Published OnlineFirst March 2, 2010; DOI: 10.1158/1541-7786.MCR-09-0232

Cancer Genes and Genomics

Molecular Cancer Research

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 G_1 and S phases of the cell cycle (1). $p27^{Kip1}$ overexpression triggers cell cycle arrest in G_1 (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 $p27^{Kip}$ 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 Thr^{187} by the cyclin E–Cdk2 complex in S phase (8-10). Thr^{187}-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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Note: Supplementary data for this article are available at Molecular Cancer Research Online (http://mcr.aacrjournals.org/).

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doi: 10.1158/1541-7786.MCR-09-0232

Published OnlineFirst March 2, 2010; DOI: 10.1158/1541-7786.MCR-09-0232

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 Thr^{187}, 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). F_1 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. F_1 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

Published OnlineFirst March 2, 2010; DOI: 10.1158/1541-7786.MCR-09-0232

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

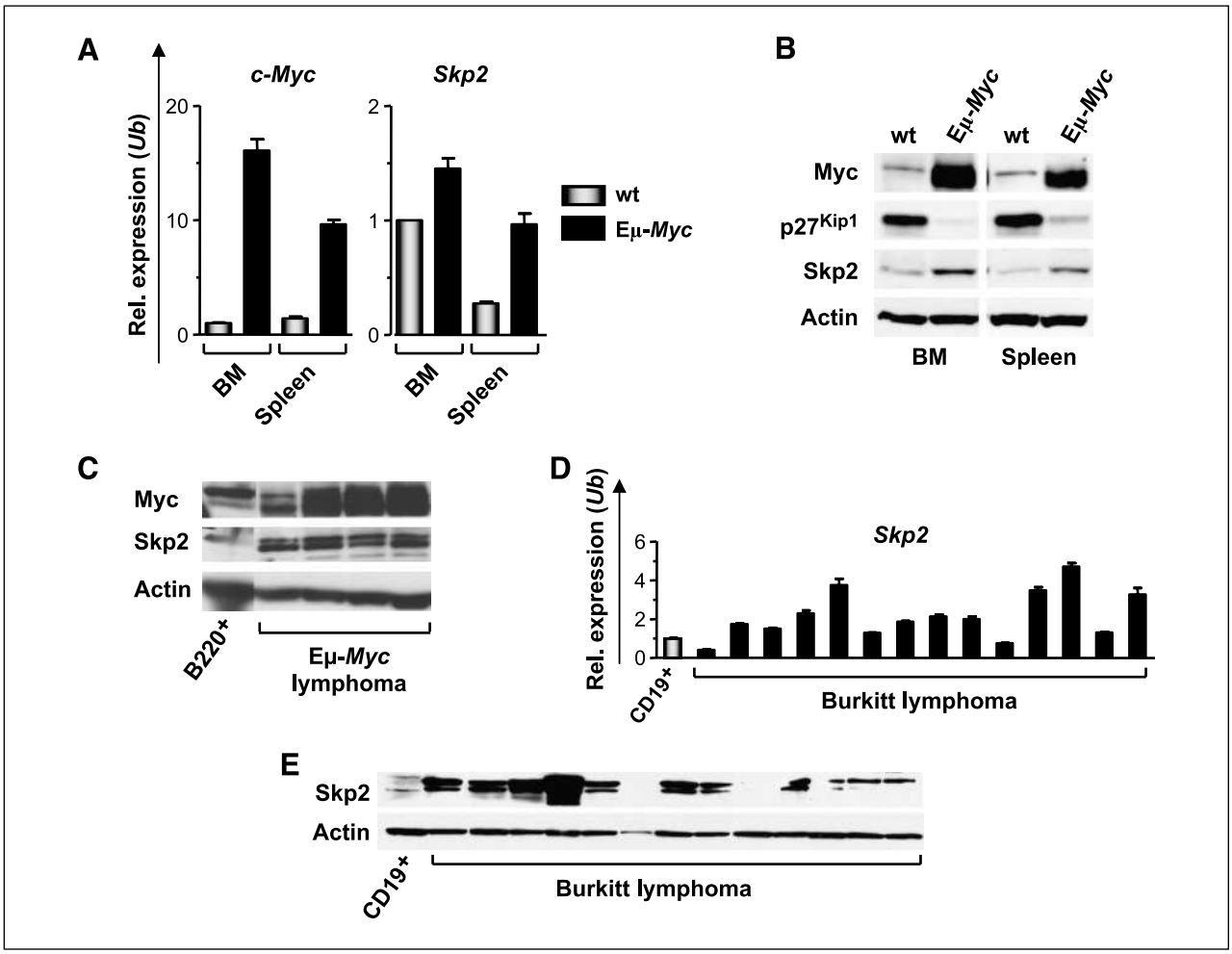

FIGURE 1. Skp2 expression is elevated in precancerous Eμ-*Myc* B cells and in Myc-induced lymphomas. A, SYBR Green real-time PCR analysis of levels of *c-Myc* and *Skp2* transcripts in bone marrow (BM) and splenic (Spleen) $B220^+$ B cells from 4-wk-old wild-type (wt; gray columns) or Eμ-*Myc* (black 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.

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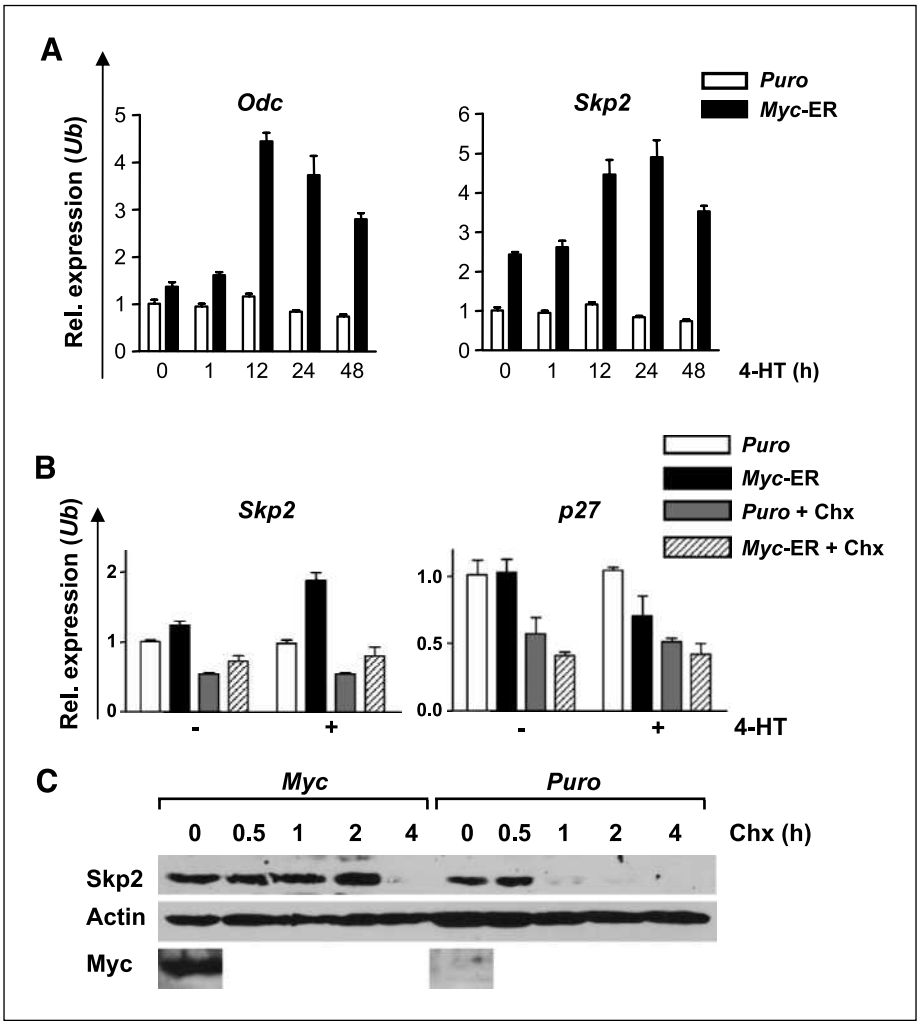

FIGURE 2. Myc regulation of *Skp2* is indirect. A, SYBR Green real-time PCR analysis of *Odc* and *Skp2* RNA levels in primary early-passage MEFs infected with pBabe-*Myc*-ER^{TAM}-IRES-puromycin (*Myc*-ER, black columns) or pBabe-IRES-puromycin (*Puro* control) retroviruses (white columns). Puromycin-resistant 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

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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 ± 3.2 × 10^3/μL, versus Eμ-*Myc;Skp2$^{+/+}$*, 11.6 ± 2.6 × 10^3/μL; Fig. 4A, left), and there were corresponding reductions in lymphocyte numbers (Eμ-*Myc;Skp2$^{-/-}$*, 4.3 ± 1.4 × 10^3/μL, versus Eμ-*Myc;Skp2$^{+/+}$*, 7.6 ± 1.0 × 10^3/μL; $P < 0.05$; 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

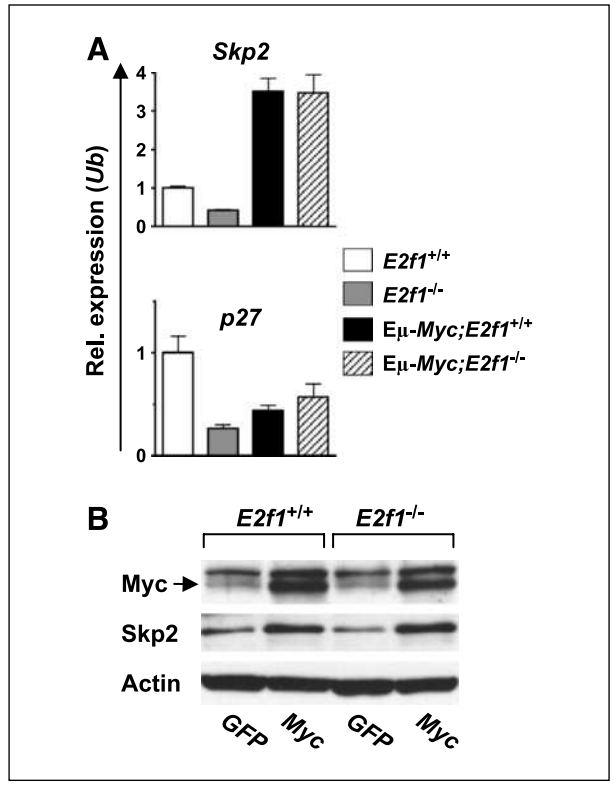

FIGURE 3. Myc regulation of Skp2 is independent of E2f1. A, SYBR Green real-time PCR analysis of *Skp2* and *p27* RNA expression in splenic B220$^+$ B cells from 4-wk-old nontransgenic and Eμ-*Myc* transgenic mice of the indicated *E2f1* genotypes. Levels of RNA were standardized to *Ub*. B, immunoblot 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.

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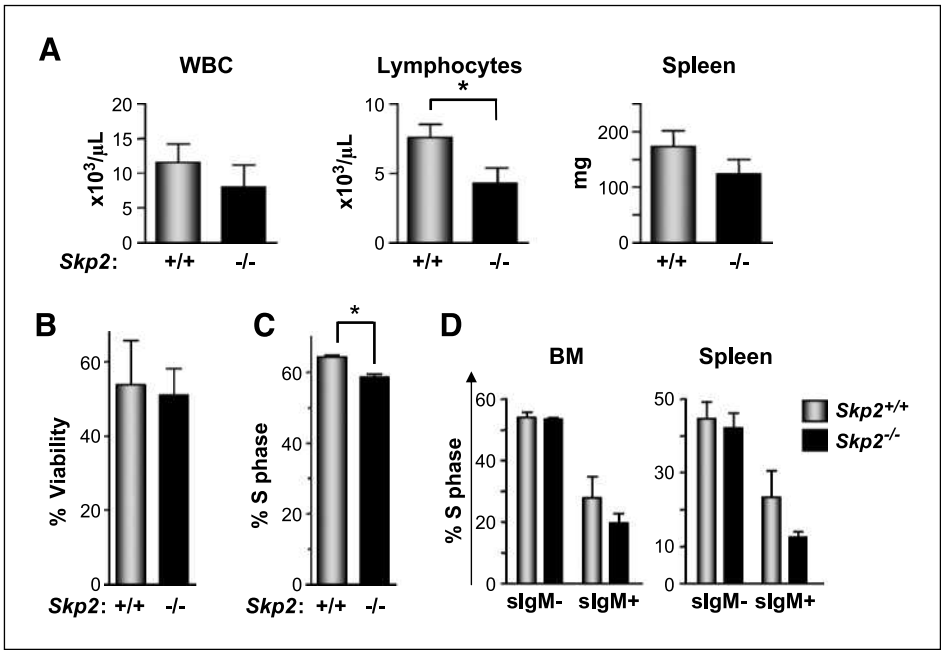

FIGURE 4. The effects of the *Skp2* deficiency on Myc-induced proliferation. A, precancerous (4-wk-old) Eμ-*Myc* transgenic mice of the indicated *Skp2* genotype were analyzed for WBC (left) and lymphocyte numbers in the peripheral blood (middle) and for weights of their spleens (right). *, $P < 0.05$. 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 the 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*$^{-/-}$ 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 Eμ-*Myc;Skp2*$^{+/+}$ and Eμ-*Myc;Skp2*$^{-/-}$ genotypes were not statistically significant.

Eμ-*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 Eμ-*Myc* transgenic mice.

The premalignant B220$^+$ B cells of Eμ-*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$^+$ 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 littermates 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 littermates 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* heterozygozity 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 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 Eμ-*Myc;Skp2*$^{+/+}$ and Eμ-*Myc;Skp2*$^{-/-}$ littermates.

Published OnlineFirst March 2, 2010; DOI: 10.1158/1541-7786.MCR-09-0232

Notably, the ability of Myc to suppress $p27^{Kip1}$ protein levels was essentially cancelled in *Skp2*-deficient Eμ-*Myc* B cells, both *in vivo* (Fig. 6A) and *ex vivo* (Fig. 6B). By contrast, $p27^{Kip1}$ mRNA was suppressed in all Eμ-*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 Eμ-*Myc;Skp2*$^{+/+}$ littermates, nearly all Eμ-*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 essentials role in regulating Myc protein levels, a prediction was that Myc protein levels would be elevated in *Skp2*-deficient Eμ-*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 Eμ-*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 Eμ-*Myc;Skp2*$^{+/+}$ versus Eμ-*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 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 $p27^{Kip1}$ 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 Eμ-*Myc* B cells. These findings, along with those showing that $p27^{Kip1}$ 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 $p27^{Kip1}$ 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 be 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 Eμ-*Myc* B cells

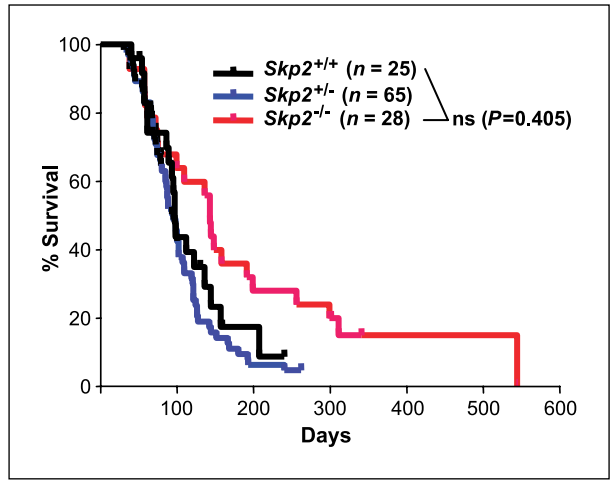

FIGURE 5. *Skp2* loss has no significant effect on Myc-driven lymphomagenesis compared with wild-type controls. The survival of Eμ-*Myc* transgenic littermates of the indicated *Skp2* genotypes is shown. The differences in the rates of tumor incidence between the *Skp2*$^{+/+}$ and the *Skp2*$^{-/-}$ 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.

Published OnlineFirst March 2, 2010; DOI: 10.1158/1541-7786.MCR-09-0232

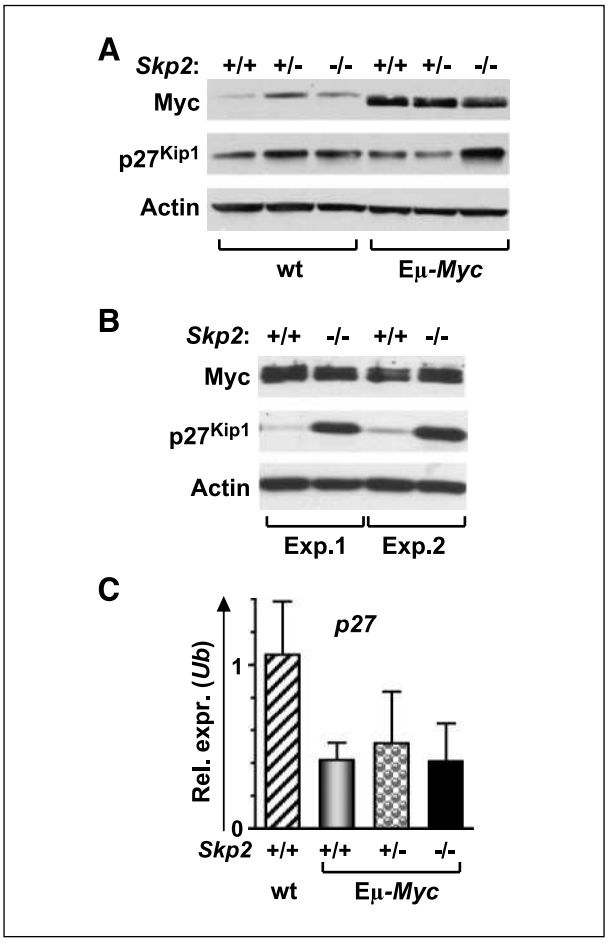

FIGURE 6. Skp2 is required for Myc-mediated suppression of $p27^{Kip1}$. A, immunoblot analyses of Myc and $p27^{Kip1}$ protein levels in precancerous (4-wk-old) splenic $B220^+$ B cells from nontransgenic (wt) and Eμ-*Myc* transgenics of the indicated *Skp2* genotypes. B, immunoblot analysis of Myc 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.

Acknowledgments

We thank Sara Norton, Chunying Yang, and Elsie White for expert technical assistance; the Animal Resource Center, the Hartwell Center, and the FACS Core Facility of St. Jude Children's Research Hospital for animal care and technical support; Tibor Schuster (Institute for Medical Statistics and Epidemiology, TU München, Munich, Germany) for statistical analyses; Kristian Helin (Copenhagen, Denmark) for providing ER-E2f1 plasmid; Roland M. Schmid (Munich, Germany) for providing the CMV-E2f1 plasmid and the Skp2-promoter reporter construct; Michael Deininger (Portland, OR) for providing the Skp2 cDNA; and Drs. Mihaela Onciu and John Sandlund (Memphis, TN) for providing Burkitt lymphoma samples.

Grant Support

NIH grant CA76379 (J.L. Cleveland), Cancer Center Core grant CA21765, American Lebanese Syrian Associated Charities of St. Jude Children's Research Hospital, and State of Florida to Scripps Florida. U.B. Keller was supported by Deutsche Forschungsgemeinschaft grant SFB TRR54. J.B. Old was supported by National Research Service Award grant F32 CA099478.

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Received 05/28/2009; revised 12/15/2009; accepted 01/18/2010; published OnlineFirst 03/02/2010.

Published OnlineFirst March 2, 2010; DOI: 10.1158/1541-7786.MCR-09-0232

References

1. Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G_1-phase progression. Genes Dev 1999;13:1501–12.
2. Coats S, Flanagan WM, Nourse J, Roberts JM. Requirement of p27Kip1 for restriction point control of the fibroblast cell cycle. Science 1996;272:877–80.
3. Kiyokawa H, Kineman RD, Manova-Todorova KO, et al. Enhanced growth of mice lacking the cyclin-dependent kinase inhibitor function of p27(Kip1). Cell 1996;85:721–32.
4. Fero ML, Rivkin M, Tasch M, et al. A syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis, and female sterility in p27(Kip1)-deficient mice. Cell 1996;85:733–44.
5. Slingerland J, Pagano M. Regulation of the cdk inhibitor p27 and its deregulation in cancer. J Cell Physiol 2000;183:10–7.
6. Philipp-Staheli J, Payne SR, Kemp CJ. p27(Kip1): regulation and function of a haploinsufficient tumor suppressor and its misregulation in cancer. Exp Cell Res 2001;264:148–68.
7. Fero ML, Randel E, Gurley KE, Roberts JM, Kemp CJ. The murine gene p27Kip1 is haplo-insufficient for tumour suppression. Nature 1998;396:177–80.
8. Muller D, Bouchard C, Rudolph B, et al. Cdk2-dependent phosphorylation of p27 facilitates its Myc-induced release from cyclin E/cdk2 complexes. Oncogene 1997;15:2561–76.
9. Montagnoli A, Fiore F, Eytan E, et al. Ubiquitination of p27 is regulated by Cdk-dependent phosphorylation and trimeric complex formation. Genes Dev 1999;13:1181–9.
10. Malek NP, Sundberg H, McGrew S, Nakayama K, Kyriakides TR, Roberts JM. A mouse knock-in model exposes sequential proteolytic pathways that regulate p27Kip1 in G_1 and S phase. Nature 2001;413:323–7.
11. Bartek J, Lukas J. Cell cycle. Order from destruction. Science 2001; 294:66–7.
12. Carrano AC, Eytan E, Hershko A, Pagano M. SKP2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27. Nat Cell Biol 1999;1:193–9.
13. Ganoth D, Bornstein G, Ko TK, et al. The cell-cycle regulatory protein Cks1 is required for SCF(Skp2)-mediated ubiquitinylation of p27. Nat Cell Biol 2001;3:321–4.
14. Spruck C, Strohmaier H, Watson M, et al. A CDK-independent function of mammalian Cks1: targeting of SCF(Skp2) to the CDK inhibitor p27Kip1. Mol Cell 2001;7:639–50.
15. Kudo Y, Kitajima S, Sato S, Miyauchi M, Ogawa I, Takata T. High expression of S-phase kinase-interacting protein 2, human F-box protein, correlates with poor prognosis in oral squamous cell carcinomas. Cancer Res 2001;61:7044–7.
16. Shim EH, Johnson L, Noh HL, et al. Expression of the F-box protein SKP2 induces hyperplasia, dysplasia, and low-grade carcinoma in the mouse prostate. Cancer Res 2003;63:1583–8.
17. Nakayama K, Nagahama H, Minamishima YA, et al. Targeted disruption of Skp2 results in accumulation of cyclin E and p27(Kip1), polyploidy and centrosome overduplication. EMBO J 2000;19:2069–81.
18. Henriksson M, Luscher B. Proteins of the Myc network: essential regulators of cell growth and differentiation. Adv Cancer Res 1996; 68:109–82.
19. Nilsson JA, Cleveland JL. Myc pathways provoking cell suicide and cancer. Oncogene 2003;22:9007–21.
20. Welcker M, Orian A, Jin J, et al. The Fbw7 tumor suppressor regulates glycogen synthase kinase 3 phosphorylation-dependent c-Myc protein degradation. Proc Natl Acad Sci U S A 2004;101:9085–90.
21. Liu J, Levens D. Making myc. Curr Top Microbiol Immunol 2006;302: 1–32.
22. Boxer LM, Dang CV. Translocations involving c-myc and c-myc function. Oncogene 2001;20:5595–610.
23. Cavalieri F, Goldfarb M. Growth factor-deprived BALB/c 3T3 murine fibroblasts can enter the S phase after induction of c-myc gene expression. Mol Cell Biol 1987;7:3554–60.
24. Roussel MF, Cleveland JL, Shurtleff SA, Sherr CJ. Myc rescue of a mutant CSF-1 receptor impaired in mitogenic signalling. Nature 1991;353:361–3.
25. Coppola JA, Cole MD. Constitutive c-myc oncogene expression blocks mouse erythroleukaemia cell differentiation but not commitment. Nature 1986;320:760–3.
26. Askew DS, Ashmun RA, Simmons BC, Cleveland JL. Constitutive c-myc expression in an IL-3-dependent myeloid cell line suppresses cell cycle arrest and accelerates apoptosis. Oncogene 1991;6: 1915–22.
27. Zindy F, Eischen CM, Randle DH, et al. Myc signaling via the ARF tumor suppressor regulates p53-dependent apoptosis and immortalization. Genes Dev 1998;12:2424–33.
28. Eischen CM, Woo D, Roussel MF, Cleveland JL. Apoptosis triggered by Myc-induced suppression of Bcl-X(L) or Bcl-2 is bypassed during lymphomagenesis. Mol Cell Biol 2001;21:5063–70.
29. Eischen CM, Weber JD, Roussel MF, Sherr CJ, Cleveland JL. Disruption of the ARF-Mdm2-p53 tumor suppressor pathway in Myc-induced lymphomagenesis. Genes Dev 1999;13:2658–69.
30. Vlach J, Hennecke S, Alevizopoulos K, Conti D, Amati B. Growth arrest by the cyclin-dependent kinase inhibitor p27Kip1 is abrogated by c-Myc. EMBO J 1996;15:6595–604.
31. Keller UB, Old JB, Dorsey FC, et al. Myc targets Cks1 to provoke the suppression of p27(Kip1), proliferation and lymphomagenesis. EMBO J 2007;26:2562–74.
32. Polyak K, Lee MH, Erdjument-Bromage H, et al. Cloning of p27Kip1, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. Cell 1994;78:59–66.
33. Yang W, Shen J, Wu M, et al. Repression of transcription of the p27 (Kip1) cyclin-dependent kinase inhibitor gene by c-Myc. Oncogene 2001;20:1688–702.
34. Baudino TA, Maclean KH, Brennan J, et al. Myc-mediated proliferation and lymphomagenesis, but not apoptosis, are compromised by E2f1 loss. Mol Cell 2003;11:905–14.
35. DeGregori J, Kowalik T, Nevins JR. Cellular targets for activation by the E2F1 transcription factor include DNA synthesis- and G_1/S-regulatory genes. Mol Cell Biol 1995;15:4215–24.
36. Bouchard C, Thieke K, Maier A, et al. Direct induction of cyclin D2 by Myc contributes to cell cycle progression and sequestration of p27. EMBO J 1999;18:5321–33.
37. Hermeking H, Rago C, Schuhmacher M, et al. Identification of CDK4 as a target of c-MYC. Proc Natl Acad Sci U S A 2000;97: 2229–34.
38. Pagano M, Tam SW, Theodoras AM, et al. Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. Science 1995;269:682–5.
39. O'Hagan RC, Ohh M, David G, et al. Myc-enhanced expression of Cul1 promotes ubiquitin-dependent proteolysis and cell cycle progression. Genes Dev 2000;14:2185–91.
40. Martins CP, Berns A. Loss of p27(Kip1) but not p21(Cip1) decreases survival and synergizes with MYC in murine lymphomagenesis. EMBO J 2002;21:3739–48.
41. Adams JM, Harris AW, Pinkert CA, et al. The c-myc oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice. Nature 1985;318:533–8.
42. Yamasaki L, Jacks T, Bronson R, Goillot E, Harlow E, Dyson NJ. Tumor induction and tissue atrophy in mice lacking E2F-1. Cell 1996; 85:537–48.
43. Yokoi S, Yasui K, Mori M, Iizasa T, Fujisawa T, Inazawa J. Amplification and overexpression of SKP2 are associated with metastasis of non-small-cell lung cancers to lymph nodes. Am J Pathol 2004;165: 175–80.
44. Maclean KH, Keller UB, Rodriguez-Galindo C, Nilsson JA, Cleveland JL. c-Myc augments γ irradiation-induced apoptosis by suppressing Bcl-XL. Mol Cell Biol 2003;23:7256–70.
45. Bello-Fernandez C, Packham G, Cleveland JL. The ornithine decarboxylase gene is a transcriptional target of c-Myc. Proc Natl Acad Sci U S A 1993;90:7804–8.
46. Blackwood EM, Eisenman RN. Max: a helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with Myc. Science 1991;251:1211–7.
47. Zeller KI, Jegga AG, Aronow BJ, O'Donnell KA, Dang CV. An integrated database of genes responsive to the Myc oncogenic

Published OnlineFirst March 2, 2010; DOI: 10.1158/1541-7786.MCR-09-0232

transcription factor: identification of direct genomic targets. Genome Biol 2003;4:R69.
48. Latres E, Chiarle R, Schulman BA, et al. Role of the F-box protein Skp2 in lymphomagenesis. Proc Natl Acad Sci U S A 2001;98:2515–20.
49. Zhang L, Wang C. F-box protein Skp2: a novel transcriptional target of E2F. Oncogene 2006;25:2615–27.
50. Vigo E, Muller H, Prosperini E, et al. CDC25A phosphatase is a target of E2F and is required for efficient E2F-induced S phase. Mol Cell Biol 1999;19:6379–95.
51. Kim SY, Herbst A, Tworkowski KA, Salghetti SE, Tansey WP. Skp2 regulates Myc protein stability and activity. Mol Cell 2003;11:1177–88.
52. von der Lehr N, Johansson S, Wu S, et al. The F-box protein Skp2 participates in c-Myc proteosomal degradation and acts as a cofactor for c-Myc-regulated transcription. Mol Cell 2003;11:1189–200.
53. Frank SR, Schroeder M, Fernandez P, Taubert S, Amati B. Binding of c-Myc to chromatin mediates mitogen-induced acetylation of histone H4 and gene activation. Genes Dev 2001;15:2069–82.
54. Lewis BC, Shim H, Li Q, et al. Identification of putative c-Myc-responsive genes: characterization of rcl, a novel growth-related gene. Mol Cell Biol 1997;17:4967–78.
55. Morris MC, Kaiser P, Rudyak S, Baskerville C, Watson MH, Reed SI. Cks1-dependent proteasome recruitment and activation of CDC20 transcription in budding yeast. Nature 2003;423:1009–13.
56. Yu VP, Baskerville C, Grunenfelder B, Reed SI. A kinase-independent function of Cks1 and Cdk1 in regulation of transcription. Mol Cell 2005;17:145–51.
57. Westbrook L, Manuvakhova M, Kern FG, Estes NR II, Ramanathan HN, Thottassery JV. Cks1 regulates cdk1 expression: a novel role during mitotic entry in breast cancer cells. Cancer Res 2007;67:11393–401.
58. Zhan F, Colla S, Wu X, et al. CKS1B, overexpressed in aggressive disease, regulates multiple myeloma growth and survival through SKP2- and p27Kip1-dependent and -independent mechanisms. Blood 2007;109:4995–5001.
59. Yang G, Ayala G, De Marzo A, et al. Elevated Skp2 protein expression in human prostate cancer: association with loss of the cyclin-dependent kinase inhibitor p27 and PTEN and with reduced recurrence-free survival. Clin Cancer Res 2002;8:3419–26.
60. Salghetti SE, Kim SY, Tansey WP. Destruction of Myc by ubiquitin-mediated proteolysis: cancer-associated and transforming mutations stabilize Myc. EMBO J 1999;18:717–26.
61. Sidman CL, Denial TM, Marshall JD, Roths JB. Multiple mechanisms of tumorigenesis in Eμ-myc transgenic mice. Cancer Res 1993;53:1665–9.
62. Yada M, Hatakeyama S, Kamura T, et al. Phosphorylation-dependent degradation of c-Myc is mediated by the F-box protein Fbw7. EMBO J 2004;23:2116–25.
63. Adhikary S, Marinoni F, Hock A, et al. The ubiquitin ligase HectH9 regulates transcriptional activation by Myc and is essential for tumor cell proliferation. Cell 2005;123:409–21.
64. Reichert M, Saur D, Hamacher R, Schmid RM, Schneider G. Phosphoinositide-3-kinase signaling controls S-phase kinase-associated protein 2 transcription via E2F1 in pancreatic ductal adenocarcinoma cells. Cancer Res 2007;67:4149–56.
65. Wang IC, Chen YJ, Hughes D, et al. Forkhead box M1 regulates the transcriptional network of genes essential for mitotic progression and genes encoding the SCF (Skp2-Cks1) ubiquitin ligase. Mol Cell Biol 2005;25:10875–94.

Published OnlineFirst March 2, 2010; DOI: 10.1158/1541-7786.MCR-09-0232

Molecular Cancer Research

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Mol Cancer Res 2010;8:353-362. Published OnlineFirst March 2, 2010.