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14-3-3 proteins are involved in the regulation of mammalian cell proliferation

Rapid Communication

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Summary. The 14-3-3 proteins are a family of abundant, widely expressed acidic polypeptides. The seven isoforms interact with over 70 different proteins. 14-3-3 isoforms have been demonstrated to be involved in the control of positive as well as negative regulators of mammalian cell proliferation. Here we used the approach of inactivating 14-3-3 protein functions via overexpression of dominant negative mutants to analyse the role of 14-3-3 proteins in mammalian cell proliferation. We found 14-3-3 dominant negative mutants to downregulate the proliferation rates of HeLa cells. Overexpression of these dominant negative mutants triggers upregulation of the protein levels of the cyclin-dependent kinase inhibitor p27, a major negative cell cycle regulator. In addition, they downregulate the protein levels of the important cell cycle promoter cyclin D1. These data provide new insights into mammalian cell proliferation control and allow a better understanding of the functions of 14-3-3 proteins.

Keywords: 14-3-3 – Dominant negative mutants – Cell proliferation

Introduction

The 14-3-3 proteins are a family of abundant, widely expressed 28–33 kDa acidic polypeptides. They are expressed in all eukaryotic cells and are highly conserved in amino acid sequences in a wide range of organisms, including higher eukaryotes, invertebrates and plants. Seven isoforms encoded by seven distinct genes are identified in mammals. Variants of 14-3-3 proteins assemble in homo- and heterodimers. They bind to phosphoserine-containing motifs in a sequence-specific manner and function as adaptor molecules modulating interactions/functions of components involved in signal transduction and in cell cycle control. Localization of 14-3-3 proteins to cytoplasm, nucleus, various membranes, and cytoskeletal and centrosome structures has been reported. The results of an increasing number of studies provide evidence for a pathophysiological importance of changes in 14-3-3 expression and localization in conditions such as cancer and neurodegenerative diseases (Baldin, 2002; Tzivion and Avruch, 2002).

Until now the interaction of 14-3-3 proteins with over 70 proteins has been described and these are likely to represent only a fraction of the physiological 14-3-3 partners. 14-3-3 isoforms are known to mediate both, negative and positive effects for their binding partners. 14-3-3 proteins participate in the regulation of target proteins via different modes: 1) they can alter the ability of the target to interact with other partners; 2) they can affect cytoplasmic/nuclear localization of the target protein(s); 3) 14-3-3 isoforms can regulate the intrinsic catalytic activity of enzymes; 4) they harbour the potential to protect target protein(s) from proteolysis and/or dephosphorylation; 5) a 14-3-3 protein can also serve as a adapter/scaffold to bridge two proteins (Tzivion and Avruch, 2002).

An aspect of special interest is the known role of 14-3-3 isoforms in regulating cell cycle components. It has been demonstrated that overexpression of 14-3-3σ obstructs cell cycle entry by inhibiting cyclin-dependent kinase activity. 14-3-3σ associates with the cyclin-dependent kinase 2 (Laronga et al., 2000). However, it was also demonstrated that 14-3-3θ, 14-3-3ε and 14-3-3η, but not 14-3-3β or 14-3-3ζ bind to the cyclin-dependent kinase inhibitor p27. This binding depends on phosphorylation

of p27 by Akt. This interaction of p27 and 14-3-3 suppresses the nuclear localization of p27, which is important for p27 to mediate its anti-proliferative effects. Importin α3 and α5 transport p27 into the nucleus together with importin β. 14-3-3 binds p27 and prevents it from binding to importin α, resulting in the inhibition of nuclear import of p27 (Fujita et al., 2002, 2003; Sekimoto et al., 2004). 14-3-3 isoforms have also been demonstrated to regulate the activity of other cell cycle regulators such as e.g. Cdc25C. Cdc25C is a phosphatase, which activates cyclin B/cdc2 by dephosphorylation in the nucleus and thus triggers the transition from G2 phase into mitosis. Binding of 14-3-3 protein increases the cytoplasmic localization of Cdc25C by negatively regulating nuclear import in human cells (Graves et al., 2001).

Since 14-3-3 isoforms have been demonstrated to be involved in the control of positive as well as negative regulators of mammalian cell proliferation it was of interest to investigate the effects of modulated 14-3-3 activity on cell proliferation. Here we found 14-3-3 dominant negative mutants to downregulate the proliferation rates of HeLa cells. Overexpression of these dominant negative mutants triggers upregulation of p27 protein levels and downregulation of cyclin D1 protein levels. These data suggest an important role for 14-3-3 proteins in the regulation of mammalian cell proliferation.

Materials and methods

Cell transfections

HeLa cells (human cervical carcinoma cells), obtained from the American Type Culture Collection (Manassas, VA), were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum and antibiotics (30 mg/l penicillin, 50 mg/l streptomycin sulphate). The cultures were kept at 37°C and 7% CO_2. For transfections the following plasmids were used: the empty CMV-expression vector pcDNA3 as a control; pcDNA3 harboring the myc-tagged dominant negative mutant 14-3-3ηDN (Thorson et al., 1998); pcDNA3 harboring the myc-tagged dominant negative mutant 14-3-3βDN (Li et al., 2002); pcDNA3 harboring wildtype mouse p27 cDNA; pcDNA3 harboring wildtype human TSC1; or pcDNA3 harboring wildtype human TSC2. Cell transfections were performed using the Lipofectamine reagent obtained from Invitrogen (Life Technologies, Lofer, Austria) following the transfection protocol provided by the manufacturer (for a recent detailed description of this approach see Rosner and Hengstschläger, 2004).

Western blot analyses

Protein extracts were prepared in buffer containing 20 mM HEPES, pH 7.9, 0.4 M NaCl, 2.5% glycerol, 1 mM EDTA, 1 mM PMSF, 0.5 mM NaF, 0.5 mM Na_3VO_4, 0.02 μg/ml leupeptin, 0.02 μg/ml aprotinin, 0.003 μg/ml benzamidinchloride, 0.1 μg/ml trypsin inhibitor and 0.5 mM DTT. Cells were lysed by freezing and thawing, the extracts were centrifuged and the supernatants were stored at −70°C. Protein concentrations were determined using the Bio-Rad (Hercules, CA) protein assay reagent with bovine serum albumin as the standard. Proteins were run on an SDS-polyacrylamide gel and transferred to nitrocellulose. Blots were stained with Ponceau-S to control equal amounts of loaded protein. Immunodetection was performed using the anti-14-3-3 antibody (K-19, St. Cruz), which recognizes all 14-3-3 isoforms, anti-p27 antibody (C-19, St. Cruz), anti-hamartin antibody (2197, kindly provided by M. Nellist, compare Miloloza et al., 2000), anti-tuberin antibody (C-20, St. Cruz), anti-cyclin D1 antibody (M-20, St. Cruz), or anti-α-tubulin antibody (Ab-1, Oncogene). Signals were detected using the enhanced chemiluminescence method (Amersham, Little Chalfont, UK).

Proliferation assays

To study the effects of gene overexpression on cell proliferation, logarithmically growing cells were transfected as described above. Selection for transfected cells was started 24 hours after transfection. During the first two days of selection the G418 concentration was 0.7 mg/ml, thereafter the G418 concentration was set at 1.5 mg/ml. Starting with equal cell numbers (harvesting and reseeding), selected cell pools were grown under further selection, and cell number was determined on the CASY cell counter and analyser at the indicated time points. For these analyses, cells growing in Petri dishes were washed with buffer and harvested by trypsinization (Miloloza et al., 2000; Rosner et al., 2003).

Statistics

The significance of the observed differences was determined by Student's *t*-test (paired, two-tails) using GraphPad INSTAT software. *P*-values >0.05 are defined as not significant.

Results and discussion

Logarithmically growing HeLa cells were transfected with the dominant negative mutant 14-3-3ηDN or with the dominant negative mutant 14-3-3βDN. Each of these mutants has been shown to downregulate/inhibit the intracellular functions mediated by all 14-3-3 isoforms (Thorson et al., 1998; Li et al., 2002). Proliferation assays with selected transfected cell pools revealed that both dominant negative mutants downregulated the proliferation rates of HeLa cells (Fig. 1A, B; $P < 0.05$; Student's *t*-test).

To allow further evaluation of this potential we wanted to compare the effects of 14-3-3DN mutants with the effects of well known cell cycle inhibitors. In the mammalian cell cycle, the transition from the G0/G1 phase to S phase, in which DNA replication occurs, has been shown to be regulated by cyclin-dependent kinases (cdks). Mitogen-dependent D-type cyclins (cyclin D1, cyclin D2, cyclin D3) are expressed first during early G1. They associate with cdk4 or cdk6 to form an active kinase complex that phosphorylates the retinoblastoma protein and thereby activates E2F transcriptional activity. Among the genes activated via members of the E2F protein family are cyclin E, which complexed with cdk2 promotes the G1/S transition by phosphorylation of different sub-

strates, and cyclin A, which activates cdk2 to further initiate DNA replication (Sherr, 2000). In addition, two families of cdk inhibitors are known: the INK4 family (p15, p16, p18, and p19) regulating cdk4 and cdk6, and the Cip/Kip family (p21, p27, and p57) inhibiting a broader range of cdks (Sherr and Roberts, 1999). Next,

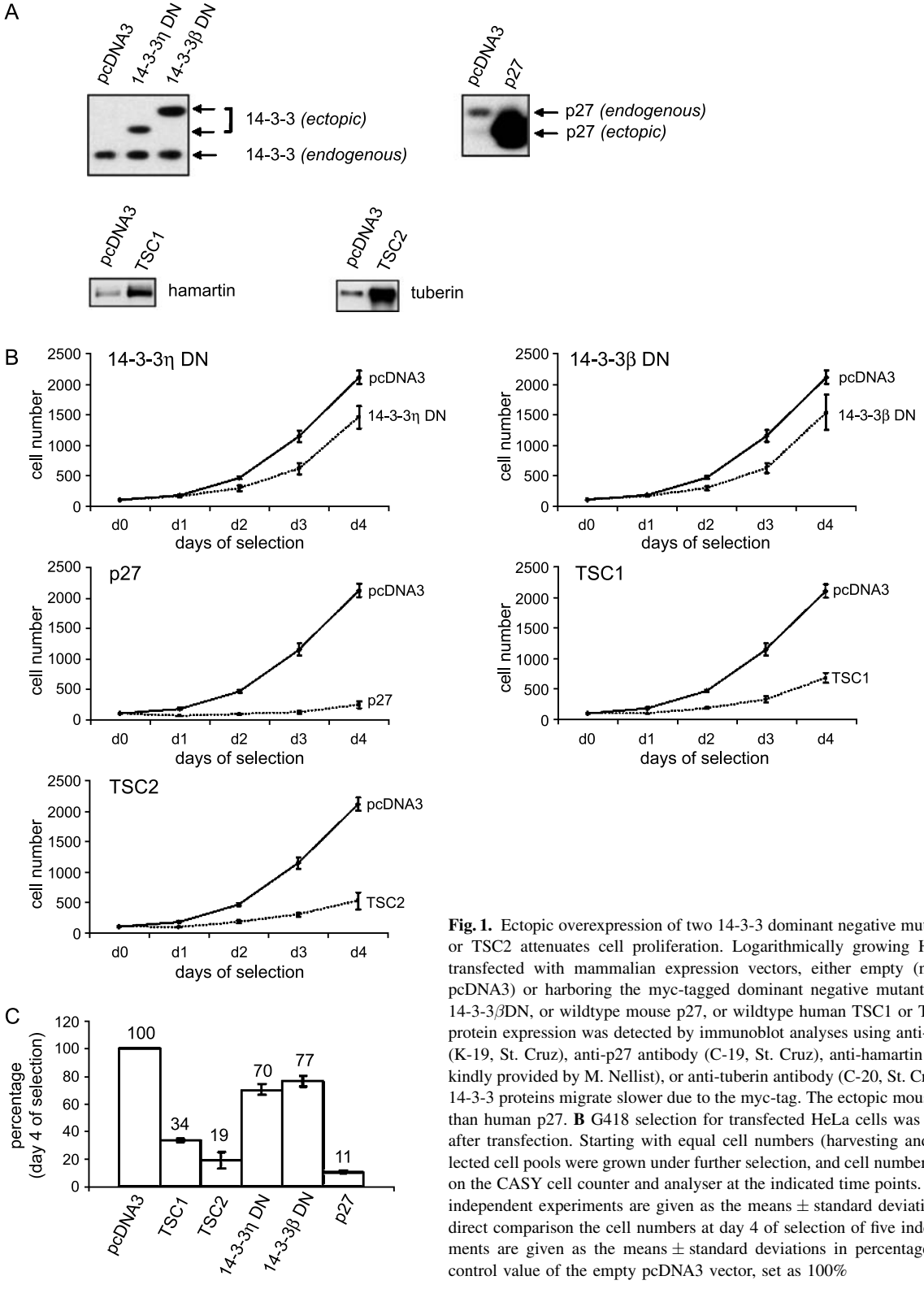

Fig. 1. Ectopic overexpression of two 14-3-3 dominant negative mutants, p27, TSC1 or TSC2 attenuates cell proliferation. Logarithmically growing HeLa cells were transfected with mammalian expression vectors, either empty (negative control, pcDNA3) or harboring the myc-tagged dominant negative mutants 14-3-3ηDN or 14-3-3βDN, or wildtype mouse p27, or wildtype human TSC1 or TSC2. **A** Ectopic protein expression was detected by immunoblot analyses using anti-14-3-3 antibody (K-19, St. Cruz), anti-p27 antibody (C-19, St. Cruz), anti-hamartin antibody (2197, kindly provided by M. Nellist), or anti-tuberin antibody (C-20, St. Cruz). The ectopic 14-3-3 proteins migrate slower due to the myc-tag. The ectopic mouse p27 is smaller than human p27. **B** G418 selection for transfected HeLa cells was started 24 hours after transfection. Starting with equal cell numbers (harvesting and reseeding), selected cell pools were grown under further selection, and cell number was determined on the CASY cell counter and analyser at the indicated time points. The data of four independent experiments are given as the means ± standard deviations. **C** To allow direct comparison the cell numbers at day 4 of selection of five independent experiments are given as the means ± standard deviations in percentage relative to the control value of the empty pcDNA3 vector, set as 100%

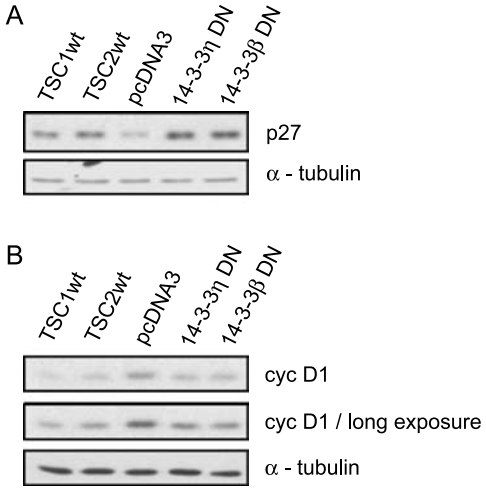

Fig. 2. Ectopic overexpression of two 14-3-3 dominant negative mutants, TSC1 or TSC2 upregulates the cyclin-dependent kinase inhibitor p27 and downregulates cyclin D1. Logarithmically growing HeLa cells were transfected with mammalian expression vectors, either empty (negative control, pcDNA3) or harboring wildtype human TSC1 or TSC2, or the myc-tagged dominant negative mutants 14-3-3ηDN or 14-3-3βDN. **A** Endogenous p27 protein expression was detected by immunoblot analyses using anti-p27 antibody (C-19, St. Cruz). To prove equal loading of proteins the blot was reprobed with anti-α-tubulin antibody (Ab-1, Oncogene). **B** Endogenous cyclin D1 protein expression was detected by immunoblot analyses using anti-cyclin D1 antibody (M-20, St. Cruz). To prove equal loading of proteins the blot was reprobed with anti-α-tubulin antibody (Ab-1, Oncogene)

we overexpressed p27 in HeLa cells. Proliferation assays demonstrated that p27 harbors a higher potential to downregulate cell proliferation than the analysed 14-3-3DN mutants (Fig. 1A–C; $P<0.05$; Student's *t*-test).

Tuberous sclerosis (TSC) is an autosomal dominantly inherited tumor syndrome occuring in about 1 in 6000 live births. It is characterized by mental retardation, epilepsy, and by the development of different growths, including cortical tubers, affecting e.g. kidneys, heart, lung and skin. TSC1 on chromosome 9q34 encodes a 130 kDa protein, named hamartin and TSC2 on chromosome 16p13.3 encodes a 200 kDa protein, named tuberin. TSC patients carry a mutant TSC1 or TSC2 gene in each of their somatic cells. Tumor development is assumed to be the result of somatic ''second hit'' mutations according to Knudson's tumor suppressor model. Loss of heterozygosity has been documented in a wide variety of TSC tumors, such as hamartomas, giant cell astrocytomas or renal carcinomas. Since their identification a variety of different functions of tuberin and hamartin have been reported (Gomez et al., 1999; Cheadle et al., 2000; Rosner et al., 2004). Tuberin and hamartin have been demonstrated to regulate cell size control due to their involvement in the insulin signaling pathway (Li et al., 2003; Pan et al., 2004). Tuberin (and hamartin) also regulates cell cycle progression via binding to the cyclin dependent kinase inhibitor p27 and, thereby, preventing p27 degradation via its SCF-type E3 ubiquitin ligase complex (Soucek et al., 1997, 1998; Miloloza et al., 2000; Rosner and Hengstschläger, 2004). After transfection of TSC1 or TSC2 followed by proliferation assays performed as described above, direct comparison revealed that the TSC genes also mediate stronger effects on cell proliferation than the 14-3-3DN mutants (Fig. 1A–C; $P<0.05$; Student's *t*-test).

That ectopic overexpression of the TSC genes triggers upregulation of p27 accompanied by a downregulation of cyclin D1 has already been described earlier (Soucek et al., 1997, 1998; Miloloza et al., 2000; Rosner and Hengstschläger, 2004). We confirmed these findings under the here used experimental conditions (Fig. 2A, B). To obtain further insights into the molecular events triggered by overexpression of 14-3-3 dominant negative mutants we analysed p27 and cyclin D1 protein levels in transfected cells. These experiments demonstrated that downregulation/inhibition of intracellular functions of 14-3-3 proteins triggers upregulation of the cell cycle inhibitor p27 accompanied by downregulation of the cell cycle promoter cyclin D1 (Fig. 2A, B).

Different reports have been published showing an interaction between tuberin and all different members of the 14-3-3 protein family (Nellist et al., 2002; Li et al., 2002; Liu et al., 2002; Shumway et al., 2003). Here we report that both, the TSC genes and the 14-3-3 proteins, are involved in the regulation of cell proliferation. These findings make it tempting to analyse in future, whether this interaction is of relevance for their potential to control proliferation.

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References

Baldin V (2002) 14-3-3 proteins and growth control. Prog Cell Cycle Res 4: 49–60

Cheadle JP, Reeve MR, Sampson JR, Kwiatkowski DJ (2000) Molecular genetic advances in tuberous sclerosis. Hum Genet 107: 97–114

Fujita N, Sato S, Katayama K, Tsuruo T (2002) Akt-dependent phosphorylation of p27 promotes binding to 14-3-3 and cytoplasmic localization. J Biol Chem 277: 28706–28713

Fujita N, Sato S, Tsuruo T (2003) Phosphorylation of p27 at threonine 198 by p90 ribosomal protein S6 kinase promotes its

binding to 14-3-3 and cytoplasmic localization. J Biol Chem 278: 49254–49260

Gomez MR, Sampson JR, Whittemore VH (1999) Tuberous sclerosis complex, 3rd edn. Oxford University Press, New York

Graves PR, Lovly CM, Uy GL, Piwnica-Worms H (2001) Localization of human Cdc25C is regulated both by nuclear export and 14-3-3 protein binding. Oncogene 20: 1839–1851

Laronga C, Yang H-Y, Neal C, Lee M-H (2000) Association of the cyclin-dependent kinase and 14-3-3 sigma negatively regulates cell cycle progression. J Biol Chem 275: 23106–23112

Li Y, Inoki K, Yeung R, Guan K-L (2002) Regulation of TSC2 by 14-3-3 binding. J Biol Chem 277: 44593–44596

Li Y, Corradetti MN, Inoki K, Guan K-L (2003) TSC2: filling the GAP in the mTOR signalling pathway. Trends Biochem Sci 29: 32–38

Liu MY, Cai S, Espejo A, Bedford MT, Walker CL (2002) 14-3-3 interacts with the tumor suppressor tuberin at Akt phosphorylation site(s). Cancer Res 62: 6475–6480

Miloloza A, Rosner M, Nellist M, Halley D, Bernaschek G, Hengstschläger M (2000) The TSC1 gene product, hamartin, negatively regulates cell proliferation. Hum Mol Genet 9: 1721–1727

Nellist M, Goedbloed MA, de Winter C, Verhaaf B, Jankie A, Reuser AJJ, van den Ouweland A, van der Sluijs P, Halley DJJ (2002) Identification and characterization of the interaction between tuberin and 14-3-3 ζ. J Biol Chem 277: 39417–39424

Pan D, Dong J, Zhang Y, Gao X (2004) Tuberous sclerosis complex: from Drosophila to human disease. Trends Cell Biol 14: 78–85

Rosner M, Hengstschläger M (2004) Tuberin binds p27 and negatively regulates its interaction with the SCF component Skp2. J Biol Chem 279: 48707–48715

Rosner M, Hofer K, Kubista M, Hengstschläger M (2003) Cell size regulation by the human TSC tumor suppressor proteins depends on PI3K and FKBP38. Oncogene 22: 4786–4798

Rosner M, Freilinger A, Hengstschläger M (2004) Proteins interacting with the tuberous sclerosis gene products. Amino Acids 27: 119–128

Sekimoto T, Fukumoto M, Yoneda Y (2004) 14-3-3 suppresses the nuclear localization of threonine 157-phosphorylated p27. EMBO J 23: 1934–1942

Sherr CJ (2000) The Pezcoller lecture: cancer cell cycle revisited. Cancer Res 60: 3689–3695

Sherr CJ, Roberts JM (1999) CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev 13: 1501–1512

Shumway SD, Li Y, Xiong Y (2003) 14-3-3β binds to and negatively regulates the tuberous sclerosis complex 2 (TSC2) tumor suppressor gene product, tuberin. J Biol Chem 278: 2089–2092

Soucek T, Pusch O, Wienecke R, DeClue JE, Hengstschläger M (1997) Role of the tuberous sclerosis gene-2 product in cell cycle control. J Biol Chem 272: 29301–29308

Soucek T, Yeung RS, Hengstschläger M (1998) Inactivation of the cyclin-dependent kinase inhibitor p27 upon loss of the tuberous sclerosis complex gene-2. Proc Natl Acad Sci USA 95: 15653–15658

Thorson JA, Yu LWK, Hsu AL, Shih N-Y, Graves PR, Tanner JW, Allen PM, Piwnica-Worms H, Shaw AS (1998) 14-3-3 proteins are required for maintenance of Raf-1 phosphorylation and kinase activity. Mol Cell Biol 18: 5229–5238

Tzivion G, Avruch J (2002) 14-3-3 proteins: active cofactors in cellular regulation by serine/threonine phosphorylation. J Biol Chem 277: 3061–3064

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