

Skp2 Directs Myc-Mediated Suppression of p27^{Kip1} yet Has Modest Effects on Myc-Driven Lymphomagenesis

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Abstract

The universal cyclin-dependent kinase inhibitor p27^{Kip1} functions as a tumor suppressor, and reduced levels of p27^{Kip1} connote poor prognosis in several human malignancies. p27^{Kip1} levels are predominately regulated by ubiquitin-mediated turnover of the protein, which is marked for destruction by the E3 ubiquitin ligase SCF^{Skp2} complex following its phosphorylation by the cyclin E–cyclin-dependent kinase 2 complex. Binding of phospho-p27^{Kip1} is directed by the Skp2 F-box protein, and this is greatly augmented by its allosteric regulator Cks1. We have established that programmed expression of c-Myc in the B cells of Eμ-Myc transgenic mice triggers p27^{Kip1} destruction by inducing Cks1, that this response controls Myc-driven proliferation, and that loss of *Cks1* markedly delays Myc-induced lymphomagenesis and cancels the dissemination of these tumors. Here, we report that elevated levels of Skp2 are a characteristic of Eμ-Myc lymphomas and of human Burkitt lymphoma that bear *MYC/Immunoglobulin* chromosomal translocations. As expected, Myc-mediated suppression of p27^{Kip1} was abolished in *Skp2*-null Eμ-Myc B cells. However, the effect of Skp2 loss on Myc-driven proliferation and lymphomagenesis was surprisingly modest compared with the effects of Cks1 loss. Collectively, these findings suggest that Cks1 targets, in addition to p27^{Kip1}, are critical for Myc-driven proliferation and tumorigenesis. *Mol Cancer Res*; 8(3); 353–62. ©2010 AACR.

Introduction

The cyclin-dependent kinase (Cdk) inhibitor p27^{Kip1} binds to and inactivates cyclin-Cdk complexes to restrict the traverse of cells through the G₁ and S phases of the cell cycle (1). p27^{Kip1} overexpression triggers cell cycle arrest in G₁ (2), whereas loss of p27^{Kip1} in mice increases rates of cell proliferation (3, 4). Patients with tumors having low or undetectable levels of p27^{Kip1} protein have a very poor outcome (5, 6), yet unlike other tumor suppressors, p27^{Kip1} is only rarely directly mutated. Further, mice heterozygous for p27^{Kip1} develop spontaneous tumors late in life, yet these retain and still express the normal p27^{Kip1}

allele (7). Finally, the subcellular localization of p27^{Kip1} also has prognostic significance, where high cytoplasmic p27^{Kip1}, which is driven by activated Akt, is associated with poor outcome (Liang et al., 2002).

Signals that control p27^{Kip1} protein levels include its phosphorylation on Thr¹⁸⁷ by the cyclin E–Cdk2 complex in S phase (8–10). Thr¹⁸⁷-phosphorylated p27^{Kip1} is targeted to the proteasome by the SCF^{Skp2} ubiquitin ligase complex that is composed of Skp1, Cullin-1 (Cul1), Rbx1, Cks1, and the F-box protein Skp2 (11). Cks1 and Skp2 form the recognition element of the SCF^{Skp2} complex for phospho-p27^{Kip1} (11–14), and their binding then leads to the ubiquitylation and destruction of p27^{Kip1}. Accordingly, elevated levels of Skp2 in human cancer correlate with low p27^{Kip1} levels (15), and enforced Skp2 expression in transgenic mice reduces p27^{Kip1} levels and induces proliferation (16). By contrast, the targeted deletion of *Skp2* leads to p27^{Kip1} accumulation, reduced proliferation, and nuclear abnormalities (17), which are also features of *Cks1* loss (14).

Myc oncoproteins that are activated in cancer are members of a basic/helix-loop-helix/leucine zipper transcription factor family that coordinates cell growth, division, and metabolism, as well as differentiation, cell migration, and apoptosis (18, 19). Accordingly, in normal cells, Myc levels are tightly regulated, and this occurs at the levels of transcription and the turnover of its mRNA and protein, as well as at the level of translation (20, 21). Myc genes are overexpressed in ~70% of all rapidly dividing tumors by virtue of chromosomal amplifications or

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translocations or through mutations in pathways that normally restrict Myc expression (22). Expression of Myc at levels found in cancer cells is sufficient to drive primary quiescent cells into S phase (23), to accelerate rates of cell proliferation (24), and to prevent withdrawal from the cell cycle (25, 26). However, these aberrant proliferative responses are harnessed by apoptotic checkpoints that are induced by Myc, including the Arf-p53 tumor suppressor pathway (27, 28) and the Bcl2 family of apoptotic regulators (28). Accordingly, mutations that inactivate these apoptotic checkpoints are found in most tumors induced by Myc (28, 29).

Myc accelerates the rates of cell proliferation, at least in part, through its ability to downregulate the expression of the Cdk inhibitor p27^{Kip1} (30, 31), which inactivates cyclin E-Cdk2 and cyclin A-Cdk2 complexes that orchestrate entry and progression through S phase (1, 6, 32). Myc suppresses p27^{Kip1} expression at the level of transcription (33), but its effects on p27^{Kip1} protein levels *in vivo* are more profound (31). First, Myc induces the expression of E2f1 (34), which then promotes *cyclin E* transcription (35), thus activating cyclin E-Cdk2 complexes (30). Moreover, Myc induces the transcription of both *cyclin D2* and its catalytic partner *Cdk4*, and this holoenzyme sequesters p27^{Kip1}, thus relieving inhibition of cyclin E-Cdk2 complexes (36, 37). Under either scenario, activated cyclin E-Cdk2 complexes then phosphorylate p27^{Kip1} on Thr¹⁸⁷, allowing its recognition by the SCF^{Skp2} complex, ubiquitination, and degradation by the proteasome (8, 9, 38). Finally, Myc induces the expression of some of the components of the SCF^{Skp2} complex, including Cul1 (39) and Cks1, and at least the latter is required for downregulation of p27^{Kip1} (31).

p27^{Kip1} is a key regulator of Myc-induced proliferation and tumorigenesis. First, loss of p27^{Kip1} accelerates lymphoma development in Eμ-Myc transgenic mice (40), a mouse model of human Burkitt lymphoma (41). Further, loss or heterozygosity of *E2f1*, or loss of *Cks1*, effectively cancels the ability of Myc to suppress p27^{Kip1} protein (but not p27^{Kip1} mRNA) levels, impairs Myc-induced proliferation, and markedly delays lymphoma onset and triples the life span of Eμ-Myc mice (31, 34). Here, we report that Myc also induces the expression of the Skp2 F-box component of the SCF^{Skp2} complex in B cells and fibroblasts and that Skp2 is expressed at high levels in Myc-driven lymphomas of mice and man. As expected, *Skp2* loss abolishes the suppression of p27^{Kip1} protein in Eμ-Myc B cells. However, quite surprisingly, the effects of the *Skp2* deficiency on Myc-induced proliferation and tumorigenesis are at most modest, suggesting that Cks1 has targets, in addition to p27^{Kip1}, that contribute to lymphomagenesis.

Materials and Methods

Mice and Tumor Analysis

Skp2-null mice (C57BL/6; ref. 17) were bred with Eμ-Myc transgenic mice (C57BL/6; ref. 41). F₁ Eμ-Myc;*Skp2*^{+/-}

offspring were bred to *Skp2*^{+/-} mice to obtain Eμ-Myc;*Skp2*^{+/+}, Eμ-Myc;*Skp2*^{+/-}, and Eμ-Myc;*Skp2*^{-/-} littermates. Animals were observed for signs of morbidity and tumor development. Tumors were harvested after sacrifice of mice, snap frozen in liquid nitrogen, and processed for analysis of DNA, RNA, and protein. *E2f1*-null mice (42), again on a C57BL/6 background, were bred with Eμ-Myc transgenic mice. F₁ Eμ-Myc;*E2f1*^{+/-} offspring were bred to *E2f1*^{+/-} to obtain Eμ-Myc;*E2f1*^{+/+}, Eμ-Myc;*E2f1*^{+/-}, and Eμ-Myc;*E2f1*^{-/-} littermates.

With institutional review board approval, and following informed consent, tumors from 14 Burkitt lymphoma patients were banked. RNA and protein were extracted from these tumors. As a control, pooled peripheral blood mononuclear cells from healthy donors were enriched using CD19 microbeads according to the manufacturer's instruction (Miltenyi Biotech), and RNA and protein were prepared.

Cell Culture

Primary bone marrow-derived pre-B cells were cultured as described previously (29). Mouse embryo fibroblasts (MEF) from E13.5 to E14.5 embryos were cultured and infected with MSCV-Myc-ER^{TAM}-IRES-GFP, MSCV-Myc-IRES-GFP, pBabe-Myc-ER^{TAM}-IRES-puromycin, pBabe-ER^{TAM}-E2f1-puromycin, or control retrovirus as described (27). To evaluate consequences of Myc activation, cells were treated with 2 μmol/L 4-hydroxytamoxifen (4-HT) and harvested for protein and RNA preparation. To assess whether Myc induction of Skp2 was direct, Myc-ER-expressing cells or control cells were pretreated with 1 μg/mL cycloheximide (Sigma Chemicals) for 30 min (which inhibited >95% of protein synthesis) before adding 4-HT. For analysis of *Skp2* RNA half-life, MEFs were cultured in the presence of 1 μg/mL actinomycin D (Sigma-Aldrich) and harvested at the indicated time. To estimate Skp2 protein half-life, MEFs were cultured in the presence of 10 μg/mL cycloheximide and harvested at the indicated time.

Fluorescence-Activated Cell Sorting Analysis and Magnetic-Activated Cell Sorting of B Cells

Rates of proliferation or apoptosis of B cells were determined using a Flow kit as described by the manufacturer (BD Biosciences Pharmingen). Bone marrow and spleen cells were incubated with B220 microbeads and enriched by magnetic cell sorting for B cells according to the manufacturer's instructions (Miltenyi Biotech) and used for immunoblot or real-time PCR analysis.

RNA Preparation and Analyses

RNA was prepared from cultured MEFs, magnetic-activated cell sorting-sorted B cells, or lymphomas using the RNeasy kit (Qiagen). For real-time PCR, cDNA was prepared from 1 μg RNA using the iScript cDNA Synthesis kit (Bio-Rad). Real-time PCR was done using an

iCycler machine (Bio-Rad) and the iTaq SYBR Green kit (Bio-Rad). Data analyses were done by comparing C_t values with a control sample set as 1. Sequences for primers are available on request.

Immunoblotting

Protein extracts (20 or 50 μ g per lane) were separated electrophoretically on a SDS-PAGE gel, transferred to membranes (Protran, Schleicher & Schuell), and blotted with antibodies specific for Skp2 (Zymed Laboratories, Inc.), p27^{Kip1} (BD Biosciences Transduction Laboratories), c-Myc and E2f1 (Santa Cruz Biotechnology), and β -actin (Sigma Chemicals).

Statistical Analyses

The statistical analysis of survival differences in E μ -Myc transgenics of *Skp2*^{-/-} versus *Skp2*^{+/+} genotype was done using a Cox regression analysis with a multiple cohort comparison Bonferroni adjusted. The statistics done to analyze differences in the *ex vivo* and *in vivo* B-cell proliferation and apoptosis indices involved paired *t* tests.

Results

Myc Induces Skp2 Expression

Myc suppresses p27^{Kip1} expression primarily by provoking ubiquitin-mediated destruction of p27^{Kip1} protein (8).

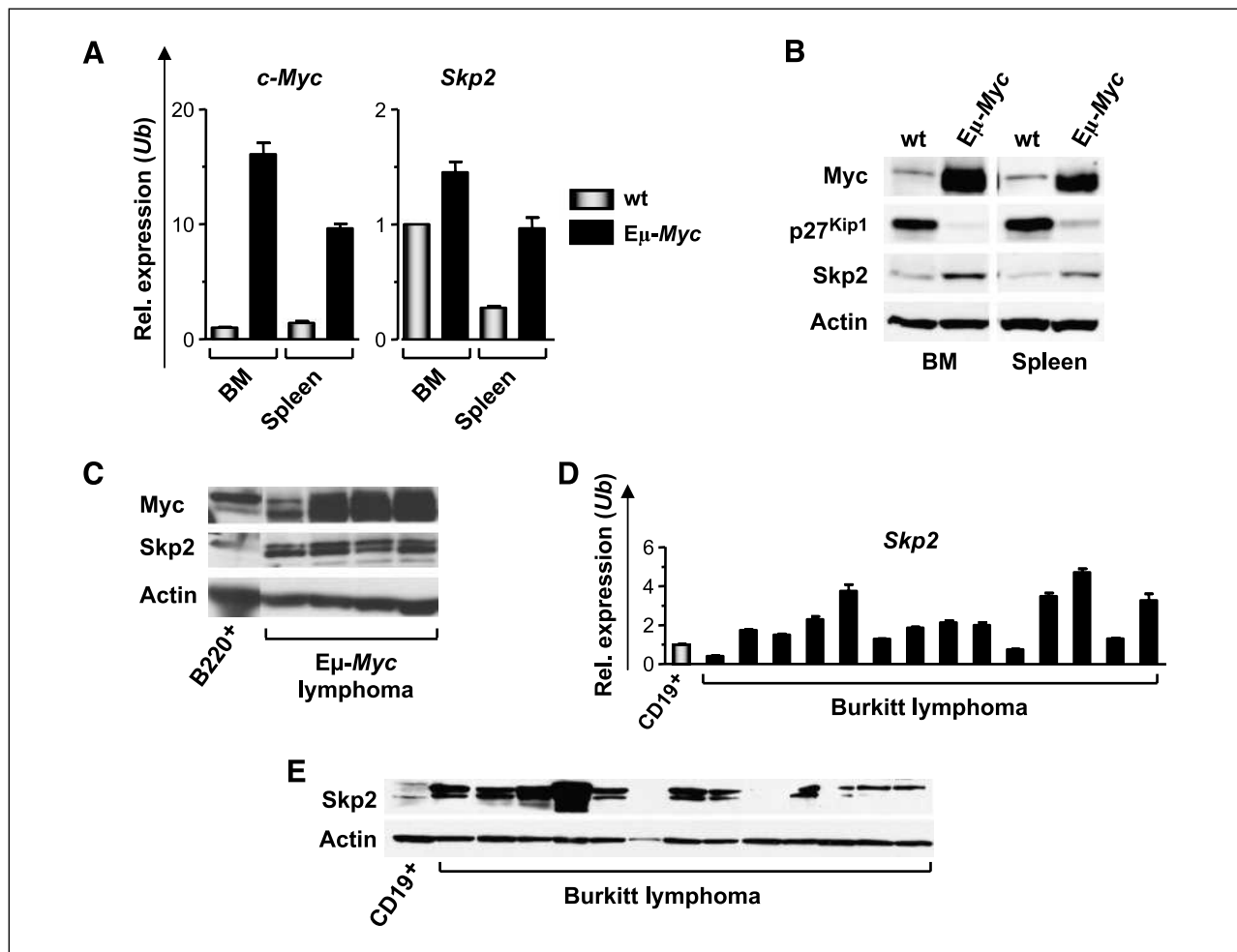


FIGURE 1. Skp2 expression is elevated in precancerous E μ -Myc B cells and in Myc-induced lymphomas. **A**, SYBR Green real-time PCR analysis of levels of *c-Myc* and *Skp2* transcripts in bone marrow (BM) and splenic (Spleen) B220⁺ B cells from 4-wk-old wild-type (wt; gray columns) or E μ -Myc (black columns) littermate mice. Levels of mRNA were standardized to the expression of *ubiquitin (Ub)*, which is not regulated by Myc. **B**, immunoblot analyses of p27 and Skp2 expression in B220⁺ B cells from 4-wk-old wild-type or E μ -Myc transgenic littermate mice. Levels of c-Myc protein are also shown and Actin served as a loading control. **C**, Skp2 protein expression is elevated in E μ -Myc lymphoma. E μ -Myc lymphoma samples were analyzed by immunoblotting for Skp2 expression. B220⁺ B cells were used as a control. Actin immunoblotting served as a loading control. **D**, *SKP2* expression is elevated in Burkitt lymphoma. Real-time PCR analysis of 14 Burkitt lymphoma samples (black columns) compared with normal human CD19⁺ B cells (gray column). Levels of mRNAs were standardized to the expression of *Ub*. **E**, Skp2 protein levels are elevated in Burkitt lymphoma. Thirteen human Burkitt lymphoma samples were analyzed by immunoblotting for Skp2 expression. CD19⁺ peripheral B cells were used as a control. Actin immunoblotting served as a loading control.

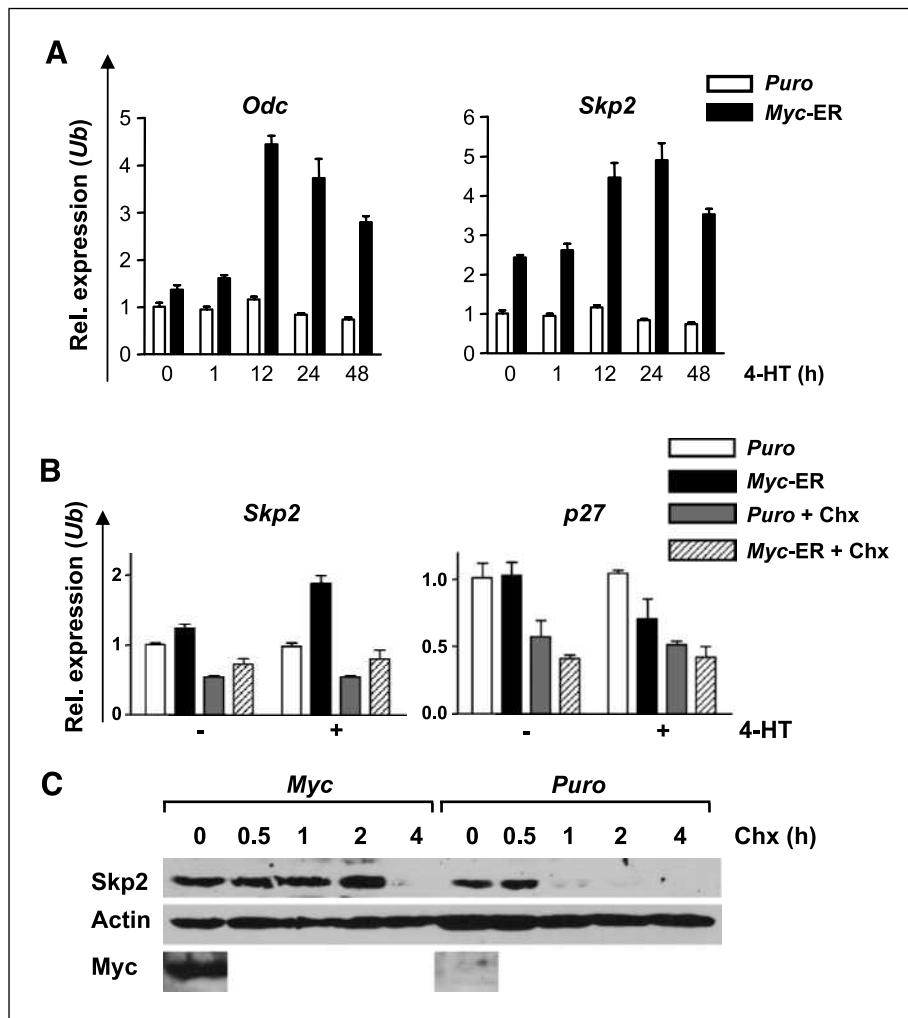


FIGURE 2. Myc regulation of *Skp2* is indirect. **A**, SYBR Green real-time PCR analysis of *Odc* and *Skp2* RNA levels in primary early-passage MEFs infected with pBabe-Myc-ER^{TAM}-IRES-puromycin (Myc-ER, black columns) or pBabe-IRES-puromycin (Puro control) retroviruses (white columns). Puromycin-resistant cells were treated with 2 μmol/L 4-HT to activate the Myc-ER^{TAM} transgene. Levels of mRNA were standardized to the expression of *Ub*. **B**, SYBR Green real-time PCR analysis of *Skp2* and *p27* RNA levels in primary early-passage MEFs infected with pBabe-Myc-ER^{TAM}-IRES-puromycin (Myc-ER) or pBabe-IRES-puromycin (Puro control) retroviruses. Puromycin-resistant cells were pretreated with cycloheximide (Chx) for 30 min and then treated with 2 μmol/L 4-HT for the times indicated. RNA was isolated from the cells and analyzed by real-time PCR. Levels of *Skp2* and *p27* mRNA were standardized to the expression of *Ub*. **C**, primary early-passage MEFs were infected with MSCV-IRES-puromycin (Puro) or MSCV-Myc-IRES-puromycin (Myc) retroviruses, puromycin selected, and treated with cycloheximide (10 μg/mL) for the indicated time. Protein levels were then assessed by immunoblotting.

Mechanistically, this occurs through Myc-mediated induction of upstream activators of the cyclin E-Cdk2-p27^{Kip1} pathway such as E2f1 (34) and of the Cul1 and Cks1 components of the SCF^{Skp2} complex that directs p27^{Kip1} degradation (31, 39). Specifically, Cks1 levels are markedly elevated in the premalignant B cells of Eμ-Myc transgenic mice, whereas the expression of the Skp1, Rbx1, and Cul1 components of the SCF^{Skp2} complex is similar to those expressed in B cells from wild-type littermates (31). However, the expression of the Skp2 F-box protein that binds to phospho-p27^{Kip1} is also elevated in precancerous Eμ-Myc B cells (Fig. 1A and B). Furthermore, Skp2 levels are also markedly elevated in lymphomas that arise in Eμ-Myc mice (Fig. 1C; ref. 31) and in human Burkitt lymphoma, where 12 of 14 Burkitt lymphoma samples analyzed expressed elevated levels of Skp2 mRNA and protein compared with control B cells (Fig. 1D and E). Increased levels of Skp2 were not due to *Skp2* amplification as previously noted in lung cancers (43), as assessed by Southern blot analyses of Eμ-Myc lymphomas (data not shown). Thus, Skp2 expression is augmented by Myc *in vivo*, and high levels of Skp2 are a hallmark of Myc-driven lymphoma.

To determine if Skp2 is also induced by Myc in other cell contexts, wild-type MEFs were infected with an MSCV-based retrovirus encoding the conditional Myc-ER^{TAM} transgene and the puromycin resistance (*Puro*^R) gene (44). As a control, MEFs were infected with a retrovirus only expressing the *Puro*^R gene. Puromycin-resistant cells were expanded in culture and then treated with the estrogen receptor (ER) agonist 4-HT, which selectively activates Myc-ER^{TAM}. As expected, Myc activation led to the induction of the direct Myc target gene *Ornithine decarboxylase* (*Odc*; ref. 45) and also led to the induction of *Skp2*, although the magnitude of the *Skp2* response was not as robust (Fig. 2A).

Myc activates the majority of its transcription targets by binding, in conjunction with its requisite dimerization partner Max, to E-box elements harboring CACGTG or CACATG recognition elements (46, 47). The mouse (and human) *Skp2* promoter-regulatory regions lack such sites, suggesting that Myc might regulate *Skp2* expression in an indirect fashion. To address this issue, Myc-ER^{TAM}-expressing MEFs were pretreated (for 30 minutes) with cycloheximide to block *de novo* protein synthesis. Activation

of Myc-ER^{TAM} failed to induce *Skp2* mRNA in the presence of cycloheximide, whereas *p27^{Kip1}* transcripts were still suppressed (Fig. 2B). To assess the possibility that elevated Myc levels affect the half-life of Skp2 transcript or protein, we analyzed Myc-expressing early-passage MEFs that were treated with actinomycin D, which blocks RNA synthesis or cycloheximide to block *de novo* protein synthesis. No increase in RNA half-life was detected (Supplementary Fig. S1A), whereas Skp2 protein half-life was significantly prolonged on ectopic Myc expression (Fig. 2C). Therefore, the regulation of *Skp2* by Myc is indirect and involves transcriptional as well as posttranslational mechanisms.

Skp2 Induction by Myc Is Independent of E2f1

E2f1 is necessary for Myc to suppress *p27^{Kip1}* protein levels, and *E2f1* is induced by Myc (34). Skp2 and E2f1 are both elevated in Ras-induced lymphomas (48) and *Skp2* has been identified as an E2f1 transcription target (49). Indeed, in wild-type MEFs infected with a retrovirus encoding ER-*E2f1*, a conditionally activatable ER fusion of E2f1 (50), treatment with 4-HT induced *Skp2* transcripts as well as the well-characterized E2f1 target genes *thymidine kinase* and *dihydrofolate reductase* (Supplementary Fig. S1B; ref. 35). Furthermore, *Skp2* promoter activity was significantly induced following cotransfection of an E2f1 expression plasmid (Supplementary Fig. S1C). Thus, we predicted that Myc would induce *Skp2* via the agency of E2f1 and tested this hypothesis by evaluating the expression of Skp2 in the precancerous B220⁺ B cells of *Eμ-Myc;E2f1^{+/+}* versus *Eμ-Myc;E2f1^{-/-}* littermates. As expected, *E2f1* transcripts were elevated in precancerous *Eμ-Myc* B cells (34), and *Skp2* mRNA levels were elevated 3- to 4-fold in *Eμ-Myc* B cells compared with levels expressed in the B cells of nontransgenic littermates. Surprisingly, similarly increased levels of *Skp2* transcripts were evident in *Eμ-Myc;E2f1^{-/-}* B cells (Fig. 3A); thus, the induction of *Skp2* expression by Myc, at least in this cell context, is E2f1 independent. Furthermore, the lymphomas that arose in *Eμ-Myc;E2f1^{-/-}* mice actually expressed somewhat higher levels of Skp2 protein than those expressed in *Eμ-Myc;E2f1^{+/+}* lymphomas (data not shown). Finally, Skp2 protein levels were similarly elevated in *E2f1^{+/+}* versus *E2f1^{-/-}* MEFs transduced with MSCV-*Myc-IRES-GFP* retrovirus (Fig. 3B). Therefore, the induction of Skp2 by Myc is E2f1 independent.

Loss of Skp2 Does Not Significantly Delay Myc-Induced Lymphoma Onset

Cks1 loss triples the life span of *Eμ-Myc* transgenics (31). The markedly increased levels of Skp2 in *Eμ-Myc* transgenic B cells and Myc-driven lymphomas (Fig. 1) suggested that Skp2 might also play critical roles in Myc-induced tumorigenesis. To test this hypothesis, we initially coexpressed Skp2 or Cks1 with Myc in immortalized BALB/c-3T3 fibroblasts. However, there were no appreciable effects of enforced expression of either Skp2 or Cks1 on Myc-induced colony formation in soft agar (Supplementary Fig. S2A-C).

To directly assess the role of Skp2 in Myc-induced tumorigenesis, *Eμ-Myc* transgenics (C57BL/6) were mated to *Skp2^{-/-}* mice (17) and *Eμ-Myc;Skp2^{+/-}* F1 offspring were bred to *Skp2^{+/-}* mice to obtain the desired *Eμ-Myc;Skp2^{+/+}*, *Eμ-Myc;Skp2^{+/-}*, and *Eμ-Myc;Skp2^{-/-}* cohort. These littermates were followed for lymphoma onset, and 4-week-old mice were assessed for hallmarks of the precancerous phase of the disease, including lymphocytosis and splenomegaly. WBC numbers and spleen weights of *Skp2^{-/-}* mice were similar to those of *Skp2^{+/+}* littermates (data not shown). As expected, *Eμ-Myc;Skp2^{+/+}* mice showed elevated numbers of WBC and lymphocytes, as well as obvious splenomegaly. Notably, there were moderate reductions in total WBC numbers in *Skp2*-null *Eμ-Myc* transgenics (*Eμ-Myc;Skp2^{-/-}*, $8.0 \pm 3.2 \times 10^3/\mu\text{L}$, versus *Eμ-Myc;Skp2^{+/+}*, $11.6 \pm 2.6 \times 10^3/\mu\text{L}$; Fig. 4A, left), and there were corresponding reductions in lymphocyte numbers (*Eμ-Myc;Skp2^{-/-}*, $4.3 \pm 1.4 \times 10^3/\mu\text{L}$, versus *Eμ-Myc;Skp2^{+/+}*, $7.6 \pm 1.0 \times 10^3/\mu\text{L}$; $P < 0.05$; Fig. 4A, middle). Finally, the spleens of *Eμ-Myc;Skp2^{-/-}* mice were smaller than those of *Eμ-Myc;Skp2^{+/+}* littermates (spleen sizes, 174 ± 29 mg versus 123 ± 24 mg for *Eμ-Myc;Skp2^{+/+}* versus

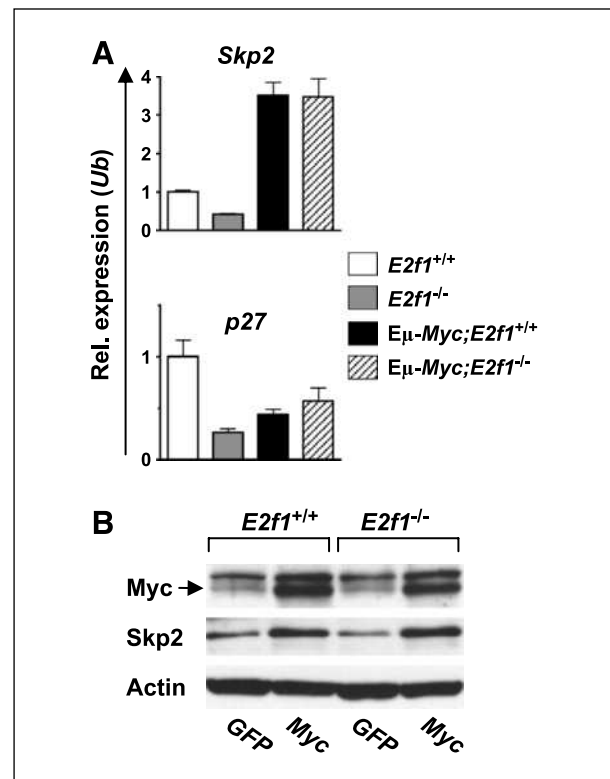


FIGURE 3. Myc regulation of Skp2 is independent of E2f1. A, SYBR Green real-time PCR analysis of *Skp2* and *p27* RNA expression in splenic B220⁺ B cells from 4-wk-old nontransgenic and *Eμ-Myc* transgenic mice of the indicated *E2f1* genotypes. Levels of RNA were standardized to *Ub*. B, immunoblot analyses of the levels of c-Myc, Skp2, and actin in FACS-sorted GFP-expressing primary *E2f1^{+/+}* and *E2f1^{-/-}* MEFs infected with MSCV-*Myc-IRES-GFP* (*Myc*) or MSCV-*IRES-GFP* (*GFP*) retroviruses.

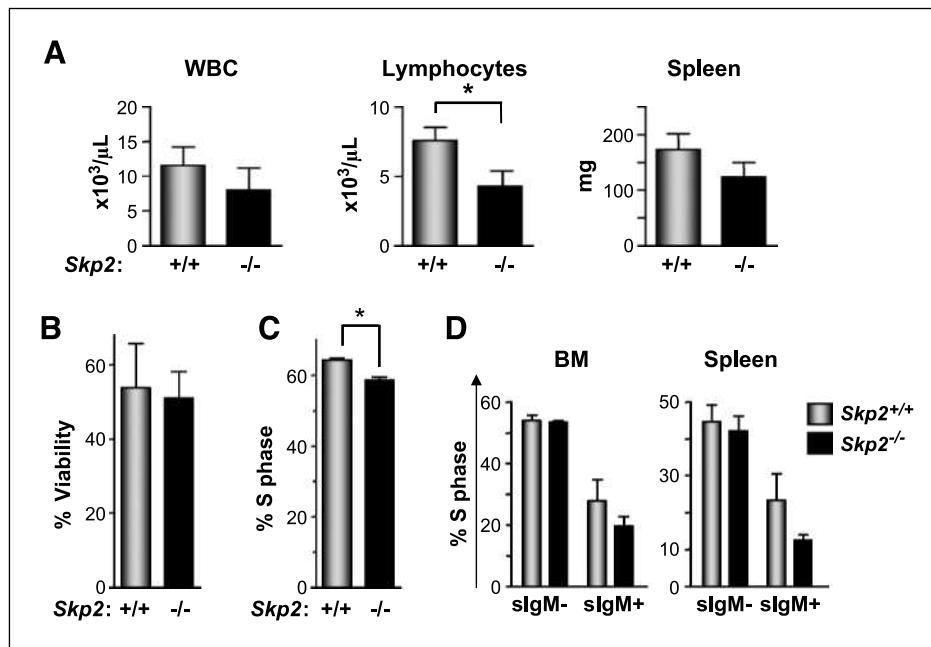


FIGURE 4. The effects of the *Skp2* deficiency on Myc-induced proliferation. A, precancerous (4-wk-old) $E\mu$ -Myc transgenic mice of the indicated *Skp2* genotype were analyzed for WBC (left) and lymphocyte numbers in the peripheral blood (middle) and for weights of their spleens (right). *, $P < 0.05$. B, B cells from *ex vivo* cultured bone marrow of $E\mu$ -Myc;*Skp2*^{+/+} and $E\mu$ -Myc;*Skp2*^{-/-} mice were assessed for their spontaneous apoptotic index ($n = 3$). C, bromodeoxyuridine incorporation into DNA (S phase) was used to assess the S-phase indices of B cells cultured *ex vivo* from the bone marrow of precancerous $E\mu$ -Myc;*Skp2*^{+/+} versus $E\mu$ -Myc;*Skp2*^{-/-} mice ($n = 3$). Columns, mean; bars, SE. *, $P < 0.05$. D, $E\mu$ -Myc;*Skp2*^{+/+} and $E\mu$ -Myc;*Skp2*^{-/-} littermates were injected with bromodeoxyuridine, and cells from bone marrow and spleen were harvested after 12 h. Bromodeoxyuridine incorporation was then determined by FACS. Columns, mean of three independent experiments; bars, SE. The differences between $E\mu$ -Myc;*Skp2*^{+/+} and $E\mu$ -Myc;*Skp2*^{-/-} genotypes were not statistically significant.

$E\mu$ -Myc;*Skp2*^{-/-} cohorts; Fig. 4A, right). There were essentially no effects of *Skp2* heterozygosity on these parameters (data not shown). Therefore, loss of *Skp2* moderately attenuates the precancerous phase of disease in $E\mu$ -Myc transgenic mice.

The premalignant B220⁺ B cells of $E\mu$ -Myc transgenics have high proliferative indices, but this response is counterbalanced by the activation of apoptotic checkpoints in these cells (29, 34). There was no difference in the apoptotic indices of precancerous B220⁺ $E\mu$ -Myc;*Skp2*^{+/+} and $E\mu$ -Myc;*Skp2*^{-/-} B cells *in vivo* by Annexin V⁺ fluorescence-activated cell sorting (FACS) analyses (data not shown). Further, the apoptotic indices of $E\mu$ -Myc;*Skp2*^{+/+} and $E\mu$ -Myc;*Skp2*^{-/-} B cells cultured *ex vivo* in medium supplemented with interleukin-7 were similar (Fig. 4B). Loss of *Cks1* markedly impairs the hyperproliferative response of $E\mu$ -Myc B cells (31). Thus, we predicted that *Skp2* loss would similarly affect the proliferative response of Myc. Indeed, *Skp2*-deficient $E\mu$ -Myc B cells had significantly slower growth indices than B cells derived from the bone marrow of their wild-type transgenic littermates when cultured *ex vivo* (Fig. 4C). However, these differences were not manifest *in vivo*, where the proliferative indices of $E\mu$ -Myc;*Skp2*^{+/+} and $E\mu$ -Myc;*Skp2*^{-/-} B220⁺ B cells were similar (Fig. 4D). Therefore, unlike *Cks1* (31), *Skp2* does not contribute to the proliferative response of Myc in B cells *in vivo*.

$E\mu$ -Myc transgenic mice succumb to aggressive, disseminating pre-B/immature B-cell lymphoma, generally within 4 months of age (41). Quite remarkably, *Cks1* loss nearly triples the life span of $E\mu$ -Myc mice (31). Nontransgenic littermates lacking *Skp2* showed no signs of tumor development throughout their life span. Surprisingly, $E\mu$ -Myc;*Skp2*^{-/-} transgenic mice showed an only moderately delayed course of lymphoma development, with a median survival of 143 days compared with median survival of 97 days of their $E\mu$ -Myc;*Skp2*^{+/+} littermates ($P = 0.405$, not significant; Fig. 5). There was no effect of *Skp2* heterozygosity on survival (median survival of 95 days; Fig. 5). The lymphomas that arose in *Skp2*-null $E\mu$ -Myc transgenics were phenotypically identical (pre-B and immature B-cell lymphomas) to those that arose in wild-type $E\mu$ -Myc transgenic littermates (data not shown). Thus, in sharp contrast to *Cks1* loss, *Skp2* loss has very moderate, statistically nonsignificant effects on Myc-driven lymphomagenesis.

Skp2 Loss Abolishes the Ability of Myc to Suppress p27^{Kip1}

Given the ability of Myc to induce *Skp2* expression while repressing p27^{Kip1} protein levels (Fig. 1), and the well-established role of the SCF^{Skp2} complex in directing p27^{Kip1} degradation (12, 17), we evaluated p27^{Kip1} RNA and protein levels in splenic B220⁺ B cells from precancerous $E\mu$ -Myc;*Skp2*^{+/+} and $E\mu$ -Myc;*Skp2*^{-/-} littermates.

Notably, the ability of Myc to suppress p27^{Kip1} protein levels was essentially cancelled in *Skp2*-deficient E μ -Myc B cells, both *in vivo* (Fig. 6A) and *ex vivo* (Fig. 6B). By contrast, p27^{Kip1} mRNA was suppressed in all E μ -Myc B cells, regardless of their *Skp2* status (Fig. 6C). Thus, like Cks1 (31), Skp2 is specifically required for Myc-mediated downregulation of p27^{Kip1} protein levels. Finally, unlike lymphomas that arose in E μ -Myc;*Skp2*^{+/+} littermates, nearly all E μ -Myc;*Skp2*^{-/-} lymphomas maintained high levels of p27^{Kip1} protein expression (data not shown).

Skp2 has been suggested to regulate Myc ubiquitination and stability and to function as an essential coactivator of Myc-mediated transactivation (51, 52). If Skp2 were to play essential role in regulating Myc protein levels, a prediction was that Myc protein levels would be elevated in *Skp2*-deficient E μ -Myc transgenic B cells. This was clearly not the case, as *Skp2* loss had essentially no effect on the steady-state levels of Myc protein in E μ -Myc transgenic B cells, either *in vivo* or *ex vivo* (Fig. 6A and B). We also addressed if Skp2 affected the transcriptional activity of Myc analyzing the expression of the established Myc target genes *Cad* (53) and *Rcl* (54) in precancerous B220⁺ B cells from E μ -Myc;*Skp2*^{+/+} versus E μ -Myc;*Skp2*^{-/-} littermates. There were essentially no changes in the levels of *Rcl* transcript in this cohort, and, if anything, the levels of *Cad* mRNA were elevated by *Skp2* heterozygosity or loss (Supplementary Fig. S3). Therefore, at least the induction of these two bona fide transcription targets of Myc is independent of Skp2.

Discussion

Myc promotes cell cycle entry and accelerates the rates of proliferation by suppressing the levels of p27^{Kip1}, a key cell cycle inhibitor (31, 34, 40). The SCF^{Skp2} allosteric regulator Cks1 is a target induced by Myc that clearly plays major roles in the proliferative response of Myc and in Myc-driven tumorigenesis in the E μ -Myc transgenic mouse model of human B-cell lymphoma. Further, Cks1 overexpression is a hallmark of Myc-driven lymphomas in mouse and man, and is absolutely required for Myc to suppress p27^{Kip1} protein levels *in vivo* (31). Here, we report Skp2 as yet another component of the SCF^{Skp2} ubiquitin ligase complex that is regulated by Myc, and in other cell contexts, the SCF^{Skp2} Cul1 scaffold protein is induced by Myc (39). Thus, Myc orchestrates the ubiquitin-mediated degradation of p27^{Kip1} by affecting highly specific (Skp2 and Cks1; ref. 13, 14, 17, 31) and rather ubiquitously expressed components (Cul1; ref. 39) of the SCF^{Skp2} ubiquitin ligase.

Skp2 was revealed, as predicted, to be essential for Myc-mediated suppression of p27^{Kip1} levels in E μ -Myc B cells. These findings, along with those showing that p27^{Kip1} deficiency accelerates lymphoma onset in E μ -Myc transgenics (40) and that *Cks1* loss impairs Myc-induced proliferation and lymphomagenesis (31), strongly suggested that *Skp2* loss would cancel the proliferative response of Myc and thus impair Myc-induced lymphoma development. Sur-

prisingly, this was not the case, where despite fully restoring p27^{Kip1} levels the effects of *Skp2* loss on Myc-induced proliferation and lymphoma development were at most moderate compared with those manifest in the *Cks1* deficiency (31). These findings suggest that at least in this context Cks1 has functions other than that as a regulator of p27^{Kip1} that also contribute to Myc-induced proliferation and tumorigenesis. Indeed, Cks1 has functions as a regulator of transcription in yeast (55, 56) and human cancer cells (57) and seems to have Skp2/p27^{Kip1}-independent functions in controlling human multiple myeloma cell growth and survival (58).

The importance of Skp2 in cancer has been documented, where Skp2 expression is highly elevated in several malignancies and where this is associated with reduced p27^{Kip1} levels, high proliferative rates, and poor outcome (15, 59). Furthermore, Skp2 cooperates with oncogenic N-Ras in promoting anchorage-independent growth of rodent fibroblasts *in vitro* and in promoting lymphomagenesis *in vivo* (48). In contrast, Skp2 does not augment Myc-induced soft agar growth of fibroblasts (Supplementary Fig. S2), and *Skp2* loss has no significant effects on Myc-driven lymphomagenesis despite canceling the ability of Myc to suppress p27^{Kip1} protein levels. We conclude that there are context-specific effects of Skp2 in tumorigenesis.

Myc oncoproteins are short-lived, and Myc turnover occurs through the ubiquitin-proteasome pathway (60). Skp2 has been suggested to bind to c-Myc, to promote its ubiquitination and degradation, and to also augment its transactivation functions (51, 52). If this scenario were operational in B cells, then *Skp2* loss should have at least led to increased levels of Myc protein in E μ -Myc B cells

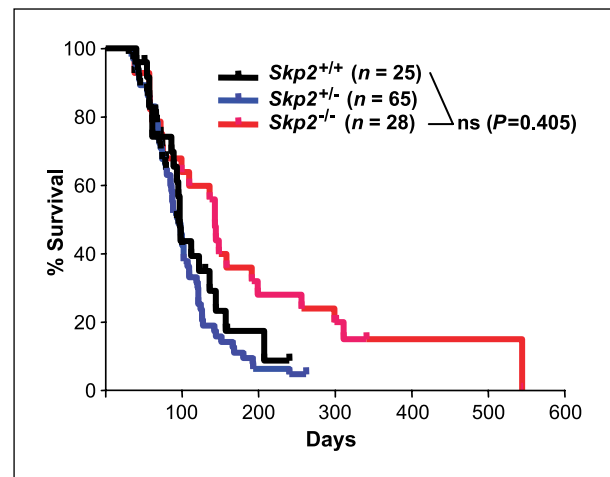


FIGURE 5. *Skp2* loss has no significant effect on Myc-driven lymphomagenesis compared with wild-type controls. The survival of E μ -Myc transgenic littermates of the indicated *Skp2* genotypes is shown. The differences in the rates of tumor incidence between the *Skp2*^{+/+} and the *Skp2*^{-/-} group are not statistically significant (Cox regression analysis: significance, 0.405; hazard ratio, 0.61; 95% confidence interval, 0.33-1.16). A statistically significant difference arises when all three cohorts are included in a log-rank (Mantel-Cox) test ($P = 0.017$). Numbers of animals per group are given in parentheses.

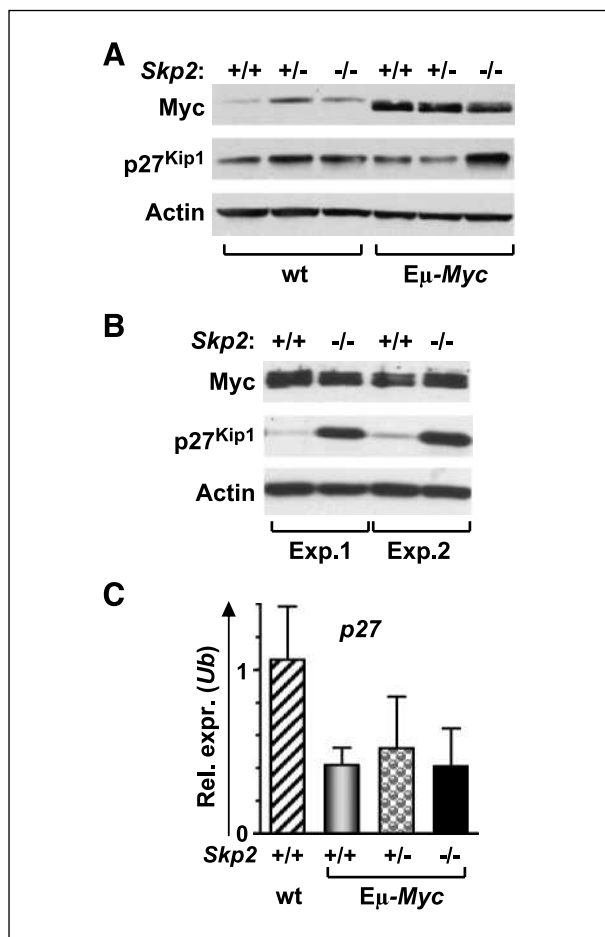


FIGURE 6. Skp2 is required for Myc-mediated suppression of p27^{Kip1}. A, immunoblot analyses of Myc and p27^{Kip1} protein levels in precancerous (4-wk-old) splenic B220⁺ B cells from nontransgenic (wt) and Eμ-Myc transgenics of the indicated *Skp2* genotypes. B, immunoblot analysis of Myc and p27^{Kip1} levels in *ex vivo* cultured B cells from precancerous Eμ-Myc; *Skp2*^{+/+} versus Eμ-Myc; *Skp2*^{-/-} mice. Two separate experiments of B cells cultured from different paired littermates are shown. C, SYBR Green real-time PCR analysis of *p27* mRNA levels in splenic B cells from precancerous Eμ-Myc; *Skp2*^{+/+}, Eμ-Myc; *Skp2*^{+/-}, and Eμ-Myc; *Skp2*^{-/-} mice compared with a nontransgenic wild-type littermate. Levels of mRNAs were standardized to the expression of *Ub*.

and thus perhaps to accelerated disease, as homozygous Eμ-Myc transgenics develop more rapid lymphomas than hemizygous Eμ-Myc littermates (61). Neither of these responses was, however, evident in Eμ-Myc; *Skp2*^{-/-} mice, and the expression of at least some established Myc targets was also unaffected in *Skp2*-deficient Eμ-Myc B cells. Our findings are thus more in accord with those of others indicating that the F-box protein Fbw7 (20, 62) or HectH9 (63) regulates Myc turnover.

In normal cells, the ability of Myc to accelerate proliferation is harnessed by the activation of apoptotic pathways, and disabling this response, by loss-of-function mutations in the Arf-p53 tumor suppressor pathway (29), dramatically accelerates the course of Myc-induced malignancies. The effects of biallelic loss of *p27*^{Kip1} on lymphoma onset in

Eμ-Myc mice are less dramatic (40), yet the *Skp2*/Cks1-p27^{Kip1} pathway is affected in all Myc-driven lymphomas (this report and ref. 31). Myc-mediated induction of *Skp2* is indirect, suggesting that Myc may work through the agency of other transcription factors to induce *Skp2*. One candidate was E2f1, as Myc induces *E2f1* and because E2f1 is required for Myc-mediated repression of p27^{Kip1} in Eμ-Myc B cells (34). Further, in immortal fibroblasts and some tumor cell lines, E2f1 promotes p27^{Kip1} degradation via its induction of *Skp2* (49), and in pancreatic cancer, the malignant phenotype is associated with E2f1-dependent induction of *Skp2* (64). The finding that Myc-induced expression of *Skp2* is independent of E2f1 was thus surprising, and a role for FoxM1, another activator of *Skp2* transcription (65), seems also unlikely, as FoxM1 expression is reduced in Eμ-Myc B cells and lymphomas (31). Thus, other transcriptional regulators downstream of Myc must control *Skp2* expression. The complexity of *Skp2* regulation is further documented by an increase in *Skp2* protein half-life that points to posttranslational effects of Myc. The fact that the effects of *Skp2* loss on Myc-induced lymphomagenesis are moderate at best, however, strongly points toward Cks1 roles besides SCF^{Skp2} and p27^{Kip1} control.

Skp2 overexpression in cancer has heretofore been linked to *SKP2* gene amplification, E2f1, and FoxM1. Our findings strongly suggest that Myc regulates *Skp2* expression to control p27^{Kip1} levels. The effect of *Skp2* loss on Myc-driven proliferation and lymphomagenesis was surprisingly modest. Given differences in the magnitude of the effects of *Cks1* versus *Skp2* loss on Myc-mediated tumorigenesis suggest other Cks1 targets (e.g., not linked to SCF^{Skp2}) that can be exploited in cancer therapeutics.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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