($\alpha\beta$ TCR) complex after rearrangement at the TCRα locus, which allows engagement by intrathymic peptide/major histocompatibility complex (MHC) ligands. CD4 and CD8 molecules interact, respectively, with class II and I MHC molecules, thereby stabilizing or enhancing the interaction of TCRs with their MHC ligands. Co-expression of CD4 and CD8 at the DP stage allows thymocytes to receive optimal signals through either class I or II MHC-specific TCRs. Negative selection leads to death by apoptosis, while positive selection leads to thymocyte activation as evidenced by the upmodulation of activation markers, such as CD5 and CD69, and differentiation into SP T cells. Some thymocytes at the DP stage undergo signals to differentiate into natural killer T (NKT) cells. Gene expression analysis in thymocytes reveals that Bcl11b is upregulated at the transition from DN1 to DN2 and the expression is maintained in cells of $\alpha\beta$ T cell lineages.

Immature DN thymocytes can be further divided into four subpopulations based on the surface expression of CD44 (or CD117/c-kit in case) and CD25, with the developmental progression being $CD44^+CD25^-$ (DN1) to $CD44^+CD25^+$ (DN2) to $CD44^-CD25^+$ (DN3) and then to $CD44^-CD25^-$ (DN4) cells (Fig. 2A). To make the developmental transition from DN3 to DP, proteins that are produced from productively rearranged genes at the TCRβ locus must be assembled into the preTCR complex and expressed on cell surface, which consists of a TCRβ-chain, the invariant pTα-chain, and CD3 components. At the DN4 stage, thymocytes re-enter into the cell cycle and rapidly proliferate.[53),54)] Since the preTCR confers survival signals for DN3/DN4 cells, only cells that have acquired a functional preTCR can transit from DN3 to DP, a process known as β-selection. Thymocyte progenitor cells at the DN1/DN2 stage retain the capability to generate cells of non-T-cell lineages such as myeloid and natural killer (NK) cells.[1),51)] However, DN3 thymocytes are committed T cells and have lost potentials to differentiate to other non-T-cell lineages.[55),56)]

Figure 2B shows flowcytomeric analysis of *Bcl11b*$^{KO/KO}$ thymocytes using CD4/CD8 and CD25/CD44 markers. The *Bcl11b*$^{KO/KO}$ thymus harbors DN and ISP cells but fail to produce DP thymocytes.[3)] Further analysis showed that thymocytes at the DN3 stage retain normal cellularity but not at the DN4 stage, and the DN4 thymocytes exhibit apoptosis, accompanying low expression of anti-apoptotic proteins, Bcl-xL and Bcl-2.[57)] As expected, *Bcl11b*$^{KO/KO}$ DN4 thymocytes lack preTCR on the surface, though containing some within the cell. These indicate that developmental progression is impaired before the DP stage and probably around the DN3 stage where cells start to express the preTCR complex on cell surface. *Bcl11b*$^{KO/KO}$ thymocytes exhibit less efficiency in DNA rearrangement of the TCRβ locus.[57)] Recombination between D and J segments normally occurs while subsequent recombination between V and DJ segments is reduced, which could contribute in part to the lack of expression of preTCR complex. The lack of preTCR expression in DN4 thymocytes may be the cause of the apoptosis, which has been considered as a mechanism to eliminate deleterious cells within the thymus.

Loss of genes encoding a component of the preTCR complex in mice lacks the preTCR signaling and leads to the developmental arrest at DN3 stage As expected, introduction of the lost gene restores the ability to produce DP and SP cells.[58)–60)] In those mice, interestingly, deletion of the apoptosis-promoting gene *p53* abrogates the developmental arrest to produce DP cells.[61)–63)] As for *Bcl11b*$^{KO/KO}$ mice, introduction of functional *TCRβ* gene did not restore the developmental arrest.[64)] This suggests that the preTCR complex formation alone cannot compensate the deficiency of Bcl11b. Furthermore, introduction of *p53* deficiency into *Bcl11b*$^{KO/KO}$ mice did not affect the developmental arrest and failed to inhibit apoptosis.[64)] This suggests that p53-related apoptosis is not the reason for the failure of T-cell development upon loss of Bcl11b. These results suggest that *Bcl11b*$^{KO/KO}$ thymocytes have defects in not only the pre-TCR signaling but some other signaling required for survival and transition to DP stage of development. Thus, the exact cause of T-cell defects in Bcl11b-lacking mutant mice remains unresolved.

Bcl11b also plays a role in the differentiation from DP to SP cells. This was demonstrated by analysis of CD4-Cre;*Bcl11b*$^{flox/flox}$ mice, where loss of Bcl11b occurs in thymocytes after the DP stage.[39),43)] The mice exhibited the developmental arrest at DP stage and did not produce SP cells and NKT cells. This indicates that Bcl11b is required for DP cells to differentiate to SP and NKT cells. The DP thymocytes underwent rearrangement at the TCRα gene normally but they failed to display proximal TCR signaling that is required for initiation of positive selection. Thus, the DP cells lacking TCR signaling underwent apoptosis during the process of positive selection. Interestingly, susceptibility to the

apoptosis in those DP cells was at least in part independent of the anti-apoptotic factor Bcl2, because the introduction of *Bcl2* transgene to CD4-Cre;*Bcl11b*$^{flox/flox}$ mice did not fully prevent apoptosis of thymocytes.[43] These results indicate that Bcl11b plays critical roles in the establishment of TCR signaling in DP cells that is required for producing precursor cells of CD4 and CD8 lineages and also thymic NKT precursors.[43]

Recently, three independent reports showed that deletion of Bcl11b blocks the progression from DN2 cells to DN3 committed T cells, indicating a role for Bcl11b at an early stage in T-cell development and maintenance of T-cell lineage commitment.[22),46),47] NK-like cells were generated in *Bcl11b*$^{KO/KO}$ mice, and they may be converted from cells of T-cell lineage. This suggests that Bcl11b plays a role on the maintenance of T cell lineage identity in T cell lineage committed thymocytes, and its absence leads to reprogram T cells into the NK cell lineage. Hence, Bcl11b may regulate the cell fate choice between cells of T cell lineage and NK cells. One of the papers by Ikawa *et al.* has succeeded in establishing a culture system that continuously cultures developmentally arrested and proliferating DN2 thymocytes in the presence of Delta-like 4 and the cytokine IL-7. Of importance, those DN2 thymocytes retain the potential to differentiate into multiple cell types, T cells, NK cells, dendritic cells, and macrophages. They discovered that the expression of Bcl11b in the proliferating DN2 cells leads to the relief of the differentiation arrest to differentiate into cells of T cell lineage. Also, they showed that simply reducing the concentration of IL-7 in the culture system stimulates robust T cell differentiation, suggesting that IL-7 controls the expression of Bcl11b and that Bcl11b is the critical T cell promoting transcription factor. Their study also includes the finding that identifies Bcl11b as a sensor that links cytokine signaling thresholds and T cell lineage commitment in early thymocyte progenitors.

4-2. Neuron. As for CNS, expression of Bcl11b/Ctip2 was first detected in subcerebral projection neurons of the cerebral cortex, including developing corticospinal motor neurons (CSMN). Developmental analysis of *Bcl11b*$^{KO/KO}$ mice showed defects in axonal extension and pathfinding by the projection neurons, resulting in failure of the neurons to connect to the spinal cord.[4] This indicates a critical role for Bcl11b in the development of corticospinal motor neurons. Interestingly, *Bcl11b*$^{KO/+}$ heterozygous mice also show some subtle defects in CSMN fasciculation, suggesting haploinsufficiency of Bcl11b leading to phenotypic consequences. Further study showed expression of Bcl11b in GABAergic medium-sized spiny neurons (MSN) within the striatum that are derived from progenitors located in the germinal zone of the developing lateral ganglionic eminence.[65] Loss of Bcl11b function results in the failure of differentiation of MSN, leading to disruption of the patch-matrix organization of MSN. This suggests roles for Bcl11b in the differentiation of MSN and establishment of cellular architecture of the striatum.[65] Strial-enriched expression of Bcl11b was also demonstrated in adulthood,[42] suggesting that Bcl11b plays important roles in the functioning and maintenance of mature medium spiny neurons.

Recent study has shown that Bcl11b is also expressed in the developing vomeronasal system in the accessory olfactory bulb of the mouse as well.[66] The vomeronasal system detects pheromones to mediate social and reproductive behaviors in terrestrial vertebrates. In *Bcl11b*$^{KO/KO}$ mice, vomeronasal sensory neurons (VSNs) are generated during development in the correct number but selectively die due to apoptosis. As a consequence, the mice display various phenotypes such as disorganization of layer formation of the accessory olfactory bulb, impaired axonal projections of VSNs, and defective mature differentiation of VSNs. The VSNs can be classified into two major types of neurons having different receptors. Interestingly, loss of Bcl11b function results in an impaired balance of cells of the two VSN types, suggesting that Bcl11b regulates the cell fate choice between the two different VSN types of neuronal cells.

4-3. Skin. Bcl11b/Ctip2 is highly expressed in some cell types in the developing epidermis whereas it is expressed at a lower level in the dermis. The expression can be also detected in adult skin but the level of expression is much lower. The development of the skin epidermis begins with the commitment of the primitive ectoderm to the keratinocyte cell fate. The subsequent processes of cellular proliferation, stratification and differentiation result in formation of the multilayered structure of epidermis. During embryonic development, keratinocytes of the innermost layer of the epidermis, the proliferative basal cell layer, undergo a program of the terminal differentiation, then exit the basal cell layer and migrate upward to the surface of the skin.[67] *Bcl11b/Ctip2*$^{KO/KO}$ mice exhibit a hypoplastic epidermis with late differentiation events.[5] The epidermis

shows impairments in keratinocyte proliferation and the development of the epidermal permeability barrier. These indicate that Bcl11b plays critical roles during skin development. *Bcl11b*$^{KO/KO}$ mice exhibit an interesting feature, i.e., born with eyes open. The hypoplastic epidermis described above may account for this open eyelid.

4-4. Ameloblast in tooth. Bcl11b/Ctip2 is expressed in the ectodermal components of the developing tooth, including enamel epithelial cells and cells of the ameloblast lineage. *Bcl11b*/*Ctip2*$^{-/-}$ mice exhibit multiple defects at the bell stage of embryonic tooth development.[6] The early bell stage (embryonic day 15.5–16.5) is characterized by continued epithelial expansion and differentiation into the inner and outer enamel epithelium, stratum intermedium, and stellate reticulum.[68] Mutant incisors and molars are reduced in size and exhibit hypoplasia of the stellate reticulum that probably harbors stem cells. A well known hallmark of mouse incisors, different from human incisors, is an asymmetric enamel formation, which results from differential distribution of ameloblasts around incisors during development. As a result, the lingual side of mouse incisors lacks ameloblasts and enamel. Interestingly, mutant incisors possess an ameloblast-like cell population at the lingual side. This suggests that Bcl11b functions as a critical regulator of epithelial cell fate and differentiation during tooth morphogenesis. Despite developmental roles delineated in incisors and molars, roles for Bcl11b in maintenance and homeostasis of tooth in adult remain open.

5. *Bcl11b* tumor suppressor gene

Tumor suppressor genes act as inhibitory signals for uncontrolled cell growth and some play a role in DNA repair or cell survival.[69] p53 is one of the most important tumor suppressors, often called the guardian of the genome because of its central role in maintaining the integrity of the cell's DNA by controlling cell cycle inhibition, repair and apoptosis.[70] APC, another important tumor suppressor, is a negative regulator on the Wnt/β-catenin signaling pathway.[71] The signaling in intestines is required for differentiation of enterocytes and secretory cells and also for maintaining stem cells and progenitors within the intestinal crypt.[71)–74)] *APC* inactivating mutations or activating β-catenin mutations impair the balance between cell proliferation, differentiation and apoptosis that affects the net number of cells in the tissue. As a consequence, it leads to formation of benign polyps or adenoma cells.

Bcl11b is a tumor suppressor and loss of a *Bcl11b* allele contributes to thymic lymphoma development. On the other hand, Bcl11b is a lineage-specific transcription factor probably responsible for turning on cell type-specific genes. Regulated expression of Bcl11b is important for differentiation of T cells and other types of cells, as described above. There are many other transcription factors affecting both cell differentiation and cancer development, and a well known precedent is β-catenin. Bcl11b and β-catenin have similar properties in cell proliferation, differentiation and apoptosis, and hence deregulation of these properties might be the contribution of loss of a *Bcl11b* allele to lymphomagenesis. In the following sections I will describe haploinsufficiency of Bcl11b for tumor suppression and how Bcl11b is involved in apoptosis, proliferation and differentiation.

Although *Bcl11b* was identified as a tumor suppressor gene in the mouse model, genetic changes were also observed in human malignancy.[32),75),76)] Mutations or deletion of *BCL11B* gene were found in approximately 10%–16% of human T-cell acute lymphoblastic leukemia (T-ALL).[32),76),77)] This indicates the involvement of Bcl11b in human malignancy as well.

6. Haploinsufficiency of Bcl11b for tumor suppression

As for tumor suppressor genes, loss of two alleles normally contributes to tumorigenesis, and this is known as the Knudson' two-hit theory. However, the Bcl11b tumor suppressor gene is exceptional, belonging to a class of haploinsufficient tumor suppressor genes.[78),79)]

Figure 3A shows cumulative incidences of thymic lymphomas in *Bcl11b*$^{+/+}$ mice and *Bcl11b*$^{KO/+}$ mice after γ-irradiation.[2)] The thymic lymphoma incidence was much higher in *Bcl11b*$^{KO/+}$ mice than wild-type mice. Figure 3B shows cumulative incidences of spontaneously developed thymic lymphomas in *Bcl11b*$^{KO/+}$ mice, *p53*$^{KO/+}$ mice and *Bcl11b*$^{KO/+}$*p53*$^{KO/+}$ doubly heterozygous mice. The incidences in *Bcl11b*$^{KO/+}$ mice and *p53*$^{KO/+}$ mice were low until one year of the age whereas the incidence in *Bcl11b*$^{KO/+}$*p53*$^{KO/+}$ mice was very high. These results suggest that loss of one *Bcl11b* allele does not affect lymphomagenesis in basal conditions but contributes to lymphomagenesis in radiation-induced injury conditions or in the *p53*$^{KO/+}$ heterozygous genetic background. One characteristic of the tumor suppressor gene *Bcl11b* is that its suppressive capacity is haploinsufficient, one wild-type allele being insuffi-

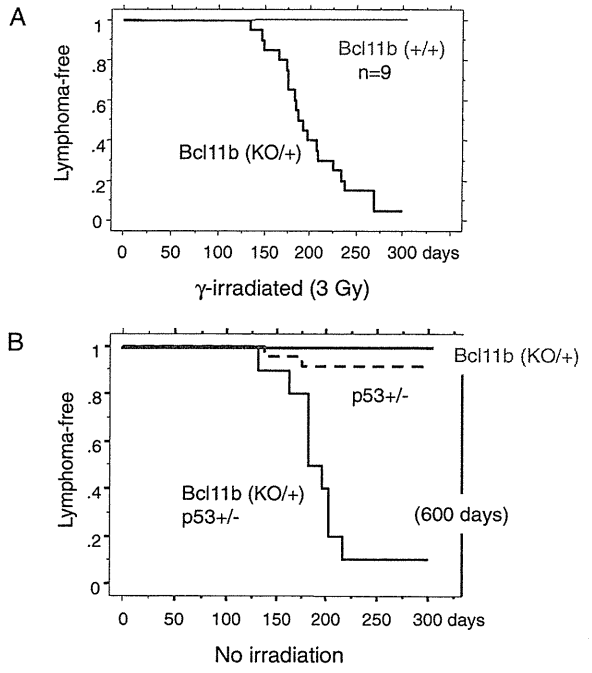

Fig. 3. The cumulative frequency distributions of thymic lymphomas (Kaplan-Mier analysis). (A) Analyzed are the thymic lymphomas that were induced by 3Gy γ-irradiation in *Bcl11b*$^{KO/+}$ mice and *Bcl11b*$^{+/+}$ mice. The curve of *Bcl11b*$^{KO/+}$ mice is shown in black and that of *Bcl11b*$^{+/+}$ mice in gray. (B) Thymic lymphomas spontaneously developed in *Bcl11b*$^{KO/+}$*p53*$^{+/-}$ mice at a high frequency in black, but vary rare in *Bcl11b*$^{+/+}$*p53*$^{+/-}$ mice in black dashed line or *Bcl11b*$^{+/-}$*p53*$^{+/+}$ mice in gray.

cient for tumor suppression. This is based on the finding that most of the thymic lymphomas developed in *Bcl11b*$^{KO/+}$ mice retained the wild-type allele although the thymic lymphomas developed in wild-type mice showed loss of one *Bcl11b* allele at a high frequency.[2),80)] The retention of the wild-type allele was also observed in spontaneously developed thymic lymphomas in *Bcl11b*$^{KO/+}$*p53*$^{KO/+}$ mice.[81)] Of importance, the retention of one *BCL11B* allele was also observed in human T-ALLs having mutations on the *BCL11B* gene.[32),76)] Because loss of one allele is an easier event and much more frequent than loss of two alleles, the haploinsufficiency of Bcl11b/BCL11B tumor suppressor is important from the point of etiological view.

7. Bcl11b and apoptosis

Keeping one *Bcl11b* wild-type allele in most thymic lymphomas may be related to the apoptosis that is often seen in cells with loss of two *Bcl11b* alleles. As described above apoptosis occurs in not all but certain types of thymocytes in *Bcl11b*-knockout mice. An anti-apoptotic property with Bcl11b has been demonstrated using Bcl11b knock-down (KD) Jurkat cells, a T-cell culture line. When Bcl11b expression was heavily reduced using siRNA method, Bcl11b-KD cells underwent apoptosis.[82),83)] On the other hand, we obtained KD cell lines retaining Bcl11b expression at certain levels that were viable and able to proliferate in the serum concentration of 5%.[82)] When the serum concentration was increased from 5% to 10% serum, the Bcl11b-KD cell lines showed growth inhibition due to cell death, accompanying decreased expressions of the CDK inhibitor p27 and the anti-apoptotic protein Bcl-xL. This suggests that the level of apoptosis is related to the cell proliferation rate, which can be controlled by serum concentration in the culture medium.

Further analysis showed that apoptosis occurred in S phase of the cell cycle and impaired activation of the cell-cycle checkpoint kinase Chk1. Activated Chk1 through phosphorylation, which is induced by DNA replication stress and subsequent formation of single-stranded DNA, leads to the arrest of cell cycle progression to allow DNA repair. Failure of the cell cycle arrest may be a cause of apoptosis in Bcl11b-KD cells by inducing proapoptotic signals. Consistently, radiation with UV, an agent producing single-stranded DNA, enhanced apoptosis more in those Bcl11b-KD Jurkat cell lines than in control Jurkat cells. Therefore, the apoptosis may be a reflection of deregulated cell cycle progression leading to DNA replication stress and of an accumulation of DNA damages during the S phase. These suggest that Bcl11b plays a role in the recovery for DNA replication stress. The anti-apoptotic property of Bcl11b may contradict the tumor suppressor function because apoptosis has been considered as a mechanism to eliminate deleterious cells with damaged DNA. However, a different interpretation is also possible. Hyperplastic or dysplastic cells in premalignant lesions often exhibit apoptotic phenotype together with high mitotic index.[84),85)] Therefore, the apoptosis may be a phenotype of precancerous cells, and the cells susceptible to apoptosis can be progressed to a rapidly progressive tumor when they acquire the ability to escape apoptosis.

8. Premalignancy and cell proliferation

Premalignant conditions are recognizable lesions that are strongly associated with the development of malignant neoplasia. They differ from normal conditions and hence may possess properties unique to

them. Normal cells possess checkpoint function that can perceive and arrest aberrant cell cycle triggered by cancer-promoting stimuli. The checkpoint functions as an inducible barrier against clonal cell expansion and genomic instability leading to tumor development. Accordingly, premalignancy might be related to impairment or inability of the checkpoint barrier function. Indeed, premalignant legions in human tissues exhibit signatures of persistent functioning of checkpoint, for instance, elevated protein expressions relevant for DNA damage responses.[84),85)]

γ-irradiation to *Bcl11b*$^{KO/+}$ mice confers the thymus atrophic, the cell number being reduced approximately one tenth, and the atrophic thymus will develop thymic lymphomas. This implies that the atrophic thymus is in a premalignant condition and comprises a lesion harboring premalignant cells. Supporting evidence is that the mice that had received thymocytes from the atrophic thymus developed thymic lymphomas at a high frequency.[86),87)] Other studies also show the presence of premalignant cells in atrophic thymus.[88),89)] In general, a small thymus with increased apoptosis and an expanded proportion of immature DN thymocytes characterizes the premalignant cells. For instance, transgenic mice expressing the oncogene *Lmo2* develop T-cell leukemia after a long latency period, keeping atrophic thymus before the development.[90),91)] Of note, the induction of atrophic thymus is caused by γ-irradiation but not a direct consequence of irradiation, because the thymus is recovered from damages within one week after γ-irradiation and atrophy of the thymus begins approximately three weeks after.[92)] To characterize γ-ray induced atrophic thymus is important to elucidate how loss of Bcl11b contributes to premalignancy and tumor development at initial stages.

Cell proliferation of clonal origin is a hallmark of premalignant cells. Clonal proliferation of certain thymocytes was observed in about a half of atrophic thymuses in *Bcl11b*$^{KO/+}$ mice at as early as 60 days after irradiation.[92)] Clonality was determined by assaying specific V(D)J rearrangements with three primer sets designed for the *TCRβ* locus. Recombination leading to the V(D)J rearrangements occurs in thymocytes at DN3 stage before β-selection. Figure 4A shows positions of primers and band patterns of PCR products in gel electrophoresis. Normal thymocytes within a thymus exhibit a DJ rearrangement pattern of all six distinct bands reflecting polyclonal origin of thymocytes. However, atrophic thymuses showed a few prominent band patterns of rearrangement (Fig. 4A), indicating clonal expansion of a few parental thymocytes having passed β-selection. The thymus is here designated as C-type thymus (C stands for clonal expansion) and the other thymuses are called T-type thymus (T stands for normal thymus).

The C-type thymus or clonal expansion was also detected in atrophic thymuses that were γ-ray induced in *Bcl11b*$^{+/+}$ wild-type mice.[93)] In this case, irradiation is required 4 times of 2.5 Gy at one-week interval for efficient lymphoma development, because *Bcl11b*$^{+/+}$ mice are much less susceptible to thymic lymphomas than *Bcl11b*$^{KO/+}$ mice. Approximately a half of those C type thymuses showed allelic loss at *Bcl11b* locus, suggesting that the allelic loss contributes to lymphomagenesis and possibly to clonal expansion of thymocytes. However, those atrophic thymuses did not exhibit the activation of DNA damage checkpoints such as γH2AX, Chk1, Chk2 or p53,[93)] which is a hallmark of human precancerous cells.[84),85)] This was an unexpected result to us. Further study is necessary to elucidate relationship among the atrophic thymus, premalignancy and the activation of checkpoint function. Collectively, these results suggest that loss of one *Bcl11b* allele in *Bcl11b*$^{KO/+}$ mice contributes to clonal cell proliferation at an early stage of thymic lymphoma development.

9. Bcl11b and cell differentiation

Figure 4B shows flowcytometry of T-type and C-type thymuses. Despite clonal cell expansion, a half of the C-type *Bcl11b*$^{KO/+}$ thymuses comprised thymocyte subtypes in the same proportion as normal thymus, mostly consisting of CD4$^+$CD8$^+$ DP cells. This indicates their retention of the capability to differentiate from DN3 cells to DP cells and further to SP cells. Accordingly, the C-type thymocytes have properties to undergo many rounds of cell division cycle within the thymus and to capable to differentiate. These capabilities must have been acquired at a developmental stage after β-selection. It is because, if not, the clonally proliferating thymocytes would have shown normal, but not skewed, distribution of the V(D)J recombination patterns.

The other half of the C-type *Bcl11b*$^{KO/+}$ thymuses consisted mostly of immature thymocytes with differentiation arrest at DN or/and ISP stages (the third low in Fig. 4B). This suggests that this class of C-type thymocytes lacks the capability to differentiate to DP cells. The differentiation arrest, a

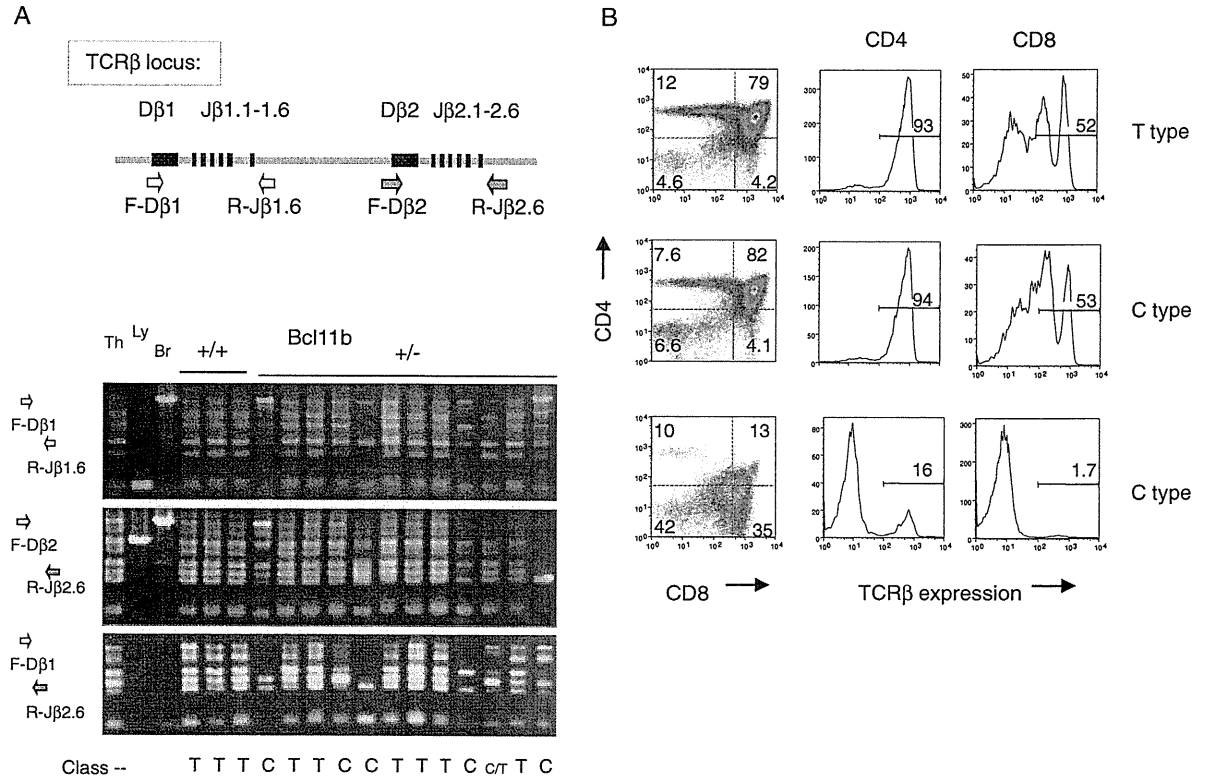

Fig. 4. Clonal proliferation of thymocytes in atrophic thymuses after γ-irradiation of *Bcl11b*$^{KO/+}$ mice. (A) The upper diagram shows part of the *TCRβ* locus and the relative location of PCR primers. The lower panel shows gel electrophoresis of PCR products with three different sets of primers, F-Dβ1 and R-Jβ1.6 (top), F-Dβ2 and R-Jβ2.6 (middle), and F-Dβ1 and R-Jβ2.6 (bottom). Thymuses are classified into two group, T type thymus (similar to normal thymus) and C type thymus (showing clonal expansion) depending on band patterns of PCR products. The T type thymus shows all six different bands as normal thymus does, whereas the C type thymus shows skewed band patterns, a few bands being more prominent than the other bands. The latter pattern indicates the presence of clonally proliferating thymocytes. (B) Flowcytometry of CD4, CD8 and TCRβ expression on thymocytes, showing differences in differentiation level. The vertical axis shows CD4 expression and the horizontal axis displays CD8 expression (left); the vertical axis shows cell number and the horizontal axis displays TCRβ expression of thymocytes in the CD4 quadrant (middle) and in the CD8 quadrant (right). Some C type thymuses showed differentiation arrest and lower expression of TCRβ on surface.

hallmark of cancer, was found only in this subgroup of the *Bcl11b*$^{KO/+}$ C-type thymocytes, but not seen in the *Bcl11b*$^{+/+}$ C-type thymocytes.[91] Of note, nevertheless approximately a half of the *Bcl11b*$^{+/+}$ C-type thymocytes lost one allele of *Bcl11b*. This suggests that acquired loss of a *Bcl11b* allele did not affect the differentiation arrest in those thymocytes. Though it is not clear at which developmental stage the acquisition of loss occurred, it is likely that the *Bcl11b* allelic loss took place at the stage after V(D)J recombination because of limited V(D)J recombination patterns detected. Li *et al.*[46] demonstrated that the expression of Bcl11b begins at the early DN1 cell stage in thymus. The DN1 cell population is multipotent and has the potential to differentiate into macrophage, NK cells, T cells and others. Actual T cell precursors within the DN1 population are CD117^{high}, whereas CD117^{low} DN1 cells are precursors for NK and $\gamma\delta$T cells or others.[94] Bcl11b expression was observed in CD117^{low} DN1 progenitor cells but not in CD117^{high} DN1 cells.[46] Hence, *Bcl11b* heterozygosity can affect lymphomagenesis at CD117^{high} DN2 stage after CD117^{high} DN1 stage. Though impaired differentiation is a hallmark of cancer, it would not alone be sufficient for DN or ISP thymocytes to acquire malignancy. The number of thymocytes in C-type thymuses is low (one tenth in average relative to normal thymus) and hence the C-type thymocytes are obviously not fully malignant. To establish full malignancy, some of the C-type

thymocytes must acquire an additional genetic or epigenetic change(s). Together, these findings suggest that the differentiation arrest is related to *Bcl11b*$^{+/-}$ genotype and may be ascribed to Bcl11b loss in immature thymocytes after early CD117^{low} DN1 stage and possibly before the ISP or DP stage.

10. Cancer stem cells and C-type thymocytes

Recently, McCormack *et al.* have shown an interesting finding of premalignant thymocytes in atrophic thymus, using the *Lmo2*-transgenic mouse model.[90] The *LMO2* oncogene causes a subset of human T cell lymphoblastic leukemias. They used a combination of *in vivo* cell fate mapping, in which the *Lmo2* gene was constitutively expressed in the thymus but not in the bone marrow (BM), and transplantation of thymocytes from young *Lmo2*-transgenic mice. As a result, they found self-renewing thymocytes in atrophic thymus of *Lmo2*-transgenic mice that were committed T cells at the DN3 stage. Of interest, these self-renewing DN3 cells possessed many features of cancer stem cells, including the ability to serially transplant, the ability to generate mature T cells, and the expression of several genes typical of hematopoietic stem cells including stem cell marker. Thymic atrophy in *Lmo2*-transgenic mice is probably caused by loss of the entry of progenitor cells from BM due to the development of self-renewing cells within the thymus. They also showed that the *Lmo2*-induced premalignant thymocytes can survive after a high-dose irradiation, consistent with cancer stem cell hypothesis. The importance of cancer stem cells in leukemia therapy has been pointed out in relapsed acute lymphoblastic leukemia in humans.[91),95)]

As described above, some of the C-type thymocytes in atrophic thymus have two properties, to undergo many rounds of cell cycle within the thymus and to capable to differentiate into DP and SP cells. In the respect of self-renewal and differentiation, they are similar to the *Lmo2*-induced premalignant thymocytes. Analyses of *Bcl11b*$^{KO/KO}$ mice[22),46)] showed that the arrested Bcl11b-KO DN2 cells started to self-renew. This may implicate Bcl11b deficiency in the generation of cancer stem cells or premalignancy. Interestingly, a subtype of human leukemias, CML (chronic myeloid leukemia), possesses self-renewal and cell lineage capabilities, and thereby the cells are assumed to be leukemia-initiating or cancer stem cells.[95),96)] Some of the C-type thymocytes have the phenotypes of CML. Therefore, those C-type thymocytes might be lymphoma stem cells. If so, they will be a model for therapy.

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Competing financial interests

The authors declare no completing interests.

References

1) Liu, P., Li, P. and Burke, S. (2010) Critical roles of Bcl11b in T-cell development and maintenance of T-cell identity. Immunol. Rev. **238**, 138–149.
2) Wakabayashi, Y., Inoue, J., Takahashi, Y., Matsuki, A., Kosugi-Okano, H., Shinbo, T., Mishima, Y., Niwa, O. and Kominami, R. (2003) Homozygous deletions and point mutations of the *Rit1/Bcl11b* gene in γ-ray induced mouse thymic lymphomas. Biochem. Biophys. Res. Commun. **301**, 598–603.
3) Wakabayashi, Y., Watanabe, H., Inoue, J., Takeda, N., Sakata, J., Mishima, Y., Hitomi, J., Yamamoto, T., Utsuyama, M., Niwa, O., Aizawa, S. and Kominami, R. (2003) Bcl11b is required for differentiation and survival of αβ T lymphocytes. Nat. Immunol. **4**, 533–539.
4) Arlotta, P., Molyneaux, B.J., Chen, J., Inoue, J., Kominami, R. and Macklis, J.D. (2005) Neuronal subtype-specific genes that control corticospinal motor neuron development in vivo. Neuron **45**, 207–221.
5) Golonzhka, O., Liang, X., Messaddeq, N., Bornet, J.M., Campbell, A.L., Mertzger, D., Chambon, P., Ganguli-Indra, G., Leid, M. and Indra, A. (2009) Dual role of COUP-TF-interacting protein 2 in epidermal homeostasis and permeability barrier formation. J. Invest. Dermatol. **129**, 1459–1470.
6) Golonzhka, O., Metzger, D., Bornert, J.M., Bay, B.K., Gross, M.K., Kioussi, C. and Leid, M. (2009) Ctip2/Bcl11b controls ameloblast formation during mammalian odontogenesis. Proc. Natl. Acad. Sci. U.S.A. **106**, 4278–4283.
7) Okumura, H., Miyasaka, Y., Morita, Y., Nomura, T., Mishima, Y., Takahashi, S. and Kominami, R. (2011) *Bcl11b* heterozygosity leads to age-related hearing loss and degeneration of outer hair cells of the mouse cochlea. Exp. Anim. **60**, 355–361.
8) Matsumoto, Y., Kosugi, S., Shinbo, T., Chou, D., Ohashi, M., Wakabayashi, Y., Sakai, K., Okumoto, M., Mori, N., Aizawa, S., Niwa, O. and Kominami, R. (1998) Allelic loss analysis of γ-ray-induced mouse thymic lymphomas: two candidate tumor suppressor gene loci on chromosomes 12 and 16. Oncogene **16**, 2747–2754.
9) Shinbo, T., Matsuki, A., Matsumoto, Y., Kosugi, S., Takahashi, Y., Niwa, O. and Kominami, R. (1999) Allelic loss mapping and physical delineation of a

region harboring a putative thymic lymphoma suppressor gene on mouse chromosome 12. Oncogene **18**, 4131–4136.
10) Okano, H., Saito, Y., Miyazawa, T., Shinbo, T., Chou, D., Kosugi, S., Takahashi, Y., Odani, S., Niwa, O. and Kominami, R. (1999) Homozygous deletions and point mutations of the *Ikaros* gene in γ-ray-induced mouse thymic lymphomas. Oncogene **18**, 6677–6683.
11) Georgopoulos, K., Moore, D.D. and Derfler, B. (1992) Ikaros, an early lymphoid-specific transcription factor and a putative mediator for T cell commitment. Science **258**, 808–812.
12) Winandy, S., Wu, P. and Georgopoulos, K. (1995) A dominant mutation in the *Ikaros* gene leads to rapid development of leukemia and lymphoma. Cell **83**, 289–299.
13) Satterwhite, E., Sonoki, T., Willis, T.G., Harder, L., Nowak, R., Arriola, E.L., Liu, H., Price, H.P., Gesk, S., Steinemann, D., Schlegelberger, B., Oscier, D.G., Siebert, R., Tucker, P.W. and Dyer, M.J.S. (2001) The *BCL11* gene family: involvement of *BCL11A* in lymphoid malignancies. Blood **98**, 3413–3420.
14) Liu, P., Keller, J.R., Ortiz, M., Tessarollo, L., Rachel, R.A., Nakamura, T., Jenkins, N.A. and Copeland, N.G. (2003) Bcl11a is essential for normal lymphoid development. Nat. Immunol. **4**, 525–532.
15) Avram, D., Fields, A., Pretty On Top, K., Nevrivy, D.J., Ishmael, J.E. and Leid, M. (2000) Isolation of a novel family of C_2H_2 zinc finger proteins implicated in transcriptional repression mediated by chicken ovalbumin upstream promoter transcription factor (COUP-TF) orphan nuclear receptors. J. Biol. Chem. **275**, 10315–10322.
16) Wang, L.H., Tsai, S.Y., Cook, R.G., Beattie, W.G., Tsai, M.J. and O'Malley, B.W. (1989) COUP transcription factor is a member of the steroid receptor superfamily. Nature **340**, 163–166.
17) Shibata, H., Nawaz, Z., Tsai, S.Y., O'Malley, B.W. and Tsai, M.J. (1997) Gene silencing by chicken ovalbumin upstream promoter-transcription factor I (COUP-TFI) is mediated by transcriptional corepressors, nuclear receptor-corepressor (N-CoR) and silencing mediator for retinoic acid receptor and thyroid hormone receptor (SMRT). Mol. Endocrinol. **11**, 714–724.
18) Cismasiu, V.B., Adamo, K., Gecewicz, J., Duque, J., Lin, Q. and Avram, D. (2005) BCL11B functionally associates with the NuRD complex in T lymphocytes to repress targeted promoter. Oncogene **24**, 6753–6764.
19) Topark-Ngarm, A., Golonzhka, O., Peterson, V.J., Barrett, B.Jr., Martinez, B., Crofoot, K., Filtz, T.M. and Leid, M. (2006) CTIP2 associates with the NuRD complex on the promoter of *p57KIP2*, a newly identified CTIP2 target gene. J. Biol. Chem. **281**, 32272–32283.
20) Guo, P., Hirano, M., Herrin, B.R., Li, J., Yu, C., Sadlonova, A. and Cooper, M.D. (2009) Dual nature of the adaptive immune system in lampreys. Nature **459**, 796–801.
21) Bajoghli, B., Guo, P., Aghaallaei, N., Hirano, M., Strohmeier, C., McCurley, N., Bockman, D.E., Schorpp, M., Cooper, M.D. and Boehm, T. (2009) Evolution of genetic networks underlying the emergence of thymopoiesis in vertebrates. Cell **138**, 186–197.
22) Ikawa, T., Hirose, S., Masuda, K., Kakugawa, K., Satoh, R., Shibano-Satoh, A., Kominami, R., Katsura, Y. and Kawamoto, H. (2010) An essential developmental checkpoint for production of the T cell lineage. Science **329**, 93–96.
23) Nakamura, T., Yamazaki, Y., Saiki, Y., Moriyama, M., Largaespada, D.A., Jenkins, N.A. and Copeland, N.G. (2000) *Evi9* encodes a novel zinc finger protein that physically interacts with BCL6, a known human B-cell proto-oncogene product. Mol. Cell. Biol. **20**, 3178–3186.
24) Menzel, S., Garner, C., Gut, I., Matsuda, F., Yamaguchi, M., Heath, S., Foglio, M., Zelenika, D., Boland, A., Rooks, H., Best, S., Spector, T.D., Farrall, M., Lathrop, M. and Thein, S.L. (2007) A QTL influencing F cell production maps to a gene encoding a zinc-finger protein on chromosome 2p15. Nat. Genet. **39**, 1197–1199.
25) Uda, M., Galanello, R., Sanna, S., Lettre, G., Sankaran, V.G., Chen, W., Usala, G., Busonero, F., Maschio, A., Albai, G., Piras, M.G., Sestu, N., Lai, S., Dei, M., Mulas, A., Crisponi, L., Naitza, S., Asunis, I., Deiana, M., Nagaraja, R., Perseu, L., Satta, S., Cipollina, M.D., Sollaino, C., Moi, P., Hirschhorn, J.N., Orkin, S.H., Abecasis, G.R., Schlessinger, D. and Cao, A. (2008) Genome-wide association study shows *BCL11A* associated with persistent fetal hemoglobin and amelioration of the phenotype of β-thalassemia. Proc. Natl. Acad. Sci. U.S.A. **105**, 1620–1625.
26) Sankaran, V.G., Menne, T.F., Xu, J., Akie, T.E., Lettre, G., Van Handel, B., Mikkola, H.K., Hirschhorn, J.N., Cantor, A.B. and Orkin, S.H. (2008) Human fetal hemoglobin expression is regulated by the developmental stage-specific repressor *BCL11A*. Science **322**, 1839–1842.
27) Sankaran, V.G., Xu, J., Ragoczy, T., Ippolito, G.C., Walkley, C.R., Maika, S.D., Fujiwara, Y., Ito, M., Groudine, M., Bender, M.A., Tucker, P.W. and Orkin, S.H. (2009) Developmental and species-divergent globin switching are driven by BCL11A. Nature **460**, 1093–1097.
28) Borg, J., Papadopoulos, P., Georgitsi, M., Gutiérrez, L., Grech, G., Fanis, P., Phylactides, M., Verkerk, A.J., van der Spek, P.J., Scerri, C.A., Cassar, W., Galdies, R., van Ijcken, W., Özgür, Z., Gillemans, N., Hou, J., Bugeja, M., Grosveld, F.G., von Lindern, M., Felice, A.E., Patrinos, G.P. and Philipsen, S. (2010) Haploinsufficiency for the erythroid transcription factor KLF1 causes hereditary persistence of fetal hemoglobin. Nat. Genet. **42**, 801–805.
29) Zhou, D., Liu, K., Sun, C.W., Pawlik, K.M. and Townes, T.M. (2010) KLF1 regulates BCL11A expression and γ- to β-globin gene switching. Nat.

Genet. **42**, 742–744.
30) Avram, D., Fields, A., Senawong, T., Topark-Ngarm, A. and Leid, M. (2002) COUP-TF (chicken ovalbumin upstream promoter transcription factor)-interacting protein 1 (CTIP1) is a sequence-specific DNA binding protein. Biochem. J. **368**, 555–563.
31) Cismasiu, V.B., Duque, J., Paskaleva, E., Califano, D., Ghanta, S., Young, H.A. and Avram, D. (2009) BCL11B enhances TCR/CD28-triggered NF-κB activation through up-regulation of Cot kinase gene expression in T-lymphocytes. Biochem. J. **417**, 457–466.
32) Gutierrez, A., Kentsis, A., Sanda, T., Holmfeldt, L., Chen, S.C., Zhang, J., Protopopov, A., Chin, L., Dahlberg, S.E., Neuberg, D.S., Silverman, L.B., Winter, S.S., Hunger, S.P., Sallan, S.E., Zha, S., Alt, F.W., Downing, J.R., Mullighan, C.G. and Look, A.T. (2011) The *BCL11B* tumor suppressor is mutated across the major molecular subtypes of T-cell acute lymphoblastic leukemia. Blood **118**, 4169–4173.
33) Marban, C., Redel, L., Suzanne, S., Van Lint, C., Lecestre, D., Chasserot-Golaz, S., Leid, M., Aunis, D., Schaeffer, E. and Rohr, O. (2007) Recruitment of chromatin-modifying enzymes by CTIP2 promotes HIV-1 transcriptional silencing. EMBO J. **26**, 412–423.
34) Senawong, T., Peterson, V.J., Avram, D., Shepherd, D.M., Frye, R.A., Minucci, S. and Leid, M. (2003) Involvement of the histone deacetylase SIRT1 in chicken ovalbumin upstream promoter transcription factor (COUP-TF)-interacting protein 2-mediated transcriptional repression. J. Biol. Chem. **278**, 43041–43050.
35) Senawong, T., Peterson, V.J. and Leid, M. (2005) BCL11A-dependent recruitment of SIRT1 to a promoter template in mammalian cells results in histone deacetylation and transcriptional repression. Arch. Biochem. Biophys. **434**, 316–325.
36) Cismasiu, V.B., Ghanta, S., Duque, J., Albu, D.I., Chen, H.M., Kasturi, R. and Avram, D. (2006) BCL11B participates in the activation of *IL2* gene expression in $CD4^+$ T lymphocytes. Blood **108**, 2695–2702.
37) Cherrier, T., Suzanne, S., Redel, L., Calao, M., Marban, C., Samah, B., Mukerjee, R., Schwartz, C., Gras, G., Sawaya, B.E., Zeichner, S.L., Aunis, D., Van Lint, C. and Rohr, O. (2009) $p21^{WAF1}$ gene promoter is epigenetically silenced by CTIP2 and SUV39H1. Oncogene **28**, 3380–3389.
38) Obata, M. Kominami, R. and Mishima, Y. (2012) BCL11B tumor suppressor inhibits *HDM2* expression in a p53-dependent manner. Cell. Signal. **24**, 1047–1052.
39) Kastner, P., Chan, S., Vogel, W.K., Zhang, L.J., Topark-Ngarm, A., Golonzhka, O., Jost, B., Le Gras, S., Gross, M.K. and Leid, M. (2010) Bcl11b represses a mature T-cell gene expression program in immature $CD4^+CD8^+$ thymocytes. Eur. J. Immunol. **40**, 2143–2154.
40) He, X., He, X., Dave, V.P., Zhang, Y., Hua, X., Nicolas, E., Xu, W., Roe, B.A. and Kappes, D.J. (2005) The zinc finger transcription factor *Th-POK* regulates CD4 versus CD8 T-cell lineage commitment. Nature **433**, 826–833.
41) Setoguchi, R., Tachibana, M., Naoe, Y., Muroi, S., Akiyama, K., Tezuka, C., Okuda, T. and Taniuchi, I. (2008) Repression of the transcription factor Th-POK by Runx complexes in cytotoxic T cell development. Science **319**, 822–825.
42) Tang, B., Di Lena, P., Schaffer, L., Head, S.R., Baldi, P. and Thomas, E.A. (2011) Genome-wide identification of Bcl11b gene targets reveals role in brain-derived neurotrophic factor signaling. PLoS One **6** (9), e23691, doi:10.1371/journal.pone.0023691.
43) Albu, D.I., VanValkenburgh, J., Morin, N., Califano, D., Jenkins, N.A., Copeland, N.G., Liu, P. and Avram, D. (2007) BCL11B is required for positive selection and survival of double-positive thymocytes. J. Exp. Med. **204**, 3003–3015.
44) Zhang, S., Rozell, M., Verma, R.K., Albu, D.I., Califano, D., VanValkenburgh, J., Merchant, A., Rangel-Moreno, J., Randall, T.D., Jenkins, N.A., Copeland, N.G., Liu, P. and Avram, D. (2010) Antigen-specific clonal expansion and cytolytic effector function of $CD8^+$T lymphocytes depend on the transcription factor Bcl11b. J. Exp. Med. **207**, 1687–1699.
45) Albu, D.I., VanValkenburgh, J., Morin, N., Califano, D., Jenkins, N.A., Copeland, N.G., Liu, P. and Avram, D. (2011) Transcription factor Bcl11b controls selection of invariant natural killer T-cells by regulating glycolipid presentation in double-positive thymocytes. Proc. Natl. Acad. Sci. U.S.A. **108**, 6211–6216.
46) Li, P., Burke, S., Wang, J., Chen, X., Ortiz, M., Lee, S.C., Lu, D., Campos, L., Goulding, D., Ng, B.L., Dougan, G., Huntly, B., Gottgens, B., Jenkins, N.A., Copeland, N.G., Colucci, F. and Liu, P. (2010) Reprogramming of T cells to natural killer-like cells upon *Bcl11b* deletion. Science **329**, 85–89.
47) Li, L., Leid, M. and Rothenberg, E.V. (2010) An early T cell lineage commitment checkpoint dependent on the transcription factor *Bcl11b*. Science **329**, 89–93.
48) Weber, B.N., Chi, A.W., Chavez, A., Yashiro-Ohtani, Y., Yang, Q., Shestova, O. and Bhandoola, A. (2011) A critical role for TCF-1 in T-lineage specification and differentiation. Nature **476**, 63–68.
49) Maillard, I., Fang, T. and Pear, W.S. (2005) Regulation of lymphoid development, differentiation, and function by the Notch pathway. Annu. Rev. Immunol. **23**, 945–974.
50) Taghon, T.N., David, E.S., Zúñiga-Pflücker, J.C. and Rothenberg, E.V. (2005) Delayed, asynchronous, and reversible T-lineage specification induced by Notch/Delta signaling. Genes Dev. **19**, 965–978.
51) Rothenberg, E.V., Moore, J.E. and Yui, M.A. (2008) Launching the T-cell-lineage developmental programme. Nat. Rev. Immunol. **8**, 9–21.
52) Rothenberg, E.V., Zhang, J. and Li, L. (2010)

Multilayered specification of the T-cell lineage fate. Immunol. Rev. **238**, 150–168.
53) Gounari, F., Aifantis, I., Khazaie, K., Hoeflinger, S., Harada, N., Taketo, M.M. and von Boehmer, H. (2001) Somatic activation of β-catenin bypasses pre-TCR signaling and TCR selection in thymocyte development. Nat. Immunol. **2**, 863–869.
54) Xue, L., Nolla, H., Suzuki, A., Mak, T.W. and Winto, A. (2008) Normal development is an integral part of tumorigenesis in T cell-specific PTEN-deficient mice. Proc. Natl. Acad. Sci. U.S.A. **105**, 2022–2027.
55) Wada, H., Masuda, K., Satoh, R., Kakugawa, K., Ikawa, T., Katsura, Y. and Kawamoto, H. (2008) Adult T-cell progenitors retain myeloid potential. Nature **452**, 768–772.
56) Bell, J.J. and Bhandoola, A. (2008) The earliest thymic progenitors for T cells possess myeloid lineage potential. Nature **452**, 764–767.
57) Inoue, J., Kanefuji, T., Okazuka, K., Watanabe, H., Mishima, Y. and Kominami, R. (2006) Expression of TCRαβ partly rescues developmental arrest and apoptosis of αβT cells in *Bcl11b*$^{KO/KO}$ mice. J. Immunol. **176**, 5871–5879.
58) Shinkai, Y., Rathbun, G., Lam, K.P., Oltz, E.M., Stewart, V., Mendelsohn, M., Charron, J., Datta, M., Young, F., Stall, A.M. and Alt, F.W. (1992) RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. Cell **68**, 855–867.
59) Mombaerts, P., Clarke, A.R., Rudnicki, M.A., Iacomini, J., Itohara, S., Lafaille, J.J., Wang, L., Ichikawa, Y., Jaenisch, R.M., Hooper, L. and Tonegawa, S. (1992) Mutations in T-cell antigen receptor genes α and block thymocyte development at different stages. Nature **360**, 225–231.
60) Shinkai, Y., Koyasu, S., Nakayama, K., Murphy, K.M., Loh, D.Y., Reinherz, E.L. and Alt, F.W. (1993) Restoration of T cell development in RAG-2-deficient mice by functional TCR transgenes. Science **259**, 822–825.
61) Bogue, M.A., Zhu, C., Aguilar-Cordova, E., Donehower, L.A. and Roth, D.B. (1996) p53 is required for both radiation-induced differentiation and rescue of *V(D)J* rearrangement in scid mouse thymocytes. Genes Dev. **10**, 553–565.
62) Jiang, D., Lenardo, M.J. and Zuniga-Pflucker, J.C. (1996) p53 prevents maturation to the CD4$^+$CD8$^+$ stage of thymocyte differentiation in the absence of T cell receptor rearrangement. J. Exp. Med. **183**, 1923–1928.
63) Haks, M.C., Krimpenfort, P.J., van den Brakel, H. and Kruisbeek, A.M. (1999) Pre-TCR signaling and inactivation of p53 induces crucial cell survival pathways in pre-T cells. Immunity **11**, 91–101.
64) Okazuka, K., Wakabayashi, Y., Kashihara, M., Inoue, J., Sato, T., Yokoyama, M., Aizawa, S., Aizawa, Y., Mishima, Y. and Kominami, R. (2005) p53 prevents maturation of T cell development to the immature CD4$^-$CD8$^+$ stage in *Bcl11b*$^{-/-}$ mice. Biochem. Biophys. Res. Commun. **328**, 545–549.
65) Arlotta, P., Molyneaux, B.J., Jabaudon, D., Yoshida, Y. and Macklis, J.D. (2008) *Ctip2* controls the differentiation of medium spiny neurons and the establishment of the cellular architecture of the striatum. J. Neurosci. **28** (3), 622–632.
66) Enomoto, T., Ohmoto, M., Iwata, T., Uno, A., Saitou, M., Yamaguchi, T., Kominami, R., Matsumoto, I. and Hirota, J. (2011) *Bcl11b/Ctip2* controls the differentiation of vomeronasal sensory neurons in mice. J. Neurosci. **31** (28), 10159–10173.
67) Mack, J.A., Anand, S. and Maytin, E.V. (2005) Proliferation and cornification during development of the mammalian epidermis. Birth Defects Res. C Embryo Today **75**, 314–329.
68) Tummers, M. and Thesleff, I. (2003) Root or crown: a developmental choice orchestrated by the differential regulation of the epithelial stem cell niche in the tooth of two rodent species. Development **130**, 1049–1057.
69) Kinzler, K.W. and Vogelstein, B. (1996) Lessons form hereditary colorectal cancer. Cell **87**, 159–170.
70) Vousden, K.H. and Prives, C. (2009) Blinded by the light: the growing complexity of p53. Cell **137**, 413–431.
71) Clevers, H. (2006) Wnt/β-catenin signaling in development and disease. Cell **127**, 469–480.
72) Polakis, P. (2000) Wnt signaling and cancer. Genes Dev. **14**, 1837–1851.
73) Fevr, T., Robine, S., Louvard, D. and Huelsken, J. (2007) Wnt/β-catenin is essential for intestinal homeostasis and maintenance of intestinal stem cells. Mol. Cell. Biol. **27**, 7551–7559.
74) Li, L. and Clevers, H. (2010) Coexistence of quiescent and active adult stem cells in mammals. Science **327**, 542–545.
75) Przybylski, G.K., Dik, W.A., Wanzeck, J., Grabarczyk, P., Majunke, S., Martin-Subero, J.I., Siebert, R., Dölken, G., Ludwig, W.D., Verhaaf, B., van Dongen, J.J., Schmidt, C.A. and Langerak, A.W. (2005) Disruption of the *BCL11B* gene through inv(14)(q11.2q32.31) results in the expression of *BCL11B-TRDC* fusion transcripts and is associated with the absence of wild-type *BCL11B* transcripts in T-ALL. Leukemia **19**, 201–208.
76) De Keersmaecker, K., Real, P.J., Gatta, G.D., Palomero, T., Sulis, M.L., Tosello, V., Van Vlierberghe, P., Barnes, K., Castillo, M., Sole, X., Hadler, M., Lenz, J., Aplan, P.D., Kelliher, M., Kee, B.L., Pandolfi, P.P., Kappes, D., Gounari, F., Petrie, H., Van der Meulen, J., Speleman, F., Paietta, E., Racevskis, J., Wiernik, P.H., Rowe, J.M., Soulier, J., Avran, D., Cavé, H., Dastugue, N., Raimondi, S., Meijerink, J.P., Cordon-Cardo, C., Califano, A. and Ferrando, A.A. (2010) The *TLX1* oncogene drives aneuploidy in T cell transformation. Nat. Med. **16**, 1321–1327.
77) Paganin, M. and Ferrando, A. (2011) Molecular pathogenesis and targeted therapies for NOTCH1-induced T-cell acute lymphoblastic leukemia.

Blood Rev. **25**, 83–90.
78) Kumar, M.S., Pester, R.E., Chen, C.Y., Lane, K., Chin, C., Lu, J., Kirsch, D.G., Golub, T.R. and Jacks, T. (2009) *Dicer1* functions as a haploinsufficient tumor suppressor. Genes Dev. **23**, 2700–2704.
79) Alimonti, A., Carracedo, A., Clohessy, J.G., Trotman, L.C., Nardella, C., Egia, A., Salmena, L., Sampieri, K., Haveman, W.J., Brogi, E., Richardson, A.L., Zhang, J. and Pandolfi, P.P. (2010) Subtle variations in *Pten* dose determine cancer susceptibility. Nat. Genet. **42**, 454–458.
80) Ohi, H., Mishima, Y., Kamimura, K., Maruyama, M., Sasai, K. and Kominami, R. (2007) Multi-step lymphomagenesis deduced from DNA changes in thymic lymphomas and atrophic thymuses at various times after γ-irradiation. Oncogene **26**, 5280–5289.
81) Kamimura, K., Mishima, Y., Obata, M., Endo, T., Aoyagi, Y. and Kominami, R. (2007) Lack of Bcl11b tumor suppressor results in vulnerability to DNA replication stress and damages. Oncogene **26**, 5840–5850.
82) Kamimura, K., Ohi, H., Kubota, T., Okazuka, K., Yoshikai, Y., Wakabayashi, Y., Aoyagi, Y., Mishima, Y. and Kominami, R. (2007) Haploinsufficiency of *Bcl11b* for suppression of lymphomagenesis and thymocyte development. Biochem. Biophys. Res. Commun. **355**, 538–542.
83) Grabarczyk, P., Przybylski, G.K., Depke, M., Völker, U., Bahr, J., Assmus, K., Bröker, B.M., Walther, R. and Schmidt, C.A. (2007) Inhibition of *BCL11B* expression leads to apoptosis of malignant but not normal mature T cells. Oncogene **26**, 3797–3810.
84) Bartkova, J., Horejsí, Z., Koed, K., Krämer, A., Tort, F., Zieger, K., Guldberg, P., Sehested, M., Nesland, J.M., Lukas, C., Ørntoft, T., Lukas, J. and Bartek, J. (2005) DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. Nature **434**, 864–870.
85) Gorgoulis, V.G., Vassiliou, L.V., Karakaidos, P., Zacharatos, P., Kotsinas, A., Liloglou, T., Venere, M., Ditullio, R.A. Jr., Kastrinakis, N.G., Levy, B., Kletsas, D., Yoneta, A., Herlyn, M., Kittas, C. and Halazonetis, T.D. (2005) Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. Nature **434**, 907–913.
86) Sado, T., Kamisaku, H. and Kubo, E. (1991) Bone marrow-thymus interactions during thymic lymphomagenesis induced by fractionated radiation exposure in B10 mice: Analysis using bone marrow transplantation between Thy 1 congenic mice. J. Radiat. Res. **32**, 168–180.
87) Kominami, R. and Niwa, O. (2006) Radiation carcinogenesis in mouse thymic lymphomas. Cancer Sci. **97**, 575–581.
88) Larson, R.C., Lavenir, I., Larson, T.A., Baer, R., Warren, A.J., Wadman, I., Nottage, K. and Rabbitts, T.H. (1996) Protein dimerization between Lmo2 (Rbtn2) and Tal1 alters thymocyte development and potentiates T cell tumorigenesis in transgenic mice. EMBO J. **15**, 1021–1027.
89) Chervinsky, D.S., Lam, D.H., Melman, M.P., Gross, K.W. and Aplan, P.D. (2001) *scid* Thymocytes with TCRβ gene rearrangements are targets for the oncogenic effect of *SCL* and *LMO1* transgenes. Cancer Res. **61**, 6382–6387.
90) McCormack, M.P., Young, L.F., Vasudevan, S., de Graaf, C.A., Codrington, R., Rabbitts, T.H., Jane, S.M. and Curtis, D.J. (2010) The Lmo2 oncogene initiates leukemia in mice by inducing thymocyte self-renewal. Science **327**, 879–883.
91) Curtis, D.J. and McCormack, M.P. (2010) The molecular basis of Lmo2-induced T-cell acute lymphoblastic leukemia. Clin. Cancer Res. **16**, 5618–5623.
92) Go, R., Hirose, S., Morita, S., Yamamoto, T., Katsuragi, Y., Mishima, Y. and Kominami, R. (2010) *Bcl11b* heterozygosity promotes clonal expansion and differentiation arrest of thymocytes in γ-irradiated mice. Cancer Sci. **101**, 1347–1353.
93) Yamamoto, T., Morita, S., Go, E., Obata, M., Katsuragi, Y., Fujita, Y., Maeda, Y., Yokoyama, M., Aoyagi, Y., Ichikawa, H., Mishima, Y. and Kominami, R. (2010) Clonally expanding thymocytes having lineage capability in γ-ray induced mouse atrophic thymus. Int. J. Radiation Oncology Biol. **77**, 235–243.
94) Lai, J.C., Wlodarska, M., Liu, D.J., Abraham, N. and Johnson, P. (2010) CD45 regulates migration, proliferation, and progression of double negative 1 thymocytes. J. Immunol. **185**, 2059–2070.
95) Calabretta, B. and Perrotti, D. (2004) The biology of CML blast crisis. Blood **103**, 4010–4022.
96) Clarke, M.F., Dick, J.E., Dirks, P.B., Eaves, C.J., Jamieson, C.H., Jones, D.L., Visvader, J., Weissman, I.L. and Wahl, G.M. (2006) Cancer stem cells-perspectives on current status and future directions: AACR Workshop on cancer stem cells. Cancer Res. **66**, 9339–9344.

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Profile

Ryo Kominami was born in 1947 and graduated from Okayama University School of Medicine at 1971. He started his research career at the Department of Biochemistry under the direction of Professor Masami Muramatsu at Tokushima University School of Medicine in 1972 and he received his PhD in 1976. After two years of study as a postdoctoral fellow at Frederic Cancer Research Institute near Washington DC in U.S.A., and then returned to Cancer Institute in Tokyo in 1979 and served as Researcher. He became Assistant Professor at the University of Tokyo in 1982 and was promoted to Associate Professor in 1983. He studied repetitive sequences, including minisatellites that were useful for individual identification. He is Professor of Biochemistry at Niigata University School of Medicine since 1987 and serving as a Vice Dean of the Graduate School of Medical and Dental Sciences at Niigata University. He isolated the *Bcl11b* tumor suppressor gene by analyzing radiation-induced mouse thymic lymphomas and found a regulatory role for Bcl11b in T-cell development. He was awarded the Japan Radiation Research Society Award in 2007.

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BCL11B tumor suppressor inhibits *HDM2* expression in a p53-dependent manner

Miki Obata, Ryo Kominami, Yukio Mishima *

Department of Molecular Genetics, Niigata University Graduate School of Medical and Dental Sciences, 1-757 Asahimachi-dori, Chuo-ku, Niigata 951-8510, Japan

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ABSTRACT

BCL11B is a C_2H_2 zinc finger transcription factor that acts as a haploinsufficient tumor suppressor. Mutations and deletion in the human orthologue *BCL11B* have been identified in human T-cell acute lymphoblastic leukemia (T-ALL) and a mouse model of thymic lymphomas. *Bcl11b*$^{KO/+}$*p53*$^{KO/+}$ doubly heterozygous mice, but not *Bcl11b*$^{KO/+}$ heterozygous mice, spontaneously develop thymic lymphomas at a high frequency, suggesting cooperativity of BCL11B and p53 in cancer development. In this study, we have examined whether or not BCL11B directly affects the p53 signaling pathway including HDM2, a ubiquitin ligase for p53 degradation. The p53 pathway regulates cell proliferation and the response to DNA damages to maintain genome integrity. Here we show that BCL11B binds to human *HDM2*-P2 promoter by ChIP (chromatin immuno-precipitation) assay and inhibits HDM2 expression in a p53-dependent manner. Deletion of the distal p53 responsive element in *HDM2* promoter region or the lack of p53 in HCT116 cells greatly reduced the repressive effect of BCL11B on *HDM2*-P2 promoter activity. The repressive activity was alleviated in γ-ray induced DNA damage conditions that activate p53, suggesting interaction between BCL11B and p53 for *HDM2* expression. These date suggest that BCL11B affects the activity of the p53-HDM2 feedback loop in basal and irradiated conditions. This may be a mechanism underlying the leukemic transformation in T-ALL and in *Bcl11b*$^{KO/+}$*p53*$^{KO/+}$ mouse thymocytes.

1. Introduction

The E3 ubiquitin ligase HDM2 (human homologue of MDM2, mouse double minute 2) and the tumor suppressor p53 form a negative feedback loop, a balanced regulatory network of proteins that controls cell cycle progression and commitment to apoptosis [1,2]. When cells undergo γ-irradiation that induces DNA damages, p53 proteins increase by phosphorylation, which in turn activates transcription of *HDM2* leading to p53 degradation. Thus, HDM2 acts as a negative regulator of p53. HDM2 binds to several other proteins such as p14^{ARF} and ribosomal protein RPL26, positive regulators of p53, and their degradation also leads to downregulation of p53 [1,2]. As for *HDM2* transcription, AP-1, Ets and Smad2/3 activate the transcription in a p53-independent manner under oncogenic pathways such as the growth factor TGFβ [2,3]. *HDM2* transcription is regulated at two distinct promoters of P1 and P2 [2–8], and P1 promoter is located upstream of the first exon of the *HDM2* gene whereas P2 promoter is situated within the first intron. P1 promoter controls basal expression of *HDM2*, and P2 promoter is highly regulated through multiple response elements within the promoter [2,3]. An activation of P2 promoter occurs via p53 in response to DNA damage agents such as γ-radiation [7,8].

Bcl11b (B cell leukemia/lymphoma-11b), also known as *Rit1* and *CTIP2*, belongs to a C_2H_2 zinc-finger transcription factor [9,10]. *Bcl11b* is expressed in various tissues including thymocytes [10–14], neurons [15,16], skin [17] and tooth [18], exhibiting critical roles in development of those organ systems. BCL11B is identified as a transcriptional repressor [19,20] by mediating either directly binds to a GC-rich consensus sequence of target genes including *p21* and *p57* and/or interacts with nucleosome remodeling and histone deacetylase (NuRD) complex [21,22].

Bcl11b was originally identified as a tumor suppressor gene in mouse thymic lymphomas, a model of human T-cell acute lymphoblastic leukemia (T-ALL) [10,23,24]. Recently, mutations and deletion of *BCL11B* have been identified in T-ALL [25–28]. Although *Bcl11b*$^{KO/KO}$ knockout mice die shortly after birth, *Bcl11b*$^{KO/+}$ heterozygous mice rarely develop thymic lymphomas [24]. However, *Bcl11b*$^{KO/+}$*p53*$^{KO/+}$ doubly heterozygous mice develop thymic lymphomas at a high frequency [23]. This suggests cooperativity between BCL11B and p53 in cancer development. In this study, we have addressed this issue by examining whether or not BCL11B directly affects the p53 and HDM2 feedback loop. Here we show that BCL11B directly interacts with P2 promoter region of *HDM2* and inhibits *HDM2* promoter activity in a p53-dependent manner.

2. Materials and methods

2.1. Plasmid construction

Bcl11b coding sequences were cloned into pcDNA3.1 (Invitrogen) as described previously [10]. Lentivirus vectors expressing shRNA for

* Corresponding author. Tel.: +81 25 227 2082; fax: +81 25 227 0757.
E-mail address: ymishima@med.niigata-u.ac.jp (Y. Mishima).

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BCL11B and full-length *Bcl11b* were constructed using BLOCK-iT lentiviral Pol II miR RNAi expression system (Invitrogen). Target sequence for *BCL11B* was described previously [29]. Promoter fragments of *HDM2* were amplified from genomic DNA of Jurkat and MOLT-4 cells and inserted into the NheI and HindIII sites of pGL3-Basic plasmid (Promega). DNAs from Jurkat cells and MOLT-4 cells carry wild-type sequence (T) and the SNP sequence (G) (the 309th nucleotide in the first intron), respectively [30]. Primers used for the *HDM2*-P1 and *HDM2*-P2 driven luciferase reporter plasmids are listed in the Supplementary Table 1. *HDM2*-P2 deletion mutants were generated using primeSTAR mutagenesis basal kit (Takara) by using primer sets listed in the Supplementary Table 1.

2.2. Cell culture, transfection and luciferase assays

Human T cell line Jurkat (p53 deficient) and MOLT-4 (p53 wild) cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS) (Nitirei). For recombinant lentivirus production, HEK293FT cells were used according to the protocol recommended by the manufacturer (Invitrogen).

HCT116 ($p53^{+/+}$ or $p53^{-/-}$) cells were cultured in DMEM medium (Sigma), supplemented with 10% FBS (Nitirei). The HCT116 ($p53^{-/-}$) cell line was kindly provided by Drs. B. Vogelstein and K.W. Kinzles (Johns Hopkins University). All transfections were performed with FuGENE 6 Reagent (Roche) according to the manufacturer's instructions. HCT116 ($p53^{+/+}$ or $p53^{-/-}$) cells were grown in 12-well plate. Plasmid DNA expressing wild-type BCL11B and indicated *HDM2* promoter-driven *Photinus pyralis* luciferase reporter plasmid (Promega) were transfected the next day. Cells were lysed after 24 h using luciferase lysis buffer (Toyo Ink), and luciferase activities were measured using the Dual-luciferase-reporter system according to the manufacturer's instructions. Transfection efficiency in luciferase reporter assays was controlled and normalized by including a constant amount of *TK-Renilla reniformis* luciferase reporter plasmid in all transfections. The activity is mostly expressed in the percent of wild-type BCL11B versus the BCL11B del Z4-6 mutant lacking activity (BCL11B del Z4-6; deleted C-terminal three zinc finger domains). The data shown were of three independent triplicate experiments as the mean ± standard deviations (SD) of the ratio between the *Photinus* and *Renilla* reporters.

γ-irradiation (10–15 Gy) was performed using a Cs^{137} irradiator.

2.3. RT-PCR

Total RNA was prepared from Jurkat, MOLT-4 or HCT116 cells by the RNA Easy Mini kit (Quiagen) according to the protocol recommended by the manufacturer. cDNA was synthesized from 5 μg of total RNA with a random primer using SuperScript II reverse transcriptase (Invitrogen) and a 10-fold dilution aliquot was used for PCR using primers described in the Supplementary Table 2. PCR program was: 1 min at 94 °C, followed by 32 cycles (30 s at 94 °C, 30 s at 54 °C, 1 min at 72 °C), followed by 5 min extension at 72 °C. PCR products were separated by 5% polyacrylamide gel electrophoresis (PAGE) and visualized as bands by staining with ethidium bromide. The intensity of bands was analyzed with Molecular Imager FX (Bio-Rad). Comparison in the band intensity was done between pLent-sh*SC* and pLent-sh*BCL11b* transfected MOLT4 cells or between pLent-*GFP* and pLent-*Bcl11b* transfected HCT116 cells.

2.4. Western blot analysis

Western blotting was performed as previously described [29]. Antibodies used were rabbit anti-BCL11B-Z [11], anti-MDM2 (R & D Systems, AF1244), anti-p53 (Cell Signaling, #2524), anti-ACTIN (Santa Cruz, sc-1615), HRP (horseradish peroxidase)-anti-goat IgG (Santa Cruz, sc-2020), HRP-anti-rabbit IgG (GE Healthcare Amersham, NA-934) and HRP-anti-mouse IgG (GE Healthcare Amersham, NA-931). Protein bands were visualized using chemiluminescent detection (ECL plus, GE Healthcare Amersham), and the intensity of bands was analyzed with Molecular Imager FX (Bio-Rad). The protein levels are shown relative to ACTIN used as a control, and are compared between pLent-sh*SC* and pLent-sh*BCL11b* transfected MOLT4 cells or between pLent-*GFP* and pLent-*Bcl11b* transfected HCT116 cells.

2.5. Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assay was performed using a ChIP assay kit (#17-295; Milopore, Upstate). Briefly, MOLT-4 and Jurkat cells were fixed with formaldehyde and sonicated. The collected chromatin solutions were incubated with anti- BCL11B antibodies (Abcom ab18465) or normal IgG (Immuno Biological Laboratories, Co. Ltd., Japan 17312) at 4 °C overnight. The immunoprecipitated complexes were eluted followed by removal of cross-links. The resulting immunoprecipitated DNA and input DNA (1% of total DNA) were subjected to PCR using primer sets described in the Supplementary Table 3. PCR products were separated by 5% PAGE and visualized by staining with ethidium bromide.

2.6. Statistical analyses

Data from at least three independent experiments were analyzed for statistical significance by Student's *t*-test. All values are expressed as mean ± SD and the value of $P<0.01$, shown by *, was considered statistically significant.

3. Results

3.1. BCL11B suppresses HDM2-P2 promoter activity

MOLT-4 cells highly expressed BCL11B but expressed HDM2 and p53 at low levels in basal conditions. We introduced pLent-sh*BCL11B* or control pLent-sh*SC* viruses into the MOLT-4 cells and examined changes in the expression of HDM2 and p53 in *BCL11B*-knockdown (KD) MOLT-4 cells. KD cells showed an increase in expression of HDM2 but not of p53 (Fig. 1A). Supplementary Fig. 1A shows failure in the specific binding of BCL11B to p53 promoter, which indicated no effect of BCL11B on p53 transcription (Supplementary Fig. 1B). Fig. 1B shows the location of P1 and P2 promoters in *HDM2* gene [2–8]. KD cells showed an increase in RNA transcripts from P2 promoter but not from P1 promoter (Fig. 1C). Supplementary Fig. 2A shows a γ-ray induced increase in RNA transcripts from P2 promoter but not from P1 promoter, and Supplementary Fig. 2B shows γ-ray induced stabilization of p53 but no change in expression of BCL11B or HDM2 proteins. These results demonstrated that reduced expression of BCL11B in MOLT-4 cells resulted in upregulation of *HDM2* transcription and that γ-irradiation affected p53 expression but not BCL11B or HDM2 expression.

We next examined BCL11B effect in HCT116 cells, which expressed HDM2 and p53 but not BCL11B. We infected viruses introducing BCL11B expression (pLent-*Bcl11b*) and control viruses (pLent-*GFP*) to HCT116 cells, and examined expressions of HDM2 and p53. Over-expression of BCL11B reduced HDM2 expression at the protein and RNA levels (Fig. 1D and E, respectively). These results suggest that BCL11B downregulates *HDM2* transcription in HCT116 cells, consistent with the repressor activity of BCL11B for *HDM2* expression found in *BCL11B*-KD MOLT-4 cells.

3.2. BCL11B binds to HDM2 promoter

To examine whether or not BCL11B associates with *HDM2* P2 promoter, we performed chromatin immunoprecipitaion (ChIP) assay in MOLT-4 and Jurkat cells expressing BCL11B. Five sets of primers were

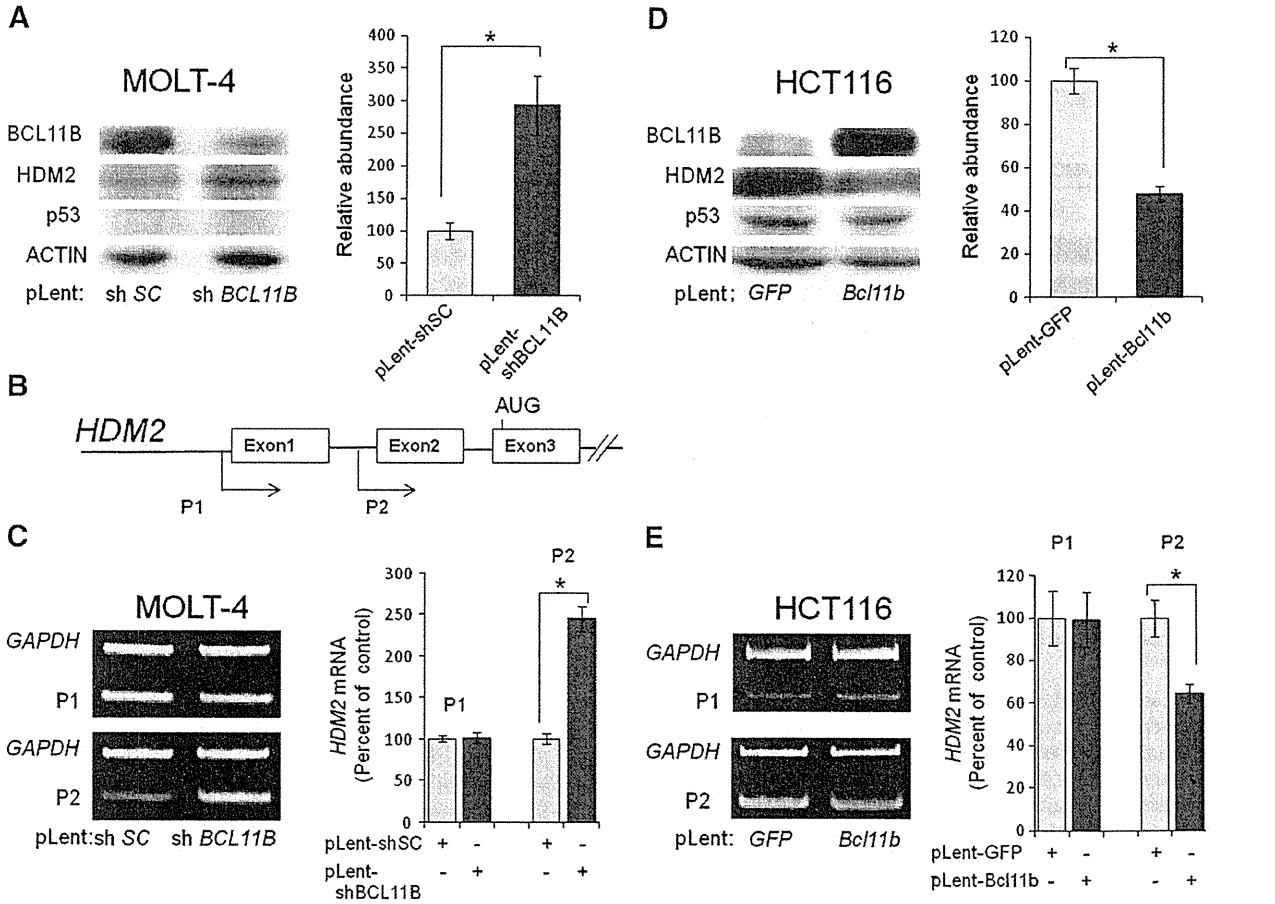

Fig. 1. BCL11B reduces *HDM2* expression by inhibiting *HDM2* promoter activity. (A) Protein expression levels in MOLT-4 cells that were transduced with control (pLent-sh*SC*) or lentivirus vector expressing shRNA for BCL11B (pLent-sh*BCL11B*) were analyzed by Western blotting at 6 days after transduction. The graph in the right shows a significant difference of HDM2 levels between pLent-sh*SC* and pLent-sh*BCL11B*. * indicates $P<0.01$. The levels are shown relative to ACTIN used as a control. (B) Schematic representation of *HDM2*-P1 and *HDM2*-P2 promoters. (C) RT-PCR analysis of *HDM2* RNA expression levels in treated cells as in (A). The graph shows comparison of *HDM2* mRNA levels transcribed from P1 and P2 promoters between pLent-sh*SC* and pLent-sh*BCL11B*. The mRNA levels are shown relative to *GAPDH* used as an internal control. * indicates $P<0.01$. (D) Protein expression levels in HCT116 cells that were transduced with control (pLent-*GFP*) or lentivirus vector expressing BCL11B (pLent-*Bcl11b*) were analyzed by Western blotting at 48 h after transduction. The graph shows comparison of *HDM2* protein levels as in (A). * indicates $P<0.01$. (E) RT-PCR analysis of *HDM2* RNA expression levels in treated cells as in (D). The graph shows comparison of *HDM2* mRNA levels. * indicates $P<0.01$. Data are presented as the mean ± SD.

used in different positions encompassing the promoter site (Fig. 2A). DNA fragments of P2 promoter region were enriched in the precipitant by BCL11B antibodies in both cell lines, whereas 5′-upstream and 3′-downstream regions were not enriched (Fig. 2B). Primers for control regions on *β-ACTIN* or on *IL-7R* genes did not show enrichment either. These results suggest that BCL11B directly binds to the *HDM2*-P2 promoter region.

3.3. *BCL11B inhibits HDM2-P2 promoter activity in a p53-dependent manner*

To further characterize the repressor activity of BCL11B for *HDM2* expression, we performed a luciferase reporter gene assay in *p53*-proficient HCT116 cells and *p53*-lacking HCT116 cells. We used two kinds of *HDM2*-P2 promoter sequences for the construct carrying wild-type T sequence at the 309th nucleotide position in the first intron and the variant G sequence at the position, because the variant-309 P2 promoter was reported to have a higher promoter activity by enhancing the binding of transcription factor Sp1 [30]. Transfection of BCL11B expression vector showed that BCL11B strongly inhibited the activity of both wild-type and variant *HDM2*-P2 promoters in HCT116 cells (Fig. 3A). The inhibition levels (about 20% of the control for each) were similar between wild-type and variant promoters, which was different from the initial prediction. Inhibition was also detected in p53-lacking HCT116 cells though it was less efficient (about 58% of the control). In contrast, BCL11B expression did not inhibit *HDM2*-P1 promoter activity in either cell line (Fig. 3A). These results suggest that BCL11B inhibits *HDM2* expression by suppressing the *HDM2*-P2 promoter activity and this suppression depends on p53 expression.

There are two p53-binding elements within the *HDM2*-P2 promoter that affect P2 promoter activation [4,5]. We generated five deletion constructs of *HDM2*-P2 promoter region, two of which lacked one of the two elements and one lacked both the p53 responsive elements (Fig. 3B). The construct lacking only proximal p53-binding element (Del-4) retained the promoter activity, suggesting no requirement of the proximal p53 binding element for promoter activity, whereas the construct lacking both elements (Del-5) showed a severe decrease to about 5% of the wild type activity (Fig. 3C). This decrease may be due to lack of the distal p53 binding element. The remaining three mutants showed decreases to 37–58%. Those decreases may be ascribed to lack of binding by other transcription factors than p53 such as AP-1, Ets and Smad2/3 [2,3]. These results suggest that *HDM2*-P2 promoter requires the distal p53 responsive element for

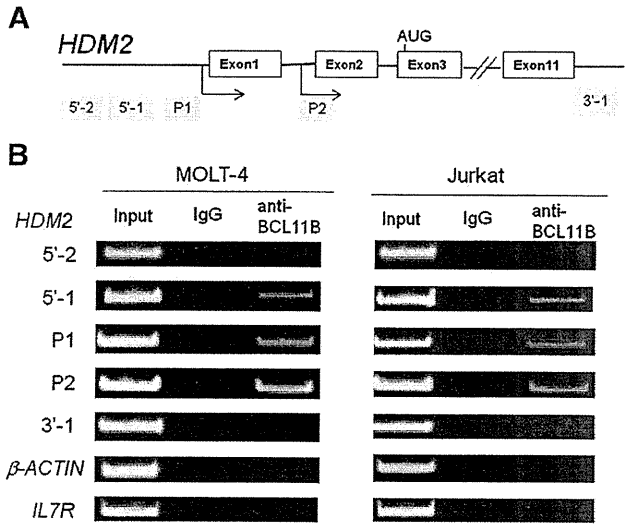

Fig. 2. BCL11B associates with the *HDM2* promoter. (A) Schematic representation of the *HDM2* gene region and positions of PCR primers used (Supplementary Table 3). (B) PCR products for input and ChIP-precipitaed DNA were separated on 5% PAGE and stained with ethidium bromide. Formaldehyde treated MOLT-4 and Jurkat cells were subjected to ChIP using antibodies against BCL11B. *β-ACTIN* gene and *IL7R* promoter were used as negative controls.

efficient transcription, though full activity of the promoter requires other sequence region in addition to the distal element.

We examined the effect of BCL11B on *HDM2*-P2 promoter using those deletion constructs. BCL11B failed to repress P2 promoter activity in the construct lacking both p53 responsive elements, whereas BCL11B efficiently repressed the activity in the three constructs retaining the distal p53 responsive element (Fig. 3D). Of note, the construct (Del-3) lacking the distal p53 responsive element possessed an intermediate activity. These results suggest that the distal element is required and plays a key role for the repression activity by BCL11B, consistent with that BCL11B suppresses the activity of the *HDM2*-P2 promoter in a p53-dependent manner.

3.4. BCL11B suppresses HDM2-P2 promoter with a less efficiency after DNA damages conditions

We examined the effect on *HDM2* expression of γ-irradiation that stabilize and activate p53 activity. We transfected the *HDM2* luciferase reporter vector into p53-proficient and p53-lacking HCT116 cells, and 4 h later those cells were exposed with and without 10 Gy of γ-irradiation. Twenty hours after, we measured the luciferase activity. *HDM2*-P2 promoter but not *HDM2*-P1 promoter activity was increased in response to γ-irradiation in both HCT116 cells (Fig. 4A). This suggests that *HDM2*-P2 promoter is activated by γ-irradiation and the activation is both p53 dependent and p53 independent. The result may be compatible with elevated RNA expressions in MOLT-4 (p53 proficient) and Jurkat (p53 deficient) cells after γ-irradiation (see Supplemental Fig. 2). However, the promoter activity in HCT116 cells was almost 10-fold higher than that in p53-lacking HCT116 cells, suggesting that p53-dependent activation has more impact than p53-independent one.

We next examined BCL11B repressor activity in DNA damage conditions induced by γ-irradiation (Fig. 4B). BCL11B expression inhibited the *HDM2*-P2 promoter activity in p53-proficient HCT116 cells, though the inhibition (about 38% of the control) was less than that (20%) in unirradiated basal conditions (see Fig. 3A). As in p53-lacking HCT116 cells, inhibition by BCL11B was also much less efficient (about 57% of the control), similar to that in unirradiated conditions. These demonstrated a reduced efficiency of BCL11B attenuation for *HDM2* promoter activity in radiation-induced DNA damage conditions. This suggests cooperativity between BCL11B and p53 on *HDM2* promoter activity.

4. Discussion

HDM2 downregulates p53 proteins whereas p53 activates transcription of *HDM2*, which is called the HDM2-p53 feedback loop. In this paper, we have examined the effect of BCL11B on the feedback loop and demonstrated that BCL11B negatively regulates the *HDM2* transcription mainly in a p53-dependent manner in basal conditions. The suppression of *HDM2* transcription may be mostly through the distal p53-binding element in *HDM2* promoter region, because only loss of the distal element alleviated the effect of BCL11B. We observed the repressor activity of BCL11B also in radiation-induced DNA damage conditions that activate p53. However, in the conditions the suppression activity by BCL11B was weaker than that in basal conditions. This suggests competitive interaction present between the activated p53 and BCL11B in the effect on *HDM2* promoter activity.

Gene repression plays an important role in controlling cell differentiation and proliferation [31]. Repression usually depends on sequence-specific DNA-binding repressors that recruit nuclear cofactors termed corepressors, which are directly responsible for silencing transcription [32]. The majority of known corepressors are components of multiprotein complexes that possess enzymatic activities such as deacetylation of histone tails and ATP-dependent remodeling of chromatin [33]. BCL11B acts as a transcriptional repressor [19,20] probably by binding to a GC-rich consensus sequence (GGCCGG) of target genes including the cyclin-dependent kinase inhibitors *p21* and *p57* [21,22]. BCL11B is also known to be associated with a nucleosome remodeling and histone deacetylase (NuRD) [20,21]. There are two GGCCGG sequence motifs within the *HDM*-P2 promoter (287 and 388 relative to the first nucleotide of intron 1). However, those motifs do not seem to serve for BCL11B function, because our results showed that the deletion mutant lacking the motifs (see Del-2 in Fig. 3B) underwent BCL11B-mediating repression. This suggests that BCL11B acts through other *HDM*-P2 promoter region including the distal p53-binding element. Corepressors have been generally considered to be ubiquitous proteins that are subject to little regulation. BCL11B may be included in a group of proteins subject to little regulation, because its expression was not affected in response to γ-irradiation.

p53 and HDM2 are both regulated in response to signals on oncogenic and tumor suppressor pathways. A variety of stress signals regulate p53 and result in p53 stabilization through preventing its degradation by HDM2. p53 is known to transcriptionally activate *HDM2* expression but there are other transcription factors such as AP-1, Ets and Smad2/3 that regulate *HDM2* expression in a p53-independent manner [2,3]. Signaling via Ras and the Erk pathway results in the binding of both AP-1 and Ets family members to a response element in *HDM*-P2 promoter that is immediately adjacent to the p53 responsive elements [2,3,6]. TGFβ has been shown to regulate HDM2 expression via the interaction of Smad2/3 with an element in *HDM*-P2 promoter as well [2]. Another regulator is $p14^{ARF}$ (the product of the alternate reading frame in *INK4A*, $p19^{ARF}$ in mice) that negatively regulates HDM2 at the protein level. $p14^{ARF}$ binds to HDM2 protein, and this binding inhibits the E3 ligase activity of HDM2.

A question on how BCL11B contributes to *HDM2* transcription remains. Since BCL11B expression seems to be not markedly regulated, BCL11B may be a constitutive component participating in the p53-HDM2 feedback loop and differs from regulatory proteins such as p53, AP-1, Ets and Smad2/3. One possible hypothesis is that BCL11B or possibly NuRD complex comprising BCL11B exists near the distal p53-binding element region in basal and irradiated conditions and maintains the alleviated *HDM2* transcription. Loss or decrease of BCL11B abrogates the alleviation, leading to increased HDM2

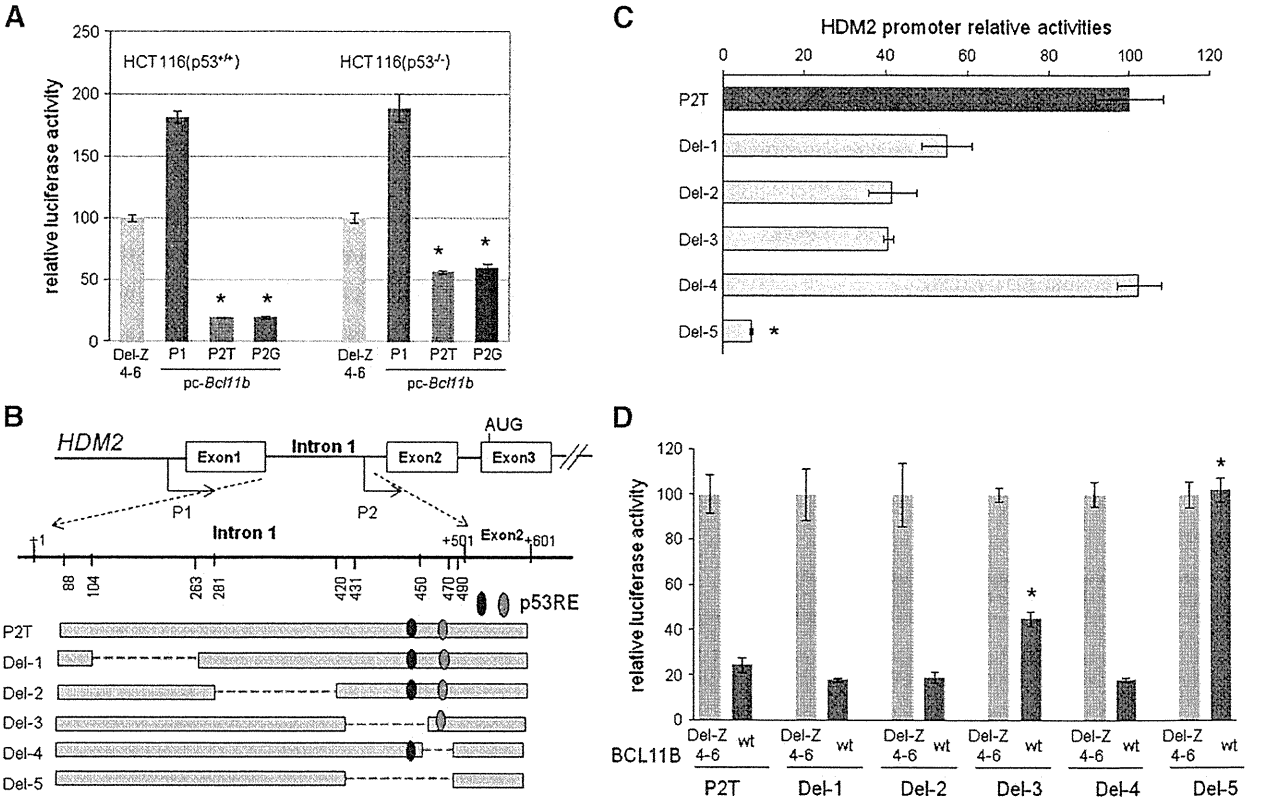

Fig. 3. Inhibition of *HDM2* transcription by BCL11B is dependent on p53. (A) Relative luciferase activities of *HDM2*-P1 and *HDM2*-P2 promoters are shown in HCT116 ($p53^{+/+}$) and HCT116 ($p53^{-/-}$) cells. The activity is expressed in the percent of wild-type BCL11B versus the del Z4-6 mutant lacking BCL11B activity (del Z4-6; deleted C-terminal three zinc finger domains of BCL11B). * indicates $P<0.01$. (B) Schematic representation of *HDM2*-P2 promoter deletion constructs. The distal p53 responsive element is shown as black oval and the proximal p53 responsive element is as gray oval. Nucleotide numbers relative to the first nucleotide (+1) of the first intron of *HDM2* gene are indicated. Del-2 construct lacks the two GGCCGG sequence elements, a putative motif for BCL11B binding. (C) Promoter activity of each deletion mutant is shown relative to P2T construct. * indicates $P<0.01$ (between P2T and Del-5). (D) Relative luciferase activities are shown as in (A). Data are presented as the mean ± SD. * indicates $P<0.01$ (comparison between P2T and Del-3, and between P2T and Del-5).

expression and p53 inactivation. When p53 is activated, p53 activator complex may replace or inactivate the BCL11B repressor complex leading to the activation of *HDM2* promoter.

Bcl11b was originally identified as a haploinsufficient tumor suppressor gene in mouse thymic lymphomas, a model of T-ALL [10,23,24]. Recently, haploinsufficiency has been also observed in T-ALL [27,28]. Another well-known haploinsufficient tumor suppressor is p53 [34]. Of note, $Bcl11b^{KO/+}$ heterozygosity alone gives a weak effect on development of thymic lymphomas, but its effect increases in the $p53^{KO/+}$ heterozygous genetic background [23]. This genetic evidence suggests cooperativity between BCL11B and p53 in lymphoma development.

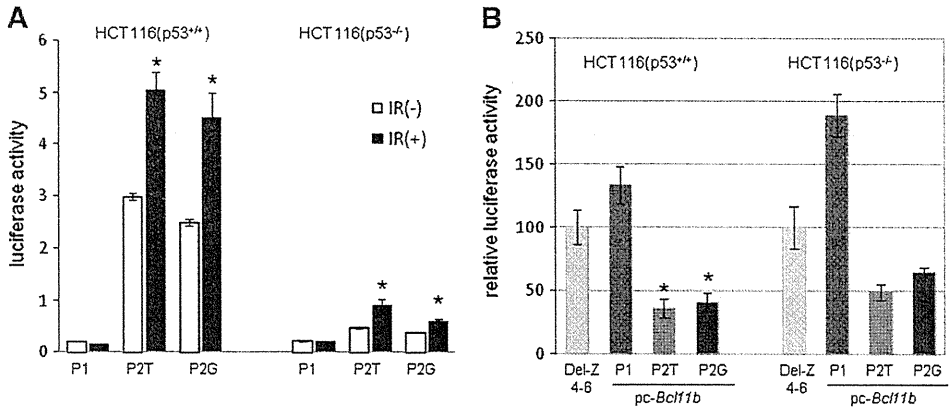

Fig. 4. Attenuation of BCL11B suppression activity for *HDM2* transcription after γ-irradiation. (A) Normalized luciferase activities of *HDM2*-P1 and *HDM2*-P2 promoters after γ-irradiation are shown in HCT116 ($p53^{+/+}$) and HCT116 ($p53^{-/-}$) cells. *HDM2* promoter-driven plasmid was transfected into HCT116 ($p53^{+/+}$) and HCT116 ($p53^{-/-}$) cells, and 4 h later those cells were exposed with and without 10 Gy of γ-irradiation. After 24 h, the luciferase activity was measured. Open columns show unirradiated cells and filled columns indicated irradiated cells. Data are presented as the mean ± SD. * indicates $P<0.01$ (between unirradiated and irradiated cells). (B) Relative luciferase activities after γ-irradiation are shown in HCT116 ($p53^{+/+}$) and HCT116 ($p53^{-/-}$) cells. *HDM2* luciferase reporter vector was transfected together with and without the BCL11B expression vector into HCT116 ($p53^{+/+}$) and HCT116 ($p53^{-/-}$) cells, and 4 h later those cells were exposed to 10 Gy of γ-irradiation. The luciferase activity was measured 24 h after the exposure. The inhibition in HCT116 ($p53^{+/+}$) cells (38% in average of P2T and P2G) was less than that (20%) in unirradiated cells in Fig. 3A (*$P<0.01$).

5. Conclusions

We found that BCL11B binds to human *HDM2*-P2 promoter and inhibits HDM2 expression in a p53-dependent manner. The repressive activity was alleviated in γ-ray induced DNA damage conditions that activate p53. Therefore, our results provide supporting evidence for the cooperativity between BCL11B and p53 in lymphomagenesis. These findings will help better understanding of T-ALL and other cancer pathogenesis and give a clue for therapeutic strategy.

Supplementary materials related to this article can be found online at doi: 10.1016/j.cellsig.2011.12.026.

Acknowledgments

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References

[1] B. Vogelstein, D. Lane, A.J. Levine, Nature 408 (2000) 307–310.
[2] J.J. Manfredi, Genes & Development 24 (2010) 1580–1589.
[3] M. Phelps, M. Darley, J.N. Primrose, J.P. Blaydes, Cancer Research 63 (2003) 2616–2623.
[4] T. Juven, Y. Barak, A. Zauberman, D.L. George, M. Oren, Oncogene 8 (1993) 3411–3416.
[5] A. Zauberman, D. Flusberg, Y. Haupt, Y. Barak, M. Oren, Nucleic Acids Research 23 (1995) 2584–2592.
[6] X. Tian, Y. Chen, W. Hu, M. Wu, Cellular Signalling 23 (2011) 193–200.
[7] Y. Barak, E. Gottlieb, T. Juven-Gershon, M. Oren, Genes & Development 8 (1994) 1739–1749.
[8] W. Duan, L. Gao, X. Wu, Y. Zhang, G.A. Otterson, M.A. Villalona-Calero, Experimental Cell Research 312 (2006) 3370–3378.
[9] D. Avram, A. Fields, K. Pretty On Top, D.J. Nevrivy, J.E. Ishmael, M. Leid, The Journal of Biological Chemistry 275 (2000) 10315–10322.
[10] Y. Wakabayashi, J. Inoue, Y. Takahashi, A. Matsuki, H. Kosugi-Okano, T. Shinbo, Y. Mishima, O. Niwa, R. Kominami, Biochemical and Biophysical Research Communications 301 (2003) 598–603.
[11] Y. Wakabayashi, H. Watanabe, J. Inoue, N. Takeda, J. Sakata, Y. Mishima, J. Hitomi, T. Yamamoto, M. Utsuyama, O. Niwa, S. Aizawa, R. Kominami, Nature Immunology 4 (2003) 533–539.
[12] P. Li, S. Burke, J. Wang, X. Chen, M. Ortiz, S.C. Lee, D. Lu, L. Campos, D. Goulding, B.L. Ng, G. Dougan, B. Huntly, B. Gottgens, N.A. Jenkins, N.G. Copeland, F. Colucci, P. Liu, Science 329 (2010) 85–89.
[13] L. Li, M. Leid, E.V. Rothenberg, Science 329 (2010) 89–93.
[14] T. Ikawa, S. Hirose, K. Masuda, K. Kakugawa, R. Satoh, A. Shibano-Satoh, R. Kominami, Y. Katsura, H. Kawamoto, Science 329 (2010) 93–96.
[15] P. Arlotta, B.J. Molyneaux, J. Chen, J. Inoue, R. Kominami, J.D. Macklis, Neuron 45 (2005) 207–221.
[16] P. Arlotta, B.J. Molyneaux, D. Jabaudon, Y. Yoshida, J.D. Macklis, The Journal of Neuroscience 28 (2008) 622–632.
[17] O. Golonzhka, X. Liang, N. Messaddeq, J.M. Bornert, A.L. Campbell, D. Metzger, P. Chambon, G. Ganguli-Indra, M. Leid, A.K. Indra, Journal of Investigative Dermatology 129 (2008) 1459–1470.
[18] O. Golonzhka, D. Metzger, J.M. Bornert, B.K. Bay, M.K. Gross, C. Kioussi, M. Leid, Proceedings of the National Academy of Sciences of the United States of America 106 (2009) 4278–4283.
[19] D. Avram, A. Fields, T. Senawong, A. Topark-Ngarm, M. Leid, The Biochemical Journal 368 (2002) 555–563.
[20] V.B. Cismasiu, K. Adamo, J. Gecewicz, J. Duque, Q. Lin, D. Avram, Oncogene 24 (2005) 6753–6764.
[21] T. Cherrier, S. Suzanne, L. Redel, M. Calao, C. Marban, B. Samah, R. Mukerjee, C. Schwartz, G. Gras, B.E. Sawaya, S.L. Zeichner, D. Aunis, C. Van Lint, O. Rohr, Oncogene 28 (2009) 3380–3389.
[22] A. Topark-Ngarm, O. Golonzhka, V.J. Peterson, B. Barrett Jr., B. Martinez, K. Crofoot, T.M. Filtz, M. Leid, The Journal of Biological Chemistry 281 (2006) 32272–32283.
[23] K. Kamimura, H. Ohi, T. Kubota, K. Okazuka, Y. Yoshikai, Y. Wakabayashi, Y. Aoyagi, Y. Mishima, R. Kominami, Biochemical and Biophysical Research Communications 355 (2007) 538–542.
[24] H. Ohi, Y. Mishima, K. Kamimura, M. Maruyama, K. Sasai, R. Kominami, Oncogene 26 (2007) 5280–5289.
[25] S. Nagel, M. Kaufmann, H.G. Drexler, R.A. MacLeod, Cancer Research 63 (2003) 5329–5334.
[26] G.K. Przybylski, W.A. Dik, J. Wanzeck, P. Grabarczyk, S. Majunke, J.I. Martin-Subero, R. Siebert, G. Dölken, W.D. Ludwig, B. Verhaaf, J.J. van Dongen, C.A. Schmidt, A.W. Langerak, Leukemia 19 (2005) 201–208.
[27] K. De Keersmaecker, P.J. Real, G.D. Gatta, T. Palomero, M.L. Sulis, V. Tosello, P. Van Vlierberghe, K. Barnes, M. Castillo, X. Sole, M. Hadler, J. Lenz, P.D. Aplan, M. Kelliher, B.L. Kee, P.P. Pandolfi, D. Kappes, F. Gounari, H. Petrie, J. Van der Meulen, F. Speleman, E. Paietta, J. Racevskis, P.H. Wiernik, J.M. Rowe, J. Soulier, D. Avran, H. Cavé, N. Dastugue, S. Raimondi, J.P. Meijerink, C. Cordon-Cardo, A. Califano, A.A. Ferrando, Nature Medicine 11 (2010) 11231–12327.
[28] A. Gutierrez, A. Kentsis, T. Sanda, L. Holmfeldt, S.C. Chen, J. Zhang, A. Protopopov, L. Chin, S.E. Dahlberg, D.S. Neuberg, L.B. Silverman, S.S. Winter, S.P. Hunger, S.E. Sallan, S. Zha, F.W. Alt, J.R. Downing, C.G. Mullighan, A.T. Look, Blood 118 (2011) 4169–4173.
[29] K. Kamimura, Y. Mishima, M. Obata, T. Endo, Y. Aoyagi, R. Kominami, Oncogene 26 (2007) 5840–5850.
[30] G.L. Bond, W. Hu, E.E. Bond, H. Robins, S.G. Lutzker, N.C. Arva, J. Bargonetti, F. Bartel, H. Taubert, P. Wuerl, K. Onel, L. Yip, S.J. Hwang, L.C. Strong, G. Lozano, A.J. Levine, Cell 119 (2004) 591–602.
[31] S. Gray, M. Levine, Current Opinion in Cell Biology 8 (1996) 358–364.
[32] A.J. Courey, S. Jia, Genes & Development 15 (2001) 2786–2796.
[33] M.G. Rosenfeld, V.V. Lunyak, C.K. Glass, Genes & Development 20 (2006) 1405–1428.
[34] C.J. Lynch, J. Milner, Oncogene 25 (2006) 3463–3470.

Development/Plasticity/Repair

Bcl11b/Ctip2 Controls the Differentiation of Vomeronasal Sensory Neurons in Mice

Takayuki Enomoto,[1] Makoto Ohmoto,[3] Tetsuo Iwata,[1] Ayako Uno,[1] Masato Saitou,[1] Tatsuya Yamaguchi,[1] Ryo Kominami,[4] Ichiro Matsumoto,[3,5] and Junji Hirota[1,2]

[1]Department of Bioengineering, Graduate School of Bioscience and Bioengineering, and [2]Center for Biological Resources and Informatics, Tokyo Institute of Technology, Yokohama 226-8501, Japan, [3]Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo 113-8657, Japan, [4]Department of Molecular Genetics, Graduate School of Medical and Dental Sciences, Niigata University, Niigata 951-8122, Japan, and [5]Monell Chemical Senses Center, Philadelphia, Pennsylvania 19104-3308

The transcription factor Bcl11b/Ctip2 plays critical roles in the development of several systems and organs, including the immune system, CNS, skin, and teeth. Here, we show that *Bcl11b/Ctip2* is highly expressed in the developing vomeronasal system in mice and is required for its proper development. *Bcl11b/Ctip2* is expressed in postmitotic vomeronasal sensory neurons (VSNs) in the vomeronasal epithelium (VNE) as well as projection neurons and GABAergic interneurons in the accessory olfactory bulb (AOB). In the absence of *Bcl11b*, these neurons are born in the correct number, but VSNs selectively die by apoptosis. The critical role of Bcl11b in vomeronasal system development is demonstrated by the abnormal phenotypes of *Bcl11b*-deficient mice: disorganization of layer formation of the AOB, impaired axonal projections of VSNs, a significant reduction in the expression of vomeronasal receptor genes, and defective mature differentiation of VSNs. VSNs can be classified into two major types of neurons, vomeronasal 1 receptor (V1r)/Gα_{i2}-positive and vomeronasal 2 receptor (V2r)/Gα_o-positive VSNs. We found that all Gα_{i2}-positive cells coexpressed Gα_o during embryogenesis. This coexpression is also observed in newly differentiated neurons in the adult VNE. Interestingly, loss of Bcl11b function resulted in an increased number of V1r/Gα_{i2}-type VSNs and a decreased number of V2r/Gα_o-type VSNs, suggesting that Bcl11b regulates the fate choice between these two VSN types. These results indicate that Bcl11b/Ctip2 is an essential regulator of the differentiation and dichotomy of VSNs.

Introduction

Most terrestrial vertebrates possess a vomeronasal system, which detects pheromones to mediate social and reproductive behaviors (Keverne, 1999; Dulac and Torello, 2003; Halpern and Martínez-Marcos, 2003; Brennan and Zufall, 2006). In the mouse, pheromone signals are detected by the vomeronasal sensory neurons (VSNs), which generally express vomeronasal receptor (VR) genes that encode a putative seven-transmembrane domain protein (Dulac and Axel, 1995; Herrada and Dulac, 1997; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997). There are two classic types of VSN, which are distinguished by the location of the cell body within the vomeronasal epithelium (VNE), the class of VR expressed, the type of G-protein subunit expressed, and their axonal target site in the accessory olfactory bulb (AOB). VSNs located apically in the VNE coexpress vomeronasal 1 receptor (V1r) genes along with Gα_{i2} and project their axons to the anterior half of the AOB. In contrast, basally located VSNs coexpress vomeronasal 2 receptor (V2r) genes along with Gα_o and project their axons to the posterior half of the AOB (Berghard and Buck, 1996; Jia and Halpern, 1996). The cell bodies of V1r/Gα_{i2}-positive and V2r/Gα_o-positive VSNs form nonoverlapping layers in the VNE. Although both types of VSNs are generated from Mash1-positive progenitor cells (Murray et al., 2003), the genetic mechanisms that regulate the differentiation and dichotomy of VSNs are not well understood; only a few critical transcription factors are known to regulate the development of VSNs (Murray et al., 2003; Ikeda et al., 2007; Duggan et al., 2008).

The *Bcl11b* gene (also known as *Ctip2* or *Rit1*) encodes a C2H2 zinc finger transcription factor that is predominantly expressed in the immune system, CNS, and the embryonic olfactory system in mice (Avram et al., 2000; Wakabayashi et al., 2003a; Leid et al., 2004). Several studies of *Bcl11b*-deficient (*Bcl11b*$^{-/-}$) mice have demonstrated that Bcl11b is required for T-cell development, the axonal projections of corticospinal motor neurons, the differentiation of medium spiny neurons of the striatum, skin morphogenesis, and odontogenesis (Wakabayashi et al., 2003b; Arlotta et al., 2005, 2008; Albu et al., 2007; Golonzhka et al., 2009a,b; Ikawa et al., 2010; Li et al., 2010a,b). In this study, we investigated the expression and function of *Bcl11b* in the developing vomeronasal system *in vivo*. We found that the expression of *Bcl11b* changed

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Correspondence should be addressed to Junji Hirota at the above address. E-mail: jhirota@bio.titech.ac.jp.

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dynamically during the development of the VNE and AOB from embryogenesis to adulthood. The loss of function of Bcl11b resulted in severe abnormalities in the vomeronasal system, including the disorganization of layer formation in the AOB, impaired axonal projection of VSNs, a severe reduction of *VR* gene expression, defects in mature differentiation of VSNs, and distinct changes in the expression of several genes in VSNs. In addition, a deficiency of Bcl11b disturbs the balance between the two types of VSNs produced, suggesting that Bcl11b regulates the fate choice between these cell types, indicating that Bcl11b is a critical regulator during functional development of the vomeronasal system.

Materials and Methods

Mutant mice. *Bcl11b*$^{-/-}$ and *Mash1*$^{-/-}$ mice were generated as described previously(Guillemot et al., 1993; Wakabayashi et al., 2003b). Mutant and wild-type mice/embryos of either sex were used. For embryo staging, mid-day of the day of the vaginal plug was designated as embryonic day (E) 0.5. The day of birth was designated postnatal day (P) 0. All mouse studies were approved by the Institutional Animal Experiment Committee of the Tokyo Institute of Technology, and were performed in accordance with institutional and governmental guidelines.

In situ *hybridization.* Probes for *Mash1*, *Ngn1*, *NeuroD*, *SCG10*, *GAP43*, and *OMP* were prepared as previously described (Hirota and Mombaerts, 2004). Probes for $G\alpha_{i2}$, $G\alpha_o$, *V1rb1*, *V1rd16*, *V1re4*, *V2ra*, *V2rb*, and *V2rc* were provided by P. Mombaerts, Max-Planck Institute of Biophysics, Frankfort, Germany (Rodriguez et al., 2002; Ishii et al., 2003). Other probes were prepared from the cDNA of *Bcl11b* (nucleotides 1501-2560, GenBank accession number BC19503), *Vil1* (nucleotides 356-2634, NCBI reference sequence NM_009509), *Tcfap2e* (nucleotides 916-1875, NCBI reference sequence NM_198960), *Cart* (nucleotides 96-788, NCBI reference sequence NM_001081493), *Big2/Contactin4* (nucleotides 1189-2575, NCBI reference sequence NM_173004.3), *Mef2b* (nucleotides 500-1255, NCBI reference sequence NM_00445484), *Panx3* (nucleotides 165-1598, NCBI reference sequence NM_172454), *Meis2* (nucleotides 453-1354, NCBI reference sequence NM_001136072.2), and *Olig1* (nucleotides 971-1919, NCBI reference sequence NM_016968.4). Single-color and two-color *in situ* hybridization (ISH) was performed according to a method described previously (Ishii et al., 2003, 2004). For two-color ISH, tyramide signal amplification (TSA)-biotin, TSA-dinitrophenyl, and TSA-plus biotin systems (PerkinElmer) were used. The images were taken on an Olympus BX51 microscope with a DP71 digital CCD camera for bright-field images and a Leica SP or SPE confocal microscope for fluorescent images.

Immunohistochemistry. Immunohistochemistry (IHC) was performed according to a previously described method (Hirota et al., 2007). The following primary antibodies and dilutions were used: rabbit anti-Bcl11b (1:200) (Wakabayashi et al., 2003b); goat anti-Ki67 (1:1500 or 1:3000, catalog #SC-7846, Santa Cruz Biotechnology); goat anti-OMP (1:5000, catalog #544-10001, Wako); guinea pig anti-GABA (1:1000 or 1:10,000, catalog #AB175, Millipore); rabbit anti-Tbx21 (1:5000, a gift from Y. Yoshihara, RIKEN, Wako, Japan) (Yoshihara et al., 2005); guinea pig anti-Tbx21 (1:1000, a gift from Y. Yoshihara) (Yoshihara et al., 2005); mouse anti-NCAM (1:500, catalog #C-9672, Sigma); mouse anti-synaptophysin (1:1000, catalog #MAB5258, Millipore); rabbit anti-protocadherin 21 (1:1000, a gift from Dr. Yoshihara) (Kaneko-Goto et al., 2008); and rabbit anti-active caspase-3 (1:500 or 1:5000, catalog #C92–605, BD Pharmingen). The following appropriate secondary antibodies were used: the Alexa series (Invitrogen) and Cy3- and Cy5-conjugated goat anti-rabbit IgG (Jackson Immuno Research Laboratories); biotin-conjugated donkey anti-goat IgG (Rockland); biotin-conjugated goat anti-guinea pig IgG and goat anti-rabbit IgG (Vector Laboratories) antibodies. For the immunostaining that used the anti-Ki67 or anti-active caspase-3 antibodies, the sections were treated with the Vector Avidin/Biotin Blocking Kit (Vector Laboratories) and incubated with primary antibodies. Immunostaining was performed using the Vectastain Detection Kit (Vector Laboratories) in combination with the TSA-biotin system (PerkinElmer) to amplify signals. Anti-Ki67 immunoreactivity was detected using the DAB-chromogenic method with streptavidin-HRP, and anti-active caspase-3 was detected with streptoavidin-Alexa488 fluorescence. For immunostaining with the mouse anti-NCAM and anti-synaptophysin antibodies, the sections were pretreated with the Vector M.O.M. Immunodetection Kit (Vector Laboratories) before treatment with the primary antibody. For immunostaining of the nuclear protein, we performed antigen-retrieval pretreatment in 10 mM sodium citrate for 10 min or in HistoVT One (Nacalai Tesque) at 70°C for 15 min using a microwave oven.

Quantitative analyses. To quantify the number of DAPI-positive cells, *VR*-expressing cells, $G\alpha_{i2}$ and $G\alpha_o$-expressing cells, proliferating cells, and apoptotic cells, every fifth or tenth coronal section (10 μm thickness), or every third coronal section (20 μm thickness) throughout the vomeronasal organ (VNO) was collected for each staining experiment, and the number of positive cells was counted.

DiI tracing experiment. DiIC18(3) (DiI) crystal (Invitrogen) was heat melted to coat a glass capillary. After dissection of the heads of *Bcl11b*$^{-/-}$ and wild-type mice, the nose tip was cut to expose the rostral vomeronasal organ, and the DiI-coated glass capillary was placed into the lumen of the VNO. Samples were incubated in PBS for 2 h at 37°C, in 0.5% PFA/PBS overnight, and in 4% PFA/PBS for a month at room temperature to allow for the diffusion of DiI from the VSNs to the axonal termini. The DiI fluorescence of sagittally transected heads was imaged using an Olympus BX51 fluorescence microscope with a DP71-digital CCD camera.

Affymetrix microarrays. VNOs were obtained from *Bcl11b*$^{-/-}$ and wild-type mice at P0 and stored in RNA*later* (Ambion). The total RNA of each preparation was extracted using the RNeasy Mini Kit (Qiagen). Biotinylated cRNA was synthesized using 10 ng of total RNA with the Two-Cycle Target Labeling and Control Reagents Kit (Affymetrix), fragmented, and hybridized to the DNA microarrays (Mouse Genome 430 2.0 Array). To ensure reproducibility, microarray analyses were performed with RNA samples from six *Bcl11b*$^{-/-}$ and five wild-type mice. The microarray data were linearly normalized with the GAPDH (Probe ID: 1418625_s_at) signal of each preparation using GeneChip operating software (Affymetrix). The statistical significance of gene expression differences between *Bcl11b*$^{-/-}$ and wild-type was analyzed using GeneSpring version 7.3 (Agilent Technologies), and differentially expressed genes with a false discovery rate <0.25 were extracted from the data for further experiments.

Results

Bcl11b is expressed in the developing vomeronasal epithelium

The expression of *Bcl11b*/*Ctip2* has been reported in the embryonic main olfactory epithelium (MOE) (Leid et al., 2004), but its expression in the VNE has not been studied. Because the MOE and the VNE are derived from the same olfactory placode, we assumed that *Bcl11b* would also be expressed in the VNE. Therefore, we examined the expression of *Bcl11b* in the developing VNE using ISH. The ISH studies revealed that *Bcl11b* was expressed in the VNE during the course of fetal development to adulthood, and its expression levels and patterns changed dynamically (Fig. 1*A*). The expression of *Bcl11b* was observed in the vomeronasal groove/VNE, and the MOE at the earliest time point examined, E11.5, which is shortly after the olfactory pits invaginate to develop the VNE and the MOE (Cuschieri and Bannister, 1975; Garrosa et al., 1998). The expression of *Bcl11b* increased gradually in level and in the number of cells during embryogenesis (Fig. 1*A*). From E16.5 to P0, a strong expression of *Bcl11b* was detected in the VSN layer but not in the sustentacular cell layer. Expression levels in individual cells were not uniform; rather, high-, low-, and nonexpressing cells were intermingled in the embryonic VNE. After birth, *Bcl11b* expression changed dynamically: *Bcl11b* expression gradually decreased and was restricted to the marginal region of the VNE (Fig. 1*A*, arrows), where neuronal progenitor/precursor cells and immature neurons localize. This result indicates that *Bcl11b* is expressed mainly in proliferating cells and/or immature neurons. Because proliferating cells and differentiating and differentiated neurons intermingle in the VNE during embryogenesis and for several days postnatally, the differential expression levels