Statistical analyses

The Mann–Whitney *U*-test was used to compare the differences in colony formation as well as the effects of ALK knockdown on cell growth between wild-type and ALK mutants. Phosphorylation signals of downstream molecules were evaluated by Student's *t*-test.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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DNMT3A mutations are rare in childhood acute myeloid leukaemia, myelodysplastic syndromes and juvenile myelomonocytic leukaemia

Acute myeloid leukaemia (AML) is a complex disease caused by mutations and deregulated gene expression, leading to increased proliferation and decreased differentiation of haematopoietic progenitor cells. Contemporary treatments have resulted in 5-year event-free survival rates of almost 60% for paediatric AML (Pui *et al*, 2011).

Recently, a whole genome sequencing study of AML uncovered recurrent mutations of an epigenetic regulator, the *DNA methyltransferase 3A* (*DNMT3A*) gene, in approximately 20% of adult AML patients (Ley *et al*, 2010; Yamashita *et al*, 2010; Yan *et al*, 2011). In these studies, *DNMT3A* mutations were frequently associated with *FLT3*-internal tandem duplication (ITD), *nucleophosmin 1* (*NPM1*) and *isocitrate dehydrogenase 1* (*IDH1*) mutations (Ley *et al*, 2010; Yan *et al*, 2011). *DNMT3A* mutations were also found in adult myelodysplastic syndromes (MDS) (8%, 12/150) (Walter *et al*, 2011), AML secondary to myeloproliferative neoplasms (MPNs) (14%, 5/35), myelofibrosis (15%, 3/20) and polycythaemia vera (7%, 2/30) (Stegelmann *et al*, 2011).

DNMT3A is involved in epigenetic regulation of genes by enzymatic de novo addition of methyl groups to the cytosine residue of CpG dinucleotides. *DNMT3A* mutations were significantly enriched with a cytogenetic profile associated with intermediate risk, including a normal cytogenetic profile, as well as the M4 and M5 subtypes, according to the French-American-British (FAB) classification system (Ley *et al*, 2010; Yan *et al*, 2011). In AML patients with a normal karyotype and *FLT3*-ITD, patients with *DNMT3A* gene mutations showed a worse prognosis than those without *DNMT3A* gene mutations (Ley *et al*, 2010; Yan *et al*, 2011); however, the frequency and clinical impact of *DNMT3A* gene mutations in paediatric AML and myeloproliferative neoplasms (MPN) remain uncertain. We searched for *DNMT3A* gene mutations in 149 AMLs who were treated on the Japanese Childhood AML Cooperative protocol, AML 99 (range: 0–15 years old, M0: 5, M1: 23, M2: 44, M3: 13, M4: 22, M5: 21, M6: 1, M7: 17, unclassified: three patients), 40 juvenile myelomonocytic leukaemias (JMMLs; range: 2 months to 8 years), 24 myelodysplastic syndromes (MDSs) and 20 paediatric therapy-related leukaemia/MDSs (t-Leuk/MDSs, range: 1–17 years). *FLT3*-ITD and *NPM1* gene alterations have been reported in these 149 AML patients (Shimada *et al*, 2007, 2008).

Total RNA extracted from the bone marrow or peripheral blood samples at diagnosis was reverse transcribed to cDNA with a cDNA Synthesis Kit (Amersham Bioscience, Tokyo, Japan). *DNMT3A* mutations were thus far reported to be almost exclusively involved in exons 16-23 (especially codon R882 in exon 23) (Ley *et al*, 2010; Yamashita *et al*, 2010; Stegelmann *et al*, 2011; Walter *et al*, 2011; Yan *et al*, 2011); thus, we confined our analysis to these exons. cDNA was amplified using the following primers: *DNMT3A* cDNA 15F, 5′-CAGGTGCTTTTGCGTGGAGTGT-3′ and 19R, 5′-ATGC AGGAGGCGGTAGAACTCA-3′, 17F, 5′-AAGATCATGTAC-GTCGGGGA-3′ and 22R, 5′-CTTTGCCCTGCTTTA TG-GAG-3′ and 20F, 5′-CCCTGTGATGATTGATGCCA-3′ and 23R, 5′-GTATTTCCGCCTCTGTG-GTT-3′ for AML samples. For JMML, MDS and t-Leuk/MDS, we confined our analysis to exon 23, including the hotspot of codon R882, of the *DNMT3A* gene using the following primers: *DNMT3A* DNA 23F, 5′-AGAACTAAGCAGGGCC-TCAGAGGA-3′ and 23R, 5′-GTATTTCCGCCTCTGTGGTT-3′. Subsequently, direct sequencing was performed on a DNA sequencer (ABI 310; Applied Biosystems, Foster City, CA, USA) using a BigDye terminator cycle sequencing kit (Applied Biosystems). The study adhered to the principles of the Helsinki Declaration, and was conducted under the regulations enacted by the Ethics Board of Gunma Children's Medical Centre.

No *DNMT3A* mutations were detected in any AML patients in our study. Recently, *DNMT3A* mutations have been reported in paediatric AML patients (Ho *et al*, 2011; Thol *et al*, 2011). Only two patients were identified (both 15 years old). Combined with these and our data, the frequency of *DNMT3A* mutations is extremely rare (2/524, 0·4%) in childhood AML. Furthermore, we did not identify *DNMT3A* mutations in MDS, JMML or paediatric t-Leuk/MDS. These findings were not compatible with those of adult MDS and MPN, suggesting that the frequency of *DNMT3A* gene mutations depends on age.

On the other hand, we found *FLT3*-ITD in 20 (13%) of 149 AML patients; however, no *NPM1* mutations were found (Shimada *et al*, 2007, 2008). Nine AML patients with *FLT3*-ITD were found to lack *DNMT3A* mutation. *DNMT3A* mutations have been correlated with *FLT3*-ITD and *NPM1* in adult AML, but not in paediatric AML. Although patients with *DNMT3A* mutations have been associated with FAB-M4, M5, especially *MLL*-negative M5, no mutations in these paediatric M4/M5 patients were found in this study. *DNMT3A* mutations have not been detected in any adult AML with favourable cytogenetics, including *t*(8;21) and inv(16) (Ley *et al*, 2010; Yan *et al*, 2011). Higher frequencies of *t*(8;21) and inv(16) in

paediatric than in adult AML patients may be associated with rare *DNMT3A* mutations in paediatric AML. These data suggest that the pathology of paediatric AML may be different from that of adult AML. We concluded that *DNMT3A* mutations, as well as *NPM1* mutations, may be infrequent in paediatric AML and MDS patients, especially those <15 years old.

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Authorship

TT and YH designed the study. AS, MS, SA, AT, KH and MT provided critical reagents and samples. NS and MP performed the experiments. RH, IT and HA supervised the work. NS and MP analysed the results. NS, TT, and YH wrote the paper and all the authors critically reviewed and revised it.

Conflict of interest

The authors declare no conflicts of interest.

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ZNF385B is characteristically expressed in germinal center B cells and involved in B-cell apoptosis

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We previously identified zinc finger (ZF) protein ZNF385B as a molecule specifically expressed in Burkitt's lymphoma (BL) among hematologic malignancies. Here, we investigated ZNF385B expression in healthy B cells in a variety of hematological tissues by RT-PCR and immunohistochemistry. ZNF385B expression was found to be limited to a subset of GC B cells, the healthy counterpart to BL B cells. To elucidate the function of ZNF385B in healthy B cells, we established a tetracycline-controlled protein-inducible system in B-cell lines and observed that ectopic expression of the longest transcript variant of ZNF385B, possessing four ZF domains, induced upregulation of *PERP* and *FAS/CD95*, a downstream target of p53, and activation of caspase, resulting in apoptosis induction. However, a ZNF385B deletion mutant with three ZF domains corresponding to shorter isoforms, did not induce upregulation; rather it inhibited apoptosis induced by CD20 cross-linking and BCR stimulation. The direct binding of ZNF385B with p53 has suggested the involvement of ZNF385B in B-cell apoptosis via modulation of p53 transactivation; our data indicate that ZNF385B characteristically expressed in GC B cells has both proapoptotic and antiapoptotic activities depending on the type of isoform and should be a novel player in GC B-cell selection.

Keywords: Apoptosis · B-cell development · Cell survival · DNA repair mechanisms · Lymphoid organs

Supporting Information available online

Introduction

The essential role of GCs within peripheral lymphoid organs is to produce high-affinity Ab-secreting B cells against foreign antigens, although the process of Ab affinity maturation also poses

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a significant risk of generating unintended autoreactive clones and oncogenic mutations [1]. Therefore, B cells have integrated control systems to expand high-affinity clones against exogenous antigens and eliminate inappropriate clones in a developmental stage-dependent manner [2]. In addition to the BCR, which plays a crucial role in clonal selection and clonal expansion [3], the involvement of a number of molecules in this regulatory system has been identified, although the details have yet to be fully clarified.

B-cell malignancies are thought to be related to B cells at specific developmental stages and retain, at least in a part, the characteristics of their healthy counterparts; therefore, it may be useful to investigate the molecular characteristics of B-cell development [4]. For example, sporadic form of Burkitt's lymphoma (BL) has been postulated to originate from early centroblasts in the GC and exhibits some of their features [5–8].

In the process of investigating the molecules specifically expressed in childhood B-cell malignancies, we preliminarily identified *ZNF385B* as a BL-specific gene. ZNF385B, also called ZNF533, is supposed to be a protein that possesses zinc-finger (ZF) domains. Notably, *ZNF385B* was expressed only in BL cells among hematopoietic malignancies and not expressed even in diffuse large B-cell lymphoma (DLBCL), another B-cell malignancy originating from GC B cells.

To date, three isoforms of ZNF385B have been identified and isoform (IF)-1 is the longest transcript variant, possessing four ZF domains, while IF-2/3 are shorter transcript variants with three ZF domains. Although ZNF385A, also called the hematopoietic zinc finger, consisting of three ZF domains [9] and having high homology with ZNF385B IF-2/3, is known to be involved in apoptotic regulation [10], the function and biological significance of ZNF385B have not been clarified thus far. Considering the specific expression of ZNF385B in GC-derived BL cells, and that GC B cells possess developmental stage-dependent mechanisms of cell proliferation and death, ZNF385B might be involved in the apoptotic regulation in GC B cells.

Therefore, we attempted to elucidate the functional role of ZNF385B in B cells using a tetracycline-controlled protein-inducible system. In the present study, we demonstrate that ZNF385B is characteristically expressed in a subset of GC B cells and involved in B-cell apoptosis. The molecular basis of ZNF385B function in apoptotic regulation has been further investigated.

Results

Expression of ZNF385B in BL cell lines and GC B cells

In an attempt to characterize the molecules that are specifically expressed in BL in comparison with those in other pediatric lymphoid malignancies, we screened genes by employing a microarray system using clinical materials and identified several candidates. Among the genes selected, we preliminarily observed that *ZNF385B* was expressed in the vast majority of BL cases, but not in cases of DLBCL, B-cell precursor, and T acute lymphoblastic leukemia (data not shown). We therefore examined the expression of *ZNF385B* in hematologic malignancy cell lines. As shown in Fig. 1A, RT-PCR analysis revealed that *ZNF385B* was expressed in all nine cell lines of BL, but not in other types of leukemia/lymphoma cell lines, including DLBCL, B-cell precursor and T acute lymphoblastic leukemia, hairy cell leukemia, and multiple myeloma.

As described above, BL has been postulated to originate from early centroblasts in GCs. Thus we examined whether *ZNF385B* is expressed in GC B cells or not. When we tested the expression of *ZNF385B* in healthy human hematopoietic tissues in which B cells are contained by RT-PCR, *ZNF385B* was found to be expressed in spleen, lymph node (Fig. 1B), and tonsil (data not shown) but not in bone marrow and peripheral blood leukocytes. We further confirmed the expression of *ZNF385B* in purified human $CD20^+$ B cells derived from spleen (Fig. 1B). In contrast, *ZNF385A*, having high homology with ZNF385B IF-2/3, was not expressed in peripheral lymphoid organs (data not shown).

We also investigated the expression of *ZNF385B* isoforms in BL cell lines and healthy human lymphoid tissues by real-time PCR and observed that each isoform was expressed variably in each tissue or cell line. As shown in Fig. 1C, although healthy lymphoid tissue mainly expressed IF-2/3, some BL cell lines predominantly expressed IF-1.

We next examined the protein expression of ZNF385B in peripheral lymphoid organs. As shown in Fig. 1D, immunohistochemical analysis of lymph nodes clearly indicated that the ZNF385B-positive cells were limited to the germinal center. Together with the results of dual staining with anti-ZNF385B and anti-CD20, it was indicated that a subset of GC B cells expressed ZNF385B (Fig. 1E).

Therefore, we intended to characterize further a subset of GC B cells expressing ZNF385B. Upon using the online available raw gene expression data of tonsil B cells separated by means of CXCR4 expression [11]] (submitted by Caron G. et al., http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc, accession no. GSE15271), we found that $CXCR4^+$ tonsil B cells expressed *ZNF385B*, whereas $CXCR4^-$ tonsil B cells did not (Supporting Information Fig. 1). In addition, we observed that only a proportion of ZNF385B-positive cells possess activated caspase-3 by immunohistochemical dual staining with anti-ZNF385B and anti-cleaved caspase-3 (Fig. 1F).

We also examined the expression of BCL6 and p53 in peripheral lymphoid organs. As shown in Fig. 1G, the BCL6-positive cells were limited to GC, while p53-positive cells were presented in both inside and outside of GC.

Effect of ZNF385B expressed in B-cell lines

We next examined the function of ZNF385B. Since all BL cell lines that we tested were found to express *ZNF385B*, we established a tetracycline-dependent protein-inducible system for both ZNF385B IF-1 and ZNF385B IF-1/DEL (Fig. 2A) in BJAB cells that lack *ZNF385B*. As presented in Fig. 2A, ZNF385B IF-1 is

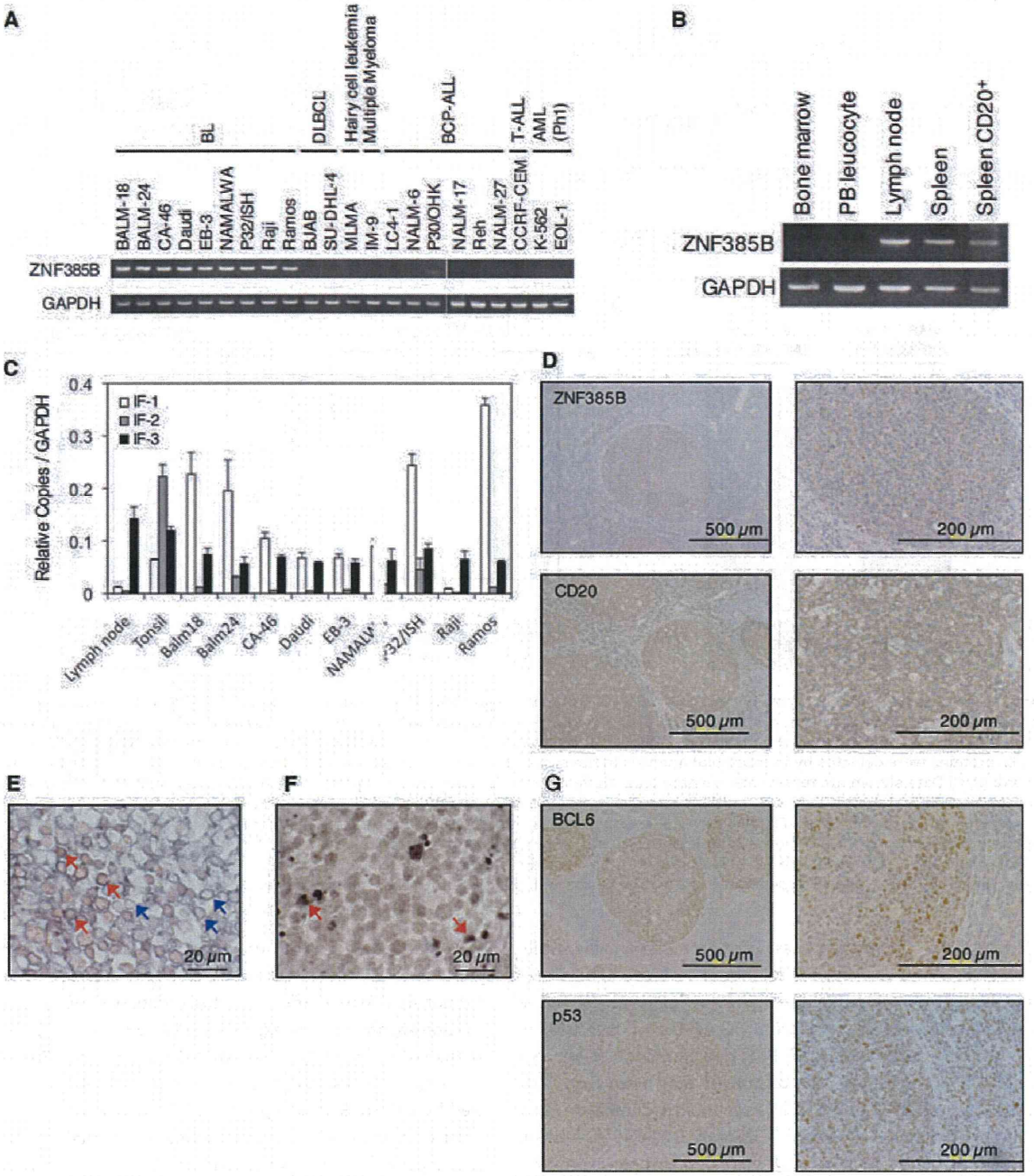

Figure 1. Expression of *ZNF385B* in hematological malignancy cell lines and healthy tissues. (A) The expression of *ZNF385B* in hematological malignancy cell lines, Burkitt's lymphoma (BL), diffuse large B-cell lymphoma (DLBCL), hairy cell leukemia, multiple myeloma, B-cell precursor acute lymphoblastic leukemia (BCP-ALL), T acute lymphoblastic leukemia (T-ALL) and acute myeloid leukemia (AML), was examined by RT-PCR. (B) The expression of ZNF385B in healthy human hematopoietic tissues and nontumorous mononuclear cells separated from autopsied spleen was examined as in (A). (A, B) GAPDH was used as a control. (C) Real-time PCR analyses of *ZNF385B* isoform expression in healthy human tissues and BL cell lines were performed. The y-axis represents the relative copy numbers quantified by control vector. Data are shown as mean/median + SD/SEM of triplicates and are representative of at least three independent experiments. (D) Immunohistochemical staining for ZNF385B and CD20 was performed using formalin-fixed, paraffin-embedded tissue specimens from nontumorous reactive lymphadenitis and visualized with DAB (brown, original magnification, 100× at left and 400× at right). (E) The same sample specimen as in (D) was stained with anti-ZNF385B (NovaRED, red, nuclei) and anti-CD20 (DAB-Ni, gray, cell membrane) simultaneously (original magnification, 1000×). The red and blue arrows indicate typical $CD20^+$ $ZNF385B^+$ double-positive and $CD20^+$ single-positive cells, respectively. (F) The same sample specimen as in (D) was stained with anti-ZNF385B (NovaRED, red) and anticleaved caspase-3 (DAB-Ni, gray) simultaneously (original magnification, 1000×). The red arrows indicate typical cleaved $caspase\text{-}3^+$ $ZNF385B^+$ double-positive cells. (G) Immunohistochemical staining for BCL6 and p53 was performed as in (D). Data shown are representative of at least three independent experiments.

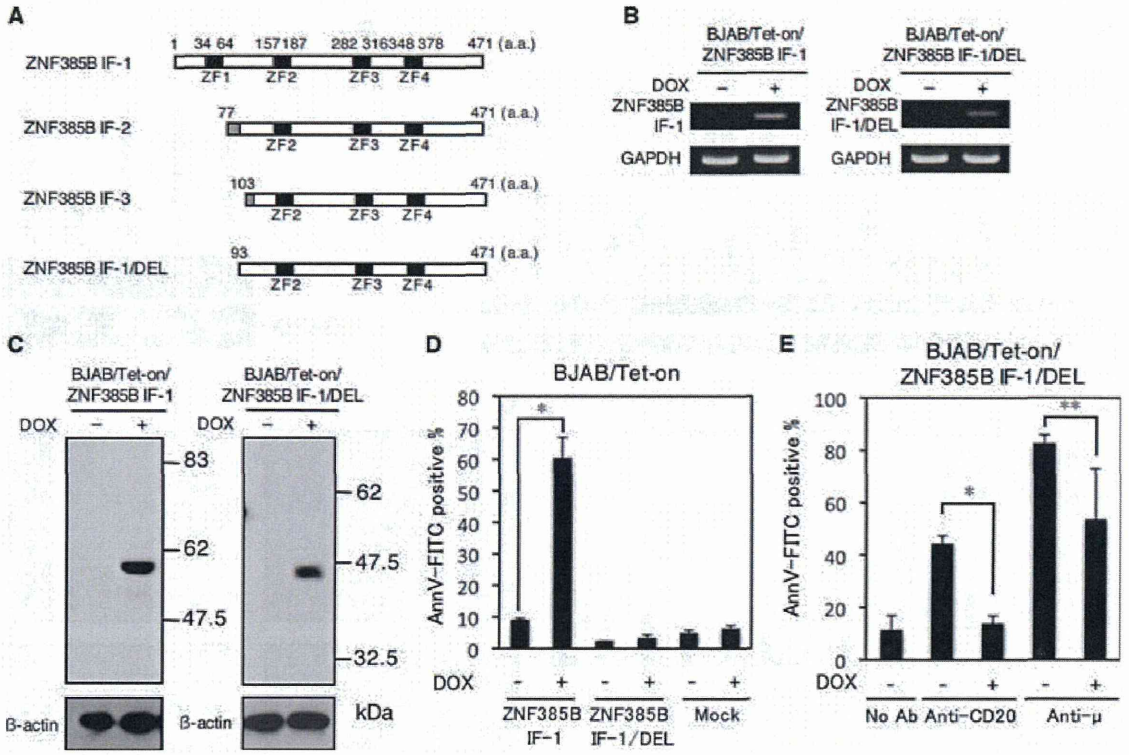

Figure 2. The effect of ZNF385B expression in BJAB cells. (A) Schematic representation of transcript variants of ZNF385B and constructed mutant. (B) The Tet-on inducible expression of *ZNF385B IF-1* and *IF-1/DEL* genes in BJAB transfectants was confirmed by RT-PCR. The cells were treated with or without 1 μg/mL DOX for the indicated periods. As an internal control, human *GAPDH* gene was also detected. (C) The ZNF 385B IF-1 and IF-1/DEL proteins were detected by immunoblot analysis in the same sample specimens as in (B). As an internal control, human β-actin was also detected. (B, C) Data shown are representative of at least three independent experiments performed. (D) BJAB Tet-on ZNF385B IF-1 and IF-1/DEL cells were treated with or without DOX for 96 h and apoptotic cells were identified by binding with FITC-labeled Annexin V (AnnV-FITC). The y-axis represents the ratio of AnnV-FITC-positive cells. As a negative control, BJAB Tet-on cells transfected with empty control vector (Mock) were simultaneously tested. (E) BJAB Tet-on ZNF385B IF-1/DEL cells were treated with or without DOX for 24 h followed by treatment with anti-CD20 and anti-mouse IgG Abs or anti-μ Ab for 48 h. Apoptotic cells were identified as in (D). (D, E) Data are shown as mean + SEM of triplicates and are representative of at least three independent experiments performed. * $p < 0.01$; ** $p < 0.05$, compared with control using Student's *t*-test.

the longest transcript variant possessing four ZF domains, while ZNF385B IF-1/DEL corresponds to shorter isoforms IF-2/3 possessing three ZF domains and have high homology with ZNF385A. Inducible expression of both ZNF385B IF-1 and IF-1/DEL in BJAB cells was confirmed by RT-PCR and immunoblot analysis (Fig. 2B and C). We have also confirmed that more than 90% of cells expressed ZNF385B IF-1 by immunohistochemistry (data not shown). We then examined the effect of ZNF385B expression on apoptosis and found that DOX-mediated ectopic expression of ZNF385B IF-1 itself induced apoptosis in 96 h in BJAB cells as assessed by binding of AnnexinV-FITC (Fig. 2D). In contrast, when ZNF385B IF-1/DEL and control vector were similarly examined, no significant apoptosis was occurred in BJAB cells (Fig. 2D), indicating the specific effect of ZNF385B IF-1 on apoptosis induction. We also examined another ZNF385B-negative cell line MD901 and observed identical results, indicating that the effect of ZNF385B on apoptosis modulation does not specifically occur in BJAB cells but is common in ZNF385B-negative B-cell lines.

We also examined the effect of ZNF385B expression on apoptosis induced by Ab-mediated BCR and CD20 stimulation. Although 48 h of incubation with anti-μ and anti-CD20 Abs induced apoptosis in BJAB cells, DOX-mediated expression of ZNF385B IF-1 did not significantly affect apoptosis (data not shown). However, it is noteworthy that the expression of ZNF385B IF-1/DEL relieved apoptosis mediated by BCR and CD20 stimulation (Fig. 2E).

Next, we investigated the molecular basis of apoptosis mediated by ZNF385B IF-1 expression. When we examined the alteration of mitochondrial transmembrane potential by employing MitoCapture™ Apoptosis Detection Kit, ectopic expression of ZNF385B IF-1 caused fluorescence shift from red to green, indicating the disruption of the mitochondrial transmembrane potential (Fig. 3A). Furthermore, activation of caspase-3 and -8 was induced after induction of ZNF385B IF-1 (Fig. 3B).

Effect of ZNF385B on the expression of genes transactivated by p53

Since ZNF385A, which has high homology with ZNF385B IF2/3, is reported to affect the expression of genes transactivated by p53

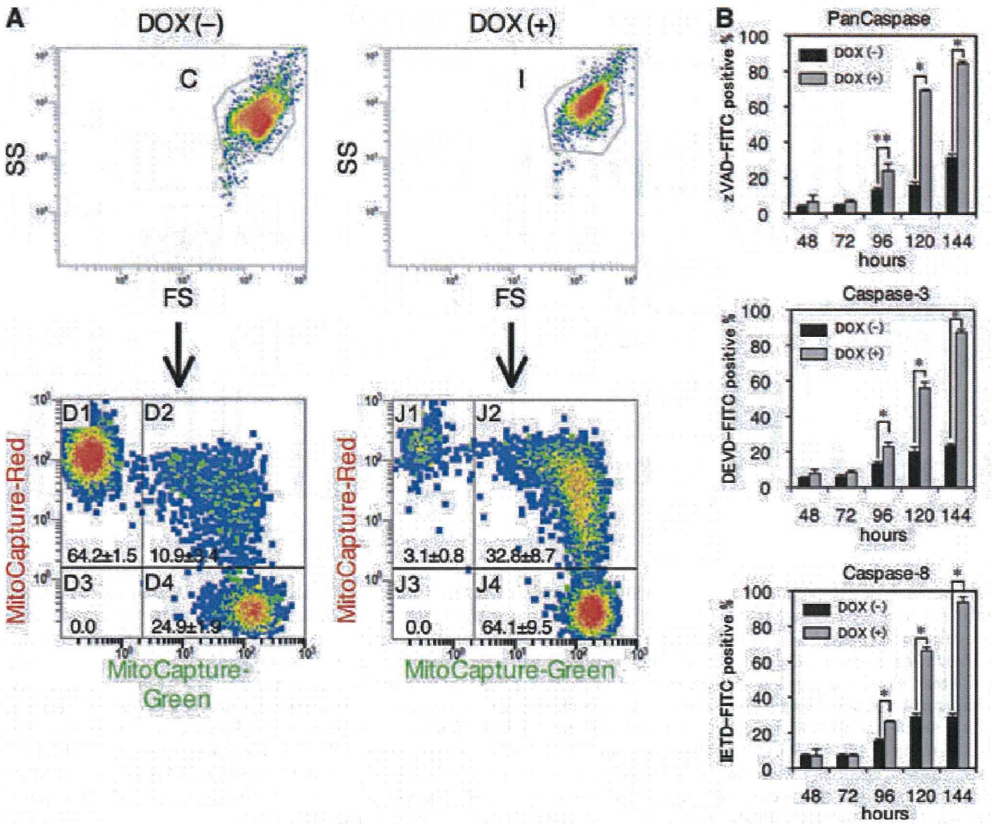

Figure 3. Analysis on ZNF385B IF-1-mediated apoptosis. (A) Mitochondrial transmembrane potential in BJAB transfectants treated with or without DOX for 6 days was determined using the MitoCapture Mitochondrial Apoptosis Detection Kit. The cationic dye MitoCapture was used to test the disruption of mitochondrial membrane potential. In the side scatter (SS) versus forward scatter (FS) plots, cells were gated by regions C and I. In viable cells, the dye aggregates in mitochondria and produces red fluorescence (FL2, y-axis), whereas in cells with altered membranes, it remains in the cytoplasm in monomeric form and produces green fluorescence (FL1, x-axis). The regions D4 and J4 represent the cell population with disrupted membrane potential. Representative plots are shown from three independent experiments. The experiment was performed in triplicate, and the means ± SEM of percentage for each region of a quadrant are presented. (B) The activation of pancaspase, caspase-3 and caspase-8 after DOX treatment for the indicated periods was detected by flow cytometry. The y-axis represents the ratio of zVAD-, DEVD-, and IETD-FITC-positive cells, respectively. Data are shown as mean + SEM of triplicates and are representative of at least three independent experiments. $^{*}p < 0.01$, $^{**}p < 0.05$, compared with control using Student's t-test.

[10], we then examined the effect of ZNF385B on the expression of some of apoptosis-related genes regulated by p53 [12] using real-time RT-PCR. As shown in Fig. 4A, the expression of ZNF385B IF-1 increased *PERP* and *FAS/CD95* expression, but not that of *BAX*, *BCL2*, *BIM*, *NOXA*, *p53TG1*, *14–3-3* σ, *BCL6*, *PUMA*, or *CDKN1A* (Supporting Information Fig. 2). In contrast, ZNF385B IF-1/DEL did not affect the expression of PERP and *FAS/CD95* (Fig. 4A). Increased PERP expression was also confirmed at the protein level as assessed by immunoblot analysis (Fig. 4B). Furthermore, we observed that shRNA-mediated knockdown of *PERP* partially inhibited apoptosis induced by ZNF385B IF-1 expression (Fig. 4C, Supporting Information Fig. 3).

We next tested whether ZNF385B interacts with p53. For this purpose, BJAB Tet-on ZNF385B IF-1 cells treated with DOX for 48 h were lysed and immunoprecipitation was performed using p53-specific mAb. As shown in Fig. 5A, we observed that ZNF385B IF-1 was coimmunoprecipitated with p53, indicating the immunocomplex formation of ZNF385B IF-1 and p53. We further investigated the direct interaction between ZNF385B and p53 by means of a yeast two-hybrid assay. When ZNF385B protein fused to the Gal4 DNA-binding domain (pAS2–1-ZNF385B IF-1 and pAS2–1-ZNF385B IF-1/DEL) and p53 DNA-binding domain fused to the Gal4 transactivation domain (pACT2-p53) were coexpressed in Y187 yeast strain, both ZNF385B IF1 and ZNF385B IF-1/DEL induced colored colonies (Fig. 5B), indicating direct interaction between ZNF385B and p53.

Discussion

In this study, we demonstrated that the expression of ZNF385B is limited to a subset of GC B cells and their tumor counterpart BL cells among hematopoietic cells. Analysis using online available raw gene expression data indicated that *ZNF385B* was expressed only in $CXCR4^{+}$, but not $CXCR4^{-}$, tonsil B cells (Supporting Information Fig. 1). Since Caron et al. [11] demonstrated that

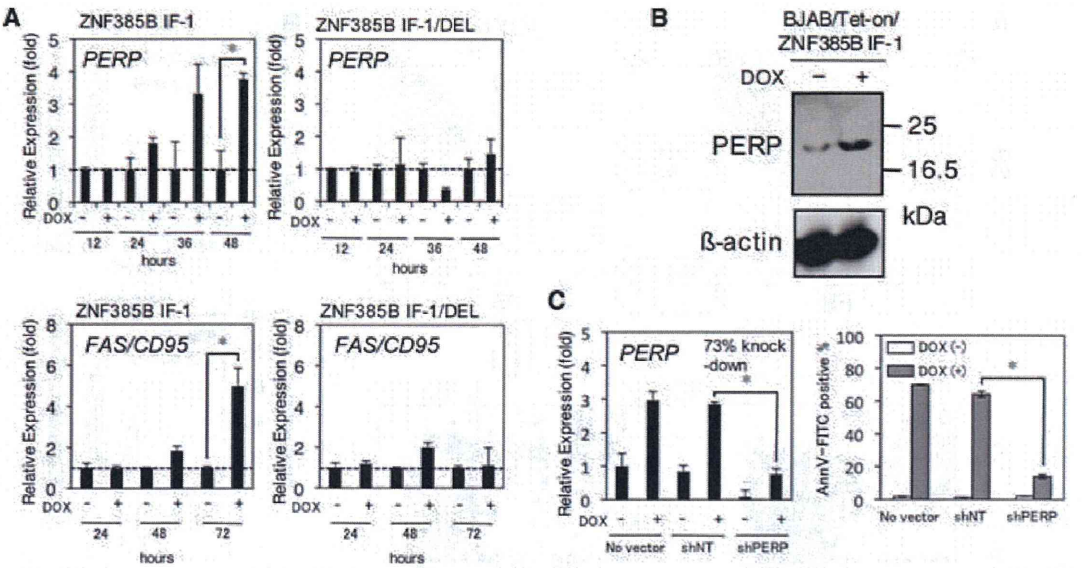

Figure 4. The expression of ZNF385B IF-1 increased PERP expression. (A) The expression analysis of p53 downstream genes (*PERP* and *FAS/CD95*) after DOX treatment was performed by real-time RT-PCR analysis using BJAB Tet-on ZNF385B IF-1 and IF-1/DEL cells. Signal intensity was normalized using that of a control housekeeping gene (human *GAPDH* gene). The y-axis represents the relative expression to that of DOX-negative cells. Data are shown as mean + SEM of triplicates and are representative of three independent experiments. * $p < 0.05$, compared with control using Student's *t*-test. Error bars indicate SEM. (B) The protein expression of PERP in the same sample specimens as in (A) was detected by immunoblot analysis. Actin served as a loading control. Data shown are representative of three experiments performed. (C) BJAB Tet-on ZNF385B IF-1 cells were transfected with *PERP* shRNA vector and the knockdown efficiency of *PERP* message by shRNA was assessed by real-time RT-PCR (the left panel). Then the cells were treated with or without DOX for 96 h and apoptotic cells were identified by binding with FITC-labeled Annexin V (AnnV-FITC) as presented in the right panel. As a negative control, BJAB Tet-on ZNF385B IF-1 cells and BJAB Tet-on ZNF385B IF-1 cells were transfected with nontarget shRNA vector (NT) and simultaneously tested. The y-axis represents the ratio of AnnV-FITC-positive cells; data are shown as mean + SEM of triplicates and are representative of three independent experiments.

CXCR4 expression could properly separate human centroblasts expressing activation-induced cytidine deaminase from centrocytes, it is suggested that ZNF385B was specifically expressed in centroblasts but not centrocytes. Furthermore, the ectopic expression of ZNF385B modulates the apoptotic induction in B cells. Interestingly, ZNF385B IF-1, the longest transcript variant, mediated apoptosis induction by itself but deletion mutant corresponding to ZNF385B IF-2/3, the shorter transcript variants, inhibited apoptosis induced by CD20 cross-linking and BCR stimulation in B cells.

ZNF385A, which has high homology with ZNF385B IF-2/3, was reported to be a p53-inducible gene product that is induced in response to genotoxic and oncogenic stress and inhibits the maintenance of G2 phase arrest following ionizing radiation, thereby sensitizing cells to DNA damage [13, 14]. In addition, ZNF385A was further reported to determine cell survival upon genotoxic stress by modulating p53 transactivation in an autoregulatory feedback loop [10]. Considering these findings together with our data, ZNF385A and B might be a protein family involved in p53-related DNA damage checkpoint machinery. Since we observed the absent expression of *ZNF385A* in peripheral lymphoid organs, ZNF385B possibly plays a role in the regulation of sensitization of GC B cells to DNA damage mediated by somatic hypermutation and immunoglobulin class switch recombination.

It is generally accepted that the expression of p53 in GC B cells is suppressed by BCL6 to allow toleration of the physiological DNA breaks required for somatic hypermutation and class switch recombination without inducing a p53-dependent apoptotic response [15], whereas complete loss of p53 function should increase the risks of the generating unintended autoreactive clones and oncogenic mutations. Margalit et al. [16] demonstrated that BCL6 transcription is positively regulated by p53, postulating that p53 is activated in response to the breaks formed in the genomic DNA due to somatic hypermutation and class switch recombination in GC B cells, and p53-mediated BCL6 expression forms an autoregulatory loop to modulate p53 function. Actually, our data indicate the expression of p53 in a subset of B cells in peripheral lymph nodes. Therefore, interdependent regulation of p53 and BCL6 functions should play a crucial role in appropriate control for clonal expansion and clonal deletion of GC B cells. In this context, ZNF385B should be involved in the regulatory machinery of p53 function and contribute to clonal selection of GC B cells. Further studies to elucidate the role of ZNF385B in the development of GC B cells are now under way.

As we also presented, the expression of ZNF385B IF-1 activated caspase-8 and -3 and disrupted the mitochondrial membrane potential. Caspase-8 was found to be mainly activated by death receptor apoptotic signaling, such as Fas/CD95 and TNFRs [17], whereas p53-mediated upregulation of PERP also led to increased levels of cleaved caspase-8 forms, as well as to reduction of its full-length substrate Bid [18]. Caspase-3 is in the downstream signaling pathway of caspase-8 and known to be an effector caspase. As

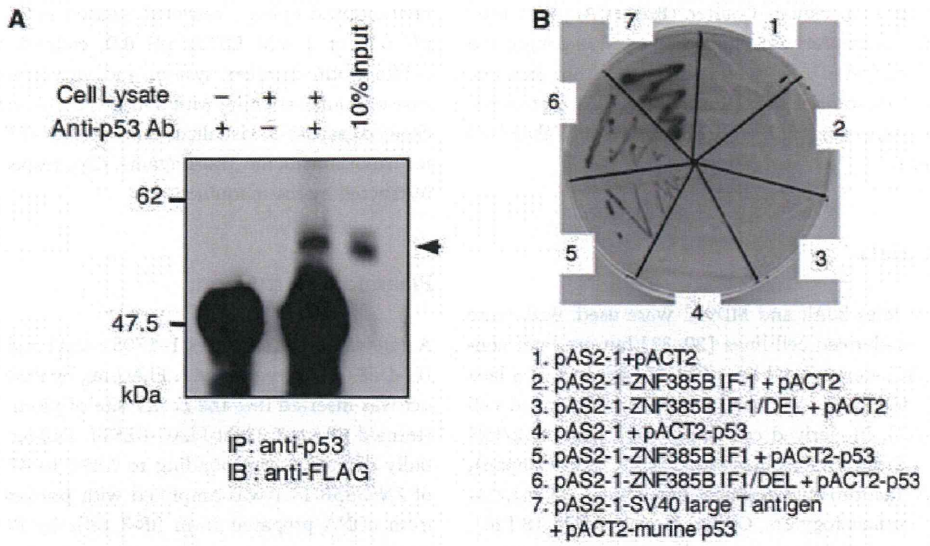

Figure 5. Binding of ZNF385B with p53. (A) Immunoprecipitation (IP) and Western blot analysis of BJAB Tet-on ZNF385B IF-1 treated with 1 μg/mL DOX for 48 h were performed using anti-p53 Ab and anti-Flag Ab, respectively. The bands corresponding to ZNF385B IF-1 protein are indicated by an arrow. (B) Yeast two-hybrid assay to show direct interaction between ZNF385B IF-1 and p53. Yeast two-hybrid assay was performed using the combination of plasmids as indicated. The colored colony formation indicates the binding between the proteins introduced into the yeasts. Representative data are shown from at least three independent experiments.

we observed upregulation of *PERP* and *FAS/CD95*, these molecules are candidate mediators of ZNF385B IF-1-induced activation of caspase-8. Indeed, our data that partial inhibition of ZNF385B IF-1 induced apoptosis by *PERP* knockdown mediated by shRNA (Fig. 4C) indicate the involvement of PERP in the apoptotic process. Since the transcription of both *PERP* and *FAS/CD95* is regulated by p53 [12], it is reasonable to speculate that ZNF385B directly modulates p53 transactivation for *PERP* and *FAS/CD95*, resulting in caspase-8 activation. The fact that FAS/CD95 is reported to be required for clonal selection within GC and establishment of the memory B-cell repertoire [19] and B-lymphocyte homeostasis [20] should support the notion of involvement of ZNF385B-mediated *FAS/CD95* upregulation in GC B-cell development.

As mentioned above, ZNF385B has both proapoptotic and antiapoptotic activities depending on the type of isoform. Since both longest and shorter isoforms can bind to p53 (Fig. 5B), the additional ZF domain located at the *N*-terminus of IF-1 (Fig. 2A) should confer opposite function to shorter isoforms, although the precise mechanism involved is still under investigation. They might act together as a dominant-negative system in the regulation of p53 function and are involved in the apoptotic regulation of GC B cells.

Although healthy lymphoid tissue mainly expressed IF-2/3, some BL cell lines predominantly expressed IF-1 (Fig. 1C). Since high expression of proapoptotic IF-1 seems to be disadvantageous for tumor genesis, predominant expression of IF-1 in BL cell lines looks inconsistent. However, considering this together with the fact that loss and inactivation of p53 proteins mediated by gene mutations are frequently observed in BL cells [21], it can be speculated that the cell origin of BL cells is GC centroblasts destined for elimination by negative selection, but that acquire resistance to apoptosis.

In conclusion, zinc-finger protein ZNF385B characteristically expressed in GC B cells had both proapoptotic and antiapoptotic activities depending on the type of isoform. ZNF385B IF-1 directly interacts with p53 and induces apoptosis via upregulation of PERP and *FAS/CD95*, followed by activation of caspase-8 and caspase-3. Although further analysis to elucidate the function of ZNF385B is clearly needed, our observation should shed light on the functional role of this zinc finger protein in the course of B-cell development in GCs.

Materials and methods

Reagents

Mouse and rabbit polyclonal Abs against ZNF385B (Sigma-Aldrich, St. Louis, MO, cat # SAB1408384, and ABGENT, San Diego, CA, cat # AP5790b, respectively) were used. The mouse mAbs used for the immunohistochemical and the biochemical analysis were anti-CD20 from Dako (Glostrup, Denmark), anti-BCL6 from Leica Biosystems Newcastle Ltd. (Newcastle, UK, cat # NCL-BCL-6), anti-actin from Sigma-Aldrich, anti-FLAG from Invitrogen (Carlsbad, CA), and anti-p53 (Bp53–12) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The Rabbit mAbs used for the immunohistochemical analysis was anti-active caspase-3 from BD Biosciences (San Jose, CA). Purified anti-μ chain rabbit polyclonal Ab from Jackson Immuno Research Laboratories Inc. (West Grove, PA) was used to cross-link BCRs. Mouse

