

ORIGINAL ARTICLE

Phosphorylation site mutated RB exerts contrasting effects on apoptotic response to different stimuli

A Masselli and JYJ Wang

Division of Hematology-Oncology and Moores Cancer Center, Department of Medicine, UCSD School of Medicine, Health Sciences Drive, University of California San Diego, La Jolla, CA, USA

The retinoblastoma tumor-suppressor protein (RB) is an important regulator of cell cycle and apoptosis. RB is phosphorylated by cyclin-dependent protein kinase during cell cycle progression. A phosphorylation site mutated (PSM)-RB has previously been shown to cause G1 arrest and to interfere with S phase progression. In this study, we examined the effect of inducible PSM-RB expression on the apoptotic response to three different death stimuli: doxorubicin (DOXO), staurosporine (STS) and tumor necrosis factor (TNF) in Rat-16 cells. Induced expression of PSM-RB attenuated caspase activation by DOXO as a result of cell cycle arrest. STS has been shown to cause RB-dependent G1 arrest or apoptosis; however, expression of PSM-RB did not prevent caspase activation by STS. Surprisingly, induced expression of PSM-RB stimulated the apoptotic response to TNF in Rat-16 cells, which mostly undergo necrosis in the absence of PSM-RB. These results show that PSM-RB exerts disparate effects on apoptotic response to different stimuli, and that cell cycle arrest does not always associate with resistance to apoptosis.

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Introduction

Retinoblastoma tumor-suppressor protein (RB), the product of the retinoblastoma-susceptibility gene (*RB*), was the first tumor-suppressor protein to be identified. Bi-allelic loss of *RB* in embryonic retinoblasts underlies the development of retinoblastoma in early childhood. The tumor-suppressor function of *RB* is associated with its critical role in cell cycle regulation: RB inhibits cell cycle progression by repressing the transcription of genes required for DNA synthesis through its interaction with the E2F family of transcription factors and the

recruitment of chromatin-modifying enzymes (Nevins *et al.*, 1997; Harbour, 2000; Nielsen *et al.*, 2001). Mitogenic signaling drives cell cycle progression through the activation of cyclin-dependent kinases (CDKs), which phosphorylate and thereby inactivate RB (Morgan *et al.*, 1998). In particular, hyperphosphorylation of RB by cyclin D-CDK4/6 complexes in mid G1 disrupts its transcriptional repressor activity by blocking the interaction with E2F and chromatin modifying enzymes (Bremner *et al.*, 1995; Knudsen and Wang, 1996; Mittnacht, 1998).

The knockout of *Rb* in mice causes embryonic lethality (around E13.5). *Rb*-null embryos suffer from ectopic proliferation and massive apoptosis in the central nervous system and exhibit defects in the terminal differentiation of muscles and erythrocytes (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992). The *Rb*-knockout phenotypes and several other lines of evidence support the ability of RB to promote differentiation (Lipinski and Jacks, 1999; Ferguson and Slack, 2001) and to suppress apoptosis (reviewed in Chau and Wang, 2003). Together, these studies revealed an intriguing dual role of RB: as an inhibitor of both cell growth and death (Chau and Wang, 2003).

In addition to E2F transcription factors and chromatin modifying enzymes, RB interacts with a variety of cellular proteins (Morris, 2001). Three distinct peptide-binding pockets have been identified in RB to bind (a) the C-terminal region of E2F (Xiao *et al.*, 2003), (b) the LxCxE peptide motif, for example, in viral E7 protein or cellular histone methyl-transferase (Lee *et al.*, 1998; Robertson *et al.*, 2000) and (c) the PENF homology motif, for example, in cellular proteins such as c-Abl tyrosine kinase and Serpin 2B (Darnell *et al.*, 2003). Each of these peptide-binding pockets is inhibited by phosphorylation (Knudsen and Wang, 1996). Mutation of at least nine of the 16 CDK sites is required to create a RB variant with constitutive growth-suppressing function that is not inactivated by CDK-phosphorylation (Knudsen and Wang, 1997). This variant, termed PSM-RB (phosphorylation site mutated RB), lacks seven phosphorylation sites in the C-region and two phosphorylation sites in the insert region of RB (Knudsen and Wang, 1997). PSM-RB and several other RB variants with phosphorylation site mutations are potent inhibitors of proliferation; they prevent S phase entry and inhibit S phase progression by interfering with

Correspondence: Professor JYJ Wang, Department of Medicine, Division of Hematology-Oncology and Moores Cancer Center, UCSD School of Medicine, 3855 Health Sciences Drive, University of California San Diego, La Jolla, CA 92093-0820, USA.
E-mail: jywang@ucsd.edu

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DNA replication (Chew *et al.*, 1998; Knudsen *et al.*, 1998; Sever-Chroneos *et al.*, 2001).

RB is also inactivated by protein degradation. RB is a substrate of caspase and caspase cleavage induces its degradation during apoptosis (Tan and Wang, 1998). Two caspase cleavage sites have been identified in RB, one at the C-terminus (DEAD⁸⁸⁶G) and the other in the N-terminal domain (DSID³⁴⁹) (Fattman *et al.*, 1997; Tan *et al.*, 1997; Fattman *et al.*, 2001). Substitution mutation of the C-terminal caspase site generates RB-MI (mutated in ICE-site), which is resistant to caspase cleavage (reviewed in Tan and Wang, 1998). Expression of RB-MI protects fibroblasts from TNF-induced apoptosis (Tan *et al.*, 1997) and cultured neurons from apoptosis caused by potassium deprivation (Boutillier *et al.*, 2000). The MI mutation has been introduced into the mouse *Rb-1* gene to create *Rb^{MI/MI}* 'knockin' mice (Chau *et al.*, 2002). In these animals, Rb-MI conferred tissue-specific protection from endotoxin-induced apoptosis (Chau *et al.*, 2002). Fibroblasts derived from these animals were protected from TNF- α type I receptor (TNFRI)-induced apoptosis, but remained sensitive to DNA damage-induced cell death (Chau *et al.*, 2002). These observations raise the question whether the constitutively active PSM-RB also acts as an inhibitor of apoptosis.

To assess whether PSM-RB affords protection from apoptosis, we employed the Rat-16 cell line that provides tight control of gene expression through a tetracycline-regulated promoter (TET off system) (Sever-Chroneos *et al.*, 2001). In this system, we compared the effect of inducible expression of RB and PSM-RB on the cellular response to three different apoptotic stimuli, namely doxorubicin (DOXO), staurosporine (STS) and tumor necrosis factor- α (TNF). Our results show that the growth-suppressing PSM-RB is not a constitutive inhibitor of apoptotic response.

Results

Inducible expression of wild-type RB and PSM-RB in Rat-16 cells

We established Rat-16-based cell lines expressing wild-type RB (WT-RB) and phosphorylation-site mutated RB (PSM-RB), respectively, each under the control of the TET-regulated promoter (TET off system). Upon switching to TET-free media, expression of WT-RB or PSM-RB was induced and both proteins localized correctly to the nucleus (Figure 1a–d). As previously reported (Knudsen and Wang, 1997; Knudsen *et al.*, 1998), expression of PSM-RB inhibited DNA synthesis causing the majority of the cells to arrest in G1 by 24 h after the removal of TET (Figure 1e and f). In contrast, expression of WT-RB did not interfere with the proliferation of Rat-16 cells (Figure 1f), because it could be inactivated by phosphorylation (Figure 1g). It has previously been shown that mutation of the C-terminal caspase cleavage site of RB does not alter its cell cycle regulatory function and phosphorylation

status (Tan and Wang, 1998). Accordingly, cells expressing caspase resistant RB-MI keep proliferating, while those expressing caspase resistant PSM-RB-MI arrest in G1 by 24 h after induction of the protein (Figure 1f and g).

Attenuation of DOXO-induced caspase activation in PSM-RB-arrested cells

DOXO, an inhibitor of topoisomerase II, is known to cause p53-dependent cell cycle arrest or apoptosis (Siu *et al.*, 1999; Panaretakis *et al.*, 2002). The induction of p53 protein was observed in Rat-16 cells following exposure to DOXO (Figure 1h, upper panel). DOXO treatment also caused cleavage of poly-ADP-ribose polymerase (PARP) (Figure 1h, lower panel). However, we did not observe DNA fragmentation for 48 h following DOXO treatment despite the complete loss of full-length PARP during this time course (Figure 1h, lower panel). We also did not detect any significant loss of viability, as the DOXO-treated Rat-16 cells did not become permeable to propidium iodide (PI) within 48 h (Figure 1i). These data show that Rat-16 cells do not undergo an acute death response to DOXO. This resistance to DOXO-induced acute cell death was not affected by the induced expression of WT-RB or PSM-RB (Figure 1i).

Although DOXO did not cause acute cell death, it did induce the activation of caspase as evidenced by the cleavage of PARP (Figure 1h) and of RB itself (Figure 2a, Δ RB). However, the majority of WT-RB and PSM-RB remained in DOXO-treated cells indicating that only a minor fraction was cleaved. The increase in dephosphorylated WT-RB was consistent with the notion that DOXO caused p53-dependent growth arrest in Rat-16 cells (Figure 2a). Exposure to DOXO (2 μ M) for 24 h resulted in a drastic reduction in the clonogenic survival of Rat-16 cells (Figure 2b). Virtually no cell proliferation could be detected in DOXO-treated samples, and the induced expression of PSM-RB for 24 h prior to DOXO exposure did not have a significant effect on clonogenic survival.

To determine if PSM-RB affected DOXO-induced activation of caspase, we measured DEVDase activity in cell extracts using the synthetic peptide substrate Ac-DEVD-AMC (Figure 2c). DEVDase activity increased 7–10-fold after 24 h of DOXO treatment in uninduced Rat-16 cells (Figure 2c, white bars); cleaved caspase-3 was detected by immunoblotting in these samples (Figure 2d, upper right-hand panel). The induced expression of PSM-RB for 24 h prior to DOXO treatment caused a reduction in DEVDase activation, whereas induced expression of WT-RB did not have an effect (Figure 2c, black bars). Levels of cleaved caspase-3 were reduced in cells induced for PSM-RB expression followed by DOXO treatment (Figure 2d, TET – lanes, upper right-hand panel). The processing of caspase-3 was not affected by the induced expression of RB-MI (Figure 2d, upper left-hand panel), which has no effect on cell cycle progression (Figure 1f). Expression of PSM-RB did not reduce the overall levels of

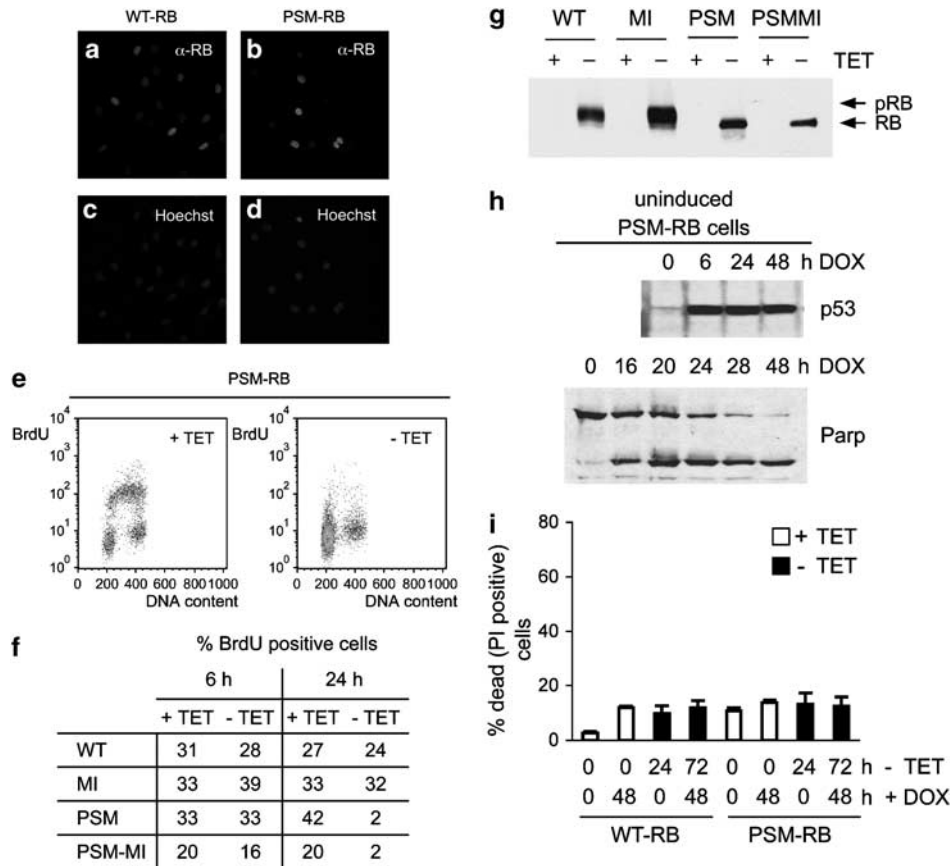


Figure 1 Effect of PSM-RB on Rat-16 cell cycle progression. (a–d) Induction and nuclear localization of WT-RB and PSM-RB in Rat-16 cells. The indicated cell lines were seeded on coverslips and cultured in the absence of tetracycline (TET) for 24 h. Cells were fixed and RB was detected by indirect immunofluorescence. Representative pictures are shown at 60 \times magnification. (e) Inhibition of BrdU incorporation by PSM-RB. PSM-RB cells were cultured for 24 h in the presence (uninduced, left panel) or absence (induced, right panel) of TET and pulse labeled with BrdU for 1 h. Cells were fixed and stained with FITC-conjugated BrdU antibody and propidium iodide (PI) and analysed by flow cytometry. A representative dot plot with FITC (Y axis) and PI (X axis) staining is shown. (f) Summary of the effects of WT-RB, PSM-RB, RB-MI and PSM-RB-MI on BrdU incorporation. The indicated cell lines were cultured for 6 h or 24 h in the presence (uninduced) or absence (induced) of TET and analysed as in (e). In total, 10 000 gated events were counted and the percentage of S phase (BrdU positive) cells was calculated. (g) Phosphorylation status of WT-RB, PSM-RB, RB-MI and PSM-RB-MI 24 h after induction. The indicated cell lines were cultured for 24 h in the presence (uninduced) or absence (induced) of TET and whole-cell lysates were prepared. Equal amounts of total protein were resolved by SDS-PAGE and RB was detected by immunoblotting. pRB, hyperphosphorylated RB; RB, unphosphorylated RB (h) Stabilization of p53 and cleavage of PARP in DOXO-treated Rat-16 cells. Uninduced PSM-RB cells were treated with 2 μ M DOXO for the indicated times. Equal amounts of protein from whole-cell lysates were resolved by SDS-PAGE and p53 (upper panel) and PARP (lower panel) were detected by immunoblotting. (i) DOXO does not induce acute cell death in Rat-16 cells. The indicated cell lines were cultured for 24 h in the presence (uninduced) or absence (induced) of TET and subsequently treated with 2 μ M DOXO for 48 h in the presence (white bars) or absence (black bars) of TET. Uptake of PI was analysed by flow cytometry. In total, 10 000 events were counted and the percentage of PI-positive cells was calculated.

procaspase-3 (Figure 2d, lower panel). Thus, PSM-RB interfered with the processing rather than the production of procaspase-3 in Rat-16 cells.

To test if cell cycle arrest was required for the reduction in caspase activation, we induced PSM-RB for 6 h prior to DOXO treatment. At this time, PSM-RB protein accumulated to a level that was already significantly higher than that of endogenous RB (Figure 3b, endogenous RB not detectable on this blot), but the percentage of cells in S phase was still the same as in uninduced cells (Figure 1f). When added 6 h after induction of PSM-RB expression, DOXO caused a level of caspase activity comparable to uninduced cells (Figure 3a). This result suggested that either cell cycle

arrest or, alternatively, an excessive level of PSM-RB was required for the reduced activation of caspase.

In order to test if cell cycle arrest is sufficient to inhibit caspase activation, we synchronized uninduced cells in quiescence by serum starvation and treated them with DOXO either after the readdition of serum (Figure 3c, white bars) or while keeping them in low serum (black bars). Cells that had re-entered the cell cycle activated DEVDase to the same level as control cells that had not been serum-starved (gray bars). In contrast, cells in quiescence showed greatly reduced caspase activation in response to DOXO.

We analysed DOXO-treated cells for red fluorescence, which increased as a result of drug uptake (Figure 3d).

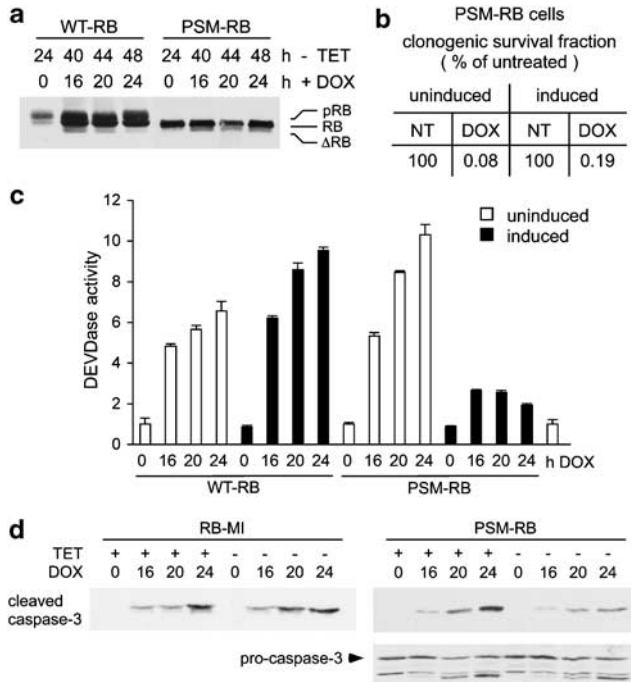


Figure 2 Doxorubicin (DOXO) induces growth arrest but not acute cell death in Rat-16 cells. (a) DOXO induces dephosphorylation and cleavage of RB. The indicated cell lines were cultured for 24 h in the absence of TET and subsequently treated with 2 μM DOXO for the indicated times. Equal amounts of protein from whole-cell lysates were resolved by SDS-PAGE and RB was detected by immunoblotting. ΔRB, truncated RB resulting from cleavage at the C-terminal DEAD sequence; pRB, phosphorylated RB; RB, unphosphorylated RB. (b) Long-term growth arrest in DOXO-treated Rat-16 cells. PSM-RB cells were cultured for 24 h in the presence (uninduced) or absence (induced) of TET and subsequently treated with 2 μM DOXO for 24 h in the presence (white bars) or absence (black bars) of TET. Cells were washed and replated in TET-containing media. Clonogenic survival was assayed after 7 days in culture using crystal violet staining. NT, no treatment; DOXO. (c) DEVDase activity in DOXO-treated Rat-16 cells. The indicated cell lines were cultured for 24 h in the presence (uninduced) or absence (induced) of TET and subsequently treated with 2 μM DOXO for the indicated times in the presence (white bars) or absence (black bars) of TET. Equal amounts of protein from whole-cell lysates were incubated with a fluorogenic caspase-3 substrate (Ac-DEVD-AMC) for 30 min. DEVDase activity was measured as fluorescence intensity and calculated as fold increase relative to uninduced, untreated control cells. (d) Levels of cleaved caspase-3 (upper panel) and procaspase-3 (lower panel) in DOXO-treated Rat-16 cells. PSM-RB cells (right-hand panels) or RB-MI cells (left-hand panel) were treated as in (a). Equal amounts of protein from whole-cell lysates were resolved by SDS-PAGE and caspase-3 was detected by immunoblotting.

Quantification of the fluorescence signals in proliferating (+ TET, uninduced) and arrested (-TET, induced) PSM-RB cells showed that DOXO-uptake was not affected by the expression of PSM-RB (Figure 3d, gray bars). The uptake of DOXO could be distinguished from the permeability to PI (Figure 3d, black bars), which was not detected in DOXO-treated cells because these cells retained membrane integrity despite the activation of caspase.

Taken together, these results suggest that PSM-RB most likely interfered with DOXO-induced caspase

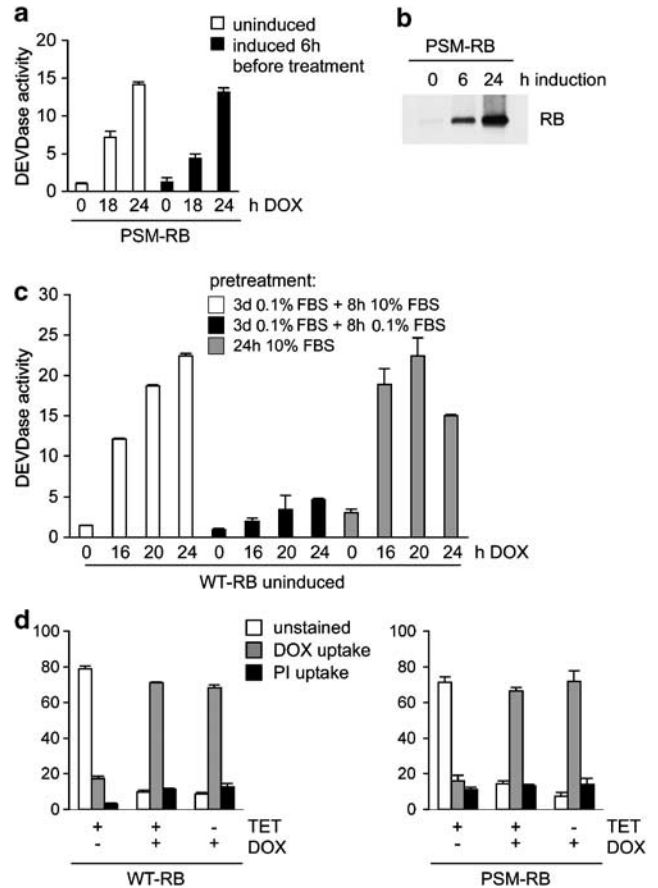


Figure 3 Inducible expression of PSM-RB inhibits caspase activation in response to DOXO. (a) DEVDase activity after DOXO treatment of unarrested PSM-RB cells. PSM-RB cells were cultured for 6 h in the presence (uninduced) or absence (induced) of TET and subsequently treated with 2 μM DOXO for the indicated times in the presence (white bars) or absence (black bars) of TET. DEVDase activity in whole-cell lysates was analysed as in (a). (b) PSM-RB protein levels 6 h and 24 h after induction. PSM-RB cells were treated as in (c). Equal amounts of protein from whole-cell lysates were resolved by SDS-PAGE and RB was detected by immunoblotting. (c) DEVDase activity in DOXO-treated WT-RB cells. WT-RB cells were cultured in the presence of TET and either serum starved for 3 days followed by 8 h culture in 10% serum (white bars), serum starved for 3 days + 8 h (black bars) or cultured in 10% serum for 24 h (gray bars). Subsequently cells were treated with 2 μM DOXO in 10% (white and gray bars) or 0.1% serum (black bars) for the indicated times. Whole-cell lysates were analysed as in (a). (d) Quiescent cells take up the same amount of DOXO as proliferating cells. PSM-cells were cultured for 24 h in the presence (uninduced) or absence (induced) of TET and subsequently treated with 2 μM DOXO for 48 h in the presence or absence of TET. Untreated and DOXO-treated cells were analysed for red fluorescence (DOXO uptake, PI uptake) by FACS analysis.

activation by blocking cell cycle progression. PSM-RB-induced cell cycle arrest did not alter the efficiency of DOXO uptake into the cell. Moreover, PSM-RB did not prevent the loss of clonogenic survival in DOXO-treated Rat-16 cultures (Figure 2c). Our results are consistent with the consensus that quiescent cells are more resistant to genotoxin-induced apoptosis, and extend it to show that cell cycle arrest can reduce the activation of procaspase-3 in cells that do not undergo acute apoptosis in response to DOXO.

Activation of caspases by STS is not inhibited by PSM-RB

STS, a nonspecific inhibitor of protein kinases, can induce G1 arrest or apoptosis. Previous studies have suggested STS-induced G1 arrest to be mediated by RB (Schnier *et al.*, 1996; Orr *et al.*, 1998; Chen *et al.*, 2000). We therefore examined whether PSM-RB-mediated growth arrest can protect Rat-16 cells from STS-induced apoptosis. To our surprise, PSM-RB did not prevent STS-induced activation of caspase activity (Figure 4a) or the cell surface exposure of phosphatidyl-serine as measured by annexin-V staining (Figure 4c). We noted that STS caused a much higher level of caspase activity than DOXO (compare Y-axis values in Figures 4a and 2c), consistent with the rapid apoptosis observed upon STS exposure. The cleavage of PSM-RB (appearance of Δ RB) was detected after 2 h of STS treatment and was followed by the gradual reduction in total PSM-RB (Figure 4b). The appearance of Δ RB was not observed with PSM-RB-MI, suggesting caspase to cleave PSM-RB at the C-terminal site disrupted by the MI-mutation. However, PSM-RB-MI was still degraded after STS treatment (Figure 4b), possibly through other caspase cleavage sites still present in PSM-RB-MI or by other proteases. Expression of PSM-RB-MI also did not prevent caspase activation (Figure 4a) or phosphatidyl-serine exposure (Figure 4c) induced by STS. Clearly, STS can efficiently activate caspase in Rat-16 cells that have undergone growth arrest

established by PSM-RB, and the MI-mutation cannot protect PSM-RB from being degraded in STS-treated Rat-16 cells.

Inducible expression of PSM-RB-enhanced apoptotic response to TNF

TNF, in combination with cycloheximide (CHX), is known to induce caspase-dependent (apoptotic) and caspase-independent (necrotic) cell death in cultured fibroblasts (Liu *et al.*, 1996; Humphreys and Wilson, 1999; Denecker *et al.*, 2001; Lin *et al.*, 2004). TNF/CHX caused viability loss of Rat-16 cells, evidenced by their permeability to PI (Figure 5a, white bars). The pan-caspase inhibitor zVAD-fmk partially inhibited this death response (Figure 5a, white bars). However, TNF/CHX did not induce nuclear condensation (Figure 5b, white bars), DNA fragmentation, as measured by sub-G1 DNA content (Figure 5c, white bars), or DEVDase activity (Figure 5d, white bars). These results suggest that uninduced Rat-16 cells mostly undergo necrotic death in response to TNF/CHX.

Following the induced expression of PMS-RB, TNF/CHX treatment did not increase levels of cell death as measured by permeability to PI (Figure 5a, black bars). However, expression of PSM-RB caused TNF/CHX to induce nuclear condensation, which was blocked by zVAD-fmk (Figure 5b, black bars) as well as DNA fragmentation measured by sub-G1 DNA content

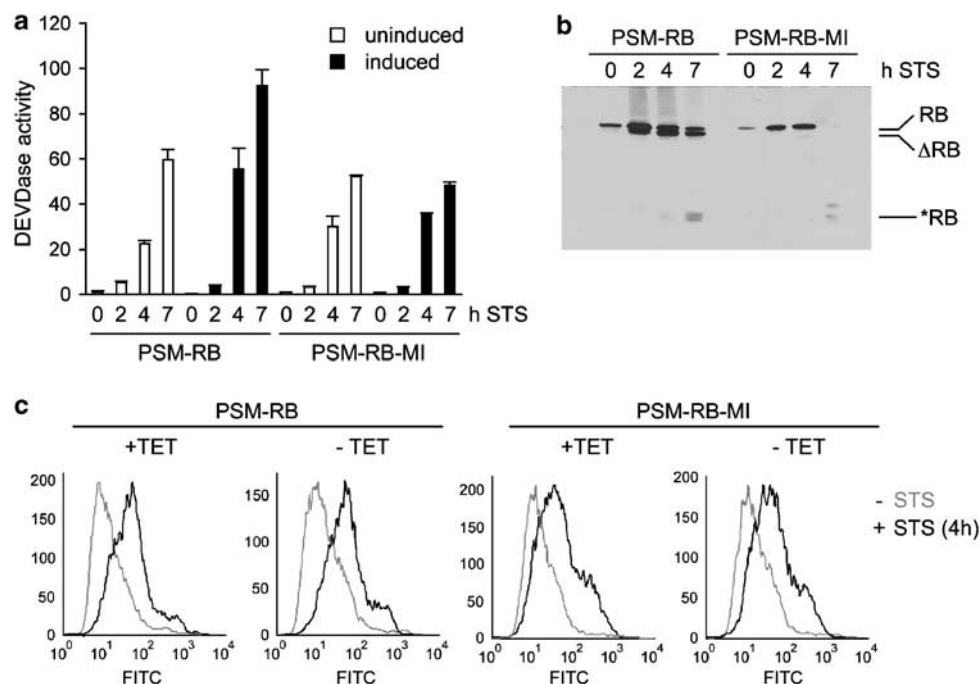


Figure 4 Staurosporine (STS)-induced caspase activation is not affected by PSM-RB. (a) DEVDase activity in STS-treated Rat-16 cells. PSM-RB and PSM-RB-MI cells were cultured for 24 h in the presence or absence of TET and subsequently treated with $1 \mu\text{M}$ STS for the indicated times in the presence (white bars) or absence (black bars) of TET. DEVDase activity was analysed as in Figure 2c. (b) PSM-RB and PSM-RB-MI protein degradation in STS-treated Rat-16 cells. PSM-RB cells were treated as in (a). Equal amounts of protein from whole-cell lysates were resolved by SDS-PAGE and RB was detected by immunoblotting. Δ RB and *RB indicate cleavage fragments of RB. (c) AnnexinV staining of STS-treated Rat-16 cells. PSM-RB and PSM-RB-MI cells were treated as in (a). Cells were trypsinized, washed, incubated with FITC-conjugated AnnexinV and analysed by FACS.

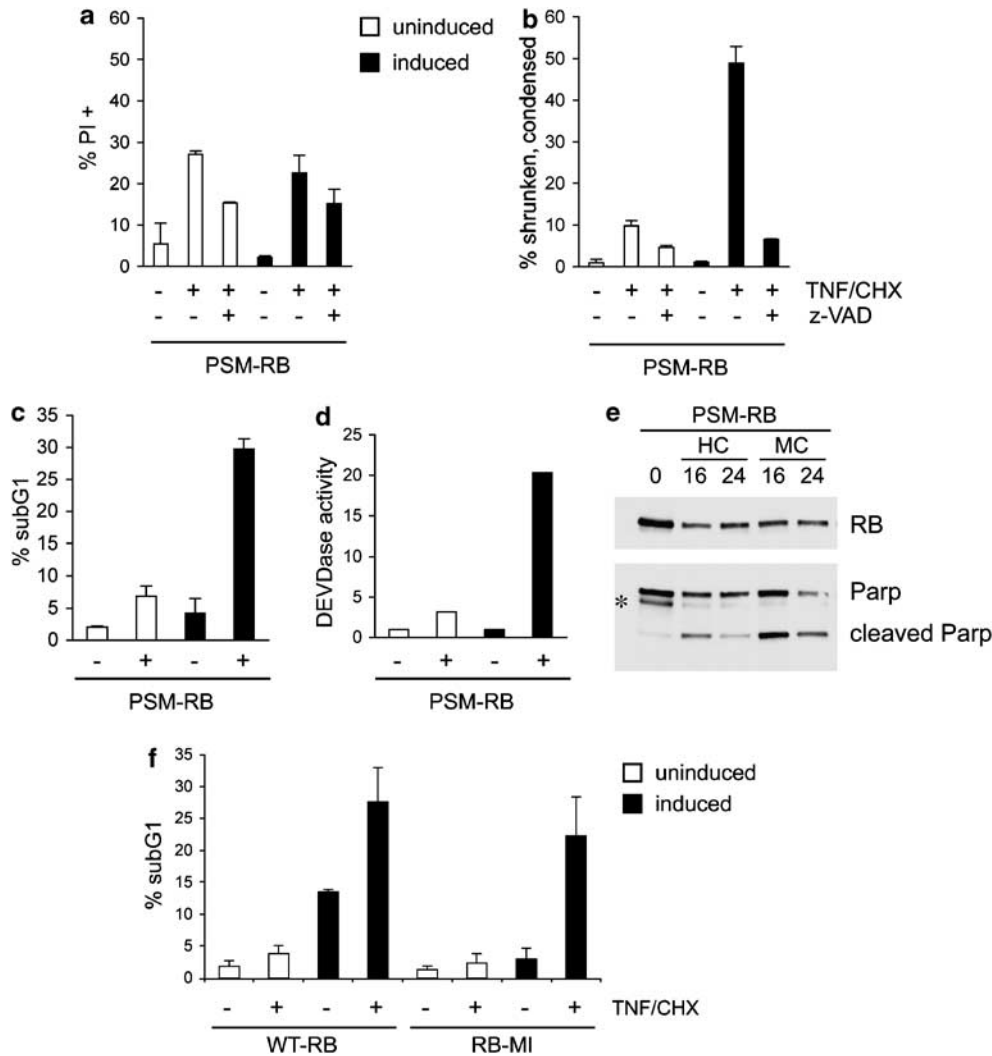


Figure 5 Induced expression of PSM-RB sensitizes to TNF-induced apoptosis. (a, b) Caspase inhibitor prevents TNF-induced cell shrinkage but not PI-uptake in PSM-RB-expressing cells. Shown are levels of PI-positive cells (a) and shrunken/condensed cells (b). PSM-RB cells were cultured for 24 h in the absence or presence of TET and subsequently treated with 20 ng/ml TNF/0.5 μ g/ml CHX for 48 h in the presence (white bars) or absence (black bars) of TET with (+) or without (-) prior addition of 50 μ M z-VAD-fmk. Uptake of PI and cell size were analysed by flow cytometry. In all, 10000 events were counted and the percentage of PI-positive (= dead) cells and shrunken/condensed cells was calculated. (c), (d) DNA fragmentation and caspase activation in TNF-treated Rat-16 cells. PSM-RB cells were treated as in (a) without addition of z-VAD-fmk. Levels of sub-G1 DNA content (c) and DEVDase activity (d) were quantified. For sub-G1 analysis cells were fixed and permeabilized after 48 h and DNA was stained with PI. Triplicate samples were counted by flow cytometry. For DEVDase analysis, lysates were prepared after 20 h and processed as in Figure 2c. (e) RB and PARP protein levels in TNF-treated PSM-RB expressing cells. PSM-RB were cultured for 24 h in the absence of TET and subsequently treated with 20 ng/ml recombinant mouse TNF plus 0.5 μ g/ml CHX (MC) or 20 ng/ml human TNF plus 0.5 μ g/ml CHX (HC) for the indicated times. Equal amounts of protein from whole-cell lysates were resolved by SDS-PAGE and RB (upper panel) and PARP (lower panel) were detected by immunoblotting on the same membrane. *Indicates faint RB bands visible due to incomplete stripping. (f) WT-RB and RB-MI sensitize to TNF-induced DNA fragmentation. WT-RB and RB-MI cells were cultured for 24 h in the absence or presence of TET and subsequently treated with 20 ng/ml TNF/0.5 μ g/ml CHX for 48 h in the presence (white bars) or absence (black bars) of TET. Cells were fixed and permeabilized after 48 h, DNA was stained with PI and sub-G1 DNA content was analysed by flow cytometry.

(Figure 5c, black bars) and DEVDase activity (Figure 5d, black bars). The enhanced DNA fragmentation response to TNF/CHX was not observed in parental Rat-16 cells upon the withdrawal of TET, thus it was not an unspecific effect of culture conditions. We observed a low level of PARP cleavage (Figure 5e, lower panel) but not a significant cleavage of PSM-RB in TNF/CHX-treated Rat-16 cells (Figure 5e, upper panel).

The manifestation of apoptotic phenotypes to TNF/CHX did not require cell cycle arrest, because induction of PSM-RB for 6 h was sufficient for this effect (Figure 6a). Moreover, we found that the induced expression of WT-RB, RB-MI or PSM-RB-MI also enhanced apoptotic phenotypes in TNF/CHX-treated Rat-16 cells (Figure 5f, and data not shown).

We found two other conditions that enhanced the apoptotic response to TNF/CHX in Rat-16 cells:

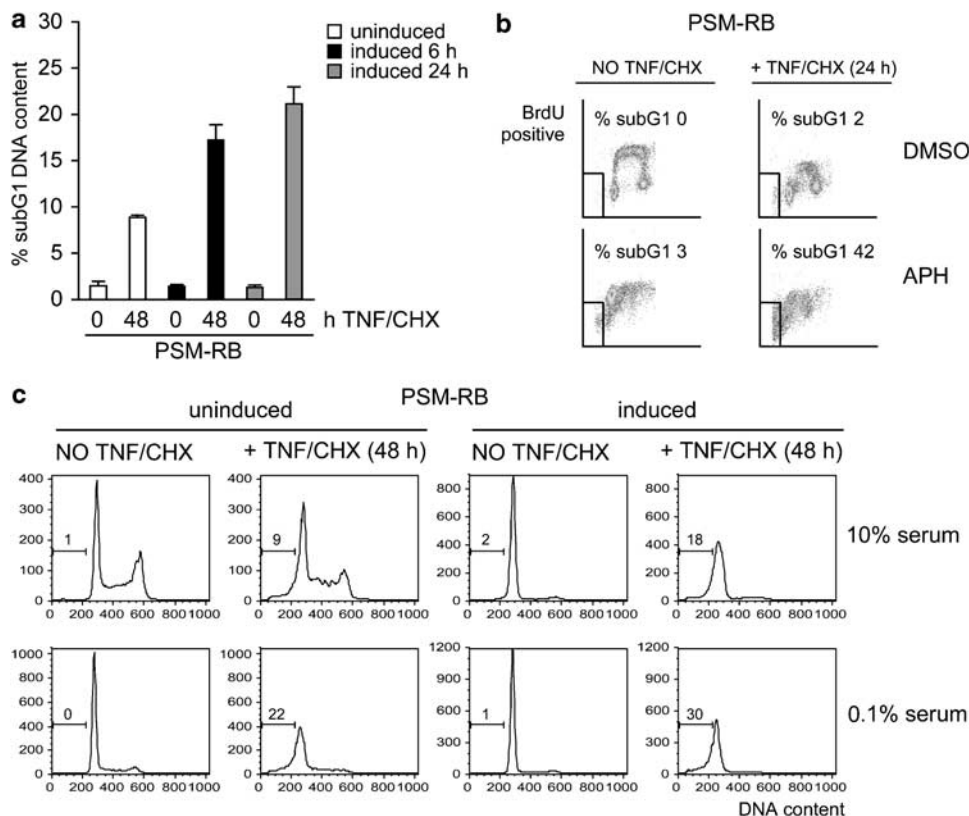


Figure 6 Sensitization to TNF-induced apoptosis by aphidicolin or serum starvation. (a) Sensitization does not correlate with PSM-RB-induced cell cycle arrest. PSM-RB cells were cultured for 6 h (black bars) or 24 h (gray bars) in the absence of TET or left uninduced (white bars) and subsequently treated with 20 ng/ml recombinant mouse TNF plus 0.5 μ g/ml CHX (TNF/CHX) for the indicated times in the presence or absence of TET. Cells were fixed and sub-G1 DNA content analysed. (b) Aphidicolin sensitizes to TNF-induced apoptosis. Uninduced PSM-RB cells were first synchronized in quiescence by culture in 0.1% serum for 3 days. After that, cells were cultured in 10% serum for 16 h and then treated with 2 μ g/ml aphidicolin (APH) (lower panel) or DMSO (upper panel) for 22 h. Subsequently, cells were treated with 20 ng/ml recombinant mouse TNF plus 0.5 μ g/ml CHX (TNF/CHX) as indicated and pulse labeled with BrdU for 1 h. Cells were fixed and stained with FITC-conjugated BrdU antibody and PI and analysed by flow cytometry. Shown are representative dot plots of 10 000 gated events with FITC (Y axis) and PI (X axis) staining. Cells with sub-G1 DNA content are indicated. (c) Sensitization by serum-starvation. PSM-RB cells were cultured in 10% serum (upper panel) or synchronized in quiescence by culture in 0.1% serum for 3 days (lower panel) in the presence of TET (uninduced). During the last 24 h, PSM-RB expression was induced in half of the samples (-TET). Subsequently, cells were treated with 20 ng/ml recombinant mouse TNF plus 0.5 μ g/ml CHX (TNF/CHX) as indicated in the presence (+TET) or absence (-TET) of tetracycline and/or serum. Cells were fixed, permeabilized and DNA was stained with PI. Cell cycle distribution and sub-G1 DNA content were determined by flow cytometry. Shown are representative histograms of 10 000 gated events.

treatment with aphidicolin or serum starvation. Synchronization of Rat-16 cells at G1/S with aphidicolin greatly enhanced TNF/CHX-induced DNA fragmentation (Figure 6b). This result is consistent with a previous report that aphidicolin sensitized fibroblastic cells to TNF-induced apoptosis (Gera *et al.*, 1993). PSM-RB has been shown to interfere with DNA replication (Sever-Chroneos *et al.*, 2001), which might enhance DNA fragmentation similar to aphidicolin. However, this cannot explain how induced expression of WT-RB accentuates apoptosis. The second condition to enhance TNF/CHX-induced DNA fragmentation was found with serum starvation (Figure 6c, lower panel). Serum starvation may limit survival factors and thus allow TNF/CHX to activate caspase-dependent cell death. We found that induced expression of PSM-RB stimulated DNA fragmentation in 10% serum to a greater extent than in 0.1% serum (Figure 6c). Perhaps the overproduction of RB and its variants could interfere with

survival signal transduction and thus sensitize Rat-16 cells to TNF-induced apoptosis.

Discussion

In this study, we have shown that inducible expression of phosphorylation-resistant PSM-RB exerts different effects on apoptotic response to three agents- DOXO, STS and TNF. PSM-RB-mediated growth arrest is associated with reduced activation of caspase by DOXO. However, PSM-RB does not inhibit the activation of caspase by STS. In addition, PSM-RB can enhance the apoptotic response to TNF in Rat-16 cells, which normally die by necrosis.

A large body of evidence has suggested that G1 arrest is antagonistic to apoptosis, particularly in response to DNA damaging agents. Our finding that PSM-RB attenuates DOXO-induced activation of caspase is

consistent with previous observations that RB promotes cell cycle arrest and inhibits apoptosis in response to DNA damage (Knudsen *et al.*, 2000; Bosco *et al.*, 2004; Mayhew *et al.*, 2004). It is well established that dephosphorylated RB (such as PSM-RB) represses E2F-regulated promoters, including those of proapoptotic genes such as caspase-3 and Apaf-1 (Muller *et al.*, 2001). In Rat-16 cells, procaspase-3 protein level was not reduced by PSM-RB, but the proteolytic cleavage of procaspase-3 was inhibited. This inhibitory effect was not observed with the expression of WT-RB, which did not cause G1 arrest. However, the inhibition of DOXO-induced caspase processing was observed in serum-starved Rat-16 cells. These results are in agreement with the conclusion that G1 arrest, triggered by serum starvation or by phosphorylation-resistant RB, is protective against DNA damage-induced apoptosis.

Similar to DNA damage, the broad-spectrum kinase inhibitor STS induces apoptosis through the mitochondria-dependent intrinsic pathway (Li *et al.*, 2000). Previous studies have shown STS to induce RB-dependent cell cycle arrest (Schnier *et al.*, 1996; Orr *et al.*, 1998; Chen *et al.*, 2000), implying that RB may also protect against STS-induced apoptosis. With Rat-16 cells, we found that PSM-RB-mediated growth arrest did not afford protection from STS-induced apoptosis. STS efficiently activated caspase in Rat-16 cells, irrespectively of the expression of PSM-RB. We observed cleavage of PSM-RB at the C-terminal caspase site, but prevention of this cleavage by the MI-mutation also did not protect from STS-induced apoptosis. Thus, RB-MI cannot block death through the intrinsic pathway, triggered by DOXO (Chau *et al.*, 2002) or STS; whereas PSM-RB can inhibit DOXO – but not STS-induced caspase activation.

TNF induces apoptosis through the type I TNF-receptor and the activation of initiator caspase (Micheau and Tschopp, 2003; Schneider-Brachert *et al.*, 2004). In addition, TNF has been shown to activate caspase-independent necrotic death mediated by oxidative stress (Los *et al.*, 2002; Cauwels *et al.*, 2003). We found that TNF/CHX induces necrosis rather than apoptosis in Rat-16 cells. We have previously shown that RB cleavage by caspase is required for TNF to induce apoptosis (Chau *et al.*, 2002). In the context of Rat-16 cells, however, RB-MI exerts no protective action on TNF/CHX-induced necrotic death. On the contrary, induced expression of RB or its variants could stimulate the apoptotic response to TNF/CHX as evidenced by DNA condensation and fragmentation. Apoptotic response to TNF/CHX in Rat-16 cells was also enhanced by aphidicolin or serum starvation. As RB and its variants were expressed at nonphysiological level in these experiments, we cannot be certain that enhancement of apoptotic death to TNF is a function of RB at physiological levels of expression. Nevertheless, a recent report has suggested RB to be required for histone deacetylase inhibitors to induce apoptosis (Wagner and Roemer, 2005). The molecular mechanism underlying the ‘proapoptotic’ function of RB remains to be determined.

Materials and methods

Construction of Rat-16 cell lines

The PSM-RB expression plasmid has been described previously (Sever-Chroneos *et al.*, 2001). WT-RB and PSM-RB expression plasmids were cotransfected with a pTK-Hyg plasmid into Rat 16 cells, which were engineered to express the TET-VP16 fusion protein and provide tight regulation of TET-regulated gene expression. These cells were kindly provided by ES Knudsen (University of Cincinnati). For each plasmid, several clones expressing WT-RB or mutant RB were isolated and one was used for the present study.

Cell culture

Cell lines harboring inducible expression of WT-RB or mutant RB were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), G418 (400 µg/ml), TET (1 µg/ml), glutamine, penicillin and streptomycin. To induce RB expression, cells were washed three times in phosphate-buffered saline (PBS) and once in TET-free media and placed into TET-free media.

Immunofluorescence microscopy

Cells were fixed with 4% para-formaldehyde (PFA) and permeabilized with 0.1% Triton X-100. RB was detected using a polyclonal antibody raised against the C-terminus (residues 768–928) of RB.

Immunoblotting

Whole-cell lysates were prepared using RIPA buffer (50 mM Tris, pH 7.4, 1% NP-40, 0.25% Na deoxycholate, 0.1% SDS, 0.5 mM EDTA, 1 mM EGTA, 150 mM NaCl, 1 mM DTT, protease inhibitors) and sonication. Lysates were clarified by centrifugation and protein concentration determined using DC-Protein assay (BioRad). Of the total protein, 50–100 µg was resolved by SDS-PAGE and transferred onto PVDF membranes. The following antibodies were used: a polyclonal antibody raised against the C-terminus (residues 768–928) of RB, anticaspase-3 (Cell Signaling), anticaspase-3 (a gift from Yuri Lazebnik, Cold Spring Harbor Laboratories), anti-Parp (Cell Signaling).

Flow cytometry

For labeling of dead cells, cells were trypsinized, washed in PBS and resuspended in PBS containing 1 µg/ml PI. Cells were incubated for 10 min at room temperature and subjected to FACS counting 10 000 events.

For cell cycle profile and sub-G1 DNA content analysis, cells were trypsinized, fixed in 70% ethanol overnight and stained with PI (20 µg/ml) in PBS plus RNase for 30 min. In total, 10 000 gated events were counted.

For BrdU incorporation analysis, cells were pulse labeled with BrdU (30 µg/ml), trypsinized and fixed in 70% ethanol overnight. Fixed cells were pelleted, resuspended in 2 N HCL containing 0.2 mg/ml pepsin and incubated 30 min at room temperature. Samples were neutralized with 3 ml 0.1 M sodium tetraborate, pH 8.5 and cells were washed once with IFA buffer (Hepes buffered saline, 4% FBS), once with IFA, 0.5% Tween 20 and stained with a FITC-conjugated anti-BrdU antibody and PI. In total, 10 000 gated events were counted.

Clonogenic survival assay

Cells were cultured for 24 h in the presence (uninduced) or absence (induced) of TET and subsequently treated with 2 µM DOXO for 24 h in the presence or absence of TET. Cells were washed and equal numbers of cells were seeded in 96-well plates in TET-containing media. After 7 days cells were stained with

0.1% crystal violet in methanol and washed with PBS. From each well, the dye was extracted with acetic acid and absorbance read at 500 nm. Untreated cells (100% survival) were used to calculate the percentage of surviving docorubicin-treated cells.

Caspase activity assay

Cells were lysed in PIPES/CHAPS buffer (20 mM PIPES, pH 7.2, 100 mM NaCl, 5 mM DTT, 2 mM EDTA, 0.1% CHAPS, 10% sucrose) and clarified by centrifugation. Of total protein, 50 μ g was incubated with 50 μ M acetylated DEVD-7-amino-4-methylcoumarin (Ac-DEVD-AMC) for 15–30 min at 37°C. Fluorometric detection of AMC was performed in triplicates (excitation 360 nm/emission 460 nm).

References

- Bosco EE, Mayhew CN, Hennigan RF, Sage J, Jacks T, Knudsen ES. (2004). *Nucleic Acids Res* **32**: 25–34.
- Boutillier AL, Trinh E, Loeffler JP. (2000). *Oncogene* **19**: 2171–2178.
- Bremner R, Cohen BL, Sopta M, Hamel PA, Ingles CJ, Gallie BL *et al.* (1995). *Mol Cell Biol* **15**: 3256–3265.
- Cauwels A, Janssen B, Waeytens A, Cuvelier C, Brouckaert P. (2003). *Nat Immunol* **4**: 387–393.
- Chau BN, Borges HL, Chen TT, Masselli A, Hunton IC, Wang JY. (2002). *Nat Cell Biol* **4**: 757–765.
- Chau BN, Wang JY. (2003). *Nat Rev Cancer* **3**: 130–138.
- Chen X, Lowe M, Herliczek T, Hall MJ, Danes C, Lawrence DA *et al.* (2000). *J Natl Cancer Inst* **92**: 1999–2008.
- Chew YP, Ellis M, Wilkie S, Mittnacht S. (1998). *Oncogene* **17**: 2177–2186.
- Clarke AR, Maandag ER, van Roon M, van der Lugt NM, van der Valk M, Hooper ML *et al.* (1992). *Nature* **359**: 328–330.
- Darnell GA, Antalis TM, Johnstone RW, Stringer BW, Ogbourne SM, Harrich D *et al.* (2003). *Mol Cell Biol* **23**: 6520–6532.
- Denecker G, Vercaemmen D, Steemans M, Vanden Berghe T, Brouckaert G, Van Loo G *et al.* (2001). *Cell Death Differ* **8**: 829–840.
- Fattman CL, An B, Dou QP. (1997). *J Cell Biochem* **67**: 399–408.
- Fattman CL, Delach SM, Dou QP, Johnson DE. (2001). *Oncogene* **20**: 2918–2926.
- Ferguson KL, Slack RS. (2001). *Neuroreport* **12**: A55–62.
- Gera JF, Fady C, Gardner A, Jacoby FJ, Briskin KB, Lichtenstein A. (1993). *J Immunol* **151**: 3746–3757.
- Harbour JWaD, DC. (2000). *Nature Cell Biol* **2**: E65–E76.
- Humphreys DT, Wilson MR. (1999). *Cytokine* **11**: 773–782.
- Jacks T, Fazeli A, Schmitt EM, Bronson RT, Goodell MA, Weinberg RA. (1992). *Nature* **359**: 295–300.
- Knudsen ES, Buckmaster C, Chen TT, Feramisco JR, Wang JY. (1998). *Genes Dev* **12**: 2278–2292.
- Knudsen ES, Wang JY. (1996). *J Biol Chem* **271**: 8313–8320.
- Knudsen ES, Wang JY. (1997). *Mol Cell Biol* **17**: 5771–5783.
- Knudsen KE, Booth D, Naderi S, Sever-Chroneos Z, Fribourg AF, Hunton IC *et al.* (2000). *Mol Cell Biol* **20**: 7751–7763.
- Lee EY, Chang CY, Hu N, Wang YC, Lai CC, Herrup K *et al.* (1992). *Nature* **359**: 288–294.
- Lee JO, Russo AA, Pavletich NP. (1998). *Nature* **391**: 859–865.
- Li K, Li Y, Shelton JM, Richardson JA, Spencer E, Chen ZJ *et al.* (2000). *Cell* **101**: 389–399.
- Lin Y, Choksi S, Shen HM, Yang QF, Hur GM, Kim YS *et al.* (2004). *J Biol Chem* **279**: 10822–10828.
- Lipinski MM, Jacks T. (1999). *Oncogene* **18**: 7873–7882.
- Liu ZG, Hsu H, Goeddel DV, Karin M. (1996). *Cell* **87**: 565–576.
- Los M, Mozoluk M, Ferrari D, Stepczynska A, Stroch C, Renz A *et al.* (2002). *Mol Biol Cell* **13**: 978–988.
- Mayhew CN, Perkin LM, Zhang X, Sage J, Jacks T, Knudsen ES. (2004). *Oncogene* **23**: 4107–4120.
- Micheau O, Tschopp J. (2003). *Cell* **114**: 181–190.
- Mittnacht S. (1998). *Curr Opin Genet Dev* **8**: 21–27.
- Morgan DO, Fisher RP, Espinoza FH, Farrell A, Nourse J, Chamberlin H *et al.* (1998). *Cancer J Sci Am* **4**(Suppl 1): S77–S83.
- Morris EJ, Dyson NJ. (2001). *Adv Cancer Res* **82**: 1–54.
- Muller H, Bracken AP, Vernell R, Moroni MC, Christians F, Grassilli E *et al.* (2001). *Genes Dev* **15**: 267–285.
- Nevins JR, Leone G, DeGregori J, Jakoi L. (1997). *J Cell Physiol* **173**: 233–236.
- Nielsen SJ, Schneider R, Bauer UM, Bannister AJ, Morrison A, O'Carroll D *et al.* (2001). *Nature* **412**: 561–565.
- Orr MS, Reinhold W, Yu L, Schreiber-Agus N, O'Connor PM. (1998). *J Biol Chem* **273**: 3803–3807.
- Panaretakis T, Pokrovskaja K, Shoshan MC, Grandeur D. (2002). *J Biol Chem* **277**: 44317–44326.
- Robertson KD, Ait-Si-Ali S, Yokochi T, Wade PA, Jones PL, Wolffe AP. (2000). *Nat Genet* **25**: 338–342.
- Schneider-Brachert W, Tchikov V, Neumeyer J, Jakob M, Winoto-Morbach S, Held-Feindt J *et al.* (2004). *Immunity* **21**: 415–428.
- Schnier JB, Nishi K, Goodrich DW, Bradbury EM. (1996). *Proc Natl Acad Sci USA* **93**: 5941–5946.
- Sever-Chroneos Z, Angus SP, Fribourg AF, Wan H, Todorov I, Knudsen KE *et al.* (2001). *Mol Cell Biol* **21**: 4032–4045.
- Siu WY, Yam CH, Poon RY. (1999). *FEBS Lett* **461**: 299–305.
- Tan X, Martin SJ, Green DR, Wang JY. (1997). *J Biol Chem* **272**: 9613–9616.
- Tan X, Wang JY. (1998). *Trends Cell Biol* **8**: 116–120.
- Wagner S, Roemer K. (2005). *Biochem Pharmacol* **69**: 1059–1067.
- Xiao B, Spencer J, Clements A, Ali-Khan N, Mittnacht S, Broceno C *et al.* (2003). *Proc Natl Acad Sci USA* **100**: 2363–2368.

Abbreviations

RB, retinoblastoma tumor-suppressor protein; CDK, cyclin-dependent kinase; TNF, tumor necrosis factor- α ; PMS, phosphorylation site mutated; PARP, poly-ADP-ribose polymerase.

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