Skp2 Directs Myc-Mediated Suppression of p27Kip1 yet Has Modest Effects on Myc-Driven Lymphomagenesis

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Abstract

The universal cyclin-dependent kinase inhibitor p27Kip1 functions as a tumor suppressor, and reduced levels of p27Kip1 connote poor prognosis in several human malignancies. p27Kip1 levels are predominately regulated by ubiquitin-mediated turnover of the protein, which is marked for destruction by the E3 ubiquitin ligase SCFSkp2 complex following its phosphorylation by the cyclin E–cyclin-dependent kinase 2 complex. Binding of phospho-p27Kip1 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 p27Kip1 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 p27Kip1 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 p27Kip1, are critical for Myc-driven proliferation and tumorigenesis. Mol Cancer Res; 8(3); 353–62. ©2010 AACR.

Introduction

The cyclin-dependent kinase (Cdk) inhibitor p27Kip1 binds to and inactivates cyclin-Cdk complexes to restrict the traverse of cells through the G1 and S phases of the cell cycle (1). p27Kip1 overexpression triggers cell cycle arrest in G1 (2), whereas loss of p27Kip1 in mice increases rates of cell proliferation (3, 4). Patients with tumors having low or undetectable levels of p27Kip1 protein have a very poor outcome (5, 6), yet unlike other tumor suppressors, p27Kip1 is only rarely directly mutated. Further, mice heterozygous for p27Kip1 develop spontaneous tumors late in life, yet these retain and still express the normal p27Kip1 allele (7). Finally, the subcellular localization of p27Kip also has prognostic significance, where high cytoplasmic p27Kip1, which is driven by activated Akt, is associated with poor outcome (Liang et al., 2002).

Signals that control p27Kip1 protein levels include its phosphorylation on Thr187 by the cyclin E–Cdk2 complex in S phase (8–10). Thr187-phosphorylated p27Kip1 is targeted to the proteasome by the SCFSkp2 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 SCFSkp2 complex for phospho-p27Kip1 (11-14), and their binding then leads to the ubiquitylation and destruction of p27Kip1 (15). Accordingly, elevated levels of Skp2 in human cancer correlate with low p27Kip1 levels (15), and enforced Skp2 expression in transgenic mice reduces p27Kip1 levels and induces proliferation (16). By contrast, the targeted deletion of Skp2 leads to p27Kip1 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
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 p27kip1 (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 p27kip1 expression at the level of transcription (33), but its effects on p27kip1 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 p27kip1, thus relieving inhibition of cyclin E–Cdk2 complexes (36, 37). Under either scenario, activated cyclin E–Cdk2 complexes then phosphorylate p27kip1 on Thr187, allowing its recognition by the SCFSkp2 complex, ubiquitination, and degradation by the proteasome (8, 9, 38). Finally, Myc induces the expression of some of the components of the SCFSkp2 complex, including Cul1 (39) and Cks1, and at least the latter is required for downregulation of p27kip1 (31).

p27kip1 is a key regulator of Myc-induced proliferation and tumorigenesis. First, loss of p27kip1 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 Cdk1, effectively cancels the ability of Myc to suppress p27kip1 protein (but not p27kip1 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 SCFskp2 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 p27kip1 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 p27kip1, 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 <sup>TAM</sup>-IRESGFP, MSCV-Myc-IRESGFP, pBabe-Myc-ER <sup>TAM</sup>-IRESPuromycin, pBabe-ER <sup>TAM</sup>-E2f1-puramycin, 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 RNAeasy 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 μg 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$^\text{Kip1}$ (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 Eμ-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$^\text{Kip1}$ expression primarily by provoking ubiquitin-mediated destruction of p27$^\text{Kip1}$ protein (8).

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**FIGURE 1.** Skp2 expression is elevated in precancerous Eμ-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 Eμ-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 Eμ-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 Eμ-Myc lymphoma. Eμ-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.
Mechanistically, this occurs through Myc-mediated induction of upstream activators of the cyclin E–Cdk2–p27Kip1 pathway such as E2f1 (34) and of the Cul1 and Cks1 components of the SCFSkp2 complex that directs p27Kip1 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 SCFSkp2 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 phospho-p27Kip1 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<sup>Tam</sup> transgene and the p27Kip1 p27Kip1 degradation (31, 39). As a control, MEFs were infected with a retrovirus only expressing the p27 transeps. 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. B, SYBR Green real-time PCR analysis of Skp2 and p27 RNA levels in primary early-passage MEFs infected with pBabe-Myc-ER<sup>Tam</sup>-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<sup>Tam</sup> 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<sup>Tam</sup>-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.

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<sup>Tam</sup>-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<sup>Tam</sup> 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<sup>Tam</sup>-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. B, SYBR Green real-time PCR analysis of Skp2 and p27 RNA levels in primary early-passage MEFs infected with pBabe-Myc-ER<sup>Tam</sup>-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.
of Myc-ER\textsuperscript{TAM} failed to induce Skp2 mRNA in the presence of cycloheximide, whereas p27\textsuperscript{Skp1} 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 expression (Fig. 2C). Therefore, the regulation of protein half-life was significantly prolonged on ectopic 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, E\textsuperscript{Myc} transgenics (C57BL/6) were mated to Skp2\textsuperscript{−/−} mice (17) and E\textsuperscript{Myc};Skp2\textsuperscript{−/−} F1 offspring were bred to Skp2\textsuperscript{−/−} mice to obtain the desired E\textsuperscript{Myc};Skp2\textsuperscript{−/−}, E\textsuperscript{Myc};Skp2\textsuperscript{+/-}, and E\textsuperscript{Myc};Skp2\textsuperscript{+/-} cohort. These littersmates 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\textsuperscript{−/−} mice were similar to those of Skp2\textsuperscript{+/-} littermates (data not shown). As expected, E\textsuperscript{Myc};Skp2\textsuperscript{+/-} 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 E\textsuperscript{Myc} transgenics (E\textsuperscript{Myc};Skp2\textsuperscript{−/−}, 8.0 ± 3.2 × 10\textsuperscript{9}/\mu L, versus E\textsuperscript{Myc};Skp2\textsuperscript{+/-}, 11.6 ± 2.6 × 10\textsuperscript{9}/\mu L); Fig. 4A, left), and there were corresponding reductions in lymphocyte numbers (E\textsuperscript{Myc};Skp2\textsuperscript{−/−}, 4.3 ± 1.4 × 10\textsuperscript{9}/\mu L, versus E\textsuperscript{Myc};Skp2\textsuperscript{+/-}, 7.6 ± 1.0 × 10\textsuperscript{9}/\mu L; P < 0.05; Fig. 4A, middle). Finally, the spleens of E\textsuperscript{Myc};Skp2\textsuperscript{−/−} mice were smaller than those of E\textsuperscript{Myc};Skp2\textsuperscript{+/-} littermates (spleen sizes, 174 ± 29 mg versus 123 ± 24 mg for E\textsuperscript{Myc};Skp2\textsuperscript{+/-} versus E\textsuperscript{Myc};Skp2\textsuperscript{−/−}).

**Skp2 Induction by Myc Is Independent of E2f1**

E2f1 is necessary for Myc to suppress p27\textsuperscript{Skp1} 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 Skp2 would be induced by Myc and tested this hypothesis by evaluating the expression of Skp2 in the precancerous E2f1−/+ cells versus E2f1−/− littermates. As expected, E2f1 transcripts were elevated in precancerous E2f1−/+ cells (34), and Skp2 mRNA levels were elevated 3- to 4-fold in E2f1−/+ versus E2f1−/− MEFs (Fig. 4A, left). Therefore, in at least this cell context, E2f1 is independent. Furthermore, the lymphomas that arose in E2f1−/+ mice actually expressed somewhat higher levels of Skp2 protein than those expressed in 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. 4B). Therefore, the induction of Skp2 by Myc is E2f1 independent.

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

Csk1 loss triples the life span of E\textsuperscript{Myc} transgenics (31), and the markedly increased levels of Skp2 in E\textsuperscript{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).

**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 E\textsuperscript{Myc} transgenic mice of the indicated E2f1 genotype. 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.
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**FIGURE 4.** The effects of the Skp2 deficiency on Myc-induced proliferation. A, precancerous (4-wk-old) Eμ-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 Eμ-Myc;Skp2+/+ and Eμ-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 bone marrow of precancerous Eμ-Myc;Skp2+/+ versus Eμ-Myc;Skp2−/− mice (n = 3). Columns, mean; bars, SE. *, P < 0.05. D, Eμ-Myc;Skp2+/+ and Eμ-Myc;Skp2−/− littersmates 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 Eμ-Myc;Skp2+/+ and Eμ-Myc;Skp2−/− genotypes were not statistically significant.

Eμ-Myc;Skp2−/− 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+ Eμ-Myc;Skp2+/+ and Eμ-Myc;Skp2−/− B cells in vivo by Annexin V+ fluorescence-activated cell sorting (FACS) analyses (data not shown). Further, the apoptotic indices of Eμ-Myc;Skp2+/+ and Eμ-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 Eμ-Myc B cells (31). Thus, we predicted that Skp2 loss would similarly affect the proliferative response of Myc. Indeed, Skp2-deficient Eμ-Myc B cells had significantly slower growth indices than B cells derived from the bone marrow of their wild-type transgenic littersmates when cultured ex vivo (Fig. 4C). However, these differences were not manifest in vivo, where the proliferative indices of Eμ-Myc;Skp2+/+ and Eμ-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.

Eμ-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 Eμ-Myc mice (31). Nontransgenic littersmates lacking Skp2 showed no signs of tumor development throughout their life span. Surprisingly, Eμ-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 Eμ-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 Eμ-Myc transgenics were phenotypically identical (pre-B and immature B-cell lymphomas) to those that arose in wild-type Eμ-Myc transgenic littersmates (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 p27Kip1

Given the ability of Myc to induce Skp2 expression while repressing p27Kip1 protein levels (Fig. 1), and the well-established role of the SCF^Skp2^ complex in directing p27Kip1 degradation (12, 17), we evaluated p27Kip1 RNA and protein levels in splenic B220+ B cells from precancerous Eμ-Myc;Skp2+/+ and Eμ-Myc;Skp2−/− littersmates.
By contrast, p27Kip1 genes analyzing the expression of the established Myc target Skp2 from Eμ-Myc-mediated downregulation of p27Kip1 protein levels. Thus, like Cks1 (31), Skp2 is specifically required for Myc proliferation by suppressing the levels of p27Kip1, a key cell cycle inhibitor (31). These findings suggest that at least in this context Cks1 has functions other than that as a regulator of p27Kip1 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/p27Kip1-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 p27Kip1 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 p27Kip1 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 Eμ-Myc B cells.

**Discussion**

Myc promotes cell cycle entry and accelerates the rates of proliferation by suppressing the levels of p27Kip1, 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 Eμ-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 p27Kip1 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 p27Kip1 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 p27Kip1 levels in Eμ-Myc B cells. These findings, along with those showing that p27Kip1 deficiency accelerates lymphoma onset in Eμ-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. Surprisingly, this was not the case, where despite fully restoring p27Kip1 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 p27Kip1 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/p27Kip1-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 p27Kip1 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 p27Kip1 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 Eμ-Myc B cells.
and thus perhaps to accelerated disease, as homozygous Eμ-Myc transgensics 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 HctH9 (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 p27Kip1 on lymphoma onset in Eμ-Myc mice are less dramatic (40), yet the Skp2/Cks1-p27Kip1 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 p27Kip1 in Eμ-Myc B cells (34). Further, in immortal fibroblasts and some tumor cell lines, E2f1 promotes p27Kip1 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<sub>Skp2</sub> and p27<sup>Kip1</sup> 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<sup>Kip1</sup> 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<sub>Skp2</sub>) that can be exploited in cancer therapeutics.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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