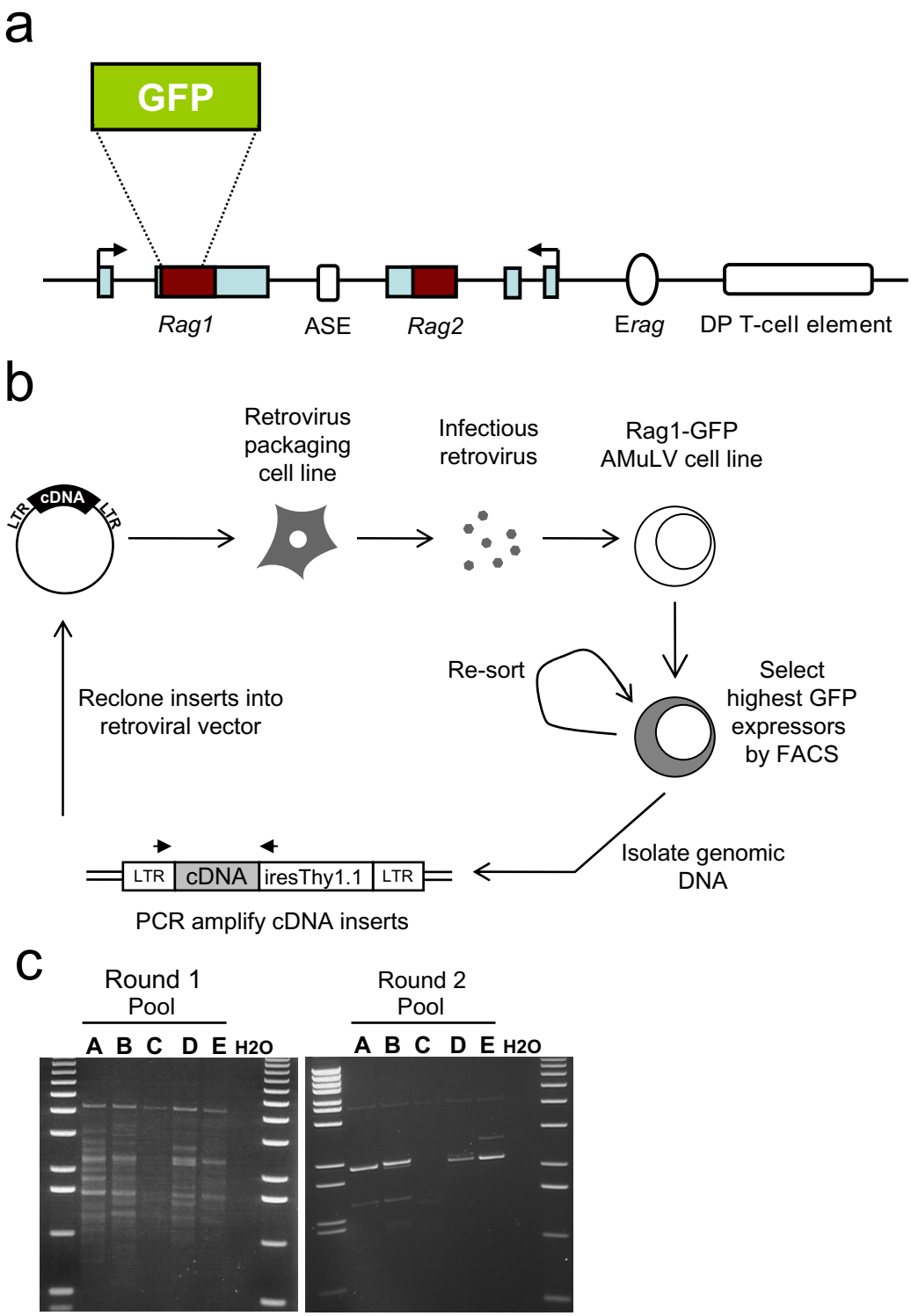
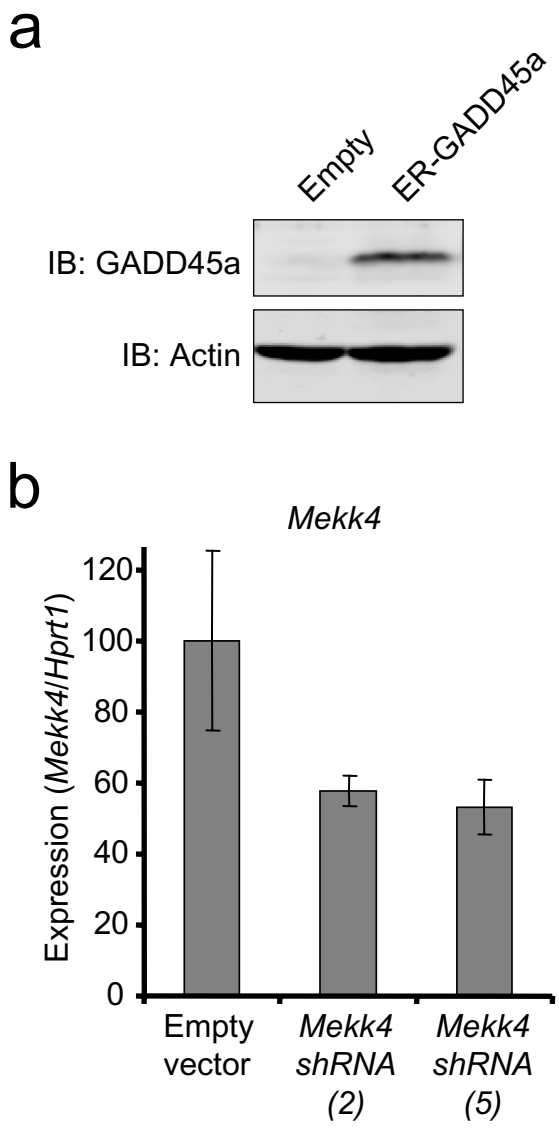
"Foxo1 directly regulates *RAG* transcription during B cell development" Amin and Schlissel 2008

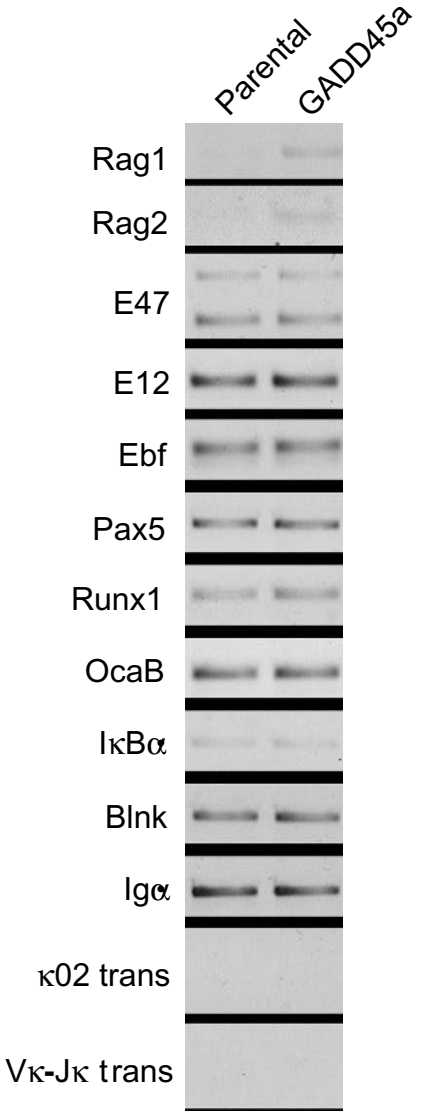


Supplementary Figure 1 AMuLV transformed *Rag1-gfp* B cells express GFP in place of *Rag1*, and can be used to screen for factors which activate the *Rag* locus. (**a**) Schematic of the *Rag* locus and the structure of the GFP knock-in mutation. *Rag1* and *Rag2* are encoded in a single locus and have convergent transcriptional orientations. Three known cis-acting elements are shown: the B-cell specific E*rag* enhancer and the double positive T cell specific silencer and anti-silencer pair (ASE). (**b**) Diagram of the cDNA library screen. (**c**) Size distribution analysis of PCR amplified retroviral cDNA inserts in genomic DNA from AMuLV transformed *Rag1-gfp* B cells infected with the retroviral cDNA library and subjected to either one or two rounds of selection as described in methods. As shown, the complexity of each pool is reduced with each round of selection.



Supplementary Figure 2 Expression of ER-GADD45a and knockdown of Mekk4 in AMuLV transformed *Rag1-gfp* B cells. (**a**) Immunoblot analysis of ER-GADD45a expression in empty-vector and ER-GADD45a-expressing virally infected *Rag1-gfp* cells. The Actin immunoblot serves as a loading control. (**b**) Quantitative PCR analysis of *Mekk4* message levels in sorted cells infected with either an empty vector control virus or a virus expressing the indicated shRNA. Values are normalized to *Hprt1* transcript levels and the expression level in empty vector infected cells was set to 1.

a



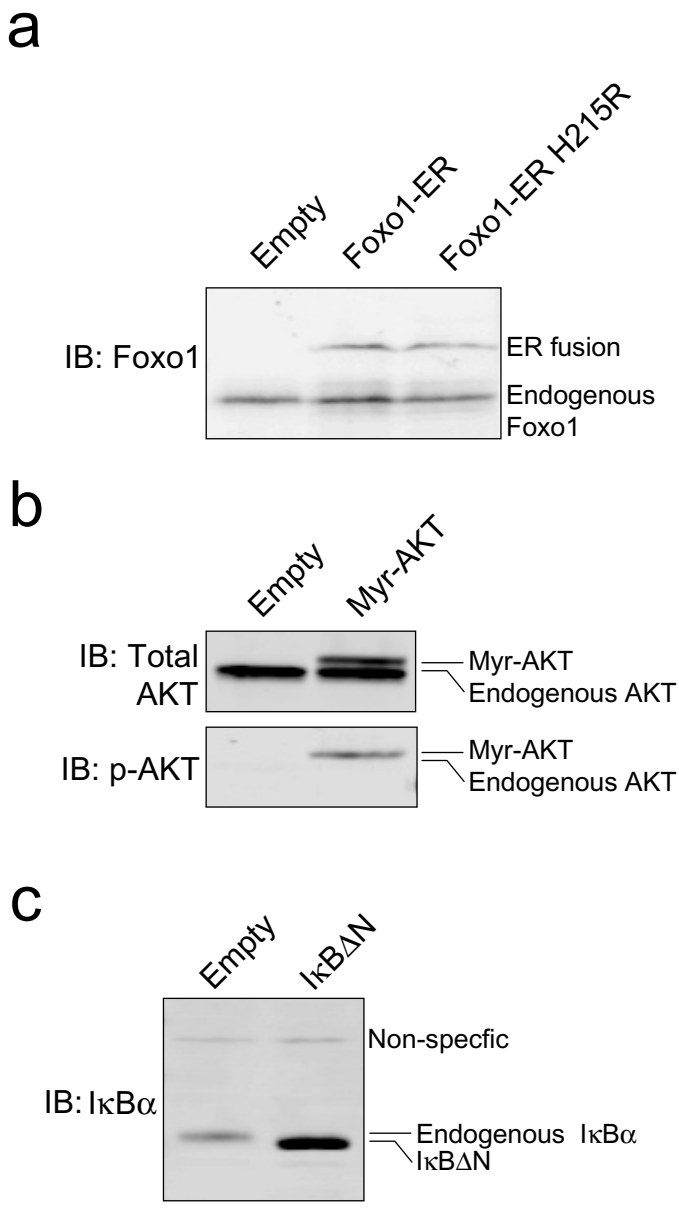
b

Average fold change	Gene
7.895716931	Rag1
6.643306238	CD36
5.687335453	similar to Ig kappa chain V-III
5.661030834	Gadd45a
5.609147533	solute carrier family 7
5.101933198	matrix metallopeptidase 13
4.748599724	immunoglobulin joining chain
4.54067799	activating transcription factor 3
4.180027333	cystathionase

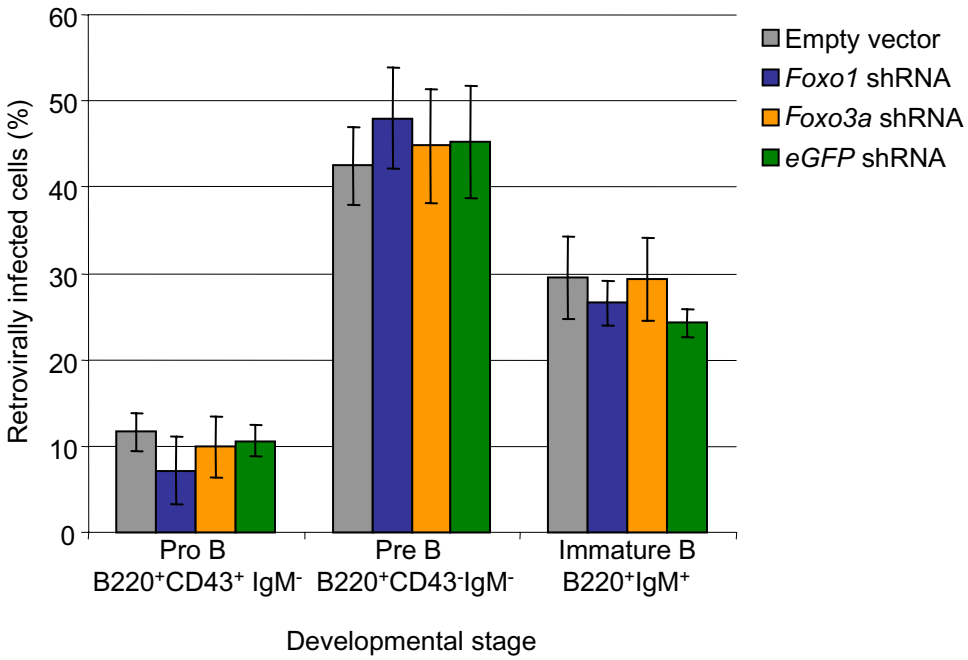
c

Average fold change	Gene
0.229877093	transglutaminase 2, C polypeptide
0.226891444	Apbb1
0.22211916	protein kinase C, theta
0.212489598	hepatocyte growth factor activator
0.194602479	dickkopf homolog 3 (Xenopus laevis)
0.183118203	COX1
0.174944943	Rab38, member of RAS oncogene family
0.169636487	H2-DMb2
0.058951318	MKK6

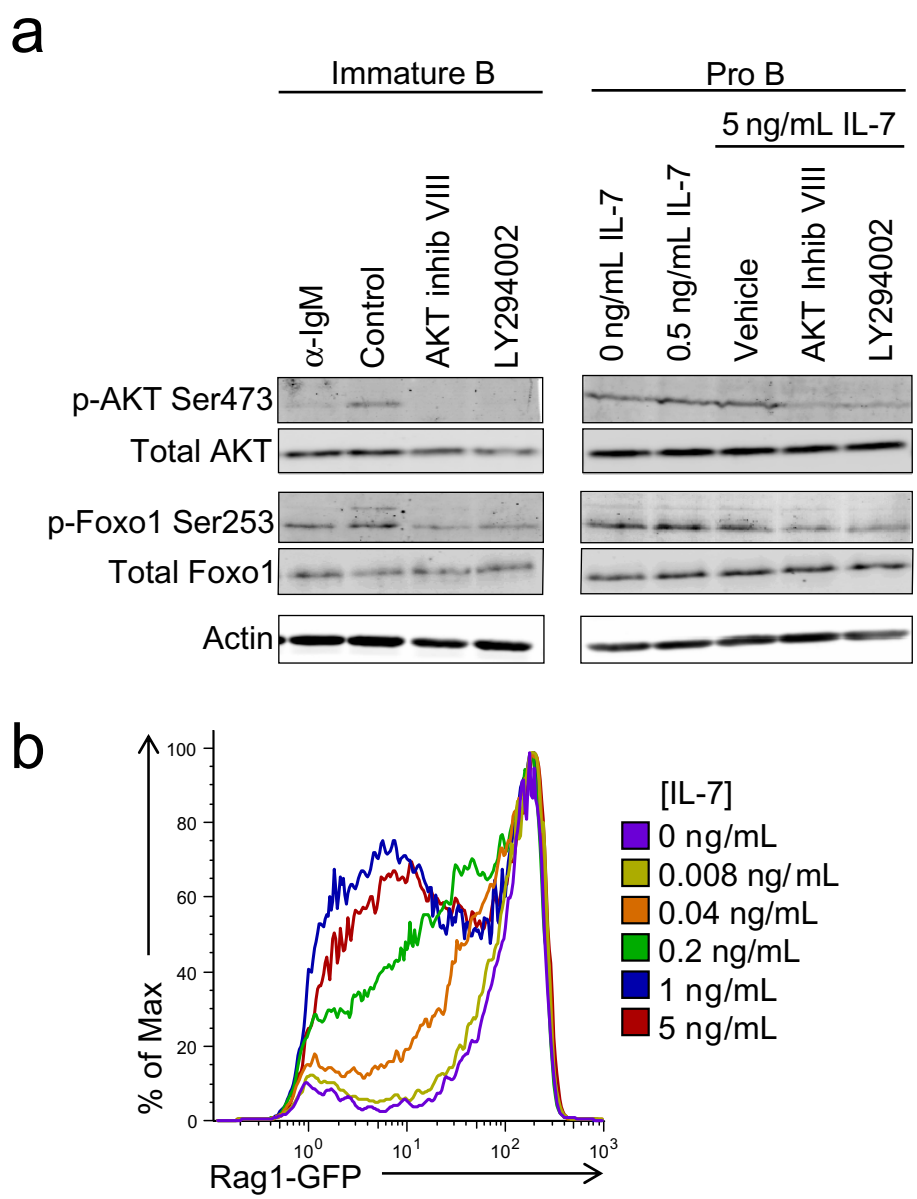
Supplementary Figure 3 GADD45a overexpression does not mimic v-Abl inhibition and alters expression of a small number of genes. (**a**) RT-PCR analysis of transcript amounts of the indicated genes in parental AMuLV transformed *Rag1-gfp* B cells or cells overexpressing GADD45a. The genes selected represent those known to be affected by v-Abl signaling in AMuLV transformed cells. (**b** and **c**) Genes whose transcript amounts were increased (b) or decreased (c) more than four-fold by GADD45a overexpression in AMuLV transformed B cells as judged by microarray hybridization and analysis.



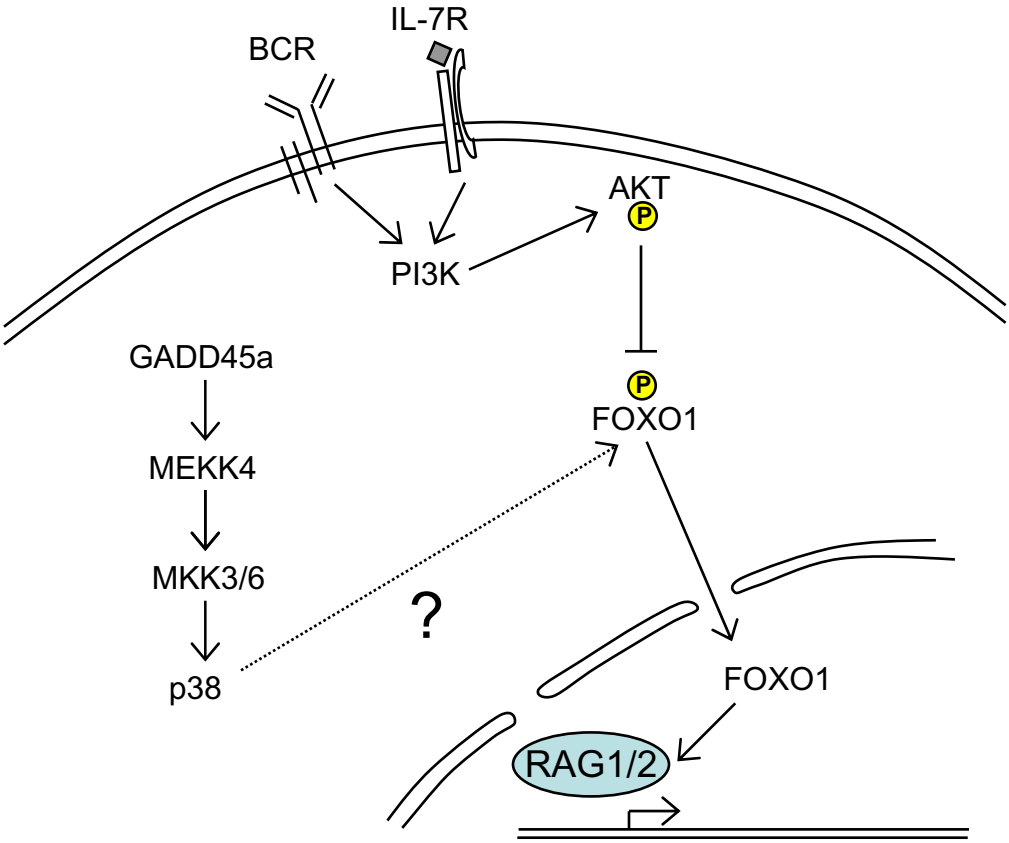
Supplementary Figure 4 Immunoblot analyses of protein overexpression in retrovirally-transduced cells. (**a**-**c**) AMuLV transformed *Rag1-gfp* B cells infected with the indicated protein encoding retroviruses compared to the same cells infected with the empty vector retrovirus. The target of the antibody used is shown at left of each blot. Where multiple bands exist, the identity of each protein species is indicated to the right. Non-specific bands or the endogenous proteins were used as loading controls. All blots are representative of at least two separate experiments.



Supplementary Figure 5 Reduction of Foxo1 or Foxo3a protein levels in primary cells does not grossly affect B cell development at the time points analyzed. Flow cytometric analyses of B cell subsets in cultured bone marrow infected with the indicated shRNA-expressing retroviruses as in Figure 5. Three to four days after retroviral infection, cells were harvested and labeled with antibodies to delineate B cell developmental subsets and mark retrovirally infected cells. Average percentages of pro B, pre B, and immature B cells (with the gating scheme shown at bottom) in the live cell gate in each infected population is given (average of the same three independent experiments as shown in Figure 5 (±s.d.)). The percentages do not add to one hundred due to non-B cells that are retrovirally infected.



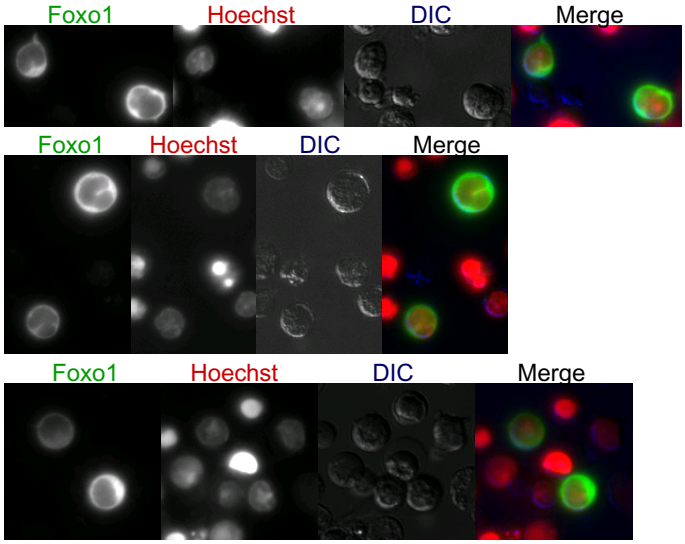
Supplementary Figure 6 IL-7R and BCR signaling repress *Rag* transcription through the PI(3)K-AKT signaling axis and phosphorylation of Foxo1. (**a**) Immunoblot analysis of total and phosphorylated Foxo1 and AKT in either immature B or pro B cells treated as indicated. Immature B cells from • HEL BCR transgenic mice were expanded in vitro and then treated with either anti-IgM antibody, control antibody, or the indicated PI(3)K or AKT chemical inhibitors for 8 hours before harvesting the cells for protein isolation. As shown, anti-IgM, LY294002, and AKT inhibitor VIII treatment reduce both AKT ser473 and Foxo1 ser253 phosphorylation in immature B cells. Pro B cells from wildtype C57BL6 mice were expanded in culture with IL-7 as above, washed and then cultured in the presence of the indicated concentration of IL-7 with or without the indicated PI(3)K or AKT inhibitors for 8 hours before harvesting the cells for protein isolation. Similar to immature cells, treatment with LY294002 or AKT inhibitor reduces AKT and Foxo1 phosphorylation in pro B cells. Reduction of IL-7 has a less dramatic effect, most likely due to the short time course of the withdrawal. Results are representative of two independent experiments. (**b**) IL-7 signaling represses *rag* transcription in pro B cells. Flow cytometric analysis of GFP expression in $B220^{+}CD43^{+}IgM^{-}$ primary pro-B cells from *Rag1-gfp* heterozygous mice cultured for five days in 2 ng/mL recombinant IL-7 and then transferred into various concentrations of IL-7 (shown as various colored lines) and cultured for an additional two days before analysis. Particular IL-7 concentrations are given on the right. All cultures were done on irradiated S17 stromal cells.



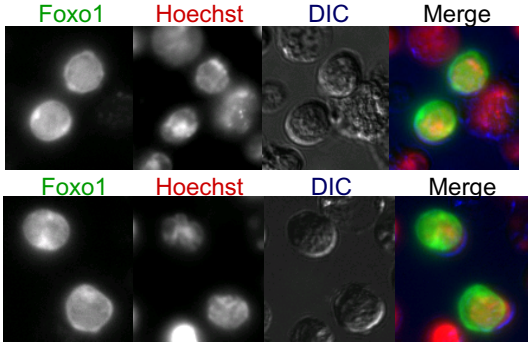
Supplementary Figure 7 Diagram of the signaling pathways considered in the main text. In primary cells, PI(3)K and AKT signaling downstream of the BCR or IL-7R leads to Foxo1 phosphorylation and cytoplasmic sequestration of the protein. In the absence of AKT activity, Foxo1 is dephosphorylated, translocates into the nucleus, and activates *Rag* transcription. In AMuLV transformed B cells, GADD45a activates a pathway ultimately leading to p38 phosphorylation. Activation of p38 positively influences Foxo1 activity by an unknown mechanism in these cells.

a

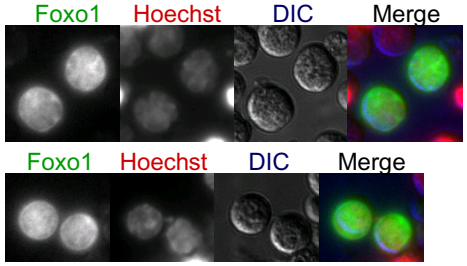
Control



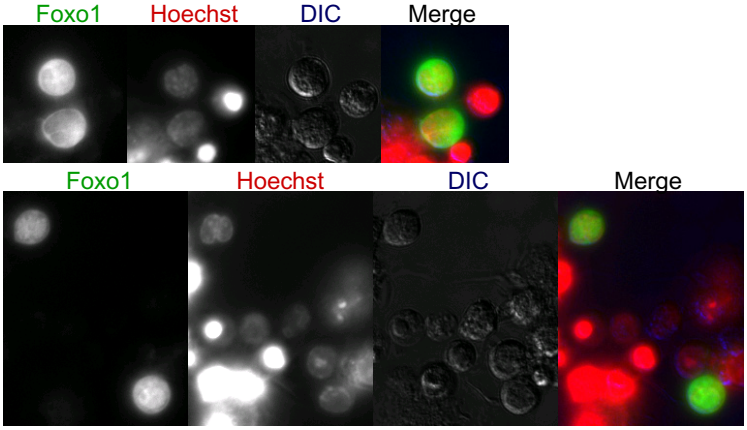
2 μM AKT Inhibitor VIII



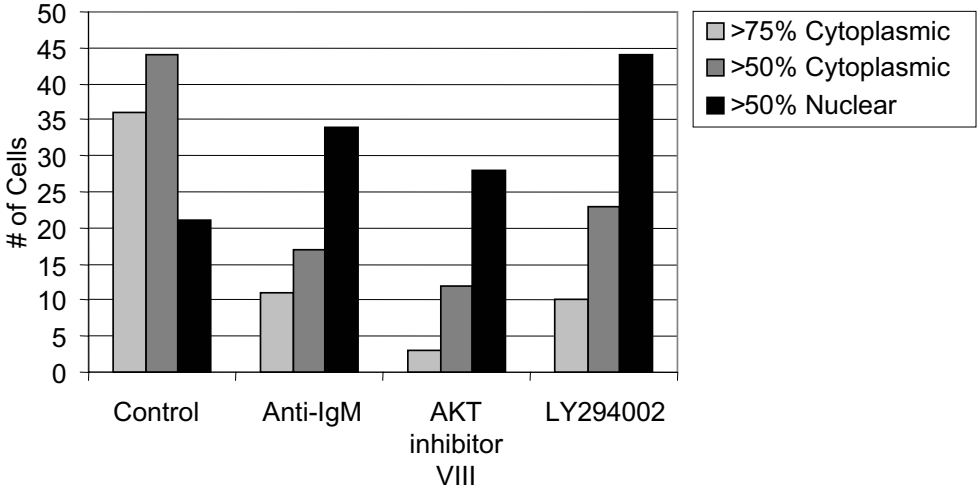
5 μM LY294002



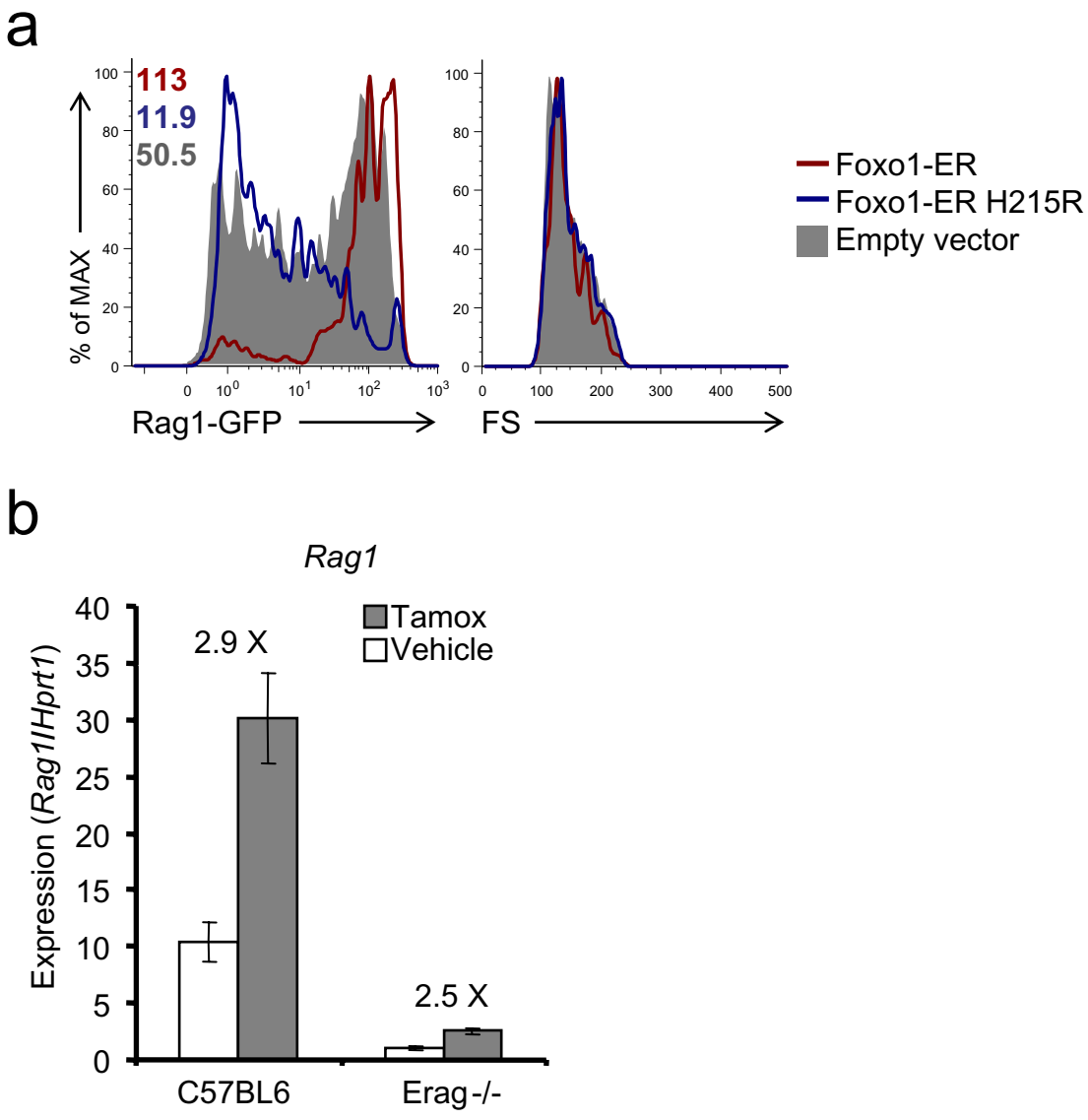
Anti-IgM



b



Supplementary Figure 8 Loss of tonic BCR signaling in immature B cells results in relocalization of Foxo1 from the cytoplasm to the nucleus. (**a**) Fluorescence analysis of YFP-tagged Foxo1 protein localization in immature B cells treated as indicated. Bone marrow from HEL BCR transgenic mice was infected with a *Foxo1-yfp* expressing retrovirus, cultured in vitro for four days and then treated with either a control antibody, anti-IgM antibody, or the indicated PI(3)K or AKT chemical inhibitors for 8 hours before addition of Hoechst nucleic acid stain and microscopic imaging. Several representative images are shown for each treatment. For each set of images Foxo1-YFP, Hoechst (which marks the nucleus), differential interference contrast (DIC) image, and a merge of all three channels are shown left to right. In the merge image green marks Foxo1-YFP, red marks Hoechst, and dark blue marks the DIC image. While Foxo1-YFP is clearly cytoplasmic in control cells, in cells treated with anti-IgM, LY294002, or AKT inhibitor VIII, Foxo1-YFP is diffusely localized throughout both the cytoplasm and nucleus. Because only a fraction of bone marrow cells are infected with the retrovirus, not all immature B cells express the Foxo1-YFP protein. (**b**) Quantification of Foxo1-YFP localization in immature B cells. Immature B cells expressing Foxo1-YFP were imaged as in (a) and the nuclear/cytoplasmic distribution of Foxo1-YFP fluorescence in each individual cell was measured. Cells in each distribution category were counted and plotted as shown. Results are from a single experiment.



Supplementary Figure 9 Foxo1-mediated regulation of *Rag* transcription requires DNA binding, but does not require the presence of the E*rag* enhancer. (**a**) Analysis of GFP expression and cell size in cultured B220+IgM– bone marrow B cells from *Rag1-gfp* heterozygous mice infected with either wildtype Foxo1, DNA binding defective Foxo1(H215R), or an empty vector control retrovirus. Three to four days after retroviral infection, cells were treated with tamoxifen for 18 hours before being harvested and labeled with antibodies to delineate B-cell developmental subsets and mark retrovirally infected cells. As shown, DNA binding defective Foxo1 does not increase GFP levels in pro or pre-B cells when compared to wildtype Foxo1, and neither protein affects cell cycling (as determined by forward scatter/cell size) at the time point analyzed. (**b**) Quantitative PCR analysis of *Rag1* mRNA levels in cultured hCD4+B220+IgM– bone marrow B cells from C57BL6 or E*rag*-/- mice infected with Foxo1-ER retrovirus and treated with either tamoxifen or a vehicle control for 15 hours. After treatment cells were harvested, labeled with appropriate antibodies, and sorted via flow cytometry for the given markers. Sorted cells were directly lysed in Trizol for RNA isolation.

Supplementary Methods:

Gene expression analysis by microarray. Two independent pools of AMuLV transformed *Rag1-gfp* B cells were infected with GADD45a expressing retrovirus and subsequently sorted into Thy1.1$^+$ and Thy1.1$^-$ populations via FACS. Both populations were grown simultaneously and at equal densities for an additional two days after sorting. 25 million cells from each population were processed to isolate total RNA using RNeasy Mini kit (Qiagen) according to manufacturer's protocol. RNA was transformed into double stranded cDNA according to protocols from NimbleGen Systems. Double stranded cDNA was then sent to NimbleGen Systems Inc. for fluorophore labeling and array hybridization. Samples were hybridized to the Nimblegen Mus musculus MM8 60mer expr (1-plex) array. Initial data acquisition and normalization was also performed by Nimblegen Systems Inc. Gene expression differences were calculated based on the average of the two independent sorts, comparing Thy1.1$^+$ to Thy1.1$^-$ cell populations. Genes for which the fold change in expression was greater than 2-fold different between the two sorts were thrown out.

Immunoblot Immunoblots were performed as described in the main text. AKT (cat #9272), phosho-AKT ser473 (cat #4058), and phospho-Foxo1 ser253 (cat #9461) antibodies were purchased from Cell Signaling Technologies. IκBα (cat #sc-371) and GADD45a (cat #H-165) antibodies were purchased from Santa Cruz Biotechnology Inc.

Anti-HEL BCR transgenic mice were purchased from Jackson Laboratories. Bone marrow from anti-HEL BCR transgenic mice was cultured in vitro in the presence of 2ng/mL recombinant IL-7 for 5 days as described in the main text. Cells were then harvested and washed extensively and placed again into culture for an additional 1-2 days to induce differentiation (>90-95% of cells were B220$^+$IgM$^+$IgD$^-$ immature B cells as judged by flow cytometry). Eight hours prior to harvesting, cells were treated with either 10 μg/mL anti-mouse IgM, control antibody, 5 μM LY294002, or 2 μM AKT inhibitor VIII. Cells were then harvested and processed for immunoblot. Pro B cells from C57BL6 mice were isolated and expanded in 2 ng/mL recombinant IL-7 for five days as described in the text (>80% of cells were B220$^+$IgM$^-$ by flow cytometry). Cells were then harvested, washed extensively, and cultured for an

additional eight hours in 0, 0.5, or 5 ng/mL IL-7 or 5 ng/mL IL-7 with 5 μM LY294002 or 2 μM AKT inhibitor VIII before harvesting for immunoblot.

Imaging analysis of Foxo1 cellular localization. Bone marrow from anti-HEL BCR transgenic mice was infected with retrovirus expressing a Foxo1-YFP fusion protein as described in the text. Infected bone marrow was expanded in vitro for 5 days in 2 ng/mL IL-7, and then harvested, washed and replated in the absence of IL-7 in wells of a chamber mounted coverglass. Cells were cultured for an additional 24 hours and then treated with either 10μg/mL anti-mouse IgM, control antibody, 5 μM LY294002, or 2 μM AKT inhibitor VIII for eight hours prior to microscopic imaging. Twenty minutes prior to imaging Hoechst nucleic acid stain was added to a final concentration of 0.2 μg/mL. Imaging was done directly on live cells in the chamber mounted coverglass using a Nikon TE2000 inverted fluorescent microscope fitted with a 37°C, 5% CO_2 controlled incubator. Foxo1-YFP localization in immature B cells was quantified by drawing bounding shapes around both the nucleus and the whole cell using the Hoechst stain and DIC images as guides. The total Foxo1-YFP fluorescence was then measured digitally using Nikon NIS-Elements AR imaging software by integrating the fluorescence intensity over these shapes. The amount of fluorescence in the cytoplasm was calculated by subtracting nuclear fluorescence from total cellular fluorescence.

Bioinformatics. Vista (http://genome.lbl.gov/vista/index.shtml) is a suite of online bioinformatics tools. The whole genome rVista program described in the text is one of these tools. The particular pre-computed alignment that was used is the Human March 2006 (hg18) genome assembly aligned with the Mouse February 2006 (mm8) assembly. The following genes were input into the program: *Rag1*, *Cd36*, *Slc7a3*, *Mmp13*, *Igj*, *Atf3,* and *Cth.*

Supplementary Table 1 FOXO binding sites are statistically overrepresented among the set of genes induced by GADD45a overexpression.

Name	Number of hits in the submitted regions	Total number of hits on genome hits on genome	-LOG10(p-value)
FOXO1	32	43570	5.6928
FOXO4	27	34073	5.4966
GATA3	27	40302	4.2495
GATA2	16	17575	4.2032
GATA	17	20534	3.9339
E2	9	7487	3.4547
VBP	14	16840	3.3689
NKX25	15	19198	3.29
POU1F1	23	40305	2.8003
TEF	18	29679	2.5896
CART1	15	23736	2.4125

Supplementary Table 2 Primers used in this study.

Primer	Sequence (5' to 3')
5’ pLIB	AGCCCTCACTCCTTCTCTAG
3’ pLIB	CGGGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG
RAG1 sense	CATTCTAGCACTCTGGCCGG
RAG1 anti-sense	TCATCGGGTGCAGAACTGAA
RAG1 probe	FAM-AAGGTAGCTTAGCCAACATGGCTGCCTC-TAMRA
RAG2 sense	TTAATTCCTGGCTTGGCCG
RAG2 anti-sense	TTCCTGCTTGTGGATGTGAAAT
RAG2 probe	FAM-AGGGATAAGCAGCCCCTC TGGCC-TAMRA
HPRT sense	CTGGTGAAAAGGACCTCTCG
HPRT anti-sense	TGAAGTACTCATTATAGTCAAGGGCA
HPRT probe	FAM-TGTTGGATACAGGCCAGACTTTGTTGGAT-TAMRA
E2A common for	GGGGAAGCCATCCTGAGGAGG
E47 specific rev	CGGCGCTCCTTCTCCCGCTCC
E12 specific rev	GGGACAGCACCTCATCTGTACTG
EBF cDNA for	GTTTGTCCACAATAACTCCAAGC
EBF cDNA rev	TGGTACCGAATATGACCTGTAAC
PAX5 for	GTCCCAGCTTCCAGTCACAG
PAX5 rev	AATAGGGTAGGACTGTGGGCC
RUNX1 for	CGGCCCCCGAGAACC
RUNX1 rev	ATGGATCCCAGGTACTGGTAGGA
OCAB for	CAACATCCTGTCACAAGCCATGCTC
OCAB rev	GAGAGCCAGCCAGCACATAATGTTC
IκBα for	GCAATCATCCACGAAGAGAAGC
IκBα rev	CGTTGACATCAGCACCCAAAG
BLNK 5’	CACCCCCCTGGACAGCGACACATC
BLNK 3’	CTGGGCTTACTGGGAAGTGTCTTGCTG
Igα 5’	TCATACGCCTGTTTGGGTCCC
Igα 3’	CCCTCATAGAGATTTTCATCTTCA
K02 for	GGAAAGGACTTGGCTTGTGC
Vκ degenerate For	GCTGCAGSTTCAGTGGCAGTGGRTCWGGRAC
Cκ rev	GTCCTGATCAGTCCAACTGTTCAGG

Supplementary Table 3 Sequences of shRNA targeting sequences described in the main text.

Target	shRNA Targeting Sequence
Gfp	GCACAAGCTGGAGTACAACTA
Mekk4 (2)	GCCGATCGAACTTGAAAGAAA
Mekk4 (5)	CGACTTCATAAGCTTATGAAT
Foxo1	CCATGGACAACAACAGTAAAT
Foxo3a	CCTTTCCCTAGCACACTTAAA
Foxo4	CCTCAGGATCTGGATCTTGAT