Identification of CIP-1-associated Regulator of Cyclin B (CARB), a Novel p21-binding Protein Acting in the G2 Phase of the Cell Cycle*

The cyclin-dependent kinase inhibitor p21cip1 regulates cell cycle progression, DNA replication, and DNA repair by binding to specific cellular proteins through distinct amino- and carboxyl-terminal protein binding motifs. We have identified a novel human gene, CARB (CIP-1-associated regulator of cyclin B), whose product interacts with the p21 carboxyl terminus. Immunocytological analysis demonstrates that the CARB protein is perinuclear and predominantly associated with the centrosome and mitotic spindle poles. In addition, CARB is also able to associate with cyclin B1, a key regulator of mitosis. However, cyclin B1-CARB complex formation occurs preferentially in the absence of p21. Unexpectedly, overexpression of CARB is associated with a growth-inhibitory and ultimately lethal phenotype in p21−/− cells but not in p21+/+ cells. These data identify a novel mechanism that may underlie the effects of p21 in the G2/M phases of the cell cycle.

Orderly progression through the cell cycle is regulated by the activity of cyclins and their catalytic partner proteins, cyclin-dependent kinases (CDKs).1 CDK-cyclin complexes phosphorylate substrates throughout the different phases of the cell cycle, thereby controlling both the duration of each phase and the transition into the next phase. The activity of CDK-cyclin complexes is in turn regulated by synthesis and degradation of cyclins, phosphorylation of the CDK subunit, and binding of CDK inhibitors such as p21cip1 (1).

The p21 protein contains motifs used for binding and inhibiting the activity of CDK-cyclins and a motif used to inhibit proliferating cell nuclear antigen-dependent DNA replication (2–5). The role of p21 in cell cycle arrest at the G1/S boundary has been well documented (reviewed in Refs. 3, 6, and 7). Recently, a number of studies have identified a role for p21 in the G2/M phase of the cell cycle (8–12); however, the mechanisms underlying this function have remained elusive.

Initiation of DNA condensation and entry into mitosis is controlled at least partially by the Cdc2-cyclin B1 kinase complex. In addition to the mechanisms regulating the activity of Cdc2, cyclin B1 is also subject to control at the level of subcellular localization by the nuclear export protein CRM-1 (13–15) and is spatially translocated during mitosis (16) (reviewed in Refs. 17 and 18). Although supraphysiological levels of p21 can inhibit the activity of Cdc2-cyclin B1 complexes in vitro (19), it is unlikely to be the primary mechanism by which p21 regulates events in the G2/M phase of the cell cycle. The role of proliferating cell nuclear antigen is also difficult to reconcile in terms of affecting G2/M timing, although it has been proposed as a mechanism (9). In addition, disruption of the p21/p53 pathway has been associated with mitotic spindle pole defects and the appearance of multiple centrosomes (20, 21). In the absence of any direct mechanistic link to the mitotic spindle poles and centrosome, recent evidence has suggested that these abnormalities may arise as a result of alterations in Cdk2-cyclin E activity at the time of centrosome duplication in S phase (22).

We speculated that additional proteins might bind the critical carboxyl-terminal region of p21, previously identified as a target of the human papilloma virus E7 oncoprotein (23, 24), and provide further insight into the activities and functions of p21. In this study, a novel protein that binds the C terminus of p21 has been identified and characterized. The protein is a component of the centrosome and associates with cyclin B1 in a p21-dependent fashion, thus providing a novel mechanism by which p21 can regulate cyclin B1 function and presumably transit through the G2/M phase of the cell cycle.

**Experimental Procedures**

Two-hybrid Screen—The cDNA sequence corresponding to amino acids 87–164 of human p21 was fused to the Gal4 DNA-binding domain of the plasmid pAS2.1 and co-transformed with a human fetal brain cDNA library expressed from pACT2 into strain CG1945 Saccharomyces cerevisiae as per the manufacturer's instructions (CLONTECH). Interacting clones were identified by growth on histidine-deficient media and the presence of β-galactosidase activity; the interaction was then rescreened in Y187 and Y190 strains.

Cloning Procedures—The 5′-end of human CARB was cloned by anchored, nested polymerase chain reaction of a fetal brain cDNA library using a primer provided to the upstream vector sequence (5′AD; CLONTECH) and three nested primers corresponding to the sequence of the clone in the reverse orientation. Sequence was also compared with sequences in dbEST at the National Center for Biotechnology Information (NCBI), and two homologous mouse clones were obtained from the I.M.A.G.E. consortium.

Protein Purification—Full-length CARB was expressed as a His-tagged protein from pET16b (Novagen) in Escherichia coli (strain BL21 DE3), purified under denaturing conditions in 8 M urea, and dialyzed into the presence of 0.1% Tris-buffered saline (pH 8.0).

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The abbreviations used are: CDK, cyclin-dependent kinase; HA, hemagglutinin; DAPI, 4′,6-diamidino-2-phenylindole; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; GST, glutathione S-transferase.

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buffered saline for immunization or immunoprecipitation experiments. GST-p21, GST-p21C, and GST-p21N were purified as described (25).

**Immunoprecipitations**—Immunoprecipitations were performed as described previously (23). Cell lysates were prepared by brief sonication in lysis buffer containing 50 mM HEPES, pH 7.5, 450 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 10% glycerol, 1 mM NaF, 0.1 mM sodium vanadate, 10 mM β-glycerophosphate, and 0.1% Tween 20. Immunoprecipitations were carried out using 800 or 1500 μg of extract for CARB-p21 or the CARB-cyclin B1 interactions, respectively, and band intensity was compared with the signal obtained from 20 μg of crude extract.

**Antibodies**—Antibodies were obtained from the following sources: anti-Cdc2 (Oncogene Science), anti-p21 (for immunoprecipitation) (D. Beach laboratory), anti-cyclin B1 (PharMingen clone GNS-11), anti-p21 C-15 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-hemagglutinin (HA) monoclonal antibody (Babco clone 16B12), anti-γ-tubulin (Babco), and anti-pericentrin (Babco).

**Cell Lines and Transfections**—HCT116 and 80S14, a p21−/− clone of HCT116 derived by homologous recombination, were a generous gift from T. Waldman and B. Vogelstein and were cultured as described (26). Full-length CARB or an N-terminally HA-tagged derivative of the cDNA clone (generated by polymerase chain reaction) were subcloned into pZeoSV2 (Invitrogen), and stable cell lines were derived by LipofectAMINE transfection (Life Technologies, Inc.) and zeocin selection. CARB and cyclin B1 were expressed from the LXS5 vector for transient transfection experiments.

**Immunofluorescence**—Cells were fixed with ice-cold methanol/acetone (1:1) and stained according to standard protocols in 5% goat serum. Staining patterns were compared with those seen with 1% paraformaldehyde and Triton X-100 permeabilization to rule out fixation artifacts. Double immunofluorescence experiments were performed using antirabbit fluorescein isothiocyanate and anti-mouse-CY3 (Jackson immunonchemicals) and recorded using a Nikon inverted microscope with Infinity optics (Meridian Instruments).

**Fluorescence-Activated Cell Sorting Analysis**—Samples were fixed in 1% paraformaldehyde in phosphate-buffered saline, stained with 100 μg/ml DAPI, and analyzed on a Becton Dickenson Vantage Turbosort with dual laser excitation (UV and 488 nm). Fluorescence resonance transfer measurements were essentially carried out on fixed cells as described (27) and compared with the results obtained with no donor or acceptor fluorochrome to control for spectral bleed-through and secondary excitation (data not shown).

**RESULTS**

**Cloning of a Novel p21-binding Protein**—Using a yeast two-hybrid strategy, a human fetal brain cDNA library was screened with the carboxyl terminus of p21 (amino acids 87–164), which encompasses the proliferating cell nuclear antigen, the second cyclin binding motif, and human papilloma virus E7 binding sites (23). A candidate cDNA was cloned, and the complete human and mouse sequences were obtained through a combination of 5′-anchored polymerase chain reaction and expressed sequence tag data base BLAST analyses (23, 28). Human and mouse sequences are highly conserved (92% identity at the amino acid level) and predict a protein of 27 kDa (data not shown). We named the protein CARB (CIP-1-associated protein-1) (30) (27% identity, 43% similarity for residues 138–232) (29) (GenBank™ accession no. AF115323). Further BLAST searches with the N terminus of CARB also revealed homology to the C-terminal region of centrosomal Nek2-associated protein-1 (30) (27% identity, 43% similarity for residues 22–158). No clear functional significance of these homologies has been described.

Association of p21 and CARB was confirmed by binding assays in vitro using proteins translated in wheat germ extracts (Fig. 1A) and bacterially produced tagged proteins (Fig. 1B) as well as coimmunoprecipitation of HA epitope-tagged CARB and p21 proteins expressed from plasmids transfected into either 293 or HCT116 cell lines (data not shown). No binding of in vitro translated p27kip1, p57kip2, or p16ink4a was observed in in vitro binding assays (data not shown).

![Fig. 1 A](image-url) binding of bacterially expressed GST-p21, the carboxyl terminus of p21 (GST-p21C), or GST alone with 32S-labeled in vitro translated CARB, human papilloma virus 16E7, or proliferating cell nuclear antigen (PCNA). Input lane indicates 5% of input. B, binding of bacterially expressed GST-p21 or truncation mutants to His-CARB. Input lane indicates 5% of input. C, co-immunoprecipitation of CARB and p21 from whole cell extracts of the HCT116 or 80S14 extracts. Left blot, immunoprecipitation of p21, Western blotted with anti-CARB; right blot, immunoprecipitation of CARB, Western blotted with anti-p21. For quantitation, see “Experimental Procedures.”

In order to study the endogenous CARB protein, a specific antiserum was raised in rabbits against hexahistidine-tagged protein expressed in E. coli. The antiserum was reactive with a protein band of 27–30 kDa in the p21 wild-type colorectal cancer cell line, HCT116, and a p21−/− cell line, 80S14, derived from HCT116 by homologous recombination (26) (Fig. 1C, lanes 1 and 3). The association of endogenous CARB and p21 in p21−/− HCT116 cells was confirmed in a co-immunoprecipitation assay (Fig. 1C, lanes 2 and 6). CARB was not immunoprecipitated with anti-p21 antiserum from lysates of p21−/− cells (lane 4). Taken together, this shows that endogenous p21 and CARB proteins associate in vivo.
Northern blot analysis indicated that a 1.3-kilobase CARB mRNA was expressed in all human tissues tested (data not shown). Analysis of CARB protein expression by immunohistochemistry in tissue sections of a developing mouse embryo confirmed widespread expression in vivo (data not shown). Two-color fluorescence-activated cell sorting analysis of the expression levels of endogenous CARB protein versus DNA content of HCT116 cells suggested that CARB levels remained constant throughout the cell cycle (data not shown). Expression of CARB was unaffected by DNA-damaging agents or cellular p21 status (Fig. 1C and data not shown).

Immunofluorescence analysis of exogenously expressed HA-CARB (Fig. 2A, left column) or the endogenous protein (Fig. 2A, upper row, middle panel, and Fig. 2B) indicated that the highest staining intensity was associated with a perinuclear structure during interphase (Fig. 2A, blue arrows) and the spindle pole bodies during mitosis (Fig. 2B, white arrow). The HA-tagged form of CARB overlapped well with endogenous CARB, although cells expressing high levels of HA-CARB had a more diffuse staining pattern throughout the cytoplasm (not shown).

Double immunofluorescence experiments, with antisera against γ-tubulin or pericentrin, key proteins of the centrosome (31, 32), demonstrated that CARB remained centrosomally associated throughout the cell cycle (Fig. 2A). To rule out fixation artifacts, this staining pattern was also compared with the staining pattern of live, unfixed, HCT116 cells transfected with a GFP-CARB fusion protein and was found to be identical (Fig. 2B).

**CARB Also Associates with Cyclin B1**—Based on observations by others (33, 34) that cyclin B1, a key regulator of mitosis, can share a similar association with the centrosome, we compared the localization of endogenous cyclin B1 and CARB in asynchronously growing HCT116 cells and their p21 null counterparts (Fig. 3A, left column). Cyclin B1 staining (red) was diffuse throughout the cytoplasm in interphase cells and only weakly associated with the centrosomal region in the p21 wild-type parental line. Surprisingly, cyclin B1 localization was altered in the p21−/− cells such that a much larger percentage of the cytoplasmic pool of cyclin B1 was associated with the CARB staining. The degree of co-localization was confirmed by fluorescence resonance energy transfer (FRET) between the secondary antibody reagents (Fig. 3A, right column). Analysis by flow cytometry demonstrated that approximately 10-fold more p21−/− cells had measurable FRET between CARB and cyclin B1 compared with p21 wild type HCT116 cells (Fig. 3B).

Consistent with the FRET observations, CARB only co-immunoprecipitated with detectable levels of cyclin B1 in lysates of asynchronously growing cells lacking p21 (Fig. 4, lanes 1 and 2), and conversely, cyclin B1 could only be detected in immunoprecipitates of CARB from p21 null cells (Fig. 4, lanes 5 and 6). Of note, cyclin B1 steady state protein levels were 2–3-fold higher in p21−/− cells (Fig. 4, lane 4), although this difference was not enough to account for the increased level of cyclin B1 in the CARB immunoprecipitates from the p21 null cells, since Cdc2 was present in cyclin B1 immunoprecipitates irrespective of p21 status (Fig. 4A, lanes 1 and 2). In accordance with the possibility that a novel C-terminal activity of p21 was regulating the association of cyclin B1 to CARB, the association was potently inhibited by the addition of either recombinant full-length GST-p21 or the C terminus of p21 fused to GST but not by the N-terminal half of p21 fused to GST (Fig. 4A, lanes 7–10). This effect was specific for p21, since the related CDK inhibitor, p27Kip1, did not inhibit the association of CARB and cyclin B1 (data not shown).

Although there was clearly an association of cyclin B1 and CARB in 80% of cells, CARB immunoprecipitates had negligible kinase activity as compared with immunoprecipitates of cyclin B1 from the same extract, although considerable amounts of cyclin B1 protein are present in CARB immunoprecipitates from the p21 null cells (Fig. 4B). However, this finding in extracts does not preclude the possibility that these complexes may have kinase activities dependent on their temporal and spatial regulation. We have not been able to show a direct interaction between CARB and cyclin B1, which could imply that additional proteins bridge the interaction.

An association between CARB and cyclin B1 was not measurable in lysates harvested from asynchronous p21−/− cells (Fig. 4A). However, the p21−/− HCT116 cell line has measurable basal p21 protein levels, and immunofluorescence analysis suggested that a small fraction of the cells expressed significant p21 levels (data not shown). This raised the possibility that the p21 level in the cell lysate could be high enough to perturb the CARB-cyclin B1 interaction. However, in an aphidicolin-synchronized population of p21−/− cells, a clear association of CARB and cyclin B1 was observed in a short period within middle to late G2 (data not shown) affecting
protein complex formation and activity. So far, the exact stimulus for the reorganization of CARB protein complexes in G2 is unclear; however, given the proteins involved and the timing of the interactions, these observations suggest that this system may be involved in the regulation of the G2/M phase of the cell cycle.

**CARB Overexpression Is Lethal in p21 Null Cells**—In stark contrast to p21 wild-type HCT116, it was not possible to derive stable CARB-overexpressing p21 null clones (Fig. 5A). Zeocin-resistant colonies arose at nearly equal frequency in vector-transfected plates. Although some zeocin-resistant clones were obtained following transfection of p2eo-HA CARB into 80S14 cells, none had detectable HA expression as judged by immunofluorescence (not shown).

To ascertain that this phenotype was not restricted to the 80S14 cell line, the effects of CARB overexpression were assayed in embryonic fibroblasts derived from p21 null mice. Consistent with the data derived from the cell lines, expression of GFP-CARB was lethal by 48 h post-transfection, in contrast to the minimal toxicity observed in p21<sup>+/+</sup> embryonic fibroblasts transfected with GFP alone (Fig. 5B).

In order to determine the effects of CARB overexpression, cells were transfected with plasmids encoding a GFP-CARB fusion protein or co-transfected with CARB and GFP on separate plasmids. Cells were analyzed at time points after transfection, and the percentage of GFP-expressing transfectants was determined by fluorescence-activated cell sorting analysis. Populations were analyzed until at least 10,000 transfected (i.e. GFP-positive) events had been recorded. Cells were stained with DAPI prior to analysis to measure DNA content, and events were gated on the basis of having a DNA content between 2 and 4N, being singlet events, and having forward and side scatter values indicative of intact cells. Since it was possible that the transfection efficiency might vary between HCT116 and 80S14 cells, each transfection of CARB and GFP was compared with a transfection with an equal concentration of GFP and the empty expression vector alone. To simplify data analysis, transfections were normalized to a control transfection of GFP and vector alone.

At 18 h post-transfection, there was no significant difference between the percentage of transfectants expressing GFP and vector or GFP and CARB regardless of p21 status (data not shown). At 48 h post-transfection, high expression of GFP and empty vector in 80S14 cells was well tolerated (Fig. 6A), but co-transfection of CARB resulted in loss of over 75% of the transfectants. Direct fusion of GFP to the N terminus of the
CARB gene produced a similar phenotype, resulting in a loss of transfectants approaching 90%. It is worth noting that although it was possible to generate stable CARB transfectants in the HCT116 line, cells expressing extremely high levels of CARB were also lost by 48 h post-transfection (data not shown).

Observations from the FRET analysis and immunoprecipitation experiments implied that CARB may affect cellular trafficking of cyclin B1, possibly limiting bioavailability in p21 null cells. We tested whether overexpression of cyclin B1 could overcome the lethality of CARB overexpression in p21 null cells. In concordance with this model, overexpression of cyclin B1 prevented the death of cells expressing high levels of GFP-CARB (Fig. 6, right-hand columns). The rescue was not complete; however, this may be a function of lower expression from the cyclin B1 expression plasmid or uneven distribution of cyclin B1 and CARB plasmids into individual cells.

Since expression of high levels of GFP-CARB completely prevented the formation of viable p21 null cell lines, we used the DAPI-based measurements to compare the ploidy of HCT116 and 80S14 cells at a time point prior to the onset of extensive cell death (Fig. 6B). Regardless of p21 status, both lines tolerated the expression of GFP and empty vector alone with a ratio of 2N to 4N DNA content of 1.7 27 h after transfection. In contrast to cells expressing GFP alone, cells expressing GFP-CARB had a larger 4N population; this effect was most marked in cells lacking p21 with a 2N/4N ratio of 0.7, suggestive of an accumulation of cells with 4N DNA content. Subsequently, at later time points (48 h and beyond) there was a rapid loss of G2 cells, suggesting that cells died in G2.

**DISCUSSION**

We have identified a novel p21 binding partner. A direct association of p21 and CARB is supported by our binding data obtained in vitro (Fig. 1, A and B) and binding of endogenous proteins from cell lysates (Fig. 1C). Furthermore, consistent with a p21-related role for CARB, overexpression is ultimately lethal in p21 null embryonic fibroblasts (Fig. 6B) or in a p21 null cell line (80S14) but is tolerated in p21 wild-type cells. We have also identified a spatial co-localization of cyclin B1 and CARB proximal to the centrosome that is most obvious in p21 null cell lines (80S14) but is tolerated in p21 wild-type cells. It is also interesting to note that not all of the cyclin B1 was associated with CARB in these cells. It is difficult to determine whether this is an accurate reflection of events in vivo, or an artifact caused by differential extraction of CARB protein after fixation with methanol/acetone, given that methanol fixation alone did not preserve the CARB signal throughout the cell cycle (data not shown). At present, it is still
unclear why cyclin B1 becomes associated with CARB at the centrosome in p21 null cells. However, the homology of CARB to members of the syntaxin family and the observations by others that cyclin B1 is subject to tight control of subcellular localization (12, 14–16, 18), suggests that CARB may be regulating cyclin B1 trafficking in the cytoplasm. Sequestration of cyclin B1 to CARB proximal to the nucleus may form a storage site for cyclin B1 from which it can be rapidly mobilized or protect cyclin B1 from proteolytic degradation.

Association of CARB and cyclin B1 is also borne out by immunoprecipitation experiments of cell extracts (Fig. 4, A and B), analyzing either cyclin B1 or CARB immunosolates by Western blotting. Consistent for a role for p21 in CARB regulation, the cyclin B1-CARB association is potently inhibited by the C terminus of p21 (Fig. 4A), suggesting that free p21 is able to dissociate this complex under certain conditions. Since CARB is either found associated to p21 or cyclin B1, but not both, it seems unlikely that the association of CARB and cyclin B1 is mediated by p21, although there is some evidence that high concentrations of p21 are able to weakly bind and inhibit cyclin B1-Cdc2 complexes in vitro (19). Furthermore, this new function of p21 is also in accordance with its recently described presence in large multiprotein complexes within centrosomes affecting the activity of apoptotic proteins before mitosis (35).

Based on our data, we propose that CARB sequesters cyclin B1 in the cytoplasm proximal to the nuclear membrane and thus may regulate the availability of cyclin B1 for nuclear import. A similar suggestion that cyclin B1 is retained in the cytoplasm by an active mechanism has been proposed previously (34), but that study did not identify a candidate molecule to tether cyclin B1. Sequestration by CARB would complement the existing control mechanisms for the mitotic kinase: phosphoregulation of Cdc2 (12), association of cyclin B1, and control of cyclin B1 subcellular transport across the nuclear membrane by the CRM-1 pathway (14, 15). CARB regulation of cyclin B1 bioavailability may regulate the onset of prophase, since availability of p21 for CARB-cyclin B1 complex disruption could only occur if p21 was not required for nucleotide excision repair or to bind other target proteins after growth arrest. This novel molecular mechanism could account for the activity of p21 in G2/M checkpoint timing (9, 11). Clearly, further experiments are warranted to confirm this idea.

The identification of the p21/CARB association may also explain recent observations from fibroblasts derived from p21 knockout mice that fail to accumulate large amounts of nuclear cyclin B1 prior to mitosis (10), an observation that fits with our hypothesis, since cells lacking p21 would be impaired in their ability to mobilize the pool of cyclin B1 bound to CARB in the cytoplasm. Thus, the lethality of CARB overexpression may arise from excessive sequestration of cyclin B1 or from titration of cyclin B1 away from other critical proteins. In accordance with this, lethality of CARB overexpression can be partially rescued by the addition of exogenous cyclin B1 (Fig. 6A), and CARB-transfected cells have a larger 4N population (Fig. 6B). It is interesting to note that p21–/– 80S14 cells have higher cyclin B1 levels (Fig. 4A, lane 4), and HCT116 CARB stable transfectants also have elevated cyclin B1 levels (data not shown), again suggesting that CARB may not only affect subcellular trafficking of cyclin B1 in a p21-dependent manner but may also affect the steady state levels of cyclin B1.

Our results suggest that the p21 carboxyl terminus can actually act in a manner to promote release of cyclin B1 from CARB. This function is distinct from the potent inhibitory activities of p21 described so far, and one interpretation of the data to date would be that p21 is actually promoting the progression into M phase, by increasing cyclin B1 bioavailability. As a consequence, the evolution of such bifunctionality would confer no selective advantage to cells that completely eliminate p21, since under DNA-damaged conditions they would be able to abrogate the G2/M damage checkpoint but be unable to efficiently initiate mitosis. Such a possibility may explain the lack of sporadic tumor formation in p21 null mice and the very low frequency of p21 gene inactivations in human cancers (7, 36).

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REFERENCES

1. Morgan, D. O. (1995) Nature 374, 131–134
2. Flores-Boza, H., Kelman, Z., Dean, F. E., Pan, Z. Q., Harper, J. W., Ellledge, S. J., O'Donnell, M., and Hurwitz, J. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8655–8659
3. Funk, J. O., and Galloway, D. A. (1998) Trends Biochem. Sci. 23, 337–341
4. Li, R., Waga, S., Hannon, G. J., Beach, D., and Stillman, B. (1994) Nature 371, 534–537
5. Waga, S., Hannon, G. J., Beach, D., and Stillman, B. (1994) Nature 369, 574–578
6. Sherr, C. J., and Roberts, J. M. (1995) Genes Dev. 9, 1149–1163
7. Harper, J. W., and Ellledge, S. J. (1996) Curr. Opin. Genet. Dev. 6, 56–64
8. Bunz, F., Dutriaux, A., Lengauer, C., Waldman, T., Zhou, S., Brown, J. P., Sedivy, J. M., Kinzler, K. W., and Vogelstein, B. (1998) Science 282, 1497–1501
9. Cayrol, C., Kniebuehler, M., and Ducommun, B. (1998) Oncogene 16, 311–329
10. Dulis, V., Steim, G. H., Far, D. F., and Reed, S. I. (1998) Mol. Cell. Biol. 18, 546–557
11. Niculescu, A. B., Chen, X., Smeets, M., Hengst, L., Prives, C., and Reed, S. I. (1998) Mol. Cell. Biol. 18, 629–643
12. Winters, Z. E., Ongkeko, W. M., Harris, A. L., and Norbury, C. J. (1998) Oncogene 17, 673–684
13. Hasting, A., Karlsson, C., Clute, P., Jackman, M., and Pines, J. (1998) EMBO J. 17, 4127–4138
14. Toyoshima, F., Morituguji, T., Wada, A., Fukuda, M., and Nishida, E. (1998) EMBO J. 17, 2728–2735
15. Yang, J., Bardes, E. S., Moore, J. D., Brennan, J., Powers, M. A., and Kornbluth, S. (1998) Genes Dev. 12, 2311–2314
16. Clute, P., and Pines, J. (1999) Nat. Cell Biol. 1, 82–87
17. Pines, J. (1999) Nat. Cell Biol. 1, E73–E79
18. Ohi, R., and Gould, K. L. (1999) Curr. Opin. Cell Biol. 11, 267–273
19. Ball, K. L., Lain, S., Fahraeus, R., Smythe, C., and Lane, D. P. (1997) Curr. Biol. 7, 71–80
20. Fukasawa, K., Choi, T., Kuriyama, R., Rulong, S., and Vande Woude, G. F. (1996) Science 271, 1744–1747
21. Mantel, C., Braun, S. E., Reid, S., Henegariu, O., Liu, L., Hangle, G., and Brumkeyer, H. E. (1999) Blood 93, 1390–1398
22. Meraldi, F., Lukas, J., Fry, A. M., Bartek, J., and Nigg, E. A. (1999) Nat. Cell Biol. 1, 88–93
23. Funk, J. O., Waga, S., Harry, J. B., Espling, E., Stillman, B., and Galloway, D. A. (1997) Genes Dev. 11, 2090–2100
24. Jones, D. L., Alani, R. M., and Munger, K. (1997) Genes Dev. 11, 2101–2111
25. Chen, J., Jackson, P. R., Kirschner, M. W., and Dutta, A. (1995) Nature 374, 386–388
26. Waldman, T., Kinzler, K. W., and Vogelstein, B. (1995) Cancer Res. 55, 5187–5190
27. Hsu, C. T., McKinnon, A. J., and Kirschner, M. W. (1995) Science 269, 147–150
28. Scheller, R. H. (1998) J. Cell Biol. 141, 1563–1574
29. Dossyee, S. J., Stein, P., Evans, L., Calarco, P. D., and Kirschner, M. (1994) Cell 76, 639–650
30. Sartena, T., Evans, L., and Kirschner, M. (1991) Cell 65, 825–836
31. Bailly, E., Pines, J., Hunter, T., and Bornens, M. (1992) J. Cell Sci. 101, 529–545
32. Pines, J., and Hunter, T. (1994) EMBO J. 13, 3772–3781
33. Li, F., Ackermann, E. J., Bennett, F., Rothermel, A. L., Plescia, J., Tognin, S., Villa, A., Marchisio, P. C., and Altieri, D. C. (2000) Nat. Cell Biol. 1, 461–466
34. Sherr, C. J. (1998) Science 274, 1672–1677