Association of class I histone deacetylases with transcriptional corepressor CtBP

T. Subramanian, G. Chinnadurai*

Institute for Molecular Virology, Saint Louis University Health Sciences Center, 3681 Park Avenue, St. Louis, MO 63110, USA

Received 6 January 2003; revised 24 January 2003; accepted 27 February 2003

First published online 21 March 2003

Edited by Gianni Cesareni

Abstract The C-terminal binding protein (CtBP) family proteins are transcriptional regulators that are conserved from worm to human. They function as corepressors of a wide array of DNA-binding transcriptional repressors. The mammalian CtBPs appear to mediate transcriptional repression in a histone deacetylase (HDAC)-dependent or -independent manner, depending on the context of the promoter. To identify the components of the CtBP corepressor complex, we isolated CtBP-containing protein complexes from the nuclear extracts prepared from HeLa cells infected with adenovirus vectors that expresses hCtBP1. Western blot analysis of these complexes suggests that hCtBP1 associates with class I HDACs, HDAC-1, HDAC-2 and HDAC-3. Some of these HDACs also interact with the *Drosophila* CtBP homolog, dCtBP. The CtBP protein complex exhibits significant HDAC activity in vitro suggesting that association of CtBP with HDACs may be functionally relevant.

Key words: C-terminal binding protein; Corepressor; Histone deacetylase

1. Introduction

Transcriptional repression plays a central role in regulating patterns of gene expression during development and differentiation. Gene-specific repression is important for controlling several cellular processes such as cell proliferation and oncogenesis. The transcriptional repressors mediate their effect through recruitment of various corepressors. The corepressors inhibit transcription by targeting components of the basal transcription machinery or by altering the chromatin structure. A number of corepressors alter the chromatin structure by covalent modification of the nucleosomal histones. These modifications of the histones are carried out by the enzymatic cofactors in the corepressor complex. The commonest type of histone modification is the removal of acetyl groups from the N-terminal tails of histones. The removal of the acetyl group is believed to cause compaction of the chromatin by facilitating contacts with adjacent nucleosomes resulting in a transcriptionally repressed state of the chromatin [1]. A number of corepressors accomplish this activity by recruiting histone deacetylases (HDACs). This class includes corepressors such as mSin3, pRb, Groucho and Mi-2/NuRD (reviewed in [2]). More recently, methylation of histones has also been shown to contribute to transcriptional repression (reviewed in [3,4]). The corepressor pRb has been reported to recruit a histone methyl transferase that methylates the histone tails [5]. Thus, the corepressors may recruit multiple cofactors to mediate concerted transcriptional silencing.

The C-terminal binding protein (CtBP) family proteins are conserved from the round worm *Caenorhabditis elegans* to human. Vertebrates contain two highly related proteins, CtBP1 and CtBP2, while invertebrates contain a single member. The founding member of this family, hCtBP1, was identified as a cellular protein that interacts with the C-terminal region of E1A [6,7]. The interaction of CtBP with the C-terminal region antagonizes a transcriptional activation function encoded by the N-terminal region of E1A [8]. Genetic and biochemical studies with several *Drosophila* repressors that function during embryo development have revealed that the *Drosophila* homolog of CtBP (dCtBP) functions as a transcriptional corepressor [9,10]. Since then it has been recognized that a large number of transcriptional repressors of higher eukaryotes mediate their activity through recruitment of CtBP [11,12]. The mechanisms by which CtBP represses transcription are not known. Transcriptional tethering studies carried out in the presence of the HDAC inhibitor trichostatin A (TSA) have revealed that CtBP may mediate transcription in a HDAC-dependent or -independent manner. The Ras-responsive transcription factor Net represses transcription from the c-*fos* serum-responsive element in a CtBP-dependent manner and the repressive effect is relieved by TSA [13]. Similarly, the cellular oncogene *Evi*-1, which represses transforming growth factor-β-responsive genes by recruitment of CtBP, has been shown to be sensitive to TSA [14]. In contrast, repression of certain cell cycle regulatory genes such as *cyclin* A and *cdc*2 mediated by the pRb family proteins (pRb and p130) appears to be HDAC-independent [15,16]. In order to understand the mechanisms by which CtBP represses transcription, we have isolated a protein complex from human cells that overexpress hCtBP1 and show that the protein complex contains class I HDACs.

2. Materials and methods

2.1. Cells and viruses

Human HeLa cells were grown in suspension cultures using minimal essential medium (Joklik's modified) containing 10% fetal bovine serum. The adenoviral (Ad) vectors, Ad-T7-CtBP (CtBP tagged with the T7 epitope at the N-terminus) and Ad-Flag-CtBP (CtBP tagged

*Corresponding author. Fax: (1)-314-577 8406.
E-mail address: chinnag@slu.edu (G. Chinnadurai).

Abbreviations: CtBP, C-terminal binding protein; HDAC, histone deacetylase; Ad, adenovirus

0014-5793/03/$22.00 © 2003 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(03)00275-8

with three tandem copies of the Flag epitope at the N-terminus) were constructed by cloning hCtBP1 coding sequences with the T7 or 3X Flag tags into *Hin*dIII and *Bam*HI sites of an adenoviral transfer vector, pLend-CMV, and cotransfected into human 293 cells along with pBHGE3 [17] as described elsewhere [18]. Recombinant adenoviruses that express CtBP were identified by Western blot analysis. The positive clones were further purified through a second round of plaque purification, amplified and titrated on 293 cells.

2.2. Nuclear extracts

One liter of HeLa cell suspension cultures (5×10^5 cells/ml) were mock infected or infected with Ad-T7-CtBP or Ad-Flag-CtBP at 100 PFU/cell and 24 h after infection, nuclear extracts were prepared [19]. Cells were harvested by centrifugation at $180\times g$ for 10 min at 4°C and washed once with ice-cold phosphate-buffered saline. The cell pellets were resuspended in 25 ml of hypotonic buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT)) and incubated in ice for 10 min. The swollen cells were again centrifuged at $180\times g$ for 10 min and the pellets were resuspended in 10 ml of buffer A. The cell suspensions were homogenized with a Dounce homogenizer with 10 strokes and the homogenates were centrifuged at $1000\times g$ for 10 min. The nuclear pellets were suspended in 2.5 ml of low-salt buffer (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 20 mM KCl, 0.5 mM DTT, 0.2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 20% glycerol) and 2.5 ml of high-salt buffer (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 1.2 M KCl, 0.5 mM DTT, 0.2 mM EDTA, 0.2 mM PMSF, 20% glycerol) was added drop by drop with gentle vortexing. The nuclear pellet suspensions were rotated for 30 min at 4°C for additional extraction. The extraction mixtures were centrifuged at $10\,000\times g$ for 30 min and the supernatants were dialyzed for 5 h against 1 l of buffer D (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 100 mM KCl, 0.5 mM DTT, 0.2 mM EDTA, 0.2 mM PMSF, 20% glycerol). The dialysates were clarified by centrifuging again at $10\,000\times g$ for 10 min and the final extracts were stored at −70°C.

2.3. Immunoaffinity binding and Western blot analysis

Samples (100 μl) of nuclear extracts from cells infected with Ad-T7-CtBP or Ad-Flag-CtBP were diluted with 900 μl of wash buffer (50 mM Tris, pH 7.6 and 150 mM NaCl) and rotated with 25 μl of agarose beads conjugated with T7 antibody (Novagen) or Flag antibody (Sigma) for 3 h. The beads were washed six times with the washing buffer, suspended in 50 μl of sample buffer, boiled for 5 min and fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE, 10%). The supernatants after pulling the CtBP complex were also analyzed by SDS–PAGE as controls. The separated proteins were transferred to nitrocellulose membrane and analyzed by Western blotting. The blots were probed with rabbit polyclonal antibodies raised against human hCtBP1 [7] or hCtIP [20] or a mouse monoclonal antibody specific for human poly(ADP-ribose) polymerase (hPARP) (Pharmingen). The HDACs were detected using goat polyclonal antibodies for HDAC-1 and 3 (Santa Cruz) and a rabbit polyclonal antibody for HDAC-2 (Santa Cruz). The blots were visualized using horseradish peroxidase-conjugated secondary antibodies (Santa Cruz) and the 'Lumi-light' detection system (Roche) according to instructions from the manufacturer.

2.4. GST pull-down assay

Glutathione *S*-transferase (GST), GST-hCtBP1 and GST-dCtBP proteins were isolated from *Escherichia coli* strain BL21(DE3) and immobilized on GST-agarose beads as described [6,7]. Nuclear extracts from uninfected cells (100 μl) was diluted to 1 ml with the wash buffer and rotated with beads containing 10 μg of GST or GST-CtBP for 3 h. The beads were collected, washed six times with the wash buffer, suspended in 50 μl of sample buffer and the associated proteins were analyzed by Western blotting.

2.5. HDAC assay

The HDAC activity was determined using a commercial kit (Upstate Biotechnology) according to the manufacturer's specification. A peptide corresponding to the N-terminal region of histone H4 (100 μg) was acetylated using [^{3}H]acetic acid (2.52 Ci/mmol; Perkin Elmer) and the BOP reagent supplied with the kit. The acetylated peptide was purified using a spin column and used as the substrate. The reaction mixture containing 40 μl of 5×HDAC buffer, 20 000 cpm [^{3}H]acetyl histone H4 peptide, 10 μl nuclear extract or CtBP protein complex (isolated from 100 μl nuclear extract) in 200 μl reaction mixture was incubated at room temperature for 18 h on a shaking platform. The reactions were also carried out in the presence of 250 mM sodium butyrate or 20 μM TSA as HDAC inhibitors. The reactions were quenched by adding 50 μl of quenching reagent and the released radioactive acetic acid was extracted with 600 μl of ethyl acetate. Samples (200 μl) of the extracted ethyl acetate were counted and the enzyme activities were plotted as cpm.

3. Results and discussion

3.1. CtBP protein complex

In order to identify the protein factors associated with CtBP, we overexpressed hCtBP1 in human HeLa cells and isolated the CtBP protein complex by immunoaffinity chromatography. For this purpose, HeLa cells were infected with adenovirus vectors that express hCtBP1 tagged either with the T7 or with the Flag epitope, nuclear extracts were prepared at 24 h after infection and fractionated on the T7 antibody matrix or the Flag antibody matrix. When the nuclear extract from cells infected with Ad-T7-CtBP was fractionated on the T7 affinity matrix and analyzed by Western blot analysis using the antibody specific for CtBP, a significant amount of CtBP was detected (Fig. 1; top panel, lane 3). In contrast, there was no detectable CtBP when the Flag-CtBP extracts were fractionated on the T7 matrix (Fig. 1; top panel, lane 4). A similar immunoaffinity specificity was also observed when the extracts were fractionated using the Flag antibody matrix (not shown). We then examined if the CtBP protein complex contains a known CtBP-binding protein, CtIP [20]. The complex from Ad-T7-CtBP-infected cells that was fractionated on the T7 matrix contained a significant amount of CtIP and there was no detectable amount when the Flag-CtBP extract was fractionated on the T7 matrix (Fig. 1; middle panel, lanes 3 and 4). The specificity was further examined by analysis of the complexes for the presence of a heterologous nuclear pro-

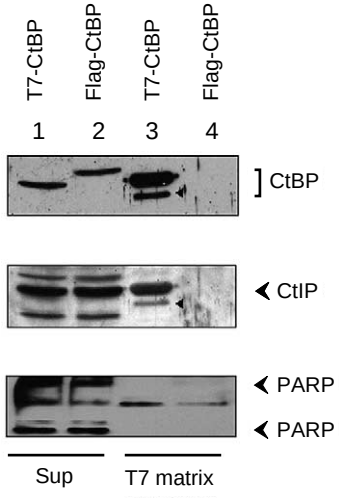

Fig. 1. Specificity of CtBP protein complex. The CtBP protein complexes were isolated from the nuclear extract of HeLa cells infected with Ad-T7-CtBP or Ad-Flag-CtBP by immunoaffinity chromatography on agarose matrix containing the T7 antibody. The supernatants and the protein complexes associated with the affinity matrix were subjected to Western blot analysis using the indicated antibodies (CtBP, CtIP and PARP). The bands indicated by arrowheads in lane 3 may correspond to degradation products of T7-CtBP or CtIP generated during chromatographic steps.

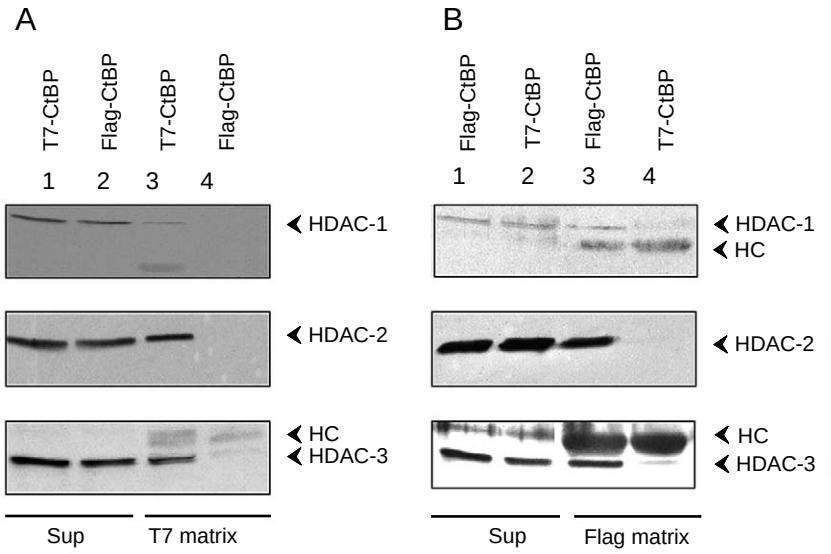

Fig. 2. Association of class I HDACs with CtBP. The CtBP protein complexes were isolated from cells infected with Ad-T7-CtBP or Ad-Flag-CtBP by immnuoaffinity chromatography on agarose matrix containing T7 (A) or Flag (B) antibody. The supernatants and the protein complexes associated with the affinity matrix were subjected to Western blot analysis using the antibodies specific to HDAC-1, 2 and 3. The bands indicated by HC represent IgG heavy chain.

tein, PARP. The supernatants of the nuclear extracts from which complexes were isolated contained significant amounts of 112 kDa and 85 kDa (proteolytically processed form) PARP polypeptides and the complexes isolated with affinity matrices did not contain detectable levels of PARP (Fig. 1; bottom panel, compare lanes 1 and 2 with lanes 3 and 4). These results indicate that the immunoaffinity approach used here is specific for the CtBP protein complex that contains a known CtBP-associated protein and not a heterologous nuclear protein.

3.2. Class I HDACs in CtBP complex

Since transcriptional studies have shown that CtBP-mediated repression of certain promoters is relieved by the HDAC inhibitor TSA [13,14], we tested if the CtBP protein complex isolated from HeLa cells contained class I HDACs. The nuclear extracts prepared from cells infected with Ad-T7-CtBP or Ad-Flag-CtBP were fractionated on the T7 or Flag antibody matrix and analyzed by Western blot analysis (Fig. 2). The CtBP complex isolated using the cognate immunoaffinity matrix contained significant amounts of HDAC-1, HDAC-2 and HDAC-3 compared to the respective controls (Fig. 2A, compare lanes 3 and 4; Fig. 2B, compare lanes 3 and 4).

We also carried out a GST pull-down study with nuclear extracts prepared from uninfected cells using GST-hCtBP1 and GST-dCtBP fusion proteins (Fig. 3). In these studies, significant interaction of HDAC-2 and 3 with GST-hCtBP1 and GST-dCtBP was detected compared to the GST control. There was no detectable interaction of HDAC-1 with both GST-hCtBP1 and GST-dCtBP fusion proteins. It is possible that HDAC-1 may interact with CtBP expressed in human cells and not with the fusion proteins purified from bacteria.

3.3. HDAC activity

We determined if HDAC activity could also be detected with the CtBP protein complex. The HDAC activity was determined in the nuclear extract prepared from HeLa cells infected with Ad-Flag-CtBP and with the CtBP protein complex isolated from the nuclear extract. The CtBP protein complex exhibited a significant amount of HDAC activity and it was inhibited by the presence of the HDAC inhibitors, sodium butyrate and TSA (Fig. 4). These results indicate that the CtBP protein complex isolated from HeLa cells is enzymatically active. We have also observed that the level of HDAC activity associated with Flag-CtBP1 was consistently more than that associated with the protein complex consisting of Flag-mSin3A protein complex prepared from cells transfected with CtBP1 or Sin3A expression plasmids (not shown).

We have shown that the transcriptional corepressor hCtBP1 expressed in human cells forms a complex with class I HDACs. Yeast two-hybrid studies have indicated that CtBP may also interact with class II HDACs [21]. While interaction with class I HDAC was readily detectable in our studies, we were unable to detect class II HDACs in the CtBP complex. It is possible that the negative results may be attributed to lack

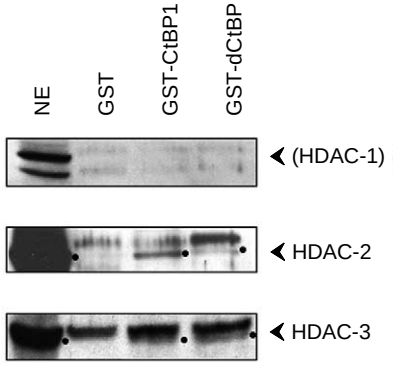

Fig. 3. Interaction of HDACs with hCtBP1 and dCtBP. The nuclear extracts prepared from uninfected HeLa cells were incubated with GST-affinity matrix containing GST or GST fusion proteins. The proteins associated with the affinity matrix were subjected to Western blot analysis as in Fig. 2. NE indicates nuclear extract. The bands corresponding to HDAC-2 and HDAC-3 are indicated by dots.

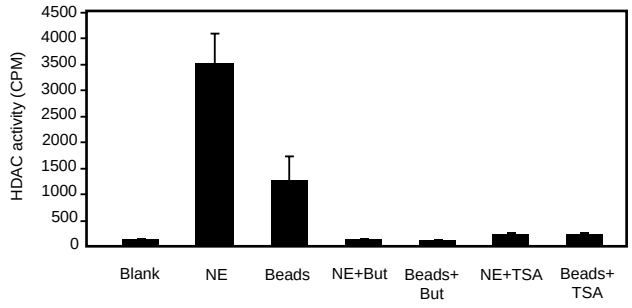

Fig. 4. HDAC activity in CtBP protein complex. Nuclear extracts were prepared from cells infected with Ad-Flag-CtBP. The HDAC activity in nuclear extract (indicated as NE) and in CtBP protein complex collected on the agarose matrix (indicated as Beads) was determined (average of two to five experiments). The reactions shown by '+But' and '+TSA' were carried out in the presence of 250 mM Na-butyrate or in the presence of 20 μM TSA as inhibitors.

of sensitive immunological reagents. In a previous coimmunoprecipitation study, interaction between transiently transfected HDAC-1 and hCtBP1 has also been detected [22]. Attempts to detect association of endogenous HDAC with CtBP1 have also revealed low levels of interaction between HDAC-2 and Flag-CtBP1 [23]. Our results that CtBP1 forms complexes with HDACs in mammalian cells would be consistent with previous transcriptional studies where CtBP-mediated repression of certain promoters has been reported to be dependent on HDAC [13,14]. However, repression of certain other promoters has been reported to be independent of HDAC [15,16]. One such HDAC-independent mode may involve recruitment of the polycomb group proteins by CtBP [16,24]. It has also been reported that one of the CtBP-binding proteins, CtIP, may also mediate in HDAC-independent transcriptional repression by targeting components of the core transcriptional machinery [25]. These reports suggest CtBP may form distinct protein complexes to mediate HDAC-dependent and -independent repression.

Recently, CtBP has been shown to possess an intrinsic NAD^+-dependent dehydrogenase (DH) activity [26]. In this respect CtBP resembles the Sir2 silencing protein that has an intrinsic NAD^+-dependent HDAC activity [27,28]. It has been reported that the DH activity of CtBP may be responsible for transcriptional repression mediated by one of the CtBP-dependent transcriptional regulators, the nuclear hormone receptor, RIP140 [26]. However, other studies have shown that mutation of a His residue critical for the catalytic activity of CtBP did not affect transcriptional repression [29,30]. Thus, it appears CtBP may mediate transcriptional repression via an intrinsic enzymatic activity as well as by formation of specific protein complexes, depending on the context of the promoter. Studies with mutant mice have suggested that CtBP deficiency results in developmental phenotypes that reflect defects in the activity of a large number of transcriptional regulators [31]. Therefore, CtBP may use multiple modes of regulation of transcription during development.

Acknowledgements: This work was supported by Research Grant CA-84941 from the National Cancer Institute. We thank Susan Parkhurst for GST-dCtBP plasmid.

References

[1] Wolffe, A.P. (2001) Essays Biochem. 37, 45–57.
[2] Knoepfler, P.S. and Eisenman, R.N. (1999) Cell 99, 447–450.
[3] Kouzarides, T. (2002) Curr. Opin. Genet. Dev. 12, 198–209.
[4] Zhang, Y. and Reinberg, D. (2001) Genes Dev. 15, 2343–2360.
[5] Nielsen, S.J. et al. (2001) Nature 412, 561–565.
[6] Boyd, J.M., Subramanian, T., Schaeper, U., La Regina, M., Bayley, S. and Chinnadurai, G. (1993) EMBO J. 12, 469–478.
[7] Schaeper, U., Boyd, J.M., Verma, S., Uhlmann, E., Subramanian, T. and Chinnadurai, G. (1995) Proc. Natl. Acad. Sci. USA 92, 10467–10471.
[8] Sollerbrant, K., Chinnadurai, G. and Svensson, C. (1996) Nucleic Acids Res. 24, 2578–2584.
[9] Nibu, Y., Zhang, H. and Levine, M. (1998) Science 280, 101–104.
[10] Poortinga, G., Watanabe, M. and Parkhurst, S.M. (1998) EMBO J. 17, 2067–2078.
[11] Chinnadurai, G. (2002) Mol. Cell 9, 213–224.
[12] Turner, J. and Crossley, M. (2001) BioEssays 23, 683–690.
[13] Criqui-Filipe, P., Ducret, C., Maira, S.M. and Wasylyk, B. (1999) EMBO J. 18, 3392–3403.
[14] Izutsu, K., Kurokawa, M., Imai, Y., Maki, K., Mitani, K. and Hirai, H. (2001) Blood 97, 2815–2822.
[15] Meloni, A.R., Smith, E.J. and Nevins, J.R. (1999) Proc. Natl. Acad. Sci. USA 96, 9574–9579.
[16] Dahiya, A., Wong, S., Gonzalo, S., Gavin, M. and Dean, D.C. (2001) Mol. Cell 8, 557–569.
[17] Bett, A.J., Haddara, W., Prevec, L. and Graham, F.L. (1994) Proc. Natl. Acad. Sci. USA 91, 8802–8806.
[18] Uhlmann, E.J., Subramanian, T., Vater, C.A., Lutz, R. and Chinnadurai, G. (1998) J. Biol. Chem. 273, 17926–17932.
[19] Dignam, J.D., Lebovitz, R.M. and Roeder, R.G. (1983) Nucleic Acids Res. 11, 1475–1489.
[20] Schaeper, U., Subramanian, T., Lim, L., Boyd, J.M. and Chinnadurai, G. (1998) J. Biol. Chem. 273, 8549–8552.
[21] Zhang, C.L., McKinsey, T.A., Lu, J.R. and Olson, E.N. (2001) J. Biol. Chem. 276, 35–39.
[22] Sundqvist, A., Sollerbrant, K. and Svensson, C. (1998) FEBS Lett. 429, 183–188.
[23] Koipally, J. and Georgopoulos, K. (2000) J. Biol. Chem. 275, 19594–19602.
[24] Sewalt, R.G., Gunster, M.J., van der Vlag, J., Satijn, D.P. and Otte, A.P. (1999) Mol. Cell. Biol. 19, 777–787.
[25] Koipally, J. and Georgopoulos, K. (2002) J. Biol. Chem. 277, 23143–23149.
[26] Kumar, V., Carlson, J.E., Ohgi, K.A., Edwards, T.A., Rose, D.W., Escalante, C.R., Rosenfeld, M.G. and Aggarwal, A.K. (2002) Mol. Cell 10, 857–869.
[27] Shore, D. (2000) Proc. Natl. Acad. Sci. USA 97, 14030–14032.
[28] Moazed, D. (2001) Curr. Opin. Cell Biol. 13, 232–238.
[29] Turner, J. and Crossley, M. (1998) EMBO J. 17, 5129–5140.
[30] Phippen, T.M., Sweigart, A.L., Moniwa, M., Krumm, A., Davie, J.R. and Parkhurst, S.M. (2000) J. Biol. Chem. 275, 37628–37637.
[31] Hildebrand, J.D. and Soriano, P. (2002) Mol. Cell. Biol. 22, 5296–5307.