The CBF1-independent Notch1 signal pathway activates human *c-myc* expression partially via transcription factor YY1

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Transcription factor Ying Yang 1 (YY1) indirectly regulates the C promoter-binding factor 1 (CBF1)-dependent Notch1 signaling via direct interaction with the Notch1 receptor intracellular domain (N1IC) on CBF1-response elements. To evaluate the possibility that the N1IC might modulate the gene expression of YY1 target genes through associating with YY1 on the YY1-response elements, we herein investigated the effect of Notch1 signaling on the expression of YY1 target genes. We found that the N1IC bound to the double-stranded oligonucleotides of YY1-response element to activate luciferase activity of the reporter gene with YY1-response elements through a CBF1-independent manner. Furthermore, the N1IC also bound to the promoter of human *c-myc* oncogene, a YY1 target gene, to elevate *c-myc* expression via a CBF1-independent pathway. The activation of reporter genes with YY1-response elements or human *c-myc* promoter by N1IC depended on the formation of N1IC–YY1-associated complex. To delineate the role of the Notch signal pathway in tumorigenesis, K562 cell lines expressing the N1IC were established. Compared with control cells, the proliferation and the tumor growth of N1IC-expressing K562 cells were suppressed. Taken together, these results suggest that the N1IC enhances the human *c-myc* promoter activity that is partially modulated by YY1 through a CBF1-independent pathway. However, the enhancement of *c-myc* expression by N1IC is insufficient to promote the tumor growth of K562 cells.

Introduction

The Notch signal pathway has been implicated in the regulation of several cellular processes such as maintenance of stem cells, cell fate decision, proliferation, differentiation and apoptosis (1–3). The aberrant Notch signal pathway was also shown to participate in human cancers as well as cancers induced by retroviral insertions in mice (4,5). However, Notch signaling may function as an oncogene or tumor suppressor that either promotes or suppresses tumorigenesis (for a review, see ref. 6). The roles of the Notch signal pathway in tumorigenesis depend on the cellular context and the crosstalk with other signal pathways (7,8). So far, the mechanisms controlling whether Notch signaling activates or suppresses tumorigenesis remain poorly understood.

Notch receptors are single-span transmembrane proteins with several functional domains, including epidermal growth factor repeats and three Lin/Notch repeats in the extracellular domain and a RAM domain, six ankyrin repeats, two nuclear localization signals, a transcriptional activator domain and a proline–glutamate–serine–threonine-rich domain in the intracellular domain. In response to ligand binding, the heterodimeric Notch receptor is activated and cleaved in order to release and translocate the Notch intracellular domain into the nucleus. The Notch intracellular domain modulates downstream target genes via both C promoter-binding factor 1 (CBF1, also called KBF2 and RBP-Jκ)-dependent and -independent pathways (9).

Previously, we found that transcription factor Ying Yang 1 (YY1) modulates the Notch1 signal pathway via its association with the high-molecular weight Notch complex (10). In addition, YY1 regulates the CBF1-dependent Notch1 signaling via the interaction with the activated Notch1 receptor [the Notch1 receptor intracellular domain (N1IC)] on CBF1-response elements. These results also raise the possibility that the N1IC might modulate gene expression of YY1 target genes through associating with YY1 on the YY1-response elements. To evaluate this possibility, we investigated the effect of Notch1 signaling on the expression of YY1 target genes in the present study. We found that the N1IC binds to the promoter of the human *c-myc* oncogene, a YY1 target gene, to regulate *c-myc* gene expression.

YY1 is ubiquitously expressed and participates in cellular processes such as embryogenesis, differentiation, replication, proliferation and tumorigenesis (for a review, see ref. 11). YY1 regulates the genes involved in these processes through its ability to initiate, activate or repress transcription. The control of these genes by YY1 depends on the context in which it binds.

The proto-oncogene, *c-myc*, encoding a transcription factor participates in the control of cell proliferation, growth and apoptosis (12). Many reports have documented that the elevated expression of Myc contributes to tumorigenesis (for reviews, see refs 13–16). c-Myc regulates a significant proportion of all genes in an organism. There is an estimated 10–15% of genes regulated by c-Myc in both human and *Drosophila* genomes (17–19). c-Myc induces a number of target genes involved in the G_1–S transition including E2Fs, CDC25A, CDK2, CDK4, Cul1, Id2 and Rb (20–27). Therefore, it was suggested that c-Myc plays a central role in the G_1–S transition as an upstream regulator of cell cycle regulatory genes (28).

Recent reports demonstrated that Notch signaling enhances the expression of c-Myc (7,29–33). Since both the Notch signal pathway and c-Myc are also shown to influence cell proliferation and tumorigenesis, we sought to address whether Notch signaling regulates the expression of the human *c-myc* oncogene which contains YY1-response elements in the promoter region. We found that the N1IC enhances human *c-myc* expression via associating with YY1 in a CBF1-independent manner.

Materials and methods

Plasmids and plasmid construction

The expression construct of pcDNA-HA-N1IC contains cDNA encoding the amino acid residues 1764–2444 of the intracellular domain of the human Notch1 receptor with an N-terminal HA tag (10). The expression constructs of pCMV-YY1 and pCMV-YY1 (1–295) contain cDNAs encoding the full length and amino acid residues 1–295 of the YY1, respectively (10,34). The cDNA of CBF1 was cloned by polymerase chain reaction (PCR) from a human liver cDNA library (Clontech, Palo Alto, CA), and the sequence was confirmed by sequencing (accession no. BC064976). The expression construct of pcDNA-CBF1-myc-His contains the cDNA encoding human full-length CBF1 with a C-terminal Myc and His tags. The expression construct of pSG5Flag-RBP-VP16 (gift of E.Manet) expresses a constitutively active RBP-Jκ mutant (35). The expression construct of secreted form of Jagged1 (pcDNA-Jagged1^{ext}-myc-His) contains the cDNA encoding amino acid residues 1–1046 of human Jagged1 with Myc and His tags at the C-terminus. The reporter plasmid containing the wild-type YY1-response elements, pYY1-RE-Luc, was described previously (34). Reporter plasmids containing the wild-type or mutant CBF1-response

Abbreviations: CBF1, C promoter-binding factor 1; ChIP, chromatin immunoprecipitation; N1IC, Notch1 receptor intracellular domain; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PI, propidium iodide; siRNA, small interfering RNA; YY1, Ying Yang 1.

elements, 4×wtCBF1Luc and 4×mtCBF1Luc, were also described previously (36). Reporter plasmid pLB1530 containing the human *c-myc* promoter (nucleotide −2328 to +961 in relation to the P2 promoter) in front of the luciferase gene in the pGL3-basic vector was a kind gift from Dr L.M.Boxer. All constructs were verified by sequencing.

For the knockdown of endogenous CBF1 and YY1, the following target sequences were constructed in small interfering RNA (siRNA) vector pLKO.1: CBF1, 5′-CCCTAACGAATCAAACACAAA-3′ and YY1, 5′-CCTCCTGATTATTCAGAATAT-3′. A RNAi vector against luciferase (pLKO.1-shLuc) was used as a negative control for knockdown validation.

Cell culture and transfection

The human erythroleukemia K562 cells, cervical carcinoma HeLa cells and COS-7 cells were cultured in RPMI 1640 and Dulbecco's modified Eagle's medium with 10% fetal bovine serum. The stable K562 cell lines expressing the HA-N1IC fusion protein (K562/HA-N1IC) and their control cells (K562/pcDNA3) were previously established (10). For transient transfection of the luciferase reporter assay, K562 cells or HeLa cells (1×10^6) were seeded onto six-well plates and transfected using the SuperFect transfection reagent (Qiagen) and luciferase activities were measured as described (10).

For the establishment of stable COS-7 cell lines expressing secreted form of human Jagged1 (COS-7/Jagged1^{ext}), COS-7 cells were transfected with the pcDNA-Jagged1^{ext}-myc-His expression plasmid using the SuperFect transfection reagent. Forty-eight hours after transfection, cells were selected with 800 μg/ml G418. The stable clones derived from single cells were screened for the expression of secreted form of Jagged1 (Jagged1^{ext}) by western blot with monoclonal anti-c-Myc antibody (Santa Cruz, CA). For the control, the pcDNA3.1-myc-His vector was also transfected into COS-7 cells to establish a stable cell line (COS-7/pcDNA3.1-myc-His).

To activate the endogenous Notch signal pathway, K562 cells transfected with reporter plasmids were used to co-culture with COS-7/Jagged1^{ext} cells or their control cells and to treat with their conditioned media. For the co-culture, COS-7/Jagged1^{ext} cells or their control cells (4×10^5) were seeded onto six-well culture plates. After 24 h of seeding, the transfected K562 cells were co-cultured with COS-7/Jagged1^{ext} cells or their control cells in RPMI 1640 medium. For the treatment of conditioned medium, COS-7/Jagged1^{ext} cells or their control cells (2×10^6) were seeded onto 10 cm culture dishes with 10 ml of Dulbecco's modified Eagle's medium and their culture media were collected after 3 days of seeding. Then the transfected K562 cells (1×10^6) in a six-well culture plate were treated with 500 μl of conditioned medium in 1 ml of RPMI 1640 medium. After 2 days of co-culture or treatment, the transfected K562 cells were harvested for luciferase reporter assay.

Western blot analysis

To prepare whole-cell lysates, cells were lysed in NETN buffer [50 mM Tris–HCl (pH 7.9), 150 mM NaCl, 0.5 mM ethylenediaminetetraacetic acid and 0.5% Nonidet P-40] containing protease and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 100 mM sodium fluoride). Laemmli's sample buffer was added to the cell lysates and heated at 95°C for 5 min, and then analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Western blotting was performed with monoclonal anti-HA, monoclonal anti-c-Myc, polyclonal anti-Notch1 C-terminus (N1IC), polyclonal anti-YY1 antibodies (Santa Cruz), polyclonal anti-CBF1 (Chemicon, Temacula, CA) and polyclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Biogenesis, Poole, UK).

Oligoprecipitation

The 5′-biotinylated oligonucleotides with wild-type (5′-CGCTCCGCGGCCATCTTGGCGGCTGGT-3′) or mutant sequence (5′-CGCTCCGCGATTATCTTGGCGGCTGGT-3′) of YY1-binding sites and wild-type sequence (5′-AGATGCAGTCGCTGAGATTCTTTGGCCG-3′) or mutant sequence (5′-AGATGCAGTCGCCTGCAGTCTTTGGCCG-3′) of CBFl-binding sites were annealed with their complementary oligonucleotides for oligoprecipitation as described (10).

Chromatin immunoprecipitation assay

The procedure for chromatin immunoprecipitation (ChIP) assay of K562/HA-N1IC cells and transfected HeLa cells using protein A–Sepharose-bound antibodies was also described previously (10). In total, 10% of the precipitated DNA was used for PCR. The GLprimer1 and GLprimer2 primers for PCR amplification were used to amplify the 334 bp DNA fragment containing the thymidine kinase promoter of herpes simplex virus type 1 and YY1-response elements in the reporter plasmid, pYY1-RE-Luc. The specific primers 5′-GAGGAGCAGCAGAGAAAGG-3′ and 5′-TCCCCCACGCCCTCTGC-3′ for PCR amplification were used to amplify the 210 bp DNA fragment (nt −409 to −200 in relation to the P2 promoter) of the *c-myc* promoter in reporter plasmid pLB1530 and chromosome DNA. The specific primers 5′-CAAGACCAAAGCGGAAAGAA-3′ and 5′-GGATCCTGTGTGATCCCTAGGC-3′ for PCR amplification were used to amplify the 312 bp DNA fragment of the Hes1 promoter in chromosome DNA.

Real-time PCR analysis

Total RNA was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Total RNA (2 μg) was used to synthesize cDNA using reverse transcriptase M-MLV (New England BioLabs, Beverly, MA) with an oligo $(dT)_{18}$ primer as described by the manufacturer. The 478 bp cDNA of human *c-myc* was amplified with the primers 5′-TACCCTCTCAACGACAGCAG-3′ and 5′-TCTTGACATTCTCCTCGGTG-3′. The 540 bp internal control β-actin cDNA was amplified with the primers 5′-GTGGGGCGCCCCAGGCACCA-3′ and 5′-CTCCTTAATGTCACGCACGATTTC-3′. Quantitative real-time PCR was performed on a LightCycler system with LightCycler FastStart DNA MasterPLUS SYBR Green I (Roche Diagnostics GmbH, Penzberg, Germany). The PCR amplification conditions were as follows: 95°C for 5 min and then 40 cycles of 95°C for 10 s, 56°C for 5 s and 72°C for 12 s. Relative quantification of the *c-myc* RNA expression level was normalized to that of β-actin and corrected to a calibrator using the RelQuant software (Roche). Data shown are representative of the mean values and standard deviations from three independent experiments performed in triplicate.

Flow cytometry

Cells were washed with ice-cold phosphate-buffered saline (PBS) and then fixed in 70% ethanol at −20°C overnight. After washing by PBS, cell pellets were re-suspended in 1.0 ml of PBS containing 100 μg/ml of RNase A and incubated at 37°C for 30 min. Finally, 100 μl of 200 μg/ml propidium iodide (PI) was added and allowed to stand on ice for 30 min. Fluorescence emitted from the PI–DNA complex was quantified after laser excitation of the fluorescent dye using FACSCalibur flow cytometry (Becton Dickinson, Mountain View, CA).

Tumorigenicity assay in nude mice

Male BALB/c nu/nu mice aged 5 weeks were purchased from the National Science Council Animal Center (Taipei, Taiwan) and allowed free access to food and water; all animal experiments were carried out with ethical committee approval. Nude mice were inoculated with 5×10^6 viable K562/HA-N1IC or K562/pcDNA3 cells in a total volume of 0.1 ml of PBS by subcutaneous injection into both hind limbs. The length (L), width (W) and depth (D) of the tumors were measured with calipers at the times indicated. The tumor volume (V) was estimated using the formula $V = (L \times W \times D \times \pi)/6$.

Results

The N1IC induces activity of the luciferase reporter gene with the YY1-response elements

Previously, YY1 was demonstrated to associate with the N1IC in suppressing the transactivation activity of Notch1 receptor (10). The complex of the N1IC and YY1 was also shown to bind on CBF1-response elements in cells. To investigate additional roles for the N1IC–YY1-associated complex, a luciferase reporter gene assay was performed. K562 cells were cotransfected with a luciferase reporter plasmid containing two copies of the wild-type YY1-response elements (pYY1-RE-Luc), the HA-N1IC fusion protein-expressing construct (pcDNA-HA-N1IC) and the YY1-expressing construct (pCMV-YY1) or their control vectors. Two days after transfection, cells were harvested and assayed for luciferase activity.

When cells were cotransfected with the reporter and HA-N1IC expression plasmids, there was a 4.6-fold enhancement of luciferase activity compared with the transfection with control vector pcDNA-HA (Figure 1A). Because the N1IC does not contain any DNA-binding domain, it could indirectly bind on YY1-response elements to regulate the activity of the reporter gene via the association with YY1 that is ubiquitously expressed in growing, differentiated and growth-arrested cells.

When the YY1 expression plasmid was cotransfected with the reporter plasmid containing the YY1-response elements into K562 cells, the luciferase activity was slightly inhibited by 0.8-fold. After the cotransfection of the reporter, N1IC and YY1 expression plasmids, the activity of the reporter gene was enhanced by 4.1-fold. These results suggest that the N1IC might activate the reporter gene containing the YY1-response elements.

Furthermore, we also checked whether this effect occurs in stable K562 cell lines expressing the HA-N1IC fusion protein

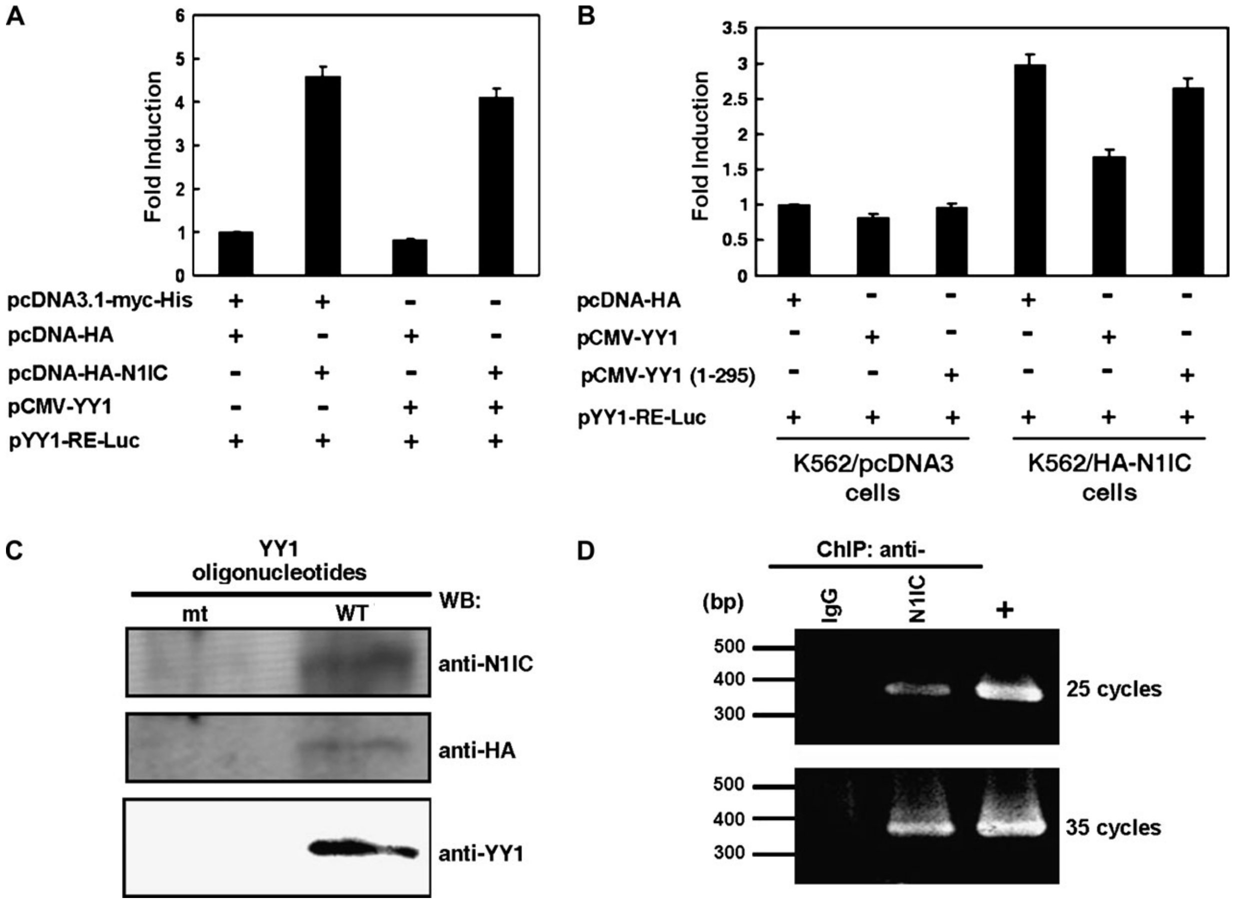

Fig. 1. The N1IC–YY1-associated complex binds to the YY1-response elements to enhance activity of the luciferase reporter gene. (**A**) A reporter plasmid containing the wild-type YY1-response elements (pYY1-RE-Luc) was cotransfected with the plasmids expressing the indicated proteins or their control vectors into K562 cells. (**B**) Reporter plasmid pYY1-RE-Luc was cotransfected with plasmids expressing the full-length YY1 (pCMV-YY1) or truncated YY1 [pCMV-YY1 (1–295)] into stable K562 cell lines expressing the HA-N1IC fusion protein (K562/HA-N1IC) or their control cells (K562/pcDNA3). After 48 h, luciferase activity was determined from whole-cell extracts, and the basal promoter activity of the reporter construct was set to unity. Mean values and standard deviations from at least four independent experiments are shown. (**C**) Nuclear extracts of K562/HA-N1IC cells were incubated with the 5′-biotinylated double-stranded oligonucleotides of wild-type (WT) or mutant (mt) YY1-response elements, and then precipitated with streptavidin–agarose beads. The precipitated proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and analyzed by western blotting using anti-N1IC antibody (upper panel), anti-HA antibody (middle panel) or anti-YY1 antibody (lower panel). (**D**) HeLa cells were transfected with luciferase reporter plasmid pYY1-RE-Luc containing wild-type YY1-response elements. Twenty-four hours after transfection, transfected cells were harvested for the ChIP assay using mouse anti-IgG or anti-N1IC antibodies. The immunoprecipitated DNA was used to amplify a 334 bp PCR product by specific primers for the region of the YY1-response elements in the reporter plasmid. The (+) PCR-positive control used 4 ng of pYY1-RE-Luc plasmid as the DNA template. The immunoblots and gel electophoresis shown here are representative of four to five independent experiments.

(K562/HA-N1IC). When the reporter plasmid containing the YY1-response elements was cotransfected with control vector pcDNA-HA, the activity of the reporter gene was enhanced by 3.0-fold in K562/HA-N1IC cells as compared with K562/pcDNA3 cells (Figure 1B). When cotransfected with reporter and full-length YY1 expression plasmids in K562/HA-N1IC cells, the luciferase activity was enhanced by 1.7-fold.

As described previously (10), YY1 suppressed the CBF1-dependent transactivation activity through association with the N1IC. Based on the findings described above, we further investigated whether the N1IC activates expression of the reporter gene with the YY1-response elements via its association with YY1. As shown in Figure 1B, the truncated YY1 (1–295), without the N1IC-binding domain (10), did not significantly suppress the YY1-dependent luciferase activity in K562/HA-N1IC cells. Therefore, these results suggest that the association between N1IC and YY1 is essential for the modulation of YY1-dependent reporter gene activity by N1IC.

The N1IC binds to the wild-type YY1-response element, but not to the mutant one

The aforementioned data showed that the formation of N1IC–YY1-associated complex is necessary for activation of the YY1-depedent reporter gene by N1IC. We surmised that the N1IC–YY1-associated complex might bind to the DNA containing the YY1-response elements to modulate reporter gene expression. To assess this possibility, we examined the DNA-binding ability of YY1 and the N1IC on the YY1-response element using oligoprecipitation and ChIP assay.

In K562/HA-N1IC cells, the N1IC and YY1 were precipitated together with the 5′-biotinylated double-stranded oligonucleotides of the wild-type YY1-response element, but not with the mutant one (Figure 1C). Additionally, the ChIP assay was also used to examine the specific association of the N1IC and DNA with YY1-response elements in the context of living cells. HeLa cells were transiently transfected with the pYY1-RE-Luc reporter plasmid containing the wild-type YY1-response elements. Twenty-four hours after transfection, cells were harvested for the ChIP assay using anti-mouse IgG or anti-N1IC antibodies. The amplified PCR product of 334 bp was only present in the samples immunoprecipitated by the anti-N1IC antibody (Figure 1D), but not in those immunoprecipitated by the control antibody. These data suggest that the N1IC binds to the YY1-response element via associating with YY1.

The binding of the N1IC on the YY1-response element induces activity of reporter gene in a CBF1-independent manner

In the prevailing model of the Notch signal pathway, N1IC modulates the target genes via both CBF1-dependent and -independent pathways (9). The association with N1IC and recruitment of co-activator change the transcription factor CBF1 from a transcription suppressor to an activator. To further delineate whether the enhancement of the activity of the reporter gene with YY1-response elements by the N1IC is CBF1-dependent, we constructed a CBF1-expressing plasmid to elucidate the effect of CBF1 on the regulation of YY1-dependent reporter gene activity. Using the luciferase reporter gene assay, the reporter plasmid 4×wtCBF1Luc containing four copies of the wild-type CBF1-response elements and the CBF1 expression construct (pcDNA-CBF1-myc-His) or its control vector were cotransfected into K562 cells. The activity of the CBF1-dependent reporter gene was suppressed to ~0.6-fold after the cotransfection of the CBF1 expression construct (Figure 2A, left). Transactivation of the reporter gene with the CBF1-response elements by the N1IC was dose dependently inhibited by the cotransfection of the CBF1 expression construct in K562 cells (Figure 2A, right). Therefore, the exogenous CBF1 expressed by the expression construct, pcDNA-CBF1-myc-His, may dilute the functional N1IC–CBF1 complex or recruit more co-repressors to modulate Notch signaling.

To analyze the effect of CBF1 on the regulation of YY1-dependent reporter gene activity, K562 cells were cotransfected with the reporter plasmid pYY1-RE-Luc, HA-N1IC fusion protein expression construct and CBF1 expression construct. The results showed that the exogenous CBF1 did not significantly affect the YY1-dependent reporter gene activity transactivated by the N1IC (Figure 2B). Furthermore, the oligoprecipitation was performed in HeLa cells transfected with the CBF1 expression construct to analyze the possibility of CBF1 binding onto the YY1-response element. The CBF1 was found to bind to the wild-type CBF1-response element, but not to the mutant one (Figure 2C). The endogenous YY1 also bound to the wild-type YY1-response element, but not to the mutant one. However, exogenous CBF1 bound neither to the wild-type YY1-response element nor to the mutant one.

To further evaluate the role of CBF1 on the regulation of the YY1-dependent reporter gene activity, the expression construct of pSG5Flag-RBP-VP16 expressing a constitutively active RBP-Jκ-VP16 fusion protein was transfected to induce the CBF1-dependent reporter gene activity (35). The expression of RBP-Jκ-VP16 fusion protein dose dependently enhanced activity of the luciferase reporter gene with wild-type CBF1-rersponse elements, but not with the mutants (data not shown). However, the constitutively active RBP-Jκ-VP16 fusion protein did not affect the YY1-dependent reporter gene activity (data not shown). These data suggest that the N1IC–YY1-associated complex modulates the YY1-dependent reporter gene activity via the CBF1-independent pathway.

YY1 further promotes the c-myc promoter activity enhanced by N1IC through a CBF1-independent pathway

Based on the results described above, we further investigated the role of the N1IC–YY1-associated complex in the control of YY1 target

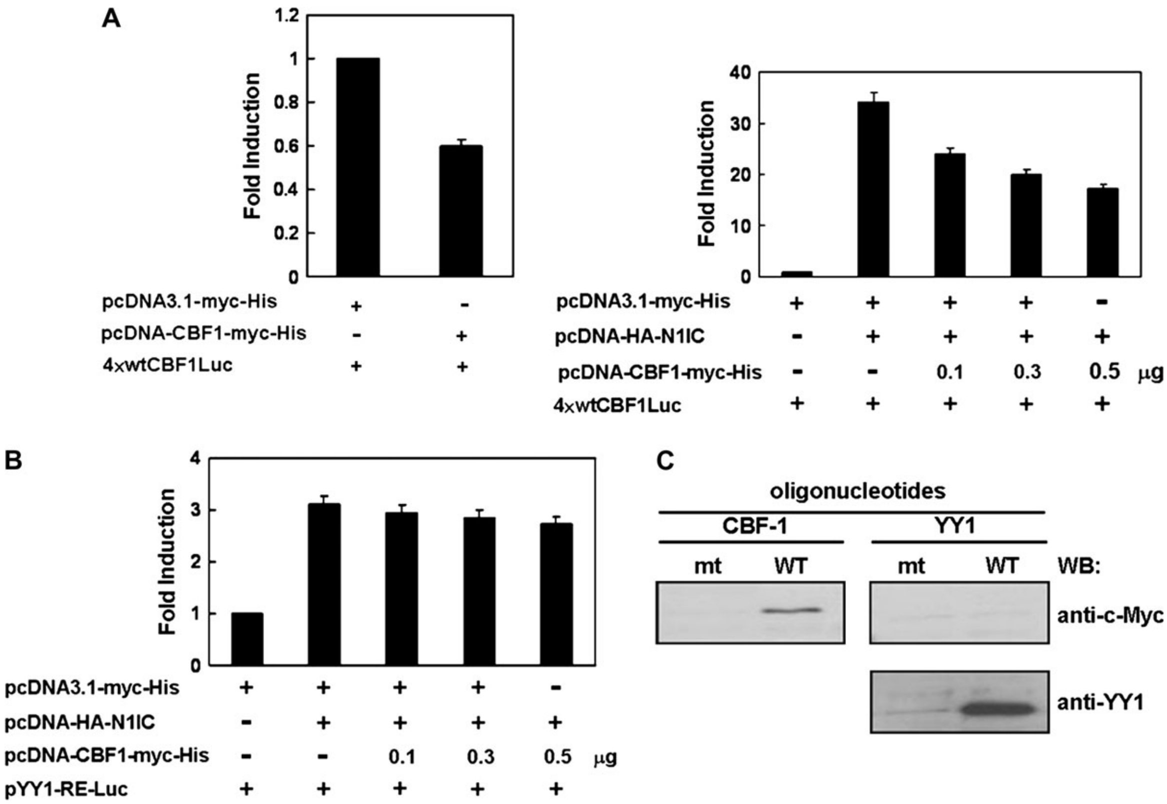

Fig. 2. The N1IC induces activity of the luciferase reporter gene with YY1-response elements through a CBF1-independent pathway. (**A**) The luciferase reporter plasmid 4×wtCBF1Luc containing four copies of the wild-type CBF1-response elements was cotransfected with the CBF1 expression construct pcDNA-CBF1-myc-His into K562 cells (left). The reporter plasmid 4×wtCBF1Luc was also cotransfected with the HA-N1IC fusion protein-expressing construct pcDNA-HA-N1IC and various amounts of CBF1 expression construct pcDNA-CBF1-myc-His into K562 cells (right). (**B**) Reporter plasmid pYY1-RE-Luc was cotransfected with the pcDNA-HA-N1IC expression construct and various amounts of the pcDNA-CBF1-myc-His plasmid into K562 cells. (**C**) The nuclear extracts of HeLa cells transfected with the pcDNA-CBF1-myc-His expression construct were incubated with the 5′-biotinylated wild-type (WT) or mutant (mt) oligonucleotides of CBF1-response elements and YY1-response elements, and then the oligonucleotides were precipitated with streptavidin–agarose beads. The precipitated proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and analyzed by western blotting using anti-c-Myc antibody (upper panel) or anti-YY1 antibody (lower panel). The immunoblots shown here are representative of four independent experiments. Luciferase reporter gene activity was determined as described in the legend to Figure 1.

genes. Among the target genes of YY1 (37), the *c-myc* oncogene also plays the same critical roles as the Notch1 signaling and YY1 in proliferation, differentiation and apoptosis. Additionally, YY1 was shown to regulate *c-myc* expression in the mouse (38). There are YY1-binding sites in the human *c-myc* promoter (39). Therefore, it is possible that the N1IC–YY1-associated complex binds to the YY1-response elements in the human *c-myc* promoter to regulate *c-myc* expression. To examine the possibility that *c-myc* is the target gene of the N1IC–YY1-associated complex, the luciferase reporter plasmid containing the human *c-myc* promoter (pLB1530) was cotransfected with the YY1- and HA-N1IC fusion protein-expressing constructs in both K562 cells and HeLa cells (Figure 3A, left and right, respectively). The N1IC increased the luciferase reporter gene activity of the *c-myc* promoter, and this increment was further promoted by the full-length YY1 but not the truncated one. Therefore, this enhancement of the *c-myc* promoter activity by YY1 is dependent on the interaction between N1IC and YY1.

To check whether activation of the *c-myc* promoter by the N1IC is CBF1-dependent or -independent, the CBF1 expression construct (pcDNA-CBF1-myc-His) was cotransfected with reporter plasmid pLB1530 to evaluate the effect of CBF1 on the activity of the *c-myc* promoter. Although the amount of transfected CBF1-expressing construct was increased, the activity of the reporter gene containing the *c-myc* promoter was not significantly affected in HeLa cells (data not shown). Moreover, cotransfection of the pLB1530 reporter plasmid and the pcDNA-HA-N1IC expression plasmid with various amounts of the pcDNA-CBF1-myc-His expression construct into K562 cells showed that exogenous CBF1 did not influence the transactivation activity of the N1IC on the *c-myc* promoter (data not shown). The constitutively active RBP-Jκ-VP16 fusion protein also did not promote reporter gene activity of the *c-myc* promoter (Figure 3B). Therefore, both exogenous CBF1 protein and RBP-Jκ-VP16 fusion protein did not act on the human *c-myc* promoter. These results suggest that the transcription factor YY1 further enhances the activity of the human *c-myc* promoter transactivated by N1IC through a CBF1-independent pathway.

The N1IC and YY1, but not CBF1, bind to the human c-myc promoter

To prove that modulation of *c-myc* promoter activity by the N1IC was not an artifact of protein over-expression, a ChIP assay was performed to study the specific interaction between the N1IC and *c-myc* promoter DNA in the context of living cells. HeLa cells were transfected with reporter plasmid pLB1530 containing the *c-myc* promoter. Twenty-four hours after transfection, transfected cells were harvested for the ChIP assay using mouse anti-IgG or anti-N1IC antibodies. According to PCR results, the amplified 210 bp DNA fragment of the *c-myc* promoter was only present in the sample immunoprecipitated with the anti-N1IC antibody, but not with the control antibody (Figure 4A). Furthermore, the ChIP assay was also used to examine the association between the N1IC and the *c-myc* promoter in chromosomal DNA of cells. The lysates of HA-N1IC fusion protein-expressing K562/HA-N1IC cells were used for immunoprecipitation by mouse anti-IgG, anti-N1IC, anti-YY1 and anti-CBF1 antibodies. The amplified PCR products of 210 bp *c-myc* promoter DNA were only present in the sample immunoprecipitated with the anti-N1IC and anti-YY1 antibodies, but not with the anti-IgG and anti-CBF1 antibodies (Figure 4B). For the control, the amplified PCR products of 312 bp Hes1 promoter DNA were only present in the sample immunoprecipitated with the anti-N1IC and anti-CBF1 antibodies, but not with the anti-IgG and anti-YY1 antibodies. This clearly proved that the N1IC and YY1, but not CBF1, bind to the *c-myc* promoter in cells. These results imply that the N1IC may bind to the YY1-response element in the *c-myc* promoter via associating with YY1. This interaction may elevate the *c-myc* promoter activity through a CBF1-independent pathway.

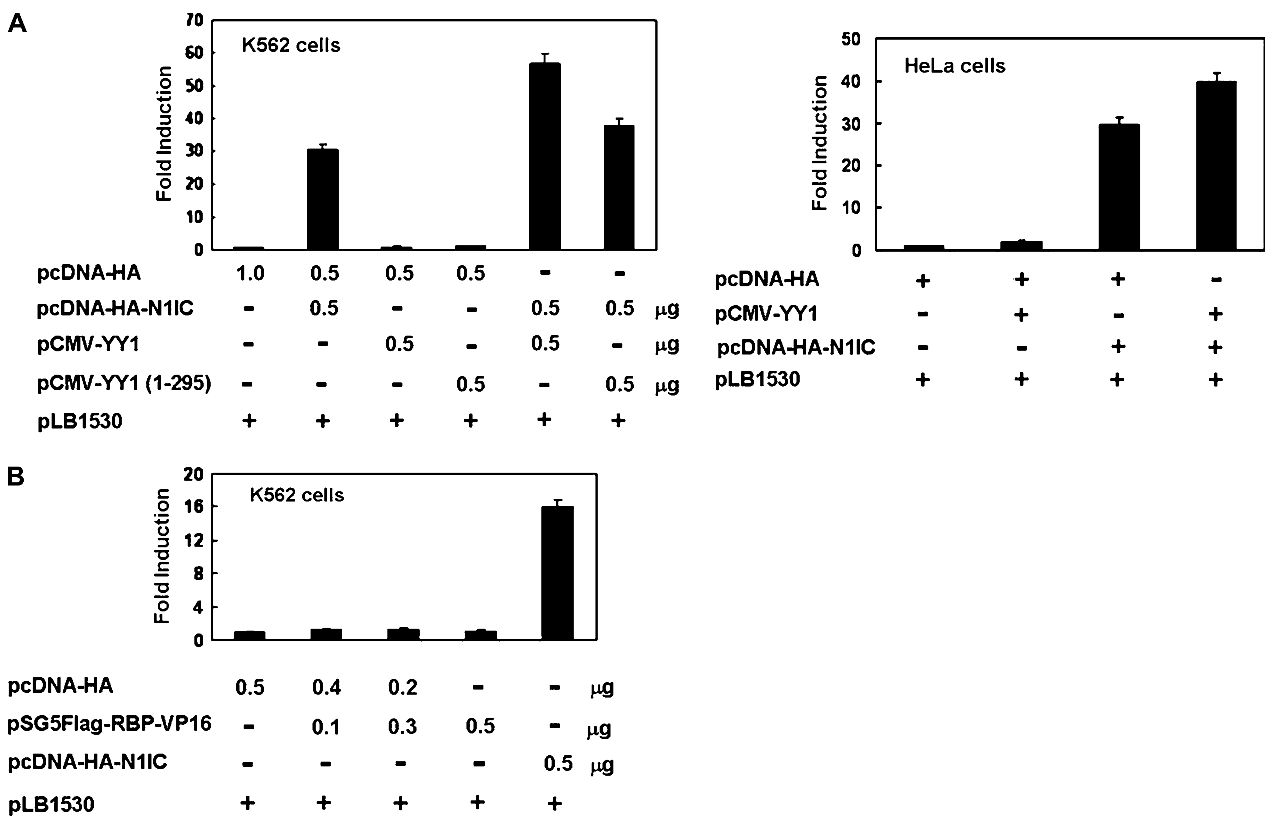

Fig. 3. The N1IC activates the *c-myc* promoter modulated by YY1 through a CBF1-independent pathway. (**A**) The reporter plasmid pLB1530 containing the human *c-myc* promoter was cotransfected with the expression constructs pcDNA-HA-N1IC and pCMV-YY1 or pCMV-YY1 (1–295) into K562 cells (left) and HeLa cells (right). (**B**) The luciferase reporter plasmid pLB1530 was cotransfected with the expression constructs pSG5Flag-RBP-VP16 or pcDNA-HA-N1IC into K562 cells. Luciferase reporter gene activity was determined as described in the legend to Figure 1.

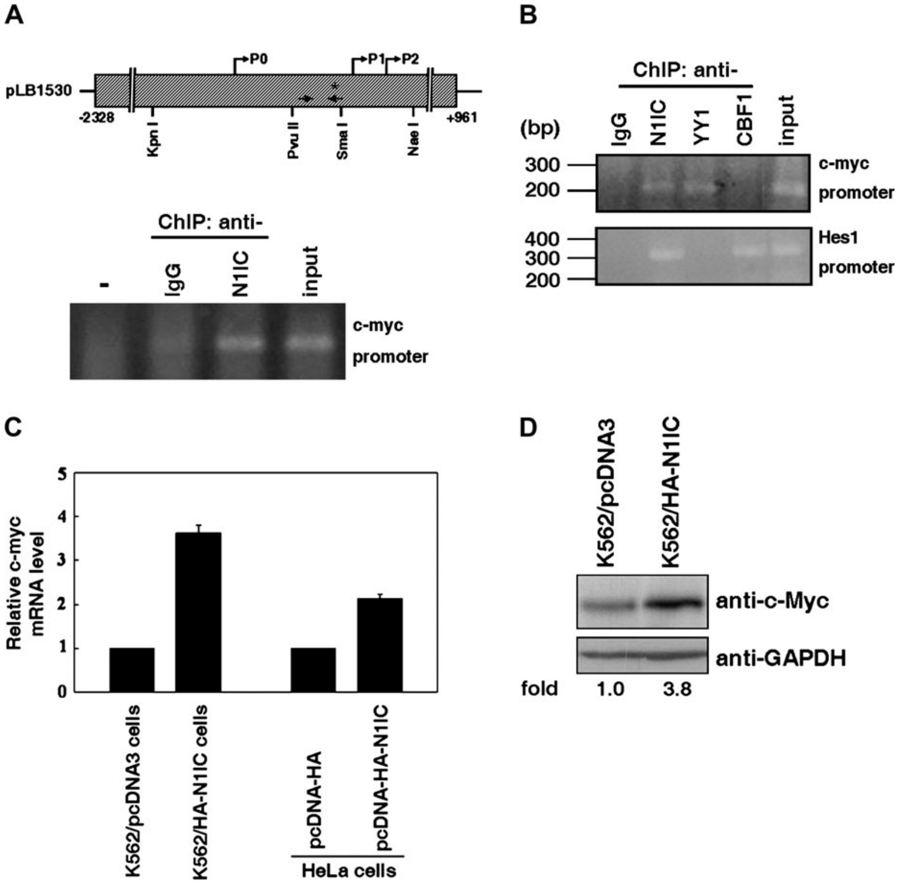

Fig. 4. The N1IC binds to the *c-myc* promoter and enhances the expressions of *c-myc* mRNA and protein. (**A** and **B**) The hatched box indicates the sequence of human *c-myc* promoter in reporter plasmid pLB1530 (upper panel). The asterisk indicates the position of the putative YY1-response element (nt 244 to −240 in relation to the P2 promoter). HeLa cells transfected with the pLB1530 reporter plasmid (**A**) and HA-N1IC-expressing K562/HA-N1IC cells (**B**) were harvested for the ChIP assay using mouse anti-IgG, anti-N1IC, anti-YY1 and anti-CBF1 antibodies. The immunoprecipitated DNA was used to amplify the 210 bp PCR product by specific primers (arrows in upper panel) for the region of the *c-myc* promoter and the 312 bp PCR product for the region of the Hes1 promoter. −, PCR-negative control without a DNA template. Input, 10% of cell lysates. (**C**) The *c-myc* transcript levels in HA-N1IC-expressing K562/HA-N1IC cells, K562/pcDNA3 control cells and HeLa cells transfected with HA-N1IC-expressing construct (pcDNA-HA-N1IC) or its control vector (pcDNA-HA) were measured by quantitative real-time PCR. The data were compared after being normalized to β-actin. (**D**) c-Myc (upper panel) and GAPDH (lower panel) in both HA-N1IC-expressing K562/HA-N1IC cells and K562/pcDNA3 control cells were measured by western blot analysis, and their intensities were quantitated. The data were compared after being normalized to internal control GAPDH. The gel electophoresis and immunoblots shown here are representative of four to five independent experiments.

The expressions of c-myc mRNA and protein are enhanced by the N1IC

As described above, the N1IC binds on the *c-myc* promoter to induce promoter activity. This induction of endogenous *c-myc* promoter activity will lead to promotion of *c-myc* expression. To check whether *c-myc* expression is increased by the N1IC, real-time PCR and western blot analysis were performed. As shown in Figure 4C and D, levels of both *c-myc* mRNA and the protein were elevated in the presence of N1IC. These results suggest that the gene expression of the human *c-myc* oncogene is activated by the N1IC.

The c-myc promoter is activated by the endogenous Notch signal pathway

To further determine whether *c-myc* promoter is activated by the endogenous Notch signaling, the single-cell-derived stable COS-7 cells constitutively expressing the secreted form of Notch ligand Jagged1 (COS-7/Jagged1^{ext}) were established to activate the endogenous Notch signal pathway. As seen in Figure 5A, the expression of Jagged1^{ext} was detected in both cell lysates and conditioned medium of COS-7/Jagged1^{ext} cells. Both the co-culture with COS-7/Jagged1^{ext} cells and treatment with their conditioned media induce the activity of reporter gene containing CBF1-response elements (data not shown).

K562 cells transfected with reporter plasmid containing human *c-myc* promoter were co-cultured with COS-7/Jagged1^{ext} cells (Figure 5B) or treated with their conditioned media (Figure 5C). The luciferase reporter gene activity driven by *c-myc* promoter was elevated in the presence of secreted form of Jagged1. Therefore, the activation of endogenous Notch signaling by Jagged1^{ext} enhances the *c-myc* promoter activity.

Over-expression of the N1IC inhibits the proliferation of K562 cells

In addition to the oncogenic role, there is mounting evidence that Notch signaling functions as a tumor suppressor. To date, the molecular mechanism controlling the Notch signal pathway, which serves as an oncogene or a tumor suppressor in tumorigenesis, remains obscure. To understand the role of the *c-myc* expression activated by N1IC in tumorigenesis, the K562/HA-N1IC cells were used in this study to evaluate their growth curves. The cumulative cell number of K562/HA-N1IC cells was lower than their control K562/pcDNA3 cells (Figure 6A).

The siRNA method was also used to check whether CBF1 and YY1 involve in this inhibition of growth in K562 cells. As presented in Figure 6B, the endogenous CBF1 and YY1 were knockdowned by siRNAs. The knockdowns by siRNAs against CBF1 and YY1 were also confirmed by reporter gene assay after the transfection with

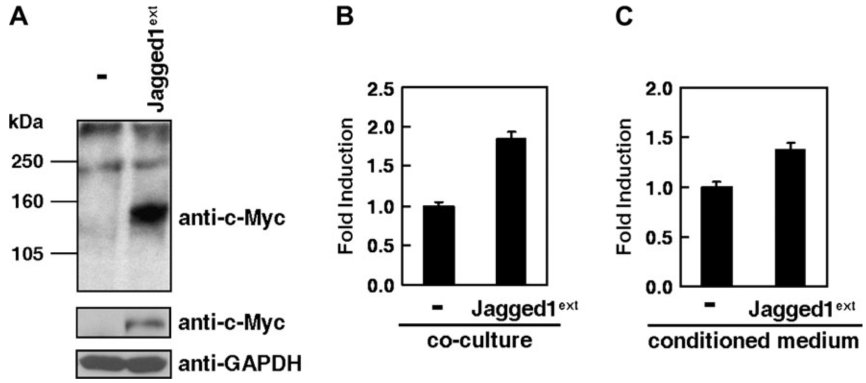

Fig. 5. The *c-myc* promoter is activated by the endogenous Notch signal pathway. (**A**) The conditioned media (upper panel) and whole-cell extracts (middle and lower panels) prepared from COS-7/Jagged1^{ext} (Jagged1^{ext}) cells and their control cells (−) were used to measure the expressions of Jagged1^{ext} and GAPDH by western blot analysis using mouse anti-c-Myc or anti-GAPDH antibodies. The immunoblots shown here are representative of four independent experiments. (**B** and **C**) K562 cells transfected with the pLB1530 reporter plasmid were co-cultured with COS-7/Jagged1^{ext} (Jagged1^{ext}) cells and their control cells (−) or treated with their conditioned media. Luciferase reporter gene activity was determined as described in the legend to Figure 1.

reporter plasmids containing CBF1- and YY1-response elements (data not shown). The data showed that the suppression of cell proliferation in K562 cells by N1IC was relieved after the knockdowns of CBF1 or YY1 (Figure 6B).

Additionally, the K562/HA-N1IC cells were slightly arrested in G_0/G_1 phase than their control cells using the PI staining and flow cytometry analysis (Figure 6C). These results suggest that the expression of the HA-N1IC fusion protein arrested K562 cells and suppressed their proliferation.

N1IC suppresses tumor growth of K562 cells in vivo

We further investigated whether N1IC also suppresses cell growth in the case of subcutaneously implanted HA-N1IC fusion protein-expressing cells or their control cells in nude mice. Tumors of HA-N1IC fusion protein-expressing K562/HA-N1IC cells were reduced to 19% of the sizes observed in the control K562/pcDNA3 cells on day 15 after tumor inoculation (Figure 6D). These results suggest that N1IC suppresses tumor growth of K562 cells *in vivo*.

Discussion

The role and control of Notch signaling in various cancer cells are very complicated and not yet fully understood. The Notch signal pathway is not exclusively oncogenic in its promotion of tumorigenesis; it can instead function as a tumor suppressor. Which of the two faces shown is dependent on the cellular context and the crosstalk with other signal pathways (6–8). Moreover, the N1IC also binds with several cellular factors to form a high-molecular weight complex to modulate its transactivation activity in the nucleus, including Mastermind-like-1, CBF1, YY1 and β_{II}-tubulin (10,40,41). In addition to the CBF1-dependent pathway, mounting evidence points to the existence of a CBF1-independent Notch signal pathway that is poorly characterized at present (42,43). These make the outcome of Notch activation difficult to predict.

In this study, we investigated the role of the N1IC–YY1-associated complex in regulation of YY1 target gene expression. We show herein that the N1IC binds to the YY1-response elements and the human *c-myc* promoter in cells. The YY1 further promotes the *c-myc* promoter activity enhanced by N1IC. This increment of the *c-myc* promoter activity by YY1 depends on the formation of the N1IC–YY1-associated complex (Figure 3A). In addition, the reporter gene activity of the human *c-myc* promoter was not affected by the exogenous CBF1/RBP-Jκ proteins (Figure 3B) and the DNA of this promoter was not detected by the ChIP assay using anti-CBF1 antibody (Figure 4B). Therefore, CBF1 cannot bind on the human *c-myc* promoter to regulate *c-myc* expression. It was speculated that formation of the N1IC–YY1-associated complex may promote the human *c-myc* promoter activity in a CBF1-independent manner. This is the first report regarding the role of the N1IC–YY1-associated complex in CBF1-independent Notch signaling.

As seen in Figure 3A, exogenous YY1 was insufficient to activate human *c-myc* promoter in the absence of N1IC. However, the *c-myc* promoter activity was enhanced in the presence of N1IC. YY1 is a ubiquitous protein abundantly expressed in K562 cells (10). It is possible that the abundant endogenous YY1 partially associates with exogenous N1IC to activate *c-myc* promoter. The transfection of YY1 expression plasmid increases the possibility of YY1 to bind on YY1-response elements in *c-myc* promoter to regulate the reporter activity. When both N1IC and YY1 expression plasmids were cotransfected, the repressing function of YY1 had changed by N1IC to activate the *c-myc* promoter. An intriguing similar situation is that the regulation of adeno-associated virus P5 promoter by the viral oncoprotein E1A and YY1 (44,45). The repressing and activating functions of YY1 are regulated by E1A mediated by the p300 (45,46).

In the present study, the N1IC was shown to regulate expression of the human *c-myc* oncogene via its association with YY1. Transcription factor c-Myc targets ∼10% of the genes involved in proliferation, growth, differentiation, metabolism and apoptosis (12,16,47). c-Myc can act as a primary regulator of the G_1–S transition and the elevated expression of Myc in tumor tissue contributes to tumorigenesis. Thus, control of *c-myc* expression by the N1IC may be an alternative way for the Notch signal pathway to regulate tumorigenesis. Therefore, the pleiotropic effects of Notch signaling complicate its role in tumorigenesis.

Indeed, the N1IC was demonstrated to cooperate with *c-myc* in inducing lymphoid tumors in mice (48). It was also found that the N1IC indirectly deregulates *c-myc* expression in Mv1Lu epithelial cells in a cell type-dependent manner (7). Satoh *et al.* (29) showed that the expression of *c-myc* was enhanced by Notch1 receptor to support the self-renewal of hematopoietic stem cells in a murine model. Moreover, the *c-myc* expression is up-regulated by Notch signaling to induce mammary tumorigenesis in mice (30). The *c-myc* is an important direct target of Notch1 receptor that contributes to the growth of T cell lymphoblastic leukemias and lymphomas in murine cells (31). These studies showed that the CBF1-dependent Notch signal pathway activates the *c-myc* expression in murine cells. Therefore, the regulation of *c-myc* promoter by Notch signaling in human is different from that in mouse.

In a murine model, YY1 was found to bind to the *c-myc* promoter and activate *c-myc* transcription (38). There are YY1-response elements in the human *c-myc* promoter (39). We also showed here that the activated Notch1 receptor bound to the human *c-myc* promoter in cells (Figure 4A and B). These imply that the N1IC may bind to the YY1-response element in the *c-myc* promoter by associating with

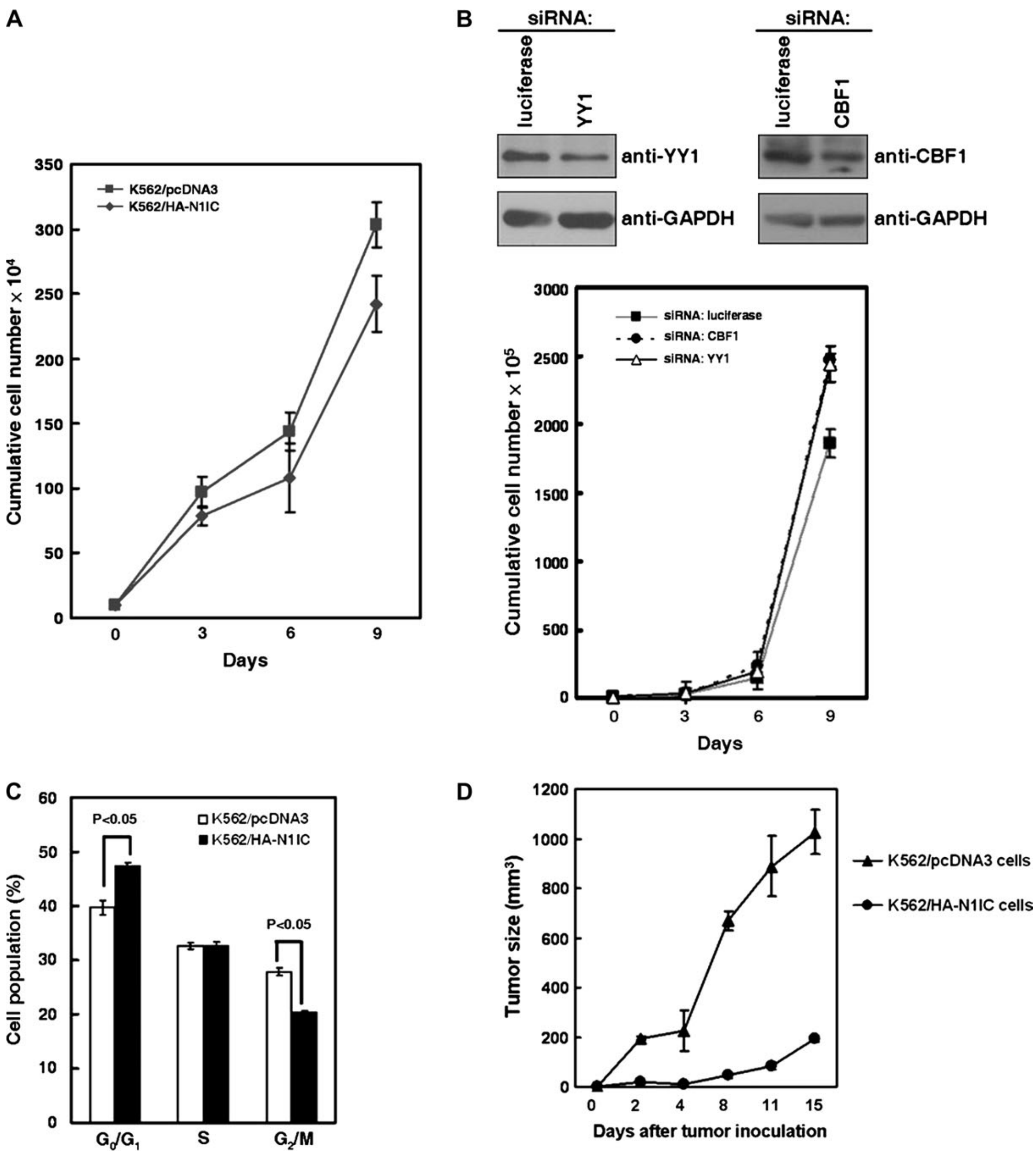

Fig. 6. N1IC inhibits the proliferation and tumor growth of K562 cells. (**A**) The stable cell clone of HA-N1IC fusion protein-expressing K562 cells (K562/HA-N1IC) and their control cells were seeded (1×10^5) and then counted the cell number by trypan blue exclusion method at the times indicated. The results are shown as mean ± SD of triplicated cultures. (**B**) HeLa cells were transfected with siRNA constructs against luciferase, YY1 and CBF1. Forty-eight hours after transfection, the transfected cells were harvested for western blotting using anti-YY1, anti-CBF1 and anti-GAPDH antibodies (upper panel). After transfection with siRNA constructs, K562/HA-N1IC cells (5×10^5) were seeded and then counted (lower panel). The results are shown as mean ± SD of triplicated cultures. (**C**) The K562/HA-N1IC cells and their control cells were stained with PI and were analyzed for DNA content by flow cytometry. A total of 10,000 cells was analyzed from each sample, and the proportion of cells in G_0/G_1, S and G_2/M phases of cell cycle was indicated. Data shown are representative of the mean values and standard deviations from three independent experiments performed in triplicate. (**D**) The viable HA-N1IC fusion protein-expressing K562 cells and their control cells were subcutaneously inoculated into nude mice. The tumor sizes were measured at the times indicated. Data shown are from five nude mice. Data points are the means of three independent experiments.

transcription factor YY1. Intriguingly, there is a putative YY1-binding site (CCATA) in the amplified 210 bp PCR product of *c-myc* promoter by ChIP assay (Figure 4A). We will further evaluate whether N1IC associates with YY1 bound on this putative YY1-binding site.

As shown in Figure 6B, the relief of growth inhibition caused by the knockdown of CBF1 may be due to the modulation of expression of cell cycle-related proteins regulated by N1IC. For example, the N1IC activates the expression of cyclin D1 through a CBF1-dependent manner (49). Therefore, the knockdown of CBF1 may affect cell growth through regulating the expression of cell cycle-related proteins controlled by N1IC.

The expression of *c-myc* induced by N1IC is insufficient to promote the cell growth and tumorigenesis of K562 cells (Figure 6). How does the N1IC induce *c-myc* expression and suppress cell proliferation in K562 cells? One possibility is that c-Myc may act as an inducer of apoptosis in the N1IC-expressing K562 cells. To check this possibility, the DNA contents of the K562/HA-N1IC cells and their control K562/pcDNA3 cells were determined by PI staining and flow cytometry analysis to evaluate apoptosis by the sub-G1 peak. The results showed that the apoptosis of K562 cells was not induced in the presence of N1IC (data not shown). Moreover, the N1IC also did not promote the apoptosis of K562 cells in 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay (data not shown).

Alternatively, the *c-myc* oncogene can drive proliferation by promoting cell cycle re-entry. Several target genes involved in the G_1–S transition are induced by c-Myc (20–27). As described previously (7,29–33), the expression of *c-myc* mRNA is activated by the oncogenic form of the Notch1 receptor (N1IC). Therefore, the induction of c-Myc by the N1IC may alter the expressions of cell cycle-related proteins, which in turn results in the control of cell growth of K562 cells. The Rb/E2F pathway also plays an important role in the control of the G_1–S transition. The phosphorylation of Rb is maintained in a hyperphosphorylated state in epithelial cells with low serum conditions and suppressed in endothelial cells by N1IC (7,50).

Based on the findings described above, we will further investigate the role of cell cycle-related proteins in the Notch-mediated growth suppression of K562 cells. To understand more about the role of Notch signaling in tumorigenesis, we would like to further screen other target genes of the N1IC–YY1-associated complex.

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