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Cell type-specific regulation of calmodulin 2 expression by mutant p53

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Abstract To identify genes that are stimulated by oncogenic forms of mutant p53, we studied, by microarray analysis and PCR-select subtractive hybridization, gene expression changes in human wild-type (wt) p53-negative immortal 041 fibroblasts infected to stably express p53 mutant 175H. In contrast to the wt p53 transactivator, 175H induced only few and weak, gene expression changes. We report here the stimulation of calmodulin 2 (*CaM 2*), but not *CaM 1* or *3*, gene expression specifically in 041 cells. The stimulation of the *CaM 2* promoter required the 5′ untranslated sequences as well as the integrity of the transactivation domain of 175H. However, direct binding of 175H to the 5′UT in vitro could not be demonstrated.

Keywords: p53; Calmodulin; Transcription; Transactivation

1. Introduction

Oncogene expression and a large number of stresses that disrupt the nucleolus can cause cell senescence or apoptosis through the stabilization and activation of the p53 transcription factor [1,2]. These tumor suppressive cellular responses are themselves suppressed in many tumors by the mutational inactivation of wt p53. Numerous observations have documented that many full-length mutant forms of p53 are not only defective for the wt functions but exert novel oncogenic activities such as the increase of tumorigenicity [3], mutation frequency [4], metastatic potential [5], and genomic instability [6,7]. It is unclear precisely how mutant p53 acts as an oncoprotein but several observations indicate that it may be able to regulate genes despite of its defective core DNA binding domain [8–11].

Calmodulin (CaM) is a small ubiquitous calcium-binding protein and a principal transducer of calcium signals that modulates a large number of essential cellular processes including cell motility and proliferation [12]. How can a single protein be spatially and temporally regulated to orchestrate so many, and sometimes even opposing, signals? One explanation may be found in the unique feature of genetic redundancy within the calmodulin gene family: Three *CaM* genes located on different chromosomes exhibit 20% divergency in their coding regions, yet no amino acid substitutions; thus, all three genes encode the same protein. By contrast, the non-coding regions including the regulatory sequences of the three genes are very different; nonetheless, each is highly conserved across vertebrate species. Based on these and further observations, a current model suggests that the local availability of CaM may be regulated through the build-up of local CaM pools from individual, differentially regulated *CaM* genes [13]. We report here that p53 mutant 175H can stimulate specifically calmodulin 2 (*CaM 2*), but not *CaM 1* or *3*, gene expression.

2. Materials and methods

2.1. Cell culture, viruses and transfection

041, H1299 and Saos-2 cells were cultured in DMEM, supplemented with 10% FCS and grown in a humidified 7% CO_2 atmosphere at 37 °C. Retroviruses were harvested from the 293GP producer lines as previously described [14]. Cells were infected with titered virus stock in the presence of 4 μg/ml polybrene (Sigma) for 4 h, and virus-infected cells were selected in 400 μg/ml G418 at 48 h after infection for approximately seven days. For transient transfection, exponentially growing cells in 12-well dishes were incubated for 4 h with 0.3 μg of total DNA, of which 0.1 μg was empty vector, 0.1 μg was the green fluorescence protein expressing plasmid pEGFP-C3 (Clontech, Palo Alto, USA), and 0.1 μg was either wild-type or mutant p53-expressing plasmid (pCMV-wtp53, pCMV-175H, pCMV-175HΔC, pCMV-175H22,23, pCMV-175HΔC22,23, pCMV-175HΔO), or empty vector (pCMVpA). As luciferase reporter plasmids either pGL2-Basic(CaM2-luc), pGL2-Basic(CaM2-(Δ5′UT)-luc) or pWaf-luc were employed. In Southwestern analyses, reporter plasmid PG13-CAT containing 13 p53-binding sites and plasmid MG15-CAT with 15 mutated sites, were additionally used. As transfection reagent, effectene from Qiagen (Hilden, Germany) was employed according to the manufacturer's recommendation for 12-well dishes. Luciferase assays were employed using the "Luciferase Assay System" (Promega, Mannheim, Germany) as specified by the manufacturer.

2.2. Antibodies and immunoblot analysis

The p53 monoclonal antibody DO-1 was purchased from Calbiochem (San Diego, USA); the Calmodulin monoclonal antibody AB-2 (2D1) was from Neomarkers (Fremont, USA), and the monoclonal ß-actin antibody was from Sigma–Aldrich (Taufkirchen, Germany). Cells from 10 cm dishes were lysed in 150 μl of a lysis buffer heated to 85 °C and containing 50 mM Tris–HCl (pH 6.8), 100 mM DTT, 2% SDS and 20% glycerol. Samples containing 15 μg of total cellular protein were subjected to 12% SDS–PAGE and transferred to a PVDF membrane (Immobilon-P, Millipore, Bedford, USA). Signals were detected upon overnight incubation of the membranes with anti-p53 (1:1000), anti-ß-actin (1:5000), or anti-calmodulin (1:500) antibodies followed by a final incubation with a peroxidase-conjugated secondary anti-mouse antibody and Renaissance Enhanced Luminol Reagents (NEN, Boston, USA), performed as specified by the supplier.

2.3. Northern and Southern blot analysis

Total RNA from cells grown on 10 cm dishes was isolated via RNeasy Mini Kit from Qiagen (Hilden, Germany) as specified by the supplier. 10 μg of DNaseI digested RNA (DnaseI: Roche, Mannheim, Germany) was electrophoresed on a formaldehyde-containing 1%

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agarose gel, or 20 μg of DNA was subjected to electrophoresis, and blotted onto a nylon membrane (SS Nytran, Schleicher Schuell, Germany) following standard procedures. The membranes were hybridized with AlkPhos-labelled probes (Amersham Biosciences, Freiburg, Germany) in hybridization buffer at 55 °C as specified by the supplier.

2.4. Subtractive hybridization

Subtractive hybridization was performed in O41 cells using the BD Clontech PCR-select cDNA Subtraction Kit (BD Biosciences Clontech, Heidelberg, Germany) as specified by Clontech. Following the first round of subtraction a subsequent PCR amplification of differentially expressed genes was performed, using the adapter specific primers 1 and 2R. PCR products were then cloned into PCRII-TOPO vector (Invitrogen, Carlsbad, USA). Selecting for the kanamycine resistance gene, clones were isolated and the cDNA probes were excised with *Eco*RI for Northern blot analysis.

2.5. Southwestern blotting

300 ng of each DNA sample was mounted onto a nitrocellulose membrane (Schleicher Schuell, Germany) and crosslinked with 120 J/m^2 of UVC light. Membranes were washed twice in PBS and blocked with 5% skim milk in PBS for 3 h. Nuclear extracts were diluted to a protein concentration of 1 mg/ml in gel-shift buffer (12.5 mM Tris–HCl pH 7.9; 3.1 mM $MgCl_2$; 25 mM KCl, 0.5 mM DTT; 10% glycerol; 0.25 mM EDTA; 0.2 mM PMSF; 10 μg/ml leupeptin; 1 μg/ml pepstatin; 10 μg/ml aprotinin and 1 mg/ml of pGL2-Basic (Promega) plasmid DNA for blocking) and were incubated overnight at 4 °C with the membrane, followed by 3 × 5 min washes in PBS-Tween (0.05%) [10]. The membrane was next incubated for 1 h with antibody DO-1, followed by 3 × 5 min washes in PBS-Tween. A final incubation with a peroxidase-conjugated secondary anti-mouse antibody and Renaissance Enhanced Luminol Reagents (NEN, Boston, USA) was performed as specified by the supplier.

3. Results

p53-negative 041 fibroblasts stem from a Li-Fraumeni patient who had inherited a deletion in one p53 allele, and have subsequently lost the remaining allele [15]. Although non-epithelial, these merely immortal rather than fully transformed cells were chosen to study gene expression changes induced by the p53 'gain-of-function' mutant 175H to minimize the chance that 175H-inducible changes have already occurred through other mechanisms in the course of transformation. Initially performed cDNA microarray studies showed that, in contrast to wt p53, 175H apparently induced only very few, and weak, gene expression changes, in accord with the mutant being DNA binding-impaired. Of four sequences identified to be stimulated more than 4-fold and 13 sequences transcriptionally overproduced at least 3-fold (the inclusion criterium), none could be verified in Northern blot analyses. Only one sequence, the *CaM 2* gene, which was stimulated only 2.1-fold and was therefore initially not further analyzed, could eventually also be identified by PCR-select subtractive hybridization (see Section 2 for details) performed with 041 cultures expressing vector-only or 175H, and be verified in Northern and immunoblots.

In Northern blots, the *CaM 2* transcript was 2.5-fold overproduced in three, at different times separately infected 175H-cultures when compared to control-infected cultures. Concomitantly, an approximately 2-fold increase in calmodulin protein expression became apparent in cells expressing 175H (Fig. 1(a)). Since mutant p53 lacks important features of classical transcription factors such as a functional core DNA binding domain, and has been documented to be able to cause gene amplification in human cells [6], we examined whether the

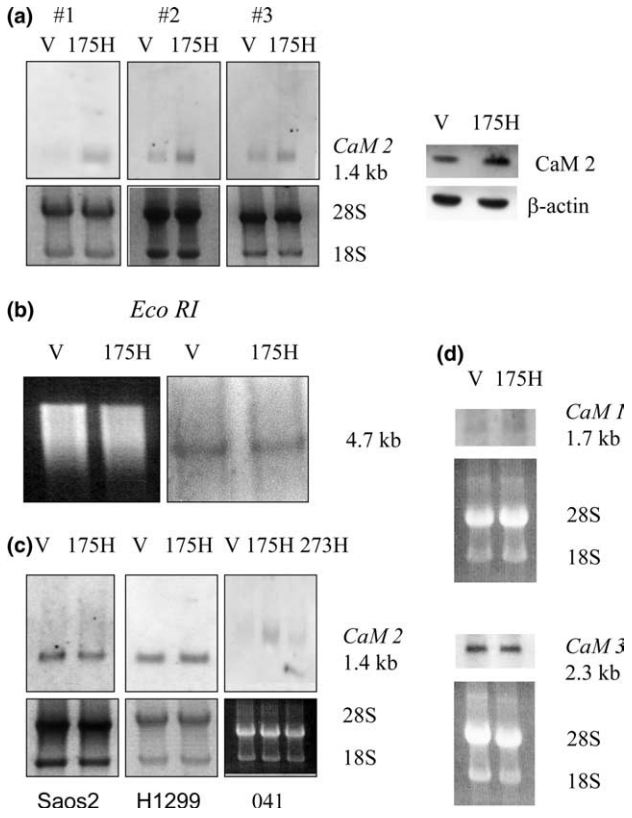

Fig. 1. Cell type-specific stimulation of CaM 2 expression in the presence of 175H. (a) Northern blot analysis of different 041 cultures bulk-infected with either empty vector (V) or 175H. 18S and 28S show rRNAs as loading controls. Right panel: Western blot analysis of 15 μg of protein from infected 041 cultures. Calmodulin antibody was used at 1:500; actin antibody at 1:5000. (b) Southern blot analysis of 20 μg DNA from 041-vector and 041-175H cultures digested with *Eco*RI. (c) Northern blot analysis of *CaM 2* expression on the cell lines Saos-2, H1299 and 041 infected with vector (V), 175H or 273H. (d) Northern blot analysis of the expression of *CaM 1* and *3* in 041-vector and 041-175H cultures.

CaM 2 gene dose was altered in 175H-cultures. Southern blot analyses revealed that equal *CaM 2* gene doses were present in cultures producing 175H and in the controls (Fig. 1(b)). Moreover, fluorescence in situ hybridizations failed to show ploidy differences between control-infected and 175H-infected cultures (data not shown). When human wt p53-negative Saos-2 osteosarcoma or H1299 adenocarcinoma cells were infected to produce 175H, or when 041 cells were infected to produce the DNA contact mutant 273H, no changes in *CaM 2* gene expression were observed (Fig. 1(c)). Finally, there were no changes in the expression of the *CaM 1* and *3* genes (Fig. 1(d)). Thus, the conformational mutant 175H can stimulate *CaM 2* gene expression in a cell type-specific manner.

To examine the effects of mutant p53 on the regulatory sequences of the *CaM 2* gene, a plasmid carrying 1.1 kb of the *CaM 2* promoter, including the 5′ untranslated sequences, in front of the luciferase gene was co-transfected with either empty vector or an effector plasmid producing 175H. All transfections included a plasmid expressing the green fluorescence protein gene from the hCMV promoter. Luciferase activity was analyzed 48 h after transfection, and the numbers of transfected cells as well as the fluorescence intensities were determined by flow cytometry and were employed to normalize

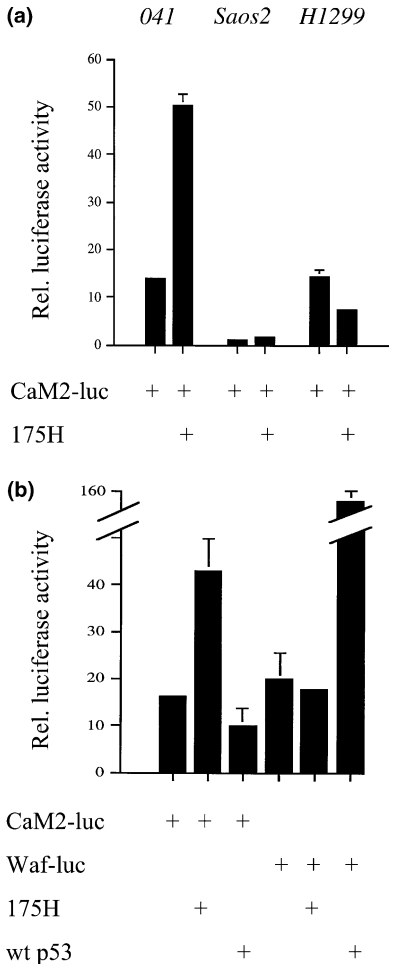

Fig. 2. Cell type-specific stimulation of plasmid-based *CaM 2* regulatory sequences by 175H. (a) Transient transfections of the denoted cell lines with the indicated reporter and effector plasmids. Luciferase assays were performed 48 h after transfection. (b) Transfections of 041 cells with CaM 2 or Waf reporter plasmids and either wt or mutant p53 effector plasmids. Error bars show SD from at least three experiments.

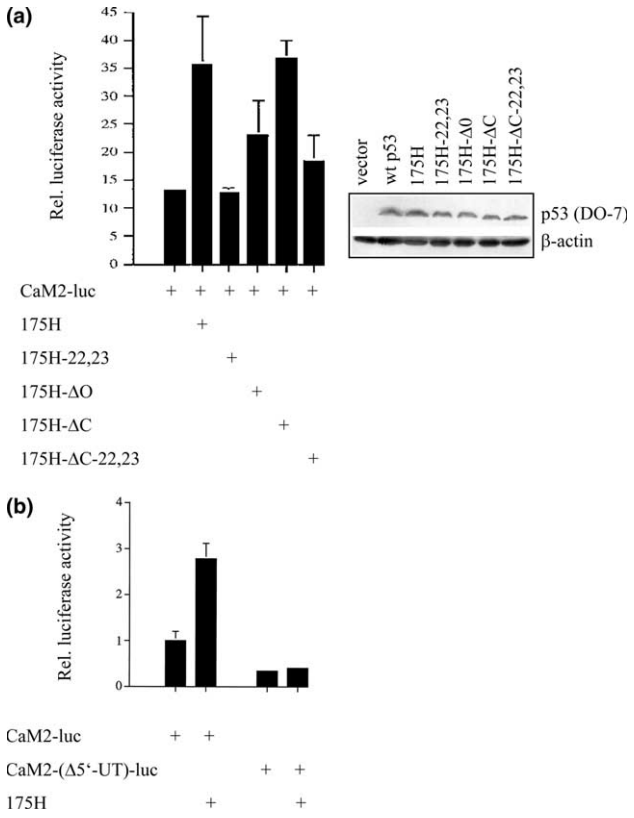

Fig. 3. CaM2-luc stimulation requires an intact transactivation domain of 175H and the 5′UT of the *CaM 2* gene. (a) Transient transfection for 48 h of 041 cultures with CaM2-luc reporter and effector plasmids producing 175H or variants of 175H. The Western blot, produced from a 12% gel and stained with anti-p53 and β-actin antibodies, documents the approximately equal expression of all used p53 effector plasmids in transient transfections. (b) Transfection for 48 h of 041 cells with the CaM2-luc reporter or a derivative lacking the 5′UT, plus either empty vector or 175H-expression plasmid.

for transfection efficiency. Fig. 2(a) shows that the *CaM 2* regulatory sequences were stimulated 2.5-fold in the presence of 175H in 041 cells, in accord with the approximately 2-fold *CaM 2* transcript overproduction observed in 175H-expressing 041 cells. In contrast, and in agreement with the failure of 175H to stimulate *CaM 2* transcript in Saos-2 and H1299 cells, luciferase activity was not increased by 175H in these cell types. When 041 cultures were transfected with the reporter plasmids CaM2-luc or pWaf-luc carrying the wt p53-responsive p21Waf/Cip1-promoter in front of a luc gene, and each reporter was co-transfected either with empty vector, wt p53-vector or 175H-expressing vector, it became apparent that 175H can stimulate the *CaM 2* but not *Waf* promoter, while wt p53 can transactivate *Waf* but not *CaM 2* sequences (Fig. 2(b)). Combined these findings indicate that the *CaM 2* promoter in the context of a plasmid is stimulated in the presence of p53 mutant 175H specifically in 041 Li-Fraumeni cells.

To obtain information about the domains of 175H required for *CaM 2* promoter stimulation, CaM2-luc reporter plasmid was transiently co-transfected into 041 cells with empty vector or effector plasmid expressing, at equal levels, one of the 175H double mutants harboring additional defects in either the transactivation, oligomerization or general nucleic acids binding domains of p53 (Fig. 3(a)). The results show that the integrity of the N-terminal transactivation domain (residues 22 and 23) is essential for *CaM 2* promoter stimulation. Tetramerization of 175H was not absolutely required, and the C-terminal general nucleic acids binding domain was dispensable. Since the core DNA binding domain is dysfunctional in 175H, the latter suggests that the effect on the *CaM 2* promoter does not involve direct DNA contact by the p53 mutant. In turn, when 041 cultures were co-transfected with CaM2-luc or CaM2-(Δ5′UT)-luc in which the 5′UT region had been deleted, plus the 175H expression plasmid, stimulation of luc expression was observed only with the complete *CaM 2* construct (Fig. 3(b)), indicating that the 5′ untranslated sequences of the *CaM 2* gene are, directly or indirectly, targeted by 175H.

It is presently unclear how mutant p53 can modulate gene expression. To study whether 175H associates with sequences in the *CaM 2* promoter or 5′UT region, an in vitro protein-DNA binding assay based on the Southwestern blotting procedure was employed [10]. For this purpose, a panel of plasmids carrying the relevant promoter sequences or not, were immobilized on nitrocellulose membranes, incubated with cell extracts transfected to express empty vector, wt p53

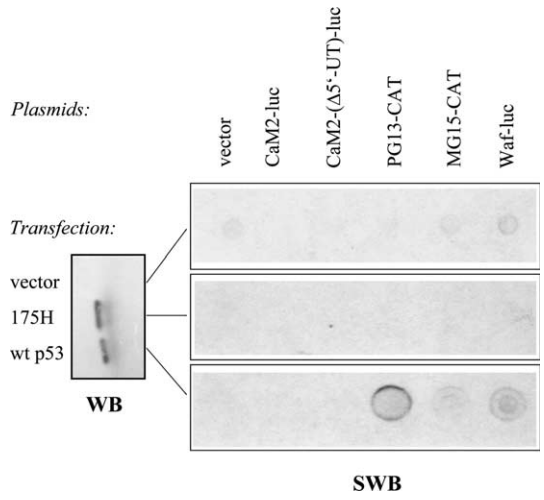

Fig. 4. In vitro-binding of p53 to plasmids with or without putative target sequences. Cultures were transfected with either empty luc vector, or 175H- or wt p53-producing plasmids. Expression of the transgenes was determined by Western blotting (WB) with anti-p53 antibody DO-1. In a Southwestern blot (SWB) procedure, membranes carrying 0.3 μg of the indicated plasmids were incubated with the extracts from the transfected cultures, and bound p53 was detected by DO-1 diluted at 1:1000.

or 175H, and were exposed to anti-p53 antibodies. As documented in Fig. 4, wt p53 extracts produced a signal with the PG13-CAT and Waf-luc plasmids but not with the luc vector, the CaM2-luc or the CaM2-(Δ5′UT)-luc vector, or with MG15-CAT, indicating that wt p53 can associate with known target sequences in this experimental setup. In contrast to wt p53 extracts, p53-null extracts produced no signal at all. 175H extracts generated no signal with the control vectors, and also no signal with CaM2-luc and CaM2-(Δ5′UT)-luc. Thus, a direct binding of 175H in vitro to *CaM 2* regulatory sequences in the context of a plasmid could not be demonstrated.

4. Discussion

We have presented here data indicating that the conformational p53 mutant 175H can stimulate the expression of the *CaM 2*, but not the *CaM 1* or *3*, gene and increase the levels of CaM protein in immortal human wt p53-null 041 Li-Fraumeni fibroblasts. 175H failed to affect *CaM* expression in the fully transformed human Saos-2 osteosarcoma or H1299 lung adenocarcinoma cells. Furthermore, *CaM 2* overexpression in 041 cells was not the result of *CaM 2* gene amplification, and stimulation of a plasmid-based *CaM 2* promoter required the transactivation domain of 175H and the 5′ untranslated sequences of *CaM 2*. Mutants of p53 have been identified as activators of a number of cellular genes, including the multi drug resistance *(MDR)-1* [8], topoisomerase I [16], *c-myc* [9], and apoptosis-inhibiting *BAG-1* genes [17]. We have initially tested, by PCR and Northern blotting, for changes in the expression of *BAG-1*, *c-myc*, the mutant p53-binding protein 1 gene *MBP-1*, and *c-fos* but have failed to observe a stimulating effect of 175H in our cell system (data not shown). So if mutant p53 can transactivate genes, how, if at all, does it associate with DNA? After all, the core DNA binding domain of the wt protein is dysfunctional in the mutants. Previous finding have indicated that mutant p53 may nonetheless be able to recognize specific DNA sequences and bind these directly [18]. Furthermore, [9] could demonstrate that the *c-myc* gene is regulated by mutant p53 directly, through the interaction of the C-terminal general nucleic acids binding domain with sequences within exon 1 of the *myc* gene. Such mechanism, however, is apparently not generally employed by mutant p53. More recent observations have shown, for instance, that the activation of the *MDR-1* gene by mutant p53 requires bridging between the *MDR-1* promoter and mutant p53 by ETS transcription factors [19]. Not least, mutant p53 may stimulate a transcription factor that subsequently regulates a number of downstream target genes and thus modulate gene expression indirectly. Our finding that the C-terminus of mutant p53 is dispensable for *CaM 2* gene stimulation excludes a mechanism like the one reported for *c-myc*. Finally, the combined domain inactivation and in vitro DNA binding data render a direct interaction between 175H and *CaM 2* sequences unlikely.

Can the cell type-specific stimulation of a two-fold overproduction of the ubiquitously highly expressed CaM protein by mutant p53 have a biological relevance? A possible clue may come from the answer to the question why one and the same CaM protein is encoded by three different genes. CaM can interact with at least 100 different proteins, and a current model suggests that the specificity and fidelity of CaM interactions may be provided by the generation of distinct subcellular CaM pools, for instance through the specific transport/local translation of different *CaM* transcripts from the uniquely regulated *CaM* genes [13]. Differential localization of *CaM* transcripts within cells has indeed been documented [20–22], and selective inhibition of individual *CaM* genes has yielded specific effects, for example on the cell cycle and proliferative capacity of cells [23–25]. The *CaM 2* gene has been suggested to be an early response gene that reacts quickly to growth factor and hormone stimulations [26,27]. Moreover, CaM has been associated with apoptosis and metastasis [28], as has mutant p53 [5,29]. Future studies will show whether part of mutant p53's 'gain-of-function' activities is mediated by calmodulin.

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References

[1] Vousden, K.H. and Lu, X. (2002) Nat. Rev. Cancer 2, 594–604.
[2] Rubbi, C.P. and Milner, J. (2003) EMBO J. 22, 6068–6077.
[3] Dittmer, D., Pati, S., Zambetti, G., Chu, S., Teresky, A.K., Moore, M., Finlay, C. and Levine, A.J. (1993) Nat. Genet. 4, 42–46.
[4] Iwamoto, K.S., Mizuno, T., Ito, T., Tsuyama, N., Kyoizumi, S. and Seyama, T. (1996) Cancer Res. 56, 3862–3865.
[5] Pohl, J., Goldfinger, N., Radler-Pohl, A., Rotter, V. and Schirrmacher, V. (1988) Mol. Cell. Biol. 8, 2078–2081.
[6] El-Hizawi, S., Lagowski, J.P., Kulesz-Martin, M., Albor, A. and Kaku, S. (2002) Cancer Res. 62, 3264–3270.
[7] Gualberto, A., Aldape, K., Kozakiewicz, K. and Tlsty, T.D. (1998) Proc. Natl. Acad. Sci. USA 95, 5166–5171.
[8] Chin, K.V., Ueda, K., Pastan, I. and Gottesman, M.M. (1992) Science 255, 459–462.
[9] Frazier, M.W., He, X., Wang, J., Gu, Z., Cleveland, J.L. and Zambetti, G.P. (1998) Mol. Cell Biol. 18, 3735–3743.

[10] Zalcenstein, A. et al. (2003) Oncogene 22, 5667–5676.
[11] Kim, E. and Deppert, W. (2003) Biochem. Cell Biol. 81, 141–150.
[12] Stull, J.T. (2001) J. Biol. Chem. 276, 2311–2312 (Epub 2000, November 28).
[13] Toutenhoofd, S.L. and Strehler, E.E. (2000) Cell Calcium 28, 83–96.
[14] Stahler, F. and Roemer, K. (1998) Oncogene 17, 3507–3512.
[15] Liu, P.K., Kraus, E., Wu, T.A., Strong, L.C. and Tainsky, M.A. (1996) Oncogene 12, 2267–2278.
[16] Albor, A., Kaku, S. and Kulesz-Martin, M. (1998) Cancer Res. 58, 2091–2094.
[17] Yang, X., Pater, A. and Tang, S.C. (1999) Oncogene 18, 4546–4553.
[18] Will, K., Warnecke, G., Wiesmuller, L. and Deppert, W. (1998) Proc. Natl. Acad. Sci. USA 95, 13681–13686.
[19] Sampath, J. et al. (2001) J. Biol. Chem. 276, 39359–39367 (Epub 2001, August 1).
[20] Zhang, S.P., Natsukari, N., Bai, G., Nichols, R.A. and Weiss, B. (1993) Neuroscience 55, 571–582.
[21] Berry, F.B. and Brown, I.R. (1996) J. Neurosci. Res. 43, 565–575.
[22] Palfi, A., Vizi, S. and Gulya, K. (1999) J. Histochem. Cytochem. 47, 583–600.
[23] Rasmussen, C.D. and Means, A.R. (1989) EMBO J. 8, 73–82.
[24] Prostko, C.R., Zhang, C. and Hait, W.N. (1997) Oncol. Res. 9, 13–17.
[25] Hou, W.F., Zhang, S.P., Davidkova, G., Nichols, R.A. and Weiss, B. (1998) Antisense Nucl. Acid Drug Dev. 8, 295–308.
[26] Bai, G. and Weiss, B. (1991) J. Cell Physiol. 149, 414–421.
[27] Dowd, D.R., MacDonald, P.N., Komm, B.S., Haussler, M.R. and Miesfeld, R. (1991) J. Biol. Chem. 266, 18423–18426.
[28] Inbal, B., Cohen, O., Polak-Charcon, S., Kopolovic, J., Vadai, E., Eisenbach, L. and Kimchi, A. (1997) Nature 390, 180–184.
[29] Li, R. et al. (1998) Oncogene 16, 3269–3277.