The Crosstalk Between Notch1 and BCL6

James Damian Hakert

Florida State University

Summary

We reported that BCL6 inhibited Notch signaling by interfering the interaction between Notch1 intracellular domain (NICD) and Mastermind-like 1 (MAML1) in the transcriptional complex during left-right body axis patterning in the amphibian, *Xenopus*. This regulatory mechanism of Notch signaling by BCL6 must be crucial for other biological events such as other developmental processes and cancer formation, because the expression of Notch1 and BCL6 overlapped in several developmental tissues and both Notch1 and BCL6 are popular oncogenes. Since activation of Notch signaling induces apoptosis in B-cell lymphocytes and the anti-apoptotic function of BCL6 has been suggested to be an important mechanism for B-cell lymphoma formation, we hypothesize that the inhibition of Notch-induced apoptosis by BCL6 leads to the formation of lymphoma. For our study, we used HeLa cells instead of B-cell lymphocytes, because Notch signaling also induces apoptosis in HeLa cells and HeLa cells are more practical for our experiments. In order to test our hypothesis, the interaction between Notch1 and BCL6 within the mammalian cells was first verified. Through Immunoprecipitation and Immunocytochemistry, the interaction between Notch1 and BCL6 was observed in the HeLa cells. Next, we tested that

BCL6 inhibited Notch-induced apoptosis in the HeLa cells. NICD and/or BCL6 DNA plasmid were transfected, followed by Hoechst staining. Apoptotic cells in the GFP-NICD and/or BCL6 transfected cells were counted, and then apoptosis in the HeLa cells was evaluated. The number of apoptotic cells, when when compared to those transfected with Notch1 alone, was reduced significantly by co-transfection with BCL6. This is indicative that BCL6 prevented apoptosis induced by Notch signaling. Taken together, BCL6 appears to interact with Notch1 directly and protect the HeLa cells from apoptosis induced by Notch signaling.

Background

Section I: Notch Signaling

The Notch signaling pathway is well conserved in both vertebrates and invertebrates, and plays a very important role in cell fate determination, cell growth, progenitor cell maintenance, boundary formation, differentiation and apoptosis in adjacent cells (1). Following an interaction between the Delta/ Serrate/Lag-2 (DSL) ligand and Notch receptor, the intracellular domain of the Notch receptor (NICD) is released from the membrane by two sequential proteolytic cleavages. When NICD translocates into the nucleus, in an attempt to activate transcription of target genes, it forms a complex with the transcription factor C-promoter binding factor 1/ Suppressor of Hairless/ Lag-1 (CSL), and the transcriptional co-activator Mastermind-like (MAML) (3).

Pathophysiologic alterations in Notch signaling have been associated with tumorigenesis. Many non–small cell lung cancers express high levels of Notch1, Notch2, and HES1 in comparison to small-cell lung cancers, while activation of the Notch signaling pathway is an early event in human pancreatic tumorigenesis (6). On the other hand, Notch has been also found to induce cell cycle arrest in various types of cells, one of which is the HeLa cell (1). Cell cycle distribution analysis has showed that the increase in G1 phase cells observed in HeLa-ICN is significant. These results demonstrated that over-expression of Notch1 (NICD) was able to substantially inhibit *in vitro* growth and proliferation of HeLa cell,s and induced G1 cell cycle arrest (3). Over-expression of Notch1 (NICD) led to a cell cycle arrest in small-cell lung cancer cells and inhibited the proliferation of various prostate cancer cells, suggesting that Notch activation can reduce the neoplastic potential of tumors (6). These findings indicate that Notch plays various roles in cancer formation.

Section II: B-Cell Leukemia/Lymphoma 6 (BCL6)

BCL6, a transcriptional repressor, encodes one of the most studied so-called 'POK (Poxviruses and Zinc-finger [POZ] and *Krüppel*)' proteins, harboring N-terminal POZ (or BTB) domain that mediates protein/protein interactions including self interactions, and a varying number (six in the case of BCL6) of C-terminal *Krüppel*-like C2H2-type zinc fingers (2). BCL6 was initially cloned by virtue of its disruption in certain types of human non-Hodgkin lymphomas (BNHL) (2). It maps to a major translocation breakpoint cluster affecting the 3q27

chromosomal region in two types of BNHL: follicular lymphomas (FL) and diffuse large B-cell lymphomas (DLCL). When BCL6 expression becomes deregulated by this translocation, in which heterogonous promoters are substituted for normal BCL6 regulatory sequences, follicular and diffuse B-cell lymphomas are largely prone to develop. Although the mechanism of the how the deregulation of BCL6 results in B-cell lymphoma still remains unclear, several possible mechanisms, including anti-apoptotic and anti-differentiation effect of BCL6, have been proposed (2). As BCL6 shields the cells against pro-apoptotic signals, the over-expression of BCL6 may turn out to be oncogenic (2).

Section III: Apoptosis

Apoptosis is 'programmed cell death', an evolutionarily evolved mechanism to prevent excessive cell maturation and unregulated cell growth, possibly leading to a carcinoma (7). Apoptosis is considered a vital component of various processes including normal cell turnover, proper development and functioning of the immune system, hormone-dependent atrophy, embryonic development and chemical-induced cell death (7). Inappropriate apoptosis (either too little or too much) is a factor in many human conditions including neurodegenerative diseases, ischemic damage, autoimmune disorders, and many types of cancer (7).

Caspases are widely expressed in an inactive proenzyme form in most cells and, once activated, can often activate other pro-caspases, allowing initiation of a protease cascade (7). Some pro-caspases can also aggregate and autoactivate

(7). This proteolytic cascade, in which one caspase can activate other caspases, amplifies the apoptotic signaling pathway and thus leads to rapid cell death (7). The extrinsic, intrinsic, and granzyme B pathways converge on the same terminal, or execution pathway (7). This pathway is initiated by the cleavage of caspase-3 and results in DNA fragmentation, degradation of cytoskeletal and nuclear proteins, cross-linking of proteins, formation of apoptotic bodies, expression of ligands for phagocytic cell receptors, and finally uptake by phagocytic cells (7). The granzyme A pathway activates a parallel, caspase-independent cell death pathway via single stranded DNA damage (7).

Section IV: Crosstalk between Notch1 and BCL6 in cancer formation

We recently reported that BCL6 interacted with Notch1 and inhibited the Notch activity in the amphibian system (3). Since these two molecules are highly conserved between the amphibian and mammalian system, this regulation of Notch signaling by BCL6 is likely conserved in the mammalian systems as well. Interestingly, the active Notch1 caused growth suppression of the cells, accompanied by apoptosis in the HeLa cell (4) and B-cell lymphocyte (2). Furthermore, an abnormal, anti-apoptotic function of over-expressed BCL6 possibly leads to the formation of B-cell lymphoma as prior mentioned (2). Based on these and our findings, BCL6 may inhibit Notch1-induced apoptosis, which could be a possible mechanism of abnormal cell growth.

Materials and Methods

1. Plasmid construction

a. PCR:

Mouse Notch1 intracellular domain or human BCL6 was amplified by the following primers and PCR condition (denaturing at 95°C for 30 seconds, annealing at 60°C for 30 seconds, extending at 72°C for 1 minute and 30 seconds, and thirty cycles):

Mouse Notch1

U 5' GGGAATTCATCCCGCAAGCGCCGGCGGCA 3'

D 5' CCGTCTAGAGCTCGAGCTGTCCAACAGGC 3'

Human BCL6

U 5' CCGAGATCTCGGCCTCGCCGGCTGACAGCTG 3'

D 5' CCGCTCGAGGCAGGCTTTGGGGAGCTCCG 3'

b. DNA purification and digestion by restriction enzymes:

PCR product was purified by UltraClean Standard Mini Plasmid Prep Kit (Mo Bio). Three times volume of Gel Binding solution was added to PCR product. This solution was applied to spin column and spin column was centrifuged. Next, spin column was washed by 300µl of Washing Buffer, and then DNA was extracted by adding 30µl of water. Following DNA purification, DNA was digested by EcoRI/Xbral or BglII/Xhol (New England Biolab) at 37°C overnight.

c. DNA purification from agarose gel and DNA ligation

After incubating DNA at 37°C overnight, DNA was applied to agarose gel

for purification. DNA was isolated from agarose gel by UltraClean Standard Mini Plasmid Prep Kit. DNA bands were cut out from agarose gel. Three times gel volume of Gel Binding solution was added to cut gel, and then incubated at 65° until gel was melted. This solution was applied to spin column and it was centrifuged. Spin column was washed by 300µl of Washing Buffer, and then DNA was extracted by 30µl of water. Following DNA purification, purified NICD or BCL6 DNA was ligated with pCS2+GFP vector or pCS2+Flag by T4 ligase (New England Biolab). Sample was incubated at 16°C for 6 hrs.

d. Transformation

Twenty-five microliters of Competent cells (DH5α *E. Coli*), to which 1.5µl of ligated plasmid was added, were kept on ice for 30 minutes. After heat shock at 42°C for 30 seconds, 100µl of LB solution was added and competent cells were incubated at 37°C for 1 hour. Sample was spread onto LB plate with Ampicillin and the plate was incubated at 37°C overnight.

c. DNA preparation:

DNA was purified by QIAprep Spin Miniprep Kit (QIAGEN). Pelleted bacteria were resuspended with 250µl of Buffer P1, 250µl of Buffer P2 was added, and then 350µl of Buffer N3 was added. After samples were centrifuged, supernatants were applied to spin column. Centrifuged spin column was washed

by 750µl of Buffer PE, and then DNA plasmid was eluted from column by 50µl of Buffer EB

Buffer EB.

2. Immunoprecipitation

a. Preparation of cell extract and Immunoprecipitation

HeLa cells were lysed in the lysate buffer (20 mM Tris-HCl [pH 8.0], 5 mM $MgCl_2$, 1 mM EDTA, 50 mM KCl, 0.1 % Triton X-100, 10% glycerol, 1mM dithiothreitol and protease inhibitors) and protein extracts were used for immunoprecipitation. Immunoprecipitation is a procedure using the physics of precipitating a specific protein through the interaction with its respective specific antibody. Thirty microliters of protein A/G agarose beads (Santa Cruz Biotechnology, Inc.), an antibody (α-Notch1 antibody [Millipore] or α-BCL6 antibody [Abcam]), and HeLa cell extract were mixed and incubated at 4°C overnight. After precipitation, samples were washed with lysate buffer three times.

b. Immunoblotting

Immunoblotting is a procedure used to detect specific proteins. 8 % SDS-PAGE gel (sodium dodecyl sulfate polyacrylamide gel electrophoresis) was used for protein electrophoresis. After running samples into SDS-PAGE gel, proteins were transferred to PVDF membrane (Millipore) in transfer buffer (25 mM Tris, 200 mM Glycine and 10 % Methanol) at 40V for 2 hours. Transferred membranes were placed into blocking buffer (5 % skim milk in PBST [1 x PBS,

0.05 % Tween-20]), and were incubated at room temperature for 1 hour. Blocking buffer was replaced by a diluted antibody solution [α-Notch1 antibody

(Millipore), α-BCL6 antibody (Abcam), α-GFP antibody (Invitrogen) or α-Flag antibody (Sigma) in the blocking buffer] and membranes were incubated at 4°C overnight. Next day, membranes were washed by PBST three times and then incubated with a diluted secondary antibody solution at room temperature for 1 hour.

After reaction with a secondary antibody, membrane was washed with PBST three times. For the chemiluminescent reaction, Immobilon Western Chemiluminescent HRP Substrate (Millipore) was used.

3. Immunocytochemistry

This is a procedure used to visualize protein expression by an antibody within the cell. Cells were washed with 1mL of PBS three times and fixed with 1mL of 3.7% formaldehyde at room temperature for 10 minutes. Cells were washed again with PBS three more times and permeablized with 1mL of PBS, containing 0.2% Triton-X-100 Buffer, at room temperature for 10 minutes. After cells were washed with 1mL of PBS three more times, cells were blocked with 100mL of blocking solution (1 x PBS, 0.1% Tween-20, 1% IgG-free ram serum) at 37°C for 30 minutes. α-Notch1 or α-BCL6 antibody was applied, and cells were incubated at 37°C for 1 hour. Cells were washed with 1mL of PBS with 0.1% Tween-20 three times at 5 minutes per wash. Secondary antibody was applied

to cells, which were then incubated at 37°C for 1 hour. After washing with 1mL of PBS with 0.1% Tween-20 three times, DAPI staining was performed.

4. Transfection

Transfection is a procedure that introduces DNA into cells. A mixture of TransIT®-LT1 Transfection Reagent (Mirus) and serum-free DMEM was added to DNA plasmid, and then incubated at room temperature for 20 minutes. Following incubation, the mixture was added to cultured HeLa cells.

5. Hoechst staining and cell counting

Hoechst staining is a fluorescence DNA staining. After transfection, HeLa cells were incubated at 37°C for 48 hours. Floating cells were collected and stained by Hoechst 33342 solution (Invitrogen) on ice for 30 minutes. After Hoechst staining, pictures of three different areas were taken, and GFP-positive and apoptotic cells were counted. All error bars shown are the standard deviation (SD) from the mean of independent triplicates.

Results

BCL6 interacts with Notch1 in the HeLa cells

In order to confirm the interaction between Notch1 and BCL6 in the mammalian system, the HeLa cells, Immunoprecipitation was performed. After protein extracts from the HeLa cells were mixed with α-Notch1 or α-BCL6 antibody, the interaction was examined via immunoblotting. α-Notch1 or α-BCL6

antibody could precipitate Notch1/BCL6 complex, respectively (Figure 1). This result indicates that BCL6 interacts with Notch1 within the HeLa cells, and this interaction is conserved among different species and different tissues.

BCL6 and NICD are expressed in the nucleus of HeLa cells

To prove that the interaction between BCL6 and Notch1 occurs in the nucleus, the localization of Notch1 and BCL6 in the HeLa cells was examined. Immunocytochemistry was performed with α-Notch1 or α-BCL6 antibody to test the expression of Notch1 and BCL6 in the HeLa cells. As expected, the expression of Notch1 and BCL6 protein was detected in the nucleus, which was stained by DAPI. This result suggests that BCL6 binds with Notch1 in the nucleus, and BCL6 inhibits the transcriptional activity of Notch signaling, similar to *Xenopus*. (Figure 2)

Flag-BCL6 and GFP-NICD were successfully over-expressed in the HeLa cells

Before we studied the anti-apoptotic function of BCL6 in the HeLa cells, we tested whether Flag-tagged BCL6 and GFP-NICD were over-expressed in the HeLa cells by immunoblotting. After transfection of pCS2+Flag-BCL6 and pCS2+GFP-NICD into the HeLa cells, protein extract was lysed and immunoblotting with α-Flag or α-GFP antibody was performed. Expected sizes of Flag-tagged BCL6 (80 KD) and GFP-NICD (70 KD) were observed (Figure 3).

This result shows that proteins from plasmids, which are newly generated for this project, were correctly expressed in the HeLa cells.

BCL6 inhibits apoptosis induced by Notch signaling in the HeLa cells

To test if BCL6 could inhibit apoptosis, which is induced by Notch signaling, the configuration of chromatin was evaluated by staining DNA with Hoechst. Chromatin condensation, as well as nuclear segregation, indicates apoptosis (Figure 4). GFP control vector, GFP-NICD, Flag-tagged BCL6 were transfected into the HeLa cells, followed by Hoechst staining. The percentages of apoptotic cells were 24.5 % (n=286) with the GFP control vector, 45.2 % (n=219) with GFP-NICD alone, and 22.3 % (n=567) with GFP-NICD/Flag-tagged BCL6, respectively (Figure 5).

The over-expression of Notch1 induced nearly double the number of apoptotic cells by control transfection. This is better than published study (6) because we used the floating cells (apposed to the attached cells) for our assay. When BCL6 was transfected with Notch1, the percentages of apoptotic cells dropped to the similar number by control transfection. This result supports our hypothesis of BCL6 suppressing Notch-induced apoptosis in the HeLa cells.

Discussion

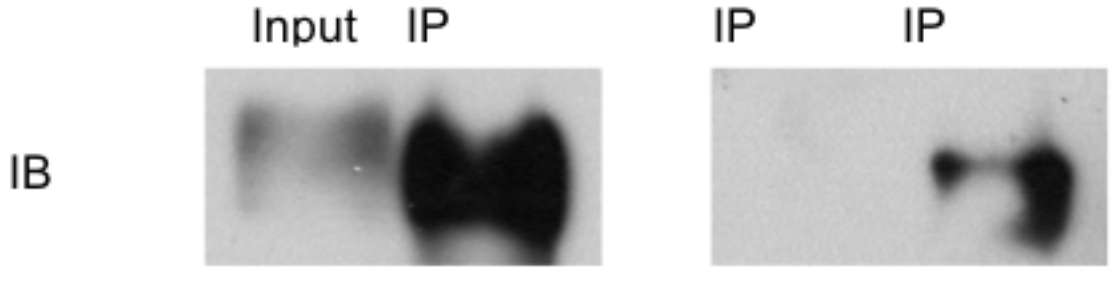
Our results show that BCL6 interacts with Notch1 in the nucleus and inhibits Notch signaling from inducing apoptosis in the HeLa cells. However, as we used only one apoptosis assay, the configuration of chromatin, other methods

such as caspase, TUNEL, or Annexin V assays must be applied to confirm our results in the future.

Previous studies showed that Notch increase the expression of p53 (6), an upregulated modulator of apoptosis, to induce apoptosis in the HeLa cells (1). In

addition, Hairy1, a chicken ortholog of HES1 and a transcriptional downstream gene of Notch signaling, mediates Notch-induced apoptosis in B-cell lymphocytes (4). Since BCL6 inhibits Notch signaling by competing with MAML1 for NICD in the transcriptional complex during *Xenopus* embryogenesis (3), it must be important to test whether the expression of HES1 is suppressed by BCL6. Furthermore, whether the increase of p53 expression by the Notch activation goes back to normal by the co-expression of BCL6 or not must be also addressed in the further studies.

The anti-apoptotic function of abnormally over-expressing BCL6 could be a potential reason for the oncogenic properties of BCL6 and could be a possible mechanism for B-cell lymphoma occurrences (2). Importantly, the over-expression of activated Notch1 could induce apoptosis in B-cell lymphocytes (4). This data and our results support the possibility that the inhibition of Notch signaling by abnormally expressing BCL6 in B-cell lymphocytes could be a mechanism of B-cell lymphoma formation. However, further studies are required to solve this vital question. Also, this novel discovery could potentially lend its hand to the possible development of therapeutics that could change the lives of those suffering with B-cell lymphoma.



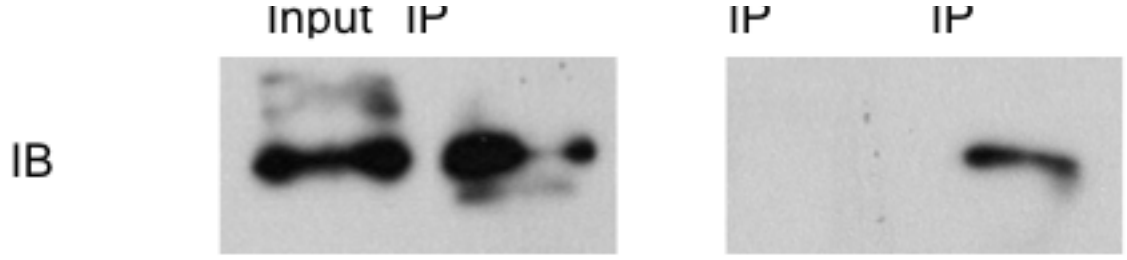


Figure 1. BCL6 interacts with Notch1 in the HeLa cells. Protein extracts from the HeLa cells were used for immnoprecipitation with α-Notch1 and α-BCL6 antibodies. After immunoprecipitation, immunoblotting with α-Notch1 and α-BCL6 antibodies was performed. The left columns show that both the isolation of proteins and the immunoprecipitaion by antibodies were done successfully. The right columns demonstrate that the Notch1/BCL6 complex was precipitated with either antibody but not without antibodies (IP Control). IB: immunoblotted, IP: immunoprecipitated.

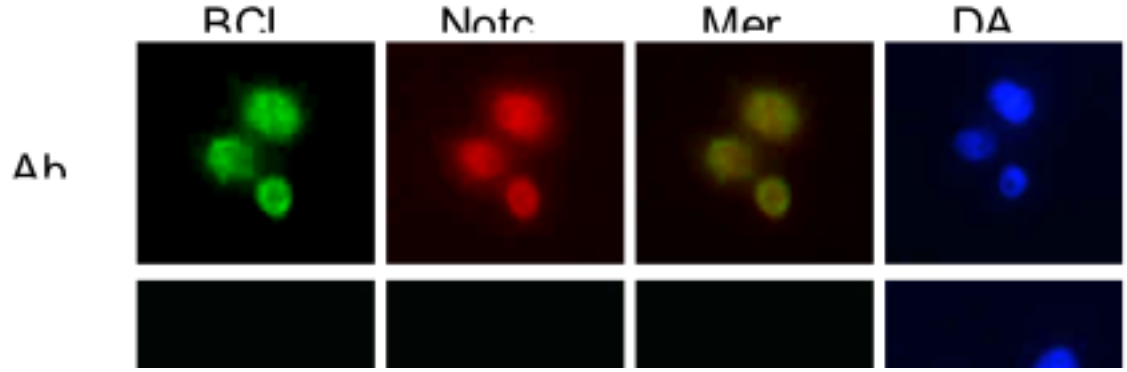


Figure 2. Both BCL6 and NICD are expressed in the nucleus. The HeLa cells were stained with α-BCL6 (FIT-C) and α-Notch1 (Texas Red) antibodies, respectively. The nuclei (blue) were stained with DAPI. Without antibodies, the expression of both proteins was not observed. Ab: antibody.

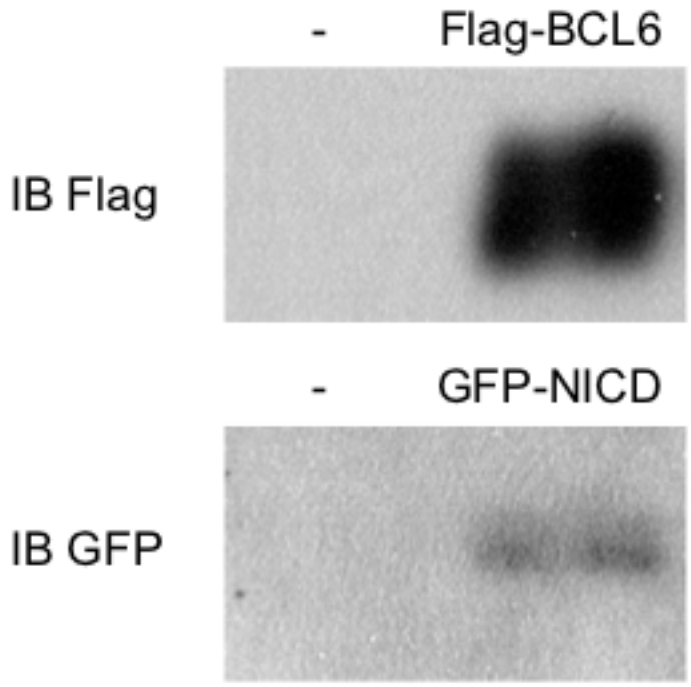


Figure 3. Flag-BCL6 and GFP-NICD were expressed. Two micrograms of each plasmid (pCS2+Flag-BCL6 or pCS2+GFP-NICD) was transfected into the HeLa cells. After 24 hours, protein extract was generated and immunoblotting was performed with α-GFP or α-Flag antibody. IB: immunoblotted.

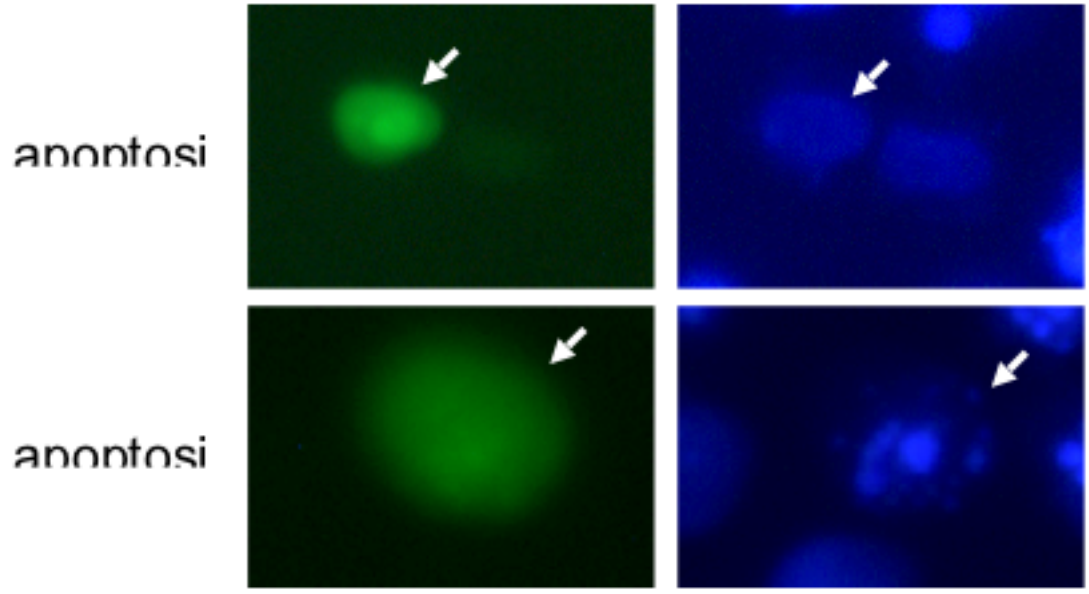


Figure 4. GFP-positive and apoptotic cells. Transfected cells were stained with Hoechst, and then the apoptotic cells were counted. GFP positive cells (green) represent transfected cells. The breaking down and condensing of the chromatin in the nucleus, indicated by an arrow in the lower panels, shows clear apoptotic activity within the cells. On the other hand, the arrows in the upper panels indicate a GFP positive and normal cell, respectively.

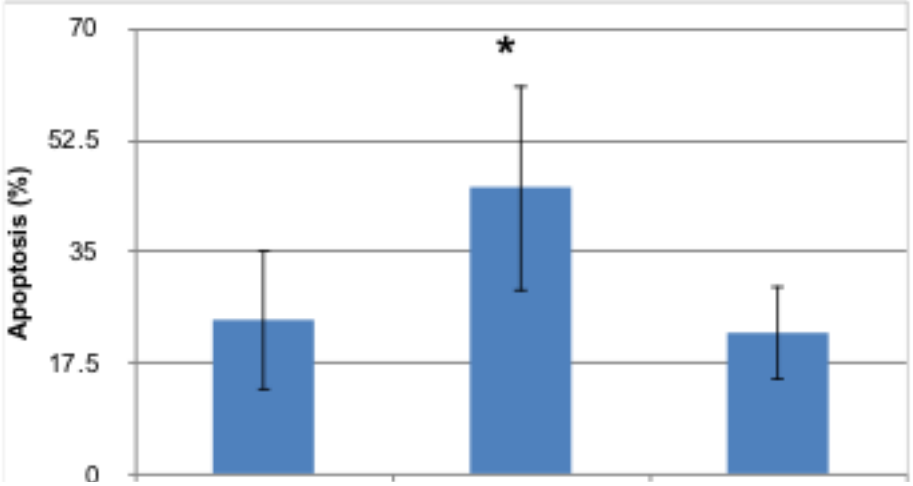


Figure 5. BCL6 inhibits apoptosis induced by Notch signaling. The mean of the percentages of apoptotic cells the total cells from three individual experiments, was calculated. All the error bars shown are standard deviations (SD) from the mean of the triplicates. Although error bars were relatively large, there is a substantial difference (about two fold) in the percent apoptotic cells of NICD when compared to both Control and NICD/BCL6 in each individual experiment. * $P<0.1$

References

1) Lai, E.C. *Notch signaling: control of cell communication and cell fate*. Development. (2004) 131: 965-973.

2) Olivier, A. C. *Ambivalent role of BCL6 in cell survival and transformation*. Oncogene. (2003) 22: 507–516.

3) Sakano D., Kato, A., Parikh, N., McKnight, K., Terry, D., Stefanovic, B., and Kato, Y. *BCL6 canalizes Notch-dependent transcription, excluding*

Mastermind-like1 from selected target genes during left-right patterning. Developmental Cell. In press.

4) Morimura, T., Goitsuka, R., Zhang, Y., Saitoi, I., Reth, M., and Kitamura, D. *Cell Cycle Arrest and Apoptosis Induced by Notch1 in B Cells*. J Biol Chem. (2000) 275(47): 36523-36531.

5) Tarnowski, B. I., Spinale, F. G., and Nicholson, J. H. *DAPI as a Useful Stain for Nuclear Quantitation*. Biotechnic and Histochemistry. (1991)

66(6): 296-302.

6) Wang, L., Qin, H., Chen, B., Xin, X., Li, J., and Han, H. *Overexpressed Active Notch1 Induces Cell Growth Arrest of HeLa Cervical Carcinoma Cells*. Int J Gynecol Cancer. (2007) 17: 1283–1292.

7) Elmore, S. *Apoptosis: A Review of Programmed Cell Death*. Toxicol Pathol. (2007) 35(4): 495-516.