Supplemental Material

Myc Targets Cks1 to Provoke the Suppression of p27<sup>Kip1</sup>, Proliferation and Lymphomagenesis

Ulrich B Keller<sup>1,5</sup>, Jennifer B Old<sup>1,6</sup>, Frank C Dorsey<sup>1,2</sup>, Jonas A Nilsson<sup>1,7</sup>, Lisa Nilsson<sup>1,7</sup>, Kirsteen H MacLean<sup>1</sup>, Linda Chung<sup>1</sup>, Chunying Yang<sup>1</sup>, Charles Spruck<sup>3</sup>, Kelli Boyd<sup>4</sup>, Steven I Reed<sup>3</sup>, and John L Cleveland<sup>1,2,*</sup>

Supplementary Online Materials

Supplemental Materials and methods

Supplementary Figure Legends

Figure S1
Figure S2
Figure S3
Figure S4
Figure S5
Figure S6
Figure S7
Figure S8
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), p27Kip1 (BD Biosciences Transduction Laboratories), E1 (Upstate), Cul1 and p19Skp1 (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\textsuperscript{35}-Translabel\textsuperscript{TM}/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\textsuperscript{35}--Translabel\textsuperscript{TM} 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\textsuperscript{TM} (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\textsuperscript{TM} (Amersham), then dried and exposed to film. Quantification of the band intensity was performed using Quantity One\textsuperscript{TM} (BioRad).

**Histology and immunohistochemistry**

Full necropsy was performed on 6 Cks1\textsuperscript{+/+} and 9 Cks1\textsuperscript{-/-} E\textsubscript{μ}-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\textsuperscript{Kip1} (BD1 Biosciences), IgG1κ or IgG2ακ (Pharmingen, negative controls), and biotinylated rabbit anti-rat or anti-mouse antibody
strepavidin 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™ 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 p19Arf expression in lymphomas arising in the indicated cohorts of mice. * Indicates those lymphomas expressing high levels of p19Arf, which is only observed in lymphomas bearing loss-of-function missense mutations in p53 (Eischen et al, 1999, 2001).

Supplementary Figure S7 p27Kip1 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 p27Kip1 in 293T cells that transiently express murine p27Kip1, right panels) or MSCV-IRES-GFP control virus and sorted for GFP by FACS. Cells were then assessed for p27Kip1 expression by immunoblot. (B) Eµ-Myc;Cks1−/− B cells expressing GFP or p27shRNA-GFP were cultured in IL-7 conditioned medium, pulse-labelled with ³H-thymidine and assessed for proliferation by measuring the ³H-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 10⁶ 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

A

<table>
<thead>
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<td>Myc</td>
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B

Wild type  Eμ-Myc

Ccnd2  Ccnd2  Ccnd2*

Rel. expression to ub

* p<0.05

cyclin D2

BM  Spleen
Supplementary Figure S2, Keller et al

A

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4-HT (hr)

B

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Supplementary Figure S3, Keller et al

![Graphs showing relative expression of various genes in different conditions.](image)

- **cdh1**
- **APC1**
- **APC2**
- **APC4**
- **APC7**
- **APC11**

Relative expression of genes in different conditions:

- **Cks1**
  - WT
  - \( E_\mu \)-Myc

- **WT**
Supplementary Figure S4, Keller et al

A

<table>
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<tr>
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![Blot showing Cks1 expression over time for different groups](image)

![Graph showing % Cks1 Remaining over time for Vector (t₁/₂ = 6.6 hr) and Myc-ER (t₁/₂ = 5.8 hr)](image)

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![Blot showing Myc-ER and β-Actin expression for Cks1⁺⁺ and Cks1⁻⁻](image)

B

<table>
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<tr>
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<td>0 60 90 120</td>
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![Graph showing intensity of Cks1 over time for Vector and Myc-ER](image)

![Bar graph comparing intensity of Vector and Myc-ER](image)
**Supplementary Figure S5, Keller et al**

### A

**ck1**

<table>
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**foxm1**

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### B

- **Eµ-Myc**
- **Eµ-Myc; E2f1+/−**
- **Eµ-Myc; E2f1−/−**

**Cks1**

**Actin**
Supplementary Figure S7, Keller et al

A

![Western blot images of c-Myc, p27, and Actin](image)

- Eµ-Myc;Cks1−/− B cells
- 293T

B

![Graph showing ³H-thymidine uptake](image)

- GFP
- p27shRNA-GFP

Eµ-Myc;Cks1−/− B cells

p = 0.0105

C

![Bar chart showing cell number](image)

- GFP
- p27shRNA-GFP

Exp. 1
Exp. 2

Eµ-Myc;Cks1−/− B cells
Supplementary Figure S8, Keller et al

A

Supplementary Figure S8, Keller et al

B

B

Supplementary Figure S8, Keller et al

B

Supplementary Figure S8, Keller et al

B

Supplementary Figure S8, Keller et al

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Supplementary Figure S8, Keller et al

B

Supplementary Figure S8, Keller et al