Notch inhibits apoptosis by direct interference with XIAP ubiquitination and degradation

Wen-Hsien Liu¹, Huey-Wen Hsiao^{1,2},
Wen-I Tsou¹ and Ming-Zong Lai^{1,2,3,*}

¹Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan, ROC,
²Graduate Institute of Microbiology and Immunology, National Yang-Ming University, Taipei, Taiwan, ROC and ³Graduate Institute of Immunology, National Taiwan University, Taipei, Taiwan, ROC

The physiological activity of Notch is a function of its ability to increase survival in many cell types. Several pathways have been shown to contribute to the survival effect of Notch, but the exact mechanism of Notch action is not completely understood. Here we identified that the regulation of cell survival by Notch intracellular domain could partly be attributed to a selective increase of X-linked inhibitor of apoptosis protein (XIAP). We further found that Notch intracellular domain inhibited the degradation of XIAP during apoptosis. The transactivation domain of Notch interacted directly with the RING region of XIAP to block the binding of E2 and prevent the *in vivo* and *in vitro* ubiquitination of XIAP. This antiapoptotic activity of Notch was abolished when XIAP was knocked down. Our results reveal a novel mechanism for Notch-selective suppression of apoptosis through an increase in the stability of a key antiapoptotic protein, XIAP.

The EMBO Journal (2007) **26**, 1660–1669. doi:10.1038/sj.emboj.7601611; Published online 22 February 2007

Subject Categories: differentiation & death; immunology
Keywords: apoptosis; Notch; ubiquitination; XIAP

Introduction

Notch is a transmembrane receptor protein that plays a major role in cell fate determination (Artavanis-Tsakonas *et al.*, 1999; Lai, 2004). Upon binding a Notch ligand, the Notch receptor is cleaved sequentially by ADAM and γ -secretase to release the Notch intracellular domain (NICD). This NICD contains a RAM domain at its N-terminal end, followed by six ankyrin repeats (ANK), a nuclear localization sequence, a transactivation domain (TAD), and a PEST domain at the C-terminal end. NICD is translocated into the nucleus, where it forms a complex with RBP-J κ (also known as CBF1, CSL, or Su(H1)) and controls transcription activation of targeted genes (Artavanis-Tsakonas *et al.*, 1999; Lai, 2004).

Deregulation of Notch signaling is tumorigenic in many cell types (Leong and Karsan, 2006; Miele, 2006). *Notch-1* gene translocation (t(7;9)), resulting in constitutive expression of the NICD, was first found in human T cell acute lympho-

blastic leukemia (T-ALL) (Ellisen *et al.*, 1991). Overexpression of NICD in mouse hematopoietic cells leads to T-cell leukemia (Pear *et al.*, 1996). Recent studies have shown that almost half of all T-ALL patients carry activation mutations of Notch-1 (Weng *et al.*, 2004; Grabher *et al.*, 2006; O'Neil *et al.*, 2006). Notably, a high fraction of these *Notch-1* mutants code for a premature stop in the PEST domain (Weng *et al.*, 2004; Grabher *et al.*, 2006; O'Neil *et al.*, 2006). Deletion of the PEST domain stabilizes NICD (Rechsteiner and Rogers, 1996) and accelerates lymphoid oncogenesis (Feldman *et al.*, 2000).

Notch signals directly modulate cell proliferation and apoptosis (Miele and Osborne, 1999; Jundt *et al.*, 2002; Leong and Karsan, 2006; Miele, 2006). The inhibition of apoptosis by Notch has been illustrated via several different mechanisms. Notch protects cells from TCR-induced apoptosis by interaction with Nur77 (Jehn *et al.*, 1999). Notch activation upregulates p21(WAF/Cip) and protects myeloma cells from chemotherapy-induced apoptosis (Nefedova *et al.*, 2004). The antiapoptotic effect of Notch has also been shown to require Akt/PKB signaling (Nair *et al.*, 2003; Sade *et al.*, 2004). Another apoptosis suppressing effect of Notch activation is the inhibition of p53 (Nair *et al.*, 2003; Beverly *et al.*, 2005; Mungamuri *et al.*, 2006). Notch signaling upregulates NF- κ B (Oswald *et al.*, 1998), a transcription factor regulating the expression of many antiapoptotic proteins. Notch further promotes survival by upregulation of Bcl-2 and Mcl-1 (MacKenzie *et al.*, 2004; Oishi *et al.*, 2004). Notch may also inhibit apoptosis through interference with JNK activation (Kim *et al.*, 2005). However, the molecular processes that mediate antiapoptotic activity of Notch are not completely understood.

The 'inhibitor of apoptosis proteins' (IAPs) are caspase inhibitors originally identified in baculoviruses for their ability to inhibit apoptosis in host cells (Crook *et al.*, 1993). In mammalian cells, IAP family members act by binding to and inhibiting caspases-3, -7, and -9 (Deveraux and Reed, 1999; Salvesen and Duckett, 2002; Riedl and Shi, 2004). The X-linked inhibitor of apoptosis protein (XIAP) is one of the best-characterized IAP members in terms of both its structure and biochemical mechanism. XIAP contains three baculovirus IAP repeat (BIR) domains, followed by a RING-finger domain with E3 ubiquitin ligase activity. The BIR 2 domain, and its N-terminal linker, binds and inhibits active caspase-3 and -7, whereas the BIR 3 domain binds and blocks caspase-9 (Deveraux and Reed, 1999; Riedl and Shi, 2004). XIAP is not a stable protein as illustrated by the downregulation of XIAP by binding of copper ion (Mufti *et al.*, 2006). During apoptosis induction, XIAP and c-IAP1 are also degraded through auto-ubiquitination (Yang *et al.*, 2000). Interestingly, human c-IAP1 and c-IAP2 are much less effective than XIAP1 at inhibition of caspase (Eckelman and Salvesen, 2006). XIAP is often overexpressed in malignant cells and elevated levels of XIAP increase resistance to apoptosis induced by both mitochondrial and death receptor cascades (for a review see Schimmer

*Corresponding author. Institute of Molecular Biology, Academia Sinica, Taipei 11529, Taiwan, ROC. Tel.: +886 2 2789 9236;
Fax: +886 2 2782 6085; E-mail: mblai@imb.sinica.edu.tw

Received: 21 August 2006; accepted: 25 January 2007; published online: 22 February 2007

et al, 2006). XIAP has thus become a promising target for the treatment of malignancy.

In the present study, we demonstrate that NICD prevents the ubiquitination and degradation of XIAP during apoptosis via a direct interaction between Notch and XIAP. Our results suggest a novel mechanism for the Notch-induced increased survival of tumor cells through reduced degradation of XIAP.

Results

We used a Notch-1 intracellular domain construct with a partially truncated PEST domain (Notch-1 1747-2444, abbreviated as NIC; the full-length Notch-1 intracellular domain is referred as NICD) to investigate the regulatory function of Notch-1 on apoptosis. Myc-tagged NIC or vector was transfected into Jurkat T leukemia cells with ecotropic receptors by retroviral infection. Successfully retroviral-infected Jurkat cells, marked by their expression of YFP, were isolated by FACS sorting. NIC-expressing Jurkat cells and control YFP cells were treated with various concentrations of anti-Fas antibody CH11. Active Notch suppressed Fas-induced apoptosis by more than 60% at all tested concentrations of CH11 (Figure 1A). Expression of NIC also effectively reduced TRAIL-, cisplatin-, and etoposide-induced apoptosis (Figure 1B, not shown for TRAIL- and cisplatin-induced apoptosis). Expression of NIC in the DO11.10 T-cell hybridoma line inhibited CD3-induced and dexamethasone-mediated apoptosis cell death in these cells (Figure 1C and D). Therefore, we conclude that Notch activation effectively inhibits apoptosis triggered by death receptors, DNA damage, and glucocorticoids.

To understand the mechanisms involved in resistance to apoptosis conferred by NIC, we examined the steady-state levels of several proapoptotic and antiapoptotic proteins in Jurkat cells. The levels of Fas and FADD were nearly identical for YFP- or NIC-expressing Jurkat cells (Figure 1E). NIC did not affect cell-surface expression of Fas receptors compared with YFP control cells (Figure 1F). Expression of antiapoptotic proteins including c-IAP1, c-IAP2, and c-FLIP_L were also similar between the YFP control and the NIC-expressing Jurkat cells (Figure 1E). In contrast, one antiapoptotic protein in particular was significantly upregulated in NIC-expressing Jurkat and DO11.10 cells: the X-linked inhibitor of apoptosis XIAP (Figure 1G and H). A nearly three-fold increase of XIAP protein was detected in NIC-expressing T cells relative to their YFP control. Because the Notch intracellular domain we used was truncated at PEST region, we also examined whether the full-length Notch intracellular domain exerted a similar effect. Overexpression of the full-length Notch intracellular domain resulted in elevated levels of XIAP, whereas the levels of c-IAP1, c-IAP2, and c-FLIP_L were not altered (Supplementary Figure 1A). Therefore, either full-length Notch intracellular domain or PEST-truncated Notch intracellular domain increases XIAP expression.

One of the main signal pathways of Notch activation is mediated by the translocation of NICD into the nucleus, followed by the binding of NICD to RBP-J κ and consequent target gene transcription. To examine whether Notch transcriptionally activates *xiap* expression, XIAP mRNA levels of YFP or NIC-expressing Jurkat cells were determined. The levels of XIAP mRNA were similar in YFP and NIC-expressing

Figure 1 Notch intracellular domain prevents T cells from apoptosis and induces XIAP expression. (A–D) Expression of Notch intracellular domain inhibited apoptosis. NIC was transduced into Jurkat-Eco cells and DO11.10 T-cell hybridomas by retroviral infection. YFP control and NIC-expressing Jurkat cells were treated with the indicated concentrations of anti-Fas antibody CH11 (A) or etoposide (B), whereas YFP control and NIC-expressing DO11.10 cells were stimulated with anti-CD3 (C) or dexamethasone (D). Cells were stained with propidium iodide, and apoptotic cells were determined by flow cytometry. Each datapoint is the mean of the triplicate within the same experiment, with standard deviation (s.d.) represented by error bars. Each experiment has been independently repeated three times with similar results. (E) The levels of Fas, FADD, c-FLIP_L, c-IAP1, and c-IAP2 were not affected by NIC expression in Jurkat cells. Total cell extracts from YFP- or NIC-expressing Jurkat cells were prepared, and the contents of Fas, FADD, c-FLIP_L, c-IAP1, and c-IAP2 were analyzed by Western blotting using their specific antibodies. (F) Surface Fas expression was normal in NIC-expressing Jurkat cells. YFP- or NIC-expressing Jurkat cells were stained with PE-conjugated anti-Fas antibodies and surface Fas expression was determined by flow cytometry. (G, H) NIC expression increased cellular XIAP contents. Protein levels of XIAP in YFP- and NIC-expressing Jurkat (G) or DO11.10 cells (H) were determined using anti-XIAP antibody. The amounts of XIAP were quantitated by densitometry and normalized against the contents of β -tubulin or Hsc70. The XIAP levels in each YFP control cells were designated as 1. The number represents average of two experiments with s.d. indicated.

Jurkat cells (Figure 2A), suggesting that increases in XIAP are not because of stimulation of XIAP transcription by NICD.

Previous reports have demonstrated that XIAP is an unstable protein (Yang *et al*, 2000). To determine whether Notch activation affected the protein stability of XIAP, YFP- or NIC-expressing Jurkat cells were treated with cycloheximide

(CHX) to block new protein synthesis. CHX treatment in YFP control cells resulted in a rapid decrease of XIAP, whereas expression of NIC significantly delayed the XIAP turnover (Supplementary Figure 2A). The protein half-life of XIAP in NIC-expressing cells was calculated to be 120 min, a four-fold increase over that in YFP control cells ($t_{1/2} = \sim 30$ min) (Supplementary Figure 2B). Because XIAP translation could be upregulated by stress (such as CHX) (Holcik *et al*, 2000), we also used pulse-chase experiment to confirm the increase on XIAP protein stability in NIC-expressing Jurkat cells treatment (Supplementary Figure 2C and D). We further generated Jurkat clones with inducible expression of NIC. Following the induction kinetics of NIC, a proportional increase of XIAP was observed (Supplementary Figure 3A). The function of the induced NIC was demonstrated by the inhibition of cell death triggered by Fas and cisplatin in the induced clone B6 (Supplementary Figure 3B and C). Together, these results suggest that NIC increases the stability of XIAP.

A major mechanism that regulates protein stability in eukaryotic cells is ubiquitin-dependent, proteasome-mediated protein degradation (Yang *et al*, 2000). To examine whether XIAP turnover was dependent on proteasome-mediated protein degradation, YFP or NIC-expressing Jurkat cells were treated with the proteasome inhibitor MG132. Levels of XIAP in both control (YFP) and NIC-expressing cells were markedly increased after treatment of MG132 (Figure 2B). MG132 treatment increased the XIAP contents in control cells to the level seen in cells expressing NIC (Figure 2B), suggesting that Notch increases XIAP stability through downregulation of proteasome-mediated protein degradation.

Akt and its downstream signaling molecules have a well-known antiapoptotic effect. Notch signals activate Akt (Sade *et al*, 2004), as shown in Supplementary Figure 1B, where NIC expression in Jurkat cells led to increased Akt phosphorylation relative to the YFP control. It has been reported that phosphorylation of XIAP by Akt protects XIAP from ubiquitination and degradation (Dan *et al*, 2004). To examine whether NIC-induced XIAP upregulation is mediated by Akt, YFP control and NIC-expressing Jurkat cells were treated with the PI-3 kinase inhibitor wortmannin. Akt phosphorylation was abolished in Jurkat cells regardless of NIC expression (Supplementary Figure 1B). Inhibition of Akt activation by wortmannin had no effect on XIAP expression in both control and NIC-expressing Jurkat cells. Therefore, upregulation of XIAP by Notch activation is not Akt-dependent.

The execution of apoptosis was accompanied by degradation of the antiapoptotic XIAP, as demonstrated by the decrease in XIAP levels in Jurkat cells after Fas ligation by CH11 (Figure 2C). Expression of NIC increased the total XIAP contents and delayed the turnover of XIAP induced by Fas (Figure 2C). The inclusion of pan-caspase inhibitor z-VAD prevented Fas-initiated degradation of XIAP (Supplementary Figure 4A). MG132 also effectively suppressed Fas-induced XIAP turnover (Supplementary Figure 4B), suggesting Fas-triggered XIAP degradation was proteasome-dependent. We further examined XIAP degradation in etoposide-triggered apoptosis during early hours and found a similar dependence on caspase and proteasome (Supplementary Figure 4C and D). Etoposide-triggered XIAP degradation was observed before significant cell death was detected, and was antagonized

Figure 2 Notch suppresses XIAP degradation and ubiquitination. (A) NIC did not affect the expression of XIAP mRNA. Total RNA from YFP- or NIC-expressing Jurkat cells was isolated. The amounts of XIAP or β -actin mRNA were determined by RT-PCR. (B) Inhibition of proteasome increased XIAP expression. YFP- or NIC-expressing Jurkat cells were treated with MG132 (50 μ M) proteasome inhibitor for 6 h and the levels of XIAP determined. (C) NIC expression reduced Fas-induced XIAP degradation. YFP- and NIC-expressing Jurkat cells were treated with 20 ng/ml of CH11. At the indicated time points, cell death was quantitated and total cell extracts were prepared, and the levels of XIAP determined. (D) NIC suppressed XIAP ubiquitination induced by etoposide. YFP- and NIC-expressing Jurkat cells were treated with etoposide (10 μ g/ml) in the presence of MG132 (25 μ M) for the indicated time. Cell extracts were heated in 1% SDS to dissociate nonspecific association, diluted with lysis buffer, immunoprecipitated using anti-XIAP antibodies (Santa Cruz), and blotted with anti-ubiquitin (Ub) and anti-XIAP (BD Transduction) antibodies. Molecular weight markers (in kDa) are indicated on the left. (E) Notch suppressed XIAP ubiquitination *in vivo*. YFP- and NIC-expressing Jurkat cells were transfected with XIAP-FLAG as indicated. Twenty-four hours after transfection, cells were untreated or treated with MG132 for 6 h, and cell lysates were prepared. XIAP-FLAG was immunoprecipitated from preheated cell extracts (as in (D)) using FLAG-M2 agarose. Immunoprecipitates were eluted with acidic glycine buffer, separated by SDS-PAGE, and blotted with anti-ubiquitin (upper panel) or anti-FLAG-M2 (middle panel) antibodies. Expression of NIC-Myc (bottom panel) in cell lysates was determined by anti-Myc antibodies.

by NIC expression (Supplementary Figure 4E). Etoposide promoted the ubiquitination of XIAP, illustrated by that immunoprecipitation of XIAP from etoposide-stimulated Jurkat cells identified the association of ubiquitin with XIAP (Figure 2D, YFP). Etoposide-triggered XIAP ubiquitination was largely abrogated by NIC expression in Jurkat cells (Figure 2D, NIC). Thus, Notch activation inhibits etoposide-triggered ubiquitination of XIAP.

XIAP is known to catalyze its own ubiquitination (Yang *et al*, 2000). To study the effect of NIC on the ubiquitination status of XIAP *in vivo*, XIAP-FLAG was expressed in Jurkat cells with or without NIC, followed by treatment with MG132. XIAP was then immunoprecipitated by anti-FLAG M2 agarose and the extent of ubiquitination was analyzed. Inhibition of proteasome activity by MG132 significantly enhanced the XIAP-FLAG protein contents (Figure 2E, lanes 7 and 8). The weak ubiquitination of XIAP detected in cells transfected with XIAP alone (Figure 2E, lane 3) was profoundly elevated in the presence of MG132 (Figure 2E, lane 7). Expression of NIC effectively suppressed the ubiquitination of XIAP (Figure 2E, lanes 4 and 8). Therefore, NIC antagonizes XIAP ubiquitination induced by XIAP overexpression *in vivo*. In another analysis, we expressed XIAP-FLAG and XIAP Δ RF-FLAG in Jurkat and NIC-Jurkat cells. Etoposide treatment of Jurkat cells led to degradation of XIAP-FLAG (Supplementary Figure 5A). In contrast, etoposide did not trigger protein turnover of XIAP Δ RF-FLAG, the XIAP mutant that cannot be autoubiquitinated (Yang *et al*, 2000) (Supplementary Figure 5B). In addition, deletion of RING domain conferred the resistance of XIAP to further NIC regulation seen in wild-type XIAP (Supplementary Figure 5A and B).

The observation that NIC prevented XIAP ubiquitination suggests a possible interaction between NIC and XIAP. A co-immunoprecipitation assay was used to evaluate the extent of any NIC-XIAP binding. In 293T cells cotransfected with XIAP-FLAG and NIC-myc, NIC-myc was detected in immunoprecipitates containing XIAP-FLAG (Figure 3A), suggesting a likely binding between XIAP and NIC. In addition, in 293T cells overexpressing NIC-myc alone, immunoprecipitation of NIC-Myc pulled down the endogenous XIAP (Figure 3B). Immunoprecipitation of the endogenous XIAP in 293T cells also brought down the overexpressed NIC-myc (Figure 3C). Similarly, in NIC-Myc-expressing Jurkat cells, immunoprecipitation by either anti-Myc or anti-XIAP identified XIAP and NIC, respectively (Supplementary Figure 6A). These results strongly suggest an association between NIC and XIAP *in vivo*.

Because the NIC-XIAP association was detected in Jurkat and 293T cells where NIC was overexpressed, we further elucidated the interaction between XIAP and the endogenously generated Notch intracellular domain. The stimulation of DO11.10 cells with Jagged-1 led to processation of the full-length Notch receptor, concomitant with the appearance of the cleaved Notch intracellular domain (Figure 4A). Generation of the endogenous NICD was peaked 2 h after Jagged-1 engagement (Figure 4A, right panel). XIAP expression was also increased after Jagged-1 treatment, with kinetics identical to that of endogenously produced NICD, indicating the XIAP stabilizing effect of the naturally generated NICD (Figure 4A). Immunoprecipitation with anti-XIAP pulled down the endogenously generated Notch intracellular

domain but not the full-length Notch receptor (Figure 4B). Immunoprecipitation of the cleaved Notch intracellular domain also brought down the endogenous XIAP. Therefore, the endogenous XIAP is associated with and stabilized by the naturally generated Notch intracellular domain *in vivo*.

We next examined whether NIC directly interacted with XIAP. GST-NIC and its mutants with deletion at different domains were constructed, including GST-NIC Δ RAM, GST-NIC Δ ANK, and GST-NIC Δ TAD (Figure 3D). In a GST pull-down assay, His-XIAP was brought down by GSH-agarose when the bacterially expressed GST-NIC was incubated with recombinant His-XIAP (Figure 3D, WT), illustrating a direct interaction between NIC and XIAP. GST-NIC Δ RAM, GST-NIC Δ ANK, and GST-NIC Δ TAD were then used to elucidate the region on NIC that interacts with XIAP. Deletion of the RAM or ANK domains did not affect the binding of NIC to XIAP (Figure 3D, Δ RAM and Δ ANK). Truncation of the TAD domain from NIC abolished its association with His-XIAP (Figure 3D, Δ TAD), indicating the TAD domain is the XIAP-binding region on NIC.

In addition, we identified the domain on XIAP that binds NIC. His-XIAP mutants with deletions of the BIR1, BIR2, ZBIR3, or RF domains were prepared (Figure 3E). The binding of GST-NIC to His-XIAP Δ BIR1, His-XIAP Δ BIR2, or His-XIAP Δ BIR3 was similar to that of His-XIAP (Figure 3E). In contrast, removal of the C-terminal RING-finger domain from XIAP prevented the association of His-XIAP with GST-NIC (Figure 3E, Δ RF). Therefore, NIC directly binds XIAP, where the C-terminal domain on NIC and the RING-finger domain of XIAP are critical for such interaction.

Because NICD is known for its transcription activity inside nucleus, we examined the intracellular localization of NIC-myc to see whether its interaction with the cytosolic protein XIAP is possible. Immunoblots on the nuclear and cytoplasmic extracts of Jurkat cells confirmed the cytosolic nature of XIAP. NIC was present both in the nuclear and cytoplasmic extracts (Supplementary Figure 6B). The intracellular colocalization of NIC and XIAP was examined by confocal microscopy illustrating the overlapping image of NIC and XIAP in 293T cells (Supplementary Figure 6C). Therefore, NIC is able to bind XIAP in cytoplasm. We further examined whether the association of XIAP with NIC affects the transcription activity of NIC. Coexpression of XIAP decreased NIC-directed HES-5 transactivation (Supplementary Figure 7A). NIC is also known to repress the transcription activation of AP-1 (Chu *et al*, 2002), which was partially alleviated by XIAP coexpression (Supplementary Figure 7B). The binding of XIAP to NIC thus interferes with the transcription regulatory activity of NIC inside nucleus.

As NIC interacts with the RING-finger domain of XIAP (Figure 3E), it is possible that NIC binding to XIAP would compete with E2 ubiquitin-conjugating enzymes targeting the same RING-finger domain. To test this possibility, His-XIAP and the E2 enzyme UbCH5 were incubated with a range of concentrations of GST-NIC and their interaction was analyzed by His pull-down assay. In the absence of NIC, Ni-NTA agarose captured His-XIAP and UbCH5 (Figure 5A), demonstrating the direct binding of E2 to XIAP. With increasing amounts of GST-NIC present in the incubation mixture, binding of UbCH5 to His-XIAP was gradually reduced (Figure 5A). The addition of 2.5 μ g GST-NIC nearly abolished the association of UbCH5 to His-XIAP. Therefore, the interac-

Figure 3 Direct binding of NIC to XIAP. (A) Interaction of NIC with XIAP *in vivo*. 293T cells were transfected with NIC-Myc and/or XIAP-FLAG as indicated. Expression of NIC-Myc and XIAP-FLAG was confirmed by anti-Myc and anti-FLAG antibodies. XIAP-FLAG was immunoprecipitated from cell lysates with anti-FLAG (M2) agarose and the association of NIC-Myc was detected by anti-Myc. (B, C) NIC interacted with endogenous XIAP in 293T cells. 293T cells were transfected with NIC-Myc or pcDNA3. Total cell extracts were prepared 24 h after transfection and immunoprecipitated by anti-Myc (B) or anti-XIAP (C) antibodies, and blotted with anti-XIAP (B) or anti-Myc (C). (D) GST pull-down analysis of GST-NIC and its mutants for their binding to His-XIAP. The upper panel is a schematic illustration of NICD mutants with deletion of different domains. Aliquots of 2 μ g of GST, GST-NIC, GST-NICΔRAM, GST-NICΔANK, and GST-NICΔTAD were loaded onto glutathione agarose beads and incubated with 2 μ g of His-XIAP. The amounts of His-XIAP brought down by GST-agarose were detected by immunoblot with anti-His antibodies. Input of GST, GST-NIC, and GST-NIC mutants were determined by anti-GST antibodies, whereas input of His-XIAP was assessed by anti-His antibodies. (E) GST pull-down analysis of GST-NIC for the binding to His-XIAP and its mutants. Upper panel shows the schematic demonstration of XIAP mutants with deletion at different domains of XIAP. Aliquots of 2 μ g of GST or GST-NIC were loaded onto glutathione agarose beads and incubated with 2 μ g of His-XIAP, His-XIAPΔBIR1, His-XIAPΔBIR2, His-XIAPΔBIR3, and His-XIAPΔRF. Bound His-tagged proteins were detected by immunoblot with anti-His antibodies. Input of His-XIAP and its mutants were determined by anti-His antibodies, whereas input of GST and GST-NIC was assessed by anti-GST antibodies.

tion of NIC with XIAP decreases the accessibility of E2 ubiquitin-conjugating enzymes to XIAP.

The effect of NIC-XIAP interaction on XIAP ubiquitination was further examined *in vitro*. Incubation of His-XIAP with cell extracts from 293T cells resulted in ubiquitination of XIAP (Figure 5B). The addition of GST-NIC suppressed the conjugation of ubiquitin to XIAP. We also tested the ability of different GST-NIC mutants (Figure 3D) to inhibit XIAP ubiquitination. The addition of GST-NICΔRAM and GST-NICΔANK, NIC mutants that interact with XIAP, prevented the ubiquitination of XIAP (Figure 5B). In contrast, GST-NICΔTAD, unable to bind XIAP, failed to block the coupling of ubiquitin to XIAP. Taken together, these results suggest that the interaction of XIAP with NICD inhibits the binding of E2 to XIAP and subsequent ubiquitination of XIAP.

We further examined whether TAD alone (Figure 6A, region 2097–2444) interacts with XIAP *in vivo*. NIC-Myc,

NICATAD-Myc, or TAD-Myc, was transduced into DO11.10 cells and their expression was confirmed by immunoblotting (Figure 6B). XIAP expression was upregulated by TAD-Myc, similar to NIC-Myc (Figure 6B, lysate, XIAP). Immunoprecipitation of NIC or TAD, but not NICATAD, pulled down XIAP, indicating that the Notch TAD region accounts for the NIC-XIAP binding (Figure 6B). TAD effectively inhibited the ubiquitination of XIAP *in vitro* (Figure 5B). In addition, TAD alone was able to protect against apoptosis induced by glucocorticoid, Fas, and etoposide (Figure 6C–E). Conversely, the removal of TAD abolished NIC antiapoptotic capacity in DO11.10 and Jurkat cells. Therefore, TAD is the functional part of NIC in both XIAP binding and apoptosis inhibition.

Because TAD domain dictates the transcription activity of NIC, known indispensable for the antiapoptotic activity of NIC, we further searched for NIC mutant that may differenti-

Figure 4 Interaction between XIAP and the endogenously generated Notch intracellular domain. (A) Endogenously generated NICD stabilized XIAP. DO11.10 cells were stimulated with immobilized Jagged1-Fc and total cell lysates prepared at 1, 2, 4, and 6 h after stimulation. The Notch1 intracellular domain generated was detected by antibody specific for the cleaved Notch1 intracellular domain (Val1744), whereas the full-length Notch1 was examined by anti-Notch1 extracellular domain (G-20). The amounts of NICD and XIAP were quantitated by densitometry, normalized against GAPDH contents, and plotted versus time of stimulation. The number is the average of two experiments. (B) Association of the endogenous NICD with XIAP. Total cell lysates from (A) were immunoprecipitated using anti-XIAP antibodies and the immune complexes analyzed by Western blot with Val1744 and G-20 antibodies. Immunoprecipitation was also performed with Val1744 antibodies and protein complexes assayed with anti-XIAP antibody.

ate the XIAP-binding ability from the transcription activity of Notch. We found that partial deletion of TAD domain retained most of the NIC transcription activity, shown by activation of the HES-5 promoter (Figure 6A, NICΔptTAD), yet completely eliminated the capacity of NIC to bind XIAP (Figure 6B, NICΔptTAD). Despite the transactivation activity, NICΔptTAD was unable to protect DO11.10 and Jurkat cells from apoptosis (Figure 6C–E). This is in direct contrast to TAD, which bound XIAP and inhibited apoptosis, yet did not activate HES5-Luc (Figure 6A). Together, the XIAP binding capacity could be dissociated from the transcription ability in the context of the antiapoptotic effect of Notch.

Our results suggest NICD inhibits apoptosis through preventing XIAP degradation. If XIAP is essential for the antiapoptotic action of Notch, the absence of XIAP should greatly impair the ability of Notch to suppress apoptosis. We tested this in cells where XIAP was downregulated by siRNA (Chawla-Sarkar *et al*, 2004). Figure 7A demonstrates that transfection of XIAP-specific siRNA effectively knocked down endogenous XIAP in control (YFP) and NIC-expressing Jurkat cells. As a control, downregulation of XIAP did not affect the level of c-IAP1. Knockdown of XIAP moderately increased the extent of apoptosis triggered by Fas, TRAIL,

Figure 5 NIC inhibits the binding of E2 and ubiquitination to XIAP *in vitro*. (A) NIC inhibited the binding of UbcH5 to XIAP. His-XIAP and UbcH5 (2 μg each) were loaded onto Ni-NTA agarose and incubated with the indicated amounts of GST-NIC. The amounts of UbcH5 pulled down by Ni-NTA agarose were detected by immunoblot with anti-UbcH5 antibodies. Input of His-XIAP and GST-NIC were determined by anti-His and anti-GST antibodies, respectively. (B) NIC inhibited XIAP ubiquitination *in vitro*. *In vitro* ubiquitination assays were performed in 30 μl reaction mixture containing ubiquitin (1 μg), His-XIAP (1 μg), 293T cell extracts (30 μg), and 1 μg of GST-NIC, GST-NICΔRAM, GST-NICΔANK, GST-NICΔTAD, or GST-TAD, as indicated. Ni-NTA agarose was added to capture His-XIAP. The Ni-NTA agarose precipitates were resolved by SDS-PAGE and the extent of ubiquitination on XIAP was determined with anti-ubiquitin antibodies (top panel). Input of His-XIAP (middle panel) and various forms of GST-NIC (bottom panel) in the reaction mixtures were assessed by anti-His and anti-GST antibodies.

etoposide, and cisplatin in control Jurkat cells (Figure 7B–E). Importantly, the NIC-mediated inhibition of death receptor- and DNA damage-induced apoptosis was not seen in Jurkat cells where XIAP was downregulated. These results support the proposed mechanism whereby NIC inhibits apoptosis by stabilization of XIAP.

Discussion

Notch activation is associated with T-ALL in humans and in mouse models. A large fraction of Notch-1 mutations observed in T-ALL are within the PEST domain of Notch-1, leading to expression of Notch-1 with a truncated PEST domain (Weng *et al*, 2004; Grabher *et al*, 2006; O'Neil *et al*, 2006). In the present study, we used Notch-1 intracellular domain with truncated PEST domain (NIC) as an oncogenic form of Notch-1 to delineate its antiapoptotic effect in tumor cells. We found that Notch targets XIAP, which leads to the survival of tumor cells. In cells with constitutive NIC, XIAP levels are selectively upregulated. Furthermore, Notch inter-

Figure 6 The TAD domain accounts for the binding of NIC to XIAP and XIAP-binding could be differentiated from transcription activity of NIC. (A) Activation of HES-5-Luc by NIC and NICΔTAD. 293T cells were transfected with HES-5-Luc, pcDNA4 vector, or different forms of NIC, and luciferase activity was determined 24 h after transfection. Results are average of two independent experiments with s.d. indicated. (B) Interaction of XIAP with TAD *in vivo* in DO11.10 cells. NIC was immunoprecipitated by anti-Myc from cell lysates of DO11.10 cells transduced with YFP, NIC-Myc, NICΔTAD-Myc, TAD-Myc, or NICΔptTAD-Myc, and the association with endogenous XIAP was analyzed. The amounts of NIC, NIC mutants, XIAP, and GAPDH in cell lysates were assessed by Western blots. (C–E) TAD accounted for the antiapoptotic activity of NIC. DO11.10 cells expressing NIC-Myc, NICΔTAD-Myc, TAD, NICΔptTAD-Myc, or YFP were treated with dexamethasone (C), whereas Jurkat cells transduced with NIC-Myc, NICΔTAD-Myc, TAD, NICΔptTAD-Myc, or YFP were stimulated with CH11 (D) or etoposide (E). Cell death was quantitated 18 h later. Each datapoint is the mean of the triplicate in the same experiment, with s.d. as error bars. Each experiment has been independently repeated twice.

acts specifically with XIAP, as we found that the binding of Notch to XIAP prevented the ubiquitination and the degradation of XIAP.

Elevated XIAP levels have been observed in several types of malignancy (Kashkar *et al*, 2003; Berezovskaya *et al*, 2005; Kater *et al*, 2005). Proteasome-mediated degradation of XIAP is essential to initiate apoptosis in cancer cells (Yang *et al*, 2000; Sohn *et al*, 2006). XIAP is ubiquitinated by its own RING domain, and deletion of the RING domain confers on XIAP a resistance to apoptosis-induced degradation (Yang *et al*, 2000). In the present study, we found that the specific binding of NIC to XIAP at the RING domain of XIAP (Figure 3E) prevented the recruitment of ubiquitin E2. This Notch–XIAP interaction thereby inhibited the ubiquitination of XIAP (Figure 5), leading to an elevated level of XIAP in

NIC-expressing cells (Figure 1G and H). The effect of NIC on the enhanced XIAP expression was most prominent during the course of apoptosis (Figure 2C). In addition, endogenously generated NICD was effective in stabilizing XIAP (Figure 4).

The TAD domain has been demonstrated to be essential for Notch-1 to induce T-cell leukemia (Aster *et al*, 2000). The TAD domain accounts for the transcription activity of NICD, whereas transcription activation by the NICD–CBF1–Mastermind complex is required for the transforming activity of Notch and growth and survival of T-ALL (Jeffries *et al*, 2002; Weng *et al*, 2003). Our observation that TAD domain of Notch-1 interacts with XIAP (Figure 3D) illustrates another important function of the TAD region by promoting the stability of XIAP. The removal of TAD abolished the binding

Figure 7 Downregulation of XIAP abrogates the antiapoptotic effects of Notch. (A) Specific knockdown of XIAP by siRNA. YFP- or NIC-expressing Jurkat cells were transfected with XIAP-specific siRNA by electroporation. After 48 h, a fraction of cells were lysed for determining the expression of XIAP, c-IAP1, and Hsc70. (B–E) XIAP knockdown abolished the anti-apoptotic capacity of Notch. Jurkat cells from (A) were treated with CH11 (B), TRAIL (C), cisplatin (D), or etoposide (E) at the concentrations indicated, and apoptosis was quantitated 18 h later. Each datapoint is the mean of the triplicate, with s.d. as error bars. Each experiment has been independently repeated twice.

of Notch-1 to XIAP and abrogated the antiapoptotic activity of Notch-1 (Figure 6B–E), whereas TAD alone was sufficient to interact with XIAP, block XIAP ubiquitination, and antagonize apoptosis (Figures 5B and 6B–E). The TAD region of Notch-1 thus directly participates in the antiapoptotic action. Notably, TAD alone failed to stimulate HES5-Luc, and together with NICΔptTAD, the mutant transactivates HES5 but did not bind XIAP (Figure 5), suggesting that the antiapoptotic effect of NIC could be detected in the absence of NIC transcription activity.

A recent study reported the upregulation of c-IAP1, c-IAP2, and c-FLIP_L in NIC-expressing Jurkat cells (Sade *et al*, 2004). This is in contrast to our observation that expression of c-IAP1, c-IAP2, and c-FLIP_L were similar between the control and the NIC-expressing Jurkat cells (Figure 1E). Instead, we found that the major effect of NIC was the upregulation of XIAP (Figure 1G and H). We do not know the exact reason for the discrepancy between our results and those of Sade *et al* (2004). We tested the effect of full-length Notch intracellular domain used by Sade *et al* (2004), but failed to detect alteration in c-IAP1, c-IAP2, and c-FLIP_L (Supplementary Figure 1). A possibility is that different subclones of Jurkat may respond differently to NIC expression. However, our results also demonstrate that in the absence of any apparent increase in c-IAP1, c-IAP2, and c-FLIP_L, Notch effectively suppressed different types of apoptosis (Figure 1), suggesting that c-IAP1, c-IAP2, or c-FLIP_L may not be critical for the antiapoptotic effect of Notch in Jurkat cells.

The upregulation of XIAP by NICD contributes to another antiapoptotic mechanism of Notch-1. Notch is known to activate NF-κB through different mechanisms, such as direct activation of NF-κB2 (p100/p52) or increased nuclear retention of NF-κB (Oswald *et al*, 1998; Shin *et al*, 2006; Vacca *et al*, 2006). Notably, XIAP stimulates the activation of NF-κB through TAK1, independent of the binding and inactivation of caspase (Hofer-Warbinek *et al*, 2000; Levkau *et al*, 2001). Notch-mediated stabilization of XIAP may thus further add to NF-κB activation.

It has to be noted that our results do not imply that blockage of XIAP ubiquitination by Notch is the sole mechanism that Notch inhibits apoptosis. Previous studies have unveiled several mechanisms for the antiapoptotic activity of Notch. Notch-1 has been shown to suppress apoptosis through Nur77, p21(WAF/Cip) upregulation, Akt/PKB signaling, inhibition of p53, activation of NF-κB, upregulation of Bcl-2/Mcl-1, and inhibition of JNK activation (Oswald *et al*, 1998; Jehn *et al*, 1999; Nair *et al*, 2003; MacKenzie *et al*, 2004; Nefedova *et al*, 2004; Oishi *et al*, 2004; Sade *et al*, 2004; Beverly *et al*, 2005; Kim *et al*, 2005; Mungamuri *et al*, 2006). XIAP is likely coordinated with all other Notch antiapoptotic machineries for an effective suppression of cell death. The physiological significance of our observation is that Notch-stabilized XIAP, in the absence of Notch downstream mediators, is still able to antagonize apoptosis. As judged from the biochemical process revealed in the present study, XIAP upregulation may be the one mechanism that is constitutively operated in most of cells once Notch intracellular domain is generated.

Many Notch-1 downstream mediators failed to fully reproduce the oncogenic or antiapoptotic activities of Notch. For example, overexpression of HES inhibits B-cell development but does not promote leukemia formation (Kawamata *et al*, 2002). Similarly, CBF1 accounts for some but not all of the antiapoptotic activity of Notch-4 (MacKenzie *et al*, 2004). Part of the reason that the antiapoptotic effects of Notch-1 cannot be completely replaced by its downstream effectors may be due to the fact that there are Notch activities absent in the Notch downstream genes. For example, the interaction of Notch with Nur77 may prevent apoptosis induction by Nur77 (Kolluri *et al*, 2003; Lin *et al*, 2004). Our observation that Notch-1 protects XIAP from degradation adds another direct antiapoptotic activity of Notch-1 protein. The exact contribution of Notch-1 protein to the overall level of apoptosis remains the goal of future investigation.

XIAP is a recent target in cancer therapy. Small-molecule inhibitors for XIAP are currently being identified (Schimmer *et al*, 2004; Kater *et al*, 2005). Given the many observations that aberrant Notch signaling is found in different types of cancer (Leong and Karsan, 2006; Miele, 2006), XIAP may thus contribute significantly to the survival of cancer cells. In cancers with a high incidence of abnormal Notch activation, the targeting of XIAP may turn out to be an effective method of treatment.

Materials and methods

Reagents

The mouse cDNA encoding Notch-1 intracellular domain was obtained by RT-PCR on RNA prepared from EL4 cells. The two primers used were: 5'-ATG TTC TTT GTG GGC TGT GGG and 5'-TGG

CAG TGA TGT TGG TAG GGC. The full-length Notch-1 intracellular domain (named NICD in this study) represents amino acids 1747–2531 of Notch-1 (number according to Kurooka *et al*, 1998). The C-terminal PEST domain (2445–2531) was partially deleted to generate an oncogenic form of Notch intracellular domain and was abbreviated as NIC in this study (aa 1747–2444). NIC-myc was obtained by subcloning NIC cDNA into pcDNA4 (Invitrogen). XIAP cDNA was isolated using RT-PCR on RNA prepared from K562 cells. The two primers used were: 5'-ATG ACT TTT AAC AGT TTT GAA and 5'-AGA CAT AAA AAT TTT TTG CTT. XIAP-FLAG was obtained by subcloning XIAP cDNA into pIRES-hrGFP-1a (Stratagene). The RT-PCR primers used to detect the mRNA level of XIAP were: 5'-ATC CAG AAT GGT CAG TAC and 5'-CTG AGT ATA TCC ATG TCC. Cisplatin, etoposide, ubiquitin, and UbC5 were purchased from Sigma (St Louis, MO). Rat Jagged-1-Fc was obtained from R&D (Minneapolis, MN). Rabbit polyclonal anti-FADD (H181), anti-XIAP (N-19), anti-Notch1 extracellular domain (G-20), and rabbit polyclonal anti-Bcl-2 (N-19) were purchased from Santa Cruz Biotech (Santa Cruz, CA). A second XIAP antibody was obtained from BD Transduction Laboratory. Anti-cleaved Notch1 (Val1744) was purchased from Cell Signaling (Beverly, MA). Rabbit anti-UbC5 was obtained from Chemicon (Temecula, CA).

Cell death measurement

Apoptosis was determined by propidium iodide (PI) (Nicoletti *et al*, 1991). T cells were treated with apoptosis inducing reagents, resuspended in fluorochrome solution (50 µg/ml PI, 0.1% sodium citrate, and 0.1% Triton X-100), and placed at 4°C in the dark overnight. DNA contents were then analyzed on a FACScan (Becton Dickinson). The fraction of cells with sub-G1 DNA content was determined using the CELLFIT software program (Becton Dickinson).

Densitometry measurements

The developed films were analyzed on a Luminescent Image Analyzer LAS-1000 (Fuji Photo Film Co., Japan) using Image Gauge software (Version 3.2, Fuji). Quant mode was used to select the reading area of XIAP/NIC protein band and to subtract background. The reading was normalized against the reading of β -tubulin or Hsc70. The normalized reading of the control T cell sample was then set as 1 (or 100%) to calculate the extent of increase in the NIC-expressing or treated T cell samples.

GST pull-down assay

GST-NIC (NIC1747-2444), GST-NIC Δ RAM (NIC1810-2444), GST-NICAANK (NIC1747-1863 and 2077-2444), and GST-NICATAD (NIC1747-2193) were generated by subcloning NIC and its mutants into pGEX-4T3 (Amersham Pharmacia). Recombinant GST fusion proteins were then purified on GSH agarose. His₆-XIAP, His₆-XIAPABIR1, His₆-XIAPABIR2, His₆-XIAPABIR3, and His₆-XIAP Δ RF

were produced by subcloning XIAP and its deletional mutants into pRSET-a (Invitrogen) for N-terminal His₆ flanking. Recombinant His₆-XIAP and its mutants were then purified on Ni-NTA agarose (Qiagen). Cell lysates for GST pull-down assay were prepared in GSH binding buffer (50 mM potassium phosphate buffer, pH 7.5, 150 mM KCl, 1 mM MgCl₂, 10% glycerol, 1% Triton X-100, 1% aprotinin, and 1 mM PMSF). GST fusion protein (5 µg) (GST-NIC or its mutants) was loaded onto GSH agarose (20 µl, 50% slurry) and incubated with 5 µg of His₆-XIAP or its mutants for 2 h at 4°C. GST agarose beads were then washed three times with binding buffer and analyzed by SDS-PAGE.

In vitro ubiquitination assay

In vitro ubiquitination was performed according to Dohi *et al* (2004) with minor modification. In short, reaction mixture contained ubiquitin (1 µg), GST-NIC (1 µg), His₆-XIAP (1 µg), and 293T cell extracts (30 µg) in 30 µl reaction buffer (40 mM Tris, pH 7.5, 50 mM NaCl, 5 mM MgCl₂, 0.5 mM ATP, 1 mM DTT, 10% glycerol, 1 mM PMSF, 10 µg/ml leupeptin, and 10 µg/ml aprotinin). The reaction was allowed to proceed at 30°C for 1 h. A 50 µl volume of reaction buffer and 20 µl of 50% slurry Ni-NTA agarose were then added, mixed at 4°C for 2 h to pull down His₆-XIAP. Ni-NTA agarose was then washed three times with ice-cold reaction buffer, resuspended in SDS sample buffer, heated at 95°C for 5 min, and resolved on SDS-PAGE. The amount of ubiquitin associated was determined by blotting with anti-ubiquitin antibody.

siRNA transfection

The siRNA for human XIAP (Chawla-Sarkar *et al*, 2004) was purchased from Dharmacon. For the expression of siRNA in T cells, 5×10^6 Jurkat cells were mixed with 1 nmole of XIAP-specific siRNA in a final volume of 0.6 ml OPTI-MEM medium (Invitrogen-Gibco) and electroporation was performed on a Bio-Rad Gene Pulser (Bio-Rad, CA). After 48 h, a fraction of cells were lysed for immunoblot analysis and the remaining cells were treated with different reagents for apoptosis measurement.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Acknowledgements

We thank Dr Gina Costa and Dr Nan-Shih Liao for pGC-IRES-YFP vector, Dr Garry P Nolan for Phoenix-Eco and Jurkat-Eco cells, and Dr Harry Wilson for editing the manuscript. This work was supported by a grant from Academia Sinica, Grant 94-2320-B001-001 from National Science Council, and Grant NHRI-EX95-9527NI from National Health Research Institute, Taiwan, ROC

References

- Artavanis-Tsakonas S, Rand MD, Lake RJ (1999) Notch signaling: cell fate control and signal integration in development. *Science* **284**: 770–777
- Aster JC, Xu L, Karnell FG, Patriub V, Pui JC, Pear WS (2000) Essential roles for ankyrin repeat and transactivation domains in induction of T-cell leukemia by notch1. *Mol Cell Biol* **20**: 7505–7515
- Berezovskaya O, Schimmer AD, Glinskii AB, Pinilla C, Hoffman RM, Reed JC, Glinsky GV (2005) Increased expression of apoptosis inhibitor protein XIAP contributes to anoikis resistance of circulating human prostate cancer metastasis precursor cells. *Cancer Res* **65**: 2378–2386
- Beverly LJ, Felsher DW, Capobianco AJ (2005) Suppression of p53 by Notch in lymphomagenesis: implications for initiation and regression. *Cancer Res* **65**: 7159–7168
- Chawla-Sarkar M, Bae SI, Reu FJ, Jacobs BS, Lindner DJ, Borden EC (2004) Downregulation of Bcl-2, FLIP or IAPs (XIAP and survivin) by siRNAs sensitizes resistant melanoma cells to Apo2L/TRAIL-induced apoptosis. *Cell Death Diff* **11**: 915–923
- Chu J, Jeffries S, Norton JE, Capobianco AJ, Bresnick EH (2002) Repression of activator protein-1-mediated transcriptional activation by the Notch-1 intracellular domain. *J Biol Chem* **277**: 7587–7597
- Crook NE, Clem RJ, Miller LK (1993) An apoptosis-inhibiting baculovirus gene with a zinc-finger-like motif. *J Virol* **67**: 2168–2174
- Dan HC, Sun M, Kaneko S, Feldman RI, Nicosia SV, Wang HG, Tsang BK, Cheng JQ (2004) Akt phosphorylation and stabilization of X-linked inhibitor of apoptosis protein (XIAP). *J Biol Chem* **279**: 5405–5412
- Deveraux QL, Reed JC (1999) IAP family proteins-suppressors of apoptosis. *Gene Dev* **13**: 239–252
- Dohi T, Okada K, Xia F, Wilford CE, Samuel T, Welsh K, Marusawa H, Zou H, Armstrong R, Matsuzawa S, Salvesen GS, Reed JC, Altieri DC (2004) An IAP-IAP complex inhibits apoptosis. *J Biol Chem* **279**: 34087–34090
- Ellisen LW, Bird J, West DC (1991) TAN1, the human homolog of the *Drosophila* Notch gene, is broken by chromosomal translocations in lymphoblastic neoplasms. *Cell* **66**: 649–661
- Eckelmann BP, Salvesen GS (2006) The human anti-apoptotic proteins cIAP1 and cIAP2 bind but do not inhibit caspases. *J Biol Chem* **281**: 3254–3260
- Feldman BJ, Hampton T, Cleary ML (2000) A carboxy-terminal deletion mutant of Notch1 accelerates lymphoid oncogenesis in E2A-PBX1 transgenic mice. *Blood* **96**: 1906–1913

- Grabher C, von Boehmer H, Look AT (2006) Notch 1 activation in the molecular pathogenesis of T-cell acute lymphoblastic leukemia. *Nat Rev Cancer* **6**: 347–359
- Hofer-Warbinek R, Schmid JA, Stehlik C, Binder BR, Lipp J, de Martin R (2000) Activation of NF- κ B by XIAP, the X chromosome-linked inhibitor of apoptosis, in endothelial cells involves TAK1. *J Biol Chem* **275**: 22064–22068
- Holcik M, Yeh C, Korneluk RG, Chow T (2000) Translational upregulation of X-linked inhibitor of apoptosis (XIAP) increases resistance to radiation induced cell death. *Oncogene* **19**: 4174–4177
- Jeffries S, Robbins DJ, Capobianco AJ (2002) Characterization of a high-molecular-weight Notch complex in the nucleus of Notch(ic)-transformed RKE cells and in a human T-cell leukemia cell line. *Mol Cell Biol* **22**: 3927–3941
- Jehn BM, Bielke W, Pear WS, Osborne BA (1999) Protective effects of notch-1 on TCR-induced apoptosis. *J Immunol* **162**: 635–638
- Jundt F, Anagnostopoulos I, Forster R, Mathas S, Stein H, Dorken B (2002) Activated Notch1 signaling promotes tumor cell proliferation and survival in Hodgkin and anaplastic large cell lymphoma. *Blood* **99**: 3398–3403
- Kashkar H, Haefs C, Shin H, Hamilton-Dutoit SJ, Salvesen GS, Kronke M, Jurgensmeier JM (2003) XIAP-mediated caspase inhibition in Hodgkin's lymphoma-derived B cells. *J Exp Med* **198**: 341–347
- Kater AP, Dicker F, Mangiola M, Welsh K, Houghten R, Ostresh J, Nefzi A, Reed JC, Pinilla C, Kipps TJ (2005) Inhibitors of XIAP sensitize CD40-activated chronic lymphocytic leukemia cells to CD95-mediated apoptosis. *Blood* **106**: 1742–1748
- Kawamata S, Du C, Li K, Lavau C (2002) Overexpression of the Notch target genes *Hes in vivo* induces lymphoid and myeloid alterations. *Oncogene* **21**: 3855–3863
- Kim JW, Kim MJ, Kim KJ, Yun HJ, Chae JS, Hwang SG, Chang TS, Park HS, Lee KW, Han PL, Cho SG, Kim TW, Choi EJ (2005) Notch interferes with the scaffold function of JNK-interacting protein 1 to inhibit the JNK signaling pathway. *Proc Natl Acad Sci USA* **102**: 14308–14313
- Kolluri SK, Bruey-Sedano N, Cao X, Lin B, Lin F, Han YH, Dawson MI, Zhang XK (2003) Mitogenic effect of orphan receptor TR3 and its regulation by MEKK1 in lung cancer cells. *Mol Cell Biol* **23**: 8651–8667
- Kurooka H, Kuroda K, Honjo T (1998) Roles of the ankyrin repeats and C-terminal region of the mouse notch1 intracellular region. *Nucleic Acids Res* **26**: 5448–5455
- Lai EC (2004) Notch signaling: control of cell communication and cell fate. *Development* **131**: 965–973
- Leong KG, Karsan A (2006) Recent insights into the role of Notch signaling in tumorigenesis. *Blood* **107**: 2223–2233
- Lin B, Kolluri SK, Lin F, Liu W, Han YH, Cao X, Dawson MI, Reed JC, Zhang XK (2004) Conversion of Bcl-2 from protector to killer by interaction with nuclear orphan receptor Nur77/TR3. *Cell* **116**: 527–540
- Levkau B, Garton KJ, Ferri N, Kloeke K, Nofer JR, Baba HA, Raines EW, Breithardt G (2001) XIAP induces cell-cycle arrest and activates nuclear factor- κ B: new survival pathways disabled by caspase-mediated cleavage during apoptosis of human endothelial cells. *Circ Res* **88**: 282–290
- MacKenzie F, Duriez P, Wong F, Nosedà M, Karsan A (2004) Notch4 inhibits endothelial apoptosis via RBP-J κ -dependent and -independent pathways. *J Biol Chem* **279**: 11657–11663
- Miele L (2006) Notch Signaling. *Clin Cancer Res* **12**: 1074–1079
- Miele L, Osborne B (1999) Arbiter of differentiation and death: Notch signaling meets apoptosis. *J Cell Physiol* **181**: 393–409
- Mufti AR, Burstein E, Csomos RA, Graf PC, Wilkinson JC, Dick RD, Challa M, Son JK, Bratton SB, Su GL, Brewer GJ, Jakob U, Duckett CS (2006) XIAP is a copper binding protein deregulated in Wilson's disease and other copper toxicosis disorders. *Mol Cell* **21**: 775–785
- Mungamuri SK, Yang X, Thor AD, Somasundaram K (2006) Survival signaling by Notch1: mammalian target of rapamycin (mTOR)-dependent inhibition of p53. *Cancer Res* **66**: 4715–4724
- Nair P, Somasundaram K, Krishna S (2003) Activated Notch1 inhibits p53-induced apoptosis and sustains transformation by human papillomavirus type 16 E6 and E7 oncogenes through a PI3K-PKB/Akt-dependent pathway. *J Virol* **77**: 7106–7112
- Nefedova Y, Cheng P, Alsina M, Dalton WS, Gabrilovich DI (2004) Involvement of Notch-1 signaling in bone marrow stroma-mediated *de novo* drug resistance of myeloma and other malignant lymphoid cell lines. *Blood* **103**: 3503–3510
- Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C (1991) A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J Immunol Methods* **139**: 271–279
- Oishi K, Kamakura S, Isazawa Y, Yoshimatsu T, Kuida K, Nakafuku M, Masuyama N, Gotoh Y (2004) Notch promotes survival of neural precursor cells via mechanisms distinct from those regulating neurogenesis. *Dev Biol* **276**: 172–184
- O'Neil J, Calvo J, McKenna K, Krishnamoorthy V, Aster JC, Bassing CH, Alt FW, Kelliher M, Look AT (2006) Activating *Notch1* mutations in mouse models of T-ALL. *Blood* **107**: 781–785
- Oswald F, Liptay S, Adler G, Schmid RM (1998) NF- κ B2 is a putative target gene of activated Notch-1 via RBP-J κ . *Mol Cell Biol* **18**: 2077–2088
- Pear WS, Aster JC, Scott ML, Hasserjian RP, Soffer B, Sklar JD, Baltimore D (1996) Exclusive development of T cell neoplasms in mice transplanted with bone marrow-expressing activated Notch alleles. *J Exp Med* **183**: 2283–2291
- Rechsteiner M, Rogers SW (1996) PEST sequences and regulation by proteolysis. *Trends Biochem Sci* **21**: 267–271
- Riedl SJ, Shi Y (2004) Molecular mechanisms of caspase regulation during apoptosis. *Nat Rev Mol Cell Biol* **5**: 897–907
- Sade H, Krishna S, Sarin A (2004) The anti-apoptotic effect of Notch-1 requires p56lck-dependent, Akt/PKB-mediated signaling in T cells. *J Biol Chem* **279**: 2937–2944
- Salvesen GS, Duckett CS (2002) IAP proteins: blocking the road to death's door. *Nat Rev Mol Cell Biol* **3**: 401–410
- Schimmer AD, Dalili S, Batey RA, Riedl SJ (2006) Targeting XIAP for the treatment of malignancy. *Cell Death Differ* **13**: 179–188
- Schimmer AD, Welsh K, Pinilla C, Wang Z, Krajewska M, Bonneau MJ, Pedersen IM, Kitada S, Scott FL, Bailly-Maitre B, Glinsky G, Scudiero D, Sausville E, Salvesen G, Nefzi A, Ostresh JM, Houghten RA, Reed JC (2004) Small-molecule antagonists of apoptosis suppressor XIAP exhibit broad antitumor activity. *Cancer Cell* **5**: 25–35
- Shin HM, Minter LM, Cho OH, Gottipati S, Fauq AH, Golde TE, Sonenshein GE, Osborne BA (2006) Notch1 augments NF- κ B activity by facilitating its nuclear retention. *EMBO J* **25**: 129–138
- Sohn D, Totzke G, Essmann F, Schulze-Osthoff K, Levkau B, Janicke RU (2006) The proteasome is required for rapid initiation of death receptor-induced apoptosis. *Mol Cell Biol* **26**: 1967–1978
- Vacca A, Felli MP, Palermo R, Di Mario G, Calce A, Di Giovine M, Frati L, Gulino A, Screpanti I (2006) Notch3 and pre-TCR interaction unveils distinct NF- κ B pathways in T-cell development and leukemia. *EMBO J* **25**: 1000–1008
- Weng AP, Ferrando AA, Lee W, Morris IV JP, Silverman LB, Sanchez-Irizarry C, Blacklow SC, Look AT, Aster JC (2004) Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. *Science* **306**: 269–271
- Weng AP, Nam Y, Wolfe MS, Pear WS, Griffin JD, Blacklow SC, Aster JC (2003) Growth suppression of pre-T acute lymphoblastic leukemia cells by inhibition of notch signaling. *Mol Cell Biol* **23**: 655–664
- Yang Y, Fang S, Jensen JP, Weissman AM, Ashwell JD (2000) Ubiquitin protein ligase activity of IAPs and their degradation in proteasomes in response to apoptotic stimuli. *Science* **288**: 874–877