment upstream of the TERT translational start site, pHTERTpromoterUC26 from Riccardo Dalla Favaer (Columbia University).

Cell cultures. LCLs 4134, 4810, 4193, 4815 and 4141 were obtained by infecting peripheral blood mononuclear cells from normal donors with EBV strain B95.8. The establishment and characterization of these cell lines have been previously described.3 We considered as ‘early LCLs’ cells within the first 50 culture passages after EBV infection, and ‘late LCLs’ cells which underwent up to 50 culture passages after EBV infection.3 Cells (4134) expressing ectopic TERT (4134/TERT+) and control cells (4134/BABE) were obtained by infecting parental telomerase-negative cells with the pBABE retroviral vector, either containing or lacking TERT CDNA, respectively.3 4134/MgRh-ICN2 and 4134/MSCVpuro-ICN2 cells were obtained by infecting 4134 cells with retroviral vectors expressing the NOTCH2-ICD (pMigRh-ICN2 or pMSCVpuro-ICN2). Retroviral vectors were generated by a transient three-plasmid vector packaging system, as previously described.3 BL41 is an EBV-negative BL cell line, kindly provided by Martin Rowe (Cancer Center, University of Birmingham, Birmingham, UK). BL41/B95.8 is the counterpart cell line infected in vitro with EBV strain B95.8 and was kindly provided by Martin Allday (Ludwig Institute for Cancer Research, London, UK). B95.8 6.8 cell line is an EBV-positive marmoset lymphoblastoma cell line, which spontaneously has around 5% of cells in the lytic cycle.53 was employed as positive control for lytic protein expression. LCLs and BL41 cells were cultured in RPMI-1640 medium (Euroclone, Milano, Italy), supplemented with 2% glutamine, 50 μM 2-mercaptoethanol, and 10% heat-inactivated fetal bovine serum (FBS; Gibco, Milano, Italy; standard medium). BL41/B95.8 and B95.8 cells were cultured in standard medium supplemented with 1 mM sodium pyruvate, 1% nonessential amino acids (Sigma), and 50 mM NaCl.

Telomerase activity assay. Telomerase activity was assessed with a PCR-based telomeric repeats amplification protocol (TRAP) as previously reported.66,67 The TRAP assay was performed with 0.250 μg of total cell lysates.

Western blot. Western blot analyses were prepared as previously reported.68 The expression of viral EA-D and cellular NOTCH2-ICD, BATF, JAG1 and α-tubulin were evaluated by monoclonal anti-EA-D (clone 6D1, Abcam, Cambridge, UK), monoclonal anti-NOTCH2 (clone C861.60DHN developed by Spyros Artavanis-Tsakonas, obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NCI and maintained by The University of Iowa, Department of Biology, Iowa City, IA, USA), monoclonal anti-BATF (1D4; Novus Biologicals, Cambridge, UK), polyclonal anti-JAG1 (H114; Santa Cruz Biotechn, Dallas, TX, USA) and clone B-512 (Sigma), respectively. Blots were incubated with an appropriate peroxidase-conjugated secondary antibody (Sigma), and stained with a chemiluminescence detection kit (SuperSignal West Pico Chemiluminescent Substrate, Pierce, Rockford, IL, USA).

Immunohistochemistry. Cytosines were fixed in cold acetone (4 °C) for 10 min. Expression of gp350 protein was analyzed using the monoclonal antibody clone O221 (Abcam). After incubation for 1 h with the primary antibody, immunostaining was performed with the avidin-biotin peroxidase complex (ABC kit; Vector Laboratories, Burlingame, CA, USA), monoclonal anti-EA-D (clone 6D1, Abcam, Cambridge, MA, USA), and 3–3 diaminobenzidine chromogen as substrate (Dako, Glostrup, Denmark). The cells were lightly counterstained with Mayer’s hematoxylin. The specificity of the staining procedure was confirmed by replacing the primary antibody with PBS.

Luciferase reporter assay. HCT116 cells (4.5 x 10⁶) were transiently transfected with 1 μg of pGL3N2PR-2327/-99, serial dilution of pEGFP-HTERT, or empty pEGFP-C1 and 0.5 μg cytomegalovirus β-galactosidase (pCMV-β-Gal). A set of experiments were also conducted with a smaller NOTCH2 luciferase promoter construct (pGL3N2PR-110) instead of the longer pGL3N2PR-2327/-99. In some experiments, cells were treated with the inhibitors XAV-933 (Selleck Chemicals LLC, Houston, TX, USA) or N-AI (EDM Millipore) at the indicated concentration 3 h before transfection. DMSO was employed as control. As positive control, HCT116 were also co-transfected with pCGN-HA-S33Y-βcatenin a plasmid known to activate the NOTCH2 promoter or with pcDNA3 as control plasmid. Lipofectamine 2000 (Invitrogen) was used as a transfection reagent according to the manufacturer's instructions.

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EBV genome quantification. EBV DNA from virus particles released in culture supernatants were quantified by real-time PCR37 after ultracentrifugation and DNase treatment, as previously reported.3
Apoptosis analysis. Apoptosis was evaluated by staining cells with annexin V and propidium iodide (PI, Roche), as previously detailed, and analyzed by flow cytometry. At least 50,000 events were acquired, data were processed with CellQuestPro software (BD Biosciences, Erembodegem, Belgium), and analyzed with Kaluza Analyzing Software v1.2 (Beckman Coulter, Brea, CA, USA). Annexin V-positive/PI-negative and annexin V-positive/PI-positive samples were classified as early and late apoptotic cells, respectively, and both fractions were included in the percentage of apoptotic cells.

Statistical analysis. For statistical comparisons, the Student’s t-test or the Mann–Whitney U test were conducted with SPSS software version 21 (IBM, Armonk, NY, USA). P-value < 0.05 was considered to be statistically significant.

Conflict of Interest

The authors declare no conflict of interest.

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