SKP2 Oncogene Is a Direct MYC Target Gene and MYC Down-regulates p27\(^{\text{KIP1}}\) through SKP2 in Human Leukemia Cells\(^*\)\(^{\dagger}\)

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Gabriel Bretones\(^{\dagger}\), Juan C. Acosta\(^{\dagger,2}\), Juan M. Caraballo\(^{\dagger}\), Nuria Ferrándiz\(^{\dagger,2}\), M. Teresa Gómez-Casares\(^{\dagger}\), Marta Albajar\(^{\dagger}\), Rosa Blanco\(^{\dagger}\), Paula Ruiz\(^{\dagger}\), Wen-Chun Hung\(^{\dagger}\), M. Pilar Albero\(^{\dagger,2}\), Ignacio Perez-Roger\(^{\dagger,2}\), and Javier León\(^{\dagger}\)

From the \(^{\dagger}\)Departamento de Biología Molecular, Instituto de Biomedicina y Biotecnología de Cantabria, Universidad de Cantabria, Consejo Superior de Investigaciones Científicas, SODERCAN (Sociedad para el Desarrollo de Cantabria), 39011 Santander, Spain, the \(^{\dagger}\)Servicio de Hematología, Hospital Dr. Negrín, 35020 Las Palmas de Gran Canaria, Spain, the \(^{\dagger}\)Servicio de Hematología, Hospital Universitario Marqués de Valdecilla-Instituto de Investigación y Formación Marqués de Valdecilla, 39009 Santander, Spain, the \(^{\dagger}\)Instituto de Biomedicinas, National Sun Yat-Sen University, Kaohsiung 804, Republic of China, and the \(^{\dagger}\)Department of Chemistry, Biochemistry and Molecular Biology, Cardenal Herrera-CEU University, 46113 Moncada, Spain

SKP2 is the ubiquitin ligase subunit that targets p27\(^{\text{KIP1}}\) (p27) for degradation. SKP2 is induced in the G\(_1\)-S transit of the cell cycle, is frequently overexpressed in human cancer, and displays transformation activity in experimental models. Here we show that MYC induces SKP2 expression at the mRNA and protein levels in human myeloid leukemia K562 cells with conditional MYC expression. Importantly, in these systems, induction of MYC did not activate cell proliferation, ruling out SKP2 up-regulation as a consequence of cell cycle entry. MYC-dependent SKP2 expression was also detected in other cell types such as lymphoid, fibroblastic, and epithelial cell lines. MYC induced SKP2 mRNA expression in the absence of protein synthesis and activated the SKP2 promoter in luciferase reporter assays. With chromatin immunoprecipitation assays, MYC was detected bound to a region of human SKP2 gene promoter that includes E-boxes. The K562 cell line derives from human chronic myeloid leukemia. In a cohort of chronic myeloid leukemia bone marrow samples, we found a correlation between MYC and SKP2 mRNA levels. Analysis of cancer expression databases also indicated a correlation between MYC and SKP2 expression in lymphoma. Finally, MYC-induced SKP2 expression resulted in a decrease in p27 protein in K562 cells. Moreover, silencing of SKP2 abrogated the MYC-mediated down-regulation of p27. Our data show that SKP2 is a direct MYC target gene and that MYC-mediated SKP2 induction leads to reduced p27 levels. The results suggest the induction of SKP2 oncogene as a new mechanism for MYC-dependent transformation.

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1 Supported by fellowships from Spanish Ministerio de Ciencia e Innovacion.

2 Present address: MRC Clinical Sciences Centre, Imperial College Faculty of Medicine, Hammersmith Hospital Campus, London, United Kingdom.

3 To whom correspondence should be addressed: Departamento de Biología Molecular, Facultad de Medicina, IBBTEC, Cardenal Herrera Oria s/n, 39011 Santander, Spain. Tel.: 34-942-201952; E-mail: leoni@unican.es.

\(^{\dagger}\) c-MYC (hereafter MYC) is an onco- transcription factor of the helix-loop-helix/leucine zipper protein family. MYC exerts a wide array of biological functions in different cellular models related to cell cycle control, genomic instability, energetic metabolism, protein synthesis, intercellular communication, and control of cell differentiation (for reviews see Refs. 1–3). MYC forms heterodimers with the protein MAX that bind to E-boxes in the regulatory regions of target genes. MYC regulates ~1000 genes and binds to 15% of genomic loci (4–7).

Most target genes are activated by MYC in an E-box-dependent manner, although an important fraction of MYC target genes are repressed by MYC (reviewed in Refs. 8 and 9). Consistent with the biological activities of MYC, its expression is deregulated in a wide array of human solid tumors and in leukemia, often associated to tumor progression (10–12).

SKP2 is an oncogenic protein frequently overexpressed in human cancers. Moreover, SKP2 behaves as an oncogene in transformation assays (13, 14). SKP2 encodes the F-box protein of the ubiquitin ligase SCFSKP2 complex; this complex is comprised by three core subunits RBX1, CUL1, and SKP1, whereas the substrate is bound by SKP2 along with the small protein CKS1 (14, 15). More than 20 SKP2 substrates have been found, including several proteins involved in cell cycle control (e.g. cyclin E, p21, p57, and E2F1) (14), and SKP2 can suppress p53-mediated apoptosis (16). Nonetheless, the main oncogenic mechanism of SKP2 is attributed to degradation of the cyclin-dependent kinase (CDK)\(^4\) inhibitor p27\(^{\text{KIP1}}\) (hereafter p27) (13).

p27 was originally described as a CDK inhibitor, with cyclin E-CDK2 complexes as its primary targets (17, 18). Low p27 levels are associated with a poor prognosis in most tumors (19, 20). p27 is regulated mainly at the protein stability level. The degradation of p27 by proteosomes is preceded by ubiquityla- (21), which is mediated by the SCFSKP2 complex, in which SKP2 is the p27-recognition subunit (22–24). To be recognized

\(^{\dagger}\) The abbreviations used are: CDK, cyclin-dependent kinase; 4HT, 4-hydroxy-tamoxifen; CHX, cycloheximide; CML, chronic myeloid leukemia; TPA, phorbol-12-myristate-13-acetate; qPCR, quantitative PCR; sh, short hairpin.

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by the SCFSKP2 complex, p27 must be bound to cyclin-CDK complex and phosphorylated at Thr-187. p27 binds to cyclin E-CDK2 as a target for phosphorylation by the bound CDK2 or a second active cyclin E-CDK2 complex at Thr-187 (reviewed in Figs. 1a and 1b). The oncogenic activity of SKP2 is attributed mainly to low p27 levels, and consistently, the levels of SKP2 and p27 inversely correlate in many tumors and cell models (13, 14).

MYC and p27 show functional antagonism in proliferation: MYC and the loss of p27 cooperate in animal carcinogenesis models (27), and MYC abrogates p27 function in proliferation. The antagonistic effect of MYC is mediated through several mechanisms (reviewed in Refs. 11). MYC down-regulates murine p27 at the transcriptional level (28, 29); in addition, it induces cyclin D2 and CDK4, which sequester p27 in CDK-cyclin complexes (30, 31); finally, MYC induces expression of CUL1 (32) and CKS1 (33), both components of the SCFSKP2 complex. A correlation between MYC and SKP2 expression was recently described in a murine lymphoma model, with only modest effects on p27 levels (34). The previous studies are nonetheless hampered by the fact that SKP2 is induced in cell cycle stimulation, which is also a well-known MYC activity. This has made it difficult to distinguish whether SKP2 induction is a direct effect of MYC. Here we studied the MYC-SKP2-p27 axis in different cell models with conditional MYC expression in which MYC is induced in cell cycle-arrested cells. We show for here that SKP2 is a direct MYC target gene and that MYC-mediated SKP2 up-regulation contributes to p27 degradation.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Cell Proliferation**—K562 and K562-derived cell lines were cultured in RPMI 1640 medium with 8% fetal calf serum and antibiotics. Unless otherwise stated, the cells (2.5 × 10^5) cells/ml were treated with 75 μM ZnSO_4_200 nm 4-hydroxytamoxifen (4HT) (Sigma), 10 μM phorbol-12-myristate-13-acetate (TPA), and 1 μM imatinib. Transient transfections were performed in a nucleofector (Amaxa) following the manufacturer’s instructions. Short hairpin expression vectors used were shMyc (35) and pSR-shSKP2 (36). Kmyc are K562 cells with a MYC gene inducible by ZnSO_4_ (37). KMER4 are K562 cells stably transfected with a vector expressing the MycER construct. MycER is activated by 4HT (38). Kp27-5 cells are K562 cells stably transduced with a p27 gene inducible by Zn^2+ (39). Kp27MER are Kp27-5 cells transduced with the MycER gene (40). KmycBp53 are KmycB cells expressing a p53 mutant that is activated at 32 °C (41). KMERshSKP2 are KMER4 cells infected with retrovirus expressing pSR-shSKP2 and selected with 1 μg/ml puromycin. pSR-shSKP2 was constructed by inserting the siRNA sequence AAGGGAGTGACAAAGACTTTTCAAGAGACAAAGTCCTTGTTGCTACCCCTT (36) into the BgIII and HindIII sites of pSUPER-retro vector (the sequence of the hairpin loop is underlined). KMERpSR are KMER4 cells infected with the empty retroviral vector pSUPER-retro. TM1 are derived from the Mv1Lu lung epithelial cell line with a tetracycline-repressible MYC gene (42).

5 M. Albajar and J. Leon, submitted for publication.

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HO15.19 Myc-null rat fibroblasts were generated from parental TGR-1 by homologous recombination (43). TM1, HO15.19, and TGR1 cells were cultured in DMEM supplemented with 10% fetal calf serum and antibiotics. P493-6 cells are immortalized human B cells expressing a tetracycline-repressible MYC gene (44) and were cultured in RPMI 1640 with 10% fetal calf serum and antibiotics. Cell proliferation was determined by cell counting in a hemocytometer.

**Nude Mice Xenografts**—KMERpSR or KMERshSKP2 cells (10^6) were resuspended in 0.2 ml of RPMI/Geltrex (Invitrogen) (1:1). The cell suspension was injected subcutaneously into the right and left flanks of 6-week-old female athymic nude mice (Hsd:Athymic Nude-Foxn1 nu/nu; Harlan Laboratories models); after 21–24 days, the mice were euthanized, and the tumors were weighed.

**mRNA Analysis**—Total RNA from cell lines and bone marrow cells was isolated using the RNAesy kit (Qiagen). RT was performed with i-Script reverse transcriptase (Bio-Rad). Quantitative PCR (qPCR) was performed with the SYBRGreen PCR kit (Bio-Rad). The sequences of primers used and amplicon sizes are shown in supplemental Table S1. The data were normalized to ribosomal protein S14 mRNA levels.

**Immunoblot**—Total cell lysates and immunoblots were carried out as described (39). The blots were developed with secondary antibodies conjugated to IRDye680 and IRDye800 (Li-Cor Biosciences) and visualized in an Odyssey scanner. The antibodies used were anti-actin (goat polyclonal, sc-1616), MYC (sc-764), SKP2 (sc-7164), p27 (sc-528), α-tubulin (sc-5546) (all rabbit polyclonals from Santa Cruz Biotechnology), p27 monoclonal antibody (K-25020; Transduction Labs), and Thr(P)-187-p27 (rabbit polyclonal, 71-7700; Invitrogen).

**Chromatin Immunoprecipitation**—The cells (5 × 10^7) were fixed in 1% formaldehyde, lysed with SDS, and sonicated, essentially as described (45). ChIP was performed using Dynabeads-Protein G (Dynal Biotech) coupled to anti-MYC antibody and rabbit IgG as specificity control. Quantitative PCR of eluted DNA was performed with the primers of SKP2 and lactate dehydrogenase genes indicated in supplemental Table S1.

**Immunofluorescence Analysis**—Kp27MER cells were treated with ZnSO_4_ and/or 4HT for 24 h and immunostained with anti-SKP2 and -p27 antibodies as described and revealed with fluorescein- or rhodamine-conjugated secondary antibodies as described (40). The cells were stained with Vectashield mounting medium (Vector Laboratories) with DAPI to visualize nuclei.

**Luciferase Assays**—The cells (3 × 10^6) were electroporated with an Amaza electroporator and Mirus Ingenio (Mirus Bio LLC) transfection reagent. Kmyc cells were transfected with 3 μg of the −1148-SKP2 promoter (46) or pGL3basic vector (Promega) as control. K562 or KmycJ cells were nucleolectected with 1.5 μg of 4×Ebox-Luc or the MYC-unresponsive 4×EboxMut-Luc reporter constructs (47). K562 cells were nucleolectected with 1.5 μg of pSR-shSKP2 or pSUPER-retro vector. The cells were lysed, and luciferase activity was measured in duplicate in a dual luciferase reporter gene assay system (Promega). The data were normalized to Renilla luciferase values (0.5 μg of pRL-TK vector in each transfection).

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**RESULTS**

**Induction of MYC Is Associated to Induction of SKP2**—We studied the MYC-SKP2 relationship in cell models in which (i) ectopic MYC expression is activated or induced and (ii) MYC induction does not stimulate cell proliferation, a critical condition to avoid SKP2 up-regulation subsequent to cell cycle progression. In the Kp27MER cell line, p27 can be induced with ZnSO$_4$, and MYC can be activated with 4HT (Fig. 1A, top panel). Large scale gene expression profiling in these cells showed that MYC activation provoked up-regulation of SKP2 mRNA (40). We set out to validate this microarray data. Induction of p27 resulted in rapid growth arrest (Fig. 1A) and SKP2 expression was down-regulated (Fig. 1C), as predicted. Importantly, MYC activation did not modify p27-mediated growth arrest in this model (Fig. 1A), confirming previous results (40). MYC activation (i.e. 4HT treatment) nonetheless resulted in a 2-fold increase in SKP2 mRNA in proliferation-arrested Kp27MER cells (Fig. 1B, black bars). Moreover, MYC activation induced robust up-regulation of SKP2 protein, as detected by immunoblot (Fig. 1C, compare lanes 1 and 2 and lanes 3 and 4). The immunoblot also showed down-regulation of endogenous MYC following activation of ectopic MYC. This well known effect of ectopic MYC (49, 50) confirmed MycER activation by 4HT. In contrast to MycER-expressing cells, 4HT did not modify SKP2 expression in Kp27-5 cells, in which p27 is induced by Zn$^{2+}$ but which do not carry the MycER allele (Fig. 1B).

We used KMER4 cells treated with 1 μM imatinib, a BCR-ABL inhibitor that causes rapid down-regulation of endogenous MYC in K562 cells (51). MYC activation with 4HT in KMER4 cells did not rescue the proliferation arrest mediated by imatinib (Fig. 1D). Treatment of KMER4 with imatinib led to down-regulation of SKP2 mRNA, as detected by RT-qPCR (Fig. 1E), and protein, as detected by immunoblot (Fig. 1F). Activation of MYC in the presence of imatinib resulted in a 2-fold induction of SKP2 mRNA after 24 h (Fig. 1E) and protein (Fig. 1F, compare lanes 1 and 2 and lanes 3 and 4). Although BCR-ABL was previously reported to induce SKP2 (36, 52) and to induce MYC (51, 53), here we show that ectopic MYC up-regulates SKP2 in proliferation-arrested cells with inhibited BCR-ABL.

The KmycJ cell line is a K562 derivative that bears a ZnSO$_4$-inducible MYC allele (37). Treatment of K562 and KmycJ cells with 10 nm TPA resulted in growth arrest (Fig. 2A). TPA also induced a marked down-regulation of MYC, and as anticipated, SKP2 mRNA and protein expression were also repressed by TPA (supplemental Fig. S1). MYC induction did not rescue cells from TPA-induced growth arrest (Fig. 2A), confirming previous reports (54). We induced MYC in KmycJ cells pretreated (12 h) with 10 nm TPA, i.e. when the cells had very low endogenous MYC levels. In these TPA-arrested cells, MYC induction with Zn$^{2+}$ resulted in a notable increase in SKP2 mRNA, as assessed by RT-qPCR (Fig. 2B), and in protein, as...
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FIGURE 2. SKP2 induction by MYC in KmycJ and KmycBp53 cell lines. A, the KmycJ cell model (top panel). Proliferation rates of KmycJ cells treated with 10 nM TPA and 75 μM ZnSO₄ were measured by counting viable cells in a hemocytometer (bottom panel). B, KmycJ cells were treated with 10 nM TPA for 12 h and then induced with 75 μM ZnSO₄ as indicated. SKP2 mRNA levels were measured by RT-qPCR. C, KmycJ cells were treated as in B, and expression of SKP2, MYC, and actin (loading control) was assayed by immunoblot. D, the KmycBp53 cell model (top panel). Proliferation rates of KmycBp53 cells were treated with 75 μM ZnSO₄ and incubated at 37 or 32 °C (bottom panel). E, KmycBp53 cells were incubated (37 or 32 °C) alone or with ZnSO₄ for 24 h. SKP2 mRNA levels were determined by RT-qPCR. The values are the means ± S.E. of three experiments. F, KmycBp53 cells were treated as in E, and the levels of SKP2, MYC, and actin (loading control) in total cell lysates were assayed by immunoblot.

determined in immunoblot (Fig. 2C). The results were similar when we used a different MYC-inducible K562 cell line (KmycB) (not shown).

Finally, we assayed SKP2 induction by MYC in the KmycBp53 cell line. This is a K562-derived cell line bearing a p53 mutant that is activated at 32 °C and a Zn²⁺-inducible MYC transgene (41). MYC induction did not antagonize the p53-mediated proliferation arrest at 32 °C (Fig. 2D). We found that MYC induction with Zn²⁺ resulted in the increase of SKP2 mRNA (Fig. 2E) and SKP2 protein (Fig. 2F), despite the fact that the cells were growth-arrested by p53 at 32 °C. We conclude that MYC induction provokes SKP2 induction independently of cell cycle activation.

Silencing of MYC Is Associated with Repression of SKP2 in Several Cell Types.—To confirm the effects of MYC on SKP2 expression, we silenced MYC in K562 cells using siRNA. K562 cells were nucleofected with a short hairpin MYC vector (pRS-shMYC) (35). MYC silencing was confirmed by immunoblot and was accompanied by down-regulation of SKP2 protein (Fig. 3A) and mRNA (Fig. 3B). MYC silencing also resulted in down-regulation of B23/nucleophosmin mRNA (a MYC target gene used as positive control) but not of SKP1 mRNA, which encodes another component of the SCF²SKP2 complex used here as negative control (Fig. 2B). To confirm the effect of MYC on SKP2 expression in cell models other than K562, we studied SKP2 expression in P493-6 cells, a human lymphoblastoid cell line carrying a tetracycline-repressible MYC transgene (55). In these cells, doxycycline treatment led to rapid MYC down-regulation accompanied by down-regulation of SKP2 protein (Fig. 3C) and mRNA (Fig. 3D). We also tested TM1 cells, a mink lung epithelial cell line also carrying a tet-off human MYC allele (42).

In TM1 cells, doxycycline addition provoked MYC and SKP2 down-regulation (Fig. 3E), although the cells continued to grow (not shown).

Finally, we used the MYC-null rat cell line HO15.19, which does not express MYC (43). We analyzed MYC and SKP2 expression after serum deprivation in MYC-null and parental cells (TGR1). SKP2 levels were much lower but detectable in MYC-null cells. In contrast to parental cells, however, there was little decrease in SKP2 levels in response to serum deprivation (Fig. 3F). We conclude that MYC is a major regulator of SKP2 expression in cells from different tissues and species.

MYC Induces SKP2 mRNA in the Absence of Protein Synthesis.—To assess whether MYC directly activated transcription of the SKP2 gene, we performed the induction experiments in the presence of the protein synthesis inhibitor cycloheximide (CHX) using Kp27MER cells, in which MYC can be activated with 4HT and p27 can be induced by ZnSO₄. The cells were pretreated with 10 nM TPA (12 h) to down-regulate endogenous MYC and then treated with 10 μg/ml CHX for different periods. CHX effectiveness was confirmed by lack of induction of p27 protein in response to Zn²⁺ (Fig. 4A, compare p27 in lanes 1 and 2 versus lanes 3 and 4 in the absence of 4HT and lanes 5 and 6 versus lanes 7 and 8 in its presence). As predicted, endogenous MYC showed a high degradation rate. MycER was more stable in presence of 4HT (Fig. 4A, compare MycER levels in lanes 9 and 10 versus lanes 11 and 12), although the underlying molecular mechanism is unclear. In sharp contrast, we found that SKP2 was a long-lived protein (>6 h). SKP2 mRNA levels were determined by RT-qPCR. The results showed that 4HT activation of MycER led to increased SKP2 mRNA levels, even in the absence of synthesis of new proteins; this was prob-
ably dependent on the MycER activated by 4HT (Fig. 4B). Both MYC and MycER protein levels were very low after 6 hours of treatment, precluding analysis of SKP2 mRNA expression at longer treatment times.

MYC Activates and Binds to the Human SKP2 Promoter—We tested the ability of MYC to activate the human SKP2 promoter through promoter-luciferase assays. We transfected KmycJ cells with a luciferase reporter containing 1148 bp of SKP2 promoter (46). The results showed that the SKP2 promoter was activated by MYC (Fig. 5A, top panel). Consistent with MYC repression by TPA, MYC transactivation activity was reduced in TPA-treated cells after transfection and was elevated when MYC was induced by Zn2+/H11001. As a positive control, we used a luciferase reporter bearing four E-boxes (Fig. 5A, bottom panel). The empty luciferase construct and the reporter with four mutated E-boxes (EboxMut-Luc) were used for normalization. These results suggested that SKP2 could be a direct MYC target gene.

Sequence analysis of the human SKP2 gene revealed several E-boxes in the promoter and in the second exon. We thus used ChIP to analyze promoter occupancy by MYC. The localization of E-boxes in the human SKP2 gene and the amplicons analyzed in the ChIP experiments are shown in Fig. 5B. ChIP demonstrated MYC binding to a region with two E-boxes (CATGCG and CACGCG), mapping at 756 and 389 upstream of the transcription start site (Fig. 5C); both sequences are described as high affinity E-boxes for MYC in vivo (56, 57). As negative controls, we used K562 cells treated with imatinib and TPA, which inhibit MYC expression (Fig. 1, E and F, and supplemental Fig. S1). MYC binding was dramatically reduced after both treatments (Fig. 5C). This MYC-binding region was also detected in K562 and HeLa cells in the ENCODE genome-wide ChIP sequencing project, published by the UCSC genome browser (assembly NCBI36/hg18, Yale/UC Davis/Harvard study). MYC binding to the E-box of the lactate dehydrogenase A (58) was used as positive control (Fig. 5C). Taken collectively, our results indicate that human SKP2 is a direct MYC target gene, at least in human cells.

MYC and SKP2 Expression Correlated in Chronic Myeloid Leukemia Cells—The K562 cell line is derived from a human CML patient in blast crisis. To confirm the correlation between SKP2 and MYC expression in vivo, we used RT-qPCR to determine mRNA levels of SKP2 and MYC in the bone marrow of 31

**FIGURE 3.** SKP2 is down-regulated after MYC repression. A, K562 cells were nucleofected with a short hairpin RNA vector for MYC (shMYC) or the empty vector (Vect.), and MYC, SKP2, and actin expression were assayed by immunoblot 24 and 36 h after nucleofection. B, K562 cells were transfected as in A; at 24 h after nucleofection, the levels of SKP2, B23/nucleophosmin (positive control), and SKP1 (negative control) mRNA were determined by RT-qPCR. The values are the means ± S.E. of three independent transfections. C, human lymphoid P493-6 cells were treated with 1 μg/ml doxycycline (Dox), and SKP2, MYC, and actin expression were assayed by immunoblot. D, P493-6 cells were treated as in C, and SKP2 mRNA expression was determined by RT-qPCR. E, TM1 cells were treated with 2 μg/ml doxycycline to repress MYC for the periods indicated, and SKP2, MYC, and actin expression were determined by immunoblot. F, Myc-null HO15.19 rat fibroblasts and parental TGR1 were serum-deprived for the indicated times, and SKP2, MYC, and actin expression analyzed by immunoblot.
CML patients at diagnosis, 8 CML patients that responded to the treatment, and 4 healthy controls (Fig. 6A). Although the cohort is small, the correlation between MYC and SKP2 expression was statistically significant (Fig. 6B). To determine whether this correlation between MYC and SKP2 at the mRNA level also occurred at the protein level, we analyzed protein extracts by immunoblot; the results confirmed the MYC/SKP2 correlation in seven of eight patients tested (Fig. 6C). We used the Oncomine database to examine a possible correlation between MYC and SKP2 mRNA levels in other hematopoietic neoplasias. Three expression profiling studies showed that Burkitt lymphoma cells, in which MYC expression is deregulated as a consequence of a chromosomal translocation, also expressed higher SKP2 levels (supplemental Fig. S2). We also queried the correlation between MYC and SKP2 expression in tumor samples with the in silico transcriptomics database of the GeneSapiens system (59). CML is not included in these databases, but analyses showed a correlation between MYC and SKP2 expression in human lymphoma (supplemental Figs. S2 and S3).

MYC Identifies SKP2 Accumulation in the Cell Nucleus and p27 Phosphorylation—Previous studies firmly established that SKP2 participates in p27 degradation, and our results so far demonstrated that SKP2 is a MYC target gene in K562 cells. We thus evaluated whether MYC-induced SKP2 also resulted in p27 down-regulation in our models. p27 can be degraded in cytoplasm by a SKP2-independent pathway (60, 61), but SKP2-dependent ubiquitylation of p27 takes place in the cell nucleus (15). We thus performed immunofluorescence studies to explore the localization of both proteins in the K562 model. The results in Kp27MER cells showed that MYC acti-
vation resulted in increased nuclear SKP2 and decreased nuclear p27 levels in most cells. It is of note that some cells showing higher nuclear SKP2 levels also showed lower p27 (Fig. 7A).

The actual substrate of the SCFSKP2 complex is p27 phosphorylated at threonine 187 (26). For MYC-mediated degradation of p27 via SKP2 up-regulation, it is thus necessary that a substantial fraction of p27 be in the form of Thr(P)-187-p27. In a kinetic study to determine total p27 and Thr(P)-187-p27 in Kp27MER cells treated with Zn2+/H11001 and 4HT (4–24 h), MYC initially provoked an increase in the phosphorylated p27 fraction (10-fold after 8 h compared with control cells; Fig. 7B). At longer treatment times, the phospho-p27 fraction and the total p27 levels were reduced, as anticipated. We performed a dose-response study to analyze total p27 and Thr(P)-187-p27 in Kp27MER cells treated with 50 μM Zn2+/H9262M ZnSO4. At this ZnSO4 concentration, p27 levels were not high enough to titrate out the SCFSKP2 system, as occurs with higher ZnSO4 doses (Fig. 7B and data not shown). Immunoblot analysis showed that MYC activation with 4HT resulted in a significant decrease in p27 levels when p27 was induced with Zn2+ (Fig. 8A, compare lanes 1 and 2 and lanes 3 and 4). Consistent with the reduction in p27, the proliferation rate was augmented by MYC in Kp27MER cells treated with 50 μM ZnSO4 (data not shown and Ref. 40). In contrast, 4HT did not reduce p27 levels in parental Kp27-5 cells, which bear a Zn2+-inducible p27 but not the MycER construct (Fig. 8B). We also tested the MYC effect on the endogenous p27 that is up-regulated when the KMER4 cells reach quiescence ([H11011] 1.5 × [H11003] 106 cells/ml after 3 days of culture). Immunoblot results demonstrated that SKP2 levels were higher and p27 was lower in cells with activated MYC (Fig. 8C).

As a second approach to testing the MYC-SKP-p27 axis in our model, we infected KMER4 cells with retrovirus expressing a short hairpin SKP2 (shSKP2) vector to generate the cell line KMERshSKP2. Immunoblot results confirmed that SKP2 levels were reduced by shSKP2 and that p27 levels were concomitantly increased (Fig. 9A) compared with the KMERpSR cell line expressing the empty vector. In agreement with the increased p27 abundance, shSKP2-expressing cells grew at a
reduced rate (Fig. 9B) and showed cell accumulation in the G1 phase (not shown). This difference in growth rates was reproduced in vivo; whereas KMERpSR xenografts readily formed tumors in nude mice, KMERshSKP2 cells did not form tumors, or they were much smaller (Fig. 9, C and D). Consistent with the lack of SKP2, p27 levels were not reduced by MYC in cells expressing shSKP2 (not shown). Previous reports describe a positive effect of SKP2 on MYC stability and transcriptional activity (62, 63). Nonetheless, we found no significant changes in MYC levels after SKP2 silencing in K562 cells (Fig. 9A). We also tested MYC transcriptional activity after SKP2 silencing by the shSKP2 expression vector. SKP2 silencing was confirmed by immunoblot (Fig. 9E), but luciferase assays failed to show a significant SKP2 effect on MYC-dependent transcriptional activity (Fig. 9F). Collectively, our results show that MYC induces SKP2 and that this induction is responsible, at least in part, for p27 down-regulation.

**DISCUSSION**

MYC stimulates proliferation, and SKP2 expression is associated with cell cycle progression (actually, SKP2 stands for S phase kinase-associated protein 2). This has made it difficult to date to distinguish whether SKP2 induction is a direct or an indirect effect of MYC. Here we used four K562 cell-based models in which MYC can be induced or activated in cell cycle-arrested cells. Proliferation arrest was achieved by several approaches: ectopic p27 overexpression, p53 activation, TPA, and imatinib treatments. In all cases, MYC induction resulted in SKP2 up-regulation. In TM1 epithelial cells, MYC repression similarly provoked SKP2 down-regulation although cells continued to proliferate. SKP2 regulation by MYC was therefore not an indirect effect of MYC-induced cell cycle progression. Moreover, our data strongly suggest that SKP2 is a direct MYC target gene based on three additional observations: (i) MYC up-regulates SKP2 mRNA expression when protein synthesis is inhibited, (ii) MYC activates the SKP2 promoter in luciferase reporter assays, and (iii) MYC binds to an E-box in the 5′ regulatory region of human SKP2 in ChIP assays. Up-regulation of SKP2 was also recently reported in MYC-induced murine lym-
p27 can be degraded via SKP2-dependent and -independent systems (61, 69). The phenotype of \(Skp2^{−/−}\) mice and cells is rescued by the concomitant knock-out of the p27 (\(Cdkn1B\)) gene, however, indicating that the main transformation mechanism of SKP2 is linked to p27 degradation (70, 71). In our K562 model, SKP2 induction by MYC also results in reduced p27 levels. Moreover, Thr-187 phosphorylation is a prerequisite for SKP2 binding, and MYC promotes p27 phosphorylation at Thr-187. Although other unknown mechanisms may also operate, Thr-187 phosphorylation is likely related to the MYC-induced up-regulation of CDK2 and cyclins A and E reported in many systems (8, 11). A MYC-induced up-regulation of p27-bound CDK2 has also been reported in K562 cells (40). Thus, MYC would drive p27 degradation by increasing both the levels of SKP2 and of its substrate Thr(P)-187-27. In other models, MYC is reported to induce other SCF complex members such as CKS1 (33) or CUL1 (32), reinforcing the idea that control of p27 levels is an important MYC function.

SKP2 is reported to participate in MYC degradation and, at the same time, to activate MYC transcriptional activity (62, 63). However, in our model, the SKP2 effect on MYC levels was marginal, in contrast to the decrease observed in p27. In addition, we detected no effect on MYC transcriptional activity as measured with luciferase reporters. This discrepancy might be due to the different cell types used in our study. A recent report showed that MYC degradation in hematopoietic cells is only dependent on FBW7 ubiquitin ligase and not on SKP2 (67). Another possible explanation is exhaustion of the SKP2-dependent degradation machinery for MYC in our system (63), because the K562 cell line expresses high basal MYC levels compared with other human leukemia cell lines (72).

The demonstration of SKP2 as a MYC target gene reveals a previously unreported mechanism for the transforming activity, because SKP2 is itself an oncogenic protein. It is widely accepted that abrogation of p27 activity is one of the MYC transforming mechanisms. Here we propose another mechanism, that of p27 degradation through SKP2 induction. Our data strongly suggest that the MYC deregulation(SKP2 induction/p27 degradation axis contributes to the carcinogenic mechanism of MYC.
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