Reciprocal negative regulation between the tumor suppressor protein p53 and B cell CLL/lymphoma 6 (BCL6) via control of caspase-1 expression

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Even in the face of physiological DNA damage or expression of the tumor suppressor protein p53, B cell CLL/lymphoma 6 (BCL6) increases proliferation and antagonizes apoptotic responses in B cells. BCL6 represses TP53 transcription and also appears to inactivate p53 at the protein level, and additional findings have suggested negative mutual regulation between BCL6 and p53. Here, using Bcl6−/− knockout mice, HEK293A and HCT116 p53−/− cells, and site-directed mutagenesis, we found that BCL6 interacts with p53 and thereby inhibits acetylation of Lys-132 in p53 by E1A-binding protein p300 (p300), a modification that normally occurs upon DNA damage–induced cellular stress and whose abrogation by BCL6 diminished transcriptional activation of p53 target genes, including that encoding caspase-1. Conversely, we also found that BCL6 protein is degraded via p53-induced, caspase-mediated proteolytic cleavage, and the formation of a BCL6–p53–caspase-1 complex. Our results suggest that p53 may block oncogenic transformation by decreasing BCL6 stability via caspase-1 up-regulation, whereas aberrant BCL6 expression inactivates transactivation of p53 target genes, either by inhibiting p53 acetylation by p300 or repressing TP53 gene transcription. These findings have implications for B cell development and lymphomagenesis.

Development of memory B lymphocytes in the germinal centers (GCs)3 of secondary lymph nodes is critical for humoral immunity (1, 2). GC B cell DNA undergoes double-strand breaks required for immunoglobulin (Ig) gene class-switch recombination and somatic hypermutation (3, 4). Despite these physiological genomic stresses (which accordingly induce p53 and p53-mediated apoptosis), BCL6 promotes proliferation of GC B cells by repressing genes involved in cell-cycle arrest and/or apoptosis (5). BCL6 also represses TP53 by binding to the promoter, allowing GC B cells to circumvent physiological DNA breakage or alteration. Both BCL6 and p53 are required for GC formation and the maturation of progenitors into stable memory B cells (6).

BCL6 expression is restricted to GC B cells in the B cell lineage and is tightly regulated at both the transcriptional and post-translational levels. BCL6 expression is strongly regulated by DNA damage–induced ATM kinase, which phosphorylates BCL6 such that it interacts with the isomerase Pin1 to be degraded by the ubiquitin-proteasome system (7). Overexpressed BCL6, during GC formation, represses BCL6 gene expression by NF-κB–mediated induction of IRF4 (8–10). Repression of BCL6 is necessary for terminal B cell differentiation. Moreover, transcriptional repressor activity of BCL6 is attenuated by acetylation of its PEST domain by p300, which hinders its complexation with corepressors (11). Moreover, BCL6 expression in diffused large B cell lymphoma (DLBCL) is derepressed by promoter substitution by chromosomal translocation or point mutations. Deregulated BCL6 expression and p53 mutations act in a synergistic manner in lymphomagenesis (12–15). Recently, we found that BCL6 interacts with p53 through each other’s respective DNA-binding domains.

The tumor suppressor p53 is known as the “guardian of the genome” (16) as attested by its mutation or deletion in nearly 50% of all human cancers (17). p53 expression is tightly regulated, and it is expressed at low levels during normal physiologic conditions (18). To that end, p53 regulates genes involved in DNA-binding domain; HAT, histone acetyltransferase; Luc, luciferase; p53RE, p53 response element; 2-VAD-fmk, N-benzoylcarbonyl-Val-Ala-Asp (O-Me) fluoromethyl ketone; Casp, caspase; POZ, poxvirus zinc finger; IRF, interferon regulatory factor; ATR, ataxia telangiectasia and Rad3-related; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RIPA, radiomune precipitation assay; qChIP, quantitative ChIP; Ni-NTA, nickel-nitrilotriacetic acid; ZF, zinc finger; PCAF, p300/CREB-associated factor.
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apoptosis, cell-cycle arrest, DNA repair, metabolism, and senescence (19, 20). Specifically, genotoxic stresses increase p53 protein stability and transcriptional activity of p53 through post-translational modifications (PTMs), including phosphorylation, ubiquitination, sumoylation, and acetylation (21), resulting in regulation of p53 protein stability, target gene promoter binding, and association with other proteins (22). Additionally, specific acetylation of p53 plays important roles in stressed cell fate decisions; i.e. specific p53 PTMs are required for transcriptional activation of a group of p53 target genes controlling cell-cycle arrest, apoptosis, senescence, differentiation, etc. (23, 24). However, the mechanism of p53 mediation of cell fate decision remains incompletely elucidated.

Often, the expression and activity of tumor suppressors are negatively regulated by oncogenes, or vice versa, as is the case for MDM2-p53 (25, 26). Moreover, one of the long-standing unanswered examples is BCL6–p53 due to the peculiarity of absolute negative regulation, i.e. near-complete disappearance of p53 or BCL6 by the presence of BCL6 or p53, respectively. This negative relationship occurs at multiple levels: transcriptional repression of TP53 by BCL6 (6), opposite regulation of p53 and BCL6 expression/activity by ATM-mediated phosphorylation and binding to Pin1 (7, 26, 27), and differential regulation of p53 and BCL6 activities by p300-mediated acetylation (11, 21). In that regard, BCL6 was shown to attenuate DNA damage responses by affecting p53 pathways, e.g. acting as an inhibitor of antiproliferative ARF–p53 signaling (where ARF stands for alternate reading frame protein) (30, 31).

Caspases are a family of cysteinyI aspartate–specific proteases that themselves are activated through (often self-) proteolysis of specific asparagine residues. Active caspases specifically cleave various proteins that are implicated in apoptosis and inflammation. During apoptosis, activated “initiator” caspases initiate proteolysis and activate “effector” caspases by cleavage (32, 33). On the contrary, the nonapoptotic caspases, inflammatory caspases-1, -4, -5, -11, -12, and -14, are activated by innate immune responses and inflammatory cytokines, such as interleukin-1β (IL1β) and IL18 (34).

Caspase-1, a well-characterized inflammatory caspase that activates pro-IL1β by proteolytic cleavage in macrophages, also activates the proinflammatory cytokine IL18 (also called interferon-γ-inducing factor) (35). Caspase-1 also induces apoptosis when overexpressed in fibroblasts (36), and interestingly, p53 was shown to increase caspase expression (caspases-1, -6, and -8) by both transcription-dependent and -independent mechanisms (37–39).

With these considerations, we were particularly intrigued by previous reports showing 1) transcriptional repression of TP53 by BCL6 and 2) immortalization of GC-like B cells in the absence of p53 and opposite regulation of p53 and BCL6 expression/activity by ATM-mediated phosphorylation, Pin1, and p300-mediated acetylation. These reports suggest significant reciprocal negative regulation of expression or activities of BCL6 and p53 in germinal cancer B cells.

In this study, we found that BCL6 interacts with p53 and represses expression of p53 target genes that regulate the cell cycle while also modulating acetylation of p53 by p300 at lysine 132. In addition, p53 decreases BCL6 expression by molecular interactions involving p53–caspase-1–BCL6 complex formation.

Results

BCL6 represses p53 target genes

We first investigated whether ectopic BCL6 could repress TP53, the transcriptional coactivator p300, and p53 target genes regulating cell cycle and apoptosis. We found that mRNA and protein expression of p21/CDKN1A, MDM2, p53, and p300 was repressed potently by BCL6 in HEK293 cells (Fig. 1A). Despite this strong transcriptional repression of TP53 by BCL6, interestingly, in B cell–derived lymphoma DLBCL, including Ramos Burkitt’s lymphoma cells, BCL6 and p53 were expressed at both the mRNA and protein levels (Fig. 1B), suggesting regulatory mechanisms blocking p53 activity important for cell cycle arrest and/or apoptosis. Moreover, reverse transcription and quantitative real-time PCR (RT-qPCR) and Western blotting assays showed that p53-mediated activation of its target genes, including p21/CDKN1A, DR5, and NOXA, might be repressed by ectopic BCL6 in p53- and BCL6-null H1299 lung cancer cells (Fig. 1C and Fig. S1).

We further analyzed the functional outcome of counter-regulation of BCL6 and p53 by MTT assays. H1299 cells were transfected with p53 and/or BCL6 expression vectors, and cell proliferation was analyzed by MTT assays. The assays showed that although ectopic BCL6 expression increased cell proliferation, p53 significantly decreased cell proliferation. These results suggest that BCL6 might inhibit p53 activity important for transcriptional activation of p53 target genes, thereby blocking apoptosis and cell-cycle arrest (Fig. 1D).

BCL6 interacts with p53 and p300

We next analyzed molecular interactions between p53 and BCL6 by co-immunoprecipitation (co-IP)/Western blotting assays, finding that BCL6 interacts with p53 in HEK293 kidney and Ramos lymphoma cells. Glutathione S-transferase (GST) fusion protein pulldown assays further validated that result, showing that the two proteins interact directly via each other’s DNA-binding domains (DBDs) (Fig. 2, A–C).

Among several histone acetyltransferase (HAT) proteins that interact with and acetylate p53, p300 plays critical roles in p53-mediated DNA damage responses, such as cell-cycle arrest and apoptosis (Ref. 24 and references therein). Because BCL6 interacts with p53 and may modulate p53 activity important in DNA damage responses, we tested whether BCL6 physically interacts with p53 and p300 (or other HAT proteins) in Ramos cells exposed to the DNA-damaging agent etoposide. Co-IP and Western blotting assays showed that interaction between BCL6 and p300 was increased after etoposide treatment (Fig. 2D, left). IP with anti-p53 antibody also showed that interaction with BCL6 and p300 was increased by etoposide treatment. Although BCL6 bands appear to be similar, considering significantly lower expression by etoposide treatment, interaction among p53, p300, and BCL6 was increased (Fig. 2D, middle). In the same context, IP with anti-p300 antibody also suggested an increase in interaction between p300 and BCL6 by etoposide treatment (Fig. 2D, right). In contrast, BCL6 did not interact with the HAT Tip60 nor did PCAF (Fig. 2D).
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**BCL6 modulates p53 acetylation by p300 and decreases acetylation of p53 Lys-132**

Because BCL6 interacted with p53 and p300, we next questioned whether BCL6 might affect p300 acetylation of p53 and, if so, by what mechanism. Liquid chromatography-tandem MS (LC-MS/MS) analysis of an in vitro p53 acetylation reaction mixture revealed that BCL6 inhibited p300 acetylation of p53 Lys-132 (Fig. 3, A and B, and Fig. S2). This specific residue was previously characterized as a target site for ubiquitination (40) and possibly acetylation. We further tested whether inhibition of acetylation at p53 Lys-132 significantly affected transcription of a p53-responsive pG13-Luc reporter plasmid. Transient transfection assays revealed that potent transcriptional activation of pG13-Luc by p53 was repressed by coexpressed BCL6. p53K132R, a nonacetylatable p53 mutant, lacked the ability to transactivate the p53-responsive pG13-Luc reporter plasmid. BCL6 increased cell proliferation, and coexpression of p53 significantly attenuated cell proliferation. Coexpression of BCL6, p53K132Q, and A-485 showed potent inhibition of cell proliferation, and the cell growth–inhibitory activity of p53K132Q was not affected by the p300 inhibitor. Thus, p53K132Q is fully functional, strongly supporting p53 Lys-132 as the target of BCL6’s anti-p53 action.

RT-qPCR and Western blotting assays of spleen tissues of WT and Bcl6 knockout mice using an anti-acetylated p53 Lys-132 specific antibody revealed that BCL6 decreased acetylation of p53 at Lys-132, resulting in silencing of p21/Cdkn1a, Noxa, and Dr5 in vivo (Fig. 3F). These results suggest that inhibition of acetylation of p53 Lys-132 is important and sufficient for BCL6-mediated inactivation of p53 activity.

**p53 down-regulates BCL6 expression via caspase**

To study the correlation between BCL6 and p53 expression, H1299, HCT116 p53-null, and HEK293 cells were cotransfected with both p53 and BCL6 expression vectors. BCL6 protein expression was decreased by ectopic p53 (Fig. 4, A–C). Also, ectopic expression of p53 negatively correlated with BCL6 in Ramos lymphoma cells transfected with a p53 expression vector. However, BCL6 transcription was not affected by p53

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**Figure 1.** BCL6 represses p53 transcriptional activation of cell-cycle genes.  A, RT-qPCR and Western blot (W.B.) analyses of endogenous p53 target gene expression. HEK293 cells were transfected with pcDNA3.1-BCL6 and cultured for 48 h. GAPDH was used as a normalization control. *, *p < 0.05. B, RT-qPCR and Western blot analyses of endogenous BCL6 and p53 expression in HEK293 (1), Ramos (2), Toledo (3), Farage (4), and SU-DHL-6 (5) cells. GAPDH was used as a control. *, *p < 0.05. C, RT-qPCR and Western blot analyses of endogenous p53 target gene expression. H1299 cells were transfected with expression vectors pcDNA3.1-BCL6 and/or pcDNA3.1-p53 and cultured for 48 h. GAPDH was used as a control. *, *p < 0.05. D, MTT assay and cell growth curve of H1299 cells. Cells were cotransfected with the indicated expression vectors, and cell proliferation rates were measured. Shown is the mean value of three independent MTT assays. Error bars, S.D. *, *p < 0.05. Con, control.
Not only did BCL6 decrease p53 expression, but p53 reciprocally down-regulated BCL6 expression. Based on these findings, we investigated whether “hot spot” p53 mutations could also affect BCL6 expression. These results showed that, depending on the types of mutations (17), BCL6 expression was either decreased or unaffected (Fig. 4E). These data indicate that BCL6 and p53 negatively regulate each other’s expression or activity, thus establishing their antagonistic coexistence in B cell-derived lymphoma cells and possibly in other cell types expressing aberrant BCL6 and p53, such as those we tested, H1299, HCT116 p53-null, and HEK293 cells.

To investigate how p53 regulates BCL6 expression, H1299 cells were transfected with p53 expression vectors and analyzed for protein expression by Western blotting. BCL6 protein was significantly lower in the presence of p53 and may be degraded more rapidly (Fig. 5A). Treatment with MG132, a proteasome inhibitor, did not restore BCL6 protein expression, suggesting that p53-mediated BCL6 down-regulation was
probably not via ubiquitin-mediated proteolysis (Fig. 5B). However, treatment of the cells with the pan-caspase inhibitor Z-VAD-fmk prevented BCL6 degradation (Fig. 5C), endogenous BCL6 expression was also down-regulated by etoposide-induced p53, and that down-regulation was also inhibited by Z-VAD-fmk (Fig. 5D). These results suggest that p53 may
decrease BCL6 expression via caspase activity. Accordingly, the genetic status (WT or mutated) of p53 may affect expression of BCL6 and would therefore be likely to affect B cell proliferation and oncogenesis.

*p53 increases proteolytic cleavage of BCL6 by activating caspase-1 expression*

We next investigated which caspases cleave overexpressed BCL6 in H1299 cells or recombinant BCL6. In *vitro* caspase assays, performed using active recombinant caspases-1–10 and BCL6, showed that BCL6 was cleaved by caspases-1, -4, -5, -6, -8, and -10 (Fig. 6A). Moreover, Western blot analysis of reaction mixtures of H1299 cell lysates with ectopic BCL6 and recombinant caspases-1–10 showed that BCL6 was strongly cleaved by caspases-1, -4, -5, -8, and -10 but only weakly by caspases-2, -6, and -9 (Fig. 6B).

Among the 10 caspases tested, we assessed whether BCL6 was efficiently cleaved by caspase-1 *in vitro*. p53 potently decreased BCL6 expression, probably by endogenous caspase(s). In H1299 cells transfected with BCL6 and p53 expression vectors, knockdown of caspase-1 mRNA by siRNA rescued BCL6 from degradation (Fig. 6C). Additional Western blot analysis further showed BCL6 down-regulation by both endogenous and ectopic caspase-1, and its expression was restored by the pan-caspase inhibitor Z-VAD-fmk. Moreover, C284A, a catalytically inactive form of caspase-1, could not decrease BCL6 expression, suggesting that proteolytic cleavage of BCL6 is mainly mediated by caspase-1 (Fig. 6D).

Because caspase-1 was required for BCL6 proteolytic cleavage by ectopic p53, we investigated whether p53, BCL6, and caspase-1 might interact to form a complex using co-IP. Western blotting of the immunoprecipitates of the lysates of H1299 cells (pretransfected with BCL6 and p53 expression vectors) using anti-BCL6 antibody showed that, in the absence of Z-VAD-fmk, little p53 or caspase-1 was detected. However, following Z-VAD-fmk treatment, p53 and caspase-1 proteins were both detected in a protein complex containing BCL6, suggesting that the three proteins can form a protein complex (Fig. 6E).

One possible mechanism of p53-mediated degradation of BCL6 might be via transcriptional activation of caspase-1.
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Figure 5. p53 decreases BCL6 expression, which is reversed by the pan-caspase inhibitor Z-VA-D-FMK. A, Western blot (WB) analysis of p53 and BCL6 expression in the cell lysates prepared from H1299 cells transfected with pcDNA3.1-p53 and/or pcDNA3.1-BCL6. GAPDH was used as a control. B, Western blot analysis of p53 and BCL6 expression in H1299 cells cotransfected with the expression vectors indicated. Cells were treated with MG132 for 0.5–6 h. GAPDH was used as a loading control. C, Western blot analysis of p53 and BCL6 expression in the two cell types, suggesting this protease to be functionally significant in p53-mediated BCL6 degradation in lymphoma cells. Interestingly, etoposide increased only caspase-1, -4, -5, -11, -12, and -14 are activated by innate immune responses and inflammatory cyto-
kines (34). Because BCL6 was down-regulated by p53 and caspase-1, we investigated whether etoposide DNA damage could increase caspase-1 expression in Ramos and Farage lymphoma cells. Interestingly, etoposide increased only caspase-1 expression in the two cell types, suggesting this protease to be functionally significant in p53-mediated BCL6 degradation in lymphoma cells (Fig. 6, G and H).

GST fusion protein pulldown assays of protein interaction between BCL6 and caspase-1 and that of p53 and caspase-1 showed that the poxvirus zinc finger (POZ) domain of BCL6 and the DBD and C terminus of p53 appear to be important in the interaction with caspase-1 in vitro (Fig. 7, B and C). We further investigated the interactions among BCL6, p53, and caspase-1. Intriguingly, protein interaction between BCL6 and either caspase-1 or caspase-1 p20 (active form) was enhanced by the addition of p53 to the reaction mixture. p53 increases caspase-1 expression and facilitates the interaction between BCL6 and caspase-1. Furthermore, p53 also augmented interactions of BCL6 with caspase-4 or -5 but not with caspase-3, indicating some degree of caspase specificity in p53-mediated BCL6 degradation (Fig. 7D).

Our results demonstrate that BCL6 and p53 physically interact via each other’s DNA-binding domains. To that end, BCL6 could repress the transcriptional activation of p53 target genes controlling the cell cycle or apoptosis by decreasing p300-mediated acetylation of p53 Lys-132 following DNA damage. Conversely, p53 decreases BCL6 expression by up-regulating caspase-1 and enhancing the molecular interactions among p53, BCL6, and caspase-1. Thus, our finding of a negative regulatory mechanism for BCL6 and p53 expression or activity provides an interesting example of how tumor suppressors and oncoproteins regulate one another.

Discussion

A variety of cellular stresses, such as DNA damage, oncogene activation, and hypoxia, promote p53 expression and its PTM to influence cell fates, such as cell-cycle arrest, apoptosis, and senescence (18, 19). However, in germinal center B cells, which undergo physiological DNA breaks required for Ig gene class-switch recombination and somatic hypermutation (1–4), BCL6 represses transcription of TPS3 and inhibits DNA damage-induced apoptosis (6). Although physiological DNA damage in GC-B cells likely induces p53 expression and subsequent PTM of p53, the mechanistic details of p53 inactivation have long remained unknown. Also, p53 mRNA and protein expression is still detected in B cell–derived lymphoma, although functional inactivation of p53 by deregulated BCL6 likely contributes to lymphomagenesis.

Here, we investigated a possible mechanism for how aberrantly expressed BCL6 inactivates p53 function (Fig. 8). BCL6 modulates acetylation of p53 by p300 and decreases p53 acetylation at Lys-132, attenuating p53 transactivation of its target genes involved in cell-cycle arrest and/or apoptosis and instead stimulating cell proliferation.

Another interesting finding of our study is that p53 can decrease BCL6 protein expression via caspase-1. As a target gene of p53 and p63 (38, 40), caspase-1 induces caspase-1–dependent programmed cell death, and dysregulation of caspase-1 can potentially affect tumorigenesis as indicated by frequent loss of caspase-1 protein expression in cancer (28, 29).

For proliferating normal B cells to differentiate, they need to repress BCL6 protein expression at both the transcriptional and protein levels. CD40 represses BCL6 genes via the transcriptional repressor IRF (8). BCL6 also can repress its own gene expression by a negative feedback loop (10). Also, phosphorylation of BCL6 by mitogen-activated protein kinase and DNA damage–dependent ATR kinase leads to ubiquitination and proteasomal degradation (19). Moreover, BCL6 physical interaction with corepressors and its transcriptional repressor activity can be inhibited via its acetylation by p300. Herein, we found a novel mechanism of down-regulating BCL6 expression by proteolytic cleavage via p53 and caspase-1 (Fig. 8). Our results imply that p53 can block B cell proliferation and induce cell-cycle arrest or apoptosis by decreasing BCL6 expression.

Negative regulation of BCL6 expression by p53 depends on p53 genotypes. For example, WT p53 and the hot spot p53 mutant p53R273H decreased BCL6 expression potently. How-
ever other hot spot p53 mutants (p53R175H, p53G245S, and p53R248S) did not decrease BCL6 expression (Fig. 4E). Depending on the location of the p53 mutation site, it may be unable to activate CASP1 transcription, or it may not be able to interact with the caspase-1 protein.

In short, we were able to resolve not only the longstanding elusive oncogenic properties of BCL6 as opposed by p53 activity (30, 31) but also identified new functions of caspase-1 in BCL6 proteolytic degradation. Our findings also provide insight into how BCL6 promotes GC B cell proliferation despite significant

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**Figure 6. Caspase-1 is a critical player in BCL6 cleavage by p53.**

A, in vitro caspase assays and Western blot (WB) analysis of BCL6. Recombinant BCL6 was incubated with mock or caspases-1–10 at 37 °C for 2 h. Full-length BCL6 is indicated by arrows. Cleaved BCL6 was detected by Western blot analysis using an anti-BCL6 antibody. B, in vitro caspase assay and Western blot analysis of BCL6. H1299 cells were transfected with pcDNA3.1-BCL6 and lysed, and cell lysates (50 μg) were incubated with caspases-1–10. GAPDH and β-actin were used as loading controls. C, Western blot analysis of BCL6, p53, and GAPDH expression and RT-qPCR analysis of CASP1 mRNA expression. H1299 cells were cotransfected with the p53 and/or BCL6 expression or siRNA vectors indicated. GAPDH was used as a control. D, Western blot analysis of BCL6 and GAPDH from H1299 cells transfected with various combinations of pcDNA3.1-BCL6, pcDNA3.1-caspase-1, and pcDNA3.1-caspase-1 C284A expression vectors and further treated with Z-VAD-fmk. GAPDH was used as control. Caspase-1 C284A is an enzymatically inactive caspase-1 mutant. E, p53 increases transcription of endogenous caspase genes and enhances the interaction between BCL6 and caspase-1. Western blot analysis of the protein complex immunoprecipitated by anti-BCL6 antibody is shown. Z-VAD-fmk treatment greatly stabilized the p53–BCL6–caspase-1 protein complex. F, H1299 cells transfected with pcDNA3.1-BCL6 and/or pcDNA3.1-p53 were treated with Z-VAD-fmk. p53 vastly increased protein complex formation once caspase-1 was inhibited. G, RT-qPCR analysis of caspase-1 mRNA by ectopic p53 and p53 induced by the DNA-damaging agent etoposide. H1299 cells were transfected with a pcDNA3.1-p53 expression vector and analyzed by RT-qPCR for caspase-1, -4, and -5 mRNA expression. GAPDH was used as a normalization control. For RT-qPCR analysis of endogenous caspase-1, -4, and -5 mRNA expression, Ramos or Farage cells were treated with 50 μM etoposide for 6 h before harvest and analysis for caspase mRNA expression. Ectopic p53 could activate transcription of CASP1, CASP4, and CASP5 genes; however, p53 induced by etoposide treatment could only activate caspase-1. Error bars, S.D. Con, control.
physiological DNA breakage, p53 expression, and B cell exit into mature memory/plasma cells. BCL6 negative regulation of p53 expression via caspase activity may not be limited to B cells but may also occur in other cell types as we demonstrated in HEK293, H1299, and HCT116 cells. Our study also shows how p53 utilizes caspase-1 to suppress oncogenic BCL6 activation and may provide insight into the action of antineoplastics and dysregulation of apoptosis.

Experimental procedures

**Bcl6**−/− knockout mice

Animal experiments were approved by the Committee on Animal Investigations of Yonsei University. Dr. Masahiko Hatano of the Chiba University and Center for Animal Resource and Development (CARD) of Kumamoto University provided Bcl6−/− knockout C57BL/6J mouse populations.

**Plasmids and recombinant proteins**

The BCL6 ORF was amplified from a human cDNA library by PCR and cloned into pcDNA3.1, which has six copies of His epitopes. pG13-Luc, pGL2-(p53RE)5x-Luc, pcDNA3.1-LacZ, and pcDNA3.1-p53 were used for transcriptional analysis. Generation of GST-p53-N terminus, GST-p53-DBD, and GST-p53-C terminus has been reported elsewhere (1, 2). To prepare recombinant GST-POZ-BCL6, GST-M-BCL6, and GST-zinc finger (ZF)-BCL6 proteins, cDNA fragments encoding the POZ domain (amino acids 1–99), the middle domain (amino acids 99–518), zinc fingers (amino acids 518–681), deleted zinc fingers (amino acids 1–518), or POZ-deleted constructs (amino acids 132–681) were cloned into pGEX4T1. The recombinant GST fusion proteins were expressed in *Escherichia coli* DH5α cells. Iso-propyl 1-thio-β-D-galactopyranoside was added to a final concentration of 0.2 mM for protein induction. The active form of caspase-1 (p20 large subunit) and full-length caspases-1, -4, and -5 were amplified using PCR and cloned into pcDNA3.1. The recombinant full-length p53 and BCL6 were cloned into pET-21a, and proteins were expressed in *E. coli* BL21 (DE3) at 18 °C and purified by Ni-NTA–agarose (Qiagen). Full-length, recombinant p300 was purchased from Protein One. All plasmid constructs were verified by DNA sequencing.

**Antibodies and reagents**

Antibodies against BCL6 (D-8, sc-7388; N-3, sc-858), p53 (FL-393, sc-6243; DO1, sc-126), glyceraldehyde-3-phosphate-de-
hydrogenase (GAPDH) (FL-335, sc-25778), MDM2 (SMP14, sc-965), p21 (C-19, sc-397), DR5 (D-6, sc-166624), and NOXA (FL-54, sc-30209) were purchased from Santa Cruz Biotechnology. Antibodies against His, p300, and acetyl-lysine were purchased either from R&D Systems, Fisher Scientific, Epitomics, or Millipore. A specific human caspase-1 antibody (ab108362) was purchased from Abcam. A rabbit polyclonal antibody against acetylated p53 Lys-132 was prepared by us. The pan-caspase inhibitor Z-VAD-fmk was purchased from Promega, and the proteasome inhibitor MG132 was purchased from Calbiochem. Most of the chemical reagents were purchased from Sigma. Antibodies for flow cytometry were obtained from Vector Labs (peanut agglutinin-, FITC-conjugated) and BD Biosciences (CD45/B220, peridinin–chlorophyll–protein complex–conjugated).

Site-directed mutagenesis

Distinct p53 point mutants were constructed by site-directed mutagenesis using pcDNA3.1-p53 and pGEX4T-1-p53. The oligonucleotide primers described below were used for generating the mutant proteins p53K132R, p53R175H, p53G245S, p53R248W, and p53R273H. PCR conditions were as follows: predenaturation at 95 °C for 5 min followed by 18 cycles of 95 °C for 30 s, 55 °C for 1 min, and a final elongation step at 68 °C for 10 min. Oligonucleotide sequences used were:

- **p53K132R**: sense, 5'-CTCCCCCTGCCCTCAACAGATGTTTTGCCAACTGG-3'; antisense, 5'-CCACCTTGCGGAAAACATCCCGTGAGGCGGAGGAG-3'; p53R175H: sense, 5'-GACCGGAGTTGTAGGCACACTGCCCACCACCATGAGC-3'; antisense, 5'-GCTCATGTTGGGGCGAGTGCCTCACAACCTCGCC-3'; p53G245S: sense, 5'-CGTCCCTGACATGGGGCAGGAGCCCA-3'; antisense, 5'-TGTTGAGGATTGCCCTCCAGTTCATGCCGCCCATG-3'; p53R248W: sense, 5'-GAACAGCTTTGAGGTGACCTTGCCCTGCTCTG-3'; antisense, 5'-CAGGACAGGCGACAAACATGCACCTC-AAAGCTGATTC-3'.

Cell culture

HEK293A and HCT116 p53−/− cells were cultured in Dulbecco’s modified Eagle’s medium, whereas H1299, Ramos, Farage, Toledo, and SU-DHL-6 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin. The cells were maintained in a 5% CO2 incubator at 37 ºC. The diffuse large B cell lymphoma cell lines, Toledo, Farage, and SU-SHDL-6, were obtained from American Type Culture Collection.

MTT assays

Confluent H1299 cells grown in 10-cm culture dishes were transfected with pcDNA3.1-BCL6, and/or pcDNA3.1-p53, and/or pcDNA3.1-p53K132R, or pcDNA3.1-p53K132Q. 5 × 10⁵ cells were seeded in 96-well plates, cultured, and grown for 1–5 days. A-485 was added at 10 μM. For measuring cell proliferation, cells were incubated with 2 mg/ml MTT (Sigma) for 2–4 h at 37 °C. The supernatants were then removed, and 100 μl of DMSO (Duchefa) was added to each well. Cellular proliferation was determined by the conversion of MTT to formazan using a SpectraMAX 250 ELISA reader (Molecular Devices) set to 570 nm. Data were expressed as means ± S.D. for quadrupli-
cante measurements, and similar results were obtained from three independent experiments.

**Transient transcription analysis**

Various combinations of pG13-Luc, pGL2–5x(p53RE)-Luc, pcDNA3.1-BCL6, pcDNA3.1-p53, pcDNA3.1-p53K132R, or control vector were transiently transinfected into H1299 cells using Lipofectamine Plus reagent (Invitrogen). After transfection, cells were harvested, total RNA was extracted, and RT-PCR analysis was performed as described below. A β-gal reporter was cotransfected for normalization of transfection efficiency. Luciferase activity was measured using a SpectraMax 250 ELISA reader at 420 nm.

**Knockdown of caspase expression by siRNA**

siRNA (siCaspase-1 RNA: sense, 5′-GGUUCCGAAUUUCAUUUGAG(dTdT)-3′; antisense, 5′-CUCAAAUGAAAAUCGG-AAACC(dTdT)-3′) against caspase-1 mRNA was synthesized in duplex and purchased from Bioneer. 100 pmol of siRNA was transfected into H1299 cells using Lipofectamine iMax (Invitrogen). After transfection, cells were harvested, total RNA was extracted, and RT-PCR analysis was performed as described below.

**RT-qPCR**

Total RNA was isolated from cells using TRIzol reagent (Invitrogen). cDNAs were synthesized using 5 µg of total RNA, 10 pmol of random hexamers, and 200 units/µl Moloney murine leukemia virus reverse transcriptase in a total volume of 20 µl using a reverse transcription kit (Promega). RT-qPCR was performed using SYBR Green Master Mix (Applied Biosystems). Oligonucleotide primer sets used for RT-qPCR are listed in Tables 1 and 2. All reactions were performed in triplicate.

**Flow cytometry of splenic GC B cells**

Mice were housed in a dedicated pathogen-free environment. Three-month-old mice were immunized by intraperitoneal injection of 2% sheep red blood cells and euthanized 14 days postimmunization according to our approved protocol. Spleens were isolated and processed for flow cytometry. Splenic mononuclear cells were isolated by straining the tissue through 40-µm cell strainers in 1× PBS + 0.5% BSA followed by red blood cell lysis. Mononuclear cell suspensions were stained for antibodies and analyzed by an LSR II instrument (BD Biosciences). 10,000 events were collected per sample and analyzed with FlowJo software (FlowJo LLC).

**Western blotting**

Cells were harvested and lysed in RIPA buffer (50 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, 0.25% sodium deoxycholic acid, 150 mM NaCl, 1 mM EDTA, and Complete Mini protease mixture). Cell lysates (50 µg) were separated by 10% SDS-PAGE, transferred onto Immobilon polyvinylidene difluoride membranes (Bio-Rad), and blocked with 5% skim milk (BD Biosciences). Blotted membranes were incubated with antibodies against His tag (R&D Systems), BCL6, GAPDH, and p53 (Santa Cruz Biotechnology); washed; and then incubated with anti-mouse or anti-rabbit secondary antibodies conjugated to horseradish peroxidase (Vector Labs). Protein bands were visualized with enhanced chemiluminescence solution (PerkinElmer Life Sciences).

**ChIP-qPCR**

Molecular interactions among BCL6, p53, and p300 on p53 target promoters in H1299 cells were analyzed by following a standard ChIP assay protocol as described elsewhere (1, 2). RT-qPCR of chromatin-immunoprecipitated DNA was carried out using the following oligonucleotide primer sets designed to amplify the upstream regulatory regions around p53-binding sites of p53 target genes: CDKN1A-forward, 5′-CTGGTGCCCTCTGGATGCCTTT-3′; CDKN1A-reverse, 5′-GGGTCTTCTTAGAGTCTCCTGTC-3′; NOXA-forward, 5′-TGCCCATACCTTCTCAAGTTAG-3′; NOXA-reverse, 5′-AGGGTATTTGCGCAGACGC-3′; DR5-forward, 5′-AGAAGCCTTGTGCTCGTTGT-3′; DR5-reverse, 5′-CGGGAATTTACACCAAGTGG-3′.

**GST fusion protein purification, in vitro transcription and translation, and GST fusion protein pulldown assays**

Recombinant GST, GST-POZ-BCL6, GST-ZF-BCL6, GST-POZ deletion, and GST-zinc finger deletion fusion proteins were prepared in E. coli BL21 (DE3) cells grown for 4 h at 37 °C in LB broth containing 1 mM isopropyl 1-thio-β-d-galactopyranoside and lysed, and proteins were purified using Glutathione-Agarose 4 bead affinity chromatography (Peptron, Taejeon, Korea). The polypeptides of p53 and caspases-1, -4, and -5 were prepared by incubating 2 µg of either pcDNA3.1-p53, pcDNA3.1-p53-N terminus, pcDNA3.1-p53-DBD, or pcDNA3.1-p53-C terminus with TnT Quick-coupled Transcription/Translation Extract (Promega) containing 40 µl of TnT Quick Master Mix and 2 µl of [35S]methionine (1175.0 Ci/mol; PerkinElmer Life Sciences) at 30 °C for 90 min. Polypeptide expression levels were then analyzed by resolving 1 µl of the total mixture by 15% SDS-PAGE followed by autoradiography. To perform the GST fusion protein pulldown assays, GST fusion protein–agarose bead complexes were incubated with [35S]methionine-labeled p53 or p300 polypeptides at 4 °C for 4 h in HEMG buffer (25 mM HEPES, pH 7.9, 0.1 mM EDTA, 12.5 mM MgCl2, and 20% (v/v) glycerol). The reaction mixtures were centrifuged, pellets were rinsed, and the bound proteins were separated by 15% SDS-PAGE. Gels were then exposed to X-ray film using an image-intensifying screen (Eastman Kodak Co.).

**In vitro acetylation assays of p53 by p300**

Recombinant His-tagged full-length BCL6 (amino acids 1–681) and His-tagged full-length p53 (amino acids 1–393) proteins were expressed in E. coli DH5α cells and purified by Ni-NTA affinity chromatography. Full-length p300 was purchased from Protein One. Recombinant p53 protein (6 µg) and acetyl-CoA (0.83 mM) were incubated with p300 (200 ng) or p53 + p300 + BCL6 (6 µg) in lx HAT assay buffer (50 mM Tris-HCl, pH 8.0, 10% glycerol, 0.1 mM EDTA, and 1 mM DTT) (Fisher Scientific) at 37 °C for 1 h. Aliquots (10%) of the reaction mixtures were resolved by SDS-PAGE and analyzed by Western blotting using an anti-acetyl-lysine antibody to evaluate p53
# Table 1
Oligonucleotide primer sets used for RT-qPCR of mRNA

| Species     | Oligonucleotides       | Sequence               |
|-------------|------------------------|------------------------|
| **BCL6**    | Sense                  | 5’-AATGAGTGCTGACTGACGGCTTTCT-3’ |
|             | Antisense              | 5’-CACCAGTGATGGGCTTTTCTCTG-3’ |
| **p53**     | Sense                  | 5’-CTCTAAGTGTTGCTAGCAGAATCT-3’ |
|             | Antisense              | 5’-AACTGCTGAGTGTTGCTGCTGTAG-3’ |
| **MDM2**    | Sense                  | 5’-CTTTAAGGCTTAGCTTACCT-3’ |
|             | Antisense              | 5’-ACTGGCCAGGCTTTATTTCCT-3’ |
| **p21/CDKN1A** | Sense            | 5’-AGGGGACAGGCAGGAGGAG-3’ |
|             | Antisense              | 5’-GCTTTGAGTGATGGAAATCTG-3’ |
| **PUMA**    | Sense                  | 5’-CCCTGGTACCGCAGAAGG-3’ |
|             | Antisense              | 5’-GGAGGCTGCTGTTGACAG-3’ |
| **NOXA**    | Sense                  | 5’-ACCGCCAGTACGCGAGAAG-3’ |
|             | Antisense              | 5’-CAGGAGTCCATGAGAGGCTG-3’ |
| **DR5**     | Sense                  | 5’-CACCGTGGAGTACAGAAG-3’ |
|             | Antisense              | 5’-GCTTTGGACTGCTGTTG-3’ |
| **PERP**    | Sense                  | 5’-TGGGCTCATGGCTTGG-3’ |
|             | Antisense              | 5’-GGGAGCAGAAGAGAGGATG-3’ |
| **BID**     | Sense                  | 5’-TGGGAAGCTATACGCGTCAAG-3’ |
|             | Antisense              | 5’-GGAAGGCAACACCAGTT-3’ |
| **SURVIVIN**| Sense                  | 5’-ATCCACTGCCCCACTGAA-3’ |
|             | Antisense              | 5’-GCTCTTGAACGAAAGAACCATT-3’ |
| **GADD45A** | Sense                  | 5’-CCCCCGATAAGTGTTGGT-3’ |
|             | Antisense              | 5’-GCAGCAAACCGCCTGGA-3’ |
| **IGFBP3**  | Sense                  | 5’-GACAGCGAGCTCACAAGATTG-3’ |
|             | Antisense              | 5’-TACGCGAGGGGACATATCTC-3’ |
| **XPC**     | Sense                  | 5’-GTACGACCAAGCTGGCTAGTA-3’ |
|             | Antisense              | 5’-CTGGTCATAAATGGGCTTGTG-3’ |
| **14-3-3σ** | Sense                  | 5’-GCCATGGAGATACAGCACAAG-3’ |
|             | Antisense              | 5’-GGCTGTTGGGCGATCTGTA-3’ |
| **TSP-1**   | Sense                  | 5’-GCCACGCGCAAACACCAGG-3’ |
|             | Antisense              | 5’-TATAGTGCCAGTCAAGTGA-3’ |
| **MMP-2**   | Sense                  | 5’-CTGGCGTTTTCTCGAAATCCA-3’ |
|             | Antisense              | 5’-GTCGCGTCTTACCGTCAAAGG-3’ |
| **PTEN**    | Sense                  | 5’-GTGGGTCTGCGACCAAGATG-3’ |
|             | Antisense              | 5’-AGGTAACGCGTGAGGGAACCT-3’ |
| **MASPIN**  | Sense                  | 5’-CCAGGGCTTTTCTGGGAAATCT-3’ |
|             | Antisense              | 5’-TGGAATTCCCACCACCATCC-3’ |
| **p53R2**   | Sense                  | 5’-GATTTTCTTCACGAGAAAG-3’ |
|             | Antisense              | 5’-GAAACAGCGAGCTGTTGGG-3’ |
| **BAL-1**   | Sense                  | 5’-GGCAGAGCGAGATGTAAATG-3’ |
|             | Antisense              | 5’-CCTGCGTTTGTGAAGATTGACCTT-3’ |
| **TIGAR**   | Sense                  | 5’-AGACCGCTGACAGGAAACCC-3’ |
|             | Antisense              | 5’-TGTTCTTTTGTACGCCCTTTC-3’ |
| **SESTRIN1**| Sense                  | 5’-AGGTTAGGGCCCTCATGAAA-3’ |
|             | Antisense              | 5’-AAACGTGAAAGCAATCTCCTCTC-3’ |
| **ALDH4**   | Sense                  | 5’-CGACACCCTGCAAGGATTCTT-3’ |
|             | Antisense              | 5’-AAGTACGACCGAGAGCTCACA-3’ |
| **PIG3**    | Sense                  | 5’-TGGGCGCTTGTGGTCAAAGG-3’ |
|             | Antisense              | 5’-AGCATTTACAGCAGCTGTTG-3’ |
| **PGMI**    | Sense                  | 5’-GGGATAGCTCGTTGTGTAT-3’ |
|             | Antisense              | 5’-TCGAGATGATCCACCAATGG-3’ |
| **DRAM1**   | Sense                  | 5’-GTGCGAGCAGCCCTTCATTA-3’ |
|             | Antisense              | 5’-TGGAGGTTGGTGTTCCCGTATC-3’ |
| **GAPDH**   | Sense                  | 5’-ACCACAGTCATCGCCATCAC-3’ |
|             | Antisense              | 5’-TCCACCACTTCCTGGCTGTA-3’ |
acetylation. The remainder of the reaction mixtures was analyzed by LC-MS/MS.

**LC-MS/MS analysis of p53 post-translational modifications**

Peptides were analyzed by LC-MS/MS using a Nano Acquity UPLC system combined with an LTQ Orbitrap Elite mass spectrometer equipped with a nanoelectrospray device. An autosampler was used to load 4-μl aliquots of the peptide solutions onto a C18 trap column (inner diameter, 300 μm; length, 5 mm; particle size, 5 μm; Waters). The peptides were desalted and concentrated on the column at a flow rate of 5 μl/min. Then the trapped peptides were back-flushed and separated through a 150-mm homemade microcapillary column consisting of C18 (Aqua; 3-μm particle size) packed with 100-μm silica tubing with an orifice inner diameter of 6 μm.

Mobile phases A and B were composed of 0 and 100% acetonitrile, respectively, and each contained 0.1% formic acid. The LC gradient began with 5% B for 5 min; ramped to 15% B over 5 min, to 50% B over 55 min, and to 95% B over 5 min; and remained at 95% B over 5 min followed by 5% B for another 5 min. The column was re-equilibrated with 5% B for 15 min before the next run. The voltage applied to produce an electrospray was 2.2 kV. In each duty cycle of mass analysis, one high-mass resolution (60,000) MS spectrum was acquired using the Orbitrap analyzer (Thermo Fisher Scientific) followed by 10 data-dependent MS/MS scans using the linear ion trap analyzer. For MS/MS analysis, normalized collision energy (35%) was used throughout the collision-induced dissociation phase. All MS and MS/MS spectra were acquired using the following parameters: no sheath and auxiliary gas flow; ion-transfer tube temperature, 200 °C; ion selection threshold, 1,000 counts; activation Q, 0.25; activation time, 20 ms. Dynamic exclusion was used with a repeat count of 1, a repeat duration of 30 s, an exclusion list size of 500, an exclusion duration of 60 s, and an exclusion mass width of ±1.5 m/z.

**Analysis of MS data for acetylation of full-length p53**

MS/MS spectra were analyzed using the following software analysis protocol and the UniProt human database (41). The reversed sequences of all proteins were appended into the database for calculation of the false discovery rate. Proteome Discoverer software version 1.3 (Thermo Fisher) was used to identify the peptides using a precursor mass error of 25 ppm and a fragment ion mass error of 600 ppm. Trypsin was selected as a protease with three potential missed cleavages. Carbamidomethylation at cysteine was chosen as a static modification. Oxida-
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tion at methionine and acetylation at lysine were chosen as variable modifications. The output data files were filtered and sorted to compose the protein list with two and more peptide assignments for protein identification at a false positive rate less than 0.01.

Acetylated peptides were manually verified. To quantify the relative abundances of the acetylated peptides, label-free quantification of acetylated peptides was performed using Proteome Discoverer based on the extracted ion chromatogram. Three independent LC-MS/MS analyses of each sample (p53, p53 + p300, and BCL6 + p53 + p300) were performed for quantification of acetylated peptides.

In vitro caspase assays

Recombinant His-tagged BCL6 was expressed in *E. coli* BL21 (DE3) cells and purified by Ni-NTA affinity chromatography. Active human recombinant caspase groups I (caspases-1, -4, and -5), II (caspases-2, -3, and -7), and III (caspases-6, -8, -9, and -10) were purchased from BioVision. Alternatively, H1299 cells were transfected with a pcDNA3.1-BCL6 expression vector and lysed in RIPA buffer. Recombinant His-tagged BCL6 (2 μg) or cellular extracts (50 μg) were then incubated with 2 units of caspases-1–10 in caspase assay buffer (20 mM PIPES, 0.1 M NaCl, 5% (w/v) sucrose, 0.1% (w/v) CHAPS, and 10 mM DTT, pH 7.4) at 37 °C for 1 h. The reaction mixtures were resolved by 10% SDS-PAGE and analyzed by Western blotting using an anti-BCL6 antibody to evaluate BCL6 cleavage.

Isolation of total RNA and protein from the spleens of Bcl6 WT and Bcl6−/− knockout mice

Total RNA and protein were isolated from mouse spleen tissues by tissue homogenization in RNeasy Mini kit buffer (Qiagen) or cold RIPA buffer. cDNAs were synthesized from 0.5 μg of total RNA using a Moloney murine leukemia reverse transcriptase kit (Promega). RT-qPCR was performed using SYBR Green Master Mix. The oligonucleotide primers used for the qPCR assays are described in Table 3. Equal amounts of proteins were separated by SDS-PAGE.

Statistical analysis

Student’s *t* test was used for statistical analysis. *p* values <0.05 were considered statistically significant.

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