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Transcriptional regulator Bhlhe40 works as a cofactor of T-bet in the regulation of IFN- γ production in iNKT cell

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Abstract

Invariant natural killer T (*i*NKT) cells are a subset of innate-like T cells that act as important mediators of immune responses. In particular, *i*NKT cells have the ability to immediately produce large amounts of interferon-gamma (IFN- γ) upon activation and thus initiates immune responses in various pathological conditions. However, molecular mechanisms that control IFN- γ production in *i*NKT cells are not fully understood. Here, we report that basic helix-loop-helix transcription factor family, member e40 (*Bhlhe40*) is an important regulator for IFN- γ production in *i*NKT cells. *Bhlhe40* is highly expressed in stage 3 thymic *i*NKT cells and *i*NKT1 subsets, and level of *Bhlhe40* mRNA expression is correlated with *Ifng* mRNA expression in the resting state. Although *Bhlhe40*-deficient mice show normal *i*NKT cell development, *Bhlhe40*-deficient *i*NKT cells show significant impairment of IFN- γ production and antitumor effect. *Bhlhe40* alone shows no significant effect on *Ifng* promoter activities, but contributes to enhance T-box transcription factor Tbx21 (T-bet)-mediated *Ifng* promoter activation. Chromatin immunoprecipitation analysis revealed that *Bhlhe40* accumulates in T-box region of *Ifng* locus and contributes to histone H3-lysine 9 acetylation of *Ifng* locus which was impaired without T-bet conditions. These results indicate that *Bhlhe40* works as a cofactor of T-bet for enhancing IFN- γ production in *i*NKT cells.

Significance Statement

A unique characteristic of invariant natural killer T (*i*NKT) cells is the ability to immediately produce large amounts of interferon-gamma (IFN- γ) upon activation, which enables these cells to play critical roles in initiating immune responses in various pathological conditions. In this study, we demonstrate a novel mechanism mediated by basic helix-loop-helix transcription factor family, member e40 (Bhlhe40) for accelerating IFN- γ production in *i*NKT cells. Bhlhe40 is required for normal physiological functions of *i*NKT cells, where it positively regulates IFN- γ production. Bhlhe40 also contributes to acetylate histone H3-lysine 9 of *Ifng* locus in *i*NKT cells. These findings may help to understand the molecular mechanisms related to the biology of *i*NKT cells.

/body

Introduction

Invariant natural killer T (*i*NKT) cells are a subset of innate-like T cells that represent a small percentage of lymphocytes (1). *i*NKT cells are well defined by their unique ability to recognize glycolipid antigens such as α -galactosylceramide (α -GC), which is bound and presented by major histocompatibility complex class I-like CD1d molecule on antigen-presenting cells (APCs) (1). Upon activation, *i*NKT cells act as effector immune cells through the rapid secretion of large amounts of cytokines, such as interferon-gamma (IFN- γ), interleukin-2 (IL-2), IL-4, IL-13, IL-17, IL-21, IL-22 and tumor necrosis factor-alpha (TNF- α) (1, 2) and can also stimulate other immune cells to produce cytokines, such as IL-12 from CD1d-expressing APCs (3, 4). On the other hand, *i*NKT cells may also act as regulatory cells of other immune cells such as macrophages, dendritic cells, T cells, and natural killer cells (5). Thus *i*NKT cells has been considered as key players in the immune system which link innate and adaptive immune responses, and play important roles in many aspects of the immune system such as tumor immunity, autoimmune diseases, transplant tolerance, and immunity against infections (5). Populations of *i*NKT cells are heterogeneous and consist of multiple cell subsets characterized by distinct phenotype (Th1-like *i*NKT (*i*NKT1; IFN- γ producing *i*NKT), Th2-like *i*NKT (*i*NKT2; IL-13 producing *i*NKT), or Th17-like *i*NKT (*i*NKT17; IL-17A and IL-22 producing *i*NKT cells) (6-9). IFN- γ secreted by *i*NKT cells is considered as one of the key molecules for the augmentation of early immune responses against tumors or infections.

Despite the studies implicating *i*NKT cells as important immune cells with diverse functional roles, the molecular mechanisms of IFN- γ production in *i*NKT cells are still not fully understood. To address this important question, we have utilized the DNA

microarray analysis (expression profiles were compiled using data from RefDIC (reference database of immune cells <http://refdic.rcai.riken.jp/profile.cgi>)) for the screening of candidate genes that might be involved in the regulation of IFN- γ production in *i*NKT cells, and identified basic-helix-loop-helix family, member e40 (Bhlhe40) as a key regulator of *i*NKT cell-derived IFN- γ . Bhlhe40 belongs to basic helix-loop-helix protein family, and acts as a regulating transcription factor, which controls a wide variety of biological processes including cellular growth, proliferation, apoptosis, immune responses, in addition to the regulation of circadian rhythms (10-13).

In this study, we show that Bhlhe40 is highly expressed in stage 3 of thymic *i*NKT cells and *i*NKT1 subset, and its expression is higher than other lineages in resting state. The deficiency of *Bhlhe40* in *i*NKT cells results in remarkable decrease in IFN- γ production but does not affect cell development or proliferation, indicating that Bhlhe40 is the responsible factor for enhancing IFN- γ production in *i*NKT cells. In the analysis of molecular mechanism, we show that Bhlhe40 binds to T-box transcription factor Tbx21 (T-bet) and enhance T-bet-mediated IFN- γ promoter activation. Furthermore, Bhlhe40 accumulates in T-box region and contributed to histone H3-lysine 9 (K9) acetylation of *Ifng* locus. Together, our findings identify Bhlhe40 as one of important molecules involved in the regulation of IFN- γ production in *i*NKT cells, and give new insight into the molecular mechanism that explain the characteristics of IFN- γ -producing *i*NKT cells.

Results

***Bhlhe40* is highly expressed in *Ifng*-expressing *i*NKT subsets**

In order to investigate the role of *Bhlhe40* in *i*NKT cells, we first examined the expression levels of *Bhlhe40* mRNA in *i*NKT cells compared to other T lymphocytes. We found that splenic *i*NKT cells show high expression of *Bhlhe40* mRNA compared with CD4⁺ or CD8⁺ T cells (Figure 1A). *Bhlhe40* plays important roles in the regulation of mammalian molecular clock in a phase-dependent manner (10). Thus, we next asked if *Bhlhe40* mRNA expression in *i*NKT cells is affected by dark/light cycle. To confirm this, we compared levels of *Bhlhe40* mRNA expression in *i*NKT and CD4⁺ T cells of mice exposed to dark/light cycles. No changes in *Bhlhe40* mRNA expression were observed in *i*NKT or CD4⁺ T cells after exposure to dark/light cycles, suggesting that *Bhlhe40* mRNA expression in *i*NKT cells is independent of the circadian cycle (Figure S1). Next we examined whether *Bhlhe40* expression is induced in activated *i*NKT cells, by comparing expression levels of *Bhlhe40* mRNA in splenic *i*NKT cells after stimulation with α -CD3 monoclonal antibody (mAb) alone or combined with α -CD28 mAb. *Bhlhe40* mRNA was significantly upregulated in stimulated *i*NKT cells in a time-dependent manner relative to its expression in resting *i*NKT cells (Figure 1B). Furthermore, we evaluated *Bhlhe40* mRNA expression in thymic *i*NKT cells. *i*NKT cells development stages can be defined through differences in the expression levels of surface markers such as NK1.1 and CD44 (14) (Figure 1C). We found that the expression level of *Bhlhe40* mRNA was higher in *i*NKT cells at stage 3 (NK1.1^{hi} CD44^{hi}) compared to stage 1 (NK1.1^{lo} CD44^{lo}) or stage 2 (NK1.1^{lo} CD44^{hi}) (Figure 1D). Importantly, the high expression of *Bhlhe40* mRNA in thymic *i*NKT cells at stage 3 was accompanied with high expression levels of *Tbet* and *Ifng* mRNA compared to other

stages (Figure 1D). Additionally, iNKT cells are classified based on the expression of CD4 and IL-17 receptor B (IL-17RB) into heterogeneously different subsets (9). Consistent with previous reports, we identified four subsets of iNKT cells according to CD4 and IL-17RB staining (Figure 1E). These subsets are characterized by different cytokine patterns. *Ifng* mRNA was abundant in CD4⁺ IL-17RB⁻ subset, while *Il4* and *Il17a* mRNA were higher in CD4⁺ IL-17RB⁺ and CD4⁻ IL-17RB⁺ subsets, respectively (Figure S2). Interestingly, we also found that *Bhlhe40* was highly expressed in CD4⁺ IL-17RB⁻ subset, which is characterized by high levels of *Ifng* mRNA, compared to other subsets (Figure 1F). Together, these data indicate that *Bhlhe40* mRNA is highly expressed in *Ifng*-expressing stage 3 thymic iNKT cells and iNKT1 subsets. *Bhlhe40* mRNA expression was inducible after T-cell receptor (TCR) stimulation, and independent of the circadian cycle.

iNKT cells develop normally in *Bhlhe40*^{-/-} mice

To evaluate the contribution of *Bhlhe40* in the development of iNKT cells, we first compared frequencies of iNKT cells between wild type (WT) and *Bhlhe40*^{-/-} mice. We found that the deficiency of *Bhlhe40* did not affect the frequencies of iNKT cells in thymus, spleen and liver (Figure 2A). Furthermore, the frequencies of iNKT cells during development in the thymus were similar between WT and *Bhlhe40*^{-/-} mice (Figure 2B). Next we asked whether the deficiency of *Bhlhe40* might affect the maturation status of iNKT cells. To answer this question, thymic iNKT cells from WT or *Bhlhe40*^{-/-} mice were utilized to compare the expression of Ly49 family members, which are described as being expressed on both developing and mature iNKT cells (15). Again, no major differences were observed in the expression of Ly49 family members

between WT and *Bhlhe40*^{-/-} iNKT cells (Figure 2C). In addition to intrinsic factors, iNKT cell maturation is also determined extrinsically by the interaction between receptors on NKT common precursors and corresponding ligands expressed by CD4⁺CD8⁺ double positive (DP) thymocytes such as CD1d and signaling lymphocytic activation molecule family member 1 (SLAMF1) (16, 17). We also confirmed that CD1d and SLAMF1 expressing CD4⁺CD8⁺ DP thymocytes were comparable between WT and *Bhlhe40*^{-/-} mice (Figure 2D). Additionally, frequencies of different iNKT subsets based on CD4 and IL-17RB expressions were also comparable between WT and *Bhlhe40*^{-/-} mice (Figure 2E). Taken together, these data suggest that iNKT cells can develop normally even in the absence of *Bhlhe40*.

***Bhlhe40* enhances IFN- γ production in iNKT cells**

Bhlhe40 functions as a transcriptional regulator that is involved in cellular growth, development and immunomodulation (10-13). *Bhlhe40* showed no specific roles in the development of iNKT cells as suggested by the above results. Thus, next we asked if *Bhlhe40* might regulate iNKT cell-mediated immune responses. iNKT cells are well characterized by the ability to rapidly release large amounts of IFN- γ upon activation, which is explained by the pre-formed *Ifng* mRNA available prior to stimulation (18). As previously described, two *Ifng*-expressing iNKT subsets were identified based on CD4 and IL-17RB expression, and these two subsets expressed similar levels of pre-formed *Ifng* mRNA as compared between WT and *Bhlhe40*^{-/-} iNKT cells (Figure S2). Surprisingly, following stimulation with α -GC, *Bhlhe40*^{-/-} iNKT cells showed significant impairment in IFN- γ production compared to WT iNKT cells (Figure 3A). More interestingly, *Bhlhe40* deficiency has no significant effects on IL-4

production in splenic *i*NKT cells stimulated with α -GC (Figure 3B). We also compared the proliferation of α -GC-stimulated WT or *Bhlhe40*^{-/-} splenic *i*NKT cells, and found that *Bhlhe40*^{-/-} *i*NKT cells can proliferate normally similar to WT *i*NKT cells upon stimulation (Figure 3C). Additionally, following α -CD3/CD28 stimulation *Bhlhe40*^{-/-} *i*NKT cells showed decreased levels of induced *Ifng* mRNA compared to WT *i*NKT cells (Figure 3D), suggesting a role for Bhlhe40 in the regulation of IFN- γ expression in *i*NKT cells.

Next, we evaluated the role of Bhlhe40 in the enhancement of IFN- γ production *in vivo*. To do so, WT or *Bhlhe40*^{-/-} mice were intravenously injected with α -GC, and 1 hour after α -GC administration, splenic *i*NKT cells were stained intracellularly to measure levels of IFN- γ or IL-4 expression. Consistent with the *in vitro* data, we found that α -GC administration induced higher IFN- γ expression in WT compared to *Bhlhe40*^{-/-} *i*NKT cells; while no differences in IL-4 expression were observed between the two groups (Figure 3E). Previous reports have shown that *i*NKT cells mediate a rapid release of IFN- γ and IL-4 into the serum in response to α -GC (19). Thus, we compared IFN- γ and IL-4 concentrations in the serum of WT or *Bhlhe40*^{-/-} mice injected intravenously with α -GC. As expected, levels of serum IFN- γ were higher in WT compared to *Bhlhe40*^{-/-} mice in response to α -GC administration, while IL-4 was not altered (Figure 3F). To further confirm if decreased concentrations of serum IFN- γ was related to *Bhlhe40* deficiency in *i*NKT cells, we performed an add-back experiment. In this experiment, WT or *Bhlhe40*^{-/-} *i*NKT cells were intravenously transferred into *Ja18*^{-/-} mice, and levels of serum IFN- γ were measured at different time points within 48 hours after intravenous injection of α -GC. Again, high levels of IFN- γ were observed in the serum of α -GC-injected *Ja18*^{-/-} mice when transferred with WT but not *Bhlhe40*^{-/-}

*i*NKT cells (Figure S3), indicating that IFN- γ in the serum of α -GC-stimulated mice was derived from *i*NKT cells but not from other cells such as NK or T cells. On the other hand, levels of serum IL-4 were comparable between the two groups (Figure S3). Together, these data suggest that although WT and *Bhlhe40*^{-/-} *i*NKT cells show similar levels of pre-formed *Ifng* mRNA, IFN- γ production was significantly impaired in *Bhlhe40*^{-/-} *i*NKT cells following stimulation, which suggests that Bhlhe40 may play an important role in the enhancement of IFN- γ production in stimulated *i*NKT cells.

***Bhlhe40* deficiency impairs antitumor effect of *i*NKT cells**

Several studies have reported the contribution of *i*NKT-derived IFN- γ in the inhibition of tumor metastases in mice after α -GC stimulation (20, 21). Thus, we next aimed to evaluate the impact of *Bhlhe40* deficiency on *i*NKT-mediated antitumor effect in a lung melanoma metastasis model. Compared to the significant effects of α -GC in WT mice, we found that the treatment with α -GC showed no remarkable effects in *Bhlhe40*^{-/-} mice, as the numbers of B16 melanoma nodules were similar between α -GC and control group (Figure 4A). To further confirm that the impaired response of α -GC in *Bhlhe40*^{-/-} mice was related to *i*NKT cells but not to other cells, we performed an add-back experiment. In a liver metastasis model, *Ja18*^{-/-} mice were transferred with WT or *Bhlhe40*^{-/-} *i*NKT cells, and the effects of α -GC treatment were evaluated compared to WT mice. The treatment with α -GC showed significant effects in *Ja18*^{-/-} mice when transferred with WT *i*NKT cells, while the transfer of *Bhlhe40*^{-/-} *i*NKT cells was less effective to reduce melanoma surface area (Figure 4B). Together, these results indicate an impact of *Bhlhe40* deficiency in *i*NKT cell-mediated suppression of lung or liver melanoma metastasis.

Bhlhe40 does not enhance *Ifng* promoter activities by itself

Next, we aimed to gain insight into how Bhlhe40 enhances IFN- γ production in TCR-stimulated iNKT cells. First, we examined the effects of Bhlhe40 on *Ifng* promoter activation. In *Bhlhe40*^{-/-} mouse embryonic fibroblast (MEF) cells transfected with a control or *Bhlhe40* expression vector, we found that the overexpression of *Bhlhe40* alone in *Bhlhe40*^{-/-} MEF cells has no significant effects on *Ifng* promoter activity (Figure S4A). TCR signaling leads to IFN- γ production via several downstream transcription factors including nuclear factor-kappa B kinase (NF- κ B) and nuclear factor of activated T cells (NFAT) (22, 23). Thus we next asked if Bhlhe40 may enhance IFN- γ production by supporting the functions of NF- κ B or NFAT. However, overexpression of *Bhlhe40* in *Bhlhe40*^{-/-} MEF cells has shown no significant effects on NF- κ B responsive promoter after stimulation with phorbol 12-myristate 13-acetate 4-O-methyl ether (PMA) compared to *Bhlhe40*^{-/-} MEF cells transfected with control vector (Figure S4B). Additionally, in *Bhlhe40* shRNA expressing EL-4 cells, transfection with a construct encoding knockdown-resistant *Bhlhe40* (*Bhlhe40* shRNA^r) showed no marked effects on NFAT responsive promoter (Figure S4C). Together, these data indicate that Bhlhe40 does not mediate direct effect on *Ifng* promoter activities, and has no supportive roles for molecules acting downstream of TCR signaling such as NF- κ B or NFAT.

Bhlhe40 enhances IFN- γ expression by T-bet-mediated mechanism

Since Bhlhe40 alone showed no effects on *Ifng* promoter activities, we hypothesized that Bhlhe40 might act as a cofactor rather than being a transcription

factor for the induction of *Ifng* mRNA. Thus, we next searched for candidate molecules that may interact with Bhlhe40. Among these, we focused on T-bet as a major key molecule related to IFN- γ production and acting downstream of TCR signaling (24). First, we found that T-bet is expressed at similar levels in WT and *Bhlhe40*^{-/-} iNKT cells (Figure 5A). Interestingly, in a co-immunoprecipitation assay we found that Bhlhe40 interacts with T-bet, as seen in the lysates of Lenti-XTM 293T cells transfected with *Bhlhe40* and *T-bet* encoding vectors, which was not observed by using an unrelated HA-tagged protein encoding vector. (Figure 5B). More importantly, the interaction between endogenous Bhlhe40 and T-bet was also confirmed in iNKT cells under physiological conditions, and this interaction was enhanced after α -CD3/CD28 stimulation (Figure 5C), suggesting a possible role for Bhlhe40/T-bet interaction in the regulation of IFN- γ production in stimulated iNKT cells. Next, we utilized luciferase reporter assay to evaluate the role of Bhlhe40/T-bet interaction in the regulation of *Ifng* promoter. In *Bhlhe40*^{-/-} MEF cells that lack expression of both Bhlhe40 and T-bet, we found that the co-expression of Bhlhe40 and T-bet has augmented the activity of *Ifng* promoter observed in T-bet-transfected cells, while *Bhlhe40* alone showed no significant effects (Figure 5D). Furthermore, in EL-4 cells - where Bhlhe40 but not T-bet is expressed - shRNA-mediated knockdown of *Bhlhe40* has impaired *Ifng* promoter activating effects observed only in T-bet-transfected conditions. On the other hand, co-transfection of *Bhlhe40* shRNA with *Bhlhe40* shRNA^r resulted in a rescue of the observed changes (Figure 5E), suggesting that Bhlhe40 indeed enhance IFN- γ production via T-bet. Together, these data suggest that Bhlhe40 mediates enhancement of IFN- γ production by interaction with the transcription factor T-bet.

Bhlhe40 enhances *Ifng* expression upon IL-12 stimulation

In addition to TCR-dependent stimulation, iNKT cells can be also activated by cytokines such as IL-12 (1). To examine if Bhlhe40 is also involved in the enhancement of IFN- γ after IL-12 stimulation, we compared levels of *Ifng* mRNA expression in IL-12-stimulated WT or *Bhlhe40*^{-/-} iNKT cells. We found that *Ifng* mRNA expression induced by IL-12 was remarkably impaired in *Bhlhe40*^{-/-} compared to WT iNKT cells at different time of stimulation (Figure S5A). To unveil the related mechanism, we examined if Bhlhe40 may interacts with signal transducer and activator of transcription 4 (Stat4), a key molecule that plays critical role in IL-12-induced IFN- γ production (25). However, we could not confirm an enhancement of *Ifng* promoter activities in *Bhlhe40*^{-/-} MEF cells transfected with *Stat4* and/or *Bhlhe40* expression plasmid, nor binding between Stat4 and Bhlhe40 (Figures S5B and S5C). From these results we hypothesized that Bhlhe40 – together with T-bet – may induce chromatin changes that facilitate Stat4-mediated *Ifng* promoter activities. Thus, we next aimed to evaluate the possible involvement of Bhlhe40/T-bet interaction in chromatin remodeling of *Ifng* locus.

Impaired histone H3-lysine9 (K9) acetylation in *Bhlhe40*^{-/-} iNKT cells

To evaluate our hypothesis, we performed chromatin immunoprecipitation (ChIP) assay using polymerase chain reaction (PCR) primers that target *Ifng* locus, which includes *Ifng* promoter and the conserved noncoding sequence (CNS -5 and CNS -22) previously described (26-29) (Figure 6A, Table S1). Interestingly, we found that Bhlhe40 accumulates in T-box region of *Ifng* locus, which are reported as binding sites for T-bet (30) (Figure 6B). To identify if Bhlhe40/T-bet interaction is involved in

chromatin changes of *Ifng* locus, we next compared histone H3-K9 acetylation, histone H3-K4 dimethylation and histone H3-K4 trimethylation between splenic *i*NKT cells isolated from WT or *Bhlhe40*^{-/-} NKT clone mice by ChIP PCR assay. We found that the deficiency of *Bhlhe40* has led to impaired acetylation of histone H3-K9 in T-box region of *Ifng* locus (Figure 6C), while no significant differences were observed in H3-K4 dimethylation or trimethylation in *i*NKT cells (Figure S6A,B). Additionally, histone H3-K9 acetylation, H3-K4 dimethylation and H3-K4 trimethylation in *Il4* locus – which is known as T-bet not binding locus (30, 31) – was not affected by the deficiency of *Bhlhe40* (Figure S6C-F, Table S2) (32). Together, these results suggest that the binding of *Bhlhe40*/T-bet complex to the T-box region is important for chromatin changes in *Ifng* locus.

Next, to confirm the importance of *Bhlhe40*/T-bet interaction for the chromatin changes in *Ifng* locus, we compared the accumulation of *Bhlhe40* in the T-box region of *Ifng* locus between T-bet positive or negative *i*NKT cells. As expected, we found that *Bhlhe40* accumulation was reduced in T-bet-negative *i*NKT cells (Figure 7A). Additionally, histone H3-K9 acetylation of the *Ifng* locus in T-bet-negative *i*NKT cells resembles those observed in *Bhlhe40*^{-/-} *i*NKT cells (Figure 7B, S7). Together, these results suggest a novel function of *Bhlhe40* in *i*NKT cells as a cofactor that binds T-bet and regulates chromatin remodeling of *Ifng* locus, resulting in enhanced IFN- γ production.

Discussion

The immune response is a dynamic process, starts with a rapid response of innate immunity and passes through multiple phases controlled by cellular and molecular components of the immune system to end up with the development of specific and powerful adaptive immunity. *i*NKT cells consist a subset of innate-like lymphocytes that intermediate between innate and adaptive immunity, and play important roles in the initiation and regulation of immune responses to tumors and infectious organisms (1). *i*NKT cells have attracted attention because of its unique ability to rapidly produce large amount of IFN- γ , which induces the cytotoxic activities mediated by natural killer and CD8⁺ T cells (22, 33). Activation of *i*NKT cells by the administration of α -GC or α -GC-pulsed dendritic cells has been considered as one of the most potent and promising strategies for the eradication of tumors, as several preclinical studies in animal models in addition to clinical trials have shown encouraging results of *i*NKT cell-based immunotherapy (34). However, these studies still lack a clear understanding of the molecular mechanism of cytokine production that might be critical for the improvement of these strategies.

In this study, we identified Bhlhe40 as a key player of the molecular mechanism that explains the unique ability of *i*NKT cells to rapidly produce large amounts of IFN- γ . Bhlhe40 is a transcriptional regulator expressed in a wide range of cells and is involved in various physiological functions including cellular growth and proliferation, immune responses and circadian rhythm (11, 13). As part of the Immunological Genome Project indicated, Bhlhe40 shows high expression levels in *i*NKT cells (35); however the function of Bhlhe40 remained unclear. In this study we clarified a novel role of Bhlhe40 in the enhancement of IFN- γ production in stimulated *i*NKT cells. Bhlhe40 was found

to interact with T-bet after stimulation, and this interaction led to an enhancement in *Ifng* promoter activities. The deficiency of *Bhlhe40* in *i*NKT cells has led to impaired IFN- γ production in both glycolipid antigens and α -CD3/CD28 stimulation. Additionally, the deficiency of *Bhlhe40* led to impaired IFN- γ production in IL-12-stimulated *i*NKT cells. However, we could not confirm a direct interaction between Stat4 and Bhlhe40 in resting or IL-12-stimulated *i*NKT cells. Furthermore, Bhlhe40 did not enhance signals mediated by molecules that contribute to IFN- γ production such as NFAT and NF- κ B, emphasizing the fact that Bhlhe40 enhances IFN- γ production in *i*NKT cells through direct interaction with T-bet.

As shown in this study, Bhlhe40 was found to bind T-bet in resting *i*NKT cells, and this binding was enhanced following TCR-ligand stimulation. In addition to its role as a transcriptional repressor that directly bind to class B E-box element (36), Bhlhe40 also acts as a cofactors of members of the basal transcription machinery including transcription factor II B, TATA-binding protein, transcription factor II D and exerts transcriptional repression (37, 38). Bhlhe40 is also reported to transactivate several targets such as survivin via binding to the Sp1 sites (39). In addition to its function as a transcriptional factor of *Ifng* mRNA, T-bet is also involved in chromatin remodeling (40). Previous studies have reported a role of T-bet and CBP/P300 interaction in the induction of histone H3-K9 acetylation in *Ifng* locus (41). Thus, Bhlhe40 may have a role in the enhancement of functions mediated by T-bet-CBP/P300 complex, which have to be confirmed in next studies. As suggested above, a modest epigenetic change at the transcription start site (TSS) region of *Ifng* locus was observed in *i*NKT cells, represented by high acetylation in *Ifng* locus. In *Bhlhe40*^{-/-} *i*NKT cells, acetylation of histone H3-K9 in the T-box region of *Ifng* locus was decreased. Furthermore, in T-bet

negative iNKT cells, histone H3-K9 acetylation was impaired in correlation with reduced Bhlhe40 accumulation in the T-box region of the *Ifng* locus. T-bet is known to have an ability to recruit histone H3-K4 methyltransferase and change histone H3-K4 methylation of *Ifng* locus (42). As our data show, histone H3-K4 methylation of *Ifng* locus was reduced in T-bet-negative (Figure S6C, D) but not *Bhlhe40*^{-/-} iNKT cells (Figure S6A, B). Furthermore, no changes in *Il4* locus - which is not related to T-bet - were observed in both T-bet-negative (Figure S7F, G) and *Bhlhe40*^{-/-} iNKT cells (Figure S6E, F). Collectively, these findings suggest that the difference of histone H3-K4 methylation of *Ifng* locus between T-bet-positive and T-bet-negative iNKT cells was dependent on T-bet deficiency alone. Thus, we concluded that Bhlhe40/T-bet interaction is important for histone H3-K9 acetylation but not for histone H3-K4 methylation of *Ifng* locus. While epigenetic changes often lead to a change in basal transcription levels, those of *Ifng* mRNA in iNKT cells were not affected by *Bhlhe40* deficiency. Thus, these results raised a possibility that Bhlhe40 may not directly affect epigenetic modifications, but could be a result of reduced gene expression. Additionally, results obtained from transfected cell lines suggest that Bhlhe40 works directly on a reporter construct which does not have a chromatin structure. Together with previous concerns, this should be clarified in more details in future studies.

As suggested by other reports, the deficiency of *Bhlhe40* results in decreased IFN- γ production from Th1 cells (43). *Bhlhe40*^{-/-} mice showed a resistance to the induction of experimental autoimmune encephalomyelitis, which is mediated by Th1 and Th17 cells (43). On the other hand, T-bet-expressing natural killer cells and $\gamma\delta$ -T cells are also known to rapidly produce large amounts of IFN- γ following stimulation. Therefore, it would be of great interest to investigate the role of Bhlhe40 in these cells in association

with the epigenetic regulation of *Ifng* locus, which will be performed in our future studies.

Previous reports have suggested a circadian fashion of *Bhlhe40* expression (10). However, no significant changes in *Bhlhe40* mRNA expression were observed in *i*NKT cells of mice subjected to dark/light cycles, suggesting that the functions of *Bhlhe40* in *i*NKT cells is independent of the circadian rhythm. *Bhlhe40* was shown to control various functions of T cells, such as turning of naïve CD8⁺ T cells into memory ones, cytokine productions, or tumor infiltration, in a circadian rhythm-independent manner (43-45). Similarly, in this study we report a novel function for *Bhlhe40* as an enhancer of IFN- γ production in *i*NKT cells which is also independent of the circadian rhythm.

In conclusion, our data demonstrate the molecular mechanism of IFN- γ production in *i*NKT cells, which is mediated by the interaction between *Bhlhe40* and T-bet. The identification and further characterization of *Bhlhe40* functions in *i*NKT cells will facilitate development of improved *i*NKT cell-based immunotherapeutic strategies.

Materials and Methods

Cells and animals

Lenti-XTM 293T, B16 and EL-4 were purchased from Clontech laboratory, ATCC and RIKEN cell bank respectively and cultured in appropriate medium supplemented with 10% fetal bovine serum in 5% CO₂ incubator at 37 °C conditions.

C57BL/6 mice were purchased from SLC Japan, Inc. *Bhlhe40*^{-/-} mice, and *Ja18*^{-/-} mice were generated as previously reported (11, 46). NKT clone mice were generated from NKT-NT-ES cells by nuclear transfer of iNKT cells (47), while *Bhlhe40*^{-/-} NKT clone mice were generated by crossing *Bhlhe40*^{-/-} mice and NKT clone mice in our laboratory. Six- to eight-weeks old mice were used in this study. More detailed information is described in SI Materials and Methods.

Quantitative real-time PCR

Ifng, *Il4*, *Il17a*, *Bhlhe40*, *Tbet* and hypoxanthine-guanine phosphoribosyl transferase (*Hprt*) mRNA were analyzed by SYBR[®] green real-time PCR system, using the comparative C_T method and normalized by the internal control *Hprt*. Reagents used for the experiments were shown particularly in SI Materials and Methods.

Flow cytometric analysis and cell sorting

Cells were stained with fluorochrome-labeled mAbs, PE-labeled α -GC loaded CD1d dimer (48) or isotype-matched control immunoglobulin (Ig) after pre-incubation with Fc receptor blocker (2.4G2) and data was collected by FACS CaliburTM, FACS CantoTM II or FACS AriaTM II (BD Bioscience).

For intracellular staining, cells were fixed and permeabilized using Cytofix/CytopermTM kit (BD Bioscience). Further information is written in SI Materials and Methods.

Cell proliferation assay and Enzyme-linked immuno-sorbent assay (ELISA)

iNKT cells were purified from spleen of WT or *Bhlhe40*^{-/-} mice, and co-cultured with 35 Gy-irradiated splenocytes from *Ja18*^{-/-} mice in a 96 well round-bottom culture plate in appropriate medium and conditions. After 40 hours of α -GC stimulation, ³H-thymidine (1 μ Ci/well) was added to culture for 8 hours to measure the incorporation of ³H-thymidine. IFN- γ and IL-4 production in collected supernatants were detected by ELISA. Please refer to the SI Materials and Methods.

In vivo stimulation and cytokines measurement

WT or *Bhlhe40*^{-/-} mice were intravenously injected with α -GC, and serum levels of IFN- γ and IL-4 were evaluated using CBA kit (BD Biosciences). For add-back experiments, 2×10^6 of splenic WT or *Bhlhe40*^{-/-} iNKT cells were intravenously administrated into *Ja18*^{-/-} mice. 1 hour after iNKT cells transfer, α -GC was administrated, and serum levels of IFN- γ and IL-4 were measured as described above. In intracellular staining experiments, splenic iNKT cells from α -GC-stimulated WT or *Bhlhe40*^{-/-} mice were stained with α -IFN- γ or α -IL-4 Abs. See SI Materials and Methods for more details.

Melanoma metastasis model

In lung metastasis model, B16 melanoma cells were inoculated intravenously to WT and *Bhlhe40*^{-/-} mice, followed by α -GC intraperitoneal administration. 7 days after inoculation, lung tumor nodules were evaluated by microscopic inspection. In liver

metastasis model, B16 melanoma cells were inoculated into spleens of WT mice or *Ja18^{-/-}* mice, transferred with WT or *Bhlhe40^{-/-}* iNKT cells. Then, mice received intraperitoneal injection with α -GC. 10 days after inoculation, melanoma surface area in liver was measured. Further precise information was shown in SI Materials and Methods.

Luciferase reporter assay

Lenti-XTM 293T cells were transfected using polyethyleneimine (PEI), while EL-4 and MEF cells were transfected using Neon[®] system (Invitrogen). Luciferase reporter plasmid and a transfection control, beta-galactosidase control vector, were transfected simultaneously. Cells were stimulated with PMA 0 hour or 16 hours after transfection. Cells were stimulated with ionomycin at 0 hour after transfection. Luciferase activity was measured 24 or 48 hours after transfection. See SI Materials and Methods for more details.

Short hairpin RNA against Bhlhe40

Short hairpin (shRNA) sequences against mouse *Bhlhe40* were cloned into lentiviral plasmid. A recombinant lentiviral vector was generated by transient transfection of appropriate virus composition vectors to Lenti-XTM 293T using PEI. Supernatants containing lentiviral particles were collected after 48 hours and used for the transfection to make shRNA expressing transfectant. Please refer to SI Materials and Methods.

Co-immunoprecipitation and western blot analysis

For co-immunoprecipitation assay, Lenti-XTM 293T cells were transfected using PEI

with HA-tagged Bhlhe40 or unrelated HA-tagged protein accompanied with Myc-tagged T-bet, Stat4 or control vectors. 48 hours after transient expression, cells were harvested and lysed with lysis buffers. To analyze the interaction of endogenous Bhlhe40 and T-bet, 3×10^6 iNKT cells were isolated from NKT clone mice and stimulated with α -CD3/28 Ab for 60 minutes. Cleared lysates were labeled with α -HA, α -Myc or α -T-bet antibodies and precipitated by protein G beads. Immunoprecipitated proteins were detected with α -HA Ab or α -Myc Ab for transfected samples, while α -Bhlhe40 and α -T-bet Abs were used for endogenous samples by western blotting. Please refer to SI Materials and Methods for details.

Chromatin immunoprecipitation (ChIP)

Lysates were prepared from iNKT cells of WT or *Bhlhe40*^{-/-} mice. Chromatin samples were sonicated by ultrasonic sonicator. Antibodies used for assay was shown in SI Materials and Methods. Genomic PCR was performed by primers described in Table S1, S2.

Differentiation of bone marrow dendritic cells (BMDC) in vitro

Bone marrow progenitors were cultured with appropriate medium supplemented with granulocyte-macrophage colony stimulating factor and IL-4. On 6 day, BMDCs were collected and received 35 Gy irradiation.

Cultivation of T-bet positive and negative iNKT cells in vitro

Splenic T-bet positive iNKT cell subset (TCR β ⁺ CD1d- α -GC dimer⁺ CD4⁺ IL17RB⁻) and T-bet negative iNKT cell subset (TCR β ⁺ CD1d- α -GC dimer⁺ CD4⁺ IL17RB⁺) were

separated by FACS AriaTM II sorting. Sorted cells were separately co-cultured with irradiated BMDCs with appropriate medium supplemented with α -CD3 Ab and α -CD28 Ab for 3 days. On day 3, IL-2 and IL-7 was added to the medium. After 3 weeks, cells were used for experiments. Please refer to SI Materials and Methods.

Statistical analysis

All data are expressed as mean $\pm$ SEM. The differences between sample groups were determined by Student's t test or the two-sample t test with Welch's correction. p values were considered statistically significant when less than 0.05. NS refers to not significant values.

Author Contributions

M.K., S.K. and K.S. designed experiments and performed data analysis. M.K., S.K., H.Y., Y.U., H.H., Y.S., M.H., T.A. and M.B. performed experiments and data analysis. M.K., M.B. and K.S. wrote and integrated the manuscript. H.W., M.T., and N.S. integrated the manuscript. K.S. supervised the whole study.

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Competing financial interests

The authors declare no competing financial interests.

References

1. Taniguchi M, Harada M, Kojo S, Nakayama T, & Wakao H (2003) The regulatory role of Valpha14 NKT cells in innate and acquired immune response. *Annu Rev Immunol* 21:483-513.
2. Coquet JM, *et al.* (2008) Diverse cytokine production by NKT cell subsets and identification of an IL-17-producing CD4-NK1.1- NKT cell population. *Proc Natl Acad Sci U S A* 105(32):11287-11292.
3. Kitamura H, *et al.* (1999) The natural killer T (NKT) cell ligand alpha-galactosylceramide demonstrates its immunopotentiating effect by inducing interleukin (IL)-12 production by dendritic cells and IL-12 receptor expression on NKT cells. *J Exp Med* 189(7):1121-1128.
4. Kojo S, *et al.* (2005) Induction of regulatory properties in dendritic cells by Valpha14 NKT cells. *J Immunol* 175(6):3648-3655.
5. Terabe M & Berzofsky JA (2008) The role of NKT cells in tumor immunity. *Adv Cancer Res* 101:277-348.
6. Constantinides MG & Bendelac A (2013) Transcriptional regulation of the NKT cell lineage. *Curr Opin Immunol* 25(2):161-167.
7. Hu T, *et al.* (2013) Increased level of E protein activity during invariant NKT development promotes differentiation of invariant NKT2 and invariant NKT17 subsets. *J Immunol* 191(10):5065-5073.
8. Lee YJ, Holzapfel KL, Zhu J, Jameson SC, & Hogquist KA (2013) Steady-state production of IL-4 modulates immunity in mouse strains and is determined by lineage diversity of iNKT cells. *Nat Immunol* 14(11):1146-1154.
9. Watarai H, *et al.* (2012) Development and function of invariant natural killer T

- cells producing T(h)2- and T(h)17-cytokines. *PLoS Biol* 10(2):e1001255.
10. Honma S, *et al.* (2002) Dec1 and Dec2 are regulators of the mammalian molecular clock. *Nature* 419(6909):841-844.
 11. Miyazaki K, *et al.* (2010) The role of the basic helix-loop-helix transcription factor Dec1 in the regulatory T cells. *J Immunol* 185(12):7330-7339.
 12. Seuter S, Pehkonen P, Heikkinen S, & Carlberg C (2013) The gene for the transcription factor BHLHE40/DEC1/stra13 is a dynamically regulated primary target of the vitamin D receptor. *J Steroid Biochem Mol Biol* 136:62-67.
 13. Sun H, Lu B, Li RQ, Flavell RA, & Taneja R (2001) Defective T cell activation and autoimmune disorder in Stra13-deficient mice. *Nat Immunol* 2(11):1040-1047.
 14. Benlagha K, Kyin T, Beavis A, Teyton L, & Bendelac A (2002) A thymic precursor to the NK T cell lineage. *Science* 296(5567):553-555.
 15. Maeda M, Lohwasser S, Yamamura T, & Takei F (2001) Regulation of NKT cells by Ly49: analysis of primary NKT cells and generation of NKT cell line. *J Immunol* 167(8):4180-4186.
 16. Benlagha K, Wei DG, Veiga J, Teyton L, & Bendelac A (2005) Characterization of the early stages of thymic NKT cell development. *J Exp Med* 202(4):485-492.
 17. Griewank K, *et al.* (2007) Homotypic interactions mediated by Slamf1 and Slamf6 receptors control NKT cell lineage development. *Immunity* 27(5):751-762.
 18. Stetson DB, *et al.* (2003) Constitutive cytokine mRNAs mark natural killer (NK) and NK T cells poised for rapid effector function. *J Exp Med* 198(7):1069-1076.
 19. Kawano T, *et al.* (1997) CD1d-restricted and TCR-mediated activation of

- valpha14 NKT cells by glycosylceramides. *Science* 278(5343):1626-1629.
20. Toura I, *et al.* (1999) Cutting edge: inhibition of experimental tumor metastasis by dendritic cells pulsed with alpha-galactosylceramide. *J Immunol* 163(5):2387-2391.
 21. Smyth MJ, *et al.* (2002) Sequential production of interferon-gamma by NK1.1(+) T cells and natural killer cells is essential for the antimetastatic effect of alpha-galactosylceramide. *Blood* 99(4):1259-1266.
 22. Kojo S, *et al.* (2009) Mechanisms of NKT cell anergy induction involve Cbl-b-promoted monoubiquitination of CARMA1. *Proc Natl Acad Sci U S A* 106(42):17847-17851.
 23. Szabo SJ, Sullivan BM, Peng SL, & Glimcher LH (2003) Molecular mechanisms regulating Th1 immune responses. *Annu Rev Immunol* 21:713-758.
 24. Hwang ES, Szabo SJ, Schwartzberg PL, & Glimcher LH (2005) T helper cell fate specified by kinase-mediated interaction of T-bet with GATA-3. *Science* 307(5708):430-433.
 25. Morinobu A, *et al.* (2002) STAT4 serine phosphorylation is critical for IL-12-induced IFN-gamma production but not for cell proliferation. *Proc Natl Acad Sci U S A* 99(19):12281-12286.
 26. Bailis W, *et al.* (2013) Notch simultaneously orchestrates multiple helper T cell programs independently of cytokine signals. *Immunity* 39(1):148-159.
 27. Fields PE, Kim ST, & Flavell RA (2002) Cutting edge: changes in histone acetylation at the IL-4 and IFN-gamma loci accompany Th1/Th2 differentiation. *J Immunol* 169(2):647-650.
 28. Grenningloh R, Kang BY, & Ho IC (2005) Ets-1, a functional cofactor of T-bet,

- is essential for Th1 inflammatory responses. *J Exp Med* 201(4):615-626.
29. Lovett-Racke AE, *et al.* (2004) Silencing T-bet defines a critical role in the differentiation of autoreactive T lymphocytes. *Immunity* 21(5):719-731.
 30. Szabo SJ, *et al.* (2000) A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 100(6):655-669.
 31. Beima KM, *et al.* (2006) T-bet binding to newly identified target gene promoters is cell type-independent but results in variable context-dependent functional effects. *J Biol Chem* 281(17):11992-12000.
 32. Baguet A & Bix M (2004) Chromatin landscape dynamics of the Il4-Il13 locus during T helper 1 and 2 development. *Proc Natl Acad Sci U S A* 101(31):11410-11415.
 33. Nishimura T, *et al.* (2000) The interface between innate and acquired immunity: glycolipid antigen presentation by CD1d-expressing dendritic cells to NKT cells induces the differentiation of antigen-specific cytotoxic T lymphocytes. *Int Immunol* 12(7):987-994.
 34. Motohashi S, *et al.* (2009) A phase I-II study of alpha-galactosylceramide-pulsed IL-2/GM-CSF-cultured peripheral blood mononuclear cells in patients with advanced and recurrent non-small cell lung cancer. *J Immunol* 182(4):2492-2501.
 35. Cohen NR, *et al.* (2013) Shared and distinct transcriptional programs underlie the hybrid nature of iNKT cells. *Nat Immunol* 14(1):90-99.
 36. Li Y, *et al.* (2003) DEC1 negatively regulates the expression of DEC2 through binding to the E-box in the proximal promoter. *J Biol Chem* 278(19):16899-16907.

37. Zawel L, *et al.* (2002) DEC1 is a downstream target of TGF-beta with sequence-specific transcriptional repressor activities. *Proc Natl Acad Sci U S A* 99(5):2848-2853.
38. Shen M, *et al.* (2002) Basic helix-loop-helix protein DEC1 promotes chondrocyte differentiation at the early and terminal stages. *J Biol Chem* 277(51):50112-50120.
39. Li Y, *et al.* (2006) The expression of antiapoptotic protein survivin is transcriptionally upregulated by DEC1 primarily through multiple sp1 binding sites in the proximal promoter. *Oncogene* 25(23):3296-3306.
40. Sekimata M, *et al.* (2009) CCCTC-binding factor and the transcription factor T-bet orchestrate T helper 1 cell-specific structure and function at the interferon-gamma locus. *Immunity* 31(4):551-564.
41. Chen GY, Osada H, Santamaria-Babi LF, & Kannagi R (2006) Interaction of GATA-3/T-bet transcription factors regulates expression of sialyl Lewis X homing receptors on Th1/Th2 lymphocytes. *Proc Natl Acad Sci U S A* 103(45):16894-16899.
42. Miller SA, Huang AC, Miazgowiec MM, Brassil MM, & Weinmann AS (2008) Coordinated but physically separable interaction with H3K27-demethylase and H3K4-methyltransferase activities are required for T-box protein-mediated activation of developmental gene expression. *Genes Dev* 22(21):2980-2993.
43. Lin CC, *et al.* (2014) Bhlhe40 controls cytokine production by T cells and is essential for pathogenicity in autoimmune neuroinflammation. *Nat Commun* 5:3551.
44. Hu G & Chen J (2013) A genome-wide regulatory network identifies key

- transcription factors for memory CD8(+) T-cell development. *Nat Commun* 4:2830.
45. Liu Y, *et al.* (2013) DEC1 is positively associated with the malignant phenotype of invasive breast cancers and negatively correlated with the expression of claudin-1. *Int J Mol Med* 31(4):855-860.
 46. Cui J, *et al.* (1997) Requirement for Valpha14 NKT cells in IL-12-mediated rejection of tumors. *Science* 278(5343):1623-1626.
 47. Watarai H, *et al.* (2010) Generation of functional NKT cells in vitro from embryonic stem cells bearing rearranged invariant Valpha14-Jalpha18 TCRalpha gene. *Blood* 115(2):230-237.
 48. Watarai H, Nakagawa R, Omori-Miyake M, Dashtsoodol N, & Taniguchi M (2008) Methods for detection, isolation and culture of mouse and human invariant NKT cells. *Nat Protoc* 3(1):70-78.
 49. Szabo SJ, Gold JS, Murphy TL, & Murphy KM (1993) Identification of cis-acting regulatory elements controlling interleukin-4 gene expression in T cells: roles for NF-Y and NF-ATc. *Mol Cell Biol* 13(8):4793-4805.
 50. Ranganath S, *et al.* (1998) GATA-3-dependent enhancer activity in IL-4 gene regulation. *J Immunol* 161(8):3822-3826.

Figure legends

Figure 1: High expression of *Bhlhe40* in *Ifng*-expressing *i*NKT cells.

(A) Quantitative reverse transcriptase PCR (RT-PCR) analysis of *Bhlhe40* mRNA expression in CD4⁺ T cells, CD8⁺ T cells and *i*NKT cells isolated from mouse spleen. Level of *Bhlhe40* mRNA in CD4⁺ T cells was considered as 1. (B) Quantitative RT-PCR analysis for *Bhlhe40* mRNA expression in *i*NKT cells after α -CD3/CD28-stimulation. Level of *Bhlhe40* mRNA of no stimulation at 0 hour was considered as 1. (C) Developmental stages of *i*NKT cells in thymus determined by NK1.1 and CD44 expression gated on TCR β ⁺ CD1d- α GC dimer⁺ thymocytes (D) Quantitative RT-PCR analysis for *Bhlhe40*, *Ifng* and *Tbet* mRNA in thymic *i*NKT cells at different stages of maturation. Expression level of stage 1 thymic *i*NKT was considered as 1. (E) Classification of *i*NKT subsets based on CD4 and IL-17RB expressions (gated on TCR β ⁺ CD1d- α GC dimer⁺ splenocytes) and the related cytokine patterns. (F) Quantitative RT-PCR analysis for *Bhlhe40* mRNA in *i*NKT subsets. Level of *Bhlhe40* mRNA of CD4⁻ *Ifng*^{int} was considered as 1. Similar results were obtained in 3 independent experiments.

Figure 2: Development of *i*NKT cells is independent of *Bhlhe40* expression.

(A) Frequencies of *i*NKT cells in thymus, spleen and liver (gated on CD19⁻) compared between WT and *Bhlhe40*^{-/-} mice. (B) Comparison of *i*NKT cells developmental stage in thymus between WT and *Bhlhe40*^{-/-} mice (gated on TCR β ⁺ CD1d- α GC dimer⁺ thymocytes). (C) Expression of Ly49A, Ly49C and Ly49G2 maturation markers in thymic *i*NKT cells isolated from WT or *Bhlhe40*^{-/-} mice (gated on TCR β ⁺ CD1d- α GC

dimer⁺ thymocytes). (D) Expression of SLAMF1 and CD1d on CD4⁺ CD8⁺ double positive thymocytes compared between WT and *Bhlhe40*^{-/-} mice. (E) Comparison between iNKT subsets in WT or *Bhlhe40*^{-/-} mice based on CD4 and IL-17RB expressions (gated on TCRβ⁺ CD1d-αGC dimer⁺ cells). Data are shown as representative of 3 independent experiments.

Figure 3: *Bhlhe40* deficiency results in impaired IFN-γ production in stimulated iNKT cells.

(A) Enzyme-linked immunosorbent assay (ELISA) measurement of IFN-γ in α-GC-stimulated WT or *Bhlhe40*^{-/-} iNKT cells 48 hours after stimulation. (B) ELISA measurement of IL-4 in α-GC-stimulated WT or *Bhlhe40*^{-/-} iNKT cells 48 hours after stimulation. (C) Cell proliferation is compared between splenic iNKT cells isolated from WT or *Bhlhe40*^{-/-} mice 48 hours after stimulation with α-GC. (D) Quantitative RT-PCR analysis of *Ifng* mRNA expression in splenic iNKT cells isolated from WT or *Bhlhe40*^{-/-} mice after stimulation with α-CD3 and α-CD28 Ab. *Ifng* mRNA level of WT iNKT cells at 0 hour was considered as 1. (E) Intracellular staining of IFN-γ or IL-4 in splenic iNKT cells of WT or *Bhlhe40*^{-/-} mice 1 hour after intravenous administration of α-GC (gated on TCRβ⁺ CD1d-αGC dimer⁺ splenocytes). (F) Serum levels of IFN-γ or IL-4 in WT or *Bhlhe40*^{-/-} mice intravenously injected with α-GC. Similar results were obtained in 3 independent experiments. *P < 0.01.

Figure 4: *Bhlhe40* deficiency impairs antitumor effect of iNKT cells

(A) Experimental lung metastasis of B16 melanoma cells in WT and *Bhlhe40*^{-/-} mice (n=3 per group). B16 melanoma cells (5 × 10⁵ cells) were inoculated intravenously,

followed by the intraperitoneal administration of α -GC (4 μ g) or a vehicle. After 7 days, numbers of melanoma nodules in the lungs were determined by microscopic inspection. (B) Experimental liver metastasis of B16 melanoma cells in WT mice or *Ja18*^{-/-} mice adoptively transferred with WT or *Bhlhe40*^{-/-} iNKT cells. B16 melanoma cells (1×10^6 cells) were inoculated into the spleen to WT mice or *Ja18*^{-/-} mice (n=3 per group) adoptively transferred with 1×10^6 WT or *Bhlhe40*^{-/-} iNKT cells or vehicle, followed by the intraperitoneal administration of α -GC (2 μ g). After 10 days, livers were removed and photographs were taken to measure melanoma surface area. Similar results were obtained in 2 independent experiments. **P<0.05.

Figure 5: Bhlhe40 enhance IFN- γ production by T-bet-mediated mechanism.

(A) Intracellular staining of T-bet in iNKT cells (gated on TCR β ⁺ CD1d- α GC dimer⁺ cells) from WT or *Bhlhe40*^{-/-} mice. (B) Immunoprecipitation of Myc-tagged T-bet together with HA-tagged Bhlhe40 in lysates of Lenti-XTM 293T cells, followed by immunoblot analysis with α -HA-tag or α -Myc-tag Ab in whole cell lysates. (C) Immunoprecipitation of endogenous T-bet together with Bhlhe40 in lysates of iNKT cells stimulated with α -CD3/CD28 mAb, followed by reprobed immunoblot analysis with α -T-bet Ab. (D) Luciferase activity of *Ifng* promoter-luciferase reporter plasmid in *Bhlhe40*^{-/-} MEF cells transfected with control or *Bhlhe40* and/or *T-bet* expression plasmids or control vector 24 hours after transfection. Luciferase activity level of *Bhlhe40*^{-/-} MEF cells without stimulation was considered as 1. (E) Luciferase activity of *Ifng* promoter-luciferase reporter plasmid in EL-4 cells stably transfected with *Bhlhe40* shRNA or control shRNA, 48 hours after transfection of *T-bet* expression plasmid with/or *Bhlhe40* shRNA^r (construct encoding knockdown-resistant *Bhlhe40*). Luciferase

activity level of EL-4 cells transfected with shRNA control was considered as 1. Data are shown as representative of 3 independent experiments. **P<0.05.

Figure 6: Bhlhe40 is involved in chromatin remodeling of *Ifng* locus.

(A) A scheme describes primer positions in *Ifng* locus used for chromatin immunoprecipitation (ChIP) PCR assay. (B) Bhlhe40 binding to *Ifng* locus (C) Histone H3-lysine 9 (K9) acetylation on the *Ifng* locus were detected in WT or *Bhlhe40*^{-/-} iNKT cells by ChIP-PCR assay. Histone H3-K9 acetylation at #1 site was evaluated in different samples and thus represented by a separated graph. Data are shown as representative of 2 independent experiments.

Figure 7: Bhlhe40 accumulation and H3-K9 acetylation of *Ifng* locus is reduced without T-bet.

(A) Bhlhe40 accumulation on *Ifng* locus was detected in T-bet positive or negative iNKT cells. (B) Histone H3-K9 acetylation on *Ifng* locus was detected in T-bet positive or negative iNKT cells by ChIP-PCR assay. Data are shown as representative of 2 independent experiments.

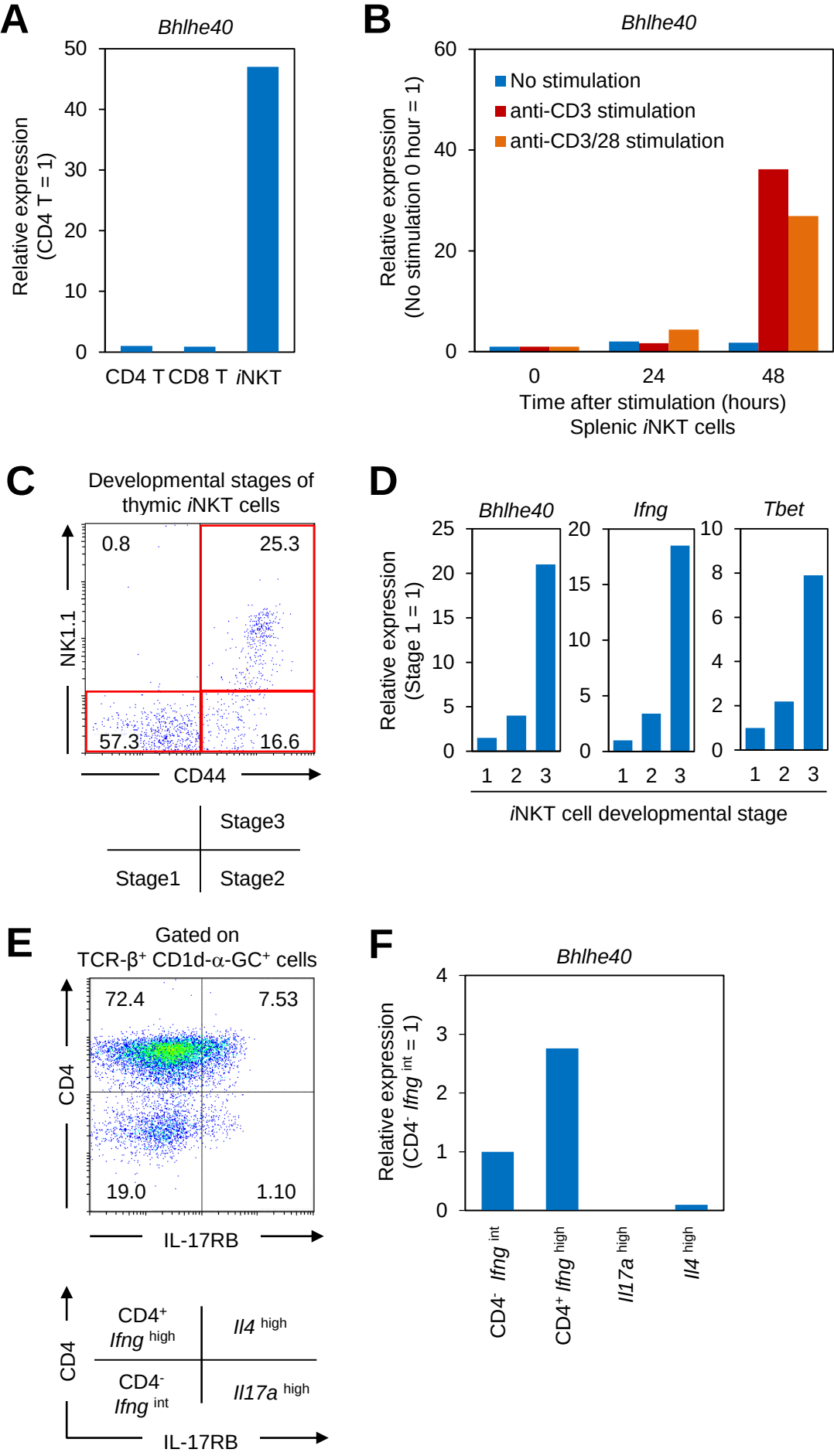


Figure 1

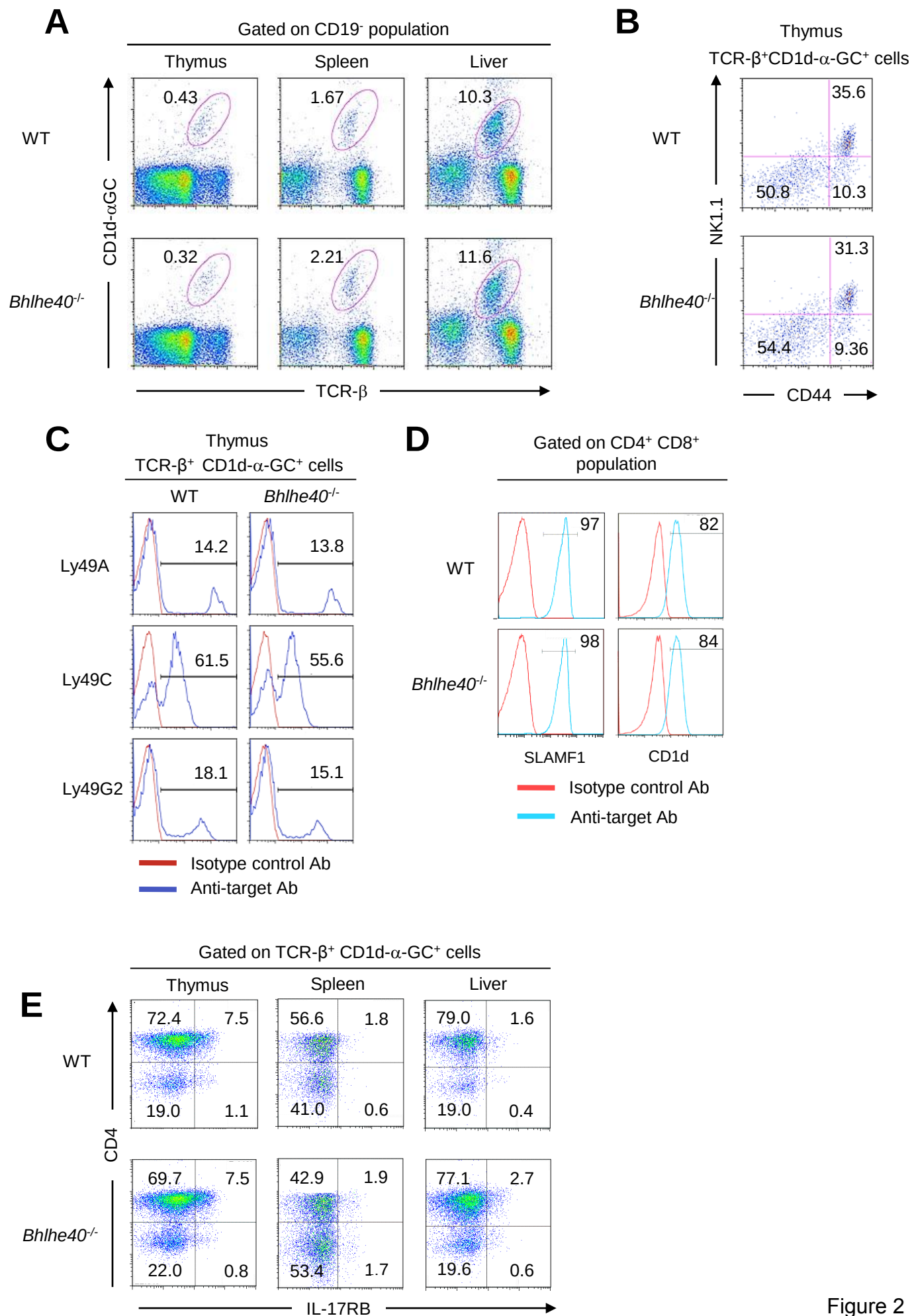


Figure 2

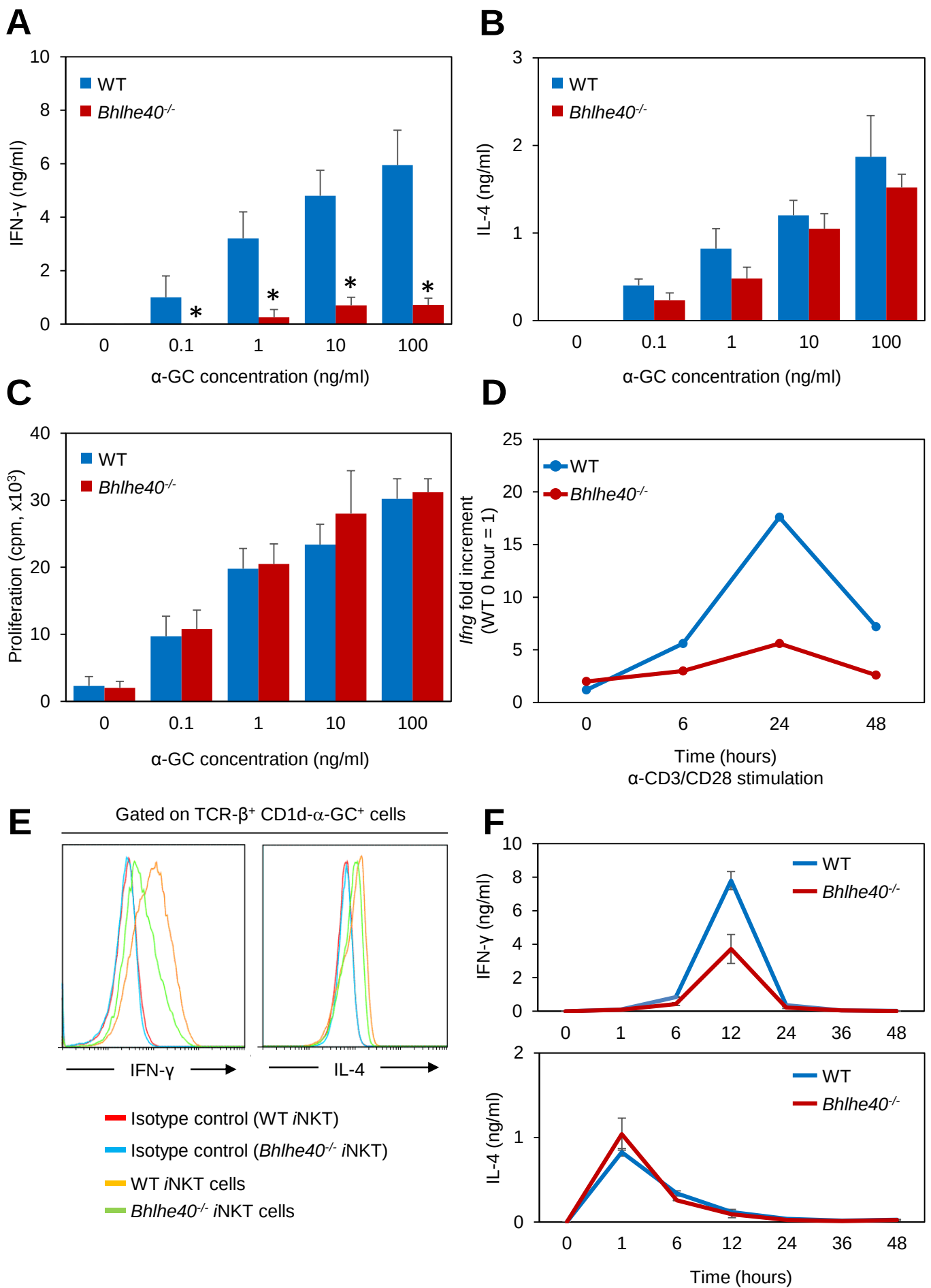
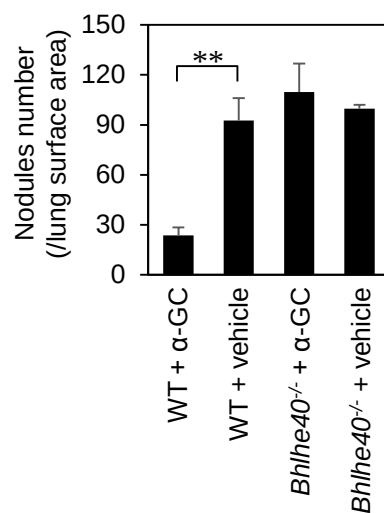
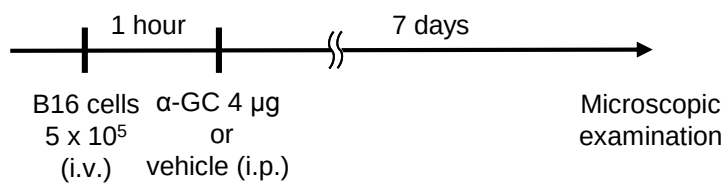
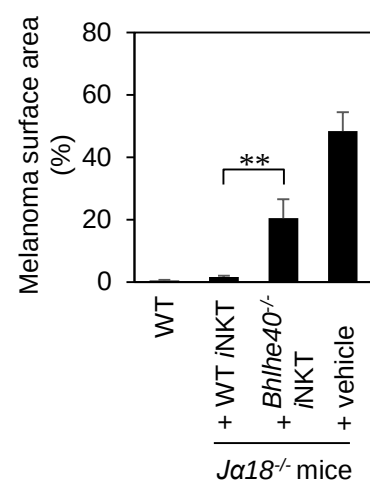
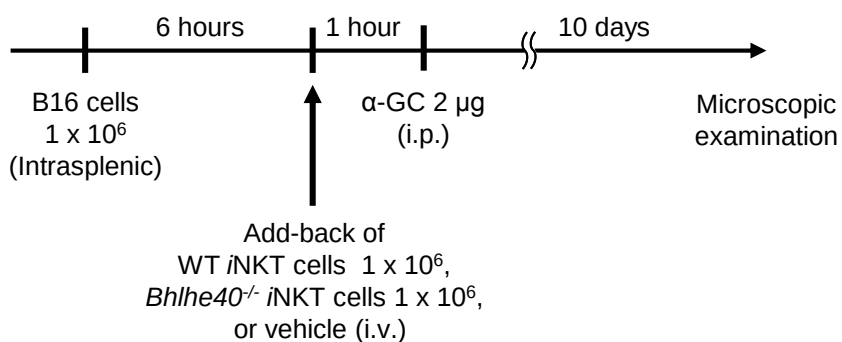


Figure 3

A**Lung metastasis model****B****Liver metastasis model***Ja18*^{-/-} mice

WT control



Add-back of WT iNKT

Add-back of *Bhlhe40*^{-/-} iNKT

Vehicle



1cm

Figure 4

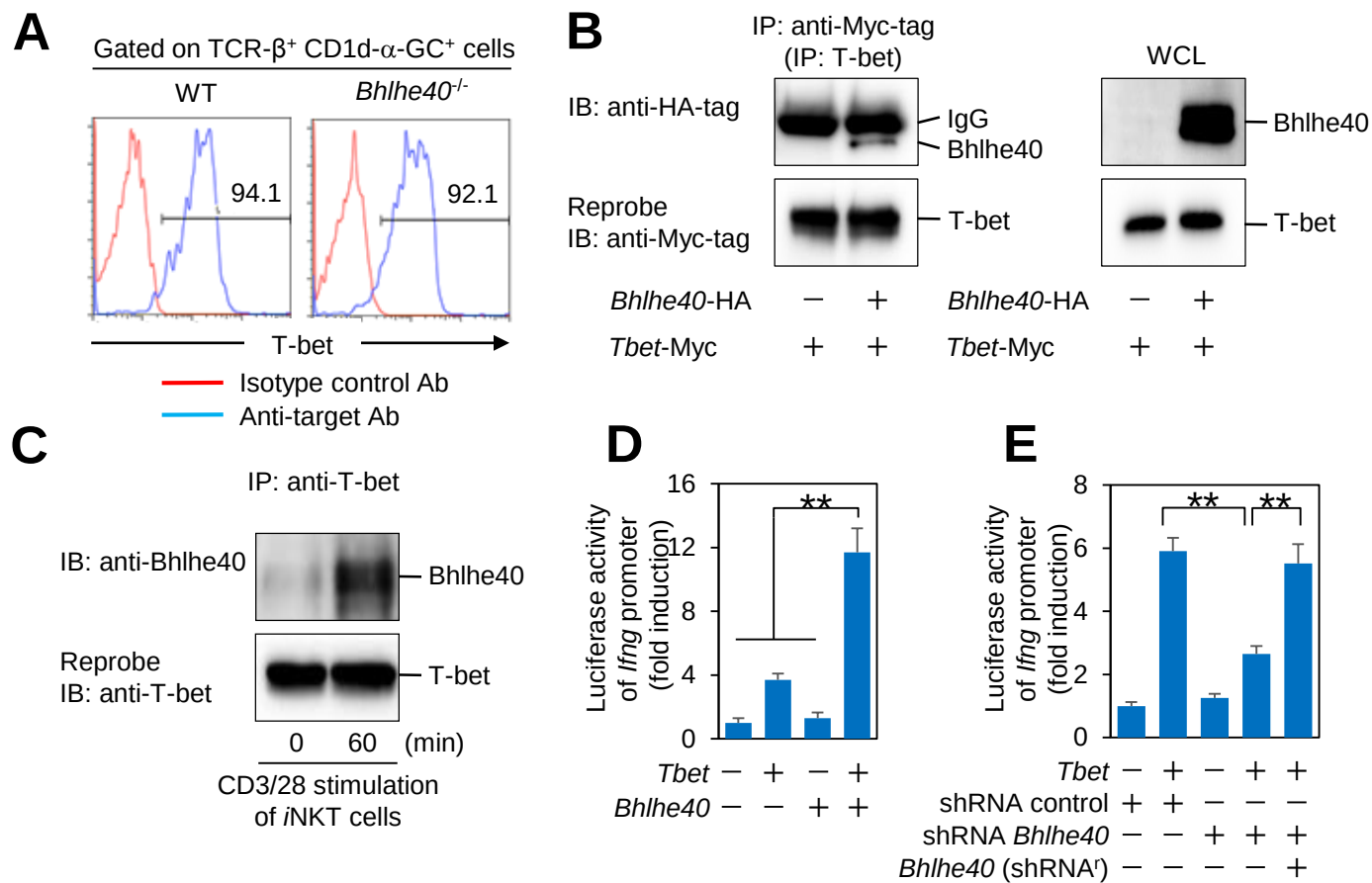


Figure 5

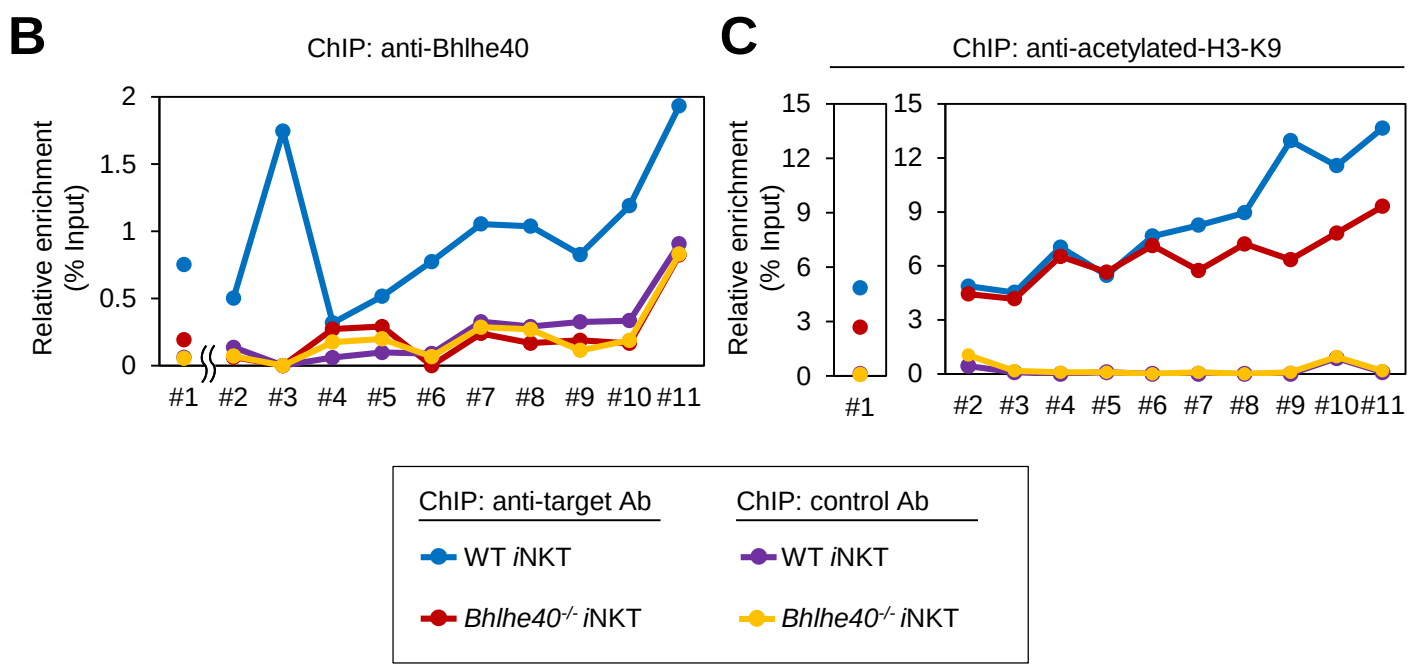
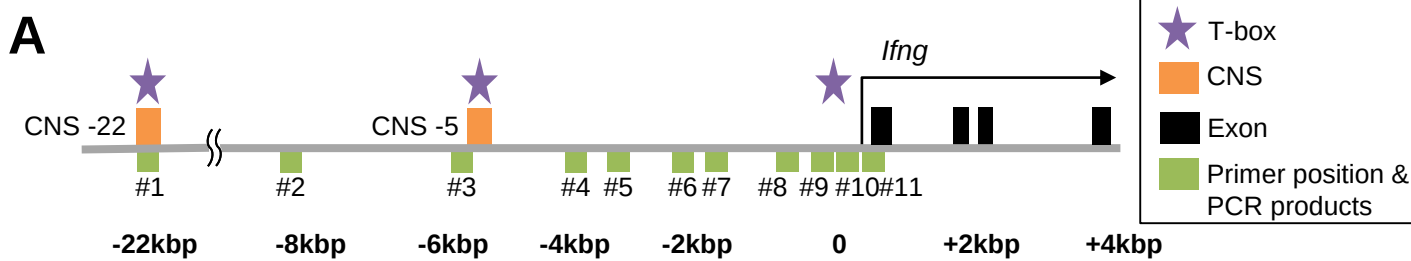


Figure 6

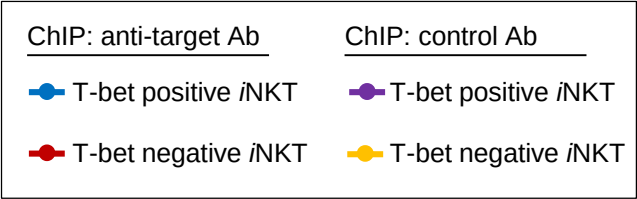
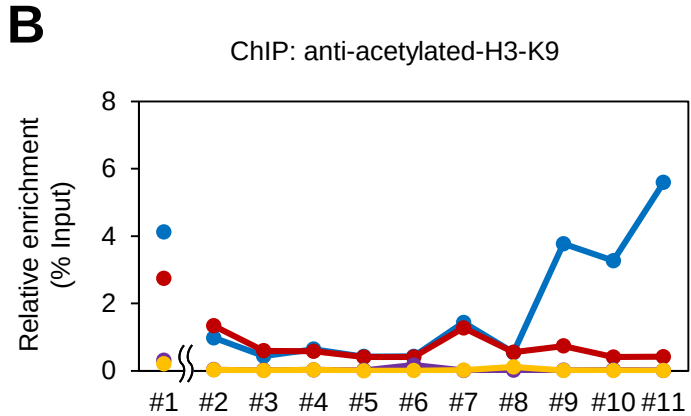
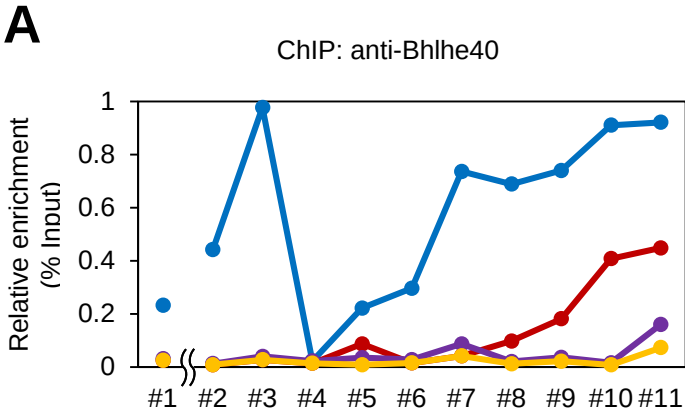


Figure 7