SUPPLEMENTAL DATA

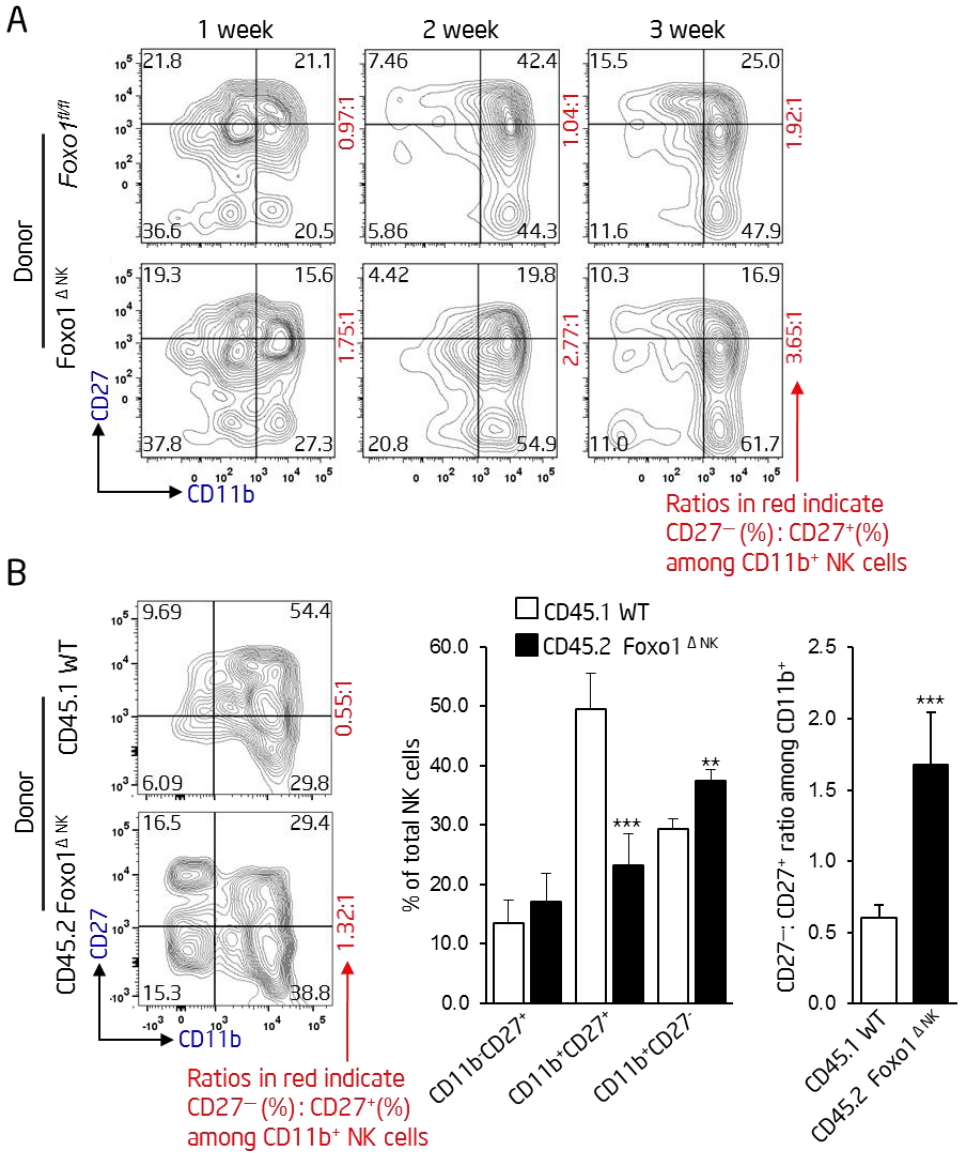


Figure S1, Related to Figure 2. Foxo1 inhibits late-stage maturation of NK cell development in a stem cell-intrinsic manner. (A) Lethally irradiated CD45.1 mice were reconstituted with 2.5 × 10^6 CD45.2 congenic *Foxo1*$^{fl/fl}$ (WT) or *Ncr1*^{iCre}*Foxo1*$^{fl/fl}$ (Foxo1$^{\Delta NK}$) mice bone marrow cells. Splenocytes were harvested at the indicated time points after reconstitution and stained with CD45.2, CD3, NK1.1, CD11b and CD27. (B) Non-irradiated *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice were reconstituted with a 1:1 ratio of bone marrow cells from CD45.1 wild-type (WT) and congenic CD45.2 Foxo1$^{\Delta NK}$ mice. Six weeks later, splenocytes were examined for CD45.1, CD45.2, CD3, NK1.1, CD11b and CD27 surface expression by flow cytometry. Left Panel: NK cells were gated as CD3$^-$NK1.1$^+$ to further determine CD11b and CD27 expression. Middle Panel: Summarized data for the left panel. Right Panel: Calculated ratios between CD27$^-$ versus CD27$^+$ cells among CD11b$^+$ NK cells based on data displayed in the middle panel. *$p < 0.05$ and ** $p < 0.01$ (Wilcoxon Rank Sum test, two-sided). Data shown are representative of two independent experiments with $n = 6$ mice per group. Error bars represent S.D.

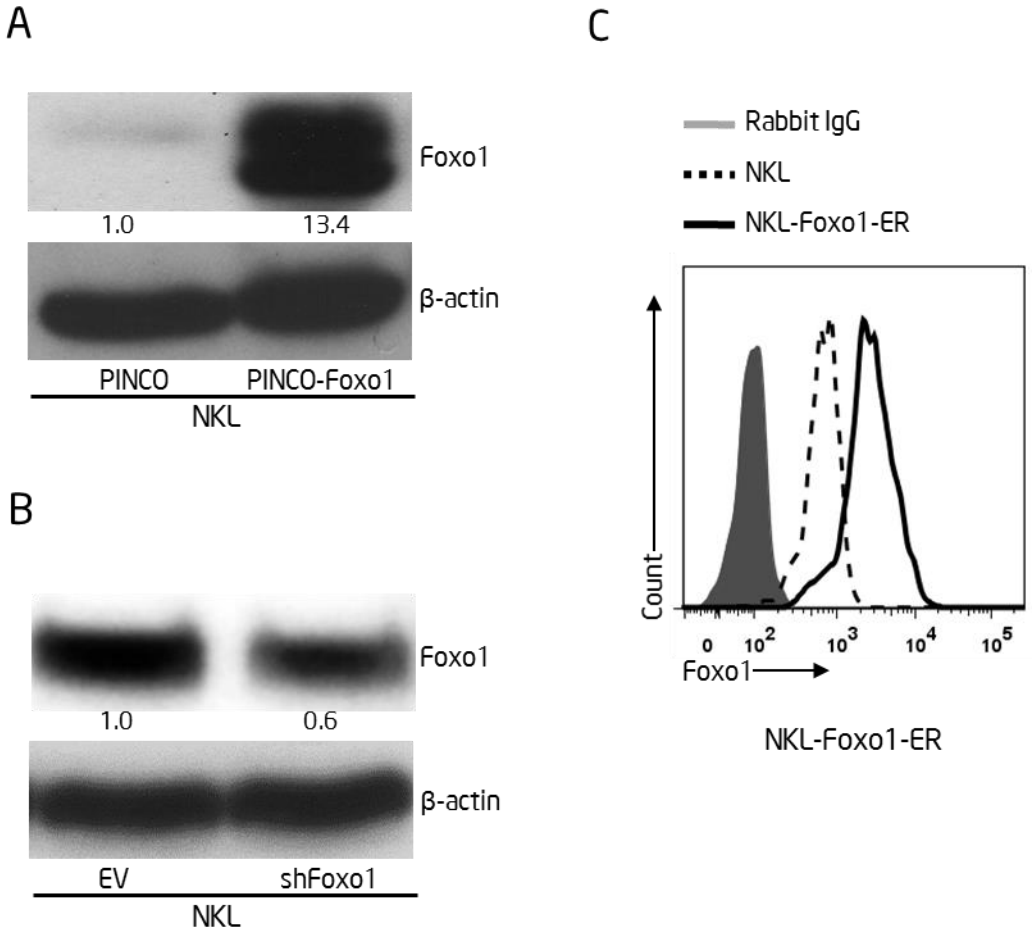


Figure S2, Related to Figures 3 and 4. Confirmation of Foxo1 over-expression and knock-down. (A) Assessment of Foxo1 protein expression, as determined by immunoblot, in NKL cells infected with either Foxo1 (PINCO-Foxo1) or the empty vector (PINCO). (B) Assessment of Foxo1 protein expression, as determined by immunoblot, in NKL cells infected with either Foxo1 shRNA (shFoxo1, knock-down) or the empty control vector (EV). Numbers under each lane represent quantification of Foxo1 via densitometry, after normalizing to β-actin (A, B). (C) Foxo1 protein expression in NKL cells expressing Foxo1-ER fusion protein (over-expression), determined by intracellular flow cytometry. Data shown in (A, B, C) are representative data of 2 independent experiments.

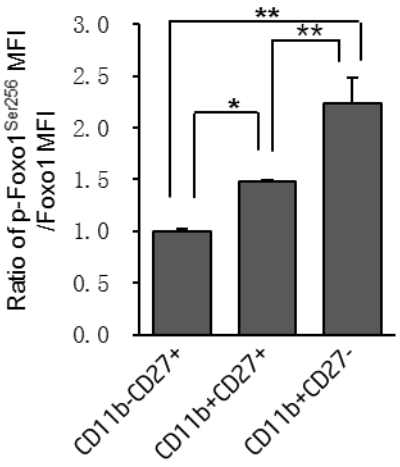
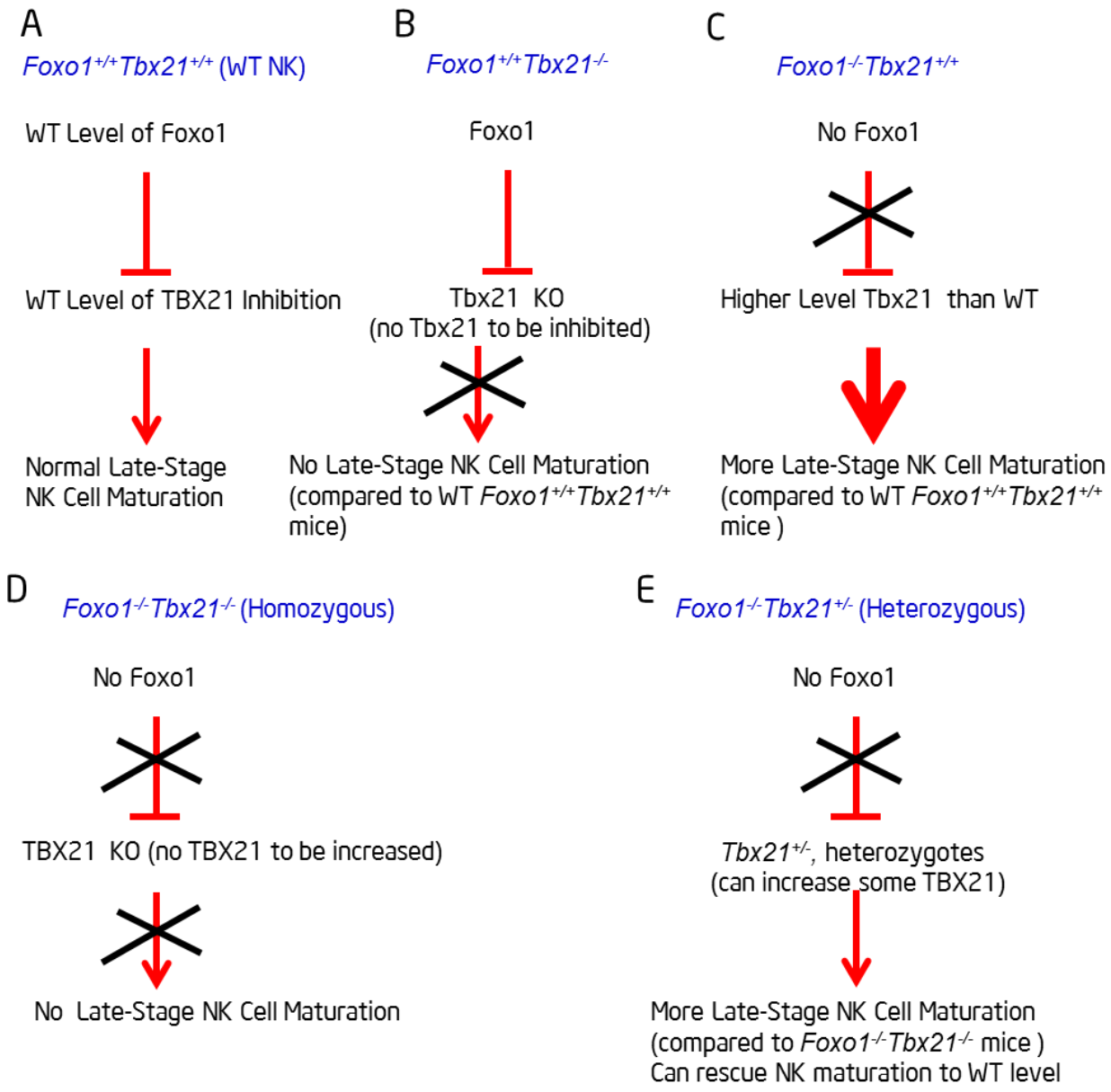


Figure S3, Related to Figure 5. The ratio of p-Foxo1^{ser256}/Foxo1 in developmental murine NK cells. CD11b^{-}CD27^{+}, CD11b^{+}CD27^{+}, and CD11b^{+}CD27^{-} mouse splenic NK cells, purified by FACS-cell sorting, were subjected to intracellular staining using p-Foxo1^{Ser256} and Foxo1 antibodies. The MFI ratios of p-Foxo1^{Ser256} to total Foxo1 are summarized. Data shown are representative of two independent experiments (for each experiment, n = 3 mice).



Note: For simplification, *Foxo1*$^{+/+}$ and *Foxo1*$^{-/-}$ are used to denote *Foxo1*$^{fl/fl}$ and *Ncr1*^{iCre}*Foxo1*$^{fl/fl}$ (Foxo1 $^{\Delta NK}$), respectively.

Figure S4, Related to Figure 7. Schemes to explain the hypothesis that Foxo1 acts upstream of Tbx21 to negatively regulate its expression.

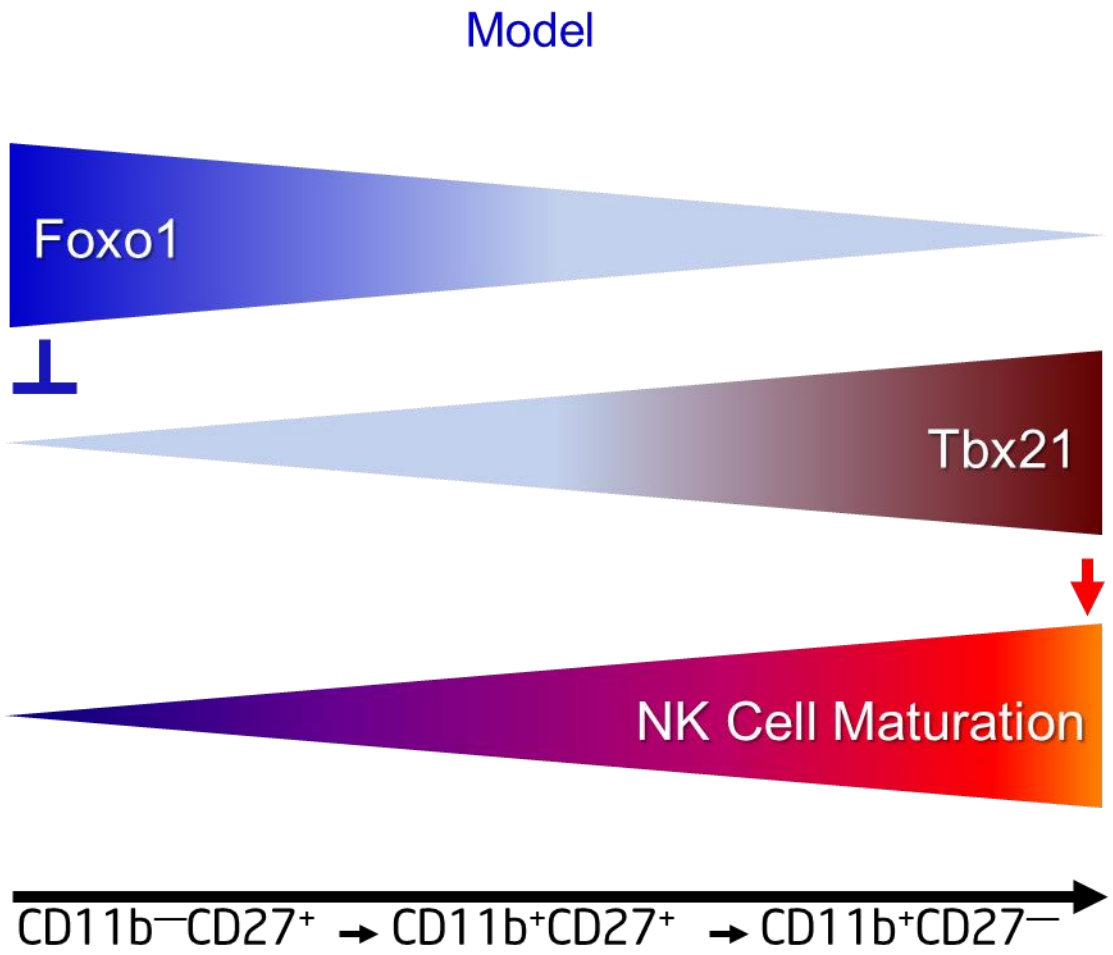


Figure S5, Related to Discussion. A model for regulation of NK cell development by Foxo1 and Tbx21. Foxo1 and Tbx21 play opposing roles in regulating NK cell development. Foxo1 plays a negative regulatory role, while Tbx21 plays a positive regulatory role. Foxo1 negatively regulates Tbx21 expression. During NK development, Foxo1 and Tbx21 expression inversely correlate with each other. Foxo1 starts at a high level while Tbx21 starts at a low level in immature NK cells. Along with NK maturation from $CD11b^{-}CD27^{+}$ to $CD11b^{+}CD27^{-}$, Foxo1 gradually diminishes and thus releases its negative control of Tbx21, leading to late-stage NK cell maturation.

SUPPLEMENTAL PROCEDURES

Primers used for chromatin immunoprecipitation (ChIP)

The primer pair used for detecting human *TBX21* promoter region was 5'-TCCATGACACCTTGTGGAGTG-3' (forward) and 5'-ACATGCTCTGAAGGGCTGCCT-3' (reverse). The primer pair for the mouse *Tbx21* promoter region was 5'-CAAGAGACTTACACTTAGGAG-3' (forward) and 5'-GACCAATGAAACTTCACTGGA-3' (reverse).

Flow cytometric analysis

Single-cell suspensions, obtained from the bone marrow, spleens, lymph nodes, blood, liver and lung tissues were used for surface staining first and then fixed and permeabilized for intracellular staining if necessary, as described previously (Yu et al., 2010). For intracellular (IC) staining of p-Foxo1^{ser256}, cells were fixed with IC fixation buffer and permeabilized with 1 mL ice-cold 90% methanol on ice for at least 30 minutes in the dark after surface staining. For cell sorting, mouse splenic NK cells were enriched using a mouse NK cell isolation kit (Miltenyibiotec) before sorting on a FACSAria II cell sorter (BD Biosciences). Antibodies were purchased from BD Biosciences or eBiosciences and were listed as follows: anti-CD3 (500A2, 145-2C11), anti-CD19 (1D3), anti-CD122 (TM-b1), anti-NK1.1 (PK136), anti-CD49b (DX5), anti-NKp46 (29A1.4), anti-CD62L (MEL-14), anti-CD27 (LG.3A10, or LG.7F9), anti-CD11b (M1/70), anti-CD43 (S7), anti-CD45.1 (A20), anti-CD45.2 (104), and anti-KLRG1 (2F1), anti-IFNγ (XMG1.2), anti-Tbx21 (4B10), anti-Foxo1 (C29H4), Foxo3 (75D8) and polyclonal anti-p-Foxo1^{ser256}.

In vitro assessment of NK function

For measuring human NK cell IFN-γ secretion, supernatant, from cultured NKL cells after stimulation with indicated cytokines for 18 hours, were harvested for ELISA analysis (Yu et al., 2006). Mouse NK cell IFN-γ production was determined by intracellular flow after

splenocytes were treated with murine IL-12 (25 ng/ml) and IL-18 (20 ng/ml), or PMA (1 μg/mL) and Ionomycin (200 ng/mL) (Sigma) for 4 hours in the presence of Brefeldin A (GolgiPlug, BD Biosciences). For analysis of natural cytotoxicity, FACS-sorted primary mouse NK cells or NKL cells were cultured at 37 °C with ^{51}Cr-labeled Yac-1 or K562 target cells (4×10^3), respectively, for 6 hours at indicated effector/target ratios in V-bottom 96-well plates. Release of ^{51}Cr into the supernatant was counted with a beta-counter.

Retroviral transduction and infection

Retroviral transduction and infection were performed as described previously (Yu et al., 2006). Briefly, human Foxo1 coding region was PCR-amplified from cDNA generated from the NK-92 cell line and was subcloned into a PINCO retroviral vector. For human Foxo1-specific shRNA, the previously reported annealed oligonucleotides (Amin and Schlissel, 2008) containing the sequence 5'-CCATGGACAACAACAGTAAAT-3' were cloned into the retroviral vector pRetroSUPER-neo-GFP (pSuper). The retroviral plasmid encoding human Foxo1-ER, a generous gift of Dr. Mark S Schlissel (University of California at Berkeley, Berkeley, CA), has been described previously (Amin and Schlissel, 2008). Retrovirus generation and infection was performed as previously described (Yu et al., 2006).

Quantitative real-time RT-PCR

Sorted total murine NK cells ($CD3^-NK1.1^+$) or $CD11b^-CD27^+$, $CD11b^+CD27^+$ and $CD11b^+CD27^-$ murine NK cell subsets or NKL cells were lysed with RLT lysis buffer, and then RNA was extracted with RNeasy Micro Kit or RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Extracted RNA was then reverse transcribed using M-MLV reverse transcriptase (Life Technologies) to generate initial cDNA for Real-time PCR according to the manufacturer's instructions. The primer pairs, used for SYBR Green real-time PCR, are available upon request.

Bone marrow chimeras

For the NK maturation kinetics study, bone marrow cells (2×10^6) from C57BL/6 CD45.2 congenic *Foxo1*$^{fl/fl}$ (WT) or *Ncr1*$^{iCre/+}$*Foxo1*$^{fl/fl}$ (Foxo1$^{\Delta NK}$) mice were adoptively transferred into lethally irradiated C57BL/6 CD45.1 mice. To determine whether the increase in mature NK cells in Foxo1$^{\Delta NK}$ mice was stem cell intrinsic or microenvironment-dependent, non-irradiated *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice were reconstituted with 2×10^6 mixed bone marrow cells at the 1:1 ratio from C57BL/6 CD45.1 WT and congenic age- and sex-matched CD45.2 Foxo1$^{\Delta NK}$ mice.

Immunoblotting

NK-92 cells were treated with IL-15 (100 ng/ml), IL-2 (150 U/ml), and IL-12 (10 ng/ml) or NKL cells expressing Foxo1 or Foxo1 shRNA or their corresponding empty vector were directly lysed in 2 × Laemmli buffer (Bio-Rad) supplemented with 2.5% β-mercaptoethonal, boiled for 5 min, and subjected to immunoblot analysis as described previously (Yu et al., 2010; Yu et al., 2006). Antibodies against Foxo1 (C29H4) and phospho (p) -Foxo1 (Ser256) were purchased from Cell Signaling. Immunoblotting with the antibody against β-actin (Santa Cruz Biotech) served as internal control.

SUPPLEMENTAL REFERENCES

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Yu, J., Mao, H.C., Wei, M., Hughes, T., Zhang, J., Park, I.K., Liu, S., McClory, S., Marcucci, G., Trotta, R., and Caligiuri, M.A. (2010). CD94 surface density identifies a functional intermediary between the CD56bright and CD56dim human NK-cell subsets. Blood *115*, 274-281.

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