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Skp2 directs Myc-mediated suppression of p27^{Kip1} yet has modest effects on Myc-driven lymphomagenesis

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Abstract

The universal cyclin-Cdk 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-Cdk2 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 impact of Skp2 loss on Myc-driven proliferation and lymphomagenesis was surprisingly modest compared to 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.

Keywords

Myc; Skp2; p27^{Kip1}; lymphomagenesis

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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), while 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 Threonine-187 by the cyclin E-Cdk2 complex in S phase (8–10). Threonine-187 phosphorylated $p27^{Kip1}$ is targeted to the proteasome by the SCF^{Skp2} ubiquitin ligase complex that is comprised 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 correlates 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 (bHLHZip) 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 accelerates 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 down-regulate the expression of the Cdk inhibitor $p27^{Kip1}$ (30–31) which inactivates cyclin E-Cdk2 and cyclin A-Cdk2 complexes that are 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 phosphorylates $p27^{Kip1}$ 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 down-regulation of $p27^{Kip1}$ (31).

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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 Myc's ability to suppress p27^{Kip1} protein (but not *p27^{Kip1}* mRNA) levels, impairs Myc-induced proliferation, and markedly delays lymphoma onset and triples the lifespan 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.

Results

Myc Induces Skp2 Expression

Myc suppresses p27^{Kip1} expression primarily by provoking ubiquitin-mediated destruction of p27^{Kip1} protein (8). Mechanistically this occurs through Myc-mediated induction of upstream activators of the cyclin E-Cdk2-to- p27^{Kip1} pathway such as E2f1 (Baudino *et al.*, 2003) 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 pre-malignant B cells of Eμ-*Myc* transgenic mice, whereas the expression of the Skp1, Rbx and Cul1 components of the SCF^{Skp2} complex are 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 pre-cancerous Eμ-*Myc* B cells (Fig. 1A, B). Furthermore, Skp2 levels are also markedly elevated in lymphomas that arise in Eμ-*Myc* mice (Fig. 1C and (31)) and in human Burkitt lymphoma (BL), where 12 of 14 BL samples analyzed expressed elevated levels of SKP2 mRNA and protein compared to control B cells (Fig. 1D, E). Increased levels of Skp2 were not due to *Skp2* amplification as previously noted in lung cancers (42), 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 mouse embryo fibroblasts (MEFs) were infected with an MSCV-based retrovirus encoding the conditional *Myc-ER^{TAM}* transgene and the *puromycin-resistance* (*Puro^{R}*) gene (43). As a control MEFs were infected with a retrovirus only expressing the *Puro^{R}* gene. Puromycin-resistant cells were expanded in culture and were then treated with the estrogen receptor (ER) agonist 4-hydroxytamoxifen (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*) (44) 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 (45–46). 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 pre-treated (for 30 min) with cycloheximide (Chx) to block *de novo* protein synthesis. Activation of Myc-ER^{TAM} failed to induce *Skp2* mRNA in the presence of Chx, 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 Chx to block *de novo* protein

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synthesis. No increase in RNA half-life was detected (Supplementary Fig. S1A), while Skp2 protein half-life was significantly prolonged upon ectopic Myc expression (Fig. 2C). Therefore, the regulation of *Skp2* by Myc is indirect and involves transcriptional as well as post-translational 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 (47) and *Skp2* has been identified as an E2f1 transcription target (48). Indeed, in wild type MEFs infected with a retrovirus encoding ER-*E2f1*, a conditionally activate-able ER fusion of E2f1 (49), treatment with 4-HT induced *Skp2* transcripts as well as the well-characterized E2f1 target genes *Thymidine kinase* (*Tk*) and *Dihydrofolate reductase* (*Dhfr*) (Supplementary Fig. S1B; (35)). Furthermore, *Skp2* promoter activity was significantly induced following co-transfection 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 pre-cancerous B220+ B cells of Eμ-*Myc;E2f1*$^{+/+}$ versus Eμ-*Myc;E2f1*$^{-/-}$ littermates. As expected, *E2f1* transcripts were elevated in pre-cancerous Eμ-*Myc* B cells (34), and *Skp2* mRNA levels were elevated 3–4-fold in Eμ-*Myc* B cells compared to levels expressed in the B cells of non-transgenic 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 lifespan of Eμ-*Myc* transgenics (Keller *et al.*, 2007). 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 co-expressed 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 pre-cancerous phase of the disease, including lymphocytosis and splenomegaly. White blood cell (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 $\times 10^3$/μl vs. Eμ-*Myc;Skp2*$^{+/+}$, 11.6±2.6 $\times 10^3$/μl, Fig. 4A, left panel) and there were corresponding reductions in lymphocyte numbers (Eμ-*Myc;Skp2*$^{-/-}$, 4.3±1.4 $\times 10^3$/μl vs. Eμ-*Myc;Skp2*$^{+/+}$, 7.6±1.0 $\times 10^3$/μl, Fig. 4A, middle panel, $p<0.05$). Finally, the spleens of Eμ-*Myc;Skp2*$^{-/-}$ mice were smaller than those of Eμ-*Myc;Skp2*$^{+/+}$ littermates (spleen sizes 174±29 mg vs. 123±24 mg for Eμ-*Myc;Skp2*$^{+/+}$ vs. Eμ-*Myc;Skp2*$^{-/-}$ cohorts, Fig. 4A, right panel). There were essentially no effects of *Skp2* heterozygosity on these parameters (data not shown). Therefore, loss of *Skp2* moderately attenuates the pre-cancerous phase of disease in Eμ-*Myc* transgenic mice.

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The pre-malignant $B220^+$ 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 pre-cancerous $B220^+$ Eμ-*Myc;Skp2*$^{+/+}$ and Eμ-*Myc;Skp2*$^{-/-}$ B cells in vivo by Annexin-V+ 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 was similar (Fig. 4B). Loss of *Cks1* markedly impairs the hyper-proliferative response of Eμ-*Myc* B cells (31). Thus, we predicted that *Skp2* loss would similarly affect Myc's proliferative response. 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 Myc's proliferative response 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 lifespan of Eμ-*Myc* mice (31). Non-transgenic littermates lacking *Skp2* showed no signs of tumor development throughout their lifespan. Surprisingly, Eμ-*Myc;Skp2*$^{-/-}$ transgenic mice showed an only moderately delayed course of lymphoma development, with a median survival of 143 days compared to 97 days median survival of their Eμ-*Myc;Skp2*$^{+/+}$ littermates (Fig. 5, p=0.405, not significant). There was no effect of *Skp2* heterozygozity on survival (95 days median survival, 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 non-significant effects on Myc-driven lymphomagenesis.

Skp2 Loss Abolishes Myc's Ability to Suppress $p27^{Kip1}$

Given Myc's ability to induce Skp2 expression whilst 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 pre-cancerous Eμ-*Myc;Skp2*$^{+/+}$ and Eμ-*Myc;Skp2*$^{-/-}$ littermates. Notably, Myc's ability 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 down-regulation 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 co-activator of Myc-mediated transactivation (50–51). 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, B). We also addressed if Skp2 affected Myc's transcriptional activity analyzing the expression of the established Myc target genes *Cad* (52) and *Rcl* (53) in pre-cancerous $B220^+$ 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.

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Discussion

Myc promotes cell cycle entry and accelerates the rates of proliferation by suppressing the levels of $p27^{Kip1}$, a key cell cycle inhibitor (Baudino *et al.*, 2003; Keller *et al.*, 2007; Martins and Berns, 2002). The SCF^{Skp2} allosteric regulator Cks1 is a target induced by Myc that clearly plays major roles in Myc's proliferative response 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, (13–14, 17, 31)) and rather ubiquitously expressed components (Cul1, (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 (Martins and Berns, 2002) and that *Cks1* loss impairs Myc-induced proliferation and lymphomagenesis (Keller *et al.*, 2007), strongly suggested that *Skp2* loss would cancel Myc's proliferative response 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 to 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 (54–55) and human cancer cells (56) and appears to have Skp2/$p27^{Kip1}$-independent functions in controlling human multiple myeloma cell growth and survival (57).

The importance of Skp2 in cancer has be documented, where Skp2 expression is highly elevated in a number of malignancies, and where this is associated with reduced $p27^{Kip1}$ levels, high proliferative rates, and poor outcome (15, 58). Furthermore, Skp2 cooperates with oncogenic N-Ras in promoting anchorage-independent growth of rodent fibroblasts in vitro, and in promoting lymphomagenesis in vivo (47). 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 Myc's ability 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 (59). Skp2 has been suggested to bind to c-Myc, to promote its ubiquitination and degradation, and to also augment its transactivation functions (50–51). 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* transgenics develop more rapid lymphomas than hemizygous Eμ-*Myc* littermates (60). 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 proteins Fbw7 (20, 61) or HectH9 (62) regulate Myc turnover.

In normal cells, Myc's ability 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

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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 (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 since 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* (48), and in pancreatic cancer, the malignant phenotype is associated with E2f1-dependent induction of Skp2 (63). 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 (64), 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 post-translational effects of Myc. The fact that the effects of *Skp2* loss on Myc-induced lymphomagenesis are moderate at best however strongly point towards 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 impact 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.

Materials and methods

Mice and Tumor Analysis

Skp2 null mice (C57Bl/6) (17) were bred with Eμ-*Myc* transgenic mice (C57Bl/6) (41). F_1 Eμ-*Myc;Skp2*$^{+/-}$ offspring were bred to *Skp2*$^{+/-}$ mice 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 (65), again on a C57BL/6 background) were bred with Eμ-*Myc* transgenic mice. F_1 Eμ-*Myc;E2f1*$^{+/-}$ offspring were bred to *E2f1*$^{+/-}$ 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 was prepared.

Cell Culture

Primary bone marrow-derived pre-B cells were cultured as described previously (29). MEFs from E13.5-E14.5 embryos were cultured and infected with MSCV-Myc-ER^{TM}-IRES-GFP, MSCV-Myc-IRES-GFP, pBabe-Myc-ER^{TM}-IRES-Puromycin, pBabe-ER^{TM}-E2f1-Puromycin or control retrovirus as described (27). To evaluate consequences of Myc activation cells were treated with 2-μM 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 pre-treated with 1μg/ml Cycloheximide (Chx, Sigma Chemicals) for 30 min (which inhibited >95% of protein synthesis), prior to adding 4-HT. For analysis of *Skp2* RNA half-life MEFs were cultured in the presence of 1μg/ml Actinomycin D (ActD, Sigma-

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Aldrich) and harvested at the indicated time. To estimate Skp2 protein half-life MEFs were cultured in the presence of 10μg/ml Chx and harvested at the indicated time.

FACS Analysis and Magnetic-Activated Cell Sorting (MACS) 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, MACS-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 performed using an iCycler machine (Bio-Rad) and the iTaq SYBR green kit (Bio-Rad). Data analyses were performed by comparing Ct values with a control sample set as 1. Sequences for primers are available upon request.

Immunoblotting

Protein extracts (20 or 50 μg per lane) were 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, Inc.) and β-Actin (Sigma Chemicals).

Statistical analyses

The statistical analysis of survival differences in Eμ-Myc transgenics of *Skp2−/−* vs. *Skp2+/+* genotype was performed using a Cox-Regression analysis with a multiple cohort comparison Bonferroni- adjusted. The statistics performed to analyze differences in the ex vivo and in vivo B cell proliferation and apoptosis indices involved paired t-tests.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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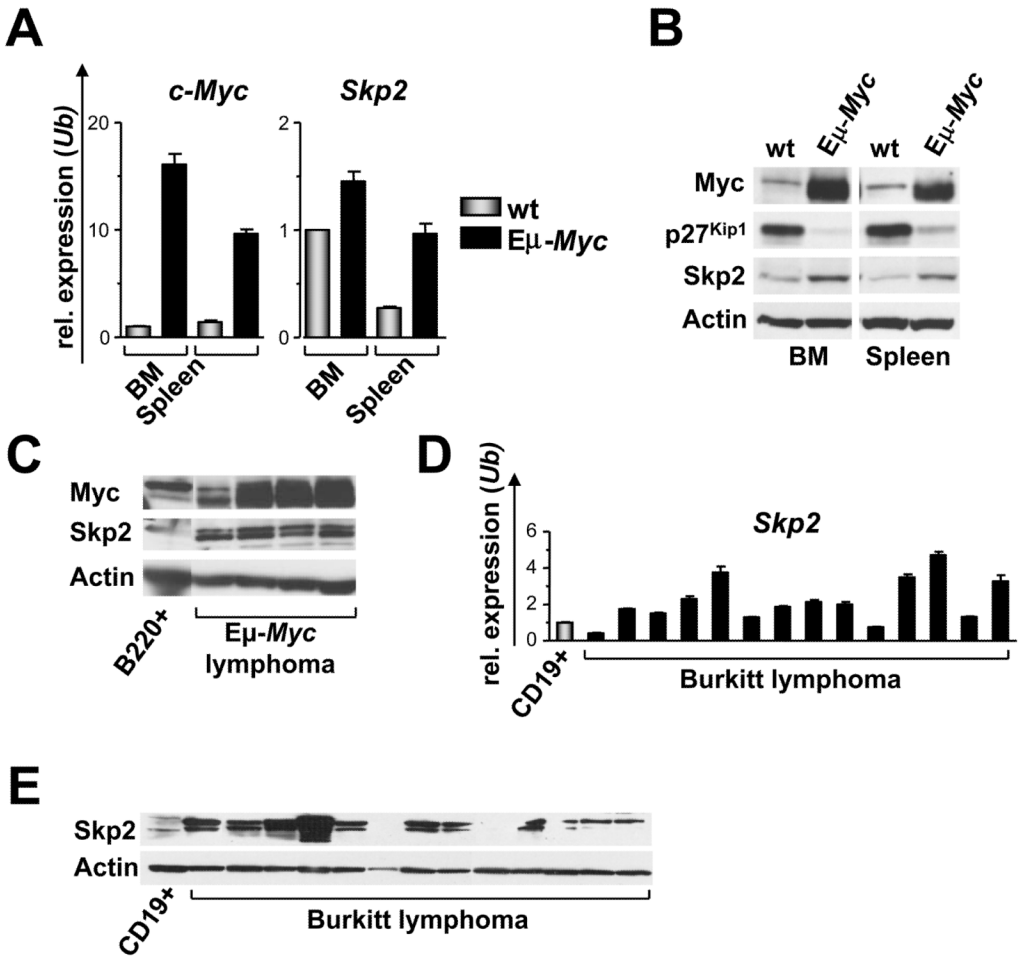

FIGURE 1.

Skp2 expression is elevated in pre-cancerous Eμ-*Myc* B cells and in Myc-induced lymphomas. **A.** SYBR-green real-time PCR analysis of levels of *c-myc*, *Skp2* and *p27* transcripts in bone marrow (BM) and splenic (spleen) $B220^+$ B cells from 4 week-old wild type (wt, gray bars) or Eμ-*Myc* (black bars) littermate mice. Levels of mRNA were standardized to the expression of *Ubiquitin* (*Ub*), which is not regulated by Myc. **B.** Immunoblot analyses p27 and Skp2 expression in $B220^+$ B cells from 4 week-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 fourteen Burkitt lymphoma samples (black bars) compared to normal human $CD19^+$ B cells (gray bar). Levels of mRNAs were standardized to the expression of *Ubiquitin* (*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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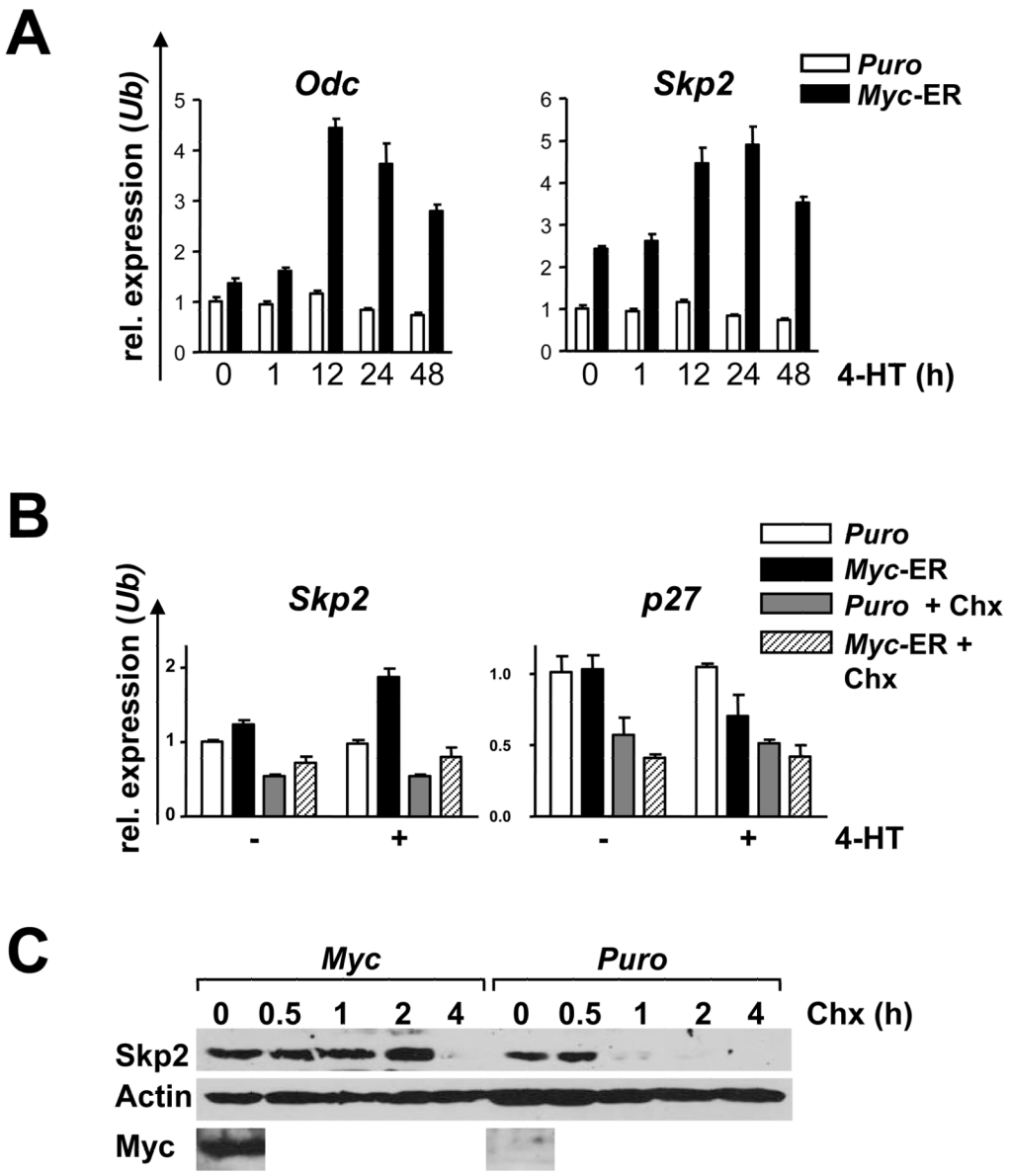

FIGURE 2.

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-Puro (*Myc*-ER, black bars) or pBabe-IRES-Puro (*Puro* control) retroviruses (white bars). Puromycin-resistant cells were treated with 2 μM 4-HT to activate the *Myc*-ER^{TAM} transgene. Levels of mRNA were standardized to the expression of *Ubiquitin* (*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-Puro (*Myc*-ER) or pBabe-IRES-Puro (*Puro* control) retroviruses. Puromycin-resistant cells were pre-treated with Chx for 30 min and were then treated with 2μm 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 *Ubiquitin* (*Ub*). **C.** Primary early passage MEFs were infected with MSCV-IRES-Puro (*Puro*) or MSCV-*Myc*-IRES-Puro (*Myc*) retroviruses, Puromycin-selected, and

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treated with Chx (10 μg/ml) for the indicated time. Proteins levels were then assessed by immunoblotting.

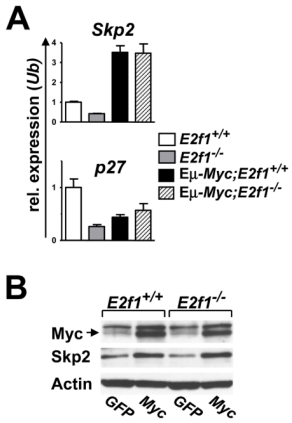

FIGURE 3.

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 week-old non-transgenic and Eμ-*Myc* transgenic mice of the indicated *E2f1* genotypes. Levels of RNA were standardized to *Ubiquitin* (*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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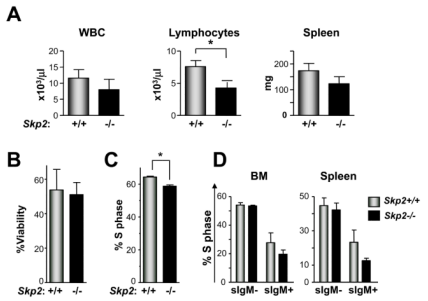

FIGURE 4.

The effects of the *Skp2* deficiency on Myc-induced proliferation. **A.** Pre-cancerous (4 week-old) Eμ-*Myc* transgenic mice of the indicated *Skp2* genotype were analyzed for white blood counts (WBC, *left panel*), and lymphocyte numbers in the peripheral blood (*middle panel*), and for weights of their spleens (*right panel*). * indicates $p<0.05$. **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.** BrdU-incorporation into DNA (S phase) was used to assess the S-phase indices of B cells cultured ex vivo from the bone marrow of pre-cancerous Eμ-*Myc;Skp2*$^{+/+}$ versus Eμ-*Myc;Skp2*$^{-/-}$ mice ($n = 3$; bars indicate mean ± SEM; * indicates $p<0.05$). **D.** Eμ-*Myc;Skp2*$^{+/+}$ and Eμ-*Myc;Skp2*$^{-/-}$ littermates were injected with BrdU, and cells from bone marrow (BM) and spleen were harvested after 12 hr. BrdU-incorporation was then determined by FACS. The bars show the mean ± SEM of three independent experiments. The differences between Eμ-*Myc;Skp2*$^{+/+}$ and Eμ-*Myc;Skp*$^{-/-}$ genotypes were not statistically significant.

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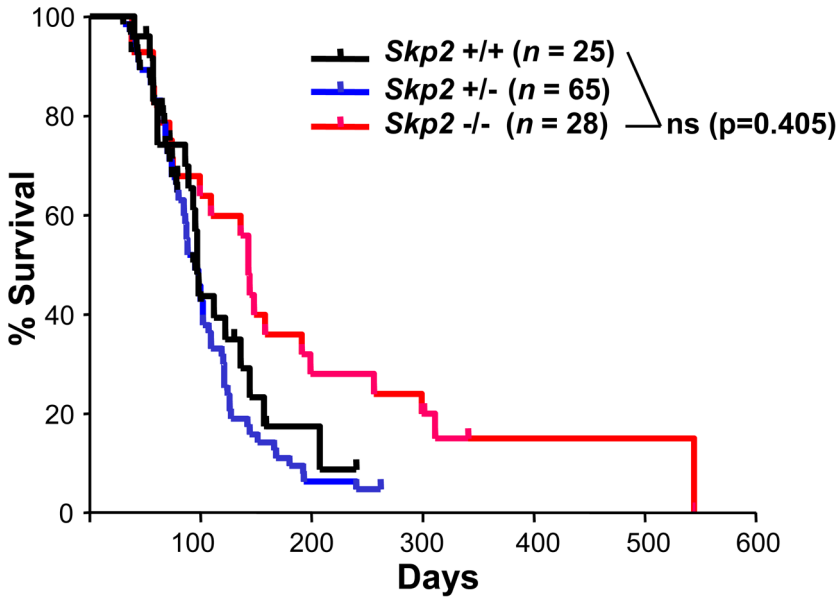

FIGURE 5.

Skp2 loss has no significant effect on Myc-driven lymphomagenesis as compared to 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.

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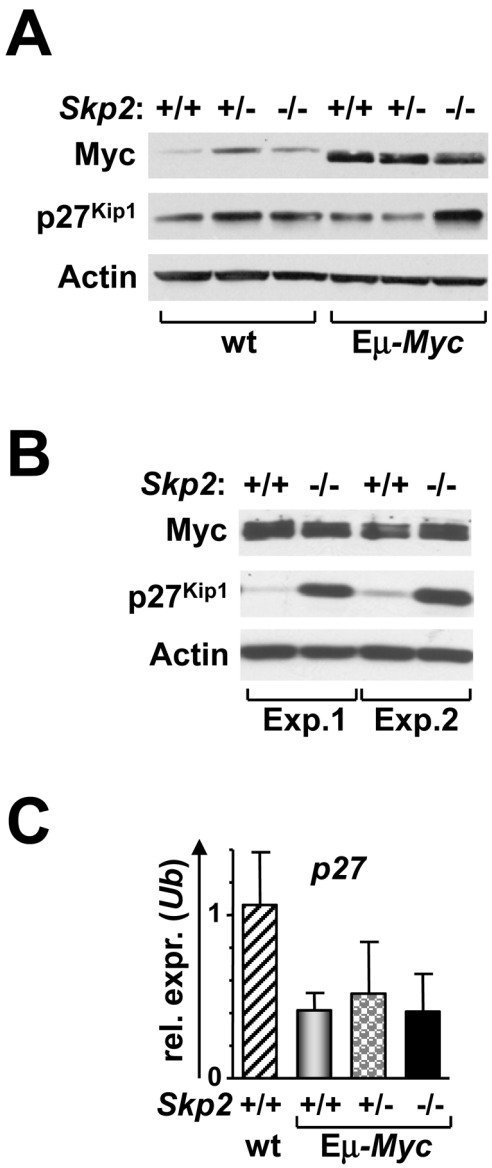

FIGURE 6.

Skp2 is required for Myc-mediated suppression of $p27^{Kip1}$. **A.** Immunoblot analyses of Myc and $p27^{Kip1}$ protein levels in pre-cancerous (4 week-old) splenic $B220^+$ B cells from non-transgenic (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 pre-cancerous 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 pre-cancerous Eμ-*Myc;Skp2*$^{+/+}$, Eμ-*Myc;Skp2*$^{+/-}$, and Eμ-*Myc;Skp2*$^{-/-}$ mice compared to a non-transgenic wild type littermate (wt). Levels of mRNAs were standardized to the expression of *Ubiquitin* (*Ub*).