Myc-enhanced expression of Cul1 promotes ubiquitin-dependent proteolysis and cell cycle progression

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The c-Myc oncoprotein plays an important role in the growth and proliferation of normal and neoplastic cells. To execute these actions, c-Myc is thought to regulate functionally diverse sets of genes that directly govern cellular mass and progression through critical cell cycle transitions. Here, we provide several lines of evidence that c-Myc promotes ubiquitin-dependent proteolysis by directly activating expression of the Cul1 gene, encoding a critical component of the ubiquitin ligase SCF^{SKP2}. The cell cycle inhibitor p27^{kip1} is a known target of the SCF^{SKP2} complex, and Myc-induced Cul1 expression matched well with the kinetics of declining p27^{kip1} protein. Enforced Cul1 expression or antisense neutralization of p27^{kip1} was capable of overcoming the slow-growth phenotype of c-Myc null primary mouse embryonic fibroblasts (MEFs). In reconstitution assays, the addition of in vitro translated Cul1 protein alone was able to restore p27^{kip1} ubiquitination and degradation in lysates derived from *c-myc*^{-/-} MEFs or density-arrested human fibroblasts. These functional and biochemical data provide a direct link between c-Myc transcriptional regulation and ubiquitin-mediated proteolysis and together support the view that c-Myc promotes G₁ exit in part via Cul1-dependent ubiquitination and degradation of the CDK inhibitor, p27^{kip1}.

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That c-Myc plays an integral role in cellular proliferation is understood from its high levels in proliferating cells and low levels in quiescent cells, its rapid increase on growth factor-stimulated proliferation (Obaya et al. 1999), and its ability to stimulate S-phase entry (Eilers et al. 1991) and shorten the cell cycle (Karn et al. 1989). It has become apparent from landmark studies in Drosophila and mammalian cells that c-Myc triggers G₁ exit in part through its ability to promote an increase in cell mass, a known physiological stimulus for G₁ exit (Killander and Zetterberg 1965; Iritani and Eisenman 1999; Johnston et al. 1999). A large body of work has also established that c-Myc can modulate the expression of genes controlling the cell cycle and that most of these Myc-responsive targets regulate the activity of the G₁ CDKs, primarily CDK2, and thereby facilitate transit through the G₁/S transition.

Activation of c-Myc in quiescent fibroblasts leads to

the rapid induction of cyclin E/CDK2 kinase activity (Steiner et al. 1995; Muller et al. 1997), findings in accord with the requirement of CDK2 activation for c-Myc to promote G₁ exit (Rudolph et al. 1996). In contrast, dominant negative mutant alleles of *c-myc* or somatic deletion of *c-myc* suppress cyclin E/CDK2 activity (Berns et al. 1997; Mateyak et al. 1997; Moreno et al., unpubl.). c-Myc activation of cyclins D1 and D2 gene expression (Daksis et al. 1994; Hoang et al. 1994) is thought to lead to D-type cyclin sequestration of p27^{kip1} from the CDK2 complex (Bouchard et al. 1999; Perez-Roger et al. 1999). The c-Myc-responsive target, *cdc25a*, (Galaktionov et al. 1996) encodes a phosphatase capable of activating both Cdk2 and Cdk4. Conversely, c-Myc can directly repress transcription of the *p27kip1* gene and appears to interfere with p27^{kip1} function (Vlach et al. 1996; Leone et al. 1997). Together, these findings indicate that c-Myc facilitates G₁ exit by positively modulating G₁ cyclin/CDK complexes and, at least in part, by negatively modulating expression of the cell cycle inhibitor p27^{kip1}.

To understand Myc's role in cell cycle progression, we conducted genome-wide expression profiles to identify cell cycle genes that are subject to direct transcriptional regulation by c-Myc. These efforts led us to one such

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Myc-responsive target, *Cullin-1* (*Cul1*; O'Hagan et al. 2000). *Cul1*, an essential component of the SCF^{SKP2} complex, was considered to be an attractive target given the link between c-Myc and CDK2 activity and the importance of SCF^{SKP2} in the regulation of p27^{kip1} protein levels (Carrano et al. 1999; Sutterluty et al. 1999; Tsvetkov et al. 1999). The regulation of p27^{kip1} polyubiquitination by the SCF^{SKP2} complex is complex and dependent on the prior phosphorylation of p27 on threonine 187 (Vlach et al. 1996; Carrano et al. 1999; Sutterluty et al. 1999; Tsvetkov et al. 1999). Depletion of any of the components of SCF^{SKP2} complex, namely Skp1, *Cul1*, or Skp2, prevents polyubiquitination and subsequent degradation of p27^{kip1} (Carrano et al. 1999; Tsvetkov et al. 1999). Moreover, targeted disruption of *skp2* leads to accumulation of p27^{kip1} (Nakayama et al. 2000). Together, these data raised the possibility that the regulation of p27^{kip1} protein levels is a critical focus of Myc's actions in the cell cycle and that *Cul1* could provide a direct link from c-Myc transactivation activity to the ubiquitination machinery responsible for p27^{kip1} regulation and to control of G₁ exit.

Results

Several studies were conducted to verify that *Cul1* is a direct Myc-responsive gene target. First, the Myc-Estrogen Receptor (Myc-ER) system was used to examine gene expression patterns following 4-hydroxy-tamoxifen (4OHT) induction of Myc under conditions of protein synthesis inhibition by cycloheximide. Addition of

4-OHT to cycloheximide-treated Myc-ER expressing IMR90 cells resulted in a threefold increase in *Cul1* mRNA levels and no change in *Cul2* gene expression (Fig. 1A), thus suggesting that Myc is capable of directly enhancing *Cul1* transcriptional activity. Second, anti-*Cul1* immunoblots of protein extracts from density-arrested Myc-ER IMR90 cells, induced for 36 hr with ethanol alone or with 4-OHT, indicated that the levels of *Cul1* protein are increased only in association with Myc-ER activation (Fig. 1B). Third, *Cul1* protein was undetectable in c-Myc-deficient MEFs in which the *c-myc* gene had been acutely deleted by Cre-mediated recombination (Moreno et al., unpubl.) (Fig. 1C). In contrast, activation of the potent Myc antagonist, Mxi1-SR, resulted in a threefold lower level of *Cul1* gene expression in the presence of cycloheximide, than was observed on activation of the weak repressor form of Mxi1, Mxi1-WR (devoid of the Sin3 co-repressor domain; Fig. 1D). Finally, *mxi1*^{-/-} MEFs expressed higher baseline levels of *Cul1* protein than *mxi1* heterozygotes (Fig. 1E).

Next, the human *Cul1* promoter was cloned and found to contain a consensus E box predicted to bind Myc and Mad(Mxi1) complexes (Blackwell et al. 1990; Fig. 1F). The *Cul1* promoter was used to drive expression of the luciferase reporter gene and assayed for Myc and Mxi1 responsiveness. Myc-ER induction was associated with up to threefold induction of *Cul1* reporter activity, while Mxi1-ER induction resulted in a maximum 10-fold repression (Fig. 1G). Mutation of the E-box abrogated Myc and Mxi1 regulation of *Cul1* promoter activity (Fig. 1G). These data, together with the above expression studies, indicate that *Cul1* is a direct transcriptional target of

Figure 1. *Cul1* is a direct transcriptional target of Myc and Mxi1. (A) *Cul1* and *Cul2* Northern blot of IMR90 Myc-ER cells untreated or treated with 1 μ M 4-OHT. GAPDH serves as an internal loading control. In addition, no change in the expression of the SCF^{SKP2} complex components SKP2 or CDC34 was detected in genome-wide expression screens for c-MYC responsive gene targets or by Northern blot analysis (data not shown). (B) *Cul1* immunoblot on IMR90 Myc-ER cells uninduced or treated with 1 μ M 4-OHT. (C) *Cul1* immunoblot on wild-type, or c-myc(-/-) MEFs. (D) *Cul1* Northern blot of IMR90 cells expressing Mxi1-SR-ER or Mxi1-WR-ER after induction with 1 μ M 4-OHT. GAPDH serves as an internal loading control. (E) *Cul1* immunoblot on *mxi1* (+/-), and *mxi1*(-/-) MEFs. (F) Schematic representation of the *Cul1* promoter. The E-box, TATA-box and start sites for transcription (arrow) and translation (ATG) were determined by sequence analysis and are indicated. (G) Histogram representing the ability of Myc and Mxi1 to regulate the *Cul1* promoter or a mutant in which the E-box CATGTG has been altered to CACTCA.

Luciferase values were determined by luminometer and corrected for transfection efficiency by β -galactosidase assay. Values shown are the mean of three experiments plus and minus the standard deviation of the mean. Northern and immunoblot experiments were performed a minimum of two times with identical results.

both Myc and Mxi1. Cul1 belongs to a family of proteins first identified in *Caenorhabditis elegans* and is implicated in regulation of the G_0/G_1 to S transition of the cell cycle (Kipreos et al. 1996). Deletion of the *Cul1* gene in mice causes arrest in early embryogenesis and accumulation of cyclin E (Dealy et al. 1999; Wang et al. 1999). Correspondingly, arrest of *cul1*($-/-$) embryos occurs at approximately the same point of early postimplantation as embryos lacking Myc superfamily function because of deletion of *max* (Shen-Li et al. 2000). To examine further the functional relationship between Cul1 and Myc in control of cell proliferation, we examined the effect of Cul1 overexpression on the growth characteristics of the slow-growing, low-S-phase *c-myc*($-/-$) MEF cultures. In control studies, transduction of *c-myc*($-/-$) MEFs with a c-Myc-encoding retrovirus led to an increase in the fraction of cells in S phase and restored significant growth potential as measured by the WST1 assay and standard growth curves (Fig. 2). The impact of enforced Cul1 expression on the S-phase fraction and growth characteristics of *c-myc*($-/-$) MEFs was equivalent to that of c-Myc (Fig. 2). As p27^{kip1} is a target for the E3-ubiquitin ligase complex SCF^{SKP2}, we also tested whether antisense neutralization of p27^{kip1} could phenocopy the effects of Cul1 or c-Myc overexpression on c-Myc-deficient MEFs. Indeed, transduction of *c-myc*($-/-$) MEFs with antisense p27^{kip1} resulted in reduction of p27^{kip1} protein levels (Fig. 2B), increase in the S-phase fraction, and restoration of proliferative activity (Fig. 2). The inability of Cyclin A overexpression to restore proliferative activity in *c-myc*($-/-$) MEFs supports the proposed specific and critical roles of Cul1 and p27 in Myc regulation of S-phase entry.

The oncogenic capacity of Myc is classically assessed through its ability to cooperate with activated RAS^{G12V} to effect the malignant transformation of primary rat embryo fibroblasts (Land et al. 1983) or MEFs null for INK4a (Serrano et al. 1997). Genome-wide expression profiling and site-directed mutation of the DNA binding domain of Myc has supported the view that oncogenic actions of Myc are highly dependent on its ability to regulate many genes governing diverse cellular processes (O'Hagan et al. 2000). Accordingly, we tested whether Cul1 and antisense-p27 could cooperate with oncogenic RAS to transform primary MEFs null for INK4a (Pomerantz et al. 1998). While c-Myc/RAS generated transformed foci in the monolayer, the cotransfection of RAS along with Cul1, antisense-p27, or the cell cycle control gene Cyclin A yielded a background number of transformed foci (data not shown). These findings reinforce the view that the transforming potential of Myc extends beyond its ability to promote G_1 exit.

The above complementation assays are consistent with the concept that Cul1-mediated p27^{kip1} degradation is a critical aspect of c-Myc regulation of G_1 exit and cellular proliferation. This relationship was further substantiated by the kinetics of Cul1 and p27^{kip1} protein levels in relation to Myc activation. First, proliferating wild-type MEFs expressed detectable Cul1 protein and low p27^{kip1} levels, while the slowly proliferating *c-myc*($-/-$) MEFs showed a reciprocal pattern of expression (data not shown). Similar results for p27^{kip1} have been reported in *c-myc* null rat fibroblasts (Mateyak et al. 1999). Second, Myc-ER activation in density-arrested IMR90 cells (which express barely detectable levels of

Figure 2. Expression of Cul1 or inhibition of p27^{kip1} expression rescues proliferation in *c-myc*($-/-$) MEFs. (A) Wild-type or *c-myc*($-/-$) MEFs infected with retroviruses expressing c-Myc, Cul1, or antisense p27^{kip1} as indicated, were plated at 1×10^5 cells per well in replicate wells and cultured for the indicated number of days. Triplicate wells were trypsinized and counted. Values shown represent the mean of three experiments performed with independently derived MEF lines. (B) p27^{kip1} immunoblot on *myc*($-/-$) MEFs infected with retrovirus expressing Myc, Cul1, or antisense p27^{kip1}. Immunoblot for Max serves as a loading control. (C) Proliferation rate of wild-type and *c-myc*($-/-$) MEFs infected with retroviruses expressing c-Myc, Cul1, or antisense p27^{kip1} as indicated. Proliferation was determined using a WST-1 cleavage assay (Boehringer Mannheim). Values shown represent the average of three independent experiments performed with independently derived MEF lines plus and minus the standard deviation of the mean. S-phase fractions were determined by FACS analysis of MEFs after staining with PI. Values shown represent the mean of three experiments with three independently derived MEF lines.

Cul1 and high levels of p27^{kip1} proteins) resulted in increased Cul1 and reduced p27^{kip1} protein levels and S-phase entry (Fig. 3A). Examination of early time points following Myc-ER activation showed that the increase of Cul1 protein, first evident at 1 hr, precedes the decline in p27^{kip1} protein levels, first evident at 2 hr.

Although these results are consistent with the scenario that Myc induces Cul1, which in turn stimulates p27^{kip1} degradation, it has also been suggested that Myc can repress transcription of the *p27kip1* gene (Li et al. 1994; Lee 1999). Thus, to verify that c-Myc also regulates p27^{kip1} on the posttranslational level, density-arrested Myc-ER-expressing IMR90 fibroblasts were labeled with 35S-Met and 35S-Cys for 1 hr. After the 1 hr pulse, 4-OHT was added to induce Myc-ER activity, and the chase was allowed to proceed for up to 36 hr to follow the kinetics of p27^{kip1} degradation. p27^{kip1} protein was present for up to 24 hr in the empty vector controls, whereas in the Myc-ER cultures, p27^{kip1} protein was undetectable after only 2 hr (Fig. 3B). These results demonstrate that Myc can regulate p27^{kip1} on the posttranslational level.

As Cul1 stimulates degradation of p27^{kip1} via ubiquitination-dependent proteolysis (Carrano et al. 1999; Sutterluty et al. 1999; Tsvetkov et al. 1999), we utilized an in vitro ubiquitination assay system to establish that Myc stimulates degradation of p27^{kip1} via ubiquitination (see Materials and Methods). In this assay, extracts from *c-myc*(-/-) MEFs or density-arrested IMR90 Myc-ER cells failed to stimulate ubiquitination of p27^{kip1} despite the addition of cyclinE/CDK2 (Fig. 4A, lanes 2,4; Fig. 4B,

lanes 2,3). In contrast, extracts from wild-type MEFs or 4-OHT-treated Myc-ER IMR90 cells stimulated robust ubiquitination of p27^{kip1} (Fig. 4A, lanes 3,5; Fig. 4B, lanes 5,6). These differences were not caused by general defects in protein ubiquitination activity as extracts from wild-type and *c-myc*(-/-) MEFs, and uninduced and induced Myc-ER IMR90 cells support the ubiquitination of p53 (Fig. 4D, cf. lanes 1 and 3 with lanes 2 and 4). Most important, addition of in vitro synthesized Cul1 protein to the extracts from *c-myc* null MEFs or density-arrested Myc-ER IMR90 cells rescued the ability of these extracts to stimulate ubiquitination of p27^{kip1} (Fig. 4A, cf. lanes 2 and 4 with lanes 6 and 8; Fig. 4B, cf. lanes 2 and 3 with lanes 8 and 9). In contrast, addition of in vitro synthesized Cul2 protein did not stimulate ubiquitination of p27^{kip1} (Fig. 4C). These data strongly suggest that Cul1 is a critical downstream target of Myc's actions on p27^{kip1}.

Discussion

Genetic studies have positioned the actions of Myc upstream of or at the RB-regulated G₁/S transition (Lahoz et al. 1994). However, our understanding of the mechanisms through which Myc engages the core components of the cell cycle machinery remains incomplete. Using genome expression profile analysis of c-Myc activation, we recently identified Cul-1 as a direct transcriptional target of c-Myc (O'Hagan et al. 2000). Here, we demonstrate that Myc-enhanced expression of Cul-1 promotes ubiquitin-dependent degradation of p27^{kip1}. Overexpression of Cul1 or antisense inhibition of p27^{kip1} rescued the slow-growth phenotype associated with *c-myc* null mouse embryo fibroblasts and was equivalent to exogenous c-Myc replacement. Moreover, Cul1 expression was sufficient for Myc-induced ubiquitination and subsequent degradation of p27^{kip1}. These data provide a direct link between c-Myc transactivation activity and the core cell cycle machinery integral to the regulation of G₁ exit.

The ubiquitin system drives the cell division cycle by the timely destruction of numerous cell cycle regulatory proteins (Elledge and Harper 1998). The SCF complex plays a critical role in this process by catalyzing substrate ubiquitination in the cell cycle (Krek 1998). It is clear that changes in expression of the F-box protein Skp2 play a major role in regulating the G₁/S transition by affecting p27^{kip1} ubiquitination (Amati and Vlach 1999; Carrano et al. 1999; Sutterluty et al. 1999; Nakayama et al. 2000). However, no change in the expression of the SCF^{SKP2} complex components SKP2, RBX1, or CDC34 was detected in genome-wide expression screens for c-MYC responsive gene targets or by Northern blot analysis (data not shown). The biological and biochemical data presented here indicate that Myc's ability to directly modulate the level of Cul1, albeit modestly, represents the critical point through which Myc promotes G₁ exit, specifically via SCF^{SKP2}-mediated polyubiquitination and degradation of p27^{kip1}. These functional and biochemical findings help clarify a key feature of the circuitry that connects c-Myc to the Rb-

Figure 3. Myc enhances expression of Cul1 and stimulates degradation of p27^{kip1}. (A) Immunoblot of Cul1 and p27^{kip1} expression on extracts from density arrested IMR90 MycER cells induced with 1 μM 4-OHT for the indicated times. Cul1 and p27^{kip1} panels were obtained from a single gel. Cell cycle profiles at each time point were determined as described in Materials and Methods. Blot illustrated is representative of three experiments. (B) p27^{kip1} protein was immunoprecipitated from density-arrested IMR90 cells containing MycER or empty vector that were metabolically labeled as described in Materials and Methods and then chased in the presence of 1 μM 4-OHT for the indicated times.

Figure 4. Cul1 is sufficient for Myc-dependent ubiquitination of p27^{kip1}. (A) Ubiquitination of ³⁵S-labeled p27^{kip1} was monitored in the presence of the indicated S100 cell extracts. Unlabeled Cul1 was added to the reactions in the lanes indicated. As a control, no purified ubiquitin was added to the reaction in lane 1. Following the in vitro ubiquitination reaction, p27^{kip1} was precipitated, resolved on SDS-PAGE, and detected by autoradiography. The three panels shown are from the same autoradiogram exposure of a single gel. Input p27^{kip1} is indicated by the arrow. (B) Ubiquitination of ³⁵S-labeled p27^{kip1} in the presence of the indicated S100 extracts. K48R-ubiquitin was added where indicated to prevent polyubiquitination. Input p27^{kip1} is indicated by the arrow. (C) Ubiquitination of ³⁵S-labeled p27^{kip1} in the presence of the indicated S100 extracts. Unlabeled Cul1 or Cul2 was added to the reactions in the lanes indicated. Input p27^{kip1} is indicated by the arrow. (D) Ubiquitination of ³⁵S-labeled p53 in the presence of S100 extracts prepared from wild-type and *c-myc*(-/-) MEFs, and IMR90 MycER cells treated as indicated. Asterisk indicates input p53. Polyubiquitinated forms are indicated as (Ubq)_n. All ubiquitination experiments were performed a minimum of three times with identical results.

regulated restriction point, the most critical decision point in the mammalian cell cycle.

It has been established that low or absent p27^{kip1} protein levels portend a poor prognosis in a variety of human carcinomas including those of the colon and breast (Catzavelos et al. 1997; Loda et al. 1997; Mori et al. 1997; Porter et al. 1997; Steeg and Abrams 1997). p27^{kip1} protein degradation activity, rather than p27^{kip1} mRNA abundance, predicts the observed differences in p27^{kip1} protein levels in the tumors examined to date. Our study raises the possibility that differences in p27^{kip1} degradation activity among human tumors reflect differential activation of c-Myc and consequent up-regulation of Cul1. If this is indeed the case, then prediction of tumor biological behavior might be improved by also examining the level of Cul1 along with its additional downstream targets.

Materials and methods

Cell culture

Embryonic day 13.5 mouse embryo fibroblasts and IMR90 and NIH 3T3 cells were grown in Dulbecco's Modified Eagle Me-

dium (DMEM) (GIBCO BRL) supplemented with 15% fetal bovine serum (FBS), 0.29 mg/ml L-glutamine, 0.03% penicillin and streptomycin, and 25 µg/ml gentamycin sulfate. Myc-ER and Mx1-ER induction studies were performed as described (O'Hagan et al. 2000).

Reporter assays

NIH 3T3 cells were transfected using LipoFectamine reagent (Life Science Technologies) with 100 ng of a luciferase reporter bearing nucleotides -1600 to +400 of the Cullin 1 promoter, 200 ng of pCMX-β-galactosidase, and either 35, 100 or 300 ng of pcDNA3 (Invitrogen) encoding c-Myc or Mx1. Empty pcDNA3 vector was included in each transfection to a total of 600 ng of DNA per transfection. β-galactosidase activity was assayed by incubation of whole-cell extracts with 400 µg/ml ONPG in buffer containing 60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, and 1 mM MgSO₄ and relative transfection efficiencies determined by reading absorbance at 415 nm.

Retroviral infection

pBABE-Myc, pBABE-Cul1, and pBABE-antisense-p27^{kip1} viruses were harvested from transiently transfected φX cell lines. All transductions with ecotropic retrovirus were carried out according to Serrano et al. (1997).

Northern blot

RNA was extracted from IMR90 cells 8 hr after induction by 4-OHT and prepared using the RNeasy Midi kit (Qiagen), followed by extraction with Triazol (Life Science Technologies), and then ethanol precipitation. Twenty micrograms of total RNA was separated by electrophoresis in a 0.8% agarose 2.2 M formaldehyde gel and transferred onto nitrocellulose. Membranes were hybridized with ³²P-labeled probes.

Immunoblots

To assay the amount of Cull1 or p27^{kip1} expressed in MEFs or IMR90 cells, cells were collected and lysed in ice-cold buffer containing 1% NP40; 50 mM Tris-HCl (pH 7.4); 400 mM NaCl; 2 μg/ml PMSF; and 1 μg/ml each of leupeptin, pepstatin, and aprotinin; and resolved by electrophoresis in 8%–16% SDS–polyacrylamide gels and transferred to PVDF membranes by electroblotting. All proteins were detected using the ECL chemiluminescence system (Amersham) and antibodies to Cull1 (NeoMarkers), p27^{kip1} (Transduction Labs), Max (Santa Cruz), or α-tubulin.

Growth curves, cell cycle, and proliferation assays

Mouse embryonic fibroblasts were isolated from individual 13.5-day embryos. *c-myc* was deleted using Cre-recombinase as described in Moreno et al. (unpubl.) and *c-Myc*, Cull1, or antisense p27^{kip1}, introduced by transient retroviral infections as described above. For growth curves, early passage MEFs were seeded at 5×10^4 cells per 60-mm dish. At the indicated times, triplicate plates of cells were trypsinized and counted. Cell cycle profiles were obtained by FACS analysis of PI-stained cells. Proliferation rates were measured using a WST-1 cleavage assay (Boehringer Mannheim) according to manufacturer's instructions.

Pulse-chase

IMR90 cells containing Myc-ER or an empty vector were density arrested for 48 hr, maintained in methionine- and cysteine-free media for 1 hr, metabolically labeled with 300 μCi ³⁵S-labeled methionine and cysteine (NEN Life Science) for 1 hr, and then chased in complete medium containing 1 μM 4-OHT for the indicated times. p27^{kip1} protein was immunoprecipitated from 500 μg total protein at each time point, resolved by electrophoresis in 8%–16% SDS–polyacrylamide gels, and transferred to PVDF membranes by electroblotting prior to autoradiography.

Preparation of S100 cell extracts

IMR90 Myc-ER cells *-/+* 4-OHT, *myc(-/-)*, and *myc(+/+)* MEFs were resuspended in ice-cold hypotonic buffer (20 mM Tris at pH 7.4, 5 mM MgCl₂, 8 mM KCl, 0.5 mM PMSF, 10 μg/ml leupeptin, 1 μg/ml pepstatin, 10 μg/ml aprotinin) for 15 min. The cells were freeze/thawed three times and pelleted by centrifugation at 14,000g for 5 min at 4°C. The resulting supernatant was then ultracentrifuged at 100,000g for 4 hr at 4°C. The supernatant (S100 fraction) was aliquoted and stored at -80°C.

In vitro ubiquitination assay.

In vitro translated ³⁵S-labeled His-tagged p27^{kip1} (3 μl) was incubated in S100 extracts (100 μg) supplemented with ubiquitin (8 μg/μl; Sigma), ubiquitin aldehyde (100 ng/μl; Boston Biochem), energy regenerating system (ERS; 20 mM Tris at pH 7.4, 2 mM ATP, 5 mM MgCl₂, 40 mM creatine phosphate, and 0.5 μg/μl creatine kinase), 1 μM okadaic acid, and 0.5 μM CyclinE/CDK2 in a reaction volume of 30 μl for 1–2 hr at 30°C. Unla-

beled in vitro translated Cull1 or Cul2 (2 μl) was added to the above reaction as described in the figures. His-tagged p27^{kip1} products were captured with Ni²⁺ agarose, resolved by SDS–polyacrylamide gel electrophoresis, and detected by autoradiography.

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Note added in proof

Ectopic expression of SKP2 was not capable of rescuing proliferation in *c-myc(-/-)* MEFs (data not shown) or in *c-myc* null Rat1a cells (Berns et al. 2000). Moreover, addition of in vitro synthesized SKP2 to S100 extracts of density-arrested Myc-ER IMR90 cells did not rescue the ability of these extracts to stimulate ubiquitination of p27^{kip1} (data not shown).

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