Supplementary Figure 1

[Image of gel electrophoresis with bands labeled 2. 4Kb and 1. 8Kb]
Supplementary Figure 2
Supplementary Figure 3
Supplementary Figure 4

The figure shows a bar graph representing the relative luciferase activity for two different promoters, AP1-Luc and NF-κB-Luc, under various conditions.

- **P/I** indicates the presence (+) or absence (−) of a particular treatment or condition.
- **pSG5** and **pSG-Carabin** represent different constructs used in the experiment.
- **[CsA] (nM)** denotes the concentration of cyclosporine A used, with values ranging from 0 to 20 nM.

The graph includes error bars to indicate the standard deviation of the data. The relative luciferase activity is shown on the y-axis, ranging from 0 to 50.
Supplementary Figure 5.
Supplementary Figure 6

GTP/GDP

GDP

GTP

PMA (40nM)
Flag-Ras
HA-Carabin
HA-Carabin (R141A)
Supplementary Figure 7

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- Phospho-p38
- Anti-p38
- Phospho-JNK
- Anti-JNK
Supplementary Figure 8

- Anti-Carabin
- Anti-tubulin

0 4 6 12 12 (h)

+CsA
Supplementary Figure 9

- pFUP2-siCarabin
- pFUP2-siRL

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Supplementary Figure 10

![Graph showing Carabin mRNA levels over time of stimulation (h)]
Supplementary Figure 11

The figure shows the expression of IL-2 mRNA over time of stimulation for two different conditions: pFUP2-RL and pFUP2-simCarabin. The x-axis represents the time of stimulation in hours (0 to 6), and the y-axis represents the IL-2 mRNA relative level (0 to 40). The data points for each condition are connected by lines, indicating a time-dependent increase in IL-2 mRNA expression.
Supplementary Figure 12
Supplementary Figure 13
Supplementary Figure 14

**a** Thymus

**b** Spleen

**c** Peripheral lymph nodes

- **WT** vs. **CarabinKD**
- **TCR⁺**, **CD4⁺**, **CD8⁺**
- Cells (x 10⁶)
Supplementary Figure 15
Supplementary Figure 16
Supplementary Figure Legends

Supplementary Figure 1: Tissue distribution of human Carabin by Northern blot analysis.
Northern blot analysis was performed on a human multi-tissue Northern blot (Clontech). A $[^{32}\text{P}]$-labeled DNA fragment of the unique C-terminal region of Carabin cDNA was used as a probe to detect Carabin mRNA (top panel) with β-actin as loading control (bottom panel). The tissues are 1, Brain; 2, Heart; 3, Skeletal muscle; 4, Colon; 5, Thymus; 6, Spleen; 7, Kidney; 8, Liver; 9, Small intestine; 10, Placenta; 11, Lung; 12, Peripheral blood leukocyte.

Supplementary Figure 2: Inhibition of the interaction between Carabin and calcineurin by CsA, FK506 and W7.
The interaction between Carabin and calcineurin is detected in a mammalian two-hybrid assay as outlined in Supplementary Figure 1b. FK506 (1nM), CsA (10 μM) and W7 (25 μM) were added to the culture for half an hour before stimulation with PMA and ionomycin for another 6 h.

Supplementary Figure 3: Mapping of the minimal calcineurin-binding domain of Carabin using GST fusion protein pull-down. Different C-terminal fragments of Carabin were produced as GST fusion proteins used in the pull-down assay. Anti-GST antibodies were used to detect GST-Carabin.
Supplementary Figure 4: Inhibition of AP-1 and NF-κB luciferase reporter genes by Carabin and CsA. The experiment was carried out in the same way as that described in Supplementary Figure 2e. CsA was added to cells 30 min prior to stimulation.

Supplementary Figure 5: The Ras GAP activity of Carabin resides in the N-terminal domain. The N- and C-terminal domains of Carabin (Carabin-N/1-366 and Carabin-C/366-446) or full length of Carabin were transfected into Jurkat T cells. After a 24-h recovery, the cells were treated with CsA (10 µM) for 30 min or left it untreated, followed by stimulation with ionomycin (1 nM) for another 30 min. The protein expression levels of the full-length and Carabin-N and Carabin-C were comparable judging from Western blot (Data not shown). The immunoprecipitated complex was subjected to GAP activity assay in vitro. The amount of HA-Carabin used in this assay is equivalent to 1 µl in Supplementary Figure 3c.

Supplementary Figure 6: Carabin decreases the Ras-GTP level in vivo. The plasmid encoding Flag-tagged Ras was transfected either alone or with HA-Carabin or HA-Carabin(R141A) into Jurkat T cells. After 24 h, the cells were labeled with [32P]-orthophosphate for 4 h, followed by stimulation with 40 nM PMA for an additional 30 min. Flag-Ras was immunoprecipitated, and bound nucleotides were eluted and resolved on a cellulose plate. The protein expression levels were comparable between HA-Carabin and HA-Carabin(R141A) (data not shown). The ratios of GTP/GDP were calculated by the formula (GTP counts/3 divided by GDP/2) and are indicated at the top of each lane.
Supplementary Figure 7: Effect of Carabin on phosphorylation of p38 and JNK in Jurkat T cells upon stimulation with anti-CD3 and CD28. The experimental procedure is similar to that described for Supplementary Figure 3e.

Supplementary Figure 8: Induction of Carabin protein in human CD4+ naive T cells stimulated by anti-CD3 and CD28. Primary human CD4+ T cells were stimulated with a combination of anti-CD3 and anti-CD28 antibodies. Cells were harvested at different times (as indicated) after stimulation. Cell lysates were prepared and subjected to Western blot analysis using anti-Carabin antibodies.

Supplementary Figure 9: Knockdown of Carabin expression by siRNA as confirmed by Western blot. Lysates prepared from primary human CD4+ naive T cells transduced with viruses harboring either control pFUP2-siRL vector or pFUP2-siCarabin were subjected to Western blot analysis using anti-Carabin (top panel) or anti-tubulin (bottom panel) antibodies.

Supplementary Figure 10: Carabin mRNA expression in mouse primary CD4+ T cells. CD4+ T cells were purified from the spleen of Clone 6.5 transgenic mice. They were stimulated with HA peptide (10 µg/ml) and APC (at a ratio of 1:10 of T cells:APC) and harvested at the indicated time. Total RNA was prepared and subjected to real time quantitative RT-PCR. The levels of Carabin mRNA was normalized against that for β-actin.

Supplementary Figure 11: Enhancement of IL-2 mRNA expression in mouse primary CD4+ T cells upon knockdown of Carabin. CD4+ T cells were purified from spleen of Clone
6.5 mice, cultured in RPMI supplemented with 10% fetal bovine serum. Two rounds of transduction were conducted by adding concentrated supernatant of lentivirus for either control (pFUP2-RL) or murine Carabin knockdown (pFUP2-simCarabin) at an MOI of 5 in the presence of 8 µg/ml polybrene. The transduction and Carabin knockdown efficiency is more than 90%, as judged by GFP expression (FACS) and Carabin protein level (Western blot) 60 h after transduction. The remaining cells were stimulated with HA peptide/APC for indicated length of time before cells are harvested for analysis of IL-2 mRNA.

**Supplementary Figure 12: Knockdown of Carabin enhances accumulation of Ras-GTP in mouse primary CD4⁺ T cells.** Primary CD4⁺ T cells were transduced with the same procedure described in Supplementary Figure 8, followed by stimulation with HA peptide/APC for additional 12 h before they were harvested for GST-RBD pull-down assay.

**Supplementary Figure 13: Knockdown of Carabin enhances the dephosphorylation of NFAT upon stimulation with ionomycin.** The mouse primary CD4⁺ T cells were transduced with the same procedure described in Supplementary Figure 8. Cells were treated with 10 µM CsA for 15 min or carrier control, followed by stimulation with ionomycin at 0.5 or 2 µM for another 20 min. Cell lysates were prepared and subjected to SDS-PAGE followed by Western blot analysis using anti-NFATc2 and anti-tubulin antibodies.

**Supplementary Figure 14: Normal T cell development in CarabinKD BMT mice.** Numbers of thymocytes (a), total mature TCR⁺, CD4⁺ and CD8⁺ T cells in the spleen (b) and peripheral
lymph nodes (c) of RL-siRNA control group (open bars) and CarabinKD group (filled bars).
Data are means ± sd of results from 4 mice in each group.

**Supplementary Figure 15:** Knockdown of Carabin protein in GFP^+CD4^+ T cells isolated from CarabinKD cells. Hematopoietic stem progenitor cells (HSC) from 6.5 TCR transgenic mice (specific for HA + I-E^d^) were transduced with lentiviral vectors expressing GFP and either Carabin-siRNA or control RL-siRNA and then transplanted into lethally irradiated syngeneic mice. After reconstitution, GFP^+CD4^+ T cells were isolated by FACS. The cell lysates were prepared and subjected to Western Blot using anti-Carabin antibodies.

**Supplementary Figure 16: Enhanced proliferation of T cells upon Carabin knockdown.**
GFP^+CD4^+ T cells were stimulated under the indicated conditions, and the incorporation of [^3H]-thymidine (in cpm) was determined 2 days later.
SUPPLEMENTARY METHODS

**Plasmids and antibodies.** DNA fragments encoding different variants of Carabin were amplified by PCR and cloned into pSG5 (Statagene), pM, pVP16 (Clontech), or pGEX-6p-2 (Amersham Pharmacia) as indicated. The most optimal pFUP2-siCarabin (human) and pFUP2-simCarabin (mouse) construct was selected from several candidates tested and was made by inserting a shRNA targeting the Carabin cDNA sequences (5’-GGCCGACCGCTATGGATTCAT-3’)/human and 5’-GGATGGAGACCATTGGCAGAG-3’)/mouse as described16. All plasmid constructs were verified by sequencing. Plasmids encoding GFP-NFAT4 (1-460) and HA-NFATc2 were provided by Drs. Frank McKeon and Anjana Rao, respectively. pSG-Carabin(R141A) mutant was generated by site-directed mutagenesis using the Quickchange XL Kit (Stratagene). Antibodies were obtained from different suppliers: phospho-p44/p42 and Erk1/2 MAP kinase, Cell Signaling Inc; pan-Ras, GST-HRP, HA, Santa Cruz Biotechnology; calcineurin, PE-conjugated antibodies, anti-CD4, anti-CD8, anti-IL2 and PE-conjugated IgG, BD PharMingen. Clone 6.5 antibodies has been described previously25. Polyclonal anti-Carabin antibodies were generated at Genemed Synthesis Inc, CA.

**Mammalian two-hybrid assay for interaction between Carabin and calcineurin.** Jurkat T cells were transfected with 5 µg of pMCNβ2, 5 µg of a pVP-Carabin plasmid, 2 µg of pCMV-βGal, and 2 µg of pG5-Luc, a luciferase reporter plasmid containing four copies of GAL4 binding sites by electroporation. After 24 h of recovery, cells were stimulated with 10 nM PMA and 1 µM ionomycin, either alone or in combination, for 6 h before cells were harvested, lysed.
and the luciferase activity was measured for each sample. Where applied, FK506 (1 nM) was added to Jurkat cells 30 min prior to the stimulation.

**GST-Carabin pull-down assay.** Cell pellets from 50 ml of *E. coli* culture expressing GST-Carabin fragments were resuspended in 2 ml of a lysis buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 10% glycerol, 2 mM β-mercaptoethanol, 0.1% Nonidet P-40, 0.5 mM EDTA, 1 mM PMSF, and 1 mM benzamidine). Glutathione-Sepharose beads (0.2 ml) that had been equilibrated with lysis buffer were added to the soluble extract, and the mixtures were incubated for 1 h at 4 °C. Resin containing bound GST-Carabin fragment was washed three times with 1 ml of the lysis buffer and was incubated with an equal amount of calcineurin lysates for 2 h at 4 °C. After washing the beads three times with 1 ml of lysis buffer, the bound proteins were eluted with 2XSDS loading buffer, analyzed by SDS/PAGE, then probed with anti-calcineurin antibody.

**Fluorescent microscopy:** Jurkat T cells were transfected with a plasmid encoding GFP-NFAT4(1-460), together with either pSG-Carabin or pSG5 empty vector, and allowed to recover for 16 h. The cells were treated with 1 µM ionomycin for 15 min, spun onto coverslips coated with polylysine, followed by fixation with 4% para-formaldehyde. Mounting was performed by using Vectashield mounting medium (Vector Laboratories), and images were captured on a Zeiss LSM510 confocal microscope.
Northern Blot. Northern blot analysis was conducted using a human Multi-tissue Northern blot (Clontech), which was probed with [$^{32}$P]-labeled human Carabin cDNA fragment as per manufacturer’s instructions.

Calcineurin phosphatase assay in vitro. The phosphatase activity assay was carried out using the Biomol Green Calcineurin Assay kit (BIOMOL International LP, Plymouth Meeting, PA) using recombinant calcineurin and RII peptide substrate contained in the kit, as per manufacturer’s instructions.

Ras-GAP activity assay in vitro. In Vitro Ras-GAP activity was determined using one of the following two methods. Method I: 10 nM GST-Ras was preincubated with 50 nM [$\alpha^{32}$P]-GTP for 30 min at room temperature in Buffer A (50 mM Na-HEPES, pH 6.8, 1 mM ATP, 1 mg/ml BSA, 1 mM DTT and 5 mM MgCl$_2$). A concentrated stock solution of Carabin was added to the reaction mixture to final concentrations of 10, 20 and 50 nM, respectively, and incubation was continued for another 20 min. An aliquot of 10 µl was removed from the reaction mixture, mixed with 10 µl of 2% SDS-40 mM EDTA (pre-warmed to 65 °C), and incubated at 65 °C for 5 min. A 1-µl aliquot was spotted on a polyethyleneimine-cellulose thin-layer chromatography (TLC) plate. The TLC plate was developed with 1M LiCl. After drying, the plate was exposed to an X-ray film. Method II: GST-Ras was expressed in the bacterial strain BL21 and purified using glutathione Sepharose 4B beads. Purified recombinant GST-Ras was loaded with [$\gamma^{32}$P]-GTP before GAP assays. Briefly, 10 µg of GST-Ras protein bound on glutathione beads was incubated with 50 µCi of [$\gamma^{32}$P]-GTP in loading buffer (20 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1 mM DTT, 0.1 mg/ml BSA) in a total volume of 20 µl. The loading reaction was stopped by
addition of MgCl$_2$ to 10 mM. Then loaded GST-Ras was eluted using glutathione and stored temporarily on ice before use. HA-Carabin or HA-Carabin(R141A) was transfected to KEK293 cells or Jurkat T cells (where indicated) and immunoprecipitated from cell lysates using an anti-HA antibody. The immune complexes were washed three times with wash buffer (20 mM Tris-HCl, pH 7.4, 500 mM NaCl, 2 mM EDTA, and 1% NP-40) and twice with GAP assay wash buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM MgCl$_2$, 1 mM DTT). For the GAP assay, 0.5 µg of $[\gamma^{32}P]$-GTP loaded GST-Ras was mixed with Carabin or Carabin(R141A) immunoprecipitated from 1 x 10$^7$ HEK293 or Jurkat T cells transfected with the corresponding expression plasmids. Reactions were carried out in 40 µl of GAP assay buffer (20 mM Tris-HCl, pH 8.0, 10 mM MgCl$_2$, and 4 mM DTT) at room temperature for 20 min. The reaction was stopped by addition of 300 µl charcoal buffer (5% charcoal, 20 mM phosphoric acid, 0.6 M HCl). The mixture was vortexed for 10 min and centrifuged at 13,000 x g for 10 min. An aliquot of 100 µl supernatant was removed, and the $[^{32}P]$-Pi released was determined by scintillation counting.

**In Vivo loading of Ras with $[\gamma^{32}P]$-GTP.** For loading of Ras with $[\gamma^{32}P]$-GTP, Jurkat T cells were washed once with phosphate-free DMEM and resuspended (2 x 10$^6$ per ml) in the same medium containing dialyzed 10% fetal calf serum and $[^{32}P]$-orthophosphate (GEH) at 50 µCi/ml. After 4-h incubation at 37 °C, labeled cells (2 x 10$^7$) were lysed with labeling lysis buffer (1% Triton X-100, 50 mM HEPES at pH 7.4, 100 mM NaCl, 5 mM MgCl$_2$, 1 mg/ml BSA, 1 mM DTT, 1 mM PMSF, 10 µg/ml leupeptin, and 10 µg/ml aprotinin). Flag-tagged Ras was immunoprecipitated. The Ras-bound nucleotides were eluted with elution buffer (2 mM EDTA, 0.2% SDS, 1 mM GDP, and 1 mM GTP) at 68 °C for 20 min. The eluted nucleotides were
subjected to thin layer chromatography using PEI cellulose plates (Baker-flex) in 0.75 M KH$_2$PO$_4$ (pH 3.4).

**Lentivirus Production.** Recombinant lentiviruses were generated using a three-plasmid system as described previous (Pan, F., Ye, Z.H., Cheng, L. and Liu, J. O. “Myocyte Enhancer Factor 2 Mediates Calcium-Dependent Transcription of the Interleukin-2 Gene in T Lymphocytes” J. Biol. Chem., 279, 14477-14480 (2004)). Virus was harvested at 48 and 72 h after transfection and titer was determined based on percentages of GFP-positive Jurkat T cells after transduction with serially diluted viral supernatant. The titer, calculated as transducing unit (TU)/ml of supernatant, was from 2 x 10$^6$ to 8 x 10$^6$ TU/ml. The virus-containing supernatant was concentrated by ultracentrifugation and stored at -80°C.

**Animals.** BALB/c mice (6-8 weeks old) were purchased from the NCI. Clone 6.5 (hemagglutinin-specific CD4$^+$ TCR transgenic) mice were obtained from H. von Boehmer (Harvard University). Mice were bred and housed at Johns Hopkins Medical Institute (JHMI); they were maintained under protocols approved at JHMI.

**Real-time RT-PCR.** For real time quantitative RT-PCR, DNA-free RNA was extracted from cells with an RNeasy Minikit (Qiagen, Valencia, CA). And 1 µg of total RNA was reverse transcribed with a First Strand cDNA Synthesis kit (Fermentas, Hanover, MD). Real-time PCR was performed in triplicate with the iCycler iQ thermal cycler and detection system (Biorad, Hercules, CA) and the PCR core regents including specific primers for IL-2 and actin were from SuperArray, MD. Relative expression was normalized against levels of β-actin. When reverse
transcriptase was omitted, threshold cycle number increased by at least ten, signifying lack of genomic DNA contamination or non-specific amplification, and the generation of the amplification product with correct size was confirmed by agarose gel electrophoresis.

**Cytokine Production, intracellular cytokine staining and FACS analysis.** IL-2 was measured using OptiEIA cytokine ELISA kit according to the manufacture’s instructions (BD). For IL-2 intracellular staining, GFP⁺CD4⁺ T cells were stimulated with HA peptide and stained according to the manufacturer’s direction (BD). The stained cells were analyzed with FACSCalibur or were sorted on a VantageSE station (BD). Data were analyzed with CellQuest software.

**Mouse bone marrow harvesting, hematopoietic progenitor cells isolation, and transduction.** All samples used for BMT were enriched for lineage antigen-negative (Lin<sup>Neg</sup>) cells using the StemSep mouse progenitor enrichment kit (StemCell Technologies). The enriched Lin<sup>Neg</sup> cells were referred to as HSCs. This enrichment depletes >97% of lineage-specific cells. For transduction, these cells were cultured overnight in RPMI 1640 supplemented with 5% FBS. Two rounds of transduction were conducted by adding concentrated virus supernatant at an MOI of 5 in the presence of 8 µg/ml polybrene.

**BMT and analysis of transgene expression.** Three days after transduction, 10<sup>5</sup> transduced Lin<sup>Neg</sup> cells were collected and transplanted intravenously into lethally irradiated (850cGy) BALB/c mice.
**Isolation of Primary CD4^+ and GFP^+ CD4^+ T cells.** Lymph nodes and spleens were harvested from transgenic mice (Clone 6.5). The tissues were mashed and the RBCs were lysed with ACK buffer. Upon centrifugation, cell pellet was resuspended in 5% RPMI media. CD4^+ T cells were purified using Dynal Mouse CD4 Negative Isolation Kit (Dynal Biotech, Oslo, Norway). The transduced GFP^+CD4^+ T cells were further isolated by cell sorting.

**Cell proliferation assay.** 1x10^5 purified GFP^+CD4^+ T cells were cultured in flat bottom 96-well plates with syngeneic spleen cells (3 x 10^5, irradiated at 1500 rads), and different doses of HA peptide for 2 days. Proliferation of T cells was determined by incorporation of [³H]-thymidine for an additional 16 h prior to harvesting.