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# The basic leucine zipper transcription factor E4BP4 is essential for natural killer cell development

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**Natural killer (NK) cells are a subset of lymphocytes crucial for innate immunity and modification of adaptive immune responses. In contrast to commitment to the T cell or B cell lineage, little is known about NK cell lineage commitment. Here we show that the basic leucine zipper (bZIP) transcription factor E4BP4 (also called NFIL3) is essential for generation of the NK cell lineage. E4BP4-deficient mice (*Nfil3*$^{-/-}$; called '*E4bp4*$^{-/-}$' here) had B cells, T cells and NKT cells but specifically lack NK cells and showed severely impaired NK cell–mediated cytotoxicity. Overexpression of *E4bp4* was sufficient to increase NK cell production from hematopoietic progenitor cells. E4BP4 acted in a cell-intrinsic manner 'downstream' of the interleukin 15 receptor (IL-15R) and through the transcription factor Id2. *E4bp4*$^{-/-}$ mice may provide a model for definitive analysis of the contribution of NK cells to immune responses and pathologies.**

NK cells represent a distinct lymphocyte subset with a central role in innate immunity, and NK cells increasingly seem to serve important functions in influencing the nature of the adaptive immune response[1,2]. Their cytotoxic function is crucial to many processes such as tumor immunosurveillance[3] and elimination of microbial infection[4]. A great deal of progress has been made in delineating the cytotoxic mechanisms of NK cell action, specifically events that control target cell recognition and receptor signaling, as well as the production of proinflammatory cytokines[5] such as interferon-γ (IFN-γ). However, the molecular basis of NK cell development is much less well understood and has been characterized as one of the most important problems to be addressed in NK cell biology[6]. Greater knowledge of how NK cells develop into functional effector cells is essential for understanding their contribution to disease processes as well as for exploiting their therapeutic potential.

The bone marrow is the main site of NK cell development in the adult mouse. In the bone marrow, CD122$^{+}$NK1.1$^{-}$ NK cell precursors (NKPs) derived from hematopoietic stem cells give rise to immature NK (iNK) cells and mature NK (mNK) cells[7]. The mNK cells can emigrate from the bone marrow and represent the main NK cell population in the peripheral lymphoid organs such as spleen and lymph nodes. NK cell development depends on the cytokine interleukin 15 (IL-15), and both *Il15*$^{-/-}$ mice[8] and IL-15 receptor α-chain (IL-15R)-deficient (*Il15ra*$^{-/-}$) mice[9] lack peripheral NK cells and detectable NK cell–mediated cytotoxicity. However, the action of IL-15 is not confined to NK cells, as both *Il15*$^{-/-}$ and *Il15ra*$^{-/-}$ mice also have considerably fewer NKT cells and CD8$^{+}$ memory T cells.

Specific transcription factors 'program' the developmental pathway from hematopoietic stem cells toward lineage-restricted differentiation[10]. These transcription factors are well characterized in B lymphocyte, T lymphocyte, erythroid and myeloid lineages, but so far no gene has been identified that specifically determines the NK lineage. Several transcription factors, such as Ets-1 (ref. 11), Id2 (ref. 12), GATA-3 (ref. 13), PU.1 (ref. 14), Mef[15], T-bet[16] and Irf-2 (ref. 17), have been reported to regulate NK cell maturation, but deletion of each of their respective genes produces additional defects in other hematopoietic cell lineages. In addition, many of these factors act after commitment to the NK cell lineage. For example, spleens of *Id2*$^{-/-}$ mice have only 10% as many mNK cells as are present in wild-type spleen but have no fewer NKPs or iNK cells in the bone marrow[12]. This block in development is later than that in *Il15*$^{-/-}$ mice, which show much less iNK cell production. Loss of *Gata3* has also been shown to hinder the development of mNK cells and lead to defective IFN-γ production[13].

NFIL3 (also called E4BP4) is a mammalian basic leucine zipper transcription factor with most similarity to the PAR (proline- and acidic residue–rich) subclass[18]. It was identified by its DNA-binding activity at the promoters of the genes encoding adenovirus E4 protein[19] and human IL-3 (ref. 20) and is proposed to mediate IL-3-dependent survival of pro–B cells[21]. Subsequent work has shown that E4BP4 is involved in transcriptional control of the mammalian circadian clock, notably in the chick pineal gland[22–24]. The involvement of E4BP4 in other cellular processes such as motorneuron growth and survival[25], regulation of von Willebrand factor expression[26] and osteoblast function[27] has also been proposed.

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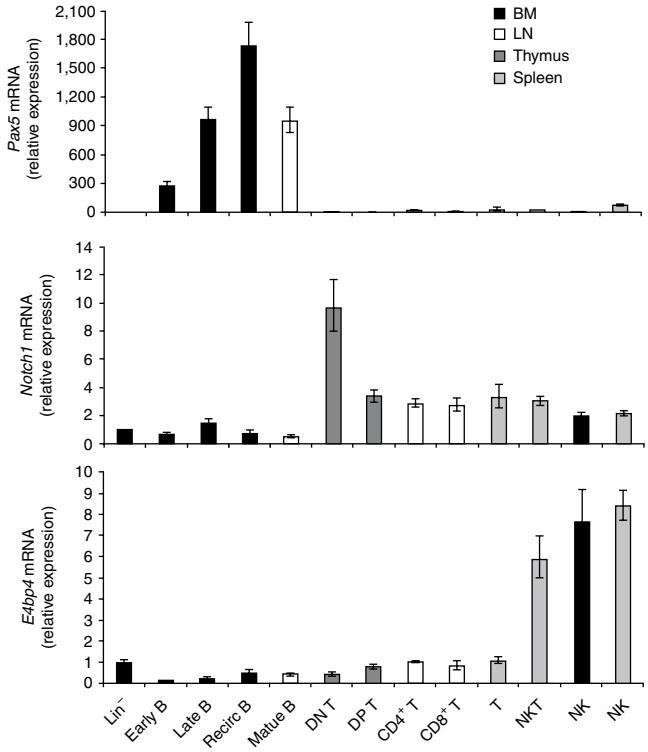


**Figure 1** Expression of *E4bp4* in mouse lymphoid populations. Real-time PCR analysis of the expression of *Pax5*, *Notch1* and *E4bp4* in Lin⁻ bone marrow cells and CD19⁺c-Kit⁺IgD⁻ B cells (Early B), CD19⁺c-Kit⁻IgD⁻ B cells (Late B) and CD19⁺IgD⁺ recirculating B cells (Recirc B) from bone marrow (BM); CD19⁺ B cells from lymph node (LN; Mature B); double-negative (CD4⁻CD8⁻) thymocytes (DN T) and double-positive (CD4⁺CD8⁺) thymocytes (DP T); mature CD3⁺ T cells from lymph node (CD4⁺ T and CD8⁺ T), splenic CD3⁺ T cells (T) and NK1.1⁺CD3⁺ NKT cells (NKT); and NK1.1⁺CD3⁻ NK cells from bone marrow and spleen (NK). Expression is normalized to that of *Hprt1* (encoding hypoxanthine guanine phosphoribosyl transferase) and is presented relative to that of Lin⁻ bone marrow. Data are representative of four experiments (mean ± s.d.).

Here we identify the gene encoding E4BP4 (*Nfil3*; called '*E4bp4*' here) as being specifically required for NK cell development. We measured *E4bp4* expression in lymphocyte populations and found that it was high in IL-15-dependent NK, NKT and CD8⁺ memory T cell populations. By generating *E4bp4*$^{-/-}$ mice, we found that this gene was critical, in a dose-dependent manner, for NK cell development but not for the production of NKT cells or CD8⁺ memory T cells. *E4bp4*$^{-/-}$ mice had no detectable mNK cell–mediated cytotoxicity. NK cell development in *E4bp4*$^{-/-}$ was blocked at the IL-15-dependent transitions from the NKP to the iNK cell stage and from the iNK to the mNK cell stage. *E4bp4* increased NK cell production when expressed in normal hematopoietic progenitor cells (HPCs). In addition, we show that E4BP4 functioned 'downstream' of IL-15R signaling and that its action was mediated via Id2.

## RESULTS

### Expression of *E4bp4* in NK and NKT cells

E4BP4 (ref. 18) is expressed in lymphoid cells and has been proposed to regulate apoptosis in both B and T cells[21,28]. To investigate a potential function for E4BP4 in lymphocyte development, we measured the expression of *E4bp4* in mouse B, T, NKT and NK cell populations by real-time PCR analysis. We confirmed efficient sorting of B cell and T cell populations by high expression of the genes *Pax5* (encoding the transcription factor Pax5) and *Notch1* (encoding), respectively. In contrast, we found higher *E4bp4* expression not in B cell or T cell populations but in NK and NKT lymphocyte subsets, in which expression was eightfold greater than that in bone marrow enriched for HPCs (lineage-negative (Lin⁻) bone marrow; **Fig. 1**).

### Loss of NK cells in *E4bp4*$^{-/-}$ mice

To investigate a potential requirement for E4BP4 in lymphocyte development, we generated mice with deletion of *E4bp4* by targeting the single coding exon of *E4bp4* in embryonic stem cells (**Supplementary Fig. 1a**). Offspring from heterozygous crosses were generated in the expected mendelian ratios, and there were no gross phenotypic abnormalities in *E4bp4*$^{-/-}$ mice. We confirmed the absence of E4BP4 expression in *E4bp4*$^{-/-}$ mice by immunoblot and RT-PCR analysis (**Supplementary Fig. 1b**).

The expression profile of *E4bp4* suggested that its deletion might 'preferentially' affect NK and NKT cell lineages. To test this hypothesis, we analyzed the main lymphocyte populations in spleens of *E4bp4*$^{+/+}$, *E4bp4*$^{+/-}$ and *E4bp4*$^{-/-}$ mice (**Fig. 2a**). The *E4bp4* genotype did not significantly alter the number of T cells (CD4⁺ or CD8⁺) or B cells (CD19⁺), but there were significantly fewer NK cells (NK1.1⁺CD3⁻) in *E4bp4*$^{+/-}$ and *E4bp4*$^{-/-}$ mice than in *E4bp4*$^{+/+}$ mice. The NK cell population, further defined as CD122⁺NK1.1⁺CD3⁻ cells (**Fig. 2b**) or NKp46⁺NK1.1⁺CD3⁻ cells (**Supplementary Fig. 2**), was almost entirely absent from the periphery of *E4bp4*$^{-/-}$ mice. *E4bp4* showed haploinsufficiency, as NK cell numbers in *E4bp4*$^{+/-}$ mice were intermediate between those found in *E4bp4*$^{+/+}$ mice and *E4bp4*$^{-/-}$ mice (**Fig. 2a**). In contrast, NKT cell numbers were not lower in either *E4bp4*$^{+/-}$ or *E4bp4*$^{-/-}$ mice (**Supplementary Fig. 3**).

To confirm the diminished NK cell cytotoxicity in *E4bp4*$^{-/-}$ mice, we measured the 'preferential' killing of major histocompatibility complex (MHC) class I–negative target cells by NK cells *in vivo*[29]. We labeled MHC class I–deficient RMA/s mouse lymphoma cells and MHC class I–expressing RMA cells with different concentrations of the cytosolic dye CFSE, then injected equal numbers of cells into mice. We collected spleens from the recipient mice after 16 h and determined the relative number of cells remaining in each population by flow cytometry. 'Preferential' elimination of RMA/s cells due to NK cell activity was 100% efficient in both *E4bp4*$^{+/+}$ and *E4bp4*$^{+/-}$ mice (**Fig. 2c**). In contrast, minimal NK activity was detectable in *E4bp4*$^{-/-}$ mice. We measured IFN-γ production by bone marrow–derived NK cells from *E4bp4*$^{-/-}$ mice and found it also to be minimal (**Fig. 2d**). Therefore, *E4bp4*$^{-/-}$ mice have effectively no peripheral NK cells and no inherent NK cell cytotoxic activity.

### *E4bp4* in NK cell development

Functional NK cells in the periphery are mostly mNK cells that developed mainly in the bone marrow from hematopoietic stem cells via NKP and iNK cell populations[7]. The much lower bone marrow NK cell numbers suggested that *E4bp4*$^{-/-}$ mice have a defect in NK development. To examine this possibility in greater detail, we compared the number of NKP, iNK and mNK cells in the bone marrow[30] across *E4bp4* genotypes (**Fig. 3a–c**). No substantial difference between genotypes in NKP (CD122⁺NK1.1⁻CD3⁻) numbers was apparent, but *E4bp4*$^{-/-}$ bone marrow had considerably fewer iNK (CD122⁺NK1.1⁺CD3⁻) cells and an even greater reduction in mNK (CD122⁺NK1.1⁺CD11b⁺) cell numbers, such that almost no mNK cells were detectable. Comprehensive analysis of differentiation markers showed that the expression of NKp46, NKG2D, DX5 and CD43 was lower on NK cells from *E4bp4*$^{-/-}$ bone marrow, as measured by mean fluorescence intensity and/or percent positivity, whereas expression of the mostly inhibitory receptors Ly49C,

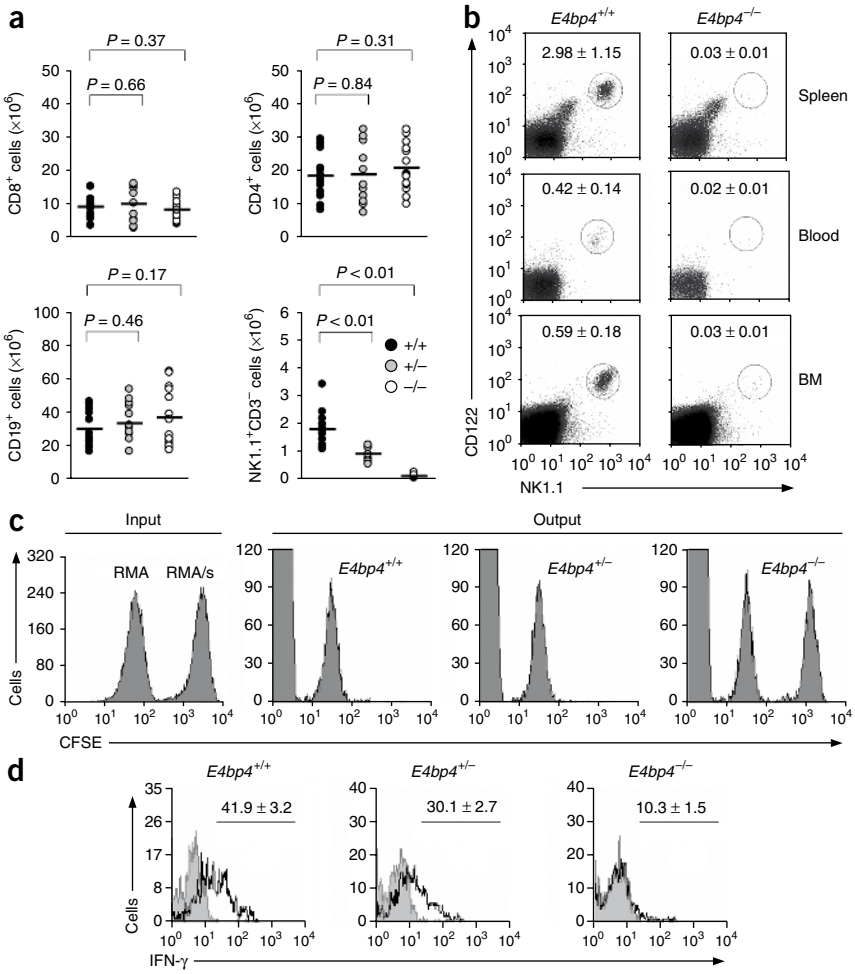


**Figure 2** Loss of peripheral NK cells and NK activity in *E4bp4*$^{-/-}$ mice. (**a**) Total viable cell numbers in peripheral splenic B cell (CD19$^+$), T cell (CD8$^+$CD3$^+$ or CD4$^+$CD3$^+$) and NK cell (NK1.1$^+$CD3$^-$) populations from *E4bp4*$^{+/+}$ and *E4bp4*$^{+/-}$ and *E4bp4*$^{-/-}$ mice ($n > 10$ per genotype, all litter-matched), determined by Trypan blue–exclusion counting and immunophenotypic analysis. Each symbol represents an individual mouse; horizontal line indicate the mean. *P* values, unpaired Student's *t*-test. (**b**) Flow cytometry of CD122$^+$NK1.1$^+$ cells in *E4bp4*$^{+/+}$ and *E4bp4*$^{-/-}$ mice. Numbers in plots indicate percent cells in outlined gate (mean ± s.d. of at least three mice per genotype). (**c**) Flow cytometry of CFSE-stained RMA/s cells (MHC class I–negative; high CFSE concentration) and RMA cells (MHC class I–positive; low CFSE concentration) injected at a ratio of 1:1 (Input) into *E4bp4*$^{+/+}$, *E4bp4*$^{+/-}$ and *E4bp4*$^{-/-}$ mice ($n = 3$ per genotype), followed by analysis of CFSE in cells from recipient spleens 16 h later (Output) to assess *in vivo* NK cell killing activity. Injection of RMA cells labeled with both high and low CFSE concentrations showed no nonspecific loss of the population labeled with a high CFSE concentration (negative control; data not shown). (**d**) Intracellular IFN-γ in NK1.1$^+$CD3$^-$ populations from total bone marrow incubated for 4 h with (black lines) or without (gray filled histograms) IL-12 and IL-18. Numbers above bracketed lines indicate percent cells in gate (mean ± s.d.; $n = 3$ mice per genotype). Data are representative of six (**a**), three (**b**), two (**c**,**d**) experiments.



Ly49I, Ly49F, Ly49H and KLRG1 was higher (**Supplementary Fig. 4**). We used real-time PCR to quantify *E4bp4* expression at each stage of NK cell development in bone marrow. A fivefold increase at the NKP-to-iNK cell transition was apparent, and the resulting higher *E4bp4* expression was maintained in mNK cells (**Fig. 3d**). Such an expression profile accurately correlates with the NKP-iNK and iNK-mNK developmental-stage defects in *E4bp4*$^{-/-}$ bone marrow. Therefore, progression through the NKP-iNK and iNK-mNK transitions requires *E4bp4* expression.

To investigate whether the requirement for *E4bp4* expression in NK cell development is cell intrinsic, we did adoptive transfer experiments with bone marrow as the source of hematopoietic stem cells. Bone marrow from wild-type (Ly5.1$^+$) mice transferred into sublethally irradiated *E4bp4*$^{-/-}$ (Ly5.2$^+$) mice repopulated the NK lineage both in the short term (4 weeks) and long term (3 months; **Supplementary Fig. 5**), which demonstrated that the *E4bp4*$^{-/-}$ niche is competent to support NK cell development. This suggested a cell-intrinsic requirement for E4BP4 in NK cell development. To confirm this hypothesis, we transferred *E4bp4*$^{+/+}$ or *E4bp4*$^{-/-}$ bone marrow (Ly5.2$^+$) at a ratio of 1:1 with competitor bone marrow (Ly5.1$^+$Ly5.2$^+$) from $F_1$ mice into lethally irradiated Ly5.1$^+$ recipients. The degree of *in vivo* reconstitution in hematopoietic cells overall (**Supplementary Fig. 6a**) and specifically for NKT (NK1.1$^+$CD3$^+$) cells (**Supplementary Fig. 6b**) was similar for both *E4bp4*$^{-/-}$ and *E4bp4*$^{+/+}$ donor bone marrow. Although *E4bp4*$^{+/+}$ and competitor bone marrow contributed similarly to long-term NK cell reconstitution, the contribution of *E4bp4*$^{-/-}$ bone marrow was only 10% as much (**Fig. 4**). Therefore, the critical requirement for *E4bp4* in NK cell production is cell intrinsic.

## Ectopic *E4bp4* increases NK cell production

NK cells can be produced *in vitro* from HPCs cultured on stromal cells such as OP9 in the presence of IL-15 (ref. 31), whereas continuous culture of HPCs in the cytokines IL-7 and the ligand for the receptor tyrosine kinase Flt3 (Flt3L) produces B cells[32]. To confirm the cell-intrinsic nature of the *E4bp4*$^{-/-}$ NK cell defect, we isolated bone marrow enriched for HPCs from *E4bp4*$^{-/-}$ and *E4bp4*$^{+/+}$ mice, cultured the bone marrow on OP9 cells with subsequent addition of cytokines and quantified lymphocyte production by flow cytometry (**Supplementary Fig. 7a**). *E4bp4*$^{-/-}$ HPCs produced effectively no NK (NK1.1$^+$) cells compared with the substantial number of NK cells produced by *E4bp4*$^{+/+}$ HPCs (**Fig. 5**). In contrast, loss of *E4bp4* resulted in twofold greater production of B (CD19$^+$) cells from HPCs (**Fig. 5**). To confirm that the IL-7 and Flt3L culture phase did not select against IL-15-dependent progenitors giving rise to NK cells, we incubated *E4bp4*$^{-/-}$ HPCs immediately after isolation with IL-15 in OP9 coculture, but this had no 'rescue' effect on NK cell production (**Supplementary Fig. 7b**). Thus, both *in vivo* and *in vitro* data indicate that NK development requires cell-intrinsic *E4bp4* expression.

To verify the direct relationship between *E4bp4* and NK development, we extended the *in vitro* system by restoring *E4bp4* expression in *E4bp4*$^{-/-}$ HPCs by retroviral transduction. As anticipated, *E4bp4*$^{-/-}$ HPCs failed to produce NK cells after transduction with

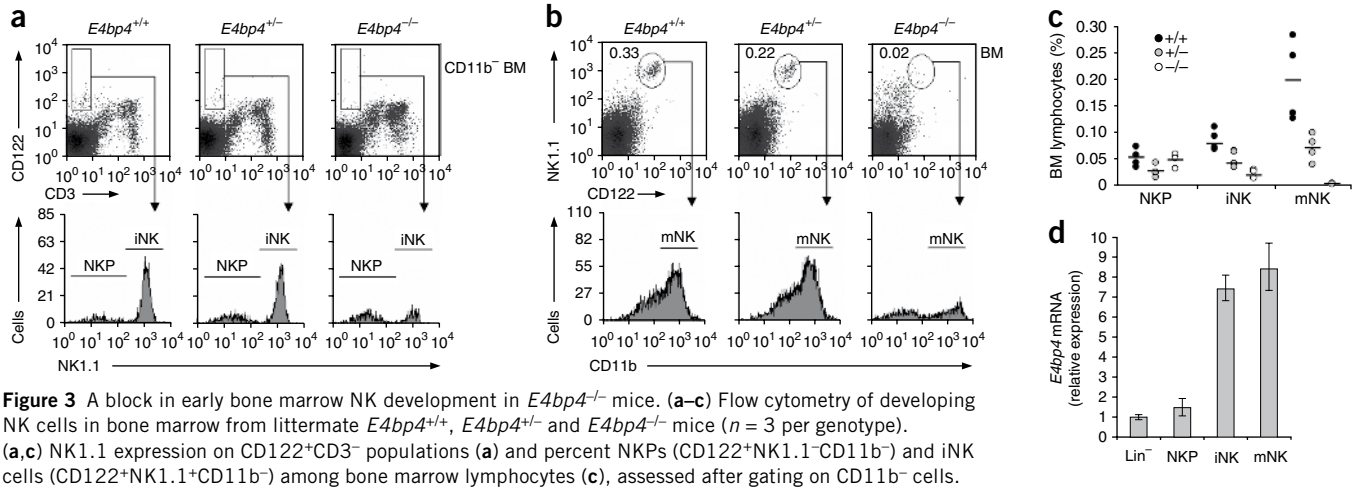


**Figure 3** A block in early bone marrow NK development in *E4bp4*$^{-/-}$ mice. (**a–c**) Flow cytometry of developing NK cells in bone marrow from littermate *E4bp4*$^{+/+}$, *E4bp4*$^{+/-}$ and *E4bp4*$^{-/-}$ mice ($n = 3$ per genotype). (**a**,**c**) NK1.1 expression on CD122$^{+}$CD3$^{-}$ populations (**a**) and percent NKPs (CD122$^{+}$NK1.1$^{-}$CD11b$^{-}$) and iNK cells (CD122$^{+}$NK1.1$^{+}$CD11b$^{-}$) among bone marrow lymphocytes (**c**), assessed after gating on CD11b$^{-}$ cells. (**b**,**c**) CD11b expression on CD122$^{+}$NK1.1$^{+}$ populations (**b**) and percent mNK cells (CD122$^{+}$NK1.1$^{+}$CD11b$^{+}$) among bone marrow lymphocytes (**c**), assessed after gating on CD3$^{-}$ cells. Each symbol represents an individual mouse; horizontal lines indicate the mean (**c**). (**d**) Real-time PCR analysis of *E4bp4* expression by Lin$^{-}$, NKP, iNK and mNK populations sorted by flow cytometry from wild-type C57BL/6 bone marrow, presented relative to *Hprt1* expression. Data are representative of three (**a–c**) or two (**d**) independent experiments.





control retrovirus (hCD2), whereas transduction with an *E4bp4*-expressing retrovirus (E4BP4-hCD2) restored NK cell production in the presence of IL-15 (**Fig. 6a**,**b**). Restoration of *E4bp4* expression to *E4bp4*$^{-/-}$ HPCs restored some NK cell production also in the absence of exogenous IL-15 (**Fig. 6a**), which suggested that E4BP4 may function 'downstream' of IL-15. Furthermore, ectopic expression of *E4bp4* in wild-type HPCs increased the proportion (**Fig. 6a**) and total number (**Fig. 6b**) of NK cells produced; thus, *E4bp4* overexpression can boost NK cell development. Efficient transduction of *E4bp4*$^{+/-}$ HPCs with a retrovirus expressing short hairpin RNA targeting *E4bp4* decreased the proportion of NK cells produced to only 30% as much (**Supplementary Fig. 8**), which further reinforced the idea of the absolute dependence of NK development on *E4bp4* expression. To confirm that *E4bp4* could 'rescue' its null mutation *in vivo*, we transduced *E4bp4*$^{-/-}$ HPCs with control or *E4bp4*-expressing retroviruses and used these cells to reconstitute lethally irradiated recipient mice. Analysis of peripheral blood 1 month after transplantation showed a partial restoration of NK cell numbers due to *E4bp4* expression (**Fig. 6c**).

Ectopic E4BP4 expression prevents the apoptosis of B cells induced by IL-3 withdrawal[21]. To test whether the function of E4BP4 in NK cell development might be explained by such an antiapoptotic action, we transduced HPCs with retroviruses expressing the genes encoding Bcl-2, Bcl-$x_L$ or Mcl-1, which are involved in NK cell survival[33]. However, this did not restore NK cell development potential in *E4bp4*$^{-/-}$ HPCs (**Supplementary Fig. 9a**). Furthermore, NK cells generated either after ectopic *E4bp4* expression in wild-type HPCs or by restoration of *E4bp4* expression to *E4bp4*$^{-/-}$ HPCs showed no change in proliferation rate compared with that of wild-type HPCs transduced with control retrovirus (**Supplementary Fig. 9b**). Together these data support the hypothesis that *E4bp4* drives NK cell production not by promoting the survival or proliferation of developing NK cells but by directly promoting NK lineage commitment.

## E4BP4 acts 'downstream' of IL-15R

IL-15 is the most important factor so far identified as regulating NK cell production, and both *Il15*$^{-/-}$ mice[8] and *Il15ra*$^{-/-}$ mice[9] lack peripheral NK cells. E4BP4 can function 'downstream' of IL-3 in B cells[21], and HPCs from *E4bp4*$^{-/-}$ mice were defective in IL-15-dependent NK cell production (**Fig. 6a**). Furthermore, increases in *E4bp4* expression in early NK cell development directly correlate with the acquisition of IL-15 dependence. Therefore, we hypothesized that E4BP4 may function 'downstream' of IL-15R in NK cell production. To test our hypothesis, we determined whether *E4bp4* could promote NK cell development in the absence of IL-15 signaling. We achieved this in the OP9 coculture system by using IL-15-blocking antibody

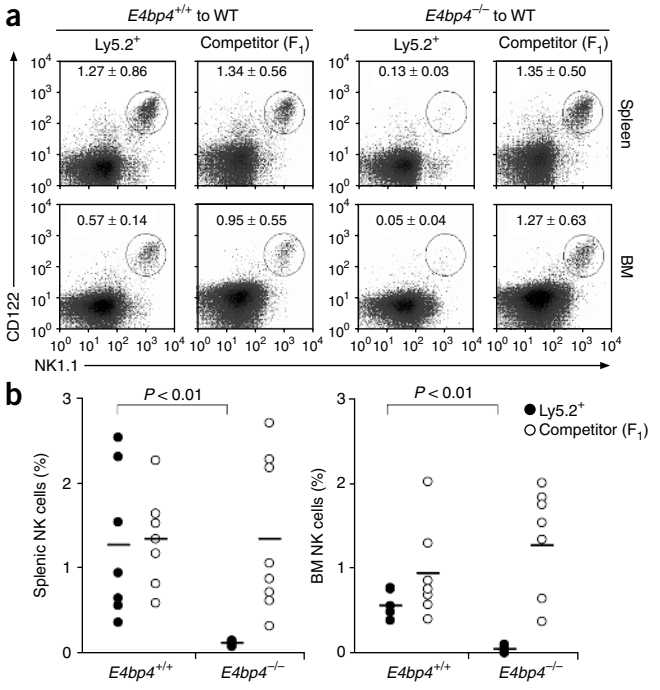


**Figure 4** *E4bp4* is a cell-instrinsic requirement for NK cell development. (**a**) Flow cytometry of CD122$^{+}$NK1.1$^{+}$ cells (gated on CD3$^{-}$ cells) from bone marrow chimeras at 4 months after injection of total bone marrow into lethally irradiated wild-type (WT) recipients. Competitor ($F_1$) bone marrow was Ly5.2$^{+}$Ly5.1$^{+}$. Numbers in plots indicate percent cells in outlined gate. (**b**) Frequency of cells in **a**; each symbol indicates an individual mouse; horizontal lines indicate the mean. *P* values, unpaired Student's *t*-test. Data are from two independent experiments (mean ± s.d. of at least seven mice per condition).

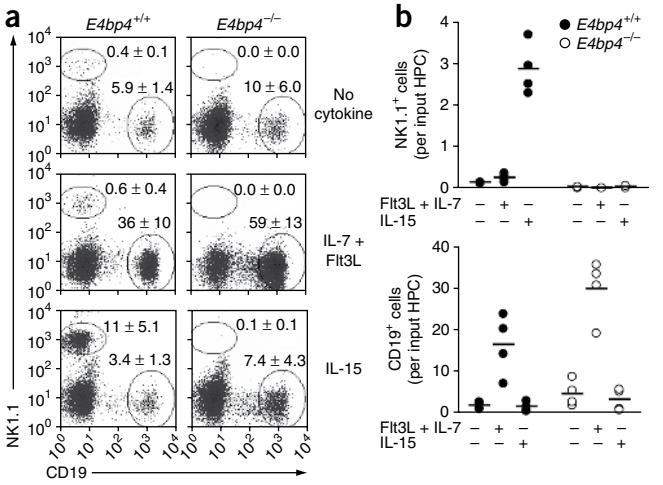


**Figure 5** IL-15-dependent NK cell production from *E4bp4*$^{-/-}$ bone marrow is impaired. (**a**) Flow cytometry of NK cell (NK1.1$^+$) and B cell (CD19$^+$) populations generated from *E4bp4*$^{+/+}$ and *E4bp4*$^{-/-}$ Lin$^-$ bone marrow on OP9–green fluorescent protein stroma in the presence or absence of exogenous cytokines (right margin). Numbers in plots indicate percent cells in outlined gates. (**b**) Frequency of cells in **a**; each symbol indicates an individual mouse; horizontal lines indicate the mean. Data are from three independent experiments (mean ± s.d.; $n = 4$ mice per genotype).

or HPCs from *Il15ra*$^{-/-}$ mice. Both antibody to IL-15 (anti-IL-15) and loss of *Il15ra* independently prevented the production of NK cells from HPCs (**Supplementary Fig. 10**). Notably, ectopic *E4bp4* expression enabled some NK cell production even in the presence of anti-IL-15 and the absence of *Il15ra*. Therefore, E4BP4 can function 'downstream' of IL-15 in NK development.

IL-15 is vital not only for NK cells but also for NKT cells and CD8$^+$ memory T cells, as indicated by the deficiency in these populations in *Il15*$^{-/-}$ and *Il15ra*$^{-/-}$ mice[8,9]. However, immunophenotypic analysis of *E4bp4*$^{-/-}$ mice showed they did not have fewer NKT cells (NK1.1$^+$CD3$^+$; **Supplementary Fig. 3**) or memory T cells (CD8$^+$CD25$^+$CD44$^{hi}$; **Supplementary Fig. 11**); in fact, both cell types were slightly greater in proportion in *E4bp4*$^{-/-}$ mice. *E4bp4*$^{-/-}$ mice challenged with influenza virus had an intact IL-15-dependent cytotoxic CD8$^+$ T cell response after restimulation (**Supplementary Fig. 12**). This result demonstrated that *E4bp4* is not ubiquitously required for IL-15-dependent cell functions and instead showed that this requirement is specific to the NK lineage.

*E4bp4* expression has been reported to be responsive to external stimuli in many cell types[21,23,28]. To investigate the effect of IL-15 on *E4bp4* expression in the NK lineage, we purified cells from bone marrow or spleen, incubated them for 3 h with or without IL-15 and quantified *E4bp4* expression by real-time PCR. Cytokine treatment increased *E4bp4* expression in NK, NKT and CD8$^+$ memory T cell populations (**Fig. 6d**) relative to the already high basal expression in each of these IL-15-dependent populations compared with that in Lin$^-$ bone marrow. However, *E4bp4* was not induced by IL-15 in NKPs (**Fig. 6d**); this may have been due to limited numbers of IL-15-responsive cells in this subset or to silencing of *E4bp4* expression.

## *E4bp4* and *Id2* promote NK cell development

Commitment to erythroid, myeloid and lymphoid lineages seems to be tightly regulated by a relatively small number of transcription factors[10]. However, no transcription factor had been identified thus far that determines commitment specifically to the NK lineage. Having found E4BP4 to be such a potential factor, we examined whether it might act through other genes known to influence lymphoid development. Real-time PCR analysis of gene expression in *E4bp4*$^{+/+}$ and *E4bp4*$^{-/-}$ bone marrow–derived HPCs showed that they had much lower *Gata3* and *Id2* expression (**Fig. 7a**), which suggested that these genes may be genetically downstream of *E4bp4*. To confirm our hypothesis, we transduced *E4bp4*$^{-/-}$ HPCs with *E4bp4*-expressing retrovirus, collected the cells after 48 h and did similar real-time PCR analysis. *E4bp4* overexpression substantially induced expression of both *Gata3* and *Id2* (**Fig. 7b**), which suggests that these genes are directly or indirectly transcriptionally regulated by E4BP4. *Id2* has been reported to be crucial in late-stage NK cell development[12]. Therefore, to assess whether *Id2* can function downstream of *E4bp4* in NK cell development, we transduced *E4bp4*$^{-/-}$ HPCs with retrovirus expressing *Id2* and monitored NK cell production. *Id2* was able to

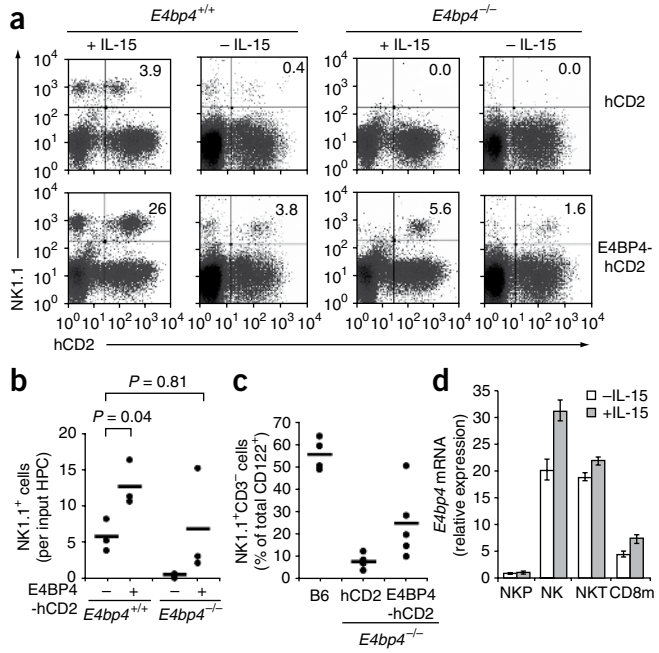


**Figure 6** Ectopic *E4bp4* expression restores *E4bp4*$^{-/-}$ NK cell development and substantially increases wild-type NK cell development. (**a**) Flow cytometry of NK cell development *in vitro* from *E4bp4*$^{+/+}$ and *E4bp4*$^{-/-}$ Lin$^-$ bone marrow transduced with control retrovirus (hCD2) or *E4bp4*-expressing retrovirus (E4BP4-hCD2) and incubated in the presence (+IL-15) or absence (–IL-15) of IL-15. Numbers in top right quadrants indicate percent NK1.1$^+$ NK cells in the hCD2$^+$ population. Data are representative of two independent experiments. (**b**) Total number of hCD2$^+$NK1.1$^+$ cells generated *in vitro* in the presence of IL-15, presented per hCD2$^+$ *E4bp4*$^{+/+}$ or *E4bp4*$^{-/-}$ progenitor input ($n = 3$ mice per condition). *P* values, unpaired Student's *t*-test. Data are representative of three experiments. (**c**) Frequency of NK cells relative to other CD122$^+$ populations in peripheral blood of C57BL/6 mice left untreated (B6) or lethally irradiated and transplanted with $2 \times 10^5$ radioprotective total bone marrow cells together with $2 \times 10^5$ *E4bp4*$^{-/-}$ bone marrow progenitors transduced with either control or *E4bp4*-expressing retrovirus, analyzed 4 weeks after transplantation ($n = 4$–6 mice per genotype). Data are representative of a single experiment. Each symbol indicates an individual mouse; horizontal lines indicate the mean (**b**,**c**). (**d**) Real-time PCR analysis of *E4bp4* expression in freshly isolated NKPs (NK1.1$^-$CD122$^+$), NK cells (NK1.1$^+$CD3$^-$), NKT cells (NK1.1$^+$CD3$^+$) and CD8$^+$ memory cells (CD3$^+$CD122$^+$; CD8m) cultured for 3 h in the presence or absence of IL-15 (30 ng/ml); results are presented relative to *Hprt1* expression. Data are representative of two experiments (mean ± s.d. of triplicates).

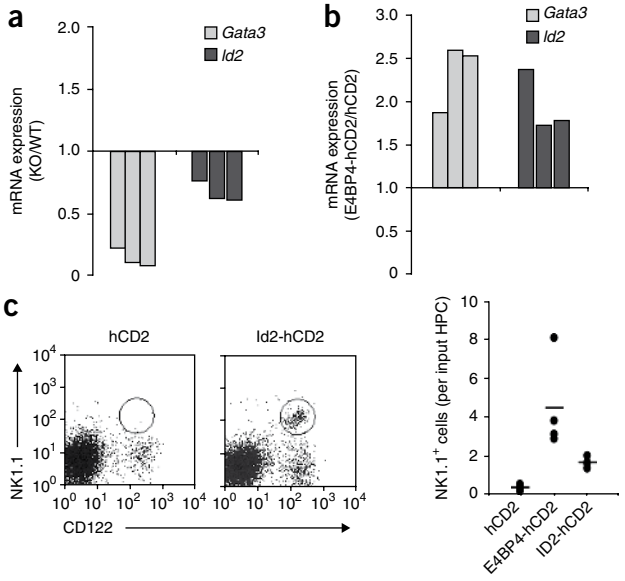


**Figure 7** *E4bp4* and *Id2* are in the genetic pathway regulating NK cell development. (**a**) Real-time PCR analysis of the expression of *Gata3* and *Id2* in *E4bp4*$^{-/-}$ Lin$^-$ bone marrow HPCs (KO), presented relative to expression in *E4bp4*$^{+/+}$ Lin$^-$ bone marrow HPCs (WT). Data are representative of three experiments (one per bar; $n = 3$ mice per genotype). (**b**) Real-time PCR analysis of the expression of *Gata3* and *Id2* in *E4bp4*$^{-/-}$ Lin$^-$ bone marrow HPCs 48 h after transduction of *E4bp4*-expressing retrovirus (E4BP4-hCD2), presented relative to expression in cells transduced with control retrovirus (hCD2). Data are representative of three experiments (one per bar; $n = 3$ mice per genotype). (**c**) Flow cytometry of NK cell development *in vitro* from *E4bp4*$^{-/-}$ Lin$^-$ bone marrow HPCs transduced with control retrovirus (hCD2) or *Id2*-expressing retrovirus (Id2-hCD2). Right, total number of NK cells produced per input HPC. Each symbol indicates an individual mouse; horizontal lines indicate the mean. Data are representative of three experiments ($n = 4$ mice per condition).

substantially restore the production of NK (NK1.1$^+$CD122$^+$) cells in the absence of *E4bp4* (**Fig. 7c**). These data indicate that *Id2* is downstream of *E4bp4* in the genetic pathway directing NK development.

## DISCUSSION

The transcriptional regulation of NK cell development is not well understood at present[6,34]. By generating mice lacking *E4bp4*, we have identified the first gene to our knowledge to specifically determine NK cell development from NKPs. We noted a block in NK production in the bone marrow of *E4bp4*$^{-/-}$ mice at the transition from NKP to iNK. The importance of *E4bp4* at this transition was strengthened by the observation that *E4bp4* expression was much higher in iNK cells than in NKPs. Although NK cell defects are also produced by deletion of genes encoding other transcription factor, such as Ets-1 (ref. 11), Id2 (ref. 12), GATA-3 (ref. 13), PU.1 (ref. 14), Mef[15], T-bet[16] and Irf-2 (ref. 17), none of these mutations result in a clearly delineated block so early in NK cell development. The only analogous block is found in *Il15*$^{-/-}$ mice that have NKPs in the bone marrow but have considerably fewer iNK cells[35]. *E4bp4* expression remains high throughout NK development, which suggests that it could also be essential for maintaining mNK cell function.

*E4bp4* has high expression in NK, NKT and CD8$^+$ memory T cells. However, only the NK cell population was deficient in *E4bp4*$^{-/-}$ mice. This observation suggests that *E4bp4* is critical for NK cells but not for other IL-15-dependent cell types, so E4BP4 function is probably influenced by additional cell type–specific factors. *E4bp4* induction by IL-15 is similar in NK cells, NKT cells and CD8$^+$ memory T cells but does not occur in the NKP population. It is possible that *E4bp4* is not inducible by IL-15 in NKPs because epigenetic marks render it transcriptionally inactive. Alternatively, IL-15 may induce *E4bp4* expression only in a subset of CD122$^+$NK1.1$^-$ cells.

We have shown here that *E4bp4* influenced the expression of *Gata3* and *Id2*, and published work has found that like *E4bp4*, both *Gata3* and *Id2* have moderate expression in NKP populations (defined as CD122$^+$NK1.1$^-$DX5$^-$) and higher expression later in NK development[36]. Such NKP populations show a 1-in-12 frequency of mNK cell production in OP9 cocultures[36]. These data collectively suggest that high *E4bp4* expression, together with the resulting high expression of *Gata3* and *Id2*, might define an IL-15-responsive 'committed NKP' subpopulation of CD122$^+$NK1.1$^-$ NKPs present in normal mouse bone marrow.

*Id2*$^{-/-}$ mice have fewer mNK cells, and this phenotype is directly related to the ability of Id2 to inhibit the function of E protein transcription factors such as E2A[12]. Our data have indicated that *Id2* is an effector of *E4bp4* function, and this suggests a position for E4BP4 at the top of the hierarchial genetic pathway controlling NK cell development. Loss of *E4bp4*-dependent *Id2* expression may also explain the greater B cell development from *E4bp4*$^{-/-}$ HPCs.

Higher expression of *E4bp4* resulted in the generation of more NK cells from HPCs. The ability to expand specific subpopulations of NK cells *ex vivo* or enhance NK cell numbers *in vivo* would be extremely powerful tools for the many potential applications of NK cells for immunotherapy[37]. The *E4bp4*$^{-/-}$ mouse is the first constitutive model to our knowledge reported to specifically lack NK cells and NK cell activity. Studies of the function of NK cells have relied on depletion with antibodies to NK1.1 or asialo-GM1. However, the expression of these markers on cells other than NK cells makes the interpretation of such experiments problematic[2]. The *E4bp4*$^{-/-}$ model should have great utility in definitively identifying the many pathologies attributed to NK cells[2] and discerning the function of NKT cells in an NK cell–free environment. The *E4bp4*-null allele is also likely to be of great value in creating compound-immunodeficient mice that now rely on the highly pleiotropic effect of *Il2rg* deletion to ablate NK cell function.

E4BP4 has been shown to regulate circadian gene expression and to be induced by light in the chick pineal gland, where it regulates the pineal clock gene *cPer2* (ref. 24). Several studies have shown that the degree of NK cell cytotoxicity is circadian in both rodents and human[38,39]. It is plausible that as E4BP4 is critical for NK development, it may also serve a central role in regulating the circadian nature of NK cell function.

## METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

*Note: Supplementary information is available on the Nature Immunology website.*

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### AUTHOR CONTRIBUTIONS

D.M.G., E.L., H.V.-F., J.d.B., O.W. and B.S. did experiments; D.M.G., O.W., M.C., D.K. and H.J.M.B. designed experiments; D.M.G. and H.J.M.B. wrote the manuscript; and H.J.M.B. directed the research.

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## ONLINE METHODS

**Generation of *E4bp4*$^{-/-}$ mice.** *E4bp4* genomic DNA spanning the coding exon (6.4 kilobases) was isolated from a 129/Sv bacteriophage P1–derived artificial chromosome. The targeting vector was generated by cloning of a neomycin-resistance gene flanked by two *lox*P sites into a ClaI site 3′ of the *E4bp4* DNA. An additional 1.9-kilobase fragment 3′ to the artificial chromosome–derived DNA was amplified by PCR from 129/Sv DNA and was directionally cloned 3′ of the neomycin-resistance cassette. The final vector contained an additional *lox*P site inserted at a SpeI site 2 kilobases upstream of the targeted exon. After electroporation and selection, correctly targeted clones were expanded, karyotyped and injected into wild-type C57BL/6 blastocysts to create chimeras. Presence of the *E4bp4*-null allele was assessed in offspring by Southern blot and PCR analysis and offspring were subsequently crossed to C57BL/6 background more than ten times. All experiments used 4- to 12-week-old mice from at least the sixth backcross.

**Mice.** All animal husbandry and experimental procedures were carried out according to UK Home Office regulations and institute guidelines. Male chimeras showing a high degree of chimerism were bred to C57BL/6 females. In male chimeras, recombination of the *lox*P sites was induced during spermatogenesis by protamine promoter–driven Cre recombinase present in PC3 embryonic stem cells (**Supplementary Fig. 1a**). The resulting litters yielded mice heterozygous for the targeted allele minus the neomycin-resistance gene. The mice were backcrossed onto the C57BL/6 background until the PC3 Cre transgene was lost. PCR genotyping was done routinely with primer 5′-CTCTGAGCTTGGCTGATGTG-3′ plus primer 5′-GCTTCAAGTCTCCACCAAGC-3′ (for amplification of the wild-type allele) or primer 5′-CCATGCTCCTGTCTTGATGA-3′ (for amplification of the null allele). Loss of expression was confirmed by immunoblot analysis of whole-cell lysates from heart tissue (prepared with radioimmunoprecipitation assay buffer) with anti-E4BP4 (C-18; Santa Cruz) and anti-β-actin (C-4; Santa Cruz) and by RT-PCR analysis of RNA (purified from total thymus with TRIzol (Invitrogen)) with the primers 5′-CGGAAGTTGCATCTCAGTCA-3′ and 5′-GCAAAGCTCTCCAACTCCAC-3′.

**Cell isolation and gene expression analysis.** Lin$^-$ HPCs were purified from bone marrow by magnetic depletion of cells stained with anti-Ter119 (TER119), anti-B220 (RA3-6B2), anti–mouse CD2 (RM2-5), anti-NK1.1 (PK136), anti-Gr-1 (RB6-8C5) and anti-CD11b (M1/70, all from eBioscience) on MACS columns (Miltenyi). For gene-expression analysis, cells were purified from 5- to 6-week-old adult female C57BL/6 mice by specific antibody staining followed by sorting on a MoFlo XDP (Beckman Coulter). Sorted populations (>97% pure) were immediately frozen in TRIzol (Invitrogen) for RNA isolation or were cultured for 3 h in RPMI medium plus 10% (vol/vol) FCS with or without IL-15 treatment before RNA isolation. For cDNA synthesis, 100 ng to 1 mg RNA was reverse-transcribed with SuperScript II (Invitrogen) and mRNA abundance was quantified by real-time PCR analysis on a 7900HT (Applied Biosystems) with probe sets recognizing *Pax5* (Mm00435501_m1), *Notch1* (Mm00435245_m1), *E4bp4* (*Nfil3*) (Mm00600292_s1), *Gata3* (Mm00484683_m1), *Id2* (Mm00711781_m1) and *Hprt1* (Mm00446968_m1; Applied Biosystems identifiers in parentheses).

**Flow cytometry.** Cells were preincubated with anti-Fc block (monoclonal antibody 2.4G2 to the FcγIII-II receptor; BD Biosciences) before being stained with conjugate antibodies to c-Kit, (2B8; BD Biosciences), KLRG1 (2F1, Abcam), NKG2D (R&D systems), CD19 (1D3), IgD (11-26), CD4 (RM4-5), CD8 (53-6.7), CD3 (145-2C11), NK1.1 (PK136), CD122 (5H4), NKp46 (29A1.4), NKG2AB6 (16a11) Ly49C/I/F/H (14B11), DX5 (DX5), CD11b (M1/70), Ly5.1 (A20), Ly5.2 (1O4), CD25 (PC61), CD44 (IM7) and human CD2 (RPA-2.10; all from eBioscience). IFN-γ production in bone marrow NK cells cultured *ex vivo* for 4 h with or without IL-12 (30 ng/ml) and IL-18 (20 ng/ml) was measured after permeabilization and staining with anti-IFN-γ (XMG1.2; eBioscience).

***In vivo* NK cell killing assay.** In experiments based on published protocols[40], $2 \times 10^8$ RMA and $2 \times 10^8$ RMA/s thymoma cells (cultured in Iscove's modified Dulbecco's medium supplemented with 10% FCS) were labeled with 16 ml CFSE (carboxyfluorescein diacetate, succinimidyl ester; Molecular Probes) at a concentration of 1 μM or 2.5 μM, respectively, and $4 \times 10^7$ cells total ($2 \times 10^7$ of each type) were injected intravenously into *E4bp4*$^{+/+}$, *E4bp4*$^{+/-}$ and *E4bp4*$^{-/-}$ recipients. After 16 h, recipients were killed, spleens were removed and CFSE positivity was determined in gated non-autofluorescent cell populations similar in size to those of input RMA and RMA/s cells.

**Adoptive cell transfer.** *E4bp4*$^{+/+}$ and *E4bp4*$^{-/-}$ recipients were irradiated with 600 rads and were transplanted intravenously with $1 \times 10^6$ bone marrow cells from C57BL/6 (Ly5.1$^+$) donors. C57BL/6 (Ly5.1$^+$) recipients were irradiated with two doses of 500 rads and were transplanted with $1 \times 10^6$ total bone marrow cells from *E4bp4*$^{+/+}$ or *E4bp4*$^{-/-}$ donors (Ly5.2$^+$) plus $1 \times 10^6$ total bone marrow competitor cells from C57BL/6 $F_1$ donors (Ly5.1$^+$ Ly 5.2$^+$). For *in vivo* 'rescue', wild-type recipients were irradiated with two doses of first 400 rads and then 500 rads and were transplanted with $2 \times 10^5$ total wild-type bone marrow cells plus $2 \times 10^5$ retrovirally transduced HPCs. Reconstitution of recipients was assessed by flow cytometry of blood at 4 weeks after transplantation.

***In vitro* lymphocyte development.** During retroviral transduction, HPCs were cultured for 4–5 d in DMEM supplemented with 10% (vol/vol) FCS (Stem Cell Technologies), Flt3L (10 ng/ml; R&D Systems), IL-7 (10 ng/ml; PeproTech) and stem cell factor (100 ng/ml; PeproTech)[31]. Cells were washed and then cultured ($3 \times 10^4$ cells per well) for 6 d on OP9 stromal cells in MEM-α medium containing 20% (vol/vol) FCS (Sigma) supplemented with either no cytokine, Flt3L plus IL-7, or IL-15 alone (30 ng/ml; PeproTech)[31]. Antibodies for blocking experiments were anti-IL-15 (500-P173; PeproTech) and normal rabbit IgG (500-P00; PeproTech), each used at a concentration of 10 μg/ml.

Retroviral transduction of progenitor cells was done as described[41]. The plasmid pMSCV-hCD2tailess-miR30 (pM2miR) was generated from pMSCV-neo (Clontech) and contains hCD2tailess fused to the miR30 micro-RNA[42]. Of three micro-RNA vectors designed, only pM2miR-1305 (5′-CCCGCACAAGCTTCGGATTAAA-3′) substantially diminished *E4bp4* expression. Total viable lymphocyte numbers were determined by Trypan blue exclusion microscopy when cells were plated onto stroma and at the end of the experiment. Proliferation of NK cells generated *in vitro* was determined by pulsing of cells with BrdU (5-bromodeoxyuridine) for 1 h before staining with anti-NK1.1 and anti-human CD2, followed by fixation, permeabilization and anti-BrdU staining according to the manufacturer's instructions (BD Biosciences).

**Statistical analyses.** An unpaired Student's *t*-test, with unequal variation assumed, was used for all statistical comparisons of means; *P* values greater than 0.05 were considered not significant.


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