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Myc targets Cks1 to provoke the suppression of p27^{Kip1}, proliferation and lymphomagenesis

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Reduced levels of the cyclin-dependent kinase inhibitor p27^{Kip1} connote poor prognosis in cancer. In human Burkitt lymphoma and in precancerous B cells and lymphomas arising in Eμ-*Myc* transgenic mice, p27^{Kip1} expression is markedly reduced. We show that the transcription of the Cks1 component of the SCF^{Skp2} complex that is necessary for p27^{Kip1} ubiquitylation and degradation is induced by Myc. Further, Cks1 expression is elevated in precancerous Eμ-*Myc* B cells, and high levels of Cks1 are also a hallmark of Eμ-*Myc* lymphoma and of human Burkitt lymphoma. Finally, loss of *Cks1* in Eμ-*Myc* B cells elevates p27^{Kip1} levels, reduces proliferation and markedly delays lymphoma development and dissemination of disease. Therefore, Myc suppresses p27^{Kip1} expression, accelerates cell proliferation and promotes tumorigenesis at least in part through its ability to selectively induce Cks1.
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Introduction

Inappropriate and/or accelerated rates of cell proliferation are hallmarks of cancer. Frequent culprits that direct this response are the *Myc* family of transcription factors (c-*Myc*, N-*Myc* and L-*Myc*), which are overexpressed in ~70% of all cancers (Oster *et al*, 2002). Dysregulation of Myc can occur either directly by chromosomal amplifications or translocations (Boxer and Dang, 2001), or indirectly through mutations in signaling pathways or tumor suppressors that control *Myc* expression. The widespread selection for Myc activation in malignancies in part reflects its essential roles in directing cell proliferation (Trumpp *et al*, 2001), cell growth (Iritani and Eisenman, 1999) and tumor angiogenesis (Pelengaris *et al*, 1999; Baudino *et al*, 2002). Indeed, overexpression of Myc prevents withdrawal from the cell cycle (Askew *et al*, 1991), accelerates rates of cell cycle traverse and is sufficient to drive quiescent cells into S phase (Roussel *et al*, 1991), properties which put the tumor cell at a considerable advantage (Evan and Vousden, 2001).

Under physiological conditions, Myc expression is tightly controlled by signaling pathways that restrict transcription and/or that control the stability of Myc transcripts or proteins (Welcker *et al*, 2004; Liu and Levens, 2006). These signals are superseded when Myc is overexpressed in cancer, but in normal cells, Myc's effects on the proliferative response are counterbalanced by apoptotic checkpoints. In particular, Myc overexpression activates the p53 apoptotic program through the nucleolar tumor suppressor Arf, which inactivates p53's endogenous inhibitor Mdm2 (reviewed in Nilsson and Cleveland, 2003; Sherr, 2004). This leads to the induction of p53's apoptotic targets, including the BH3-only Bcl-2 family protein Puma (Jeffers *et al*, 2003). Further, Myc can also suppress the expression of the antiapoptotic proteins Bcl-X_L or Bcl-2 (Eischen *et al*, 2001; Maclean *et al*, 2003). Not surprisingly then, inactivation of proapoptotic Bcl-2 family members, or Bcl-2 overexpression, accelerates the course of Myc-driven malignancies (Strasser *et al*, 1990; Eischen *et al*, 1999, 2001; Egle *et al*, 2004; Hemann *et al*, 2004), and alterations that inactivate these checkpoints occur in tumors induced by Myc (Eischen *et al*, 1999, 2001).

Myc accelerates cell proliferation, at least in part, through its ability to downregulate the expression of the cyclin-dependent kinase (Cdk) inhibitor p27^{Kip1} (Vlach *et al*, 1996; Muller *et al*, 1997), which harnesses cell cycle traverse by inhibiting cyclin E–Cdk2 and cyclin A–Cdk2 complexes that are necessary for entry and progression through S phase, respectively (Sherr and Roberts, 1999). Myc suppresses *p27^{Kip1}* transcription (Yang *et al*, 2001), although its effects on p27^{Kip1} protein levels are more profound and include effects on the half-life and localization of the protein. For example, the induction of *cyclin D2* by Myc augments levels of cyclin D2–Cdk4/6 complexes, which activate cyclin E–Cdk2 complexes by sequestering p27^{Kip1} (Bouchard *et al*, 1999; Perez-Roger *et al*, 1999). Activated cyclin E–Cdk2 then phosphorylates p27^{Kip1}, directing its association with the F-box protein Skp2 and the SCF^{Skp2} complex that ubiquitylates p27^{Kip1} and targets it for proteasomal degradation (Pagano *et al*, 1995; Montagnoli *et al*, 1999). Finally, Myc also induces the transcription of *Cullin1* (*Cul1*), a scaffolding component of SCF^{Skp2} (O'Hagan *et al*, 2000).

In Eμ-*Myc* transgenic mice (Adams *et al*, 1985), a mouse model of human Burkitt lymphoma that bears *Myc*/*immunoglobulin*

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gene translocations, the precancerous state is characterized by excessive B-cell proliferation (Baudino *et al*, 2003) that is initially offset by Myc's apoptotic programs (Eischen *et al*, 1999). Nonetheless, loss of *p27^{Kip1}* is sufficient to accelerate lymphoma development in Eμ-*Myc*;*p27$^{Kip1-/-}$* transgenic mice without affecting Myc-induced apoptosis (Martins and Berns, 2002), and *E2f1* heterozygosity or loss impairs Myc-induced proliferation and lymphoma development in Eμ-*Myc* mice, and abolishes Myc's ability to downregulate p27^{Kip1} expression. However, E2f1 alone is not sufficient to suppress p27^{Kip1} protein levels, indicating that other mediators also contribute to Myc-mediated degradation of p27^{Kip1} (Baudino *et al*, 2003).

E2f1 induces *cyclin E* expression (DeGregori *et al*, 1995) and signals that control p27^{Kip1} protein levels include its phosphorylation on Threonine-187 (Thr-187) by cyclin E–Cdk2 in S phase (Montagnoli *et al*, 1999; Malek *et al*, 2001), and on Serine-10 (Ser-10) in G_0 (Ishida *et al*, 2000; Kotake *et al*, 2005). While Ser-10 phosphorylation stabilizes p27^{Kip1} in G_0, Thr-187 phosphorylation directs p27^{Kip1} to the SCF^{Skp2} ubiquitin ligase complex that contains Skp1, Cks1, Cul1, Rbx1 and the F-box protein Skp2 (Bartek and Lukas, 2001). Skp2 binds to phosphorylated p27^{Kip1} (Carrano *et al*, 1999), but its binding is drastically reduced in the absence of Cks1 (Ganoth *et al*, 2001; Spruck *et al*, 2001), which forms part of the p27^{Kip1} phosphodegron binding site (Hao *et al*, 2005) that is necessary for efficient docking of p27^{Kip1} to the SCF^{Skp2} holoenzyme and for subsequent ubiquitylation of p27^{Kip1} (Ganoth *et al*, 2001). Consequently, the targeted deletion of *Cks1* (Spruck *et al*, 2001) or *Skp2* (Nakayama *et al*, 2000) leads to marked increases of p27^{Kip1} levels in mice, reduces cell proliferation and results in small body size, phenotypes that are the opposite of those manifest by *p27^{Kip1}* deletion (Fero *et al*, 1996; Kiyokawa *et al*, 1996; Nakayama *et al*, 1996).

p27^{Kip1} is haploinsufficient for tumor suppression (Fero *et al*, 1998), and low levels of p27^{Kip1} connote poor prognosis in human cancer (reviewed in Sgambato *et al*, 2000; Slingerland and Pagano, 2000), although how reductions in p27^{Kip1} expression occur in malignancies are largely unknown. Here, we report that Myc induces *Cks1* transcription and that elevated levels of Cks1 are a hallmark of precancerous Eμ-*Myc* transgenic B cells, and of Myc-driven lymphomas of mice and man. Further, Cks1 is shown to be necessary for Myc to repress p27^{Kip1} and to contribute to Myc-induced proliferation and lymphoma development. Therefore, the Myc-to-Cks1 pathway directs p27^{Kip1} downregulation and promotes proliferation and cancer.

Results

Myc induces Cks1 expression

Myc suppresses p27^{Kip1} expression by transcriptional repression (Yang *et al*, 2001), by provoking ubiquitin-mediated degradation of p27^{Kip1} (Muller *et al*, 1997) and through sequestering p27^{Kip1} via the induction of *cyclin D2* (Bouchard *et al*, 1999). The expression of *p27^{Kip1}* (*Cdkn1b*) transcripts (Figure 1A and B), and especially p27^{Kip1} protein (Figure 1C), was markedly reduced in the precancerous (4-week old) B cells of Eμ-*Myc* transgenic mice relative to levels expressed in the B cells of wild-type littermates. Further, suppression of p27^{Kip1} protein was evident in both mature (IgM$^+$) and especially immature (IgM$^-$) precancerous Eμ-*Myc* B cells from either spleen or bone marrow (Supplementary Figure S1A).

E2f1 is necessary for Myc-induced suppression of p27^{Kip1}, although E2f1 alone does not suppress p27^{Kip1} (Baudino *et al*, 2003); therefore, Myc regulates other components that contribute to this response. We reasoned that logical mediators could include cyclin D2 and/or components of the SCF^{Skp2} complex that directs p27^{Kip1} ubiquitylation. We therefore assessed the expression of cyclin D2 and SCF^{Skp2} components in splenic and bone marrow B220$^+$ B cells isolated from several individual precancerous (4-week old) Eμ-*Myc* transgenic mice and their wild-type littermates. Expression profiling and quantitative real-time PCR (qRT-PCR) analyses of RNA isolated from Eμ-*Myc* B220$^+$ B cells showed expected increases in the Myc target genes *Odc* and *E2f1* (Figure 1A) but revealed that *cyclin D2* (*Ccnd2*) levels were, surprisingly, reduced (Supplementary Figure S1B). By contrast, Eμ-*Myc* B cells expressed elevated levels of *Cks1* mRNA, and there were also modest increases in the levels of transcripts encoding other SCF^{Skp2} components, including *Skp2*, *Rbx1*, *Skp1* and *Cul1*; for *Rbx1*, *Skp1* and *Cul1*, these effects were usually most evident in immature (bone marrow) versus mature (spleen) B cells (Figure 1B). However, when canvassed for changes in protein levels, it was evident that Myc's effects on SCF^{Skp2} components were selective, as there was little change in the levels of Cullin1, Skp1, or Rbx1, modest increases in Skp2, but profound increases in the levels of Cks1 (Figure 1C). Therefore, Myc markedly induces the expression of Cks1 and also induces Skp2, and this is associated with a dramatic reduction in p27^{Kip1} levels in Myc-expressing B cells.

Myc's ability to induce Cks1 largely occurred through increases in *Cks1* mRNA. The *Cks1* promoter-regulatory region lacks consensus Myc responsive elements, but nonetheless could be activated through non-consensus elements. To address this issue, primary early-passage mouse embryo fibroblasts (MEFs) were infected with the MSCV-Myc-ER^{TAM}-IRES-GFP retrovirus, which harbors the Myc-ER^{TAM} transgene, a chimeric fusion of c-Myc that can be selectively activated by the ER agonist 4-hydroxy tamoxifen (4-HT; Eischen *et al*, 1999), and the gene for green fluorescence protein (GFP), expressed in *cis* from an internal ribosome entry site (IRES). Myc-ER^{TAM}- and GFP-only-expressing MEFs were left untreated or were pretreated with 1 μg/ml cycloheximide (Chx) for 30 min (which blocked protein synthesis by $>95\%$) and then were treated with 4-HT and the levels of *Cks1* transcript were assessed by real-time PCR (Figure 2A). Myc activation led to the induction of *Cks1* RNA (Figure 2A) and protein (Figure 2B), although unlike the *bona fide* Myc transcription targets *Odc* (Bello-Fernandez *et al*, 1993) and *Rcl* (Lewis *et al*, 1997), the induction of *Cks1* transcripts was blocked by Chx (Figure 2A); therefore, the induction of *Cks1* by Myc is indirect. Similar results applied to the induction of Skp2 by Myc (Figure 2A), although Skp2 induction was more delayed and less robust than that of Cks1, especially regarding changes in Skp2 protein (Figure 2B).

Myc did not activate a *Cks1*–promoter–luciferase reporter that harbors the 5′-regulatory region and exon 1 of mouse *Cks1* and, as might be expected for an indirect target, Myc also did not bind to the *Cks1* promoter-regulatory region in chromatin immunoprecipitation assays (data not shown).

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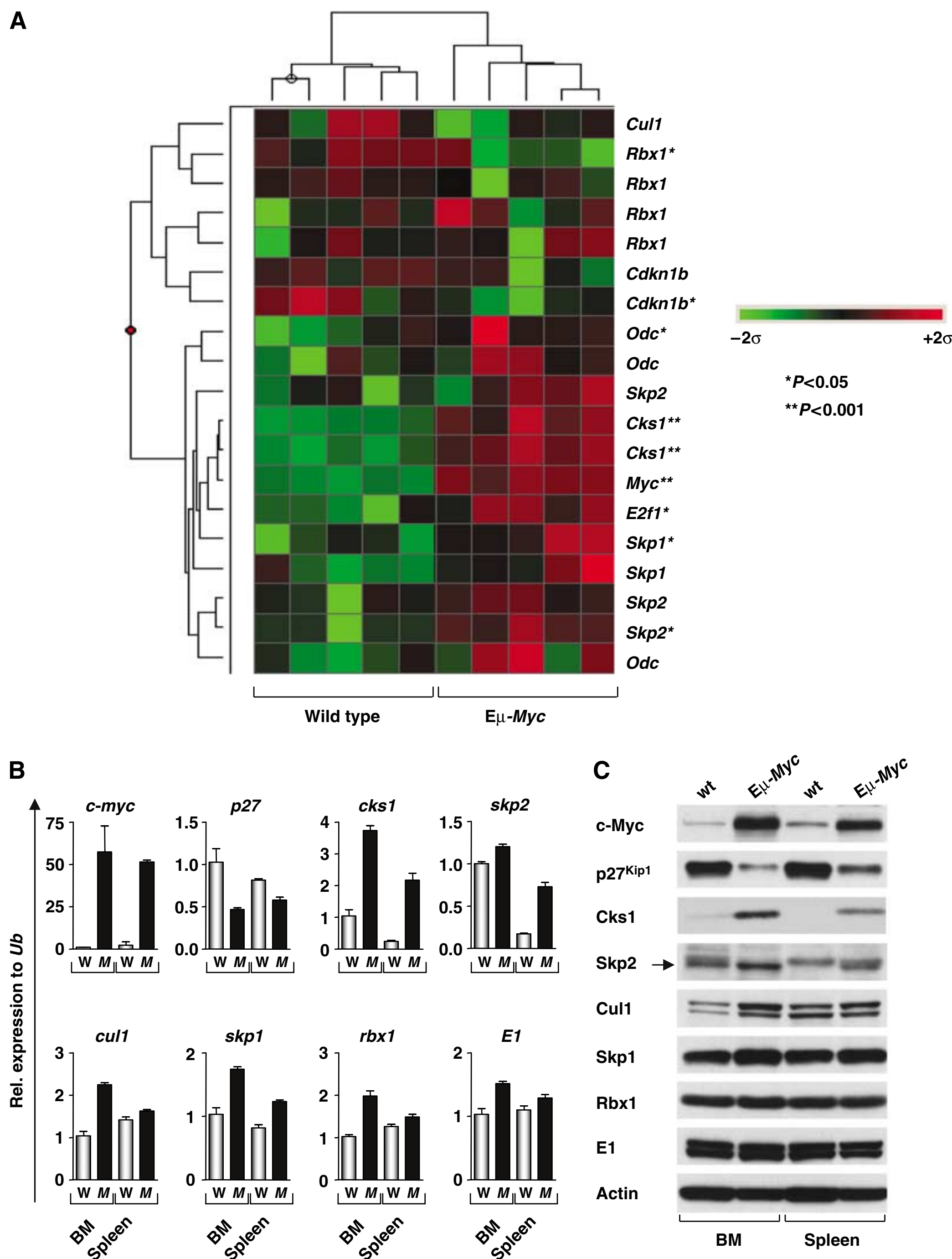

Figure 1 The expression of the Cks1 component of the SCF^{Skp2} complex is highly elevated in Eμ-*Myc* transgenic B cells. (**A**) Hierarchical clustering of components of the SCF^{Skp2} complex, *p27* (*Cdkn1b*) and the selected Myc target genes (*Odc*, *E2f1*) in $B220^+$ splenic B cells of five weanling age wild-type and five Eμ-*Myc* transgenic mice. Probe set signals were normalized to the mean across mice and values of each individual case are represented by a color, with green corresponding to s.d. (σ) below and red corresponding to s.d. (σ) above the mean, according to the scale shown. * Indicates a *P*-value of <0.05 and ***P*-value of <0.01. (**B**) SYBR-green real-time PCR analysis of $B220^+$ bone marrow (BM) and $sIgM^+$ splenic (spleen) B cells. W indicates wild-type mice and *M* denotes Eμ-*Myc* transgenic mice. Levels of mRNAs are standardized to the expression of *ubiquitin* (*Ub*), which is not regulated by Myc. (**C**) Immunoblot analysis of the indicated proteins in $B220^+$ B cells from wild-type (wt) versus Eμ-*Myc* mice. Arrow indicates the Skp2-specific (lower) band on the gels.

However, we reasoned that Myc might induce *Cks1* through the agency of other transcription factors. One obvious candidate was E2f1, which is induced by Myc and which is required for Myc-mediated suppression of $p27^{Kip1}$ protein (Baudino *et al*, 2003). We therefore assessed the effects of *E2f1* loss on the expression of *Cks1 in vivo* in splenic $B220^+$ B cells from (precancerous) 4-week old $E2f1^{+/+}$-, $E2f1^{+/-}$- and $E2f1^{-/-}$-Eμ-*Myc* transgenics and compared these to non-transgenic $E2f1^{+/+}$ and $E2f1^{-/-}$ littermates. *E2f1* loss had no effect on the markedly increased levels of *Cks1* mRNA in Eμ-*Myc* B cells and also had no effect on the induction of *Rcl* (Figure 2C). As expected, the suppression of $p27^{Kip1}$ protein

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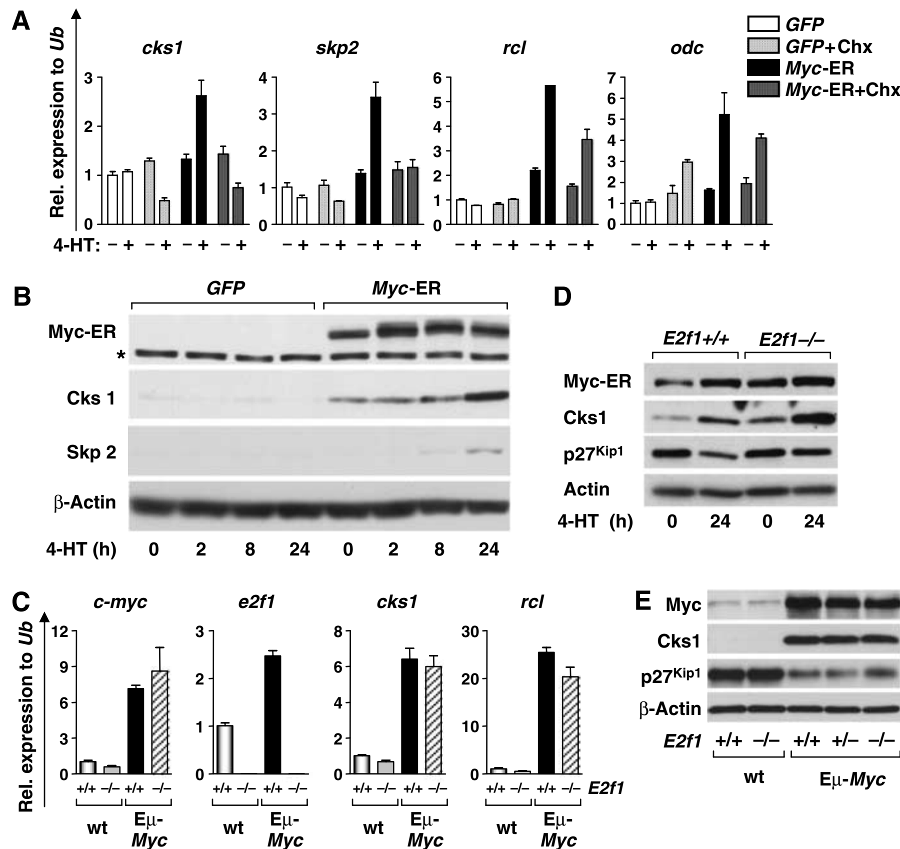

Figure 2 Myc regulation of Cks1 is indirect and independent of E2f1. (**A**) Primary early-passage MEFs were infected with MSCV-Myc-ER^{TAM}-IRES-GFP (*Myc*-ER) or MSCV-IRES-GFP (*GFP*) virus. GFP$^+$ cells were then left untreated (−) or were treated (+) with 2 μm 4-HT±Chx pretreatment (30 min) for 24 h and assessed for their expression of the indicated mRNAs by SYBR-green real-time PCR analysis. Levels of mRNA were standardized to *Ub*. (**B**) Immunoblot analysis of Cks1 and Skp2 expression upon Myc activation by 4-HT in primary MEFs. * Denotes nonspecific band detected by the Myc antiserum. (**C**) qRT-PCR analysis of the expression of the indicated mRNAs in splenic B220$^+$ B cells of 4-week-old wild-type (wt) and Eμ-*Myc* mice. The *E2f1* genotypes are indicated. Levels of mRNA are standardized to *Ub*. (**D**) Immunoblot analysis of Cks1 expression upon Myc-ER activation by 4-HT in primary, paired *E2f1*$^{+/+}$ and *E2f1*$^{-/-}$ MEFs. (**E**) Immunoblot analysis of Cks1 expression in 4-week-old splenic B220$^+$ B cells of wt and Eμ-*Myc* mice of the indicated *E2f1* genotypes.

following activation of Myc-ER^{TAM} was impaired in *E2f1*$^{-/-}$ MEFs compared with that of paired *E2f1*$^{+/+}$ MEFs. Nonetheless, there was no effect of *E2f1* loss on the magnitude of the induction of Cks1 protein by Myc in either *E2f1*-deficient MEFs (Figure 2D) or in precancerous *E2f1*$^{-/-}$-Eμ-*Myc* B cells (Figure 2E). Furthermore, E2f1 activation alone was not sufficient to induce *Cks1* and/or suppress p27^{Kip1} in early-passage MEFs engineered to express ER^{TAM}-E2f1, a chimeric form of E2f1 that activates E2f1 targets (Vigo *et al*, 1999), although E2f1 activation did, as expected, induce *cyclin E1* and *E2* (Supplementary Figure S2A). Finally, induction of *Cks1* was not associated with significant differences in the expression of *E2f2* or *E2f3*, which belong to the same E2f activator subclass and have some overlapping functions (Black *et al*, 2005), in wild-type versus splenic Eμ-*Myc* B cells (Supplementary Figure S2B). Therefore, Myc activates *Cks1* expression indirectly and in an E2f-independent manner.

At face value, the effects of Myc on Cks1 protein levels appeared more robust than effects on *Cks1* transcripts. We therefore tested whether turnover of Cks1 protein might be regulated by c-Myc, especially given that the anaphase promoting complex/cyclosome-Cdh1 (APC/C^{Cdh1}) E3 ubiquitin ligase directs the turnover of Cks1 and Skp2 (Bashir *et al*, 2004). However, the expression of Cdh1 transcripts and protein, and of other components of the APC/C^{Cdh1} complex, was similar in Eμ-*Myc* versus wild-type B cells (Supplementary Figure S3, and data not shown). Furthermore, measurements of the rate of Cks1 turnover in Myc-ER^{TAM}- versus puromycin-only-expressing MEFs by pulse–chase immunoprecipitation experiments demonstrated no significant differences in Cks1 half-life (Supplementary Figure S4A). By contrast, the rates of Cks1 biosynthesis were two- to three-fold higher in Myc-ER^{TAM}-expressing MEFs than in puromycin-only-expressing MEFs (Supplementary Figure S4B), a finding similar to the increased levels of *Cks1* transcripts observed in Myc-expressing MEFs.

Collectively, these findings suggested that the predominant mode of upregulation of Cks1 by Myc was transcriptional. To confirm this, we performed quantitative primary transcript real-time PCR (qPT-PCR) analyses, which measure the relative abundance of primary, unspliced transcripts relative to those of mature mRNA. Using *Cks1* exon 1–intron 1, intron 2–exon 2, exon 1–exon 2 and exon 2–exon 3 primer pairs for qPT-PCR, the relative abundance of immature and mature *Cks1* transcripts were similarly elevated in Eμ-*Myc* B cells (Figure 3A). qPT-PCR can also be utilized to determine the relative rates of synthesis of unspliced versus mature, spliced

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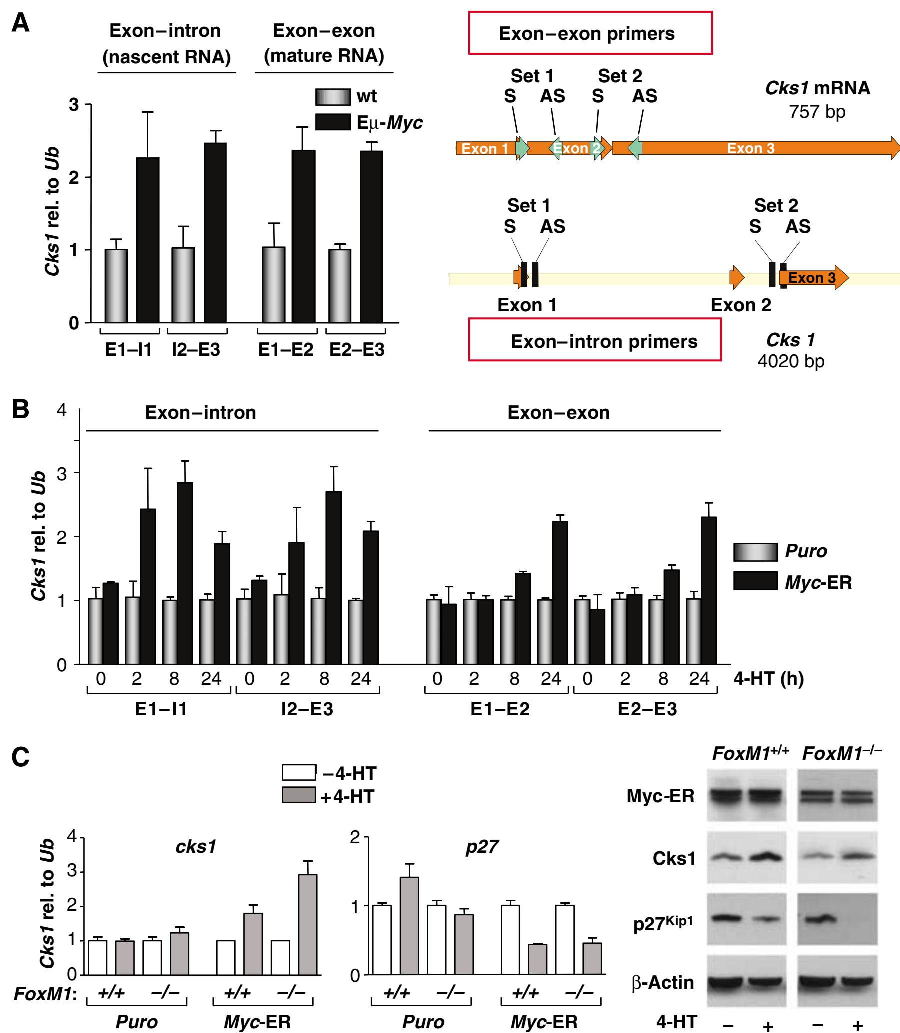

Figure 3 Myc regulates *Cks1* transcription independent of FoxM1. (**A**) Total RNA was isolated from B220$^+$ B cells from 4-week-old wild-type (wt) and Eμ-*Myc* transgenic mice and qPT-PCR analysis was performed with primers designed to detect unspliced, nascent RNA (intron–exon–PCR product) or spliced, mature mRNA only (exon–exon–PCR product). The location of the two sets of primers used to detect nascent versus mature *Cks1* mRNA is illustrated at right. Levels of *Cks1* were standardized to *Ub*. (**B**) The induction of nascent and mature RNA was analyzed in MEFs expressing Puro alone or Myc-ER^{TAM} + Puro. MEFs were treated for the indicated times with 4-HT and levels of *Cks1* were standardized to *Ub*. (**C**) Left, *FoxM1*$^{+/+}$ or *FoxM1*$^{-/-}$ MEFs expressing *Puro* alone or Myc-ER^{TAM} + *Puro* were treated with 4-HT for 24 h and RNA levels were determined by qRT-PCR. Right, extracts from the same cells were assessed for the indicated proteins by immunoblot analyses.

mRNA. We therefore assessed the rates of synthesis of immature versus mature *Cks1* transcripts in puromycin-only versus Myc-ER^{TAM}-expressing MEFs following the addition of 4-HT. As expected, the induction of unspliced *Cks1* transcripts in Myc-ER^{TAM}-expressing MEFs preceded the appearance of mature *Cks1* mRNA, which, with time, increased to levels that were similar to unspliced *Cks1* transcripts, indicating no overt effects of Myc on the splicing of *Cks1* transcripts (Figure 3B). Finally, Myc did not affect the half-life of *Cks1* transcripts in experiments assessing the effects of actinomycin-D on the turnover of *Cks1* mRNA (data not shown). Therefore, Myc provokes increases in *Cks1* at the level of transcription.

Logical mediators of the Myc-to-Cks1 response would include transcription factors that are essential for *Cks1* transcription. One potential candidate was the Forkhead box M1 transcription factor (FoxM1), as targeted deletion of *Foxm1* has revealed its role in regulating *Cks1* transcription (Wang *et al*, 2005). To address a potential role for FoxM1, paired *Foxm1*$^{-/-}$ and *Foxm1*$^{+/+}$ MEFs were transduced with the MSCV-Myc-ER^{TAM}-IRES-puro or MSCV-IRES-puro retroviruses and puromycin-resistant cultures were treated with 4-HT. Activation of Myc-ER^{TAM} led to a marked induction of *Cks1* transcripts in *Foxm1*$^{-/-}$ MEFs and also to increases in Cks1 protein, and to corresponding reductions in p27^{Kip1} protein (Figure 3C). Therefore, Myc-mediated induction of *Cks1* is also independent of FoxM1.

Alterations of the Cks1-p27^{Kip1} pathway are a hallmark of Myc-induced lymphoma

Given the effects of Myc on the expression of p27^{Kip1}, Skp2 and Cks1, we reasoned that alterations in their expression might be evident in the Myc-driven lymphomas that arise in Eμ-*Myc* transgenic mice, and in human Burkitt lymphoma. Expression of p27^{Kip1}, Skp2 and Cks1 was determined by qRT-PCR (Figure 4A) and by Western blotting of 49 lymphomas

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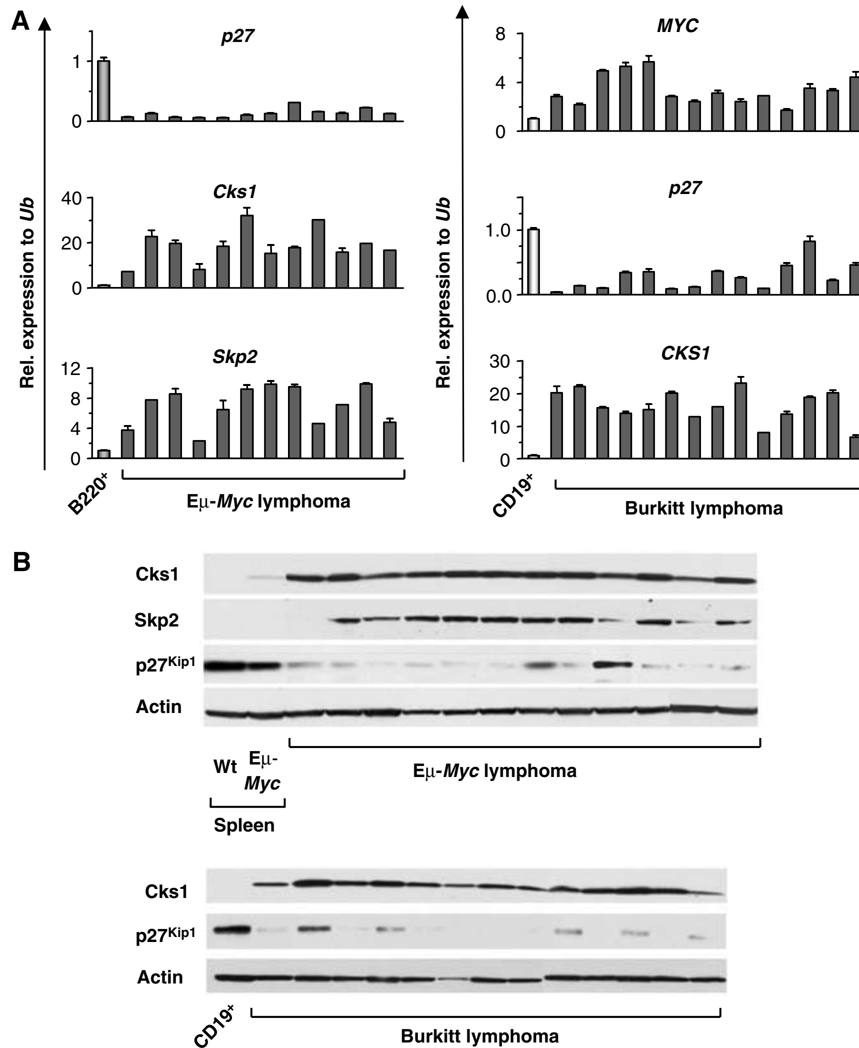

Figure 4 Alterations in the Cks1–p27^{Kip1} axis are hallmarks of Myc-induced lymphomas in mice and man. (**A**) qRT-PCR analysis of *c-Myc* (only in human samples), *p27*, *Skp2* and *Cks1* in Eμ-*Myc* lymphomas compared with wild-type B220$^+$ splenic B cells (B220$^+$, left panel), and of 14 human Burkitt lymphoma samples compared with CD19$^+$ control human B cells (right panel). Levels of mRNAs were standardized to *Ub*. (**B**) Lymphomas arising in Eμ-*Myc* transgenic mice and B220$^+$ splenic B cells from precancerous mice (Eμ-*Myc*) and wild-type controls (wt) were analyzed for expression of the indicated proteins by immunoblotting (top panel). Thirteen human Burkitt lymphoma samples were analyzed by immunoblots for expression of the indicated proteins (bottom panel). CD19$^+$ peripheral B cells from healthy donors were used as control.

from Eμ-*Myc* transgenics, and of 17 primary human Burkitt lymphoma, and was compared with their expression in normal B cells (B220$^+$ cells from wild-type littermates, or CD19$^+$ peripheral blood human B cells), and to precancerous Eμ-*Myc* splenic B220$^+$ B cells (Figure 4B). Most Eμ-*Myc* lymphomas expressed very low levels of p27^{Kip1} mRNA and protein and elevated levels of Skp2, and all tumors expressed much higher levels of Cks1 (Figure 4). Specifically, in Eμ-*Myc* mice, 94% of lymphomas had lower p27^{Kip1} levels, and Skp2 and Cks1 were greatly elevated in 85 and 95% of lymphomas, respectively (Figure 4B, representative tumors shown). Furthermore, dramatic alterations in p27^{Kip1} and CKS1 expression were also evident in all Burkitt lymphoma analyzed, which expressed reduced levels of p27^{Kip1} and much higher levels of CKS1, when compared with levels expressed in normal human B cells (Figure 4B). The marked increases in *Cks1* transcripts (Figure 4A) were not due to *Cks1* amplification, as assessed by Southern blotting (data not shown). Furthermore, the high levels of *Cks1* in Eμ-*Myc* lymphomas were not associated with corresponding changes in FoxM1 expression, as *FoxM1* transcripts were reduced in Eμ-*Myc* lymphomas compared with levels expressed in wild-type or precancerous Eμ-*Myc* B220$^+$ bone marrow cells, and markedly increased levels of Cks1 protein were also evident in lymphomas that arise in *E2f1*$^{+/-}$-, and *E2f1*$^{-/-}$-Eμ-*Myc* transgenics (Supplementary Figure S5). Therefore, the induction of Cks1 and Skp2, and the suppression of p27^{Kip1} expression, are hallmarks of Myc-induced lymphoma.

Loss of Cks1 impairs Myc-induced lymphomagenesis

The dramatic changes in p27^{Kip1} and Cks1 expression in Myc-driven lymphoma suggested that this pathway plays a critical role in tumorigenesis. Indeed, loss of *p27*^{Kip1} accelerates lymphoma development in Eμ-*Myc* mice (Martins and Berns, 2002), although the role of Cks1 in tumorigenesis is unknown. If Cks1 induction was important for Myc to suppress p27^{Kip1}, we predicted that *Cks1* loss would delay lymphoma development. To test this hypothesis, Eμ-*Myc*

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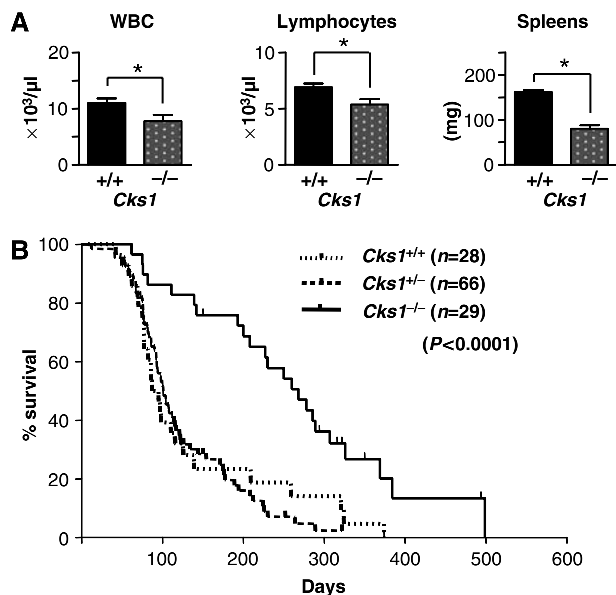

Figure 5 *Cks1* loss impairs Myc-induced lymphomagenesis. (**A**) Precancerous Eμ-*Myc* transgenic mice of different *Cks1* genotypes were analyzed for white blood counts (WBC), lymphocyte number in the peripheral blood and spleen weights. * Indicates a *P*-value of <0.05. (**B**) Kaplan–Meier survival curves of Eμ-*Myc* transgenic animals of different *Cks1* genotypes. The differences in the rates of tumor incidence between the $Cks1^{+/+}$ and the $Cks1^{-/-}$ group are highly statistically significant ($P<0.0001$).

transgenic mice were mated to $Cks1^{-/-}$ mice (Spruck *et al*, 2001) and F1 offspring were bred to obtain $Cks1^{+/+}$-, $Cks1^{+/-}$- and $Cks1^{-/-}$-Eμ-*Myc* transgenics. These littermates were then followed for the onset of disease, which is first manifest by a marked lymphocytosis and splenomegaly. To evaluate the precancerous phase, 4-week old littermates were analyzed for these parameters. Non-transgenic $Cks1^{-/-}$ mice had normal white blood cell and lymphocyte counts and no splenomegaly (data not shown). Notably, the spleens of Eμ-*Myc*;$Cks1^{-/-}$ transgenics were significantly smaller than those from Eμ-*Myc*;$Cks1^{+/+}$ littermates (spleen sizes 157 ± 7 versus 83 ± 11 mg for wild-type versus *Cks1*-null cohorts; Figure 5A, right panel). There were also obvious reductions in white blood cell numbers in Eμ-*Myc*;$Cks1^{-/-}$ transgenics (*Cks1*-null, $7.7\pm1.1\times10^6$/μl versus $Cks1^{+/+}$, $11.1\pm0.7\times10^6$/μl; Figure 5A, left panel) and corresponding reductions in lymphocyte counts (*Cks1*-null, $5.4\pm0.4\times10^6$/μl versus $Cks1^{+/+}$, $6.9\pm0.4\times10^6$/μl; Figure 5A, middle panel). Heterozygosity effects in Eμ-*Myc*;$Cks1^{+/-}$ littermates were not observed (data not shown). Thus, *Cks1* loss effectively cancels the precancerous phase of disease in Eμ-*Myc* transgenic mice.

Eμ-*Myc* transgenic mice succumb to aggressive lymphoma within 3–6 months of birth (Adams *et al*, 1985). Non-transgenic $Cks1^{-/-}$ littermates showed no signs of tumor development throughout their lifespan. Importantly, Eμ-*Myc*;$Cks1^{-/-}$ transgenic mice had significant delays in lymphoma development, with a median survival of 268 days compared with 91 and 101 days median survival of their Eμ-*Myc*;$Cks1^{+/+}$ and Eμ-*Myc*;$Cks1^{+/-}$ littermates, respectively (Figure 5B, $P<0.0001$). Thus, *Cks1* is a critical mediator of Myc-driven tumorigenesis in B cells.

Cks1 contributes to Myc's proliferative response

Loss of inhibitors of the Arf-p53 apoptotic program such as Mdm2 (Alt *et al*, 2003), or of regulators of the Myc-to-$p27^{Kip1}$ pathway such as E2f1 (Baudino *et al*, 2003), impair lymphoma development. Therefore, the profound effects of *Cks1* loss on lymphoma development in Eμ-*Myc* transgenics might reflect an amplification of Myc's apoptotic response and/or the inhibition of its proliferative response. To address the effect of *Cks1* loss on Myc-induced apoptosis, the apoptotic indices of precancerous B cells from both immature ($sIgM^-$) and mature ($sIgM^+$) $B220^+$ cells from wild-type versus *Cks1*-null Eμ-*Myc* transgenic littermates were assessed by staining with Annexin-V–FITC and propidium iodide (PI). There were no significant differences in the apoptotic indices of Eμ-*Myc*;$Cks1^{-/-}$ precancerous B cells when compared with those of Eμ-*Myc*;$Cks1^{+/+}$ littermates (Figure 6A). Further, the frequency of alterations in the Arf-p53 pathway, which occur through bi-allelic deletions of *Arf* or mutations in p53 (Eischen *et al*, 1999), were similar in wild-type, $Cks1^{+/-}$ and $Cks1^{-/-}$ Eμ-*Myc* lymphomas (Supplementary Figure S6). Therefore, the delay in lymphoma development evident in Eμ-*Myc*;$Cks1^{-/-}$ transgenic mice was not due to overt effects on Myc's apoptotic program.

To evaluate potential effects of *Cks1* loss on Myc's proliferative response, we initially assessed the rates of growth of B cells derived from transgenic littermates by *ex vivo* culture of bone marrow on S17 stromal cells in medium containing interleukin-7 (Eischen *et al*, 1999). Again, there were no differences in the apoptotic indices of Eμ-*Myc*;$Cks1^{+/+}$ versus Eμ-*Myc*;$Cks1^{-/-}$ B cells (Figure 6B). To quantify their rates of proliferation, these B cells were labeled with BrdU and analyzed by flow cytometry. Eμ-*Myc*;$Cks1^{-/-}$ B cells had much slower growth rates than B cells derived from Eμ-*Myc*;$Cks1^{+/+}$ littermates (Figure 6C). Therefore, at least *ex vivo*, the *Cks1* deficiency reduces the proliferative rates of Myc-expressing B cells.

To address whether *Cks1* loss might inhibit Myc's ability to augment cell growth (mass) (Iritani and Eisenman, 1999) and/or to accelerate rates of cell cycle traverse *in vivo*, BrdU was injected intraperitoneally into 4-week-old Eμ-*Myc* transgenic littermates of different *Cks1* genotypes. After 12 h, $B220^+sIgM^+$ and $B220^+sIgM^-$ cells were assessed for their size by forward versus side scatter, and for their S phase indices by FACS analysis. There were no significant changes in the sizes of B cells from wild-type versus *Cks1*-null transgenics, although all *Myc* transgenic cells were, as reported (Iritani and Eisenman, 1999), larger in size than non-transgenic B cells. Further, as expected (Baudino *et al*, 2003), both bone marrow- and spleen-derived Eμ-*Myc* B cells had higher proliferative rates than wild-type B cells. Loss of *Cks1* alone had only minor effects on B-cell proliferation as compared with wild-type littermate controls (Figure 6D). Importantly, *Cks1* deficiency impaired Myc's proliferative response, especially in Eμ-*Myc*;$Cks1^{-/-}$ B cells from bone marrow and pro-/pre- (IgM^-) splenic B cells, and FACS analyses demonstrated that this was associated with decreased numbers of cells in S phase and concomitant increases in the percentage of cells in G_0/G_1 (Figure 6D). Therefore, the delay in Myc-induced lymphomagenesis caused by *Cks1* deficiency is associated with defects in Myc's proliferative response. Finally, to confirm the effects of *Cks1* deficiency on Myc's ability to drive cells into S phase

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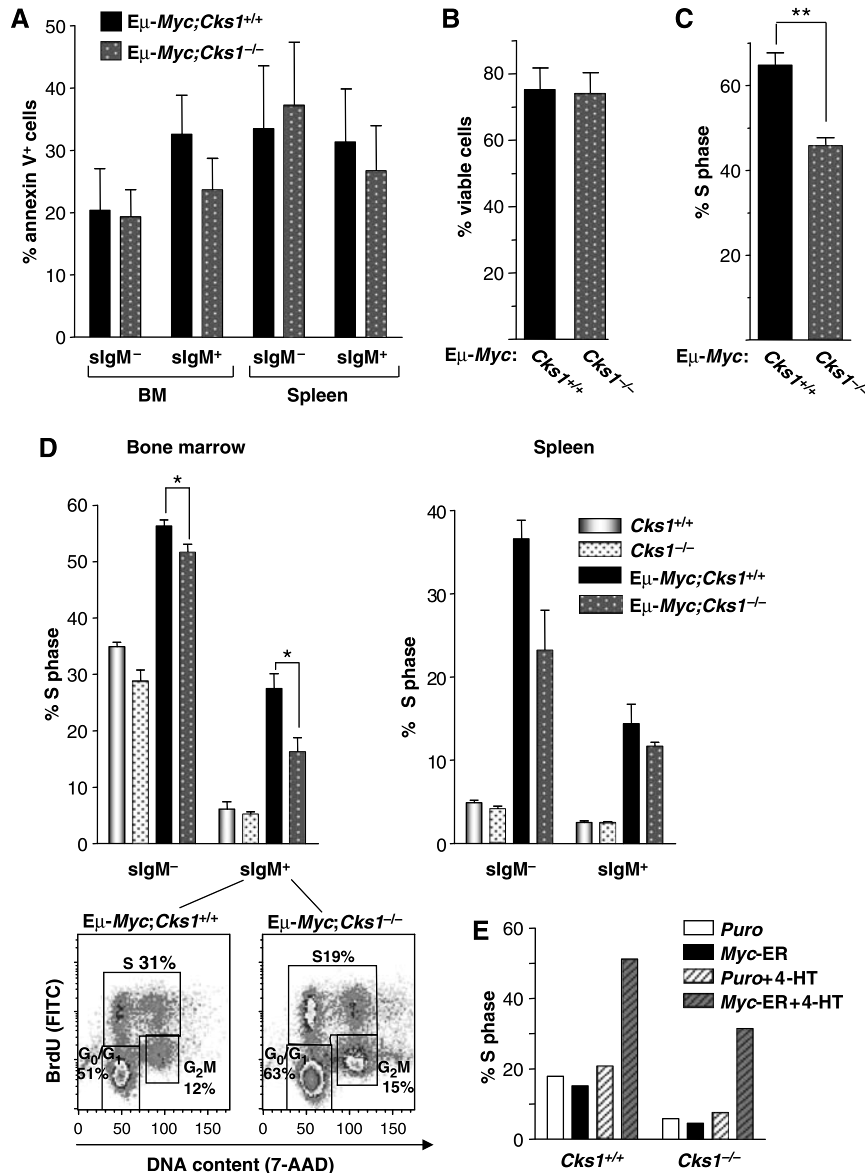

Figure 6 Loss of *Cks1* affects proliferation, but not apoptosis, of Eμ-*Myc* transgenic B cells. (**A**) Apoptosis in B220$^+$ sIgM$^-$ or sIgM$^+$ Eμ-*Myc* B cells of the indicated *Cks1* genotypes was analyzed using Annexin-V$^+$ assays. The bars represent the mean±s.e.m. ($n=3$ independent experiments). (**B, C**) B cells from *ex vivo* cultured bone marrow of littermate Eμ-*Myc* transgenic mice of different *Cks1* genotypes were analyzed for spontaneous rates of apoptosis by Annexin-V staining (B) or BrdU incorporation (C) into DNA (S phase) to assess proliferation ($n=3$; bars indicate mean±s.e.m.). ** Indicates $P<0.01$. (**D**) Wild-type and Eμ-*Myc* littermates of different *Cks1* genotypes received intraperitoneal injections of BrdU and cells from bone marrow and spleen were harvested 12 h later. BrdU incorporation was analyzed by an antibody-dependent fluorescence assay. The bars are the mean±s.e.m. of three independent experiments. * Indicates $P<0.05$. A representative FACS analysis of BrdU- and 7-AAD-stained B220$^+$ B cells from Eμ-*Myc*;*Cks1*$^{+/+}$ and Eμ-*Myc*;*Cks1*$^{-/-}$ littermates is shown below, along with the percentages of these cells in G_0/G_1, S and G_2/M phases. (**E**) Paired wild-type and *Cks1*$^{-/-}$ MEFs expressing Myc-ER were treated with 4-HT for 24 h and the percent of cells in S phase were determined by PI/FACS.

in other cell contexts, we also transduced paired early-passage *Cks1*$^{+/+}$ and *Cks1*$^{-/-}$ MEFs with *puro*- or *Myc*-ER^{TAM}-encoding retroviruses. Puromycin-resistant cells were then treated with 4-HT and evaluated by PI/FACS analyses. Again the percentage of Myc-expressing cells in S phase was markedly reduced by the *Cks1* deficiency (Figure 6E). Therefore, the *Cks1* deficiency impairs Myc's proliferative response.

Cks1 loss abolishes Myc's ability to suppress p27^{Kip1}

The targeted deletion of *Cks1* (Spruck *et al*, 2001) revealed its essential role in the SCF^{Skp2} complex that directs p27^{Kip1} degradation. Given Myc's ability to induce Cks1 while repressing p27^{Kip1}, and the effects of *Cks1* loss on Myc-induced proliferation and lymphomagenesis, we evaluated p27^{Kip1} expression in Eμ-*Myc*;*Cks1*$^{-/-}$ transgenic B cells cultured *ex vivo*. Importantly, there was a dramatic increase in p27^{Kip1} protein levels in B cells cultured from Eμ-*Myc*;*Cks1*$^{-/-}$ mice (Figure 7A). Similar changes in p27^{Kip1} expression were also evident *in vivo*, where Myc's ability to suppress p27^{Kip1} protein levels was impaired in Eμ-*Myc*;*Cks1*$^{-/-}$ B cells (Figure 7B). Interestingly, the effects of *Cks1* loss on p27^{Kip1} levels in Eμ-*Myc* B cells only occurred at the level of the protein, as levels of *p27^{Kip1}* transcripts were equally suppressed in all Eμ-*Myc* B cells, regardless of their

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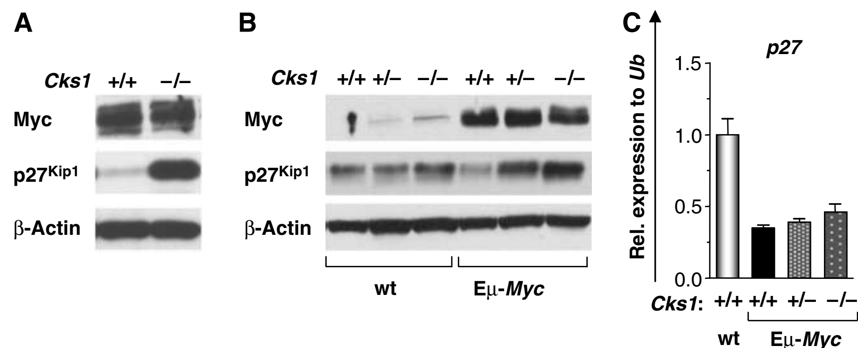

Figure 7 Cks1 is required for Myc to suppress p27^{Kip1}. (**A**) Immunoblot analysis of B cells from *ex vivo* cultured bone marrow of paired Eμ-*Myc* littermates of the indicated *Cks1* genotypes. (**B**) Immunoblot analysis of Myc and p27^{Kip1} expression was performed on splenic B220$^+$ B cells from precancerous mice of the indicated *Cks1* genotypes. (**C**) SYBR-green real-time PCR analysis of *p27* mRNA expression in splenic B cells from precancerous Eμ-*Myc* transgenics of different *Cks1* genotypes compared with non-transgenic wild-type littermates. Levels of mRNAs were standardized to *Ub*.

Cks1 status (Figure 7C). Finally, we also assessed whether the proliferative defects of Eμ-*Myc*;*Cks1*$^{-/-}$ B cells (e.g. Figure 6C) could indeed be attributed to elevated levels of p27^{Kip1} levels in these cells. To test this issue, we knocked down p27^{Kip1} expression using a p27^{Kip1}-specific shRNA by transducing Eμ-*Myc*;*Cks1*$^{-/-}$ B cells with MSCV-*p27*shRNA-IRES-*GFP* or with a control MSCV-*pBluescript*-shRNA-IRES-*GFP* virus (Nilsson *et al*, 2004). *p27*shRNA-expressing Eμ-*Myc*;*Cks1*$^{-/-}$ B cells had approximately 50% reduced levels of p27^{Kip1} protein compared with *pBluescript*-shRNA-expressing controls (Supplementary Figure S7A), and *p27*shRNA-expressing cells demonstrated a ∼20% increase in their uptake of ^{3}H-thymidine, and a 40% overall increase in their growth rate, relative to control Eμ-*Myc*;*Cks1*$^{-/-}$ B cells (Supplementary Figure S7B and C). Therefore, Cks1 is specifically required for Myc-mediated suppression of p27^{Kip1} protein levels, and this pathway contributes to Myc's proliferative response.

The Skp2 component of the SCF^{Skp2} E3 ubiquitin ligase has been suggested to regulate Myc protein stability and its transcriptional activity (Kim *et al*, 2003; von der Lehr *et al*, 2003), although Fbw7, a component of the SCF^{Fbw7} ubiquitin ligase, has been more recently been demonstrated to regulate Myc turnover (Welcker *et al*, 2004). Nonetheless, it was possible that *Cks1* status affected Myc turnover and/or function. However, *Cks1* loss had no effect on the steady-state levels of Myc protein in transgenic B cells (Figure 7A and B). Furthermore, *Cks1* loss did not affect Myc's ability to suppress *p27*^{Kip1} mRNA levels in Eμ-*Myc* transgenic B cells (Figure 7C) and also had no effect on Myc's ability to regulate a number of other well-established targets, including *rcl*, *cad* and many others (Supplementary Figure S8B), and there were no dramatic effects of *Cks1* loss on the expression of Myc target genes in the 'Myc Target gene database', http://www.myc-cancer-gene.org (Supplementary Figure S8A). Therefore, the effects of *Cks1* loss on Myc-induced proliferation and tumorigenesis appear independent of effects on Myc stability or function, and rather are specifically associated with blocking Myc's ability to downregulate p27^{Kip1} protein expression.

Loss of Cks1 impairs the metastasis of Myc-induced lymphoma

The immunophenotypes of lymphomas arising in *Cks1*-null Eμ-*Myc* transgenics were identical (pre-B and mature B-cell lymphomas) to those arising in wild-type Eμ-*Myc* transgenic littermates (data not shown). However, unlike lymphomas that arose in Eμ-*Myc*;*Cks1*$^{+/+}$ or Eμ-*Myc*;*Cks1*$^{+/-}$ littermates (Figure 8A and B), nearly all Eμ-*Myc*;*Cks1*$^{-/-}$ lymphomas maintained high levels of p27^{Kip1} protein expression (Figure 8A and C). Elevated levels of p27^{Kip1} were also evident in the late-onset lymphomas of Eμ-*Myc*;*E2f1*$^{-/-}$ mice (data not shown). Therefore, these pathways are not bypassed during lymphoma development.

Lymphomas that arise in Eμ-*Myc* transgenics are aggressive and typically display disseminated disease with invasion of all of the major organ systems (Eischen *et al*, 1999). Surprisingly, necropsy revealed marked differences in the lymphomas that arose in Eμ-*Myc*;*Cks1*$^{-/-}$ mice versus those that arose in Eμ-*Myc*;*Cks1*$^{+/+}$ littermates. Histological examination of six Eμ-*Myc*;*Cks1*$^{+/+}$ mice revealed that all suffered from disseminated disease, with diffuse bone marrow infiltrates of B220$^+$ lymphocytes (Figure 8D and F) and infiltration of the spleen, liver and the meninges (Figure 8H, and data not shown). In contrast, only one of nine diseased Eμ-*Myc*;*Cks1*$^{-/-}$ mice showed a moderate bone marrow infiltrate (Figure 8E and G show unaffected Eμ-*Myc*;*Cks1*$^{-/-}$ bone marrows), and none of these diseased mice presented liver or meningeal infiltrates (Figure 8I and J, and data not shown). Therefore, *Cks1* loss also compromises the aggressive nature of Myc-driven lymphoma.

Discussion

In normal cells, Myc activation accelerates cell cycle traverse, although this is counterbalanced by apoptotic checkpoints such as the Arf-p53 pathway, that guard against transformation (Nilsson and Cleveland, 2003). Thus, events which bypass apoptotic regulators are rate-limiting for tumor development (Eischen *et al*, 1999; Schmitt *et al*, 1999). However, these findings do not discount an important role for Myc's proliferative response in tumorigenesis. Indeed, as shown here, one key mediator of this response is Cks1, which is induced by Myc and is overexpressed in Myc-driven lymphomas, and which contributes to Myc-induced proliferation and lymphomagenesis. Strikingly, the effects of Cks1 in promoting Myc's proliferative response underscores the importance of the Myc-to-p27^{Kip1} pathway in regulating cell proliferation and tumorigenesis, where loss of *p27*^{Kip1}

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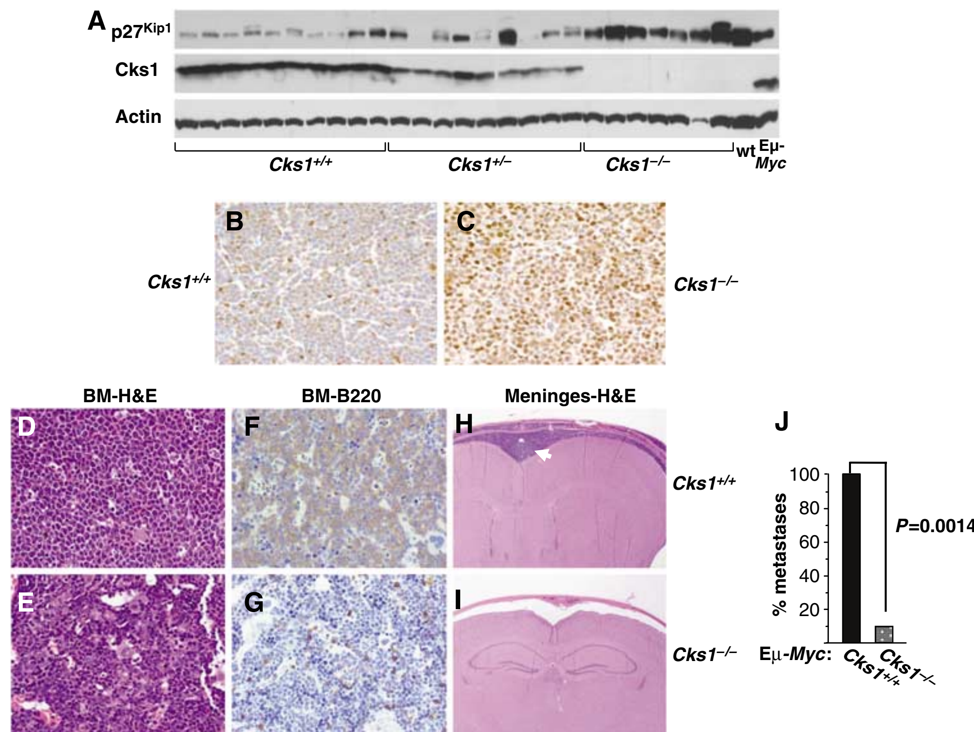

Figure 8 Lymphomas arising in *Cks1*$^{-/-}$ Eμ-*Myc* transgenic mice show a dramatic reduction of extranodal dissemination and retain elevated p27^{Kip1} protein levels. (**A**) Immunoblot analysis of lymphomas arising in mice of the indicated *Cks1* genotypes were compared with precancerous B220$^+$ B cells from wild-type (wt) and Eμ-*Myc* transgenic mice (Eμ-*Myc*). Immunohistochemical detection of p27^{Kip1} in representative lymphomas from Eμ-*Myc*;*Cks1*$^{+/+}$ (**B**) and Eμ-*Myc*;*Cks1*$^{-/-}$ (**C**) transgenic mice ($\times$40 magnification). H&E staining of bone marrow sections from sick *Cks1*$^{+/+}$ (**D**) and *Cks1*$^{-/-}$ (**E**) Eμ-*Myc* transgenic mice ($\times$40 magnification). B220—immunohistochemistry in bone marrow from sick *Cks1*$^{+/+}$ (**F**) and *Cks1*$^{-/-}$ (**G**) Eμ-*Myc* transgenic mice ($\times$40 magnification). H&E staining of brain sections from *Cks1*$^{+/+}$ (**H**) and *Cks1*$^{-/-}$ (**I**) Eμ-*Myc* transgenic mice ($\times$10 magnification). The white arrow in (H) points to the meningeal lymphoma infiltration. BM denotes bone marrow. (**J**) Nine Eμ-*Myc*;*Cks1*$^{-/-}$ mice and six Eμ-*Myc*;*Cks1*$^{+/+}$ mice were histologically evaluated for lymphoma dissemination. Shown are the percent of mice with bone marrow involvement ($P = 0.0014$).

accelerates lymphomagenesis (Martins and Berns, 2002), and where loss of *E2f1* also compromises Myc's ability to suppress p27^{Kip1} and impairs Myc-induced proliferation and lymphoma development (Baudino *et al*, 2003). Further, these studies suggest that Myc's ability to induce both E2f1 and Cks1, and perhaps also Skp2, is required in concert to suppress p27^{Kip1} expression and to accelerate cell proliferation and tumor development, and that in the absence of *Cks1*, the elevation of p27^{Kip1} levels is not bypassed during tumor development (Figure 8).

Cks1 is necessary for recognition of Thr-187-phosphorylated p27^{Kip1} by Skp2 (Ganoth *et al*, 2001; Spruck *et al*, 2001; Hao *et al*, 2005), and is thus essential to downregulate p27^{Kip1} during cell cycle traverse. Recognition of Thr-187-phosphorylated p27^{Kip1} by the SCF^{Skp2} complex occurs during S and G_2 phases (Malek *et al*, 2001), and other levels of control, for example, Ser-10 phosphorylation of p27^{Kip1} and the cytoplasmic ubiquitin ligase complex KPC, regulate p27^{Kip1} proteolysis at the G_0 and G_1 phases of the cell cycle, respectively (Ishida *et al*, 2000; Kamura *et al*, 2004; Kotake *et al*, 2005). Despite these multiple levels of control, the targeted deletion of *Cks1* revealed that it is a critical arbiter of p27^{Kip1} levels, as its loss leads to profound increases in p27^{Kip} protein (Ganoth *et al*, 2001; Spruck *et al*, 2001). Further, Cks1 has other targets that may contribute to the proliferative response, as Cks1 is also required for SCF^{Skp2}-mediated degradation of the pRb-related protein p130 (Tedesco *et al*, 2002). In turn, this leads to reduced rates of proliferation in *Cks1*-deficient cells and ultimately, in the animal, to a reduced body size (Spruck *et al*, 2001). Underscoring the importance of Cks1, recent studies have shown that Cks1 expression is highly elevated in a number of malignancies, where it is associated with reduced p27^{Kip1} levels, high proliferative rates and poor outcome (Masuda *et al*, 2003; Shapira *et al*, 2004; Slotky *et al*, 2005).

The underlying cause(s) for Cks1 overexpression in cancer are unknown, but our findings suggest that they are linked to Myc oncoproteins, which are overexpressed in a large number of malignancies. Whether tested *ex vivo* or when examined *in vivo*, Myc was revealed to upregulate Cks1 RNA and protein levels and, further, Cks1 was shown to be an essential mediator of the Myc-to-p27^{Kip1} pathway. Myc appears to induce Cks1 expression largely through effects on its transcription, but the induction of *Cks1* mRNA by Myc is E2f1 and FoxM1 independent and is indirect, suggesting that Myc regulates the expression of some other transcription factor that in turn controls *Cks1* transcription. Ultimately, defining the mediators of the Myc-to-Cks1 pathway is important, as our findings establish a crucial role for Cks1 in tumorigenesis, suggesting this pathway could be targeted in cancer prevention and/or therapeutics.

In the response to Myc, Cks1 and perhaps Skp2 are the limiting components of the SCF^{Skp2} complex that are required to degrade p27^{Kip1}, as Myc does not significantly affect the

expression of Cullin1, Skp1 or Rbx1, at least in Eμ-*Myc* transgenic B cells. Cks1's critical role is underscored by the absolute requirement for Cks1 in mediating downregulation of $p27^{Kip1}$ protein in Myc-expressing B cells, a function which is also shared by E2f1 in Myc-driven lymphomagenesis (Baudino *et al*, 2003). Since E2f1 and Cks1 are now shown to play independent and critical roles in the pathway(s) by which Myc downregulates $p27^{Kip1}$, one might predict that they are critical for proper stoichiometry of the SCF^{Skp2} holoenzyme complex. Along these lines, it is intriguing that the retinoblastoma protein (Rb) interacts with the N-terminus of Skp2 and interferes with Skp2-$p27^{Kip1}$ interactions, and with $p27^{Kip1}$ ubiquitylation (Ji *et al*, 2004). In the context of *E2f1* loss, where one should see reductions in active cyclin E/Cdk2 complexes (DeGregori *et al*, 1995), one might then expect Rb's functions to be augmented and to selectively inactivate Skp2 functions. Thus, Myc's ability to induce both Cks1 and E2f1 would ensure that proper levels of Cks1 and Skp2 are available to target $p27^{Kip1}$ to the SCF^{Skp2} holoenzyme for degradation, and it will be important to test this model. Alternatively, it is possible that Myc somehow activates signaling pathways that lead to selective phosphorylation/dephosphorylation(s) of $p27^{Kip1}$ that promote its recognition by the SCF^{Skp2} complex or by the KPC complex (Kamura *et al*, 2004).

The effect of *Cks1* loss on Myc's proliferative, but not apoptotic, response indicates that Cks1 contributes to Myc-induced pathways that regulate the rates of cell division. *In vivo*, this response increases the size of the target population of immature B cells that are most susceptible to transformation, and this is rate-limiting for tumor development. Indeed, there were essentially no effects of *Cks1* loss on Myc's apoptotic programs in B cells, as observed in *Cks1*-deficient animals (Spruck *et al*, 2001). Therefore, the effects of *Cks1* loss upon lymphomagenesis appear restricted to their effects on the proliferative rates of Myc-expressing B cells.

A striking finding revealed by the analyses of lymphomas arising in both Eμ-*Myc* transgenic mice and in human Burkitt lymphoma was that alterations in Cks1, Skp2 and $p27^{Kip1}$ expression were evident in virtually every tumor, a frequency much higher than that observed for alterations of *Arf* or *p53* (~55% of tumors in Eμ-*Myc* transgenic mice; Eischen *et al*, 1999). These findings underscore the importance of this pathway in tumor development and may indicate an important role in maintaining the tumorigenic state. Further, since typical reductions in $p27^{Kip1}$ expression do not occur in lymphomas lacking *Cks1*, and since Eμ-*Myc*;$Cks1^{-/-}$ lymphomas are much less invasive (Figure 8), these findings underscore the potential of targeting the Myc-to-Cks1 pathway in cancer therapeutics.

Materials and methods

Interbreeding of mice and tumor surveillance

Cks1-null mice (mixed background) (Spruck *et al*, 2001) were bred with Eμ-*Myc* transgenic mice (C57BL/6) (Adams *et al*, 1985). F_1 offspring were then bred to obtain $Cks1^{+/+}$, $Cks1^{+/-}$ and $Cks1^{-/-}$-Eμ-*Myc* transgenic littermates. *E2f1*-null mice (C57BL/6) were bred with Eμ-*Myc* transgenics (C57BL/6). F_1 offspring were bred to obtain $E2f1^{+/+}$, $E2f1^{+/-}$ and $E2f1^{-/-}$ Eμ-*Myc* transgenic littermates. Animals were observed for signs of morbidity and tumor development. Tumors were harvested after killing mice, snap-frozen in liquid nitrogen and processed for analysis of DNA, RNA and protein.

Burkitt lymphoma samples

With Institutional Review Board approval and following informed consent, tumors from 17 Burkitt lymphoma patients were banked. RNA and protein were extracted from these tumors. As a control, pooled peripheral blood mononuclear cells from a healthy donor were enriched using CD19-MicroBeads (Miltenyi Biotech) and RNA and protein were prepared.

Cell culture

Primary bone marrow-derived pre-B cells were cultured as described (Eischen *et al*, 1999). MEFs from E13.5–E14.5 embryos, or primary B cells, were cultured and infected with retroviruses as previously described (Eischen *et al*, 1999; Nilsson *et al*, 2004). To evaluate the consequences of Myc activation, cells were treated with 2 μM 4-HT ± 1μg/ml Chx (Sigma Chemicals) and harvested for protein and RNA preparation.

FACS analysis and magnetic-activated cell sorting (MACS) of B cells

Rates of proliferation of $B220^+sIgM^+$ and $B220^+sIgM^-$ cells were determined using a Flow kit as described (Baudino *et al*, 2003). The remainder of the bone marrow and spleen cells were incubated with B220 MicroBeads and enriched by magnetic cell sorting for B cells (Miltenyi Biotech) and used for immunoblot or real-time PCR analysis.

Methods for RNA, protein and histological analyses can be found in the Supplementary data online.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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