

Signal-induced ubiquitination of p57^{Kip2} is independent of the C-terminal consensus Cdk phosphorylation site

Marie-Pierre Leibovitch, Caroline Kannengiesser, Serge Alexandre Leibovitch*

Laboratoire de Génétique Oncologique, UMR 8125 CNRS, 94805 Villejuif, France

Received 8 April 2003; accepted 11 April 2003

First published online 1 May 2003

Edited by Jesus Avila

Abstract The cyclin-dependent kinase inhibitor p57^{Kip2} is required for normal mouse embryonic development. p57^{Kip2} consists of four structurally distinct domains in which the conserved C-terminal nuclear targeting domain contains a putative Cdk phosphorylation site (Thr³⁴²) that shares a great similitude in the adjacent sequences with p27^{Kip1} but not with p21^{Cip1}. Phosphorylation on Thr¹⁸⁷ has been shown to promote degradation of p27^{Kip1}. Although there is sequence homology between the C-terminal part of p27^{Kip1} and p57^{Kip2}, we show that the ubiquitination and degradation of p57^{Kip2} are independent of Thr³⁴². In contrast a destabilizing element located in the N-terminal is implicated in p57^{Kip2} destabilization.

© 2003 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: p57^{Kip2}; T342A; Phosphorylation; Ubiquitination

1. Introduction

Proliferation is positively regulated by cyclin-dependent kinases (Cdks), a family of highly regulated enzymes that link proliferative signals with mechanical aspects of cell duplication. In opposition to Cdks are Cdk inhibitors (Ckis) that induce cell cycle arrest in response to anti-proliferative signals, including contact inhibition, serum deprivation [1] and cell differentiation [2–5]. Ckis can be divided in two families. The Ink4 family includes p16^{Ink4a}, p15^{Ink4b}, p18^{Ink4c} and p19^{ARF}. These proteins specifically bind and inhibit Cdk4 and Cdk6 and not other Cdks such as Cdk2. p21^{Cip1}, p27^{Kip1} and p57^{Kip2}, members of the other family of inhibitors, the Cip/Kip family, have the ability to inhibit all G1/S phase cyclin–Cdk complexes [6]. However, of the Ckis, only p57^{Kip2} is required for embryonic development [7]. Loss of p57^{Kip2} results in proliferative disorders and defects in development of several tissues [8]. The amounts of Ckis expressed in cells may be crucial in controlling cell cycle progression [9], cell differentiation [10] and terminally differentiated cells in mouse embryo [11]. Expression of either p57^{Kip2} or p21^{Cip1} is a requirement for muscle differentiation as shown by double knockout of p57^{Kip2} and p21^{Cip1} that gave the same muscle-deficient phenotype as observed with myogenin (–/–) mice [12]. Up-regulation of p57^{Kip2} has been shown to stabilize

MyoD by blocking cyclin E–Cdk2 activity [10] and by direct interaction with MyoD [13] suggesting that a fine balance exists between cell cycle regulation and terminal differentiation.

The concentration of p27^{Kip1} is thought to be regulated predominantly by posttranslational mechanisms [14,15]. p27^{Kip1} is degraded by both ubiquitin-independent proteolytic cleavage and the ubiquitin–proteasome pathway [16]. Regulation of ubiquitin-mediated proteolysis is often achieved by phosphorylation of the target protein, which renders it more susceptible to degradation. p27^{Kip1} down-regulation is promoted by its phosphorylation on Thr¹⁸⁷ by the cyclin E–Cdk2 complex and by various kinases such as mitogen-activated protein kinases [17,18]. Recent data have shown that Skp2, an F-box protein that functions as the receptor component of an SCF (Skp1/Cul1/F-box protein) ubiquitin ligase complex, binds to and ubiquitinates p27^{Kip1} only when Thr¹⁸⁷ is phosphorylated [19,20]. These observations indicate that phosphorylation of p27^{Kip1} on Thr¹⁸⁷ controls its stability. Because much less is known about the role and the regulation of p57^{Kip2}, in the present report we have examined whether or not Thr³⁴² in p57^{Kip2} that is equivalent to Thr¹⁸⁷ in p27^{Kip1} is responsible for the accelerated degradation of p57^{Kip2}. We show that Thr³⁴² is not a major phosphorylation site of p57^{Kip2} in muscle cells and is not implicated in the ubiquitination and the degradation of p57^{Kip2}. Further analysis suggests that a destabilizing domain in the NH₂-terminal part of p57^{Kip2} is implicated for its degradation.

2. Materials and methods

2.1. Cell cultures and DNA transfection

The mouse skeletal muscle cell line C2C12 was maintained in growth medium supplemented with antibiotics and 20% fetal calf serum in Dulbecco's modified Eagle's medium. C2C12 cells were transfected by the calcium phosphate procedure as described previously [9].

2.2. Plasmids and cycloheximide treatment

For Gal4 fusion experiments, we first created an expression vector pCMV-Gal4-HA by inserting in frame the HindIII–ClaI filled fragment of Gal4 DNA binding domain at the EcoRV site of pCMV-3HA. Gal4_{DBD} fusion proteins were generated by PCR amplifying cDNA encoding various domains of p57^{Kip2} and ligating in frame the PCR products into pCMV-Gal4-HA. C2C12 myoblasts were transfected with pCDNA3-HA-p57^{Kip2}, pCDNA3-HA-p57^{Kip2} T342A or the various mutants in six-well plates [10]. Transfected cells were treated with cycloheximide (Sigma) at 50 µg/ml for the indicated times and harvested for Western blot analyses and p57^{Kip2} proteins were detected by immunoblotting [10]. α-Tubulin was used as an internal control. Western blot were scanned and quantified by using a GelScan (Pharmacia).

*Corresponding author. Fax: (33)-1-42 11 52 61.
E-mail address: leibovit@igr.fr (S.A. Leibovitch).

2.3. Antibodies, immunoprecipitation and Western blot analyses

For immunoprecipitation, whole cell extracts were prepared in immunoprecipitation buffer [10]. Precleared cell lysates were incubated with the indicated antibodies for 2–3 h at 4°C with gentle agitation. Immunocomplexes bound to protein G-Sepharose were then washed several times in immunoprecipitation buffer. Immunoprecipitated proteins were resolved by 10% SDS-PAGE. Electrophoretic transfer of proteins from SDS-PAGE gels to nitrocellulose membranes was blocked with Tris-HCl 50 mM, pH 7.4, 150 mM NaCl and 0.05% Tween 20 containing 5% skimmed milk and incubated overnight at 4°C with primary antibodies. Polyclonal (C20) anti-p57^{Kip2} was provided by Santa Cruz (Biotechnology, Santa Cruz, CA, USA). The monoclonal 12CA5 anti-HA antibody was provided by Boehringer (Mannheim, Germany) and anti- α -tubulin (clone DM1A) was supplied by Sigma. Membranes were washed and incubated for 1 h with a peroxidase-conjugated secondary antibody (Sigma). Membranes were incubated with an enhanced chemiluminescence system (ECL, Amersham) according to the manufacturer's instructions.

2.4. Immunofluorescence staining

Cells were cultured on coverslips and fixed in 2% paraformaldehyde for 30 min at 4°C, and permeabilized with 0.5% Triton X-100 for 5 min at room temperature. The cells were immunostained with anti-HA antibodies (12CA5). The Texas red-conjugated F(ab')₂ fragment of donkey anti-mouse IgG was used to visualize the mouse monoclonal antibodies.

3. Results

3.1. Phosphorylation of p57^{Kip2} in vivo

The C-terminal domain of mouse and human p57^{Kip2} contains a consensus Cdk phosphorylation site conserved in p27^{Kip1} together with adjacent sequences (Fig. 1A). We focused on the potential role of this site in determining the stability of p57^{Kip2} because phosphorylation of p27^{Kip1} on Thr¹⁸⁷ by Cdk2 is thought to initiate the major pathway for p27^{Kip1} proteolysis [19,20]. We generated a mammalian expression vector encoding a mutant mouse p57^{Kip2} protein in which Thr³⁴² was replaced with Ala (T342A). The phosphorylation status of p57^{Kip2} T342A was investigated by transiently expressing the HA-epitope-tagged wild-type and mutant proteins in C2C12 cells and metabolically labeling the cells with ³²Pi. p57^{Kip2} proteins were then immunoprecipitated with anti-HA and the extent of ³²P incorporation was evaluated by autoradiography and image analysis and normalized

by the amount of p57^{Kip2} protein estimated by immunoblot analysis of the immunoprecipitates with anti-p57^{Kip2} (Fig. 1B). The amount of ³²P incorporated by the T342A mutant was virtually identical to that incorporated by wild-type protein in C2C12 muscle cells. These results indicated that Thr³⁴² is not a major phosphorylation site in p57^{Kip2}. Anti-HA immunostaining of culture expressing HA-p57^{Kip2} wt and/or p57^{Kip2} T342A showed identical nuclear localization of the two proteins (Fig. 1C). Characterization of p57^{Kip2} levels in the presence of cycloheximide revealed that mutation T342A

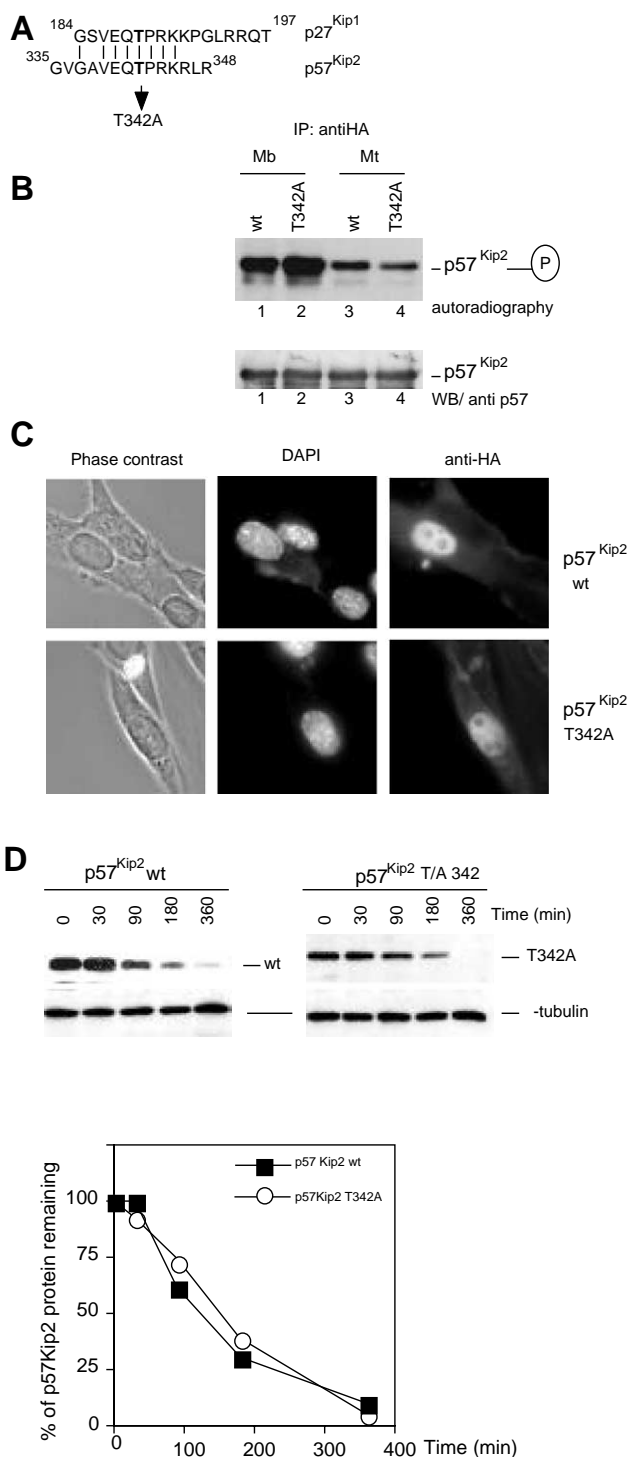


Fig. 1. A: Alignment of the putative C-terminal Cdk phosphorylation site in mouse p27^{Kip1} and p57^{Kip2}. Mutation of threonine residue in alanine is in bold (T342A). B: C2C12 myoblasts (Mb) were transfected with expression vectors encoding HA-p57^{Kip2} wild-type (wt) or HA-p57^{Kip2} T342A. Forty-eight hours after transfection duplicate transfected cells were transferred in differentiation medium for 3 days (Mt). Mb and Mt were metabolically labeled with [³²P]orthophosphate for 2 h. Cells were collected and total cell lysates were immunoprecipitated with anti-HA antibody. Immunoprecipitates were transferred onto nitrocellulose membranes. Shown is an autoradiogram of the radioactivity incorporated into p57^{Kip2} and a Western blot analysis of the same membrane probed with the anti-p57^{Kip2} polyclonal antibody. C: C2C12 myoblasts were transfected with mammalian expression plasmids encoding HA-p57^{Kip2} wt or HA-p57^{Kip2} T342A. Twenty-four hours later, the transfected cells were fixed and immunostained with anti-HA antibody and DAPI and examined by indirect immunofluorescence microscopy. D: After addition of cycloheximide to the culture medium, transfected cells were harvested at various times and processed for Western blot analysis of p57^{Kip2} proteins by using anti-HA antibody. The decay of p57^{Kip2} proteins is depicted in the graph (lower panel).

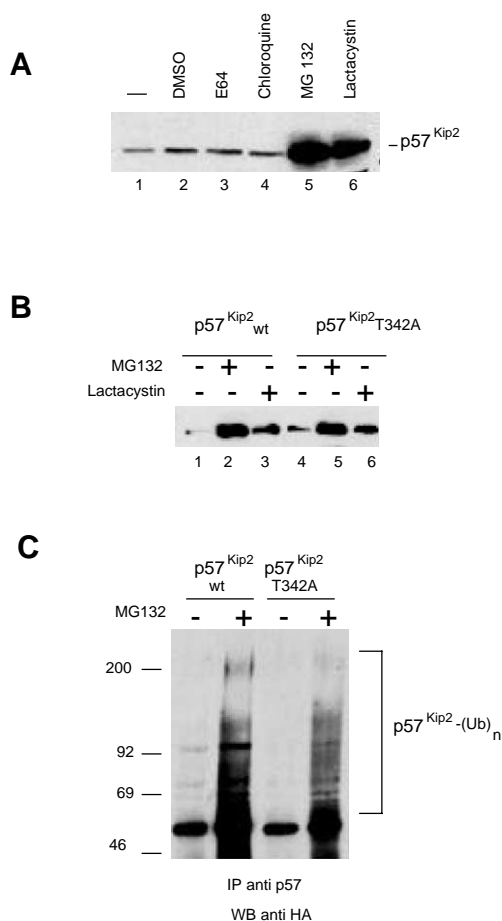


Fig. 2. Inhibition of the proteasome activity induces p57^{Kip2} protein accumulation. **A**: C2C12 myoblasts were transiently transfected by an expression vector encoding HA-p57^{Kip2} wt. Forty-eight hours later, the level of p57^{Kip2} was monitored in the absence (lane 1) or in the presence of DMSO (lane 2), the lysosomal cysteine protease inhibitor E64 (30 μ M, lane 3), the general inhibitor of lysosomal proteolysis chloroquine (100 μ M, lane 4) or the proteasome inhibitors lactacystin (10 μ M, lane 5) and MG132 (50 μ M, lane 6). Cell extracts were prepared and p57^{Kip2} accumulation was analyzed by Western blotting. **B**: C2C12 myoblasts were transiently transfected with expression vectors encoding p57^{Kip2} wt or p57^{Kip2} T342A, treated with MG132 or lactacystin and processed as in panel A. **C**: C2C12 myoblasts were transiently cotransfected with expression plasmids encoding HA-ubiquitin, HA-p57^{Kip2} wt or the mutant T342A. Forty-eight hours later, transfected cells were treated for 4 h with 20 μ M MG132 (+) or not (-) before harvesting. Cells were lysed in denaturation buffer containing 1% SDS, boiled for 5 min at 95°C. The lysates were then diluted in immunoprecipitation buffer, subjected to immunoprecipitation with anti-p57^{Kip2} antibody, and analyzed by Western blotting with anti-HA antibodies.

did not significantly modify protein stability (Fig. 1D). Altogether these data indicate that p57^{Kip2} T342A remained unaffected by mutation of the putative Cdk phosphorylation site.

3.2. p57^{Kip2} T342A is ubiquitinated and degraded by the proteasome in vivo

We investigated the effect of different protease inhibitors on the amount of p57^{Kip2} wt in asynchronous C2C12 myoblasts. Addition of MG132 or lactacystin, two specific inhibitors of the 26S proteasome, induced an accumulation of p57^{Kip2} wt protein after 4 h of treatment (Fig. 2A). In contrast, the cys-

teine protease inhibitor E64 and/or chloroquine, an inhibitor of lysosomal proteolysis, both were ineffective, showing that the proteasome pathway is mainly involved in the degradation of p57^{Kip2}. We next compared the effects of MG132 and lactacystin on the mutant p57^{Kip2} T342A protein level. Addition of MG132 (Fig. 2B, lanes 2 and 5) or lactacystin (lanes 3 and 6) but not DMSO (lanes 1 and 4) induced an accumulation of p57^{Kip2} T342A identical to the wild-type protein. Altogether, these data showed that in myoblasts the proteasome pathway degrades p57^{Kip2} independently of Thr³⁴². Ubiquitination is known to have important roles in the control of the proteasome degradation pathway. Therefore we tested whether p57^{Kip2} wt as well as mutant T342A could also be modified by ubiquitination. Expression vectors encoding the wild-type and the mutant T342A were cotransfected with a plasmid encoding HA-tagged ubiquitin. Twenty-four hours after transfection, C2C12 myoblasts were treated or not with MG132 for 4 h. p57^{Kip2} proteins were immunoprecipitated with anti-p57^{Kip2} antibody from denatured cell lysates and immunoprobbed with anti-HA to detect immunoprecipitated as well as ubiquitinated p57^{Kip2} proteins. In the presence of MG132, both wild-type and the mutant T342A showed a high degree of ubiquitination confirming that Thr³⁴² is not implicated in the ubiquitination and the degradation of p57^{Kip2} (Fig. 2C).

3.3. The NH₂ domain of p57^{Kip2} is required for rapid proteolysis

We next asked which region of p57^{Kip2} is required for its proteolysis. To do this, we constructed various p57^{Kip2} deletion mutants either cloned in frame with the Gal4_{DBD} (Fig. 3A) or not (Fig. 3B). We transiently expressed these deletion mutants in C2C12 myoblasts and measured their stability. Deletion of the amino acids 1–105 (Δ N1) greatly stabilized p57^{Kip2} by increasing its half-life from 3 h up to 6 h. Deletion of the COOH domain (Δ C1) did not affect p57^{Kip2} stability and deletion of the central part plus the COOH domain of

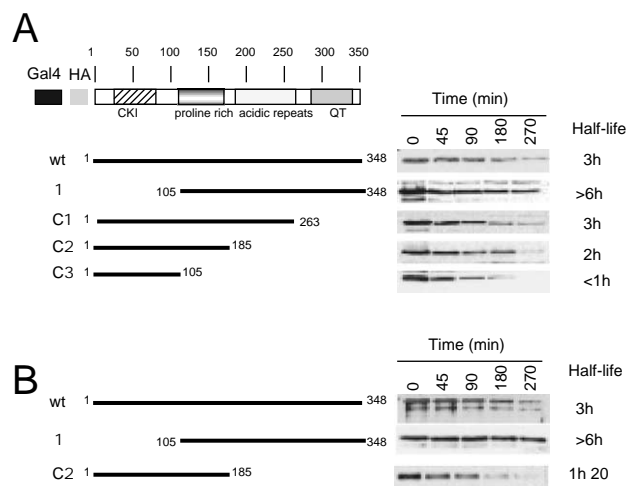


Fig. 3. The NH₂-terminal domain is implicated in the destabilization of p57^{Kip2}. **A**: Schematic diagram of the p57^{Kip2} deletion mutants indicating the main regions of the protein, which is fused to Gal4_{DBD} (left panel). C2C12 myoblasts were transfected with each indicated Gal4_{DBD}-HA-tagged p57^{Kip2} mutant and cells were then treated as in Fig. 1D. **B**: Expression vectors encoding HA-tagged p57^{Kip2} constructs were transiently transfected in C2C12 myoblasts and treated as in Fig. 1D.

p57^{Kip2} (Δ C2) reduced the half-life to ~ 2 h. On the other hand deletion of the amino acids 106 to 348 (Δ C3) greatly destabilized Gal4_{DBD}-p57^{Kip2}, by decreasing its half-life to less than 1 h suggesting that the amino acids 1–105 contain a destabilizing element. These data were confirmed in Fig. 3B. This domain in p57^{Kip2} has been shown to contain the cyclin and Cdk binding sites implicated in the inhibition of cyclin–Cdk complexes [1].

4. Discussion

The phosphorylation state of many proteins affects their stability and phosphorylation of p27^{Kip1} on Thr¹⁸⁷ is a central mechanism in the control of the stability of p27^{Kip1} by ubiquitin-mediated degradation [14–20]. Because Thr¹⁸⁷ in p27^{Kip1} and Thr³⁴² in p57^{Kip2} are located in a C-terminal conserved domain (Fig. 1A), the potential role of Thr³⁴² on p57^{Kip2} phosphorylation and stability was studied. Our results show that p57^{Kip2} is phosphorylated in vivo and is mainly degraded by the proteasome pathway. Mutation of Thr³⁴² in a non-phosphorylatable alanine residue (T342A) does not modify the phosphorylation state nor the stability of p57^{Kip2} in C2C12 muscle cells. Our data demonstrate that ubiquitination and degradation of p57^{Kip2} are independent of Thr³⁴² leading us to the hypothesis that another posttranslational modification and/or domain in p57^{Kip2} is implicated in its destabilization. This finding is strengthened by the fact that p57^{Kip2} does not physically interact with Skp2 (data not shown), the F-box protein component of the SCF^{Skp2} implicated in the ubiquitination and the degradation of p27^{Kip1} phosphorylated on Thr¹⁸⁷ [19,20]. Posttranslational modifications of target proteins are implicated in the recognition by certain SCF-type E3 ubiquitin ligases and subsequent degradation by the 26S proteasome. These include phosphorylation [21], proline hydroxylation [22], *N*-glycosylation [23]. On the other hand, ubiquitination of substrates requires direct binding of particular domain(s) between the F-box protein and the substrate [24]. By using deletion and substitution mutants, we identified a destabilizing domain in p57^{Kip2} protein located between amino acids 1 and 105. This NH₂ region contains three conserved domains: the cyclin and Cdk binding sites and the 3–10 helix motif. This latter has been shown to be implicated in the basis of a molecular mechanism by which p57^{Kip2} but not p21^{Cip1} nor p27^{Kip1} inhibits Cdk2 kinase activity [25]. This conserved domain in human and mouse p57^{Kip2} suggests that it may play an important role in the substrate recognition. Also it may be possible to use the powerful genetic approaches available for yeast to identify the components involved in the p57^{Kip2} targeting and proteolysis systems, which regulate p57^{Kip2} levels.

Acknowledgements: This work was supported by the Institut National de la Santé et de la Recherche Médicale, The Centre National de la Recherche Scientifique and grants from Ligue Nationale contre le

Cancer, Association pour la Recherche sur le Cancer (A.R.C. no. 5921) and the Institut Gustave Roussy.

References

- [1] Polyak, K., Lee, M.H., Erdjument-Bromage, H., Koff, A., Tempst, P., Robert, J.M. and Massagué, J. (1994) *Cell* 78, 59–66.
- [2] Skapek, S.X., Rhee, J., Spicer, D.B. and Lassar, A.B. (1995) *Science* 267, 1022–1024.
- [3] Parker, S.B., Eichele, G., Zhang, P., Rawls, A., Sands, A.T., Bradley, A., Olson, E.N., Harper, J.W. and Elledge, S.J. (1995) *Science* 267, 1024–1027.
- [4] Liu, M., Lee, M.H., Cohen, M., Bommakanti, M. and Freedman, L.P. (1996) *Genes Dev.* 9, 650–662.
- [5] Lee, M.H., Nicolic, M., Baptista, C.A., Lai, E., Tsai, L.H. and Massagué, J. (1996) *Proc. Natl. Acad. Sci. USA* 93, 3259–3263.
- [6] Harper, J.W. and Elledge, S.J. (1996) *Curr. Opin. Genet. Dev.* 6, 56–64.
- [7] Zhang, P., Liegeois, N.J., Wong, C., Finegold, M., Hou, H., Thompson, J.C., Silverman, A., Harper, J.W., DePinho, R.A. and Elledge, S.J. (1997) *Nature* 387, 151–158.
- [8] Yan, Y., Frisen, J., Lee, M.-H., Massagué, J. and Barbacid, M. (1997) *Genes Dev.* 11, 973–983.
- [9] Reynaud, E.G., Guillier, M., Leibovitch, M.P. and Leibovitch, S.A. (2000) *Oncogene* 19, 1147–1152.
- [10] Reynaud, E.G., Pospel, K., Guillier, M., Leibovitch, M.-P. and Leibovitch, S.A. (1999) *Mol. Cell Biol.* 19, 7621–7629.
- [11] Zhang, P., Wong, C., DePinho, R.A., Harper, J.W. and Elledge, S.J. (1998) *Genes Dev.* 12, 3162–3167.
- [12] Zhang, P., Wong, C., Liu, D., Finegold, M., Harper, J.W. and Elledge, S.J. (1999) *Genes Dev.* 13, 213–224.
- [13] Reynaud, E.G., Leibovitch, M.P., Tintignac, L.A.J., Pospel, K., Guillier, M. and Leibovitch, S.A. (2000) *J. Biol. Chem.* 275, 18767–18776.
- [14] Pagano, M., Tam, S.W., Theodoras, A.M., Beer-Romero, P., Del Sal, G., Chau, V., Yew, P.R., Draetta, G.F. and Rolfe, M. (1995) *Science* 269, 682–685.
- [15] Hengst, L. and Reed, S.I. (1996) *Science* 271, 1861–1864.
- [16] Shirane, M., Harumiya, Y., Ishida, N., Hirai, A., Miyamoto, C., Hatakeyama, S. and Nakayama, K. (1999) *J. Biol. Chem.* 274, 13886–13893.
- [17] Vlach, J., Hennecke, S. and Amati, B. (1997) *EMBO J.* 16, 5334–5344.
- [18] Montagnoli, A., Fiore, F., Eytan, E., Carrano, A.C., Draetta, G.F., Herskho, A. and Pagano, M. (1999) *Genes Dev.* 13, 1181–1189.
- [19] Carrano, A.C., Eytan, E., Herskho, A. and Pagano, M. (1999) *Nat. Cell Biol.* 1, 193–199.
- [20] Sutterluty, H., Chatelain, E., Marti, A., Wirbelauer, C., Senften, M., Muller, U. and Krek, W. (1999) *Nat. Cell Biol.* 1, 207–214.
- [21] Skowyra, D., Craig, K.L., Tyers, M., Elledge, S.J. and Harper, J.W. (1997) *Cell* 2, 209–219.
- [22] Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J.M., Lane, W.S. and Kaelin Jr., W.G. (2001) *Science* 292, 464–468.
- [23] Yoshida, Y., Chiba, T., Tokunaga, F., Kawasaki, H., Iwai, K., Suzuki, T., Ito, Y., Matsuoka, K., Yoshida, M., Tanaka, K. and Tai, T. (2002) *Nature* 418, 438–442.
- [24] Ohh, M., Park, C.W., Ivan, M., Hoffman, M.A., Kim, T.Y., Huang, L.E., Pavletich, N., Chau, V. and Kaelin, W.G. (2000) *Nat. Cell Biol.* 2, 423–427.
- [25] Hashimoto, Y., Kohri, K., Kaneko, Y., Morisaki, H., Kato, T., Ikeda, K. and Nakanishi, M. (1998) *J. Biol. Chem.* 273, 16544–16550.