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The mammalian Fizzy and Fizzy-related genes are regulated at the transcriptional and post-transcriptional levels

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Abstract The cyclosome pathway of ubiquitin-mediated proteolysis plays an essential role in cell cycle control. The multisubunit cyclosome is regulated by transient interactions with Fizzy (Fzy) and Fizzy-related (Fzr) genes. We report here that both Fzy and Fzr are transcribed in a cell cycle specific but distinct manner. Fzy transcription starts after the restriction point in late G1 and ceases upon cell division. Fzr transcription also ceases upon cell division but resumes already in mid G1, before the restriction point, and takes place also in G0. Fzr has further a striking cell cycle specific pattern of mRNA stability. During most of the cell cycle its message is fairly stable, however upon exit from mitosis it is rapidly degraded. This result is puzzling because Fzr is essential for cyclosome activity in G1, and points to a complex pattern of Fzr regulation.

Key words: Cyclosome; APC; Cdh1; Hct1; p55^{cdc}; Cdc20

1. Introduction

Ubiquitin-mediated proteolysis plays a major role in the control of the eukaryotic cell cycle [1]. Programmed proteolysis ensures that cells contain different sets of key regulators at different times of the cell cycle. Many of these key regulators control the activity and specificity of protein kinases which in turn affect the behavior of whole sets of proteins involved in DNA replication, chromosome segregation and cell division. By controlling the set of key regulators, proteolysis largely dictates the orderly succession of the different cell cycle phases.

The mitotic A and B type cyclins were the first known examples of cell cycle regulators controlled by ubiquitin-mediated proteolysis. The abrupt degradation of these proteins starts during mitosis and requires a conserved nine amino acid long 'destruction box' [2,3] in their N-terminal region. The degradation of these cyclins is essential for the cell to complete mitosis [4]. Lately several other mitotic factors: Pds1 [5], Ase1 [6], Cut2 [7], Scc1 [8], Cdc5 [9] and Cdc20 [10] have been identified as substrates of the same destruction box specific proteolysis pathway.

The specific key component for ubiquitination of destruction box substrates, is a large, 12 subunit complex of about 1500 kDa called cyclosome or anaphase promoting complex (APC) [11,12]. The cyclosome has a cell cycle specific activity which is not restricted to mitosis but persists throughout G1 and even in G0 and in differentiated cells [13].

Genetic studies in *Drosophila* [14,15] and yeast [16–18] have identified two additional proteins which play essential roles in the regulation of the cyclosome. These proteins fall into two closely related groups of WD repeat proteins [19]. One group, Fzy, includes Cdc20 (*Saccharomyces cerevisiae*), Slp1 (*Schizosaccharomyces pombe*), Fizzy (*Drosophila*) and p55^{cdc} (mammals) and the second group, Fzr, includes Cdh1/Hct1 (*S. cerevisiae*) and Fizzy related (*Drosophila*, *Xenopus* and mammals). These proteins activate the cyclosome at different phases of the cell cycle and possibly towards different substrates [20]. Fzy is involved in the degradation during the metaphase-anaphase transition and Fzr in the degradation in late mitosis and during G1. Several recent results have shown that Fzy, but not Fzr, is part of the mitotic spindle checkpoint mechanism [21,22].

The mammalian Fzy homologue p55^{cdc} has been cloned and studied [23,24]. Its protein levels oscillate throughout the cell cycle and it is a substrate of ubiquitin-mediated proteolysis [24]. The mammalian Fzr homologue has been cloned by several labs [25,26] and its protein is present throughout the cell cycle. The patterns of *Fzy* and *Fzr* transcription during Drosophila embryogenesis are very distinct. *Fzy* expression is correlated with mitotic cell cycle progression, *Fzr* is not. *Fzr* transcript levels are strongly up-regulated when cells become postmitotic. Importantly, genetic analysis has clearly demonstrated that this up-regulation is essential to arrest cell proliferation at the appropriate developmental stage [15].

We have studied the expression pattern of mouse Fzy and Fzr during the cell cycle and found that the two genes display a distinct cell cycle specific transcription pattern. Fzy transcription is very similar to that of cyclin B1 [27–29] and B2 [13] starting in late G1 or early S phase and persisting throughout mitosis. Fzy transcription ceases upon cell division and its mRNA levels decline gradually during the first hours of G1. Fzr transcription is also cell cycle specific but it follows a different and unique pattern starting in G1 before the restriction point. While it is so far unclear to what extend Fzr is transcribed in S and G2, we found that it is not transcribed in early G1. This was an unexpected observation because Fzr is essential for cyclosome activation during G1. We were even more surprised to find that Fzr mRNA, which is normally rather stable, gets specifically degraded upon cytokinesis. These results suggest that both Fzy and Fzr are regulated in a complex manner at multiple levels of synthesis and degradation.

2. Materials and methods

2.1. Fzy and Fzr cDNA clones

Two EST clones of high homology to the *Xenopus* Fzr gene

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(Y14163) were obtained from the IMAGE consortium and sequenced. EST 724-a13 coded for amino acids 1–360 and EST 2640-n23 for amino acids 388–493. The missing part of the gene was obtained by RT-PCR of HeLa cDNA. The three parts of the gene were used to construct a full length cDNA using conventional molecular biology methods [30]. A full length human Fzy ($p55^{cdc}$) EST clone (656-h22) was obtained and sequenced [31] and found to be identical to the $p55^{cdc}$ described by Weinstein et al. [23].

2.2. Tissue culture

Murine NIH3T3 fibroblasts were obtained from R. Treisman (ICRF, London) and grown in DMEM supplemented with 10% FCS, glutamine, penicillin and streptomycin, all purchased from Biological Industries, Beit Haemek. Prometaphase arrested cells were obtained by overnight treatment with 2 μM nocodazole (Sigma) followed by shake-off of the cells arrested in prometaphase. This method yields a viable and highly synchronous population of cells which, when released into fresh medium, is capable of proceeding synchronously into G1 [13]. Actinomycin D (Sigma) was dissolved in ethanol as described [32], and used at 10 μg/ml final concentration.

2.3. RNA and protein blotting

Total RNA was prepared by the Guanidinium Isothiocyanate method [33] and quantitated by optical density. One percent agarose gels were prepared with MOPS buffer supplemented with 0.66 M formaldehyde, as described [34]. Equal amounts of RNA were run for approximately 3 h in MOPS buffer without formaldehyde and subsequently blotted for 16 h by capillary transfer onto Hybond N+ charged nylon membranes (Amersham). The blots were hybridized [32] with ^{32}P-radiolabeled Fzy, Fzr and Myc specific probes. After hybridization the blots were washed and exposed to a phosphorimager screen (Fuji). Immunoblots of Fzy were performed by standard methods [35] with goat anti-Fzy ($p55^{cdc}$) antibodies (SCB).

3. Results

3.1. Mouse Fizzy and Fizzy-related genes are transcribed in cell cycle specific and distinct patterns

Cell cycle specific interactions of Fzy and Fzr with the cyclosome play an essential role in its activation to degrade different substrates. In order to study the cell cycle specific expression of these two factors we obtained human cDNA clones of both genes. The human EST clone 656-h22 was sequenced and found to be identical to the published Fzy cDNA, $p55^{cdc}$ [23]. The human Fzr was constructed of two EST clones and a short fragment amplified by RT-PCR as described in Section 2. This assembled cDNA was sequenced and the coded protein was found to be 95 and 68% identical to the *Xenopus* and *Drosophila* Fzr [15] respectively, compared to only 40% identity between the human Fzy and Fzr proteins.

Mouse NIH3T3 fibroblasts were synchronized by nocodazole arrest and shake-off which yields a highly synchronous population of prometaphase cells [13]. The cells were then released into fresh medium and harvested at 2 h intervals for FACS analysis (Fig. 1A), immunoblot with a goat anti-Fzy antibody (Fig. 1B) and RNA blot with Fzy and Fzr specific radio-labeled probes (Fig. 1C). At time point 0 almost all the cells were mitotic as judged from their rounded shape, condensed chromosomes and DNA content. Upon release into fresh medium cells divided and entered G1. They entered S phase between 8 and 10 h after release, G2 at 14 h and again G1 at 18 h.

The levels of Fzy protein, as detected by immunoblotting (Fig. 1B), oscillated sharply during the cell cycle. Fzy rapidly disappeared upon entry into G1 and remained undetectable until the onset of S phase, when it started to accumulate to peak levels in G2-M. These results are consistent with previous reports [24] and are identical to the oscillations of the cyclin B1 protein during the cell cycle [13]. Due to low concentrations of Fzr in the cell and to the lack of a good antibody we could not properly follow the changes of Fzr during the cell cycle. Fzr has however previously been reported to be present at fairly constant levels throughout the cell cycle [25].

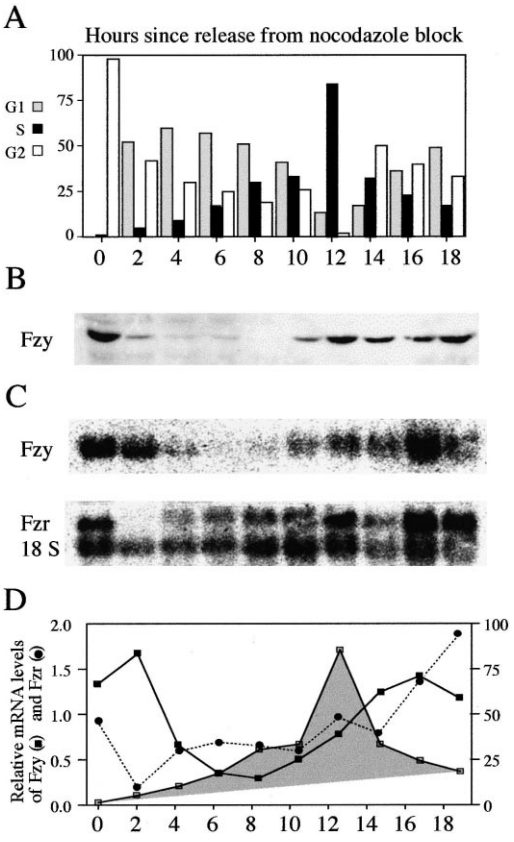

Fig. 1. Fzy protein and mRNA levels, as well as Fzr mRNA levels oscillate during the cell cycle. NIH3T3 fibroblasts were treated with nocodazole for 16 h and the prometaphase arrested cells were obtained by mitotic shake-off. Cells were then cultured in fresh medium and harvested at the indicated times. A: The cells at the different time points were analyzed by flow cytometry to assess their position in the cell cycle. B: Immunoblot of cell extracts with an anti-Fzy antibody. C: RNA blot with ^{32}P-labeled Fzy and Fzr specific probes. The Fzr specific probe was contaminated with bacterial RNA which cross-reacted with the 18S ribosomal RNA of each sample yielding a convenient standard to the mRNA loading of the different lanes of the gel. D: The relative mRNA levels of Fzy and Fzr were quantitated by a Fuji phosphorimager, normalized against the levels of 18S ribosomal RNA and plotted in relation to the percentage of S phase cells (grey shaded area).

RNA prepared from the different time points was analyzed by Northern blotting with Fzy and Fzr specific probes (Fig. 1C). Both messages oscillate sharply during the cell cycle but in a very distinct pattern (Fig. 1D). Fzy mRNA slowly declines upon release from the nocodazole arrest reaching undetectable levels within a few hours in G1 and starting to rise again in early S phase to peak levels in G2. This behavior is reminiscent of the oscillations of cyclin B [13]. The Fzr message also peaks in mitosis but declines much faster upon release from nocodazole arrest and is already undetectable after 2 h. It starts however to rise in mid G1, several hours before the Fzy message.

The difference in the initiation of Fzr transcription is clearly

manifested in the observation (Fig. 2) that Fzr mRNA is present at high levels in cells arrested in G0 by serum starvation, as well as in a highly differentiated tissue like the brain.

3.2. Fzr mRNA is rapidly degraded upon cell division

The rapid decline of Fzr mRNA after release from the nocodazole arrest (Fig. 1C) suggested that this message is very unstable. To test the stability of Fzr mRNA we treated cells released from nocodazole arrest with the transcription inhibitor Actinomycin D (ActD) and harvested them for Northern blotting. Fig. 3 shows that Fzr mRNA rapidly declined and was undetectable after 1 h. There was no difference in the decay of the message between cells treated with ActD or untreated cells, showing that Fzr indeed ceased to be transcribed upon release from the prometaphase block. Fzy message declined much slower and, like Fzr, was not affected by ActD indicating that it too was not transcribed. We reprobed our membranes a third time using a probe to detect the labile myc message and, as expected, it rapidly declined only in samples prepared from the ActD treated cells.

We next checked whether Fzr message is also labile during other phases of the cell cycle. Cells were synchronized in mitosis and released for 6, 10 or 14 h representing G1, S and G2 phase respectively. They were then treated with ActD and harvested at the indicated time points. The samples were analyzed by Northern blotting and the membranes were reprobed with the myc probe. Fig. 4 shows that the Fzr message is stable during S and G2 and slightly less stable in G1. The message was also stable in cells arrested in prometaphase by nocodazole, in telophase by the expression of indestructible cyclin B1, and obviously also in unsynchronized cells. Interestingly, also the myc message was stabilized in nocodazole, a phenomenon which has not been reported in the past.

In S and G2 there was little difference in Fzr mRNA levels between cells treated with ActD for 2 h and untreated control cells. This does not only indicate that the mRNA is very stable, but might further suggest that Fzr is not transcribed during these phases of the cell cycle. To clarify this point we are currently cloning the Fzr promoter in order to study its transcription directly.

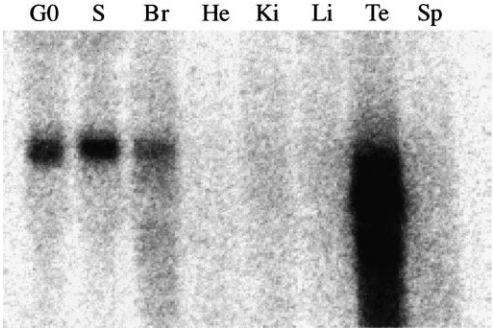

Fig. 2. Fzr is expressed at high levels in serum starved cells, in the brain and in testis. Total RNA was extracted from serum starved cells (G0), hydroxyurea arrested cells (S) and from various mouse tissues: brain (Br), heart (He), kidney (Ki), liver (Li), testis (Te) and spleen (Sp), blotted, and probed with an Fzr specific ^{32}P-labeled probe.

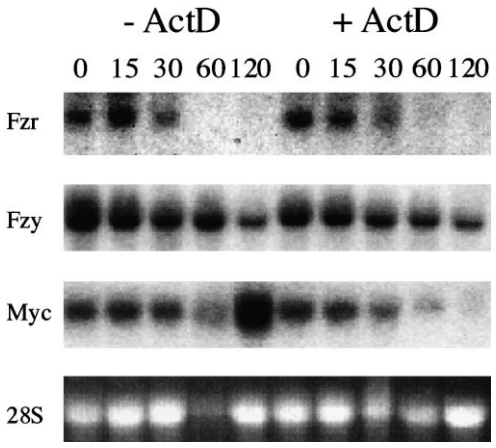

Fig. 3. Fzr mRNA is rapidly degraded upon release from a prometaphase block. NIH3T3 fibroblasts were released from a nocodazole block and cultured in fresh medium with or without ActD. Cells were harvested at the indicated time points for Northern blotting with a ^{32}P-labeled Fzr specific probe, stripped and hybridized with a ^{32}P-labeled Fzy probe and stripped again and hybridized with a ^{32}P-labeled myc probe. The image of the gel stained with ethidium bromide showing the 28S ribosomal band is included to show the relative amount of RNA in each lane.

4. Discussion

Fizzy (Fzy) and Fizzy-related (Fzr) genes interact with, and activate the cyclosome in a cell cycle specific manner ([25,26,36], T.L. and M.B., submitted). While these interactions are largely regulated by phosphorylation ([31,37,38], T.L and M.B., submitted), the role of other mechanisms, in particular of cell cycle specific expression of these genes, cannot be excluded. The level of Fzy, which oscillates sharply during the cell cycle was shown to be regulated by proteasome dependent proteolysis [24]. We show here that Fzy is further regulated by cell cycle specific transcription in a manner very similar to those of the mitotic B type cyclins.

In contrast, the observed expression pattern of Fzr mRNA and protein is, to some extent, reminiscent of that of Cdk1 ($p34^{cdc2}$) [39], both are transcriptionally regulated, the mRNA of both is degraded rapidly upon cell division and both the Fzr and the Cdk1 proteins are stable and present throughout the cell cycle. There is however at least one important difference: Cdk1 mRNA is neither present nor transcribed in cells arrested in G0 by serum starvation in contrast to Fzr which is transcribed in G0. Fzr shows therefore a novel transcription pattern which suggests that its promoter will have both cell cycle specific, as well as quiescent specific, elements. Cell cycle specific stability of mRNA has also been reported for cyclin B1 [29], however the degradation of Fzr mRNA upon cell division we report here is even more pronounced. This might be an interesting mechanism of cell cycle regulation which has hardly been addressed so far.

The cell cycle specific transcription and rapid degradation of Fzr mRNA are puzzling in view of the rather constant levels of Fzr protein during the cell cycle. Fzr activates the cyclosome in G1 [15]. A speculative explanation for the suppression of its synthesis during early G1 could therefore suggest that there is a difference between freshly synthesized and old Fzr. As wild as this speculation may sound, it has already been suggested in the past for Cdk1 which also presents a

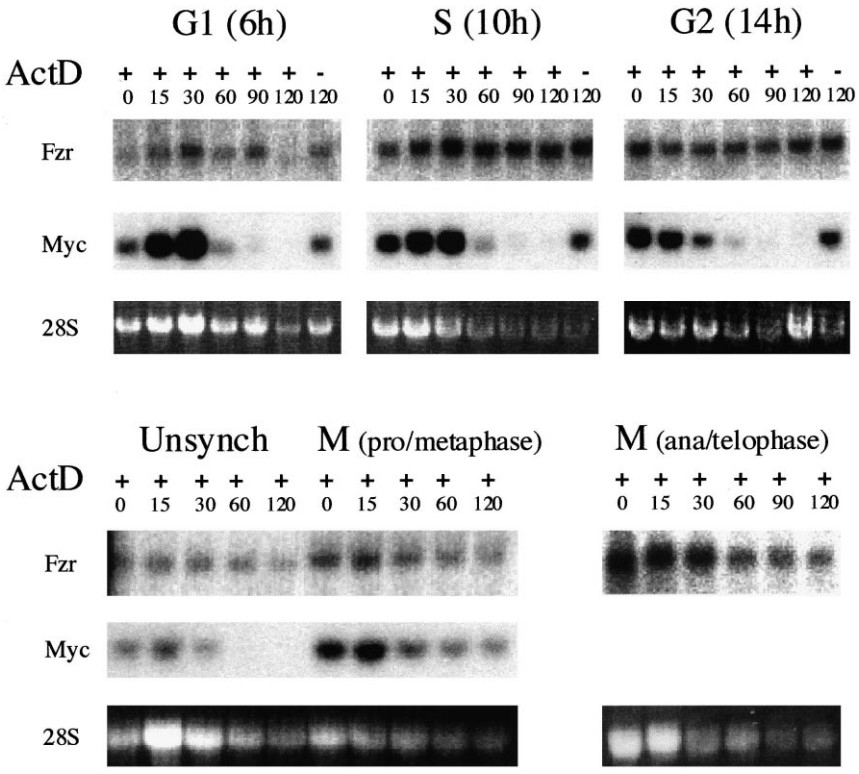

Fig. 4. Fzr mRNA is stable throughout the cell cycle. NIH3T3 fibroblasts were synchronized by nocodazole and shake-off and released into fresh medium for 6, 10 and 14 h to obtain G1, S and G2 phase cells correspondingly. Cells were then treated with ActD (+) and harvested at the indicated time points. Control cells which were not treated with ActD were allowed to proceed for 120 min (−). Total RNA was extracted from the cells and analyzed by Northern blotting with an Fzr specific ^{32}P-labeled probe. After exposure to a phosphorimager screen the filters were stripped and reprobed with a myc specific probe. The stability of Fzr mRNA was studied also in unsynchronized cells, in cells arrested in prometaphase by nocodazole, and in telophase by the expression of indestructible cyclin B1. As these cells were arrested at a fixed time point of the cell cycle a control without ActD would not have differed from time point 0 and was thus not included.

pattern of coordinated synthesis and degradation [40]. Moreover both Fzy [24] and Fzr [26] have been shown to be phosphorylated in a cell cycle specific manner. These data should therefore be kept in mind when studying Fzr which is probably controlled in a much more complex way than currently believed.

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References

[1] Townsley, F.M. and Ruderman, J.V. (1998) Trends Cell Biol. 8, 238–244.
[2] Hershko, A., Ganoth, D., Pehrson, J., Palazzo, R.E. and Cohen, L.H. (1991) J. Biol. Chem. 266, 16376–16379.
[3] Glotzer, M., Murray, A.W. and Kirschner, M.W. (1991) Nature 349, 132–138.
[4] Gallant, P. and Nigg, E.A. (1992) J. Cell Biol. 117, 213–224.
[5] Cohen-Fix, O., Peters, J.M., Kirschner, M.W. and Koshland, D. (1996) Genes Dev. 10, 3081–3093.
[6] Juang, Y.L., Huang, J., Peters, J.M., McLaughlin, M.E., Tai, C.Y. and Pellman, D. (1997) Science 275, 1311–1314.
[7] Funabiki, H., Yamano, H., Nagao, K., Tanaka, H., Yasuda, H., Hunt, T. and Yanagida, M. (1997) EMBO J. 16, 5977–5987.
[8] Michaelis, C., Ciosk, R. and Nasmyth, K. (1997) Cell 91, 35–45.
[9] Shirayama, M., Zachariae, W., Ciosk, R. and Nasmyth, K. (1998) EMBO J. 17, 1336–1349.
[10] Prinz, S., Hwang, E., Visintin, R. and Amon, A. (1998) Curr. Biol. 8, 750–760.
[11] Sudakin, V., Ganoth, D., Dahan, A., Heller, H., Hershko, J., Luca, F.C., Ruderman, J.V. and Hershko, A. (1995) Mol. Biol. Cell 6, 185–197.
[12] King, R.W., Peters, J.M., Tugendreich, S., Rolfe, M., Hieter, P. and Kirschner, M.W. (1995) Cell 81, 279–288.
[13] Brandeis, M. and Hunt, T. (1996) EMBO J. 15, 5280–5289.
[14] Sigrist, S.J., Jacobs, H., Stratmann, R. and Lehner, C.F. (1995) EMBO J. 14, 4827–4838.
[15] Sigrist, S.J. and Lehner, C.F. (1997) Cell 90, 671–681.
[16] Visintin, R., Craig, K., Hwang, E.S., Prinz, S., Tyers, M. and Amon, A. (1998) Mol. Cell 2, 709–718.
[17] Schwab, M., Lutum, A. and Seufert, W. (1997) Cell 90, 683–693.
[18] Matsumoto, T. (1997) Mol. Cell. Biol. 17, 742–750.
[19] van der Voorn, L. and Ploegh, H. (1992) FEBS Lett. 307, 131–134.
[20] Morgan, D. (1999) Nat. Cell Biol. 1, E47–E53.
[21] Lorca, T. et al. (1998) EMBO J. 17, 3565–3575.
[22] Kallio, M., Weinstein, J., Daum, J., Burke, D. and Gorbsky, G. (1998) J. Cell Biol. 141, 1393–1406.
[23] Weinstein, J., Jacobsen, F.W., Hsu-Chen, J., Wu, T. and Baum, L.G. (1994) Mol. Cell. Biol. 14, 3350–3363.
[24] Weinstein, J. (1997) J. Biol. Chem. 272, 28501–28511.
[25] Fang, G., Yu, H. and Kirschner, M.W. (1998) Mol. Cell 2, 163–171.
[26] Kramer, E.R., Gieffers, C., Holzl, G., Hengstschlager, M. and Peters, J.M. (1998) Curr. Biol. 8, 1207–1210.
[27] Cogswell, J.P., Godlevski, M.M., Bonham, M., Bisi, J. and Babiss, L. (1995) Mol. Cell. Biol. 15, 2782–2790.
[28] Hwang, A., Maity, A., McKenna, W.G. and Muschel, R.J. (1995) J. Biol. Chem. 270, 28419–28424.
[29] Maity, A., McKenna, W.G. and Muschel, R.J. (1995) EMBO J. 14, 603–609.

[30] Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1994) Current Protocols in Molecular Biology, John Wiley and Sons Inc, New York.
[31] Shteinberg, M., Protopopov, Y., Ganoth, D., Listovsky, T., Brandeis, M. and Hershko, A. (1999) Biochem. Biophys. Res. Commun. 260, 193–198.
[32] Sambrook, J., Fritsch, E. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[33] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[34] Fourney, R., Fregeau, C., Middlestadt, M., Miyakoshi, J. and Paterson, M. (1987) in: Techniques for Transfer and Binding of Proteins and Nucleic Acids to Binding Matrices (Janik, B., Ed.), pp. 135–147, Gelman Sciences, Ann Arbor.
[35] Harlow, E. and Lane, D. (1987) Antibodies, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[36] Lukas, C., Sorensen, C.S., Kramer, E., Santoni-Rugiu, E., Lindeneg, C., Peters, J.M., Bartek, J. and Lukas, J. (1999) Nature 401, 815–818.
[37] Kotani, S., Tanaka, H., Yasuda, H. and Todokoro, K. (1999) J. Cell Biol. 146, 791–800.
[38] Zachariae, W., Schwab, M., Nasmyth, K. and Seufert, W. (1998) Science 282, 1721–1724.
[39] Dalton, S. (1992) EMBO J. 11, 1797–1804.
[40] Welch, P.J. and Wang, J.Y. (1992) Proc. Natl. Acad. Sci. USA 89, 3093–3097.