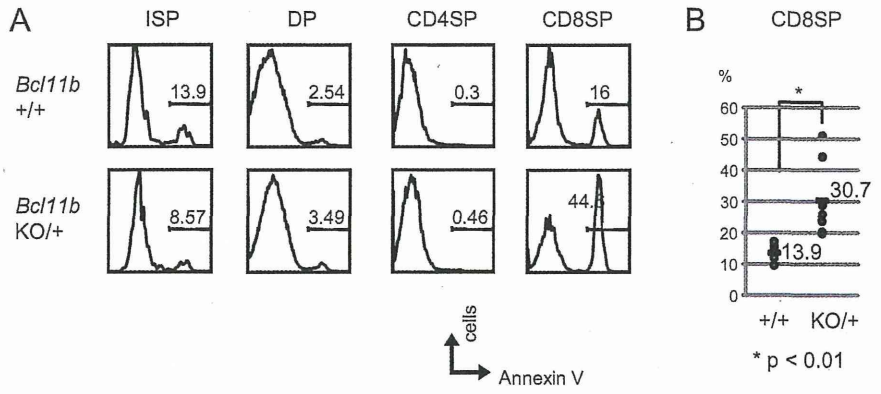

Fig. 2. Comparison of apoptosis in thymocytes from *Bcl11b*$^{+/+}$ and *Bcl11b*$^{KO/+}$ mice. (A) Flow cytometric analysis of Annexin V-positive cells in the thymocyte subsets indicated above. The vertical axis shows cell numbers and the horizontal axis shows Annexin V-expression levels. (B) The percentage of Annexin V-positive cells in thymocyte subsets. Comparison was performed between *Bcl11b*$^{+/+}$ ($n = 6$) and *Bcl11b*$^{KO/+}$ ($n = 8$) mice. *P* values for difference in ISP and CD8SP subsets are less than 1%.

percentage was very low in *Bcl11b*$^{+/+}$ and *Bcl11b*$^{KO/+}$ CD8$^+$ fractions and no marked difference was observed between them (Supplementary Fig. 2), indicating that the higher percentage of ISP cells is due to an accumulation of precursors to DP cells. On the other hand, analysis of DN cell subtypes using CD44 and CD25 markers did not show significant differences in the percentage or the cell number between *Bcl11b*$^{+/+}$ and *Bcl11b*$^{KO/+}$ mice (Supplementary Fig. 3). This suggests no impairment during development of immature DN thymocytes. Together, these results suggest developmental arrest at the two different ISP and DP stages in *Bcl11b*$^{KO/+}$ heterozygous mice.

3.2. Apoptosis and proliferation of thymocytes

T-cell development is tightly related to apoptosis. Accordingly, we examined the percentage of Annexin V-positive cells, an indicator of apoptosis (Fig. 2A). Annexin V$^+$ cells were observed in CD8SP cells at a significant level but very low in ISP, DP and CD4SP cells. Comparison between *Bcl11b*$^{+/+}$ and *Bcl11b*$^{KO/+}$ CD8SP thymocytes revealed a 3-fold increase in *Bcl11b*$^{KO/+}$ mice (Fig. 2B). This suggests that loss of a *Bcl11b* allele provides increased susceptibility to apoptosis in CD8SP cells.

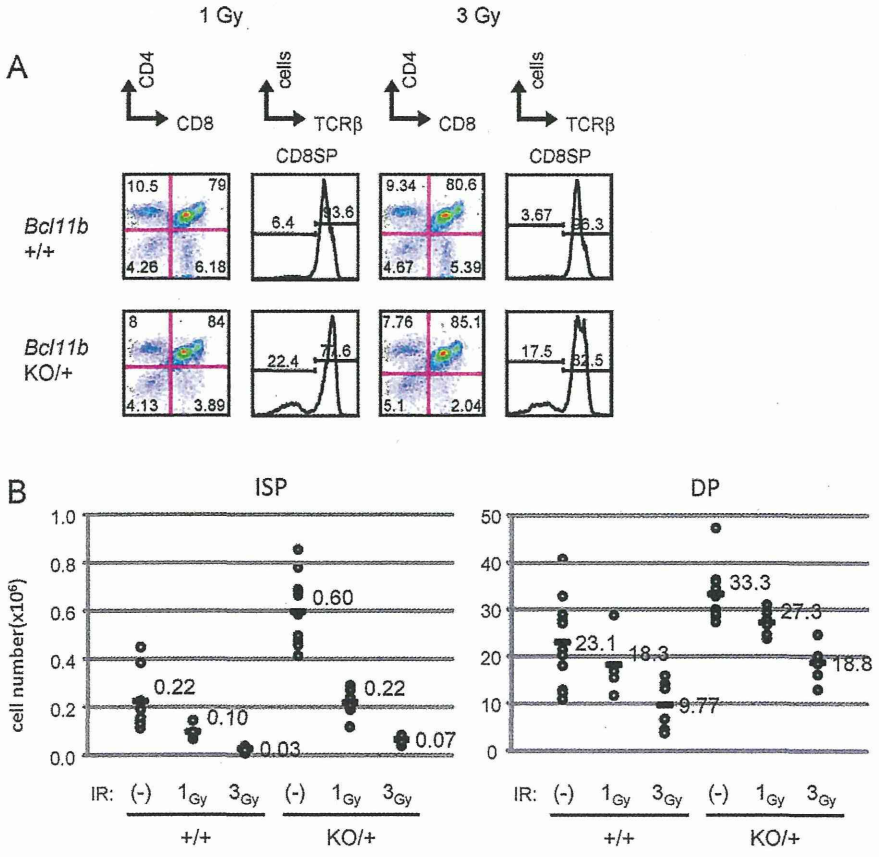

Fig. 3. Effect of γ-irradiation on the cell number in thymocytes. (A) Flow cytometric analysis of CD4, CD8 and TCRβ expression on thymocytes from 1 Gy and 3 Gy irradiated *Bcl11b*$^{+/+}$ and *Bcl11b*$^{KO/+}$ mice. Analysis was performed 4 h after irradiation. (B) The cell number of ISP and DP cells in thymic lobe after irradiation or without irradiation. The sample number is 4 in 1 Gy *Bcl11b*$^{+/+}$ mice and 6 in 1 Gy *Bcl11b*$^{KO/+}$ mice, and 6 in 3 Gy irradiated mice.

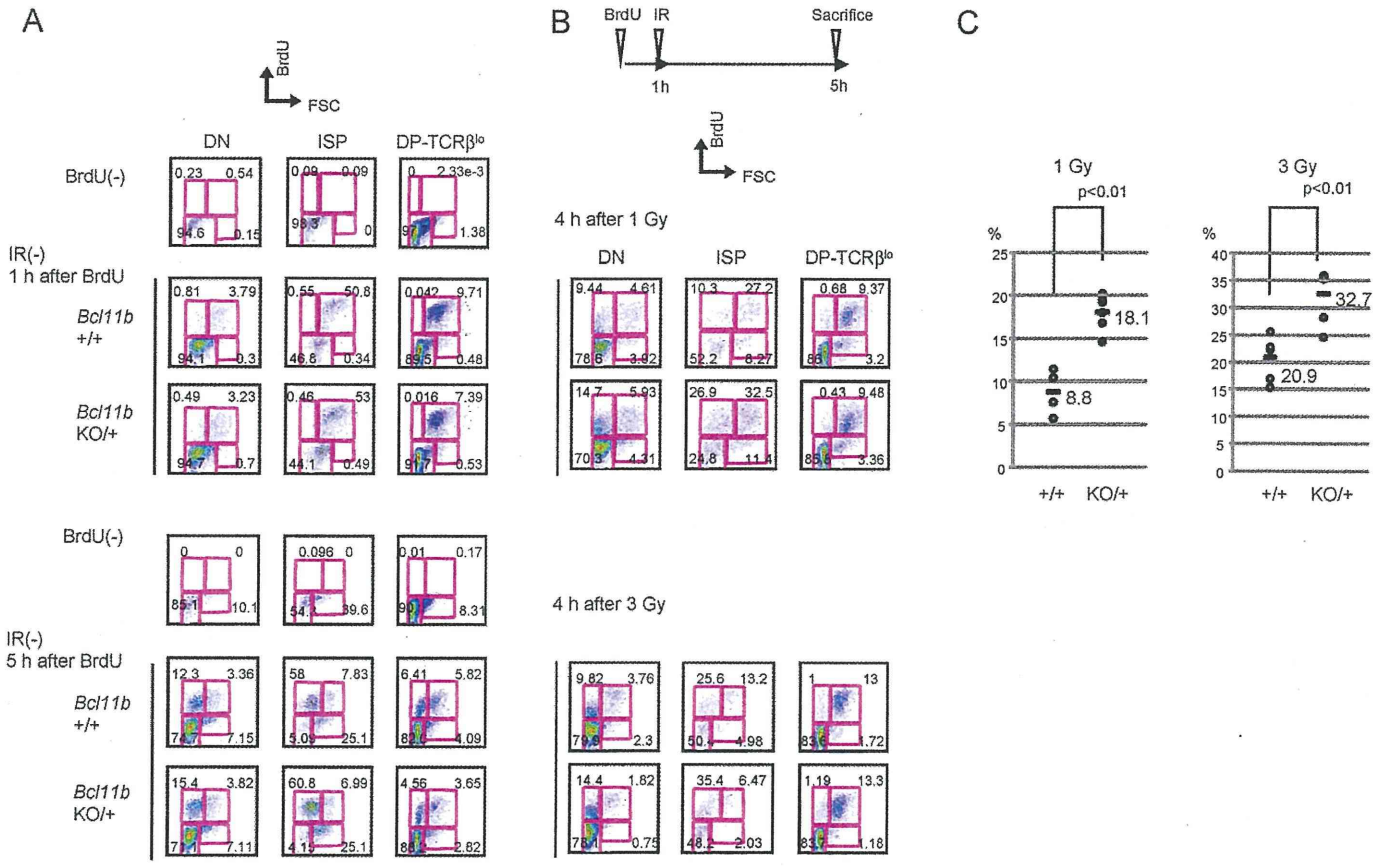

Fig. 4. Effect of γ-irradiation on cell cycle in thymocytes of *Bcl11b*$^{+/+}$ and *Bcl11b*$^{KO/+}$ mice. (A) Flow cytometric analysis of BrdU incorporation levels (vertical axis) and FSC values (horizontal axis) in thymocyte subsets in unirradiated mice. BrdU^{+}FSC^{Large} fraction represents cells in S or G2/M phases of cell cycle whereas BrdU^{+}FSC^{Small} fraction represents G1 cells that have passed S phase. (B) Flow cytometric analysis of BrdU incorporation levels and FSC values in thymocyte subsets in 1 Gy and 3 Gy irradiated mice. (C) The percentages of BrdU^{+}FSC^{S} cells in the ISP thymocyte subset in *Bcl11b*$^{+/+}$ and *Bcl11b*$^{KO/+}$ mice after 1 Gy and 3 Gy irradiation. *P* values for difference between *Bcl11b*$^{+/+}$ and *Bcl11b*$^{KO/+}$ mice after 1 Gy and 3 Gy irradiation are less than 0.5%. The sample number is 4 in 1 Gy *Bcl11b*$^{+/+}$ mice and 6 in 1 Gy *Bcl11b*$^{KO/+}$ mice, and 6 in 3 Gy irradiated mice.

Deregulation of cell cycle is a hallmark of leukemic transformation. We examined BrdU incorporation of thymocytes, an indicator of cell cycle progression, in mice 1 h after BrdU administration (Supplementary Fig. 4). About 40% of ISP cells and 8% of DP cells showed BrdU incorporation whereas very low percentages of CD4SP and CD8SP cells showed BrdU incorporation, consistent with a previous report [25]. Comparison between *Bcl11b*$^{+/+}$ and *Bcl11b*$^{KO/+}$ mice did not show significant differences in ISP and DP cells.

3.3. Effect of γ-irradiation on cellularity of thymocytes

Bcl11b$^{KO/+}$ mice developed thymic lymphomas when γ-irradiated, but not spontaneously [17]. Accordingly, of importance may be the effect of *Bcl11b*$^{KO/+}$ heterozygosity on radiation-induced cell damages. Thus, we examined cell number of thymocytes from mice 4 h after 1 Gy or 3 Gy of γ-irradiation. Fig. 3A shows expression of CD4/CD8 and TCRβ in thymocytes. Fig. 3B summarizes the cell number of ISP and DP cells in *Bcl11b*$^{+/+}$ and *Bcl11b*$^{KO/+}$ mice. One Gy irradiation led to a two-fold decrease in ISP cells and a minimal decrease in DP cells. The decreases did not differ between *Bcl11b*$^{+/+}$ and *Bcl11b*$^{KO/+}$ mice. On the other hand, 3 Gy irradiation reduced the cell number to approximately one tenth in ISP cells and to about one half in DP cells probably by inducing apoptosis differently. Comparison between *Bcl11b*$^{+/+}$ and *Bcl11b*$^{KO/+}$ mice also did not show marked difference in the decreases.

3.4. Effect of γ-irradiation on cell cycle

We next examined radiation effect on cell cycle of thymocytes. To monitor cell cycle, we administrated BrdU at 1 h before γ-irradiation. Accordingly, analysis of thymocytes was performed in mice 5 h after BrdU administration and 4 h after irradiation, and for comparison in mice 1 h and 5 h after BrdU administration without irradiation. Fig. 4A show the percentage of cells in four different areas in DN, ISP and TCRβ^{low}-DP cells in unirradiated mice. Control panels without BrdU administration are included. The four areas were determined by BrdU incorporation (vertical axis) and FSC value (horizontal axis), an indicative of cell size. BrdU^{+} FSC-large (FSC^{L}) represents cells in S or G2/M phases of cell cycle whereas BrdU^{+} FSC-small (FSC^{S}) represents G1 cells that have passed S phase after BrdU administration. BrdU^{-}FSC^{L} and BrdU^{-}FSC^{S} cells are thymocytes present in S/G2/M and G1 phase, respectively, that have not passed S phase for 5 h after BrdU administration.

In unirradiated mice 1 h after BrdU administration, the percentage of BrdU^{+}FSC^{S} cells was much less than that of BrdU^{+}FSC^{L} cells in DN, ISP, and TCRβ^{low}-DP cells irrespective of *Bcl11b*$^{+/+}$ and *Bcl11b*$^{KO/+}$ genotypes, indicating that BrdU-incorporated S-phase cells did not yet progress into G1 phase. However, in mice 5 h after, both BrdU^{+}FSC^{L} cells and BrdU^{+}FSC^{S} cells were observed at significant levels in DN, ISP, and TCRβ^{low}-DP cells. The percentage of BrdU^{+}FSC^{S} cells was more than that of BrdU^{+}FSC^{L} cells in each subset. No differences were observed between *Bcl11b*$^{+/+}$ and *Bcl11b*$^{KO/+}$

mice. These results indicated that certain fractions of thymocytes in S phase progressed to G1 phase for 4 h in those thymocyte subsets.

γ-Irradiation affected the cell cycle progression and the consequence was different depending on subsets. Fig. 4B shows the percentage of cells in four different areas in DN, ISP and TCRβ^{low}-DP cells in mice 5 h after BrdU administration and 4 h after 1 Gy or 3 Gy irradiation. TCRβ^{low}-DP cells in both irradiated mice little contained BrdU^{+}FSC^{S} cells irrespective of *Bcl11b*$^{+/+}$ or *Bcl11b*$^{KO/+}$ genotypes, indicating radiation-induced arrest at S or G2/M phase. In contrast, ISP cells comprised BrdU^{+}FSC^{S} cells at significant percentages even after irradiation, indicating the progression from S to G1 phase. One Gy irradiated mice contained more BrdU^{+}FSC^{L} cells than BrdU^{+}FSC^{S} cells whereas 3 Gy irradiated mice exhibited less BrdU^{+}FSC^{L} cells than BrdU^{+}FSC^{S} cells. Comparison between *Bcl11b*$^{+/+}$ and *Bcl11b*$^{KO/+}$ mice revealed a significant increase in the percentage of BrdU^{+}FSC^{S} cells in *Bcl11b*$^{KO/+}$ ISP cells in both 1 Gy and 3 Gy irradiated mice (Fig. 4C). Differences in radiation effect between *Bcl11b*$^{+/+}$ and *Bcl11b*$^{KO/+}$ mice were also observed in DN cells, though the DN cell fraction comprised a mixture of immature thymocytes consisting of different subsets. Together, those findings indicated that BrdU^{+}FSC^{L} ISP cells, but not BrdU^{+}FSC^{L} DP cells, progressed to BrdU^{+}FSC^{S} cells more in *Bcl11b*$^{KO/+}$ mice than *Bcl11b*$^{+/+}$ mice. This suggests that *Bcl11b*$^{KO/+}$ heterozygosity attenuates radiation-induced cell-cycle arrest at S or G2/M phase in ISP cells.

4. Discussion

This study has investigated changes in differentiation, cell survival and cell cycle during T-cell development in mice of the *Bcl11b*$^{KO/+}$ heterozygous genotype, which provides susceptibility to thymic lymphomas [4,23], a mouse model of T-ALL [15,26]. In *Bcl11b*$^{KO/+}$ mice, increases were observed in the percentage and cell number of ISP and DP cells, indicating differentiation arrest at ISP and DP stages. No impairment was observed in immature thymocytes at DN stages. Previous studies using *Bcl11b*$^{KO/KO}$ mice revealed the requirement of Bcl11b function for thymocyte differentiation at DN2 and DN3 stages [5,11–13]. These data indicate difference in the effect on differentiation between loss of one *Bcl11b* allele and loss of both alleles. The difference may be important in light of the contribution of loss of one *Bcl11b* allele alone to lymphomagenesis.

Difference in cell survival was observed between *Bcl11b*$^{KO/+}$ and *Bcl11b*$^{+/+}$ mice. The percentage of apoptotic cells in CD8SP cells was increased in *Bcl11b*$^{KO/+}$ mice. The increased apoptosis may be related to impairment in TCR (T-cell receptor) signaling that provides signaling for cell survival. This is because CD4-Cre; *Bcl11b*$^{flox/flox}$ mice lacking Bcl11b activity after DP stage fail to express a TCR complex on cell surface [8,10]. The result suggests that impairment in the TCR signaling is also present in *Bcl11b*$^{KO/+}$ mice.

TCR or preTCR signaling leads to the activation of a cascade of signaling molecules and eventually to the downstream activation of RAS-MAPK pathway, PI3K-AKT pathway and others [3,27]. Multiple components in the signaling pathway are targeted by either mutations or chromosomal translocations in T-ALL, and the genetic changes result in the activation of TCR or preTCR signaling. Accordingly the activating mutation of TCR or preTCR signaling is classified as a type B abnormality [3]. However, loss of Bcl11b results in an adverse effect, inactivation of the signaling, and hence it may not be a factor contributing to lymphomagenesis.

Difference in cell cycle was observed in radiation-induced injury conditions though not in basal conditions. In both *Bcl11b*$^{+/+}$ and *Bcl11b*$^{KO/+}$ mice, irradiation led to arrest at S and G2/M phase in DP cells but not much in ISP cells. Most ISP cells progressed from S to G1 phase after irradiation, indicating a reduced capability in radiation-induced arrest at ISP stage. Of importance is that the reduced capability of arrest was more marked in *Bcl11b*$^{KO/+}$ mice. This suggests that loss of a *Bcl11b* allele attenuates the cellular response to irradiation that results in arrest at S or G2/M phase. The attenuated cellular response leads to rapid cell cycle progression at S phase, which may decrease the time to repair and hence raise the chance for accumulation of mutations. It was reported that thymocytes at a stage after pre-TCR signaling and before completion of TCRα rearrangement, which includes ISP cells, undergo the malignant transformation in Notch1-activated conditions [28]. Therefore, ISP cells might be cells of origin in thymic lymphomas in *Bcl11b*$^{KO/+}$ mice.

Regulatory role for Bcl11b in cell cycle was studied using Bcl11b knock-down (KD) Jurkat cells, a T-cell culture line [29,30]. Bcl11b-KD cell lines showed cell death with decreased expression of the anti-apoptotic protein Bcl-xL and also a decrease in the CDK inhibitor p27. The decrease of p27 may promote cell cycle progression during S phase. Furthermore, activation of the cell-cycle checkpoint kinase Chk1 was deregulated in Bcl11b-KD cells. The activated Chk1 through phosphorylation leads to arrest of cell cycle at S phase [29]. Therefore, this deregulation may abrogate S phase checkpoint, consistent with the attenuated arrest of ISP cells in irradiated *Bcl11b*$^{KO/+}$ mice.

T-ALL is a heterogeneous disease comprising different genetic abnormalities of oncogenic and tumor suppressive genes that are associated with specific patterns of gene expressions, as determined by microarray analysis [31–33]. Type A abnormalities in T-ALL may delineate distinct molecular-cytogenetic T-ALL subgroups and are thought to cause arrest at a specific stage of normal T-cell differentiation. Results in this study demonstrated that loss of a *Bcl11b* allele in thymocytes affects T-cell differentiation leading to developmental arrest. The arrest may contribute to thymic lymphoma development. However, the consequence was not at a specific stage but at different ISP and DP stages, which may not support that *Bcl11b* mutation belongs to a group of type A abnormalities. Type B abnormalities target cellular processes such as cell cycle regulation. Our results showed that loss of a *Bcl11b* allele impairs cell cycle regulation of ISP cells in radiation-induced injury conditions. This favors that the loss of a *Bcl11b* allele belongs to the group of type B abnormalities, consistent with that *BCL11B* mutations were detected across the major molecular subtypes of T-ALL [22].

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Contributions: RG and KT contributed equally to this work and performed the majority of studies; SH performed studies in Fig. 2; YK performed studies in Fig. 3; YA and YM helped design studies in Figs. 3 and 4; RK helped to plan and direct experiments and wrote the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.leukres.2012.04.028.

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Review

Role of the transcription factor Bcl11b in development and lymphomagenesis

By Ryo KOMINAMI*1,†

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Abstract: Bcl11b is a lineage-specific transcription factor expressed in various cell types and its expression is important for development of T cells, neurons and others. On the other hand, Bcl11b is a haploinsufficient tumor suppressor and loss of a *Bcl11b* allele provides susceptibility to mouse thymic lymphoma and human T-cell acute lymphoblastic leukemia. Although there are many transcription factors affecting both cell differentiation and cancer development, Bcl11b has several unique properties. This review describes phenotypes given by loss of Bcl11b and roles of Bcl11b in cell proliferation, differentiation and apoptosis, taking tissue development and lymphomagenesis into consideration.

Keywords: Bcl11b, T-cell development, haploinsufficient tumor suppressor, T-cell leukemia, thymic lymphoma

1. Introduction

Bcl11b (B-cell CLL/lymphoma 11b) belongs to Kruppel-like C_2H_2 type zinc finger transcription proteins, the largest family of transcription factors in eukaryotes.[1] The gene encoding Bcl11b was first identified as a tumor suppressor gene by our study.[2] In fact, mice lacking one *Bcl11b* allele are susceptible to thymic lymphomas. On the other hand, mice lacking both *Bcl11b* alleles, which die shortly after birth of unknown causes, exhibit many defects in different organs of newborn mice, including immune system, central nervous system (CNS), skin, teeth, and hair cells in cochlea.[3)–6)] Therefore, Bcl11b plays critical roles in the development of those organs and possibly others.[7] Recently, Liu *et al.* have reviewed roles for Bcl11b in T-cell development and maintenance of T-cell lineage commitment.[1] Thus, this review provides a focus on the tumor suppressor role of Bcl11b rather than T-cell development.

Bcl11b is located on mouse chromosome 12 and on human chromosome 14. This gene is originally called *Rit1* (radiation-induced tumor suppressor gene 1), because *Bcl11b* was isolated by scanning γ-ray induced mouse thymic lymphomas for losses of specific chromosomal DNA.[8] More than 10 years ago, the scanning was performed for the 361 thymic lymphomas that were induced in mice crossed between BALB/c and MSM strains. The two strains belong to different mouse subspecies, *Mus musculus domesticus* and *Mus musculus molossinus*, respectively, and hence they carry many distinct alleles and DNA markers between the two. Genome-wide allelic loss or loss of heterozygosity (LOH) analysis using polymorphic DNA markers mapped several candidate tumor suppressor gene regions.[8] Further analysis localized one of the regions on mouse chromosome 12 to a 2.9 cM interval between the D12Mit53 and D12Mit279 marker positions.[9] Construction of a physical map consisting of 15 BAC clones in the vicinity contained informative boundaries of allelic losses, which allowed us to finally localize a 35 kb interval with a high frequency of allelic loss (62%). Sequence analysis of this interval led to the finding of *Bcl11b* gene, and mutation analysis identified this gene responsible for thymic lymphoma development.[2]

Another candidate region was mapped on mouse chromosome 11, which harbored *Ikaros* gene. Mutation analysis of this gene in thymic lymphomas

*1 Department of Molecular Genetics, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan.
† Correspondence should be addressed: R. Kominami, Department of Molecular Genetics, Niigata University Graduate School of Medical and Dental Sciences, Asahimachi 1-757, Chuo-ku, Niigata 951-8510, Japan (e-mail: rykomina@med.niigata-u.ac.jp).

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identified it as a tumor suppressor gene.[10] *Ikaros* is the well-known gene that plays critical roles in the development of lymphoid tissues and lymphomas.[11),12)]

2. Bcl11b and Bcl11a

Bcl11a is another member of the Bcl11 family in the mouse and human genomes.[13),14)] Although Bcl11a and Bcl11b share some sequence homology, they are located on different chromosomes and have different exon-intron structures. Bcl11a and Bcl11b are also called Ctip1 and Ctip2, respectively,[15)] because they were independently isolated for their interaction with the chicken ovalbumin upstream promoter transcription factor (COUP-TF) of orphan nuclear receptors. COUP-TF family members play important roles in development,[16)] and they usually mediate transcriptional repression by recruiting nuclear receptor co-repressor (NCoR) and/or silencing mediator for retinoid and thyroid hormone receptor (SMRT) to the template.[17)] As a transcription factor, Bcl11a and Bcl11b are also associated with the nucleosome remodeling and histone deacetylase (NuRD) complex to repress target promoters.[18),19)] However, functional association between Bcl11a/Ctip1 or Bcl11b/Ctip2 and COUP-TF remains open.

Phylogenetic analysis of Bcl11-like genes suggests that a homolog of Bcl11b first appears in cartilaginous fishes.[20)–22)] On the other hand, homologs of Bcl11a are already present in the genomes of amphioxus and sea lamprey. As previously pointed out,[21),22)] though not by Guo and Cooper *et al.*,[20)] the Bcl11 protein in sea lamprey, a jawless vertebrate, can be categorized into the Bcl11a cluster. This suggests that Bcl11b is segregated form the Bcl11a homolog at the vertebrate stage, and no Bcl11b homolog is present in invertebrates. This may be compatible with that Bcl11a is involved in the transcription of hemoglobin genes (see below) whereas Bcl11b regulates the development of T cells that are not present in sea lampreyor or in other invertebrates like sea urchin.

Bcl11a was originally called Evi9, named after a retroviral insertion site (Evi9) in myeloid leukemia tumors in BXH-2 recombinant inbred mice. Detailed analysis of the Evi9 site discovered this gene.[23)] Mice lacking *Bcl11a* exhibited neonatal lethality and impairments in B cell and lymphoid cell development.[14)] However, recent genetic studies of *BCL11A* in humans have shed new light on a complex regulatory process of fetal hemoglobin (HbF) expression. *BCL11A* is associated with persistent fetal hemoglobin in adult humans,[24),25)] which was provided by genome-wide association studies. This analysis identified *BCL11A* as a new HbF-associated gene on chromosome 2, by taking advantage of the natural variation in the level of HbF in various human populations. Subsequent studies established that BCL11A is a central mediator of γ-globin silencing and hemoglobin switching.[26)] An example of the finding in these studies is that down-regulation of BCL11A expression in adult human erythroid precursors led to robust induction of HbF,[27)] and mechanistically, BCL11A interacts with the Mi-2/NuRD chromatin remodeling complexes, as well as the erythroid transcription factors GATA1 and FOG1, in erythroid progenitors.[27)] Very recent studies revealed a network of transcription factors that the transcription factor KLF1 is a key activator of the BCL11A gene.[28),29)] Knockdown of KLF1 in human and mouse adult erythroid progenitors markedly reduced BCL11A levels and increased human gamma-globin/beta-globin expression ratios.

3. Bcl11b and trancription

Bcl11b/Ctip2 was initially identified as a transcriptional repressor that either directly bound to a GC-rich consensus sequence of target genes and/or interacts with NuRD complex.[18),19),30)] On the other hand, Bcl11b was shown to activate the transcription of NF-κB target genes,[31)] suggesting that Bcl11b acts both as a transcriptional repressor and activator in a context dependent manner. Figure 1 displays the Bcl11b structure including DNA binding and protein-interacting regions. The *Bcl11b* gene consists of 4 exons and encodes two different isoforms, α-isoform consisting of 884 and β-isoform consisting of 812 lacking exon 3 in the mouse.[2)] The long exon 4 comprises all six zinc-finger domains, and the 2nd and 3rd domains are responsible for DNA binding. Recently, structural homology modeling has been performed as to canonical DNA binding of Bcl11b zinc fingers, which is based on the high-resolution crystal structure of the zinc finger domains of the transcription factor Egr1 in complex with DNA.[32)] The result reveals that mutations identified within the 2nd and 3rd domains disrupt the structure comprising conserved amino acids that are modeled to be required for the stability of the zinc finger domain or its binding to DNA. Apart from the DNA binding region, Bcl11b possesses domains responsible for interaction with proteins and protein complexes. Their catalogue has grown recently, including histone deacetylases (HDAC1 and HDAC2), and the ubiq-

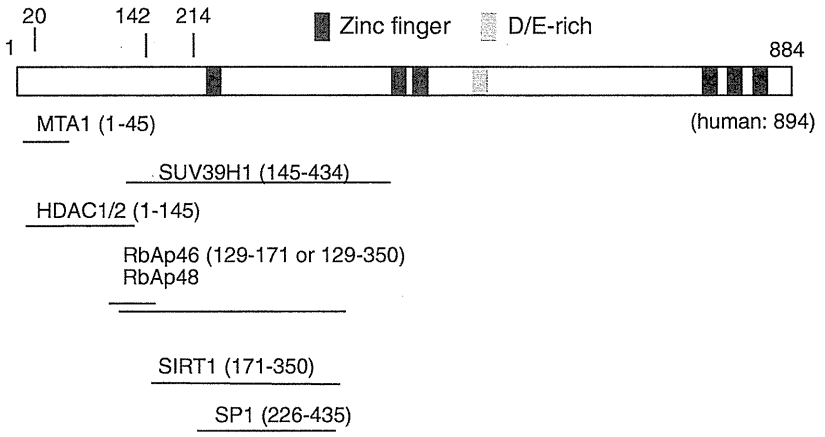

Fig. 1. Structure of Bcl11b protein. The bar represents the β isoform of Bcl11b including Zinc-finger and D/E-rich domains on the bar. All these domains are within the long exon 4, and the exons 1–3 are 214 amino acids in length. The human BCL11B is longer than the mouse one and consists of 894 amino acids in the β isoform. The 2nd and 3rd Zinc-finger domains are for DNA binding and regions shown by lines are for various proteins binding to Bcl11b.

uitous transcription factor Sp1, and the Rb-associating proteins (RbAp46 and RbAp48), the member of surtuin family proteins (Sirt1), the heterochromatin protein 1 (HP1), and the histone methyltransferase SUV39H1.[18),19),33)–36)]

Several target genes of Bcl11b were discovered in cultured cells. Genes encoding cyclin-dependent kinase inhibitiors, p21/Cip2/Waf1 and p57/Kip2, are examples, and they are transcriptinally suppresssed by Bcl11b.[19),37)] In addition, we have identified HDM2(MDM2) as a new target, a ubiquitin ligase that downregulates a key tumor suppressor p53.[38)] Interestingly, Bcl11b inhibits HDM2 expression in a p53-dependent manner and modulates responses to radiation-induced DNA damages. A study of thymocytes *in vivo* using Chip-seq (Chromatin immunoprecipitation followed by DNA sequencing) method revealed several new target sequences and genes such as Th-Pok (Zbtb7b) and Runx3,[39)] expression of which is required for immature DP thymocytes (see Section 4.1 and Fig. 2 for abbreviation of thymocyte subsets) to further differentiate into mature thymocytes (CD4SP or CD8SP cells, respectively). Those genes are upregulated in the DP thymocytes lacking Bcl11b expression, suggesting suppressive role for Bcl11b in their transcription. Induction of Th-Pok expression occurs downstream of T-cell receptor signaling,[40)] whereas Runx3 contributes to Th-Pok repression in CD8SP committed cells.[41)] Hence, these suggest essential roles for Bcl11b in early silencing of Th-Pok and Runx3 genes. It may be also possible that Bcl11b cooperatively works with ThPok or Runx in the activation or suppression of some target genes. However, it is elusive of the mechanism and whether or not these repressions by Bcl11b are done through the association with NuRD or others.

Recently, Chip-seq analysis for Bcl11b binding sequences was done in cells of striatal neurons. As a result, as many as 248 target genes were identified with the aid of gene expression profiling, which suggests the neurotrophic factor/neurotrophin signaling pathway as a primary target pathway for Bcl11b regulation.[42)]

4. Phenotypes of Bcl11b-deficient mice

Bcl11b is known to plays crucial roles in the development of several organs, including T cells, CNS, skin, and tooth. Bcl11b-deficient mice exhibit various developmental defects in these organs as follows.

4-1. T cells. As described above, the defect in T-cell development given by Bcl11b deficiency is the phenotype firstly discovered. At present, defects have been identified at several distinct stages of development of thymocytes and T cells.[3),22),43)–47)] Figure 2A illustrates T-cell development in thymus (see below for details) and indicates the stages of developmental arrest given by loss of Bcl11b. Bcl11b is a unique transcription factor that specifically functions for T-cell identity maintenance and another transcription factor of this type is Tcf1 (T-cell factor 1).[22),46)–48)] The T cell specification pathway involves many different signalings, one of which is the signaling by

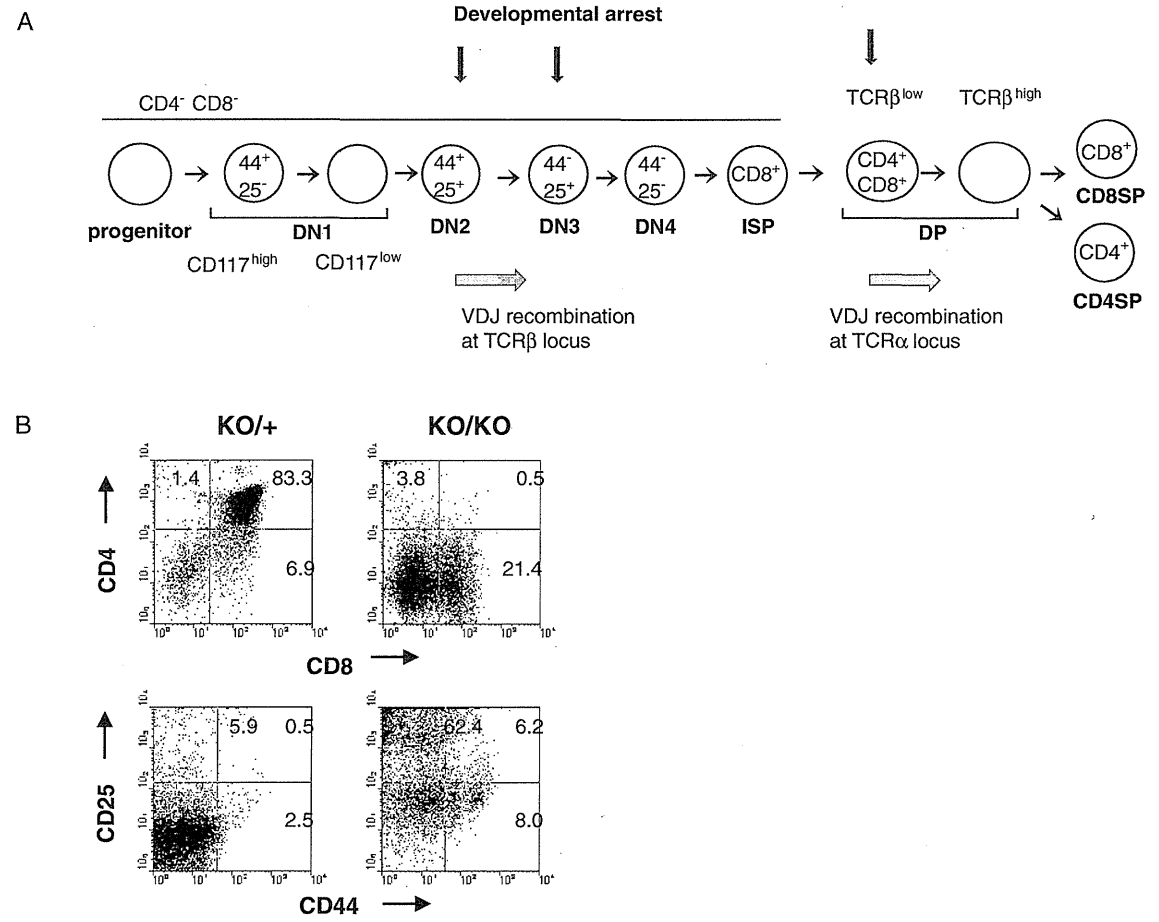

Fig. 2. (A) The diagram illustrates stages of T-cell development in thymus. Differentiation markers used here are CD117, CD44, CD25, CD4, CD8, and TCRβ on cell surface. See the text for details of development of $\alpha\beta$ T cells. In brief, it proceeds in order of maturity, $CD4^-CD8^-$ double negative (DN), $CD4^+CD8^+$ double positive (DP), and $CD4^+CD8^-$ or $CD4^-CD8^+$ single positive cells (CD4SP or CD8SP cells, respectively). Vertical arrows indicate stages of developmental arrest given by loss of Bcl11b. (B) Flowcytometry of thymocytes in $Bcl11b^{KO/+}$ and $Bcl11b^{KO/KO}$ mice using CD4, CD8, CD44, and CD25 markers. The vertical axis shows CD4 expression and the horizontal axis displays CD8 expression in total thymocytes (upper); the vertical axis shows CD25 expression and the horizontal axis displays CD44 expression in the CD4/CD8 double-negative quadrant (lower).

the Notch1 receptors. The receptors are expressed by early progenitors, and activated to function upon interaction with cognate ligands (Delta-like proteins) expressed by thymic epithelial cells.[49),50)] The review by Liu *et al.*[1)] and others[51),52)] describe tissue-specific signals that direct developmental fates of thymocyte progenitors in the thymus. To avoid redundancy, I touch briefly on thymocyte development and roles for Bcl11b in controlling thymocyte differentiation and expansion.

T cells arise from hematopoietic progenitor cells that migrate from the bone marrow to the thymus, where they proliferate as thymocytes (Fig. 2A). Development of $\alpha\beta$ T cells in the thymus proceeds through three major stages defined according to their expression pattern of CD4 and CD8 molecules on cell surface, i.e. in order of maturity, $CD4^-CD8^-$ double negative (DN), $CD4^+CD8^+$ double positive (DP), and $CD4^+CD8^-$ or $CD4^-CD8^+$ single positive cells (CD4SP or CD8SP cells, respectively). The CD4 and CD8 molecules are coreceptors of the T-cell receptor (TCR). Before DN thymocytes progress to the DP stage, they express CD8 but lack $\alpha\beta$ TCR on cell surface. Those cells are highly proliferative and called immature $CD8^+$ single positive (ISP) cells. Thymocytes at the DP stage express the $\alpha\beta$ T-cell receptor

($\alpha\beta$ 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