Notch1 oncoprotein antagonizes TGF-β/Smad-mediated cell growth suppression via sequestration of coactivator p300

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The Notch proteins constitute a family of transmembrane receptors that play a pivotal role in cellular differentiation, proliferation and apoptosis. Although it has been recognized that excess Notch signaling is potentially tumorigenic, little is known about precise mechanisms through which dysregulated Notch signaling induces neoplastic transformation. Here we demonstrate that Notch signaling has a transcriptional cross-talk with transforming growth factor-β (TGF-β) signaling, which is well characterized by its antiproliferative effects. TGF-β-mediated transcriptional responses are suppressed by constitutively active Notch1, and this inhibitory effect is canceled by introduction of transcriptional coactivator p300. We further show that this blockade of TGF-β signaling is executed by the sequestration of p300 from Smad3. Moreover, in a human cervical carcinoma cell line, CaSki, in which Notch1 is spontaneously activated, suppression of Notch1 expression with small interfering RNA significantly restores the responsiveness to TGF-β. Taken together, we propose that Notch oncoproteins promote cell growth and cancer development partly by suppressing the growth inhibitory effects of TGF-β through sequestrating p300 from Smad3. (*Cancer Sci* 2005; 96: 274–282)

The Notch pathway constitutes an evolutionarily conserved signaling pathway that mediates critical cell fate decisions, such as differentiation, proliferation and apoptosis.[1,2] In addition to the fact that Notch signaling plays pivotal roles in embryonic development, and post-embryonic growth and differentiation in multiple systems such as the immune system,[3,4] substantial evidence indicates that the constitutively activated forms of Notch family proteins are involved in tumorigenesis:[5–17] *Notch1/TAN-1* was originally identified as a recurrent chromosomal translocation, t(7;9)(q34; q34.3), in a human acute T-cell lymphoblastic leukemia,[5] resulting in the expression of an extracellular region-truncated form of Notch1 that is known to be constitutively active. The N-terminal truncated forms of the Notch1 and Notch2 proteins have been implicated in the transformation of rat kidney cells in cooperation with an adenoviral oncoprotein, E1A.[10,15] Activated forms of the Notch1[8] and Notch3 proteins[13] are capable of generating T-cell leukemia when retrovirally introduced into bone marrow cells that are transplanted into irradiated recipient mice. Also, *Int-3*, which encodes a truncated form of Notch4, has been shown to contribute to the generation of mammary carcinoma in mice.[6,11] Interestingly, recent reports have suggested that Notch1 is upregulated in Ras-transformed cells in which activation of Notch1 signaling is necessary to maintain the neoplastic phenotype.[18] Notch activation that causes human neoplasms has been shown to result not only from the truncation, based on the genetic aberration, but also Notch ligand stimulation,[18,19] suggesting that Notch activation without its own genetic abnormalities could be frequently involved in tumorigenesis.[20,21]

Despite rapidly accumulating information about the Notch signaling system, little is known about the mechanism through which excess Notch signaling triggers cellular transformation. One of the clues to this issue is the fact that Notch serves as an adaptor for molecules involved in transcriptional machinery, among which we focus on p300,[22] one of the most common transcriptional coactivator proteins.

The p300 protein interacts with molecules functioning in multiple signaling pathways. Transforming growth factor-β (TGF-β) also uses p300 through activated Smad3.[23,24] TGF-β inhibits proliferation of a wide range of cells including epithelial, endothelial and hematopoietic cells. It plays an important role in controlling tumor development, and its signaling constitutes one of the tumor-suppressor pathways.[25–27] Smads are a class of proteins that function as intracellular signaling effectors for the TGF-β superfamily, which includes TGF-β, activins and bone morphogenetic proteins (BMP).[28,29] Smad2 and Smad3 are directly phosphorylated by the type I TGF-β receptor in response to TGF-β, leading to formation of heteromeric complexes with Smad4, and are then translocated into the nucleus where they bind to the TGF-β-responsive regulatory sequences, either directly through the Smad-binding elements or in conjugation with other sequence-specific DNA-binding proteins.[30–32] It is suggested that p300 forms the bridge between the Smad complex and the transcriptional apparatus.

Here we show that constitutively active Notch1, consisting of the intracellular domain alone (ICN1), inhibits the antiproliferative activity of TGF-β via the sequestration of p300 from Smad3. We propose that conferring resistance to TGF-β signaling may, in part, be attributed to a mechanism of Notch-induced neoplastic transformation.

Materials and Methods

Plasmids. Expression vectors for Smad2-Flag, Smad3-Flag and Smad4-hemagglutinin A (Smad4-HA) were described previously (pCMV5/Smad2-Flag, pCMV5/Smad3-Flag and pCMV5/Smad4-HA).[33,34] p3TP-Lux, pcDNA3/TβRI(TD)-HA, pcDNA3/6Xmyc-Smad3 and pcDEF3/p300-Flag were kindly provided by K. Miyazono (University of Tokyo, Japan). pcDNA3/myc-ICN1 (amino acids 1747–2531 of mouse Notch1), pME18Sneo/myc-ICN, pTracerCMV/ICN1-Flag, pTracerCMV/ICN2-Flag and pTracerCMV/ICN3-Flag were described previously.[35,36] The C-terminal deletion constructs,

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pcDNA3/myc-RAMICΔC (1747–2193) and pcDNA3/myc-RAM/ANK (1747–2097), were made by digestion of pcDNA3/myc-ICN1 with *Eco*RI and *Xba*I (RAMICΔC), and with *Eco*RI and *Eco*RV (RAM/ANK), respectively. The ΔEP in-frame deletion of ICN1 was constructed by digesting pcDNA3/myc-ICN1 with *Pvu*II and *Eco*RI, and religating the plasmid. The EP mutant construct corresponds to the 2102LDE/AAA2104 mutation in the EP domain of ICN1 and was made by using an *in vitro* mutagenesis system (Stratagene). The TP-1-Luc reporter plasmid pGa981–6 was a gift from L. Strobl and U. Zimber-Strobl (GSF Institute for Clinical Molecular Biology, Germany). pEF-BOSneo-RBP-J (R218H) was kindly provided by T. Honjo (Kyoto University, Japan).

Cell culture and establishment of stable clones. HepG2, Mv1Lu, C2C12 and COS-7 cells were cultured at 37°C under 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS). CHO(r) cells were maintained in alpha-minimal essential medium containing 10% FCS. CaSki cells were cultured in RPMI 1640 containing 10% FCS. To generate stable Mv1Lu clones overexpressing ICN1, myc-ICN1 subcloned into the pME18Sneo vector was transfected using SuperFect (Qiagen) according to the manufacturer's instructions. These cells were selected in medium containing G418 (800 μg/mL). G418-resistant clones were screened for expression of myc-ICN1 by Western blotting. Two independent clones with comparable expression levels were used in further assays.

Growth inhibition assay. The stable clones derived from Mv1Lu cells were plated in duplicate at a density of 5×10^3 per well in 96-well culture plates. After 12 h, cells were treated with increasing concentrations of TGF-β1 (R and D Systems) for 48 h. During the last 4 h, the cells were labeled with 2.5 μCi/mL [^{3}H]thymidine (Amersham Pharmacia Biotech). Thereafter, the incorporation of [^{3}H]thymidine was determined by liquid scintillation counting.

For RNA interference, CaSki cells were seeded at a density of 5×10^3 per well in a 96-well culture plate 24 h after transfection with small interfering RNA (siRNA). At 12 h after seeding the cells were treated with increasing concentrations of TGF-β for 48 h, after which time the [^{3}H]thymidine incorporation assay was carried as described above.

RNA interference. The siRNA against human Notch1, 5′-AAGGUGUCUUCCAGAUCCUGA-3′, was produced by Qiagen-Xeragon (Germantown). A non-silencing siRNA, 5′-AAUUCUCCGAACGUGUCACGU-3′ (Qiagen-Xeragon), was used as a control. At 24 h after CaSki cells (1×10^5 per well) were seeded in six-well plates, the cells were transfected with siRNA at a final concentration of 50 nM using Oligofectamine transfection reagent (Invitrogen) according to the manufacturer's instructions. The cells were lysed 48 h after transfection and were subjected to Western blotting or used for the thymidine incorporation assay as described above.

Luciferase assay. For analysis of luciferase activities, HepG2 cells were seeded in 12-well culture plates at a density of 4×10^4 per well. Cells were then transfected 12 h after seeding with various amounts of effector plasmids, together with the reporter plasmids, using SuperFect (Qiagen). As an internal control of transfection efficiency, a plasmid expressing β-galactosidase was cotransfected. The cells were harvested 48 h after transfection and assayed for luciferase activity. The data were normalized to β-galactosidase activity. Cells were treated with 1 ng/mL TGF-β1 for 48 h before harvesting.

Coimmunoprecipitation and Western blotting. COS-7 cells transiently transfected with the constructs were washed and lysed in TNE buffer (10 mM Tris-HCl [pH 7.8], 150 mM NaCl, 1 mM ethylenediaminetetracetic acid, 0.5% Nonidet P-40, 0.1% aprotinin, 1 mM phenylmethylsulfonylfluoride, 12.5 mM β-glycerophosphate, 1 mM Na_3VO_4 and protease inhibitor cocktail [Sigma; 1/50 volume]). For immunoprecipitation, total cell extracts were incubated with anti-p300 monoclonal antibody (Upstate Biotechnology) for 4 h at 4°C. The samples were then incubated with protein G Sepharose (Pharmacia Biotech) for 15 min at 4°C. Immunoprecipitates were washed five times with the TNE buffer, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed by Western blotting with anti-myc antibody (9E10). Western blotting to detect intramembranously cleaved Notch1 was carried out using anticleaved Notch1 (Val 1744) antibody (Cell Signaling Technology).

Results

Active Notch1 inhibits the antiproliferative effects of TGF-β. To determine whether constitutively active Notch1 could affect the antiproliferative effects of TGF-β, we established several Mv1Lu cell lines that express ICN1 stably (N1-1 and N1-4), along with control clones (M-1 and M-2) (Fig. 1a). We carried out [^{3}H]thymidine-incorporation assays in the presence of various concentrations of TGF-β. Results are expressed as percentages relative to values obtained from control cultures in the absence of TGF-β. For example, at 1 ng/mL TGF-β, the relative [^{3}H]thymidine incorporation by M-1 and M-2 was $6.5 \pm 1.0\%$ and $5.9 \pm 0.6\%$, respectively, while that by N1-1 and N1-4 was $21.6 \pm 0.2\%$ and $28.6 \pm 0.7\%$, respectively (Fig. 1b). These results demonstrate that the growth of the mock clones was effectively inhibited by TGF-β, whereas the Mv1Lu clones that overexpress ICN1 showed reduced responsiveness to TGF-β.

Knockdown of active Notch1 expression by siRNA restores the antiproliferative effects of TGF-β. To further investigate the role of active Notch1 on the antiproliferative effects of TGF-β, we used a human cervical carcinoma cell line, CaSki. This line of cells

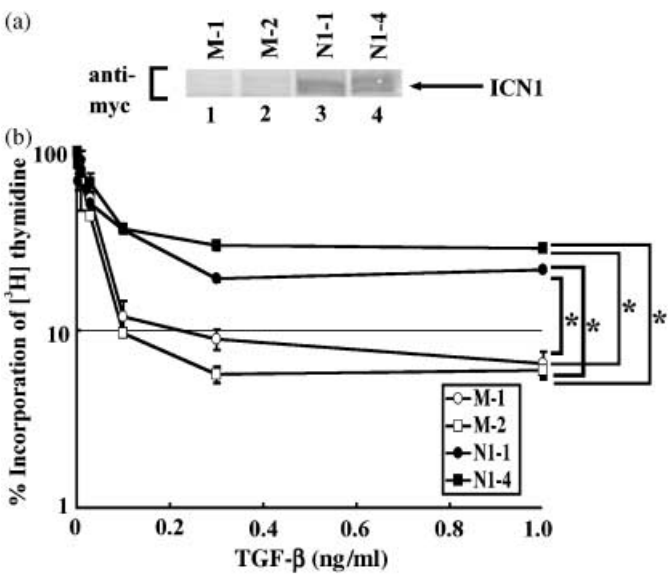

Fig. 1. Constitutive expression of active Notch1 (ICN1) in Mv1Lu cells overcomes transforming growth factor (TGF)-β-mediated growth inhibition. (a) Expression of ICN1 in stable Mv1Lu transfectants. Clones M-1 (lane 1) and M-2 (lane 2) are mock clones transfected with pME18Sneo empty vector followed by G418 selection. Clones N1-1 (lane 3) and N1-4 (lane 4) were established from cells transfected with pME18Sneo/myc-ICN1. The results of immunoblotting with anti-myc antibody are shown. (b) [^{3}H]thymidine incorporation into Mv1Lu clones was assayed in the presence of the indicated concentrations of TGF-β. Results are expressed as percentages relative to values obtained from control cultures in the absence of TGF-β. $*P < 0.01$.

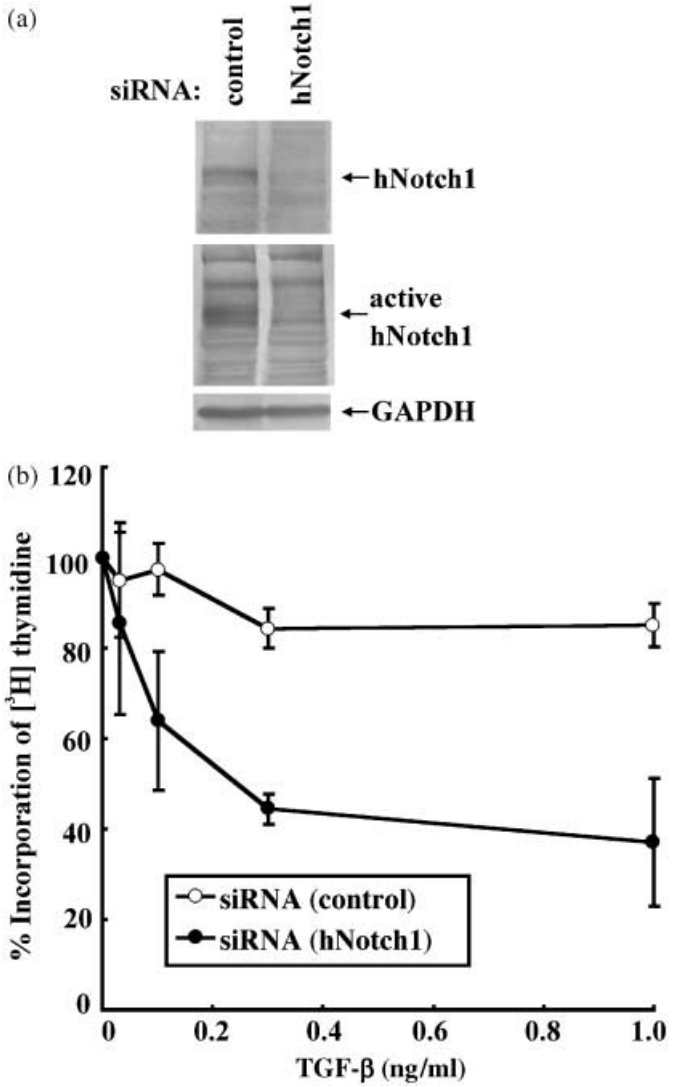

Fig. 2. Suppression of active Notch1 expression by small interfering RNA (siRNA) results in recovered responsiveness to transforming growth factor (TGF)-β. (a) Suppression of human Notch1 protein expression by siRNA. CaSki cells, spontaneously expressing truncated Notch1 protein, were transfected with control or human Notch1 siRNA, followed by immunoblotting. The upper panel, with antihuman Notch1 antibody (bTAN20); the middle panel, with anticleaved human Notch1 (Val 1744) antibody, specific to intramembranously truncated human Notch1; the lower panel, with anti-glyceraldehyde-3-phosphate dehydrogenase antibody as loading control. (b) [^{3}H]Thymidine incorporation into CaSki cells, which had been transfected with control or human Notch1 (hNotch1) siRNA, was assayed in the presence of the indicated concentrations of TGF-β. Results are expressed as percentages relative to values obtained from control cultures in the absence of TGF-β.

has been shown to endogenously overexpress Notch1, leading to spontaneous activation of Notch1 (Fig. 2a).[18] Thus, this cell line could be useful for an approach based on siRNA. We transfected CaSki cells with siRNA targeted to human Notch1, which specifically and efficiently abrogated Notch1 protein expression (Fig. 2a). By using fluorescein-labeled control siRNA, we confirmed that siRNA could be introduced into CaSki cells with almost 100% efficiency (data not shown). Interestingly, an activated form of Notch1, which is detectable only by the antibody recognizing intramembranously cleaved Notch1, was undetectable if this siRNA was introduced (Fig. 2a). We found that wild-type and control siRNA-introduced CaSki cells had poor responsiveness to TGF-β, but after repression of active human Notch1 by siRNA, CaSki cells responded to TGF-β (Fig. 2b). Taken together, these results indicate that active Notch1 functions in CaSki cells to maintain their unresponsiveness to TGF-β.

Transcriptional responses mediated by TGF-β or Smad overexpression are suppressed by Notch signaling. Next, we examined the effects of ICN1 on TGF-β-mediated transcriptional responses in HepG2 cells with reporter assays using p3TP-Lux, a TGF-β-responsive reporter plasmid. Fold increase in the luciferase activity triggered by TGF-β was repressed to 20–30% when ICN1 was introduced (Fig. 3a).

To further investigate whether ligand-induced Notch signaling also represses TGF-β signaling, we used the C2C12 cell line, which is responsive to both Notch ligand[37] and TGF-β.[38] To stimulate cells with the Notch ligand Delta1, C2C12 cells were cocultivated with irradiated CHO(r) cells expressing full-length Delta1 (CHO-fD1).[39] The increase in luciferase activity of p3TP-Lux in the presence of TGF-β was repressed by Delta1 stimulation (Fig. 3b), suggesting that ligand-stimulated Notch signaling can antagonize TGF-β signaling.

We then examined whether ICN1 inhibits the transcriptional responses induced by Smad overexpression. Transcriptional activation of p3TP-Lux induced in HepG2 cells either by Smad3 alone or a combination of Smad2 or Smad3 with Smad4 was also suppressed by cotransfection with ICN1, in a dose-dependent manner (Fig. 3c,d). Similar repression was observed when we used other Smad-responsive reporter plasmids: p800neo-Luc, which contains the natural PAI-1 promoter, and p15P113-Luc, which contains the p15 promoter (Fig. 3e,f).

In vertebrates, Notch proteins comprise a family of four transmembrane receptors (Notch1 through Notch4).[1] To examine whether constitutively active Notch proteins other than ICN1 also inhibit TGF-β/Smad signaling, we compared the effect of ICN2 and ICN3 with that of ICN1 on the TGF-β-induced activation of the p3TP reporter. We found that ICN2 and ICN3 suppress TGF-β-induced transcriptional activation just as ICN1 did (Fig. 3g), indicating that suppression of TGF-β signaling is common to the constitutively active Notch proteins.

Overexpression of p300 partially overcomes the inhibitory effect of ICN1 on Smad3-mediated transactivation. Recent studies have indicated that both Smad proteins and ICN1 bind the general transcriptional coactivator p300 to mediate their transcriptional activities.[22–24] It is known that the binding regions of the p300 protein for the partner signaling molecules are variable and that both Smads and ICN1 bind the C-terminal region of p300. This information prompted us to examine whether p300 is involved in the Notch-mediated blockade of TGF-β/Smad signaling. When p300 was exogenously introduced into HepG2 cells, we observed that the ICN1-mediated suppression of Smad3-induced p3TP-Lux transactivation was reversed in a manner dependent on the dose of introduced p300 (Fig. 4).

These observations suggest that the availability of p300 by Smad3 may be limited and reduced when ICN1 is introduced. Therefore, we speculated that p300 may be sequestered from Smad3 by ICN1 when these molecules coexist. To see whether there is a reciprocal sequestration, we investigated whether ICN1-induced transcriptional activation of the TP-1 promoter, which is well characterized as a target of Notch signaling, is suppressed by the TGF-β/Smad activation. We observed a positive, although less remarkable, reciprocal repression of the ICN1-induced TP1 transactivation by overexpression of Smad3 and Smad4 (data not shown).

ICN1 mutants defective in p300 binding fail to repress the Smad-dependent transcriptional activation. Notch1 interacts with p300 through the 'EP domain' located at the C-terminal flanking region of the ankyrin repeats (ANK).[22] To further demonstrate that p300 is involved in the ICN1-mediated repression of TGF-β/Smad-induced transactivation, we used ICN1 mutants either

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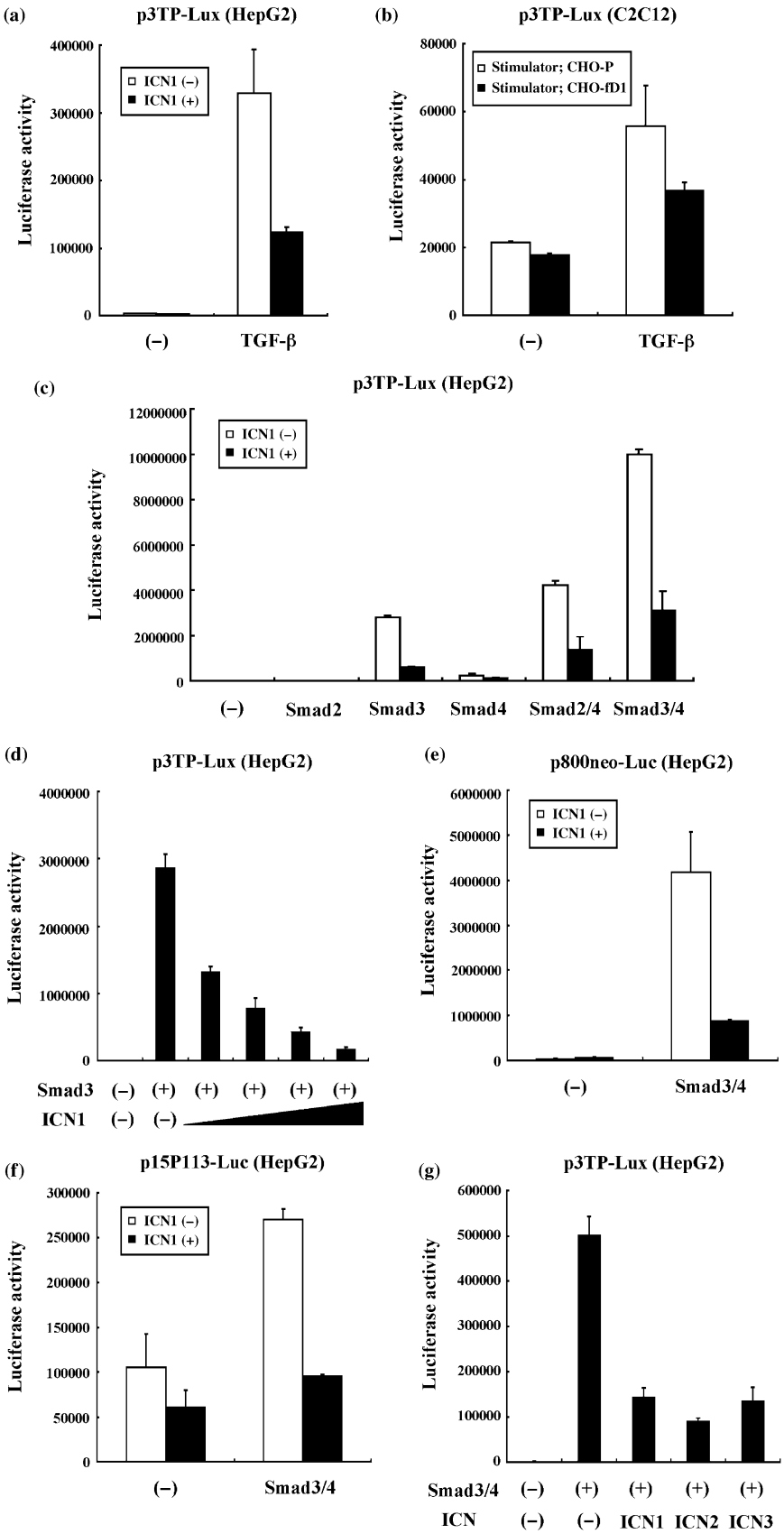

Fig. 3. Transforming growth factor (TGF)-β or Smad-mediated transcriptional responses are suppressed by Notch signaling. (a) TGF-β-induced transcriptional responses are repressed by ICN1. p3TP-Lux was transfected into HepG2 cells together with either pcDNA3 empty vector or pcDNA3-ICN1. Cells were incubated in the absence or presence of 1 ng/mL TGF-β for 48 h, and luciferase activities were measured. (b) Notch ligand stimulation also inhibits the TGF-β-mediated transcriptional responses. C2C12 cells, transiently transfected with p3TP-Lux, were cocultured with either irradiated parental CHO(r) cells (CHO-P) or irradiated CHO(r) cells expressing full-length Delta1 (CHO-fD1) in the absence or presence of 5 ng/mL TGF-β for 48 h, and luciferase activities were measured. (c) ICN-1 suppresses Smad-induced transcriptional responses. Either pcDNA3 empty vector or pcDNA3-ICN1, together with p3TP-Lux, was transfected into HepG2 cells, in combination with the indicated Smad constructs. (d) ICN1 represses Smad-induced responses in a dose-dependent manner. HepG2 cells were transfected with 3, 10, 30 or 100 ng ICN1 expression plasmid, together with p3TP-Lux and Smad3. Smad-induced transcriptional responses with (e) p800neo-Luc or (f) p15P113-Luc are also repressed by ICN1. p800neo-Luc or p15P113-Luc was transfected into HepG2 cells together with either pcDNA3 empty vector or pcDNA3-ICN1, in combination with Smad3 and Smad4. (g) ICN2 and ICN3 suppress the Smad-mediated transcriptional activation as ICN1. Either pTracerCMV empty vector, pTracerCMV/ICN1, pTracerCMV/ICN2 or pTracerCMV/ICN3 was transfected into HepG2 cells, together with p3TP-Lux, Smad3 and Smad4, and luciferase activities were measured.

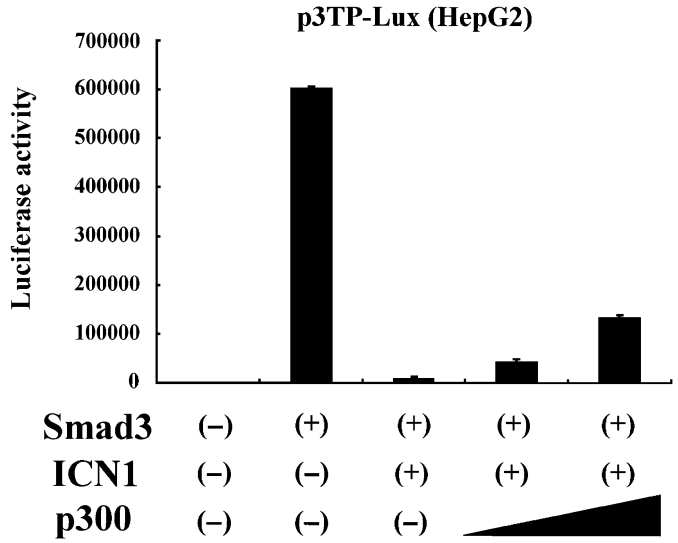

Fig. 4. Repression of Smad3-mediated transactivation by ICN1 is recovered with coactivator p300. Introduction of p300 partially rescues the ICN1-mediated suppression of Smad3-induced p3TP-Lux transactivation in a dose-dependent manner. HepG2 cells were transfected with 0.1 or 1 μg p300 expression plasmid, together with p3TP-Lux, Smad3 and ICN1, followed by a luciferase assay.

with or without the p300 interaction capability (Fig. 5a). RAMICΔC is a C-terminally truncated mutant that lacks the TAD and PEST domains but contains the EP domain. In RAM/ANK, the EP domain and all the sequence C-terminal to it are deleted. ΔEP is an internal deletion mutant lacking only 15 amino acids corresponding to the EP domain. EP (LDE/AAA) carries a three-amino acid substitution, i.e. LDE to AAA within the EP domain, which was previously demonstrated to be critical for transactivation of Notch signaling as well as for the interaction with p300.[22] As expected, RAM/ANK, ΔEP and EP (LDE/AAA), all of which lose the capacity to interact with p300, failed to fully repress transcription from the 3TP promoter induced by overexpression of Smad3 and Smad4. In contrast, RAMICΔC, which binds to p300, suppressed the TGF-β/Smad-induced transactivation just as wild-type ICN1 did (Fig. 5b). The expression levels of ICN1 and its derivatives in HepG2 cells were analyzed by Western blotting (Fig. 5b). These results suggest that the EP domain is required for the suppression of the Smad transactivation by ICN1.

ICN1 reduces the amount of p300 binding to Smad3. To determine whether ICN1 interferes with Smad3 activity through sequestration of p300, we investigated the effect of wild type and various mutants of ICN1 on the interaction between Smad3 and p300 in the presence of activated TGF-β receptor. As shown in previous reports,[24] we observed that Smad3 was coimmunoprecipitated with p300 (Fig. 6). The amount of coimmunoprecipitated Smad3, however, was markedly reduced when wild-type ICN1 was cotransfected. RAMICΔC showed a similar effect, whereas RAM/ANK, ΔEP and EP (LDE/AAA) had little or no effect on the amounts of Smad3 coprecipitated with p300 (Fig. 6). Taken together, these results suggest that the EP domain of ICN1 is essential for the sequestration of p300 from Smad3.

The RBP-J-dependent transcription of target genes is not required for the repression of TGF-β signaling by ICN1. If the inhibition of Smad3-mediated transcriptional activation by ICN1 is attributed

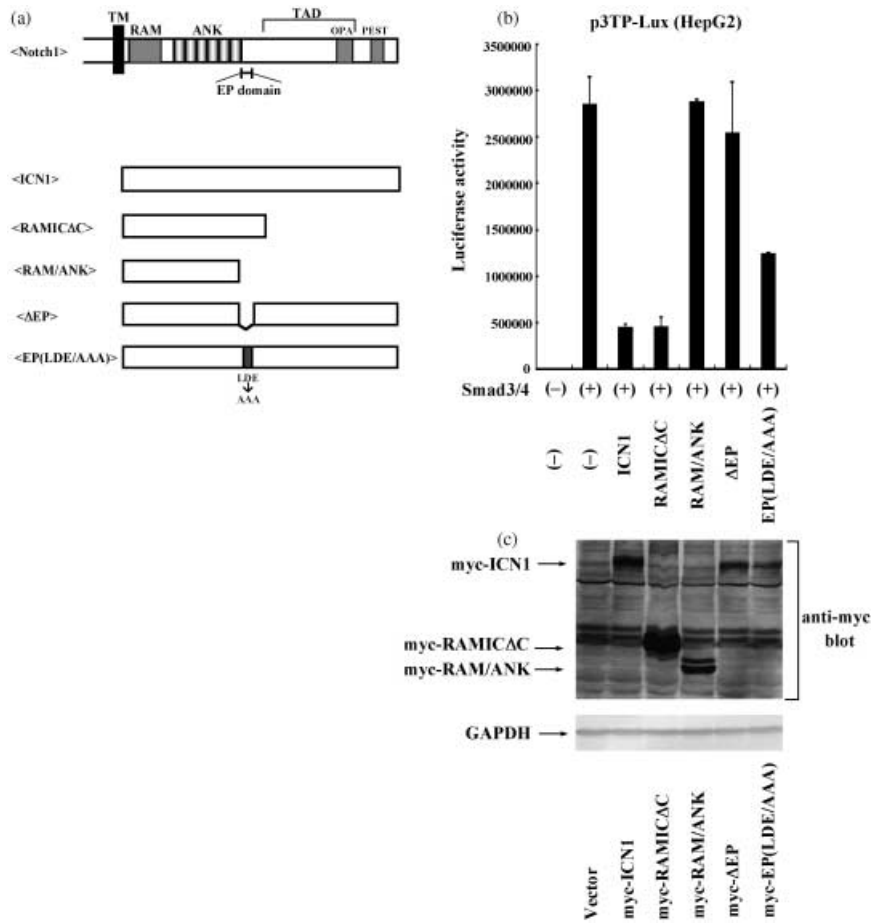

Fig. 5. The EP domain is indispensable for suppression of Smad signaling by ICN1. (a) Schematic representation of the mouse Notch1 intracellular region (ICN1) and its derivatives used in this study. The EP domain, essential for ICN1 to interact with p300, is located in the C-terminal flanking region adjacent to the ankyrin repeats of ICN1. (b) Structural requirements for repression of Smad signaling by ICN1. HepG2 cells were transfected with Smad3, Smad4 and p3TP-Lux together with wild type or each mutant of ICN1, and luciferase activities were measured. Expression levels of myc-ICN1 and its myc-tagged derivatives were evaluated in HepG2 cells. Each whole cell extract was analyzed by Western blotting using an anti-myc antibody (upper panel). The lower panel shows the glyceraldehyde-3-phosphate dehydrogenase expression as loading control.

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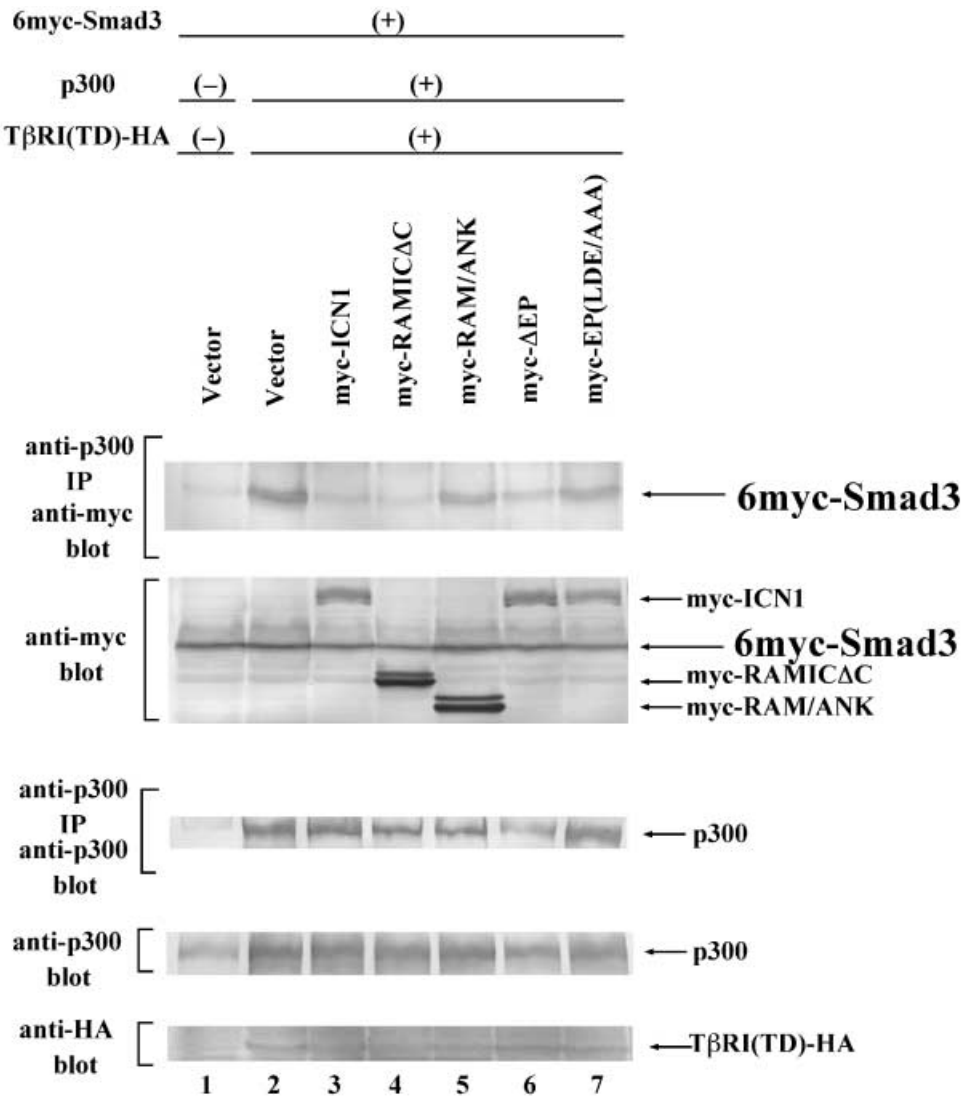

Fig. 6. Effects of ICN1 on the p300–Smad interaction. Wild type or each mutant of ICN1 was coexpressed in COS-7 cells with 6myc-Smad3, TβRI(TD)-HA and p300. The cell lysates of transfected COS-7 cells were subjected to immunoprecipitation with anti-p300 antibody followed by immunoblotting with anti-myc antibody, which detects the interaction of p300 and 6myc-Smad3. Immunoprecipitates were also blotted with anti-p300 antibody, and cell lysates were blotted with anti-myc, anti-p300 and anti-hemagglutinin A antibodies.

to the sequestration of p300 from Smad3, we hypothesized that it may not be mediated by transcription targeted by the complex of ICN1 and RBP-J (or CSL from *C*BF1/RBP-J, *S*uppressor of Hairless, *L*ag-1), a DNA-binding protein with which activated Notch proteins transactivate target genes. To clarify this possibility, we used a dominant-negative form of RBP-J (RBP-J [R218H];[40] hereafter referred to as DN-RBP), which lacks the ability to bind to DNA but still interacts with Notch1 and represses ICN1-induced transactivation of the TP-1 promoter (Fig. 7a). Reporter assays showed that DN-RBP did not reverse the ICN1-induced repression of TGF-β signaling, indicating that the RBP-J-dependent transcription of specific target genes is not required for ICN1-induced repression of TGF-β signaling (Fig. 7b).

Discussion

In this study, we have demonstrated a transcriptional cross-talk between the Notch and TGF-β signaling pathways. Because Smad proteins are important tumor suppressors, the ability of active Notch1 (ICN1) to repress TGF-β signaling could be responsible, at least partially, for the transforming activity of Notch. A recent study has reported that ICN1 blocks TGF-β-mediated growth arrest in epithelial cells.[41] In that context, ICN1 deregulates expression of c-Myc and thereby renders epithelial cells resistant to growth-inhibitory signals, suggesting a novel link between Notch and cell cycle control. In the experiments described here, we show another mechanism explaining the antagonism between the Notch and TGF-β signaling systems, that is, repression of TGF-β-mediated signaling through sequestration of coactivator p300 by ICN1, which is apparently independent from the mechanism demonstrated by Rao and Kadesch.[41]

Importantly, some investigators have demonstrated that Notch and Smad signaling show functional synergism.[42–44] More complexly, transcriptional activation of the hairy/enhancer of split (HES)-related gene Hey1 is both a direct target of Smad3 and an indirect target through Smad3-dependent transcriptional activation of Notch signaling component genes.[45] Demonstration of direct and TGF-β-dependent interactions between Smad3 and ICN1,[44] and Smad1/5 and ICN1,[42,43] indeed serves as bona-fide evidence of the cross-talk between these two signaling

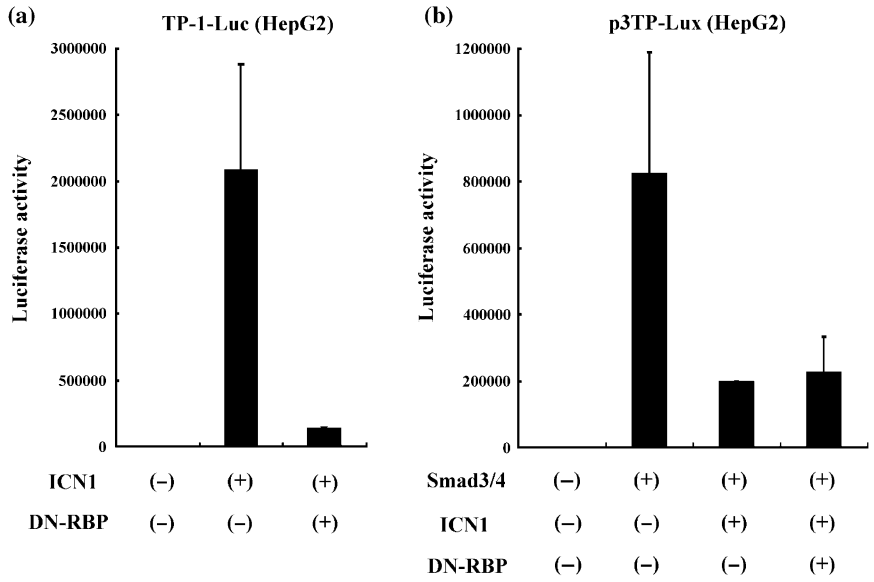

Fig. 7. The effects of DN-RBP on the transcriptional activity of ICN1 and the Smad proteins. (a) Expression of a dominant-negative form of RBP-J (DN-RBP) suppresses TP-1 activity induced by ICN1. TP-1-Luc was transfected into HepG2 cells, together with ICN1 and DN-RBP. (b) DN-RBP does not reverse the ICN1-induced repression of the Smad signaling. p3TP-Lux was transfected into HepG2 cells, together with Smad3, Smad4, ICN1 and DN-RBP.

systems. It appears that various molecular interactions could exist between these two signaling systems, most likely in a cell context-dependent manner. Indeed, there is a report showing that both synergy and antagonism could occur between the Notch and Smad signaling systems.[43]

Many transcription factors, including ICN1 and Smads, use the coactivator p300 to activate transcription.[22–24] The p300 protein is generally present at limiting concentrations within the cell nucleus, and functional antagonism between transcription factors occurs as a consequence of direct competition for binding to p300.[46–50] Domains within the p300 protein for interaction with individual transcription factors are highly variable, but both active Notch1 and Smad have been reported to bind to the C-terminal domain, which can potentially be shared. Our results suggest competition between active Notch1 and Smad for limiting quantities of complexes containing p300. Similar competition for p300 has been described for several cellular pathways, including nuclear receptor and AP-1,[46] p53 and E2F,[47] NF-κB and p53,[48] NF-κB and nuclear receptor,[49] and STAT and AP-1.[50]

Regarding Notch-induced transformation, previous studies have indicated that in baby rat kidney cells (RKE) immortalized with E1A, the minimal transforming domain includes ANK and flanking 107 C-terminal amino acids.[15] Consistent with this, our data showed that the EP domain, adjacent to ANK, is required for suppressing Smad activity by ICN1. Recently, the crystal structure revealed that the LDE motif in the EP domain not only governs the stability around this domain but also potentially contributes to direct contacts with p300,[51] supporting our result that the EP mutant (LDE/AAA) fails to sequester p300 from Smad3. Moreover, it is interesting that p300 was isolated as a cellular target of the adenoviral oncoprotein E1A,[52] which is known to block the functions of p300. Therefore, we can speculate that ICN1 may promote sequestration of p300 from Smad3 in cooperation with E1A in RKE cells.

In this study, the biological phenomenon under this transcriptional cross-talk was assessed by both upregulation and downregulation of Notch signaling. For the former, we used strategies of ligand stimulation and overexpression of constitutively active Notch1. For the latter, siRNA-based suppression of Notch1 synthesis in CaSki cells was used successfully to significantly reduce spontaneous generation of the cleaved (i.e. active form of) Notch1. We can speculate that Notch1 is spontaneously activated in CaSki cells either by ligand stimulation from neighboring cells or a cell-autonomous mechanism. In either case, we have demonstrated that spontaneous activation of Notch1 contributes to the growth of CaSki cells, and that blockade of this activation results in a recovered responsiveness to TGF-β. Taken together, we have here demonstrated that active Notch1 may serve as a positive regulator of cell growth by repressing TGF-β-induced growth inhibition. We observed, however, that CaSki cells made no response to TGF-β under treatment with γ-secretase inhibitors, chemical compounds that block Notch cleavage, despite our observation that the amount of active Notch1 was decreased and the transcriptional activation of the Notch reporter gene was suppressed when CaSki cells were treated with γ-secretase inhibitors (data not shown). This observation was apparently puzzling. However, it has since been reported that many transmembrane proteins, in addition to Notch and the amyloid precursor protein that was the substrate identified originally, could be substrates of the γ-secretase.[53] These new lines of evidence made it possible for us to speculate that γ-secretase inhibitors might influence other growth signal pathways and that the specific knockdown of Notch signaling might be achieved using the RNA interference technique, rather than with a γ-secretase inhibitor. It is of future interest to elucidate the mechanisms underlying the failure to restore responsiveness to TGF-β by γ-secretase inhibitors in CaSki cells.

Genetic and molecular studies have implicated several downstream components in the Notch signaling pathway, such as RBP-J and Deltex. As RBP-J is one of the main effectors in Notch signaling,[37,54] it is critical to determine whether the RBP-J-dependent transcription is required for the inhibitory effect of Notch signaling. If that is the case, DN-RBP, a DNA-binding mutant that perturbs Notch activity in a dominant-negative manner, should cancel this suppression. The negative result of the experiment using DN-RBP, however, suggests that RBP-J-dependent transcription of specific target genes is not required for the inhibition of TGF-β signaling.

The p300 protein functions as global transcriptional coactivator and plays important roles in a broad spectrum of biological processes, including cell proliferation and differentiation.[52] A role for p300 in tumor suppression has been proposed, and biallelic mutations of p300 have been identified in certain types of human cancers.[55,56] Furthermore, it was reported recently that reintroduction of wild-type p300 suppresses the growth of p300-deficient carcinoma cells.[57] Insufficiency of cyclic AMP response element binding protein (CBP), a coactivator closely

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related to p300, also results in both Rubinstein–Taybi Syndrome in humans, a disease characterized by an increased propensity for malignancies, and an increased incidence of leukemias in mice, suggesting that characteristics of tumor suppressors may be common to these general coactivators p300 and CBP.[52]

In summary, we propose that activated Notch represses TGF-β-mediated signaling possibly through sequestration of coactivator p300, which contributes to the mechanisms of Notch-induced neoplastic transformation. Our current results indicate that Notch oncoproteins promote cell proliferation and tumor development partly by repressing the tumor suppressor Smad.

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