

Takashi Ebihara, Wooseok Seo,
and Ichiro Taniuchi

Abstract

During hematopoiesis, a variety of cells are generated from stem cells through successive rounds of cell fate determination processes. Studies in the last two decades have demonstrated the involvement of Runx transcription factor family members in differentiation of multiple types of hematopoietic cells. Along with evolutionary conservation, the Runx

family is considered to be one of the ancestral regulators of hematopoiesis. It is conceivable that the Runx family is involved in shaping the immune system, which is then comprised of innate and acquired lymphoid cells in vertebrates. In this chapter, we will first summarize roles of Runx proteins during the development of T- and B-lymphocytes, which appeared later during evolution and express antigen specific receptors as a result of DNA recombination processes. We also discuss the recent findings that have unraveled the functions of Runx during differentiation of innate lymphoid cells (ILCs).

Keywords

Runx family • Hematopoiesis • Lymphocytes • ILCs • Transcription factor • Development

T. Ebihara
Howard Hughes Medical Institute, Department of
Medicine, Washington University School of
Medicine, 660 South Euclid Avenue,
St. Louis, MO 63110-1093, USA

W. Seo • I. Taniuchi (✉)
Laboratory for Transcriptional Regulation, RIKEN
Center for Integrative Medical Sciences (IMS),
1-7-22 Suehiro-cho, Tsurumi-ku,
Yokohama 230-0045, Japan
e-mail: ichiro.taniuchi@riken.jp

24.1 Introduction

As expected from many studies showing multiple roles of Runx family in the control of development of many types of cells, genetic ablation of either Runx1 or Cbfb resulted in lack of definitive hematopoiesis (Okuda et al. 1996; Wang et al. 1996a, b), placing Runx1/Cbfb as one of the top regulators in the development of hematopoietic cells. Studies in invertebrates such as *Drosophila* also

found that Runx family proteins play essential roles in hematopoiesis (Fossett and Schulz 2001; Lebestky et al. 2000), suggesting the ancestral function of Runx as an important regulator that controls the differentiation of hematopoietic cells (Braun and Woollard 2009). In this chapter, we summarize the roles of the Runx family in development of immune cells including lymphocytes and innate lymphoid cells (ILCs) in vertebrates.

24.2 Roles of Runx Complex in T Lymphocyte Development

24.2.1 Early Thymocyte Differentiation

After T-cell homing, precursors migrate from the fetal liver to the thymus rudiments around embryonic day 11.5–12.5 in mice, they colonize there and receive environmental cues to begin development into T-lymphoid cells. The expression of Notch ligands such as Delta-like 4 by thymic epithelial cells (TEC) is known to be a critical environmental signal for early thymocyte development (Hozumi et al. 2008; Koch et al. 2008). These early T cell progenitors (ETP) undergo sequential processes, which are controlled by transcription factor networks, in order to fully commit to the T cell lineage (Fig. 24.1) (Rothenberg et al. 2008). Since ETPs do not express CD4 and CD8 co-receptors, they are referred to as CD4⁻CD8⁻ double negative (DN) thymocytes, which are further divided into four subsets by the expression of CD25 and CD44 or CD117 (c-Kit). CD25⁻CD44⁺ DN1 subset is relatively heterogeneous (Porritt et al. 2004) and contains ETPs that differentiate into CD25⁺CD44⁺ DN2 cells. Recent studies addressing the transcriptional regulation of early T cell development have proposed that the DN2 stage is further divided into DN2a and DN2b, and that complete commitment to the T cell lineage occurs at the DN2a and DN2b transition in a Bcl11b dependent manner, based on the observation that T cell development was arrested at the DN2a stage in Bcl11b-deficient mouse (Li et al. 2010; Ikawa et al. 2010). Importantly, in host mice where T cell development is reconstituted

from Runx1-deficient hematopoietic stem cells (HSCs), a similar developmental block at the DN2 stage was observed (Fig. 24.1) (Ichikawa et al. 2004). Although the precise mechanism of how lack of Runx1 is involved in DN2a arrest is not clear, it is possible that Runx1 is necessary for induction of *Bcl11b* expression. Indeed association of Runx with a 3' downstream enhancer that plays an essential role to drive Bcl11b gene expression in T-cells was recently reported (Li et al. 2013).

There are two types of T-lymphocytes that express distinct T-cell antigen receptors, $\alpha\beta$ TCR and $\gamma\delta$ TCR. Although how and when the progenitors decide to become $\alpha\beta$ T cells or $\gamma\delta$ T cells remains controversial, there are ample studies addressing how the genes encoding α , β , δ and γ chains of TCR are activated and undergo DNA recombination, known as V(D)J recombination mediated by RAG-1/2 recombinase complexes at the antigen receptor loci. V(D)J recombination is a unique property endowed only to lymphocytes among somatic cells and is strictly controlled. Since there are other excellent reviews discussing the mechanisms of V(D)J recombination (Majumder et al. 2015), we simply focus on the effects of enhancer inactivation of each T-cell antigen gene. It has been shown that the E α enhancer, E β enhancer, E δ enhancer, and E γ enhancer at the *Tcra*, *Tcrb*, *Tcrd*, and *Tcrg* genes, respectively, are essential for this recombination. Interestingly, each enhancer contains Runx recognition motifs (Hsiang et al. 1993; Redondo et al. 1991; Takeda et al. 1990; Hernandez-Munain and Krangel 1995) and is bound by Runx complexes (Tani-Ichi et al. 2011; Oestreich et al. 2006; Hollenhorst et al. 2007). Among these enhancers, Runx sites often locate closely to Ets binding sites (Wotton et al. 1994; Hernandez-Munain et al. 1998). For instance, in the E β enhancer, two ETS-Runx elements have been identified. Targeted mutations abrogating two Runx sites within the E β enhancers eliminated the enhancer function (Majumder et al. 2015), suggesting that Runx binding is essential for enhancer activation (Fig. 24.1). However, due to the arrest of DN2 stage through lack of Runx1 activity, the role of Runx1 function in the regula-

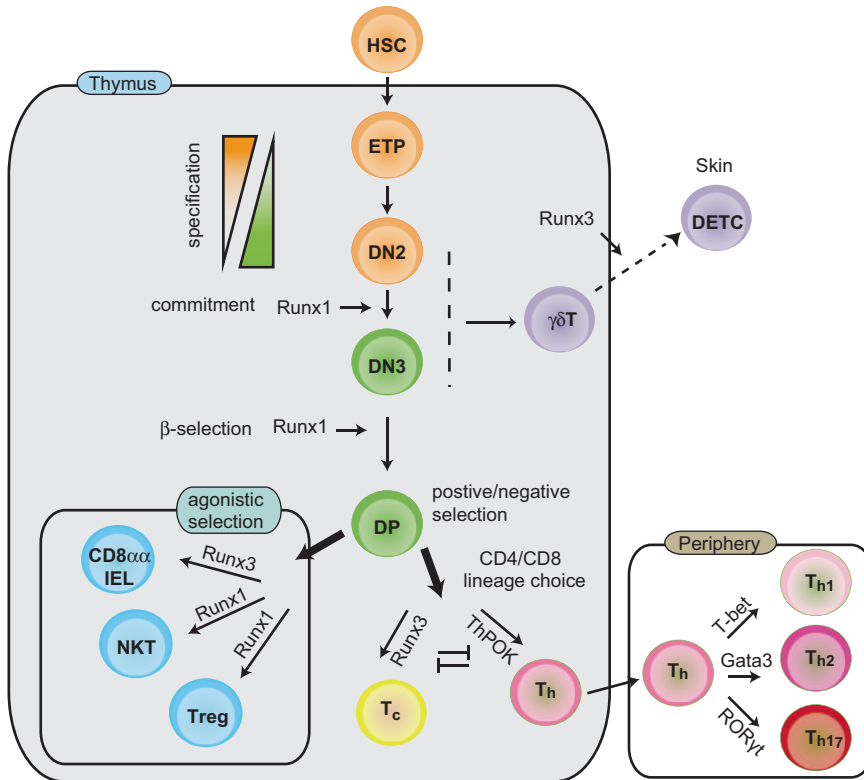


Fig. 24.1 Runx and T cell development. Upon pre-thymic acquisition of thymus homing property at some point during differentiation of hematopoietic stem cells (HSC), early thymocyte progenitors (ETP) colonize at the thymus and begin to develop into T lymphocyte-lineage by cascading activation of T cell programs as well as erasing developmental potency to alternative lineages, referred to as commitment process. Full commitment to T-lineage occurs at transition from DN2 to DN3 stage, and Runx1 is essential for this transition. $\gamma\delta$ T cells are differentiated from DN2/DN3 cells and development of skin-specific $\gamma\delta$ T cells, known as dendritic epidermal T cells (DETC), requires Runx3. Runx1 is important to pass β -selection. CD4⁺CD8⁺ DP thymocyte precursors are selected according to affinity of TCR with self-peptide (positive/negative selection) and positively selected thymocytes differentiate

into CD4 helper (Th) or CD8 cytotoxic (Tc) T cells (CD4/CD8 lineage choice). Cross-antagonism between Runx3 and ThPOK plays a central role to fine separation of two fates as well as to couple MHC specificity of TCRs with appropriate fate. Some DP precursors develop into regulatory T cells (Treg), natural killer T cells (NKT) and CD8 $\alpha\alpha$ intra epithelial cells (IEL) through process known as agonistic selection, during which Runx1 and Runx3 play important roles for their differentiation. In the periphery, upon encountering antigens and depending on environmental cues, Th cells differentiate into distinct types of effector cells such as Th1, Th2 and Th17, each of which secrete signature cytokine, IFN γ , IL-4 and IL-17, respectively. Roles of Runx proteins during effector Th subset differentiation are discussed in the Sect. 24.2.3.

tion of *Tcrb* gene activation remains unclear. It is noteworthy, however, that in addition to enhancers in the T-cell antigen receptor loci, a μ enhancer in the *Igh* locus that encodes the B-cell antigen receptor also contains ETS-Runx composite elements (Erman et al. 1998). Thus, there might be a common regulatory mechanism by which antigen receptor loci are activated through co-binding of ETS and Runx to essential enhanc-

ers in these loci. Consistent with this note, skin specific $\gamma\delta$ T cells, referred to as DETC (dendritic epidermal T cells) expressing the invariant V γ 3 chain, were absent in the epidermis of Runx3-deficient mice (Fig. 24.1) (Woolf et al. 2007). However, fewer precursors expressing the V γ 3 chain were observed in the fetal thymus of Runx3-deficient mice, suggesting that Runx3 is also involved in the processes of expansion driven

by interleukin 2 (IL-2) and migration guided by integrin CD103 for DETC differentiation after $\gamma\delta$ TCR expression.

24.2.2 Differentiation of $\alpha\beta$ T Cell Subsets from CD4⁺CD8⁺ DP Precursors

24.2.2.1 Overview

During $\alpha\beta$ T cell differentiation, there is a “check point” stage to monitor whether V(D)J rearrangement at the *Tcrb* locus successfully generates a functional TCR β chain, known as β -selection. Cells lacking TCR β chain expression fail to form pre-TCR complexes, consisting of the common pre-T α protein and the TCR β protein, are not allowed to differentiate to the next stage and are arrested at the CD25⁺CD44⁻ DN3 stage. On the other hand, signals through the pre-TCR complexes lead to cell proliferation and activate developmental programs including V(D)J rearrangement at the *Tcra* locus and the expression of CD4 and CD8 co-receptors. Thus CD4⁻CD8⁻ DN thymocytes become CD4⁺CD8⁺ DP thymocytes expressing the mature $\alpha\beta$ TCR with diverse antigen-specificity and face another selection process, known as positive/negative selection, which evaluates the quality of $\alpha\beta$ TCR in terms of their affinity to self-peptides presented on the major histocompatibility complex (MHC). Lack of TCR-mediated signals due to insufficient affinity to self-peptide–MHC complexes causes apoptosis, designated as ‘death by neglect’. Cells with TCRs that react too strongly to self-peptide–MHC complexes, are thereby thought to be potentially self-reactive lymphocytes and are eliminated through a ‘negative selection’ process, to reduce the risk of auto-immunity. Only a few DP cells that express TCRs of appropriate affinity with self-peptide–MHC complexes are selected in a process known as ‘positive selection’, and proceed to develop into mature thymocytes. The CD4 and CD8 co-receptors help the TCRs to recognize self-peptides on MHC-class-II and MHC-class-I molecules via specific interaction with class-II and class-I, respectively. After positive selection, two major $\alpha\beta$ T-lineages, helper

cells and cytotoxic cells are generated. It is well known that cells selected through MHC-class-II (thereby their TCRs are MHC-class-II specific) become helper cells and lose CD8 expression, while those selected by MHC-class-I differentiate into the cytotoxic lineage and lose CD4 expression. Thus, in addition to the specificity of TCRs to MHC classes, CD4/CD8 expression profiles also show a perfect match with helper/cytotoxic lineage choice. In addition to the helper/cytotoxic lineage dichotomy, at least three types of $\alpha\beta$ T cells, iNKT cells, regulatory T cells (Treg), and CD8 $\alpha\alpha$ intraepithelial lymphocytes (IEL) are generated from the CD4⁺CD8⁺ DP thymocytes through ‘agonist selection’ (Fig. 24.1) (Kronenberg and Rudensky 2005; Lambolez et al. 2007).

24.2.2.2 Runx and *Cd4/Cd8* Gene Regulation

As mentioned above, lineage specific expression of CD4/CD8 co-receptors after positive selection stimulated studies addressing the regulation of this lineage specific expression (Ellmeier et al. 1999). The search for critical *cis*-regulatory elements in the *Cd4* and *Cd8* loci first resulted in the identification of a transcriptional silencer in the *Cd4* locus as a critical regulatory region for helper-lineage specific CD4 expression (Sawada et al. 1994; Siu et al. 1994). A 434 bp transcriptional silencer, located at the first intron in the *Cd4* gene, is sufficient to repress reporter transgene expression in DN thymocytes and cytotoxic-lineage cells. Furthermore, following studies that removed the *Cd4* silencer from the mouse genome confirmed its relevance by revealing derepression of CD4 in CD8⁺ T cells at a comparable level to that in CD4⁺ T cells (Leung et al. 2001; Zou et al. 2001). These results from mouse genetics clearly indicated that a single transcriptional silencer is responsible for helper-lineage specific CD4 expression by repressing *Cd4* in alternative cytotoxic-lineage T cells.

On the other hand, functionally negative regulatory elements have not been identified in the *Cd8* locus. Instead, at least six enhancer elements, termed as E8I to E8VI, have been isolated (Hostert et al. 1998; Ellmeier et al. 1998;

Sakaguchi et al. 2015). The effects of each enhancer deletion on *Cd8* expression revealed redundant function within the enhancers. Along with the CD8-lineage specific activity of E8I in a reporter transgene expression assay (Ellmeier et al. 1997), a combinatory regulation of enhancers rather than active repression by silencer(s) is supposed to control the lineage specificity of *Cd8* expression.

Runx proteins are known to directly regulate both *Cd4* and *Cd8* expression. Runx1 protein was isolated from a search for a *Cd4* silencer binding protein using the yeast one-hybrid screen (Taniuchi et al. 2002). Along with the essential requirement for the Runx recognition site for *Cd4* silencer activity, conditional inactivation of Runx1 in DN2/3 thymocytes by the *Lck-Cre* transgene resulted in CD4 expression in DN3 cells as was observed by lack of *Cd4* silencer (Taniuchi et al. 2002). On the other hand, loss of Runx3 but not Runx1, severely affected *Cd4* silencer activity in CD8-lineage cells (Taniuchi et al. 2002) as was manifested by de-repression of CD4 in mature Runx3-deficient CD8⁺ T cells. Such a distinct role of two Runx proteins in *Cd4* gene repression at two stages may reflect different expression patterns of Runx1 and Runx3. While Runx1 is most highly expressed in immature DN and DP thymocytes, expression of Runx3 was nearly specific to CD8 SP thymocytes (Egawa et al. 2007). In addition to the involvement of Runx proteins in *Cd4* gene regulation, the binding of Runx3 to some enhancers in *Cd8* gene was also reported (Sato et al. 2005). Recently, the functional contribution of Runx to *Cd8* gene expression was demonstrated by the inefficient maintenance of CD8 expression in the absence of Runx3 (Hassan et al. 2011). Such dual roles of Runx3 in co-receptor gene expression, *Cd4* silencing, and *Cd8* reactivation, which comprise a key feature of cytotoxic-lineage cells predicted that induction of Runx3 expression might be a key event to activate programs that induce a cytotoxic fate. It should be noteworthy that transgene-mediated ectopic expression of Runx3 in immature thymocytes only partially redirected MHC-class-II-specific T cells to CD8⁺ T-cells (Kohu et al. 2005; Grueter et al. 2005), at

least in part via low CD4 expression on precursor cells as a result of prolonged *Cd4* silencer activity in the DN stage.

24.2.2.3 Antagonistic Interplay Between Runx and ThPOK

ThPOK is a member of the BTB/POZ transcription factor family; many members of this family have been shown to play essential roles in immune cell development (Ellmeier and Taniuchi 2014). Gain- and loss-of-function studies of ThPOK in mice have revealed that ThPOK is a master transcription factor for CD4⁺ helper T cell development (Kappes and He 2006). For instance, a natural mutation referred to as the *hd* mutation that results in substitution of glycine for arginine in the putative second zinc-finger domain of the ThPOK protein, led to a loss of CD4⁺ T cell development through fate conversion of MHC-class-II specific cells into CD4⁻CD8⁺ T cells (He et al. 2005). On the contrary, ectopic expression of ThPOK from DP precursors onwards resulted in lack of CD8⁺ T cells due to redirection of MHC-class-I-specific thymocytes to CD4⁺CD8⁻ T cells (He et al. 2005).

This striking finding that the presence or absence of a single transcription factor, ThPOK, serves as a major determinant for CD4 helper versus CD8 cytotoxic lineage separation, raised the profound question of how helper-lineage-specific expression of ThPOK is controlled. The answer was revealed during characterization of Runx mutant mice. One issue to be considered while interpreting phenotypes caused by single Runx protein ablation is the redundancy between Runx family proteins. In particular, cross-regulation between Runx1 and Runx3 sometimes underestimates the effect caused by the lack of either protein. Given that the product of a single gene *Cbfb* is the sole common subunit for all Runx proteins in mammals, inactivation of *Cbfb* has the advantage in terms of avoiding redundancy between Runx proteins. It should be noted that the Cre/loxP-mediated recombination system occasionally suffers from the expansion of a leaky population that escapes Cre-mediated recombination. In addition, protein stability is another factor to be considered in Cre/loxP medi-

ated conditional gene inactivation. Compared to Runx proteins that could be rapidly degraded by proteasomes, the Cbfb protein seemed to be present longer after the inactivation of its gene. Thus, in thymocyte differentiation, inactivation of *Cbfb* gene at the DN stage by *Lck-Cre* nearly recapitulated the compound inactivation of both *Runx1* and *Runx3* at the DP stage by *Cd4-Cre*. Analyses of T-cell development in these mice showed that loss of the Runx complex function in DP thymocytes led to severe reduction of mature thymocyte generation. Most importantly, in the remaining mature T-cell pool, CD8⁺ T cells were almost absent (Setoguchi et al. 2008). Further analyses using mice in which *Runx3* inactivation was combined over *Runx1* mutation, causing the deletion of the VWRPY motif at C-terminal end, revealed that redirection of MHC-class-I-specific T cells to CD4⁺ T-cells was the reason for the loss of CD8⁺ T cells. This was a phenocopy of the ThPOK transgenic mice, and prompted analyses of *Cbfb^{fl/fl}: Cd4-Cre* mice that retain a substantial number of CD8⁺ T cells that abnormally depress CD4. These CD4⁺CD8⁺ cells developed under the gradual loss of Cbfb protein after positive selection, expressed *Thpok* gene, providing supportive evidence that redirection of MHC-class-I-specific thymocytes to the CD4⁺ T-cell lineage in mice lacking functional Runx proteins is due to inappropriate de-repression of *Thpok*. These observations clearly indicate that Runx proteins are involved in *Thpok* repression.

Naturally, the next question would be how Runx proteins are involved in *Thpok* regulation and whether Runx proteins directly regulate the regulatory regions of the *Thpok* gene. By using a ChIP-on-chip approach, two regions in the *Thpok* gene were identified as Runx binding sequences (RBS-1 and RBS-2). Further functional analysis of RBS-1 in a reporter transgene expression assay identified transcriptional silencer activity in this sequence. At the same time, a group led by Dr. Kappes also characterized the regulatory regions in *Thpok* and identified a silencer activity in a their distal regulatory element (DRE), that perfectly overlapped with RBS-1 (He et al. 2008). These observations indicate that silencer activity in DRE/RBS-1, hereafter referred to as *Thpok*

silencer, is responsible for helper lineage specific expression via repression of *Thpok* expression in cytotoxic-lineage cells. The physiological relevance of *Thpok* silencer was confirmed by full *Thpok* de-repression as well as by the loss of CD8⁺ T cells upon its removal from mouse genomes (Setoguchi et al. 2008). Importantly, the analytical ChIP assay detected Runx binding to *Thpok* silencer in both ThPOK-expressing helper and non-expressing cytotoxic cells (Setoguchi et al. 2008). This observation indicates that Runx binding is essential but not sufficient for *Thpok* silencer activity and that uncharacterized mechanisms beyond Runx binding may serve as the switch that controls the specificity of *Thpok* silencer activity.

Thus two silencers at different loci, the *Cd4* silencer and the *Thpok* silencer, require Runx protein binding to exert their silencer activity. Interestingly, albeit this common feature, both silencers show distinct dependency for VWRPY motifs at the C-terminal end of Runx proteins. While *Cd4* silencer activity depends completely on the VWRPY motif, *Thpok* silencer still represses the *Thpok* gene to a significant extent without the VWRPY motif (Seo et al. 2012b).

Collectively, one of the most important functions of Runx proteins during cell fate decision by CD4⁺CD8⁺ DP precursors is the repression of *Thpok* as well as *Cd4* genes. On the other hand, characterization of ThPOK function revealed that ThPOK acts to restrain Runx3 expression in CD4⁺ T cells. Thus, ThPOK and Runx3 repress the expression of each other, forming an antagonistic interplay. Since such antagonism between two transcription factors, which play a central role in the development of alternative lineages, is often observed at developmental branch point (Laiosa et al. 2006), the cross antagonism between ThPOK and Runx3 serves as a central mechanism in CD4 helper/CD8 cytotoxic dichotomy (Fig. 24.1). Given that CD8-lineage specific expression of Runx3 reflects a CD8-lineage specific expression from the distal P1 promoter-driven *Runx3* transcript (Egawa et al. 2007), ThPOK should be involved in the regulatory mechanism that controls the lineage specific activity of the distal P1 promoter. IL-7 signals are

known to be important for the generation of CD8-lineage cells in the thymus and can activate *Runx3* (Park et al. 2010). In line with this finding, it was proposed that ThPOK is indirectly involved in *Runx3* repression through induction of the SOCS protein family, a strong inhibitor of the IL7 cytokine signals, thereby preventing *Runx3* induction (Luckey et al. 2014). Unfortunately, at this moment, little is known about the regulatory regions that control *Runx3* expression in T cells. Further studies are necessary to precisely understand the regulation of CD8-lineage specific *Runx3* expression.

24.2.2.4 Roles of Runx in Differentiation of Treg and iNKT Cells

Beyond their role in regulating the CD4/CD8 lineage separation, Runx proteins are known to have other important roles in development of T cell subsets. Results of *Runx1* inactivation alone showed that the efficiency of β -selection and positive selection was impaired (Egawa et al. 2007). In addition, generation of invariant natural killer T (NKT) cells expressing the invariant V α 14 chain and the reactive lipid antigen on MHC class –I related CD1d was lost by lack of *Runx1* (Egawa et al. 2005). Given that *Runx3* is also expressed in iNKT cells, *Runx3* is not likely to possess a compensatory function to support iNKT cell differentiation.

Another $\alpha\beta$ T cell subset generated from CD4⁺CD8⁺ DP precursors includes the regulatory T (Treg) cells (Ohkura et al. 2013; Josefowicz et al. 2012). Treg cells have a suppressive function and play an essential role in immune tolerance. FoxP3, a member of the forkhead box transcription factor family, is essential for the generation and function of Treg cells. Mutation of X chromosome-linked human *FOXP3* gene result in the IPEX (immunodysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome, showing multi-organ autoimmune inflammatory disease, whereas mutations in the murine *Foxp3* gene, for instance, a natural mutation in the scurfy strain or engineered loss of the functional mutation, are known to cause severe postnatal lethal autoimmune disorders

(Josefowicz et al. 2012). Thus, how the expression and functions of Foxp3 are regulated, is an essential and profound question in immunology. A study by the Sakaguchi's group has shown that RUNX1 can interact with FOXP3 protein by the immunoprecipitation assay (Ono et al. 2007). RUNX1 seemed to bind to the IL2 promoter and enhance IL-2 production upon TCR stimulation, which is repressed by FOXP3. In addition to regulation at the protein level, Runx proteins were shown to regulate *Foxp3* gene expression through binding to promoters and CNS2 enhancers (Bruno et al. 2009; Kitoh et al. 2009; Rudra et al. 2009; Klunker et al. 2009). A CNS2 enhancer undergoes Treg specific DNA demethylation processes and contributes to stable expression of the *Foxp3* gene (Zheng et al. 2010). Interestingly, CNS2 remained methylated in Treg cells lacking Cbfb and *Foxp3* expression level was reduced (Rudra et al. 2009; Kitoh et al. 2009). Results of Treg specific conditional inactivation of *Runx1* or *Runx3* genes showed that *Runx1*/Cbfb complexes are responsible for maintaining Foxp3 expression levels and thereby preventing immunological disorders such as gastritis, serum IgE elevation, and lymphadenopathy (Kitoh et al. 2009).

The third $\alpha\beta$ T cells subset generated through agonistic selection of CD4⁺CD8⁺ DP thymocytes include the CD8 $\alpha\alpha$ IEL cells due to their unique expression profile of CD8 $\alpha\alpha$ homodimers but not CD8 $\alpha\beta$ heterodimers as well as the tissue localization in the space between gut epithelial cells. Distinct from the CD8 $\alpha\beta$ heterodimer, CD8 $\alpha\alpha$ homodimers interact with the MHC class I like TL molecule (Leishman et al. 2001). By using a soluble TL tetramer, the sole reagent that is useful to separately detect CD8 $\alpha\alpha$ from CD8 $\alpha\beta$ on cells expressing both forms, it was shown that a proportion of DP thymocytes already expressed the CD8 $\alpha\alpha$ homodimer, thereby referred to as CD4⁺CD8 $\alpha\beta$ ⁺CD8 $\alpha\alpha$ ⁺ triple positive (TP) thymocytes (Gangadharan et al. 2006). TP thymocytes are supposed to be precursors for CD8 $\alpha\alpha$ IEL, and they become CD4⁻CD8⁻ DN thymocytes after agonistic selection. *Runx3* is essential for the re-expression of CD8 $\alpha\alpha$ (Pobezinsky et al. 2012), presumably through direct activation of E8I enhancers.

24.2.3 Role of Runx in Differentiation of Effector T Cells

Advances in the last decade have identified and characterized novel effector CD4⁺ T cell subsets beside the classical Th1 and Th2 cells. In addition, these subsets retain a plasticity that allows them to occasionally modulate their identity (Nakayamada et al. 2012). A characteristic difference in the functions of these subsets is the pattern of cytokine secretion conferred by the induction of specific transcription factors, as is known in the case of classical Th1/Th2 subsets, these signature cytokines and transcription factors are IFN γ /IL-4 and T-bet/Gata3, respectively. Runx proteins have also been shown to regulate effector T cell subsets and cytokine expression. During Th1 cell differentiation, expression of Runx1 and Runx3 proteins exhibited unique reciprocal expression kinetics. Runx1 expression is downregulated while Runx3 expression is induced. Importantly, in differentiated Th1 cells, Runx3 represses *Il4* gene transcription through binding to an *Il4* silencer (Naoe et al. 2007; Djuretic et al. 2007).

Discovery of Th17 cells, whose characteristic cytokine is IL17, led to a renewed view of helper T cell differentiation as well as pathogenesis of autoimmune diseases (Harrington et al. 2005; Park et al. 2005). Detailed characterization of this IL17 producing cell subset including a comparison of gene expression profiles with other immune cells, combined with mouse genetics, identified ROR γ t (retinoic-acid-receptor-related orphan receptor- γ t) as the master driver of Th17 differentiation (Ivanov et al. 2006). Enforced expression of Runx1 has been shown to induce IL17 expression, and vice versa, diminished Runx1 expression by shRNA based knockdown showed an inhibitory effect against Th17 differentiation (Zhang et al. 2008). This Runx1 activity was reported to be mediated through direct regulation of the promoter/enhancer after protein complex formation with ROR γ t.

24.3 Roles of Runx Complexes in Early B Lymphocyte Development

B cell development occurs in the bone marrow in adult mice and initiates from the common lymphoid progenitors (CLPs) (Matthias and Rolink 2005). When CLPs adopt the B cell fate by starting to express the B cell lineage marker B220 and Rag recombinases, they are referred to as pre-pro-B cells. Once V(D)J fragments of immunoglobulin heavy chain locus (*Igh*) are successfully rearranged during the pro-B cell stage, the pre-B cell receptor is formed with the surrogate light chain and drives the transition from pro-B cells to pre-B cells. Like other lineages of blood cell types, it is no exception that early B cell development requires the expression of a series of transcription factors to activate B cell programming as well as to erase the potentials for alternative lineages. A widely-accepted model for transcription factor networks for B cells involves E2A, EBF, and Pax5, and a cascading cross-regulation among these three factors is central to the network. From a simplistic point of view, E2A activates EBF (Kee and Murre 1998), and EBF activates Pax5 (Decker et al. 2009).

Disruption of E2A or EBF (or both) results in developmental arrest at the pre-pro-B cell stage (Zhuang et al. 1994; Bain et al. 1994; Lin and Grosschedl 1995). Since E2A and EBF directly regulate genes specific to early B cell progenitors such as Rag1/2, Ig α β , VpreB, and λ 5, these two factors have been considered as the main specification factors. On the other hand, inactivation of Pax5 results in blockade of B cell development at the pro-B-cell stage and such pro-B cells show the potential to follow other lineages after *in vivo* transplantation (Nutt et al. 1999; Rolink et al. 1999). Combined with the fact that Pax5 actively represses non-B-lineage genes (Mikkola et al. 2002), Pax5 has been considered to be the main commitment factor for B lymphoid-lineages. However, during recent years, other transcription factors important for B cell development in

addition to E2A, EBF, and Pax5 have been identified, which include but are not limited to Bcl11a, Runx1, and Foxo1.

By using inducible-targeting strategies to overcome embryonic lethality, Runx1 was shown to have a pivotal role mainly in priming the lymphoid lineage (both B and T cells) during hematopoiesis (Ichikawa et al. 2004; Growney et al. 2005). More specifically, these studies clearly demonstrated that Runx1 is involved in the regulation of B cell generation since almost no B cells were developed in the absence of Runx1. A clue to the molecular mechanisms underlying these observations was obtained from a study showing that E2A and EBF cooperate with Runx1 to activate B cell programs before the involvement of Pax5 (Maier et al. 2004). Specifically, progressive demethylation of the *mb-1* gene, which encodes a critical signaling component of the pre-B cell receptor CD79a, requires synergistic activities of both EBF and Runx1. This poised epigenetic change in the *mb-1* gene is necessary for Pax5 to activate the *mb-1* promoter.

A further attempt was made to decipher the precise relationship between Runx1 and EBF during B lymphopoiesis by conditionally inactivating *Runx1*, *Runx3*, and *Cbfb* genes by *mb1-cre* transgene (Seo et al. 2012a). Consistent with previous reports, inactivation of *Runx1* and *Cbfb* (but not *Runx3*) resulted in defective B cell generation from early B cell progenitors. An interesting observation from this study was that Runx1 seems to directly activate *Ebf* transcription, since the amounts of *Ebf* mRNA was dramatically reduced from the progenitors in the absence of Runx1. Importantly, B cell development of Runx1-deficient cells was rescued by over-expression of EBF at least *in vitro*, suggesting that the major reason for developmental arrest in *Runx1^{EF}: mb-1Cre* mice could be the reduced EBF expression. Therefore, it is possible that Runx1, together with E2A, is an upstream factor for EBF even though they seem to function together once expressed at the later stages (Lukin et al. 2010; Lin et al. 2010). Further studies will be required to dissect the extent to which E2A, Runx1, and EBF cooperate and the unique functions endowed to each transcription factor.

24.4 Roles of Runx Complexes in Development of Innate Lymphoid Cells (ILCs) and Conventional NK Cells

24.4.1 Overview of ILCs

Innate lymphoid cells (ILCs) are lymphocytes that reside in the mucosa and produce innate cytokines independently of antigen specificity upon infection or allergic stimulation to maintain the epithelial barrier (Sonnenberg and Artis 2015; Eberl et al. 2015; Cortez et al. 2015). ILCs do not have rearranged antigen-specific receptors but are dependent on the common γ chain of the cytokine receptor IL-2R (γ c) for their differentiation. Based on their cytokine production and transcription factor requirements, ILCs are comprised of three groups, type I ILC (ILC1 and conventional NK: cNK), type II ILC (ILC2) and type III ILC (ILC3) populations. ILC populations generally express a dimeric IL-7 receptor α chain (CD127)/ γ c complex with some exceptions as described below. However, IL-7 is required only for ILC2 and ILC3 but not for the type I ILC population which expresses an IL-2/IL-15R β chain (CD122)/ γ c complex and is dependent on IL-15 instead of IL-7 for differentiation (Sonnenberg and Artis 2015; Fuchs et al. 2013; Klose et al. 2014).

The type I ILC population expresses the transcription factor T-bet and produces the type I cytokine IFN γ in response to IL-12, IL-15, and IL-18 (Sonnenberg and Artis 2015; Fuchs et al. 2013; Klose et al. 2013, 2014). In mice, CD3⁻NK1.1⁺NKp46 (NCR)⁺ cells in tissues are recognized as type I ILC populations. Given that the type I ILC population is defined by IFN γ -producing cells, cNK cells in the spleen could also be categorized as type I ILC populations. However, recent studies suggest that cNK cells are a distinct population from ILC1 cells that are resident in mucosal tissues (Sojka et al. 2014; Klose et al. 2014; Gasteiger et al. 2015; Constantinides et al. 2014). The transcription factor Eomes is expressed only in cNK cells and is required for their differentiation while ILC1 cells in mucosal tissues are negative for Eomes (Gordon

et al. 2012; Daussy et al. 2014). cNK cells do not express CD127 which is one of the markers for ILC1 cells (Cella et al. 2014). Thus, we hereafter term cNK cells as the Eomes⁺ tissue-nonresident type I ILC population and ILC1 cells as the Eomes⁻ tissue-resident type I ILC population.

ILC2 cells respond to IL-25, IL-33 and TSLP derived from epithelial cells and produce type II cytokines, IL-5, IL-9, and IL-13 (Sonnenberg and Artis 2015; Moro et al. 2010; Neill et al. 2010). ILC2 cells are characterized by high GATA-3 expression, which is required for their differentiation and secretion of type II cytokines (Hoyle et al. 2012). In contrast, ILC1 and ILC3 cells express GATA-3 at intermediate levels (Serafini et al. 2014; Klose et al. 2014). Thus, GATA-3 deficiency affects all ILC populations. Other transcription factors such as ROR α , Gfi1, and Bcl11b are also necessary for ILC2 differentiation (Wong et al. 2012; Spooner et al. 2013; Califano et al. 2015).

ILC3 cells react to IL-1 β and IL-23 from dendritic cells and produce IL-17 and IL-22 (Sonnenberg and Artis 2015; Cella et al. 2009; Satoh-Takayama et al. 2008). ROR γ t and AHR regulate cytokine production by ILC3 cells and their differentiation (Sawa et al. 2010, 2011; Sanos et al. 2009; Lee et al. 2012; Kiss et al. 2011). According to CD4 and NCR expression, ILC3 cells in the adult intestine were originally sub-grouped into NCR⁺ ILC3 (NK-22, ILC22), CD4⁺ ILC3 and CD4⁻ NCR⁻ ILC3 cells (Sonnenberg and Artis 2015). More recently, it has been reported that ILC3 cells are comprised of two distinct populations, T-bet⁻ CCR6⁺ ILC3 (Lymphoid tissue inducer-like cells: LTi-like cells) and T-bet⁺ CCR6⁻ ILC3, because T-bet⁻ CCR6⁺ ILC3 cells do not give rise to T-bet⁺ CCR6⁻ ILC3 cells, and vice versa (Klose et al. 2013). While T-bet⁻ CCR6⁺ ILC3s are CD4⁺ or CD4⁻, T-bet⁺ CCR6⁻ ILC3 cells are mostly NCR⁺. LTi cells in the fetus highly express ROR γ t and contribute to lymphoid tissue formation including Peyer's patches and lymph nodes. LTi cells are also recognized as members of ILC3 cells (Sonnenberg and Artis 2015).

24.4.2 Developmental Pathway of ILCs

All ILC subsets develop from CLPs in the bone marrow and fetal liver (Sonnenberg and Artis 2015; Possot et al. 2011). CLPs also give rise to T- and B-lymphocytes as well as ILCs including cNK cells (Fig. 24.2). Some ILC progenitors downstream of CLPs were identified in the Lin⁻ α 4 β 7⁺ fraction of bone marrow cells. CXCR6⁺ α 4 β 7⁺ lymphoid progenitors (α LP) and early ILC progenitors (EILPs) lose the potential to become lymphocytes but maintain the capacity to differentiate into cNK, ILC1, ILC2, and ILC3 cells (Yang et al. 2015; Yu et al. 2014). EILPs are marked by the expression of the transcription factor TCF-1. Deficiency of TCF-1 leads to absence of all ILCs. Downstream of EILPs, two ILC-specific progenitor cells are designated as common helper ILC progenitors (CHILPs) expressing the transcription factor Id2, and the common precursor to ILCs (ILCPs) expressing the transcription factor PLZF (Klose et al. 2014; Constantinides et al. 2014). CHILPs give rise to ILC1, ILC2, and ILC3 cells but not cNK cells. Along with the expression of PLZF in a small proportion of CHILPs, CHILPs are supposed to be upstream of ILCPs. In line with this model, ILCPs have a limited capacity to differentiate into ILCs because LTi-like cells as well as cNK cells do not develop from ILCPs. ILC2 progenitors (ILC2Ps) are ILC2-specific progenitor cells that are downstream of ILCPs in the bone marrow. ILC1- and ILC3-specific ILC progenitor cells are ILC-lineage negative (ILCLN) cells that are characterized as Lin⁻ CD127⁺ lacking ILC markers (ILC1: NK1.1, ILC2: KLRG1, ILC3: ROR γ t) and reside in the adult intestine. ILCLN cells were suggested to be the progenitor cells that migrate to the gut and lose their homing receptor α 4 β 7. Apparently, ILCLN cells are likely to be a heterogeneous population because they differentiate into NCR⁺ ILC3 and LTi-like cells, each of which arises from a different developmental pathway (Fig. 24.2).

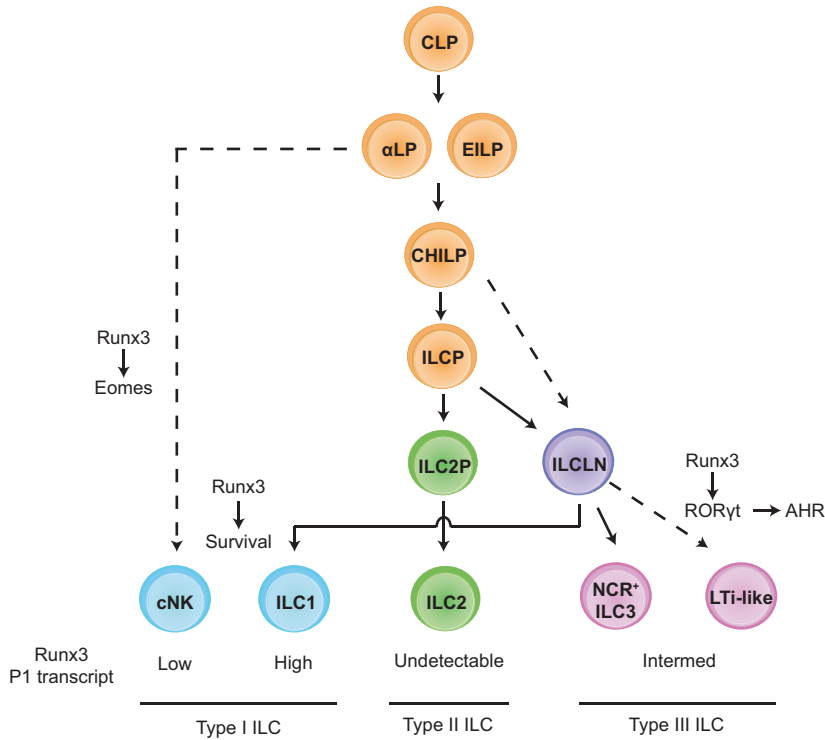


Fig. 24.2 Runx and ILC development. All ILC populations are the progeny of common lymphoid progenitors (CLPs) in the fetal liver and bone marrow. The ILC-committed precursor cells are CXCR6⁺α4β7⁺ lymphoid progenitors (αLPs) and early ILC progenitors (EILPs) that give rise to all ILC populations, but not T or B cells. The common helper ILC progenitors (CHILPs) do not differentiate into conventional NK (cNK) cells in spleen, but

maintain a capacity for other tissue-resident ILC populations. The common precursor to ILCs (ILCPs) is downstream of CHILPs and do not differentiate into LTi-like cells. ILC-lineage negative (ILCLN) cells in the intestine are the progenitor cells specific to ILC1 and type III ILC3. All ILCs are differentially characterized by the levels of *P1-Runx3* transcripts. Roles of Runx3 during ILC specification are discussed in the Sect. 24.4.4

24.4.3 Runx Expression in ILC Progenitor Cells and ILC Subsets

Expression of *Runx1*, *Runx2*, and *Runx3* is relatively low in CLPs, αLPs, and CHILPs. However, both *Runx1* and *Runx3* are expressed at extremely high levels in PLZF⁺ ILCPs (Ebihara et al. 2015). *Runx3* transcript expression from the P1 promoter (*P1-Runx3*) is also very high in ILCPs and is correlated to PLZF expression during differentiation into CHILP, suggesting a possible cross-regulation between Runx3 and PLZF expression. However, the functions of Runx complexes in

ILCPs remain to be elucidated. Given that ILC2Ps in the bone marrow and ILC2 cells in the intestine do not express *Runx3* from the P1 transcripts, it is possible that downregulation of *Runx3* expression is important for ILC2 differentiation. For ILC1 and ILC3 differentiation from ILCPs, *P1-Runx3* transcripts are reduced to intermediate level in ILCLN cells in the adult intestine. Then, upregulation of *P1-Runx3* occurs for ILC1 cells, while ILC3 cells maintain the intermediate level of it (Ebihara et al. 2015).

cNK cells develop from αLP and EILPs through NK progenitor cells (Lin⁻ CD122⁺ NK1.1⁻ DX5⁻). *P1-Runx3* transcripts are gener-

ally low throughout cNK cell differentiation compared to those in ILC1 cells in the intestine and liver (Ebihara et al. 2015; Ohno et al. 2008; Levanon et al. 2014).

ILC subsets in the intestine and cNK cells in the spleen have been well studied regarding Runx expression and function. In the intestine, cNK cells are relatively rare whereas other ILC subsets are dominant. The intestinal intraepithelial layer is enriched with ILC1 cells. ILC1, ILC2 and ILC3 cells are evenly distributed in the intestinal lamina propria and Peyer's patches. All ILC subsets including ILC1, ILC2 and ILC3 cells in the intestine predominantly express *Runx3* transcripts (Ebihara et al. 2015). However, ILC2 cells in the intestine use the P2, but not the P1 promoter although ILC1 and ILC3 cells in the intestine express only *P1-Runx3* transcripts. cNK cells express *Runx3* from both promoters, but the major transcript is the *P2-Runx3* (Ebihara et al. 2015; Levanon et al. 2014). When *P1-Runx3* transcripts are deleted in CD8⁺ T cells, Runx3 protein is barely detected in $\alpha\beta$ T cells (Egawa et al. 2007; Egawa and Littman 2008) due to the inefficient activity of Kozak sequences to initiate translation for P2-Runx3 protein (Kim et al. 2015). When *P1-Runx3* transcript level was examined by a reporter allele that specifically reflects Runx3 P1-promoter activity, reporter expression was found to be very high in ILC1 cells, intermediate in ILC3 cells, low in cNK cells, and undetected in ILC2 cells. Given that *P1-Runx3* transcripts are correlated to Runx3 protein expression at least in T cells, differential expression pattern of *P1-Runx3* mRNA should be associated with Runx3 protein expression in ILCs. However, *P2-Runx3* transcripts in ILC2 cells might be translated into protein at least to some extent because high level of Runx3 protein can be detected in cNK cells when *P2-Runx3* transcripts are abundant (Levanon et al. 2014).

Runx3 is also expressed in ILC1 or cNK cells in other tissues. Liver-resident NK cells turned out to be ILC1 cells in which the *P1-Runx3* mRNA amount was as high as those in the intestinal ILC1 cells (Ebihara et al. 2015). *Runx3* expression in skin-resident NK cells is as low as that in skin-nonresident NK cells. In the uterus,

DBA⁺ NK cells also express *Runx3* mainly from the P1 promoter (Levanon et al. 2014). Thus, among all Runx family members, Runx3 is the dominant Runx protein in all ILC subsets and is expressed highly in most type I ILC population and intermediately in ILC3 cells.

24.4.4 Roles of Runx Complexes in ILCs Development

24.4.4.1 Type I ILC

Several genetic approaches have clarified that Runx complexes are involved in cNK cell function and differentiation. Mice harboring the hypomorphic allele of Cbfb exhibited absence of NKPs and cNK cells. When Cbfb is deleted in hematopoietic cells with *Vav1-Cre* mice, LMPPs and CLPs do not emerge (Satpathy et al. 2014), suggesting the requirement of Cbfb for early differentiation of lymphocytes before commitment to cNK cells. Recently, cNK cell-specific Cbfb function has been examined by conditional *Cbfb* gene inactivation using *NCR-iCre* mice. Cbfb deletion in cNK cells leads to great reduction of NK cells in the spleen and an immature NK cell phenotype including low expression of Ly49, low DX5, and Eomes, and inefficient IFN γ production in response to IL-12 and IL-18 stimulation (Ebihara et al. 2015). Conditional deletion of Runx3 in cNK cells recapitulates the phenotypes of Cbfb-deficiency in cNK cells. However, probably due to compensation by Runx1, the phenotypes of Runx3-deficient cNK cells are generally milder than those of Cbfb-deficient cNK cells (Ebihara et al. 2015; Levanon et al. 2014). ChIP-seq and transcriptome analysis showed that products of Runx3-bound genes seemed to be associated with survival, proliferation, maturation, and migration of cNK cells (Levanon et al. 2014). Runx3 appears to function downstream of IL-15 signaling and contributes to cNK cell survival. However, the precise mechanism of Runx3 induction through IL-15 signaling is still unclear. Runx3 also positively regulates CD96 and Crtam, both of which are involved in cNK cell activity (Levanon et al. 2014). Thus, the Runx3/Cbfb complex regulates cNK cell survival and functions.

ILC1 cells in the intestine and liver also require Runx3/Cbfb β complexes for their survival and IFN γ response to IL-12 (Ebihara et al. 2015). Normal levels of T-bet expression in Cbfb β -deficient ILC1 cells in the intestine suggested that T-bet might be upstream regulator for *Runx3* expression in ILC1 cells as was observed in CD8⁺ T cells. Type I ILC populations in the skin and salivary gland were reduced in the absence of Runx3 or Cbfb β . Taken together, the Runx3/Cbfb β complex is indispensable to all type I ILC populations.

24.4.4.2 Type II ILC

ILC2 cells in the intestine express only the *P2-Runx3* transcript. Runx3 is dispensable for ILC2 differentiation in the intestine (Ebihara et al. 2015). During the differentiation of effector CD4⁺ T cell subsets, GATA-3 antagonizes Runx3 through protein-protein interactions to promote T_H2 skewing (Yagi et al. 2010), whereas Runx3 blocks GATA-3 activity for T_H1 differentiation (Kohu et al. 2009). This balance between Runx3 and GATA-3 seems to be one of the determinant factors for ILC1 and ILC2 function and differentiation as well. Runx3 expression might overwhelm GATA3 expression in ILC1 cells and GATA-3 could suppress Runx3 in ILC2 cells. Further studies will be necessary to clarify the physiological roles of Runx complexes in ILC2s.

24.4.4.3 Type III ILC

An early study showed that LTi cells in the fetal gut are reduced in mice lacking *P1-Runx1* transcripts or Cbfb β variants, resulting in a severe deficit in secondary lymphoid organ formation (Tachibana et al. 2011). LTi cells express less ROR γ t in the fetal gut of Cbfb β -deficient mice. However, the counterparts of LTi cells in the adult intestine normally express ROR γ t in those mice, suggesting the possible association of ROR γ t with Runx complexes in ILC3s. Recently, LTi cells turned out to be the progeny of CLPs which require the Runx1/Cbfb β complex for differentiation (Possot et al. 2011; Sonnenberg and Artis 2015; Constantinides et al. 2014; Klose et al. 2014; Cherrier et al. 2012; Satpathy et al. 2014). Therefore, it should be considered that

reduction of LTi cells in Cbfb β -deficient mice might reflect impaired CLP differentiation. Recently, as the ILC differentiation process has become more characterized, the roles of Runx complexes in ILC3s have been revealed (Ebihara et al. 2015). Among the Runx family members, Runx3 is predominantly expressed by all ILC3 subsets at intermediate levels, which is less than in ILC1 cells and more than in cNK cells and ILC2 cells. Runx3 ablation in all hematopoietic cells showed normal differentiation from CLPs to ILCPs stages, but resulted in accumulation of ILCLN cells in the intestine, reduction of ILC1 cells, and absence of ILC3 cells in the intestine. Runx3-deficient ILCLN cells are not apoptotic and cannot give rise to ILC3 cells *in vivo* when transferred into alymphoid mice. Fewer ILC1 cells were also developed from Runx3-deficient ILCLN cells than Runx3-competent ILCLN cells. Thus, Runx3 is necessary for ILCLN cells to differentiate into ILC1 and ILC3 cells.

Mechanistically, Runx3 directly contributes to ROR γ t expression in ILC3 cells (Ebihara et al. 2015). A reporter assay using the human NK cell line exhibited that Runx3 enhances ROR γ t promoter activity through the Runx binding site in the ROR γ t promoter. In addition, Runx3 binding to the ROR γ t promoter in ILC3 cells was confirmed by the ChIP assay, indicating that Runx3 regulates ROR γ t in ILC3 cells (Ebihara et al. 2015). The aryl hydrocarbon receptor (AHR) is another ILC3-related transcription factor that was shown to regulate ILC3 differentiation and IL-22 production together with ROR γ t. In the absence of Runx3, expression of AHR was also undetected in ILC3 cells (Ebihara et al. 2015). AHR expression in ILC3 cells was reduced to half in mice harboring half dosage of the ROR γ t gene, suggesting that ROR γ t is also involved in the mechanisms that regulate AHR expression in ILC3 cells. Although direct binding of ROR γ t to the AHR promoter in ILC3 cells has not been examined yet, ROR γ t binds to enhancer regions in the AHR promoter of T_H17 cells, counterpart α β T cells that share many features with ILC3 cells (Ebihara et al. 2015; Ciofani et al. 2012). These data support that Runx3 regulates ROR γ t and its downstream AHR. Collectively, Runx3 is

indispensable for ILCLN cells to acquire two ILC3 transcription factors, ROR γ t and AHR, for final differentiation into ILC3 cells.

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