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Supplemental Material

Myc Targets Cks1 to Provoke the Suppression of $p27^{Kip1}$, Proliferation and Lymphomagenesis

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Supplementary Online Materials

Supplemental Materials and methods

RNA preparation and analyses

RNA was prepared from cultured MEFs or MACS-sorted B cells using the RNeasy kit (Qiagen). For Affymetrix analyses, cRNA was synthesized using the One-Cycle Target Labeling and Control Reagent package (Affymetrix) and the reaction was probed to the 430A mouse Affymetrix chip. The scanned data output was imported into the Spotfire software (Spotfire). Following normalization, selected probe sets for genes indicated in Figure 1A were clustered using the Hierarchical Clustering function of Spotfire. Statistical analysis was performed in Excel (Student's t-test). For real-time PCR, cDNA was prepared from 1 μg RNA using the iScript cDNA synthesis kit (Bio-Rad). Real-time PCR was performed using an iCycler machine and the iTaq SYBR green kit (Bio-Rad). Data analyses were performed by comparing Ct values with a control sample set as 1. Sequences for primers are available upon request.

Immunoblotting

Protein extracts (20 or 50 μg per lane) were electrophoretically separated on a SDS-PAGE gel, transferred to membranes (Protran) and blotted with antibodies specific for Cks1, Skp2, and Rbx1 (Zymed Laboratories), $p27^{Kip1}$ (BD Biosciences Transduction Laboratories), E1 (Upstate), Cul1 and $p19^{Skp1}$ (BioSource), c-Myc (Santa Cruz) and β-Actin (Sigma).

Pulse-Chase/Immunoprecipitation

Primary early passage wild type and *Cks1*$^{-/-}$ MEFs were infected with either MSCV-Myc-ER^{TAM}-IRES-Puro (*Myc*-ER^{TAM}) virus or MSCV-IRES-Puro (Vector) control virus. Puromycin-resistant

cells were then plated at 500,000 cells per 10cm dish. The following day, the media was exchanged for media that contained 2μM 4-HT and all subsequent media contained 4-HT. After 12 hours in 2μM 4-HT, the cells were incubated in methionine-free media for 15 minutes and then 65μCi of S^{35}-Translabel™/ml was added to the media and incubated for 1 hour. Cells were then washed 3 times with pre-warmed complete media and incubated for the indicated times. For the production experiments, there was no subsequent wash and cells were incubated with 65μCi of S^{35}--Translabel™ per milliliter for the indicated times. Cells were then scraped, washed 3 times with PBS, and lysed with sonication in RIPA buffer containing protease inhibitors. Cell lysates from equal numbers of cells were then incubated with 7 μg of affinity-purified rabbit anti-Cks1 antibody (Zymed) bound to MagnaBind™ (Pierce) Protein-A magnetic beads for 16 hours at 4ºC. Immunoprecipitates were then washed 3 times with RIPA buffer containing protease inhibitors and proteins were then eluted in 50μl of Laemmli buffer. Samples were separated on an SDS-PAGE gel, fixed, incubated with the fluorographic substrate Amplify™ (Amersham), then dried and exposed to film. Quantification of the band intensity was performed using Quantity One™ (BioRad).

Histology and immunohistochemistry

Full necropsy was performed on 6 *Cks1*$^{+/+}$ and 9 *Cks1*$^{-/-}$ Eμ-*Myc* transgenic mice suffering from palpable or visible lymphoma. Slides of 5-6 μm sections cut from formalin-fixed paraffin-embedded tissues were deparaffinized. Immunohistochemistry assays were performed using the DAKO autostainer following the manufacturer's instructions. The reagents used were: rat anti-mouse CD45R/B220 (Pharmingen), murine anti-mouse $p27^{Kip1}$ (BD1 Biosciences), IgG1aκ or IgG2aκ (Pharmingen, negative controls), and biotinylated rabbit anti-rat or anti-mouse antibody

(Vector), streptavidin conjugated to horseradish peroxidase (DAK, DAB (3,3' diaminobenzidine tetrahydrochloride, DAKO). Slides were counterstained with hematoxylin (DAKO), dehydrated, and then coverslipped.

Supplementary Figure Legends

Supplementary Figure S1 Myc induces the expression of Cks1 in immature and mature B cells and represses the expression of *cyclin D2*. (**A**) Bone marrow (BM) and splenic cells from wild type (wt) and Eμ-*Myc* mice were FACS-sorted for $B220^{+}sIgM^{-}$ and $B220^{+}sIgM^{+}$ B cells, followed by immunoblotting for the indicated proteins. (**B**) *Left panel*: *Cyclin D2* (*Ccnd2*) gene expression analysis of $B220^{+}$ splenic B cells of five weanling-age wild type and five Eμ-*Myc* transgenic mice. Probe set signals were normalized to the mean across mice, and values of each individual case are represented by a color, with green corresponding to SD (σ) below and red corresponding to SD (σ) above the mean, according to the scale shown. *Right panel*: SYBR-green real-time PCR analysis of $B220^{+}$ bone marrow (BM) and $sIgM^{+}$ splenic (Spleen) B cells. Levels of mRNAs are standardized to the expression of *ubiquitin* (*ub*).

Supplementary Figure S2 The induction of Cks1 expression is independent of E2fs. (**A**) E2f1 is not sufficient to induce *Cks1*. Primary early passage MEFs were infected with pBabe-Puro (Puro) or pBabe-ER-E2f1-Puro (ER-E2f1) retroviruses. Puromycin-resistant cells were treated for the indicated times with 4-HT to activate ER-E2f1 and

were assessed for the expression of the indicated transcripts by real-time PCR. Levels of RNA were standardized to *ub*. (**B**) The expression of *e2f1*, *e2f2* and *e2f3* transcript levels was assessed in splenic $B220^+$ B cells of wild type (wt) and pre-cancerous Eμ-Myc transgenic mice. Levels of RNA were standardized *ub*.

Supplementary Figure S3 Myc does not affect the expression of components of the APC/C^{Cdh1} complex that directs the turnover of Cks1 (Bashir *et al*, 2004). SYBR-green real-time PCR analysis of the indicated components of the APC/C^{Cdh1} E3 ubiquitin ligase complex were determined for $B220^+$ bone marrow-derived B cells from several individual (*n* = 3 for each genotype) pre-cancerous (4-6 week old) wild type (WT) or Eμ-*Myc* transgenic littermates of the indicated *Cks1* genotypes. Levels of mRNAs were standardized to *ub*.

Supplementary Figure S4 Myc-induced increases in *Cks1* transcripts augment Cks1 biosynthesis. (**A**) Primary early passage wild-type MEFs and *Cks1*$^{-/-}$ MEFs were infected with MSCV-Myc-ER^{TAM}-IRES-Puro (*Myc*-ER^{TAM}) virus or MSCV-IRES-Puro (Vector) control virus. *Top Panel*, puromycin-resistant cells were incubated with 2μM 4-HT for 12 hours, then pulse labeled with S^{35}-Translabel^{TM} for 2 hour, and then chased with medium with excess cold methionine and cysteine. At the indicated intervals equal cell numbers were harvested, and immunoprecipitated with affinity-purified anti-Cks1-specific antibody. *Middle Panel*, the mean calculated half-life of Cks1 in *Myc*-ER- versus puromycin-expressing (Vector) MEFs, determined from four independent experiments. *Bottom Panel*, levels of the Myc-ER^{TAM} transgene in wild type and *Cks1*$^{-/-}$ MEFs was

determined by immunoblot. β-actin was assessed as a loading control. (**B**) Cks1 biosynthesis was monitored by pulse-labeling *Myc*-ER- versus puromycin-expressing (Vector) MEFs with ^{35}S-methionine/cysteine after the activation of Myc with 2μM 4-HT for 12 hours. *Top panel*, at the indicated intervals labeled cells were harvested and lysates were immunoprecipitated with affinity purified anti-Cks1-specific antibody. *Lower left panel*, a graph of the data shown in the top panel documents the increased rate of Cks1 biosynthesis, as assessed by the increase in pixel density over time*. Lower right panel*, the mean fold increase in Cks1 biosynthesis was determined by pulse-labeling with S^{35}-Translabel^{TM} after treatment of *Myc*-ER- versus puromycin-expressing (Vector) MEFs with 2μM 4-HT for 12 hours (*n* = 4).

Supplementary Figure S5 Cks1 induction is independent of FoxM1 and E2f1. (**A**) The expression of *Cks1* and *FoxM1* transcripts in B220+ bone marrow B cells from pre-cancerous (4-week old) Eμ-*Myc* mice and from wild type littermates was compared to their expression in Eμ-*Myc* lymphoma (1-10) by qRT-PCR. Levels of RNA are standardized to *ub*. (**B**) Cks1 protein is also elevated in lymphomas that arise in Eμ-*Myc*;*E2f1*$^{-/-}$ mice. Cks1 expression was determined by immunoblot analyses using extracts prepared from bone marrow B220^{+} cells from wild type (wt) and pre-cancerous Eμ-*Myc* transgenic mice and from *E2f1*$^{+/-}$-, *E2f1*$^{+/-}$-, and *E2f1*$^{-/-}$- Eμ-*Myc* lymphomas.

Supplementary Figure S6 *Cks1* deficiency does not alter the frequency of alterations in Arf and p53 during lymphomagenesis. (**A**) Southern blot hybridization of genomic DNA from lymphomas arising in the indicated cohorts of mice. The asterisk (*) indicates

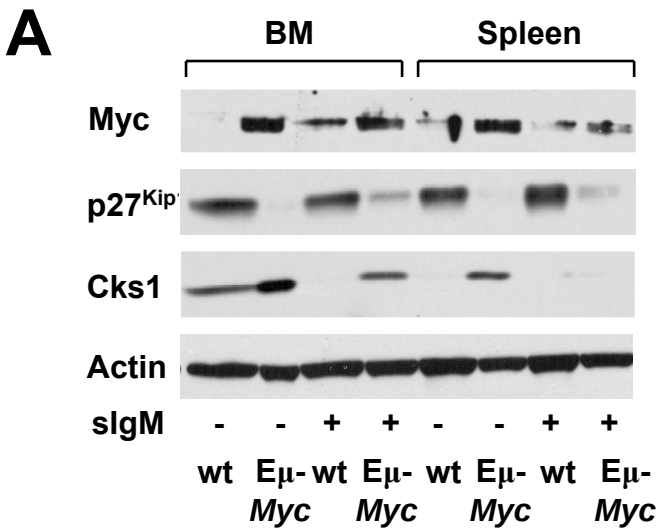
those lymphomas having bi-allelic deletions in *Arf*. None of the tumors showed a deletion in *p53*. (**B**) Western blot analysis of p53 and $p19^{Arf}$ expression in lymphomas arising in the indicated cohorts of mice. * Indicates those lymphomas expressing high levels of $p19^{Arf}$, which is only observed in lymphomas bearing loss-of-function missense mutations in p53 (Eischen *et al*, 1999, 2001).

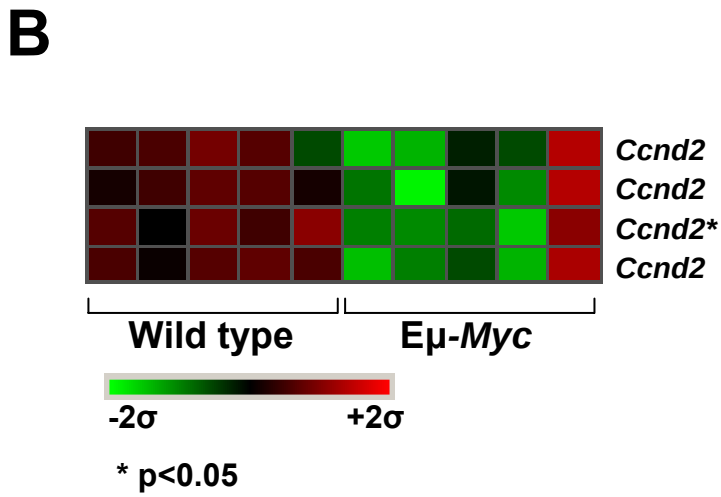
Supplementary Figure S7 $p27^{Kip1}$ knockdown partially restores the proliferative defects of pre-cancerous Eμ-*Myc*;*Cks1*$^{-/-}$ B cells. (**A**) Pre-cancerous in vitro cultured Eμ-*Myc*;*Cks1*$^{-/-}$ B cells were infected with MSCV-p27shRNA-IRES-GFP virus (validated for efficient knockdown of $p27^{Kip1}$ in 293T cells that transiently express murine $p27^{Kip1}$, *right panels*) or MSCV-IRES-GFP control virus and sorted for GFP by FACS. Cells were then assessed for $p27^{Kip1}$ expression by immunoblot. (**B**) Eμ-*Myc*;*Cks1*$^{-/-}$ B cells expressing GFP or p27shRNA-GFP were cultured in IL-7 conditioned medium, pulse-labelled with ^{3}H-thymidine and assessed for proliferation by measuring the ^{3}H-thymidine uptake. The bars show the mean value of 3 experiments ± SEM. p=0.0105. (**C**) The same cells were assessed for proliferation by counting the number of viable cells. Cells were replated at 2.5 x 10^{6} cells to ensure optimal growth conditions. Shown are the cumulative cell numbers at day 6 of culture for two independent experiments.

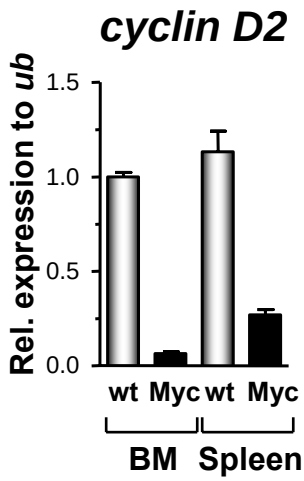
Supplementary Figure S8 (**A**) *Cks1* loss has little effect on Myc target genes in the pre-cancerous B cells of Eμ-*Myc* transgenic mice. Hierarchical clustering of Myc target genes, as annotated on the 'Myc Target Gene Database', (http://www.myc-cancer-gene.org) of $B220^{+}$ splenic B cells isolated from 4-week old mice of the indicated

genotypes was performed. Probe set signals were normalized to the mean across mice, and values are represented by a color, with green corresponding to SD (σ) below and red corresponding to SD (σ) above the mean, according to the scale shown. (**B**) *Cks1* loss does not overtly affect Myc's transcriptional activity. SYBR-green real-time PCR analysis of the indicated Myc targets were determined for $B220^{+}$ bone marrow-derived B cells from several individual (*n* = 3 for each genotype) pre-cancerous (4-6 week old) wild type (WT) or Eμ-*Myc* transgenic littermates of the indicated *Cks1* genotypes. Levels of mRNAs were standardized to the expression of *ub*. None of the differences seen in the levels of the Myc targets were statistically significant.

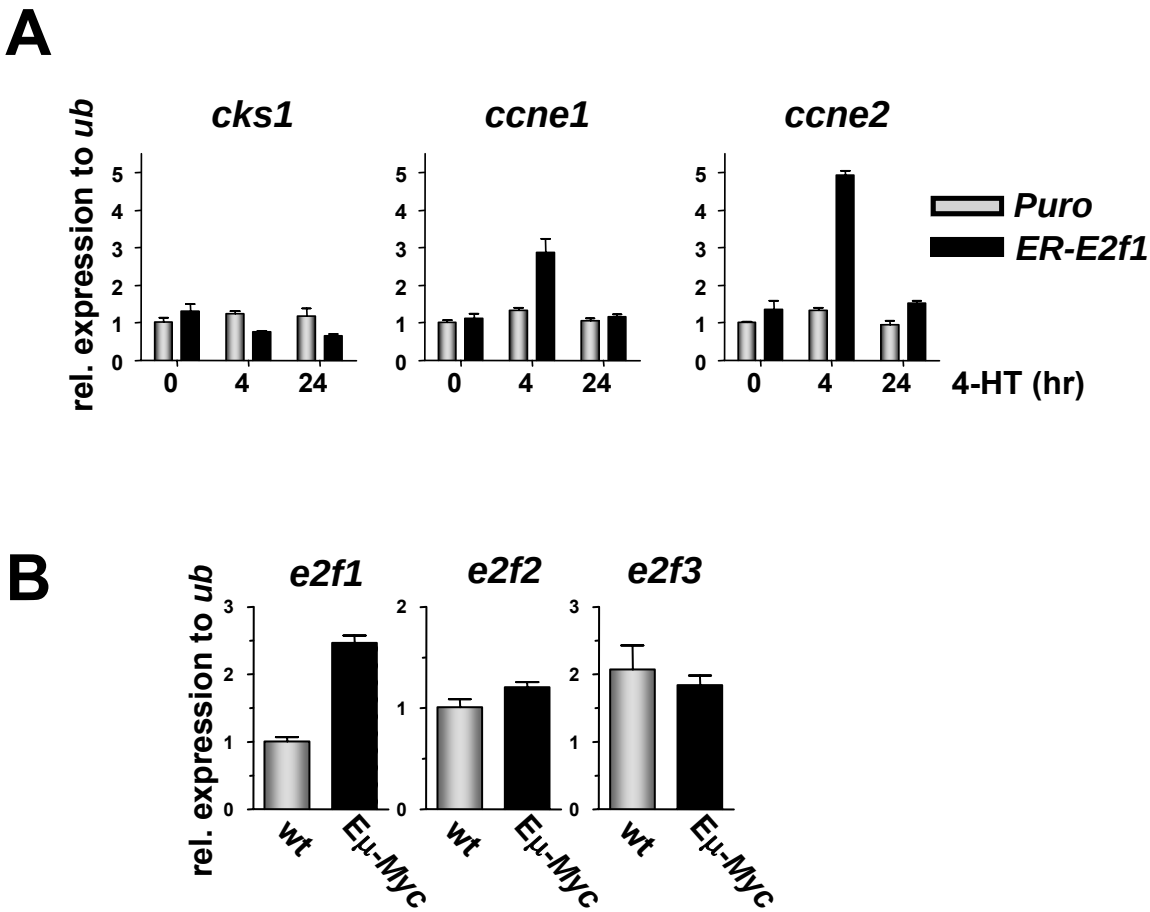
Supplementary Figure S1, Keller *et al*



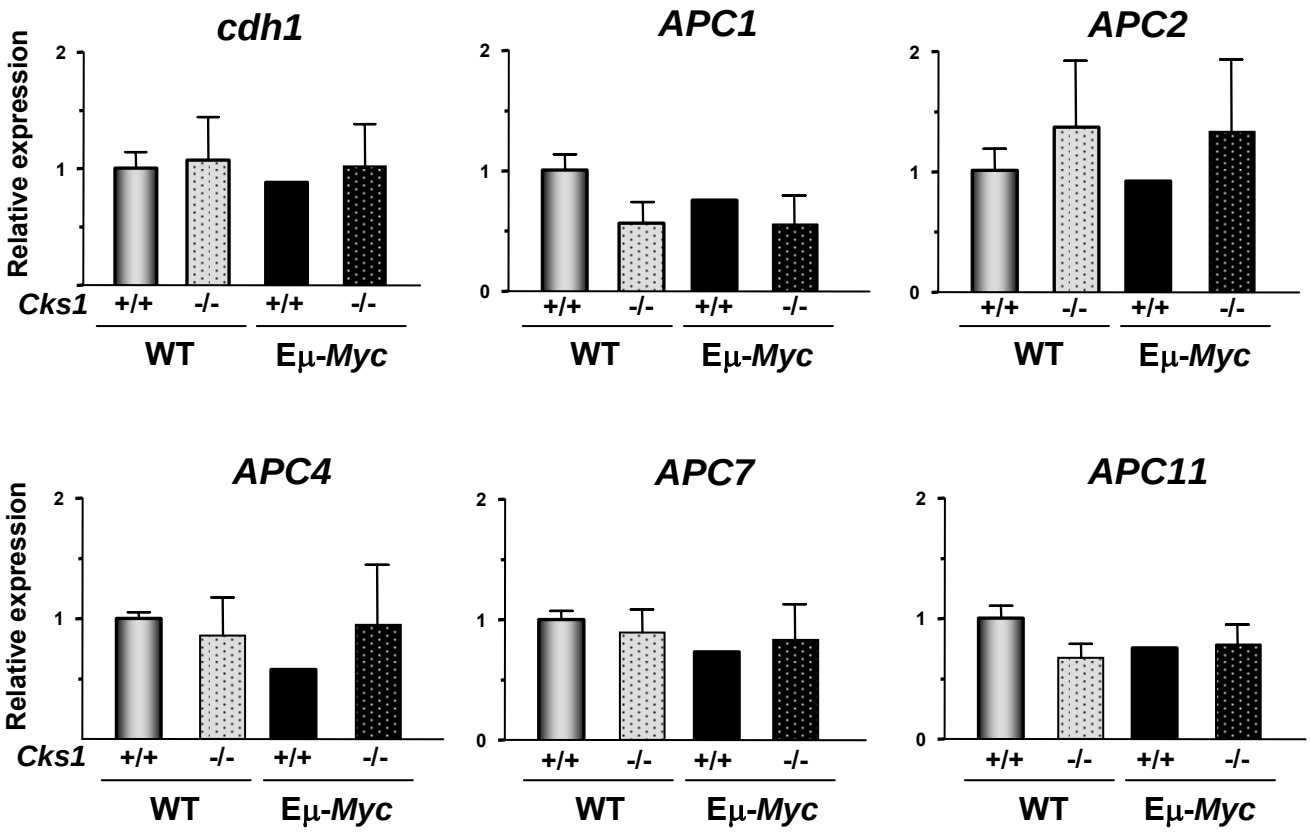




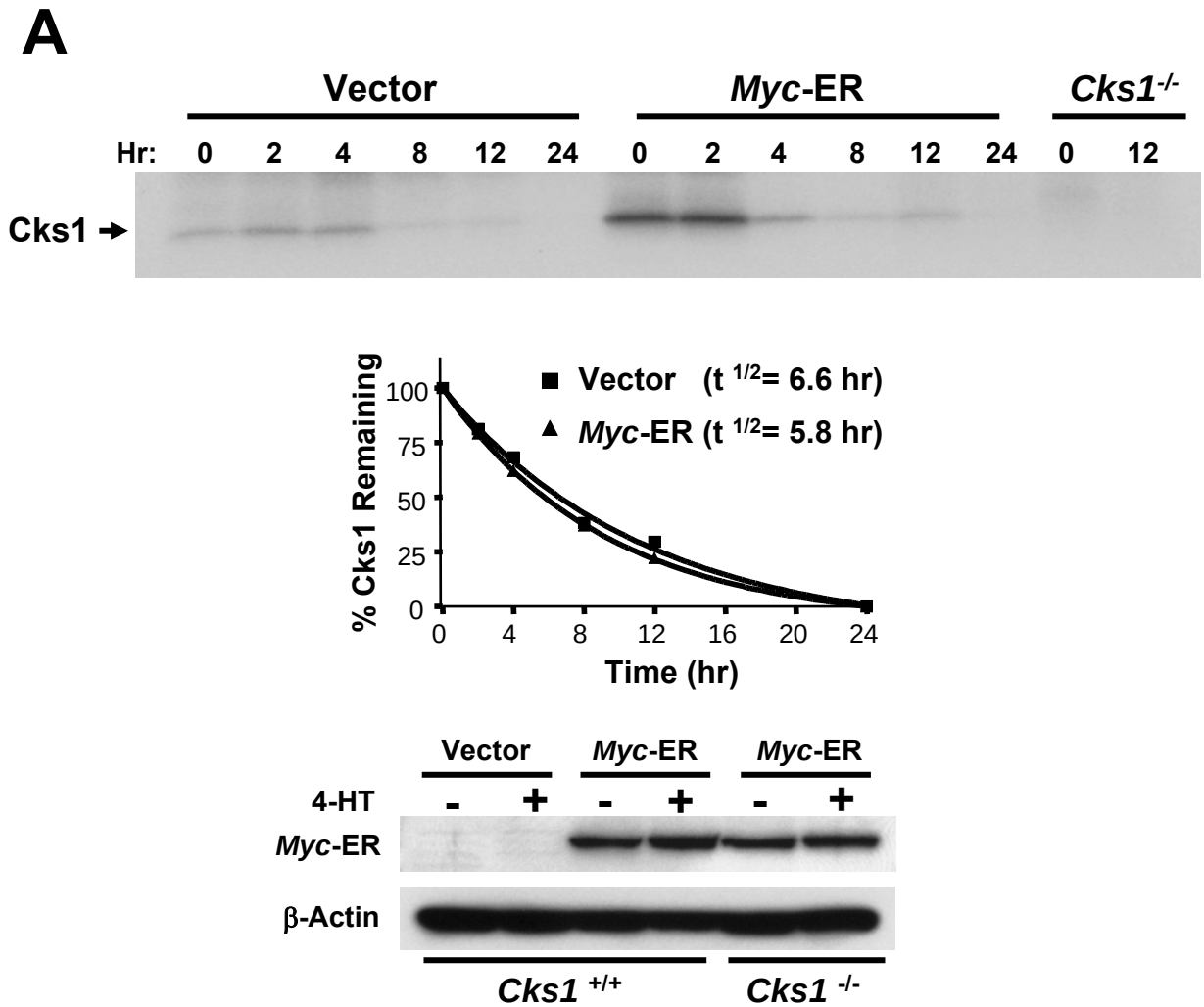
Supplementary Figure S2, Keller *et al*

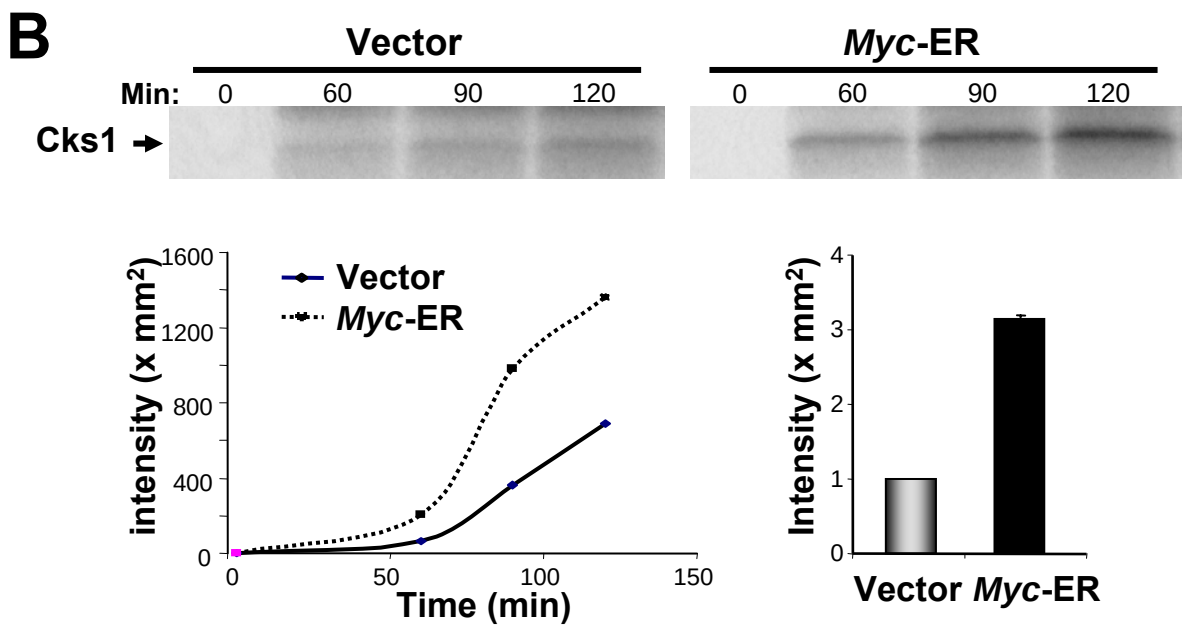


Supplementary Figure S3, Keller *et al*

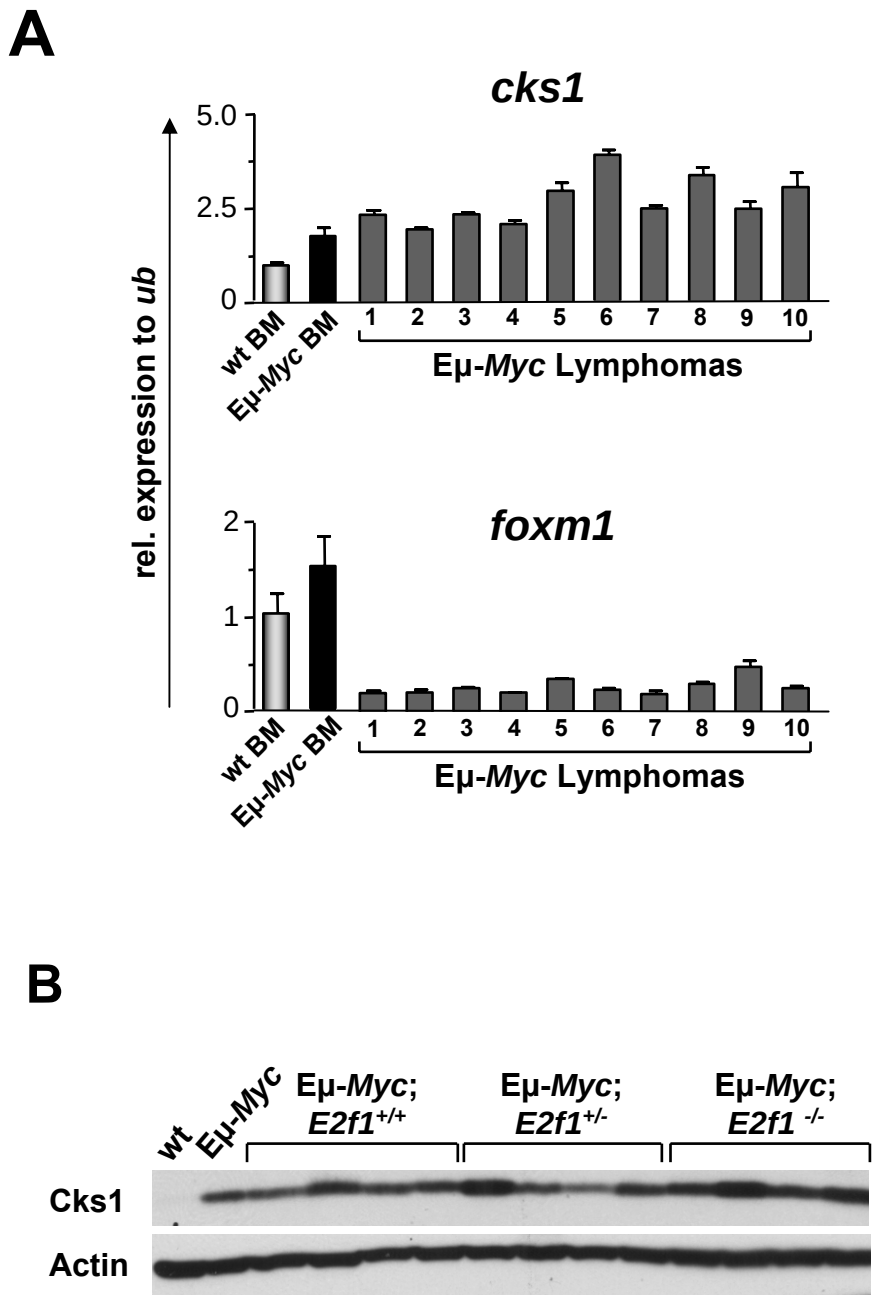


Supplementary Figure S4, Keller *et al*

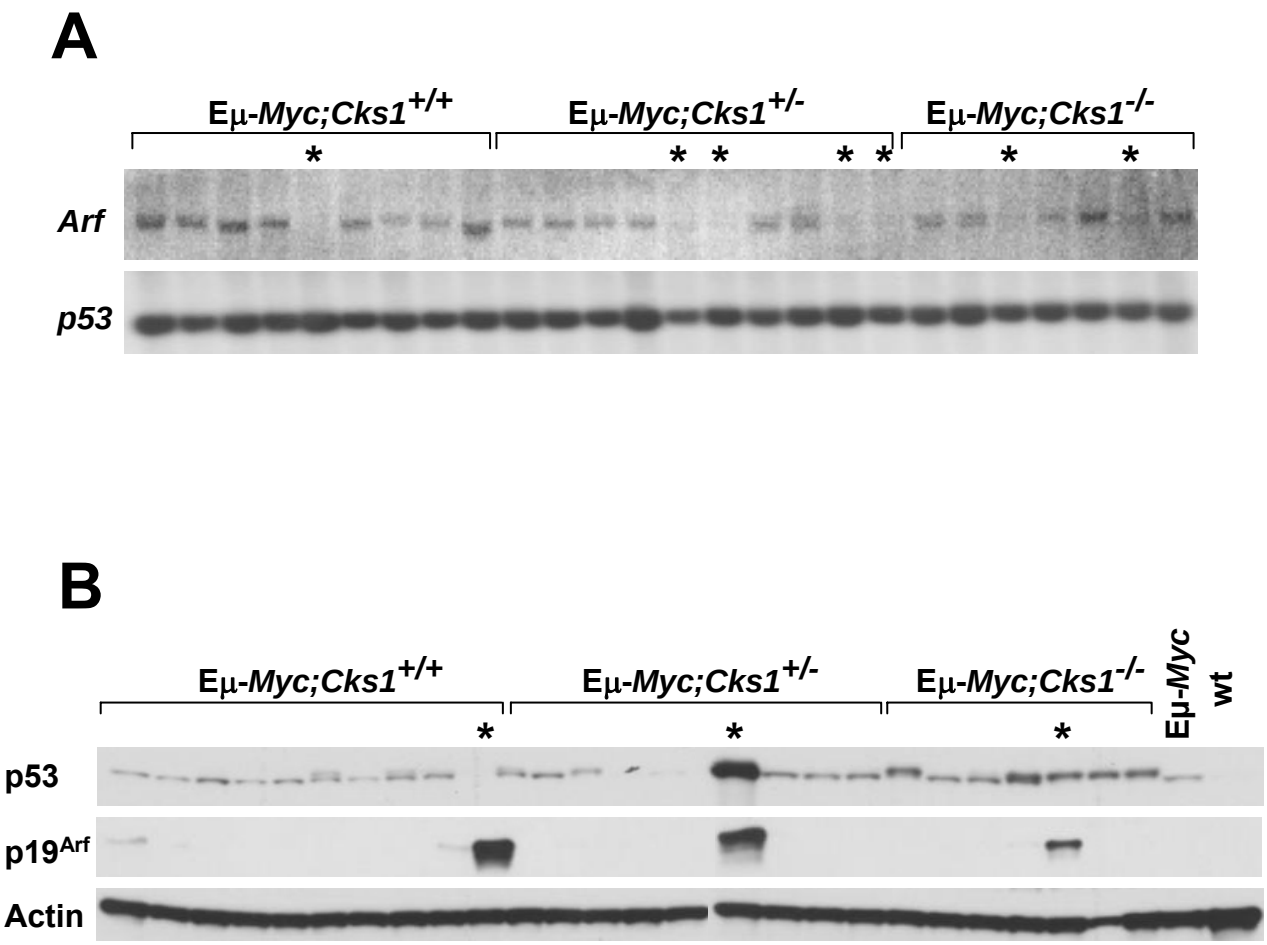




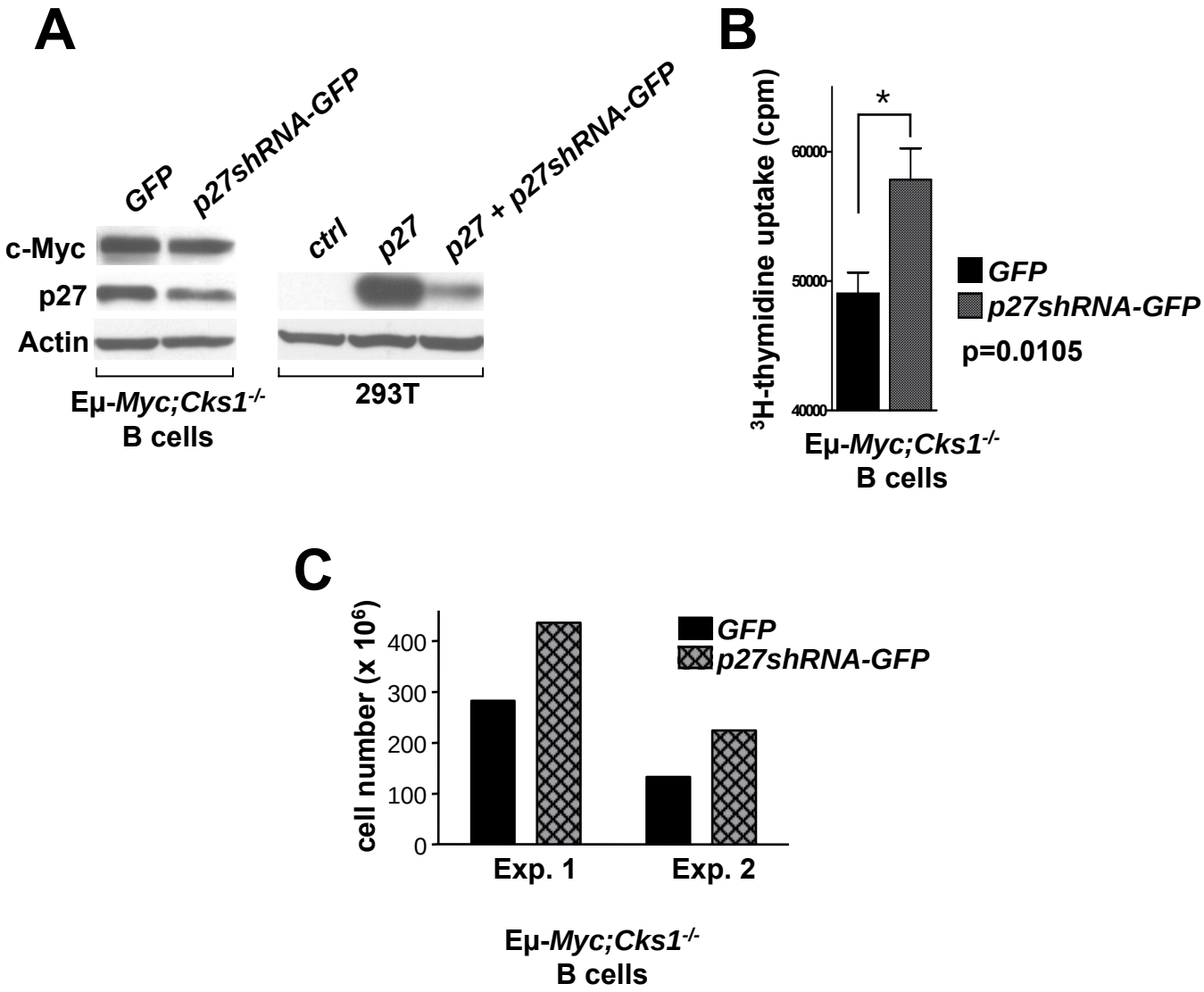
Supplementary Figure S5, Keller *et al*



Supplementary Figure S6, Keller *et al*

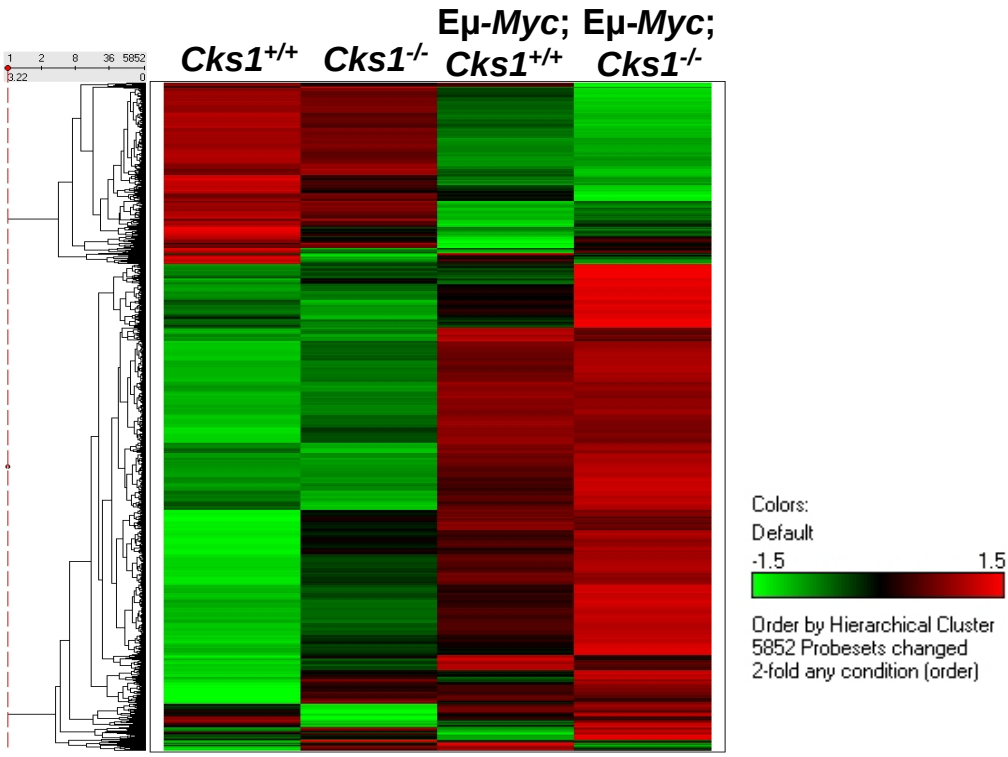


Supplementary Figure S7, Keller *et al*



Supplementary Figure S8, Keller *et al*

A



B